Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Letters to the Editor

Chemokine profiling in children and adults with symptomatic and asymptomatic respiratory viral infections

Dear Editor,

The study by Lui et al., indicated that an inflammatory immune response is associated with severe RSV infections in elderly [1,2]. In children under the age of 5 years respiratory infections are responsible for 4–20% mortality globally, and half of these may be caused by viral infections [3]. In asymptomatic young children 40–60% are positive for one or multiple viruses in the nasopharynx [4,5]. In adults this percentage is lower, but still respiratory virus can be detected by molecular diagnostics in5–20%. Molecular diagnostics are highly specific and sensitive. However, high costs, relatively long turnaround time, and restricted number of targets are important limitations.

Infection of host epithelial cells can attract and activate local surveying innate immune cells leading to the secretion of inflammatory mediators, such as chemokines and cytokines. Of all early immune signals, the production of chemokines is considered the very initial signal meant to recruit other immune cells to resolve the infection. Potentially, these chemokines could predict the presence of any virus [6]. Analysis of chemokines in the nasopharynx has the advantage that the same specimen may be used for molecular diagnostics and chemokine analyses without the need of collecting a blood sample. In addition to detecting the presence of viruses, understanding differences in the immune response between symptomatic and asymptomatic cases may increase our insight into protective immune responses and might help to find immune markers associated with symptoms and disease prognosis [1,6,7].

In this study two independent cohorts were included and analysed for viral presence and chemokine levels (see Supplementary Methods). The first cohort (POC-ID) are children hospitalized for suspected respiratory infections, obviously having very clear symptoms. POC-ID consists of 61 children of whom 30 showed viral presence in the nasopharynx (**Fig. S1, Table S1**). This group of virus-positive children consisted of 57% males with an average age of 2.7 years (95% confidence intervals 1.6–3.9) compared to 4.2 (2.8–5.7) years in the virus-negative children group. In 21 children a single virus was detected and in 9 children 2 or more different viruses were detected. Of the virus-positive children 9 were admitted to the intensive care unit. The second cohort (OKIDOKI3) are asymptomatic or symptomatic children and adults recruited from the general population. OKIDOKI3 consisted of 255 asymptomatic or symptomatic children and 198 mostly asymptomatic adults. 70% of the children and 26% of the adults were virus-positive. Virus-positive children were more often symptomatic compared to virus-negative children ($p = 0.042$). In 56% of the virus-positive children more than one virus was detected.

In all nasopharyngeal swabs used for viral diagnostics chemokines were detected, confirming that nasopharyngeal swabs could serve to analyze host immune responses (**Fig. 1A**). In the POC-ID cohort virus-infected children showed different chemokine profiles compared to children attending the hospital for respiratory symptoms without an identified infectious cause ($p = 0.016$) (**Fig. 1B**). The concentrations of CCL4, CXCL10 and CXCL11 were significantly increased in virus-positive patients compared to virus-negative patients (**Fig. 1C**).

In the independent OKIDOKI3 cohort consisting of asymptomatic or symptomatic children and adults the chemokine profile analyses revealed considerably higher concentrations in children compared to adults (**Fig. 1D**). Therefore, the age groups within OKIDOKI3 were analyzed separately. In children, significantly increased concentrations of CCL2, CCL4, CCL5, CXCL8 and CXL10 were observed when viruses were detected in the swab samples (**Fig. 1E, Table S2A**). CCL11 levels were significantly higher in virus-negative children. In symptomatic virus-positive children increased concentrations of CCL3 ($p<0.05$) and CCL4 ($p<0.01$) were observed compared to asymptomatic virus-positive children (**Fig. 1E**). CXCL10 concentrations were increased in symptomatic virus-negative children, but not in symptomatic virus-positive children. Multivariate RDA analysis in children indicates that overall the chemokine profiles were indeed significantly different between virus-positive and -negative children (**Fig. 1F**, $p = 0.001$).

In adults, CCL2, CCL5, CCL20, and especially CXCL9, CXCL10 and CXCL11 were significantly increased if virus-positive (**Fig. 2A, Table S2B**). Multivariate RDA analysis based on all chemokine responses confirmed the importance for the individual observations with CXCL9, CXCL10 and CXCL11, and overall showed a clear and significant distinction between virus-positive and -negative adults (**Fig. 2B**, $p = 0.001$). In adults the presence or absence of symptoms was not associated with altered concentrations of chemokines.

A random forest model was built to assess the importance of different markers in predicting viral presence. The multi-way importance plot shows that CCL17, CCL11, CCL2 and CXCL8 (IL-8) were the strongest predictors for the presence of viruses in children (**Fig. 2C**). Interestingly, symptoms hardly contributed to the distinction between viral positive or negative. ROC analysis (**Fig. 2D**) showed an area under the curve of 0.85, with a sensitivity of 0.94 and a specificity of 0.60, resulting in an accuracy of 0.84.

In adults the strongest signal was observed for CXCL11 (**Fig. 2E**). In addition, CXCL10, CXCL9, CXCL1 and CCL5 were important predictors in the model. The area under the ROC curve (**Fig. 2F**) was 0.73, with a sensitivity of 0.60 and a specificity of 0.86, resulting in an accuracy of 0.79.

We show that nasopharyngeal swab samples can be used to detect early markers of infection by measuring a broad chemokine

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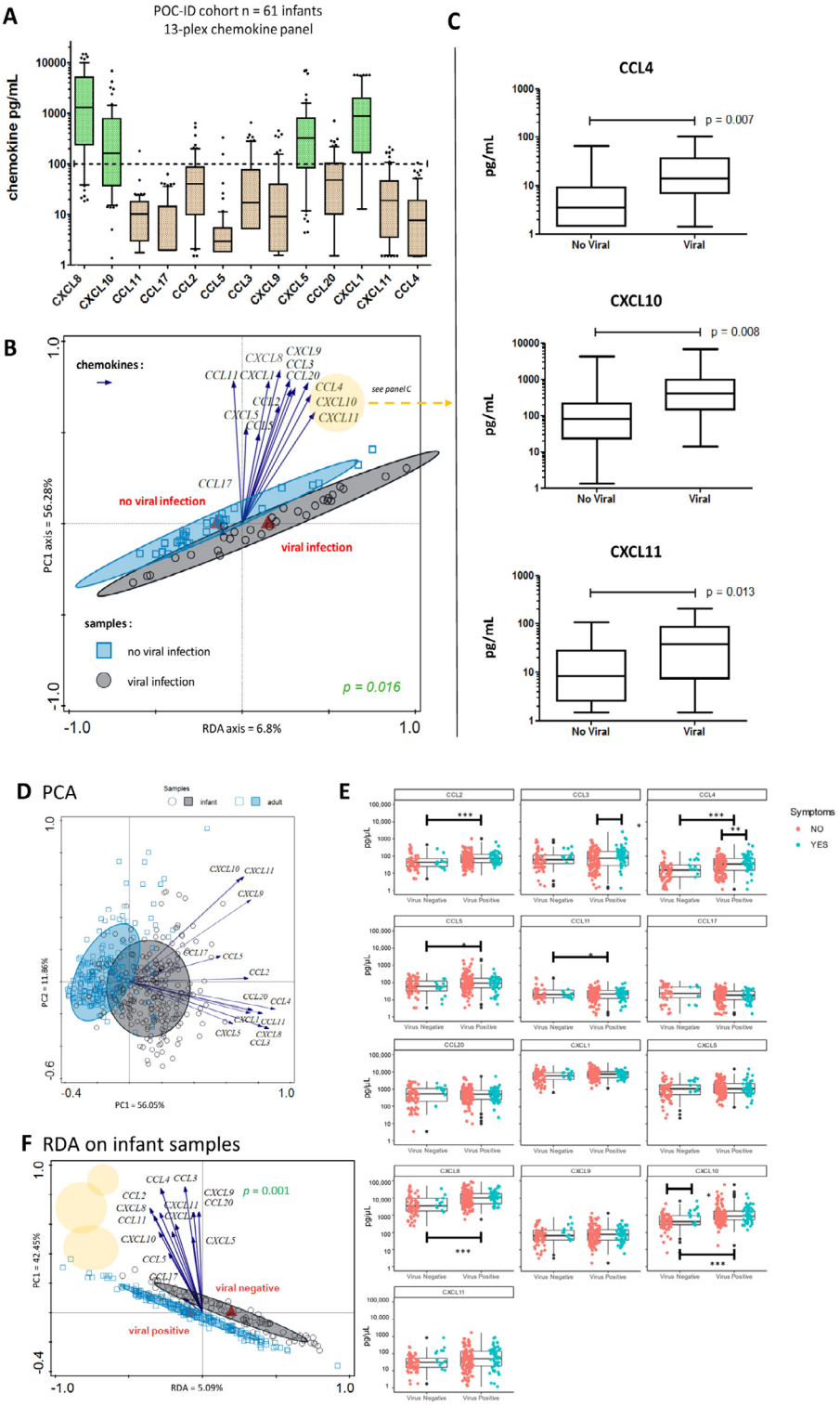

Fig. 1. Chemokine levels in children with or without a viral infection in the POC-ID cohort. (A) Concentrations of the chemokines measured in the samples of the POC-ID cohort. Box plots show median value, 50% median values an error bars 1–99% percentiles. (B) RDA analysis of the chemokine levels in virus-positive and virus-negaitve children. (C) The chemokines CCL4, CXCL10 and CXCL11 in POC-ID children with or without virus. (D) PCA of children and adults of the OKIDOKI cohort. (E) Chemokine levels in children of the OKIDOKI cohort in relation to the presence of virus or symptoms or not. Statistical test results are provided in Table S2A. (F) RDA of children in the OKIDOKI cohort relative to the presence of virus.

panel. A combination of molecular diagnostics with chemokine profiling at the site of infection will support decision making in urgent clinical situations, especially because of the prognostic value. Moreover, confirmation that the infection is likely caused by a virus, in case of an inconclusive molecular diagnostic outcome, will reduce unnecessary use of antibiotics. Increased levels of nasopharyngeal CCL4 were observed in virus-positive samples of children, but not adults. Vice versa, the chemokines CCL2 and CCL5 were observed in non-hospitalized children and adults but not in hospitalized children. Our data cannot provide conclusive evidence for the

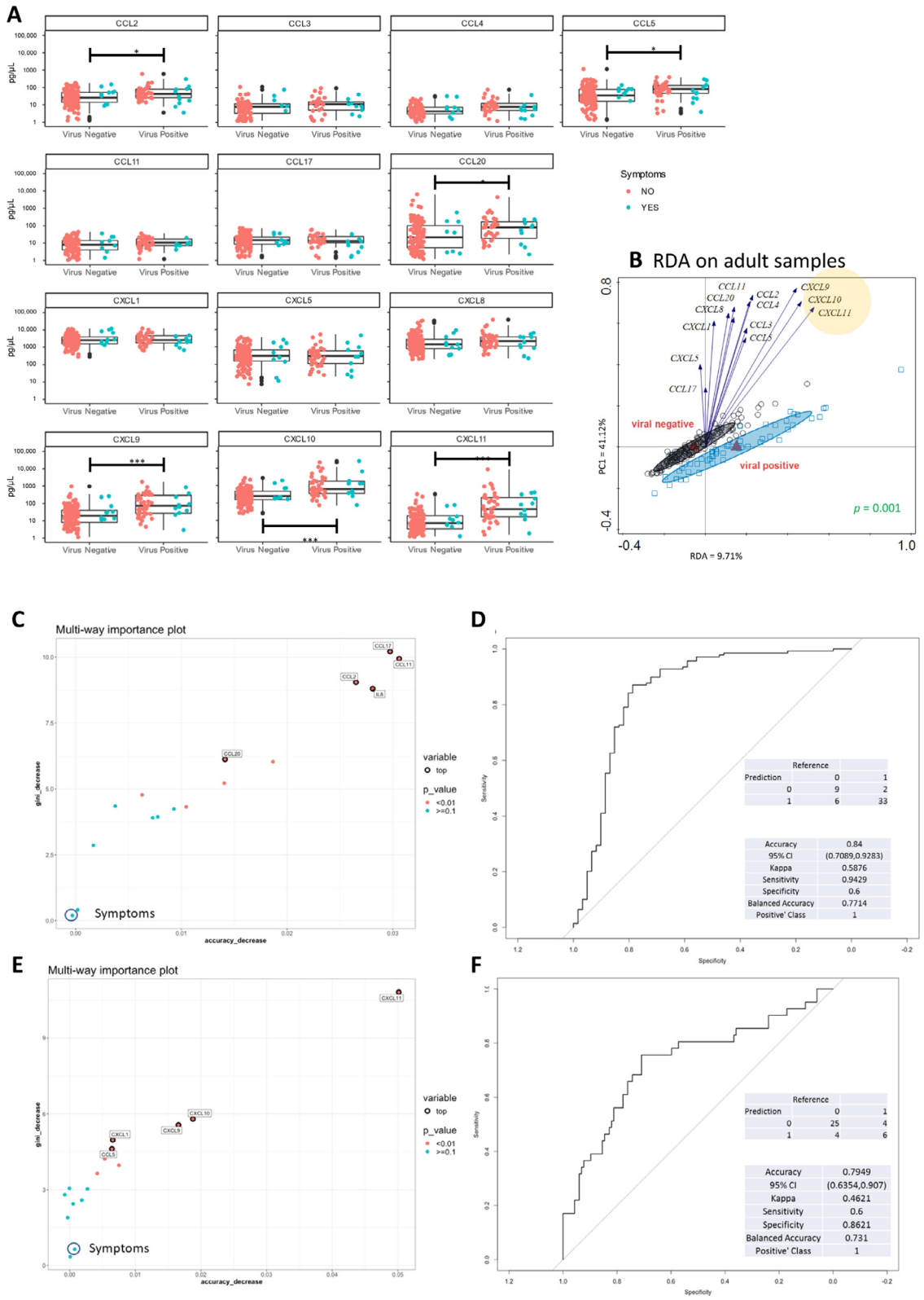

Fig. 2. Chemokine levels in adults of the OKIDOKI cohort and random forest analyses. (A) Chemokine concentrations in virus-negative and virus-positive samples of persons with and without symptoms. Statistical test results are provided in Table S2B. (B) RDA analysis of the adults in the OKIDKOI cohort relative to the presence of virus. (C–F) Random forest models to assess to ability of chemokines to predict the presence of virus. (C) Multi-way importance plot of chemokines to predict the presence of virus in nasopharyngeal samples of children in the OKIDOKI cohort. The top predictors are highlighted, and color index indicates degree of significance. (D) ROC curve for the random forest model in 'A'. (E) Multi-way importance plot of chemokines to predict the presence of virus in nasopharyngeal samples of adults in the OKIDOKI cohort. The top predictors are highlighted, and color index indicates degree of significance. (F) ROC curve for the random forest model in 'D'.

role of these specific markers in protective immune response or disease, but these findings are worth pursuing to identify whether early induction of CCL2 and CCL5 is associated with protection against more severe viral infection and to determine whether increased CCL4 leads to worse disease prognosis. A better understanding of these responses could help to understand the risk of developing (severe) disease following viral infection.

Declaration of Competing Interest

The authors declare no conflict of interest according to ICMJE criteria.

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Supplementary materials

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G. Den Hartog*[1]
Centre for Immunology of Infectious Diseases and Vaccination, National Institute for Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, Bilthoven 3721 MA, the Netherlands

T.H.A. Ederveen[1], P.B. Venkatasubramanian[1]
Center for Molecular and Biomolecular Informatics, Radboud University Medical Center, Geert Grooteplein Zuid 10, Nijmegen 6525 GA, the Netherlands

G. Ferwerda
Laboratory of Medical Immunology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Geert Grooteplein Zuid 10, Nijmegen 6525 GA, the Netherlands
Radboud Center for Infectious Diseases, Radboud University Medical Center, Geert Grooteplein Zuid 10, Nijmegen 6525 GA, the Netherlands

C.H. van den Kieboom, C.E. van der Gaast-de Jongh
Laboratory of Medical Immunology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Geert Grooteplein Zuid 10, Nijmegen 6525 GA, the Netherlands

M. Vissers
Centre for Immunology of Infectious Diseases and Vaccination, National Institute for Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, Bilthoven 3721 MA, the Netherlands

J. Zoll, W.J.G. Melchers
Radboud Center for Infectious Diseases, Radboud University Medical Center, Geert Grooteplein Zuid 10, Nijmegen 6525 GA, the Netherlands
Department of Medical Microbiology, Radboud University Medical Center, Geert Grooteplein Zuid 10, Nijmegen 6525 GA, the Netherlands

M.A. Huynen
Center for Molecular and Biomolecular Informatics, Radboud University Medical Center, Geert Grooteplein Zuid 10, Nijmegen 6525 GA, the Netherlands
Radboud Center for Infectious Diseases, Radboud University Medical Center, Geert Grooteplein Zuid 10, Nijmegen 6525 GA, the Netherlands

N. Rots
Centre for Immunology of Infectious Diseases and Vaccination, National Institute for Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, Bilthoven 3721 MA, the Netherlands

J. Rahamat-Langendoen[1]
Radboud Center for Infectious Diseases, Radboud University Medical Center, Geert Grooteplein Zuid 10, Nijmegen 6525 GA, the Netherlands
Department of Medical Microbiology, Radboud University Medical Center, Geert Grooteplein Zuid 10, Nijmegen 6525 GA, the Netherlands

M.I. de Jonge[1]
Laboratory of Medical Immunology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Geert Grooteplein Zuid 10, Nijmegen 6525 GA, the Netherlands
Radboud Center for Infectious Diseases, Radboud University Medical Center, Geert Grooteplein Zuid 10, Nijmegen 6525 GA, the Netherlands

*Corresponding author.
E-mail address: Gerco.den.hartog@rivm.nl (G.D. Hartog)

[1] These authors contributed equally to this work.

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Re-emergence of highly pathogenic avian influenza A(H5N8) virus in domestic Goose, China

Dear Editor,

Recently, increased antigenicity changes in highly pathogenic H5N6 avian influenza viruses (AIVs) have been reported in this journal.[1] Highly pathogenic avian influenza viruses (HPAIVs) of the H5 subtype remain a great concern for the poultry industry and public health. Since the first report of HPAI H5N1 influenza virus (A/Goose/Guangdong/1/1996(H5N1); Gs/GD) in Guangdong Province in China in 1996,[2] the Gs/Gd lineage virus and its genetic reassortants have continued to cause outbreaks in wild birds and poultry yielding massive mortality. The hemagglutinin (HA) gene of this Gs/GD lineage has undergone reassortments with other subtypes of AIVs and has diversified into multiple genetic clades, including 2.3.4.4.[3] HPAIVs of the H5N8 subtype of 2.3.4.4 subclade were first detected in eastern China domestic ducks in in 2010.[4] This subtype has caused outbreaks in domestic ducks and migratory birds in China and South Korea in early 2014.[5] Since then, this H5N8 virus has spread throughout Asia to Europe and the United States via migrating birds and has caused multiple outbreaks. However, the H5N6 subtype became the dominant AIVs subtype in southern China since 2014. Although the H5N8 virus reemerged in swans in 2020 in Xinjiang and Inner Mongolia, no outbreaks of H5N8 virus have been reported in domestic poultry in mainland China since 2017. In this study, we report the detection of a novel HPAI A(H5N8) in the domestic Goose in Guangdong, China in November 2020.

During a surveillance program of AIVs in waterfowl in Guangdong Province, China, from November 2020 to March 2021, we detected 8 HPAIVs (A/Goose/Guangdong/21FU001/2021-A/Goose/Guangdong/21FU008/2021) of H5N8 subtype with a HA cleavage site sequence (REKRRKR↓GLFGAI) from dead domestic geese. The liver, pancreas, and spleen tissues were mixed and homogenized and the total RNA of the homogenates was extracted using Trizol reagent (Vazyme, China), following the manufacturer's instructions. Viral RNA was then reverse-transcribed using HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, China) with the Uni12 (5′-AGCAAAAGCAGG-3′) primer. The presence of AIV was determined using an RT-PCR assay with a designed primer[6] targeting the conserved region of the M segment with an expected size of 234 bp. Full-length viral genomes were then amplified using a Phusion hot start II high-fidelity PCR mix utilizing universal primers sets described by Hoffman *et al.*[7] The viral genome sequences were then deposited at GenBank with accession numbers MZ882169-MZ882184.

All of these 8 H5N8 strains contained the 222Q and 224 G (H5 numbering) in the HA, which suggested a typical avian-like (α,2,3-SA) receptor preference. The residues E627and D701 in the PB2 protein suggest that these H5N8 viruses have not yet adapted to mammalian cells. However, residues 218Q and 223R (H5 numbering) were observed in the HA protein, indicating increased virus binding affinity to sialyl lewisX glycans and suggested the adaption of H5N8 to terrestrial birds. All H5N8 viruses detected in this study shared the amino acid substitution of T192I in the antigenic site B, which suggests potential antigenic drift. Two isolates had an amino acid substitution D128N in HA that was not found in the other H5N8 strains worldwide. The NA gene from all 8 samples had the amino acid substitution N28S compared to the human isolate A/Astrakhan/3212/2020 reported in Russia in December 2020.

Sequence comparison results suggested that the 8 isolates in this study shared a high nucleotide identity between HA and NA genes (99.2−99.9%). A BLAST search in the Global Initiative on Sharing All Influenza Data (GISAID) database suggests that these H5N8 strains shared high nucleotide identity (> 99%) with the H5N8 virus isolated from chickens in Russia in November 2020 (EPI1813345, Table 1). The BlAST (https://www.ncbi.nlm.nih.gov/blast/) search in GenBank showed that the closest genetic relative of these H5N8 viruses was isolated from a Mute Swan (*Cygnus olor*) in Shandong province in January2021 (MW960368).

The MAFFT (Multiple Alignment using Fast Fourier Transform) embedded in the UGENE software was then used to align the multiple sequences (Version 36.0).[8] The alignment lengths of each gene segment were: PB2, 2398 nt; PB1, 2252 nt; PA, 2211 nt; HA, 1750 nt; NP, 1521 nt; NA, 1452 nt; M, 1003 nt; and NS, 865 nt. The nucleotide and amino acid homology of the genes among the 8 strains were then calculated by using the Geneious software (Version 11.0.9) (www.geneious.com) using the default settings. The phylogenetic trees of genes were then constructed using the maximum likelihood (ML) method with 1000 bootstrap replicates in IQ-TREE (Version 1.6.12). The phylogenetic tree was then further annotated by FigTree (Version 1.4.3). The phylogenetic analysis then showed that these H5N8 viruses belong to the clade 2.3.4.4b (Supplementary Fig. 1). A phylogenetic analysis using the NextStrain phylogenetic pipeline[9] indicated that these H5N8 viruses share a common ancestor with H5N8 viruses circulating in Egypt during 2018–2019, and with viruses detected in eastern Europe (Belgium, Denmark, and Israel) during 2016–2018 (Fig. 1A). Sequence com-

Table 1
Top blast hit of viruses from global initiative on sharing all influenza data (GISAID) in June 2021, which shared the highest nucleotide similarity with the Guangdong H5N8 isolates reported in this study.

Segment	Virus name	Accession number	Similarity (%)
PB2	A/chicken/Omsk/0119/2020 (A/H5N8)	EPI1813382	99
	A/chicken/Omsk/0112/2020 (A/H5N8)	EPI1813342	99
PB1	A/chicken/Omsk/0112/2020 (A/H5N8)	EPI1813343	99
	A/Goose/Omsk/0113/2020 (A/H5N8)	EPI1813159	99
PA	A/chicken/Omsk/0119/2020 (A/H5N8)	EPI1813381	99
	A/chicken/Omsk/0119/2020 (A/H5N8)	EPI1813221	99
HA	A/chicken/Omsk/0112/2020 (A/H5N8)	EPI1813345	99
	A/Goose/Omsk/0113/2020 (A/H5N8)	EPI1813161	99
NP	A/Turkey/Omsk/0003/2020 (A/H5N8)	EPI1813122	99
	A/Turkey/Rostov-on-Don/332–12/2021 (A/H5N8)	EPI1848767	99
NA	A/chicken/Kostroma/304–06/2020 (A/H5N8)	EPI1848645	99
	A/chicken/Kostroma/304–04/2020 (A/H5N8)	EPI1848637	99
M	A/chicken/Kostroma/304–10/2020 (A/H5N8)	EPI1848657	99
	A/chicken/Kostroma/304–08/2020 (A/H5N8)	EPI1848649	99
NS	A/Duck/SouthwesternChina/B1904/2020(H5N8)(A/H5N8)	EPI1844105	99
	A/Duck/Northern China/ZGL/2020(H5N8)	EPI1844094	99

*Homology analysis of all influenza gene segments PB2, PB1, PA, HA, NP, NA, M and NS from Guangdong H5N8 isolates was performed on GISAID.

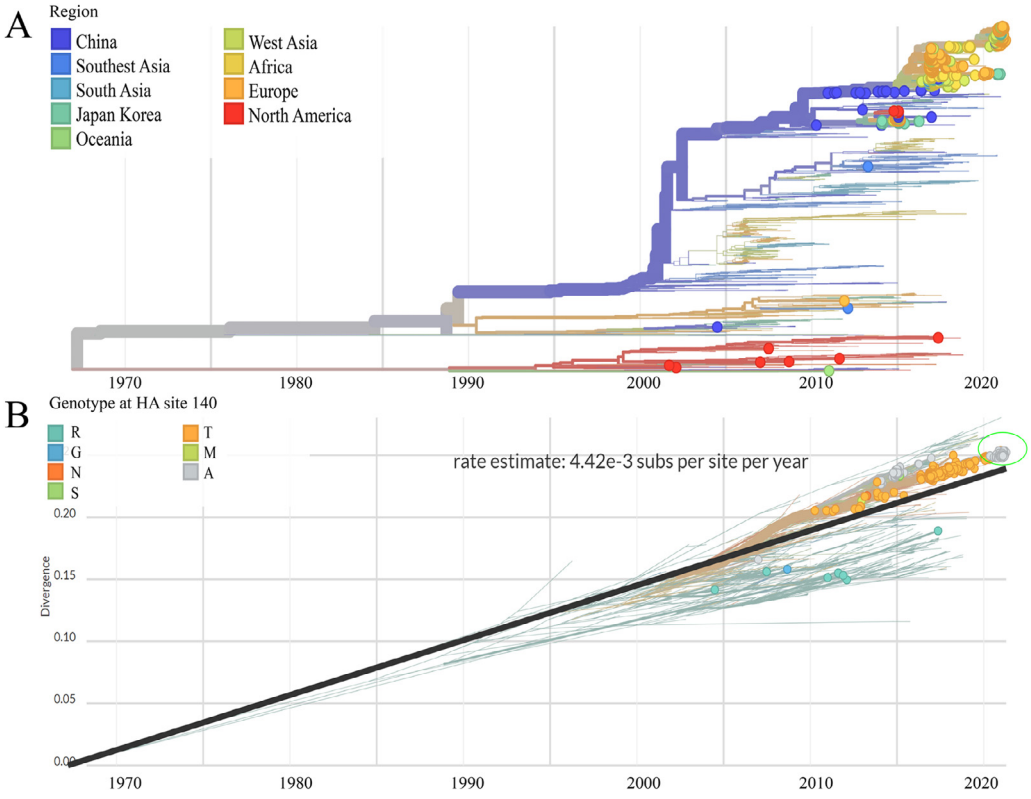

Fig. 1. Phylogenetic trees of hemagglutinin of the highly pathogenic avian influenza (HPAI) A(H5N8) viruses (A) and the diversity of residue 140 of the hemagglutinin and the diversity of residue 140. The phylogenetic analysis of H5N8 HPAIV was built using the NextStrain phylogenetic pipeline (https://nextstrain.org/).

parisons of the HA gene revealed that residue 140 had the highest diversity with an entropy of 0.818. It is to be noted that recent H5N8 isolates shared a common T140A mutation in contrast to their ancestor strains (Fig. 1B).

Africa, in particular Egypt, has been suggested as a hotspot for the global spread of the HPAI H5Nx virus.[10] Egypt, especially Lake Manzala in north Egypt, is one of the key migration spots for migratory birds crossing Africa, Europe, and Asia. Similar HPAI H5N8 virus has been reported in Mute swans (Cygnus olor) and whooper swans (Cygnus cyguns) in Netherlands and Northern China during the early winter 2020. These swans may play a key role in disseminating H5N8 viruses into domestic poultry that may pose a serious threat to public health.

In conclusion, we report the reinvasion of the HPAI H5N8 virus in domestic geese in Guangdong, China. This finding provided evidence for the rapid spreading and reassortment of the HPAI H5N8 virus in domestic poultry. The reemergence of the HPAI H5N8 virus is a serious concern for both poultry industry and human health. Hence, an enhanced surveillance of HPAI H5N8 virus in migratory birds and domestic poultry is essential to enable early warning of the potential wide dissemination of these novel H5N8 viruses.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2021.10.006.

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Jinyue Guo[1]
College of Life Science and Engineering, Foshan University, No33 Guangyun road, Shishan town, Nanhai District, Foshan, Guangdong 528231, China

Hai Yu[1]
Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai 200241, China

Congying Wang[1], Sheng Yuan
College of Life Science and Engineering, Foshan University, No33 Guangyun road, Shishan town, Nanhai District, Foshan, Guangdong 528231, China

Hailiang Sun
College of Veterinary Medicine, South China Agricultural University, Guangzhou, Guangdong 510642, China

Jing Yang, Qing Cheng
College of Life Science and Engineering, Foshan University, No33 Guangyun road, Shishan town, Nanhai District, Foshan, Guangdong 528231, China

Kaijian Luo
College of Veterinary Medicine, South China Agricultural University, Guangzhou, Guangdong 510642, China

Yong Li
College of Animal Science and Technology, Jiangxi Agricultural University, Nanchang, Jiangxi 330045, China

Zhili Li, Quan Liu, Shujian Huang, Feng Wen*
College of Life Science and Engineering, Foshan University, No33 Guangyun road, Shishan town, Nanhai District, Foshan, Guangdong 528231, China

*Corresponding author.
E-mail address: wenf@fosu.edu.cn (F. Wen)

[1] These author contributed equally to this work.

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War-torn Afghanistan - potential risk to the polio eradication efforts: A call for global concern!

Dear Editor,

In a recent article titled “Emerging polio hotspots in Pakistan: Challenges and the way forward” we reported the alarming emergence of polio hotspots in Pakistan.[1] Afghanistan, on the other hand, a polio-endemic country, is now at a potentially historic crossroads due to years of conflict and war-torn areas. Considering the political unrest in Afghanistan and the uncontrolled influx of refugees to Pakistan, the current article highlights the future consequences of polio eradication in this region.

Past tragedies have left many Afghans scarred and fearful about the country’s future. After two decades of war, more than 0.075 million Afghan military personnel and over 0.1 million civilians died, while only the United States of America (USA) spent more than $2 trillion to bring stability there. After USA troops’ withdrawal, the early fall of the Afghan government has stunned the world.[2] Although the USA trained and armed the Afghan forces with advanced technology, but they could not contain the rebels longer. Amid rising violence and security challenges in Afghanistan’s conflict-ridden regions, the World Health Organization (WHO) field reports reveal that civilians remain obvious target victims of armed conflict.[3]

Afghanistan’s health system made developments progressively over the last 17 years and increased coverage of health services. In 2018, almost 3135 health institutions were operational and accessible to > 87% population in around two hours’ drive.[4] These constructive endeavors are now at risk of downfall, while the healthcare sector is confronted with more new challenges, particularly severe budgetary constraints, which would inevitably result in devastation. International Monetary Fund (IMF) recently frozen Afghanistan’s access to reserve assets, while a few days before, they were supposed to receive almost $500 million.[5]

Keeping in view the current circumstances, Afghanistan is witnessing one of the worst health crises in the world. According to WHO, conflicts, natural catastrophes, and population displacement have left more than 3.7 million Afghan people needing emergency health assistance.[6] Dreadfully, Afghanistan’s disease surveillance and immunization programs were also not strengthened enough to detect, prevent, and control disease outbreaks at early stages. This would raise the disease burden and wreak havoc on disease management, control, and eradication efforts in this war-torn region.

There has never been a time in history when the world was so close to eliminating Wild Poliovirus (WPV). Pakistan and Afghanistan are the only countries where new WPV cases have been detected following the WHO declaration that Africa is polio-free. According to the WHO, poliovirus activity has surged dramatically, with the number of WPV cases reaching 56 in 2020, up from 29 in 2019. Meanwhile, 84 WPV cases were reported in Pakistan in 2019 (Figs. 1 & 2).[1,7,8]

Owing to this political unrest, the internal displacement crisis looms in Afghanistan, and thousands of Afghan refugees are migrating to the neighboring Pakistani areas. The geographical areas near the Pak-Afghan border were the hardest impacted by polio cases during the last two years, and appallingly, all Afghan refugees are entering Pakistan without being vaccinated. Many people have fallen between the gaps due to the enormous population inflow, particularly children, who were missed in routine immunization and did not receive supplemental immunization, which poses a severe threat to both countries’ attempts to eradicate the crippling poliovirus. Since infectious organisms do not recognize physical boundaries, unrestricted migration may transmit WPV in neighboring nations, making Pakistan and Afghanistan the world’s epicenter. According to the recent survey in 2021, In Pakistan, the immunization coverage among children age 12–23 months was Quetta (45%), Karachi (64%), Peshawar (76%), and Punjab (≥ 80%) coverage.[9]

Considering this situation, comprehensive policies and strategies are direly needed to manage the influx of refugees effectively. Primary health care, including immediate synchronized cross-border vaccination for childhood diseases, should also be a priority for refugee management activities at this time. In this manner, the possible change in disease burden may be mitigated, and any local health emergency can be remedied before it develops into public health emergency of international concern (PHEIC).

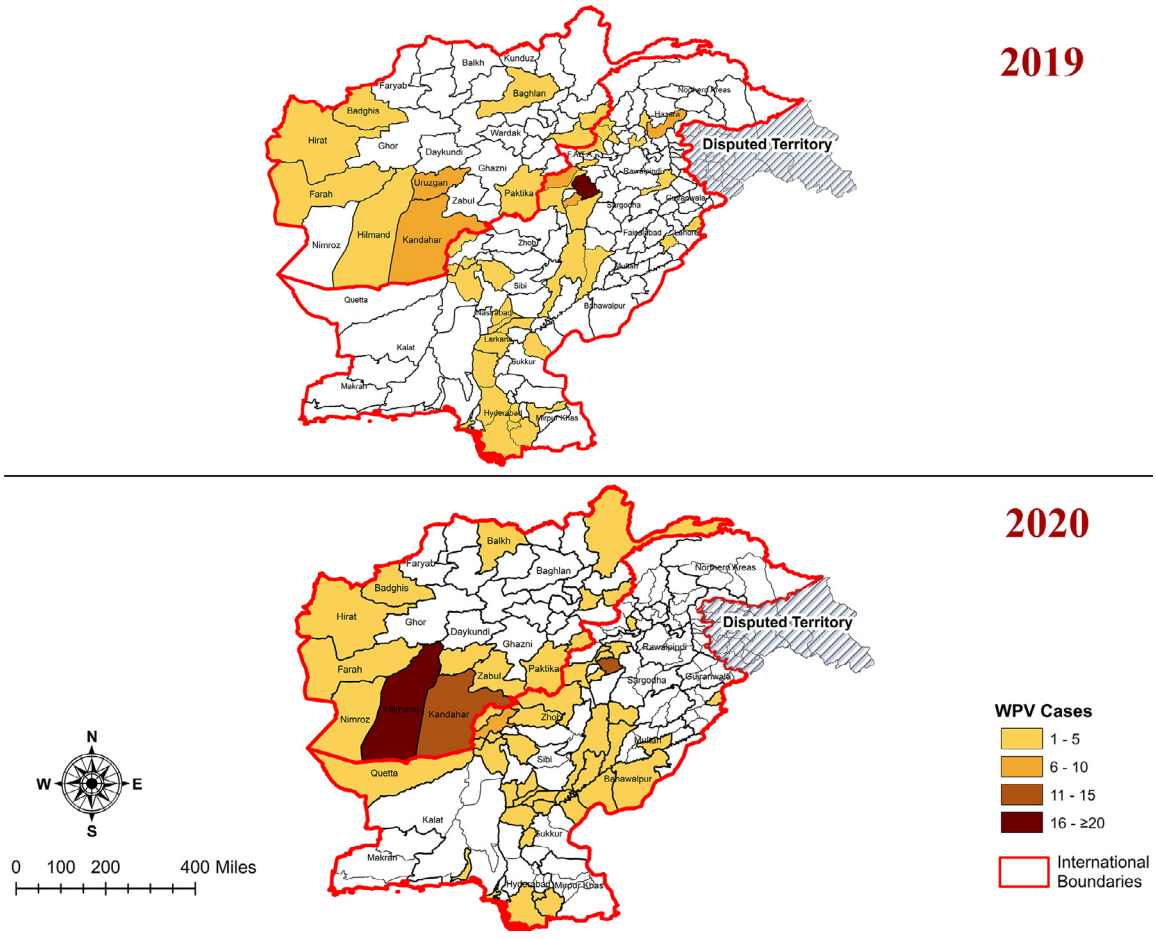

Fig. 1. This figure depicts the wild polio hotspots in Pakistan and Afghanistan. In 2020, polio cases surged alarmingly along the Pakistan-Afghan border. The massive unvaccinated influx of Afghan refugees might result in a polio outbreak in both countries.

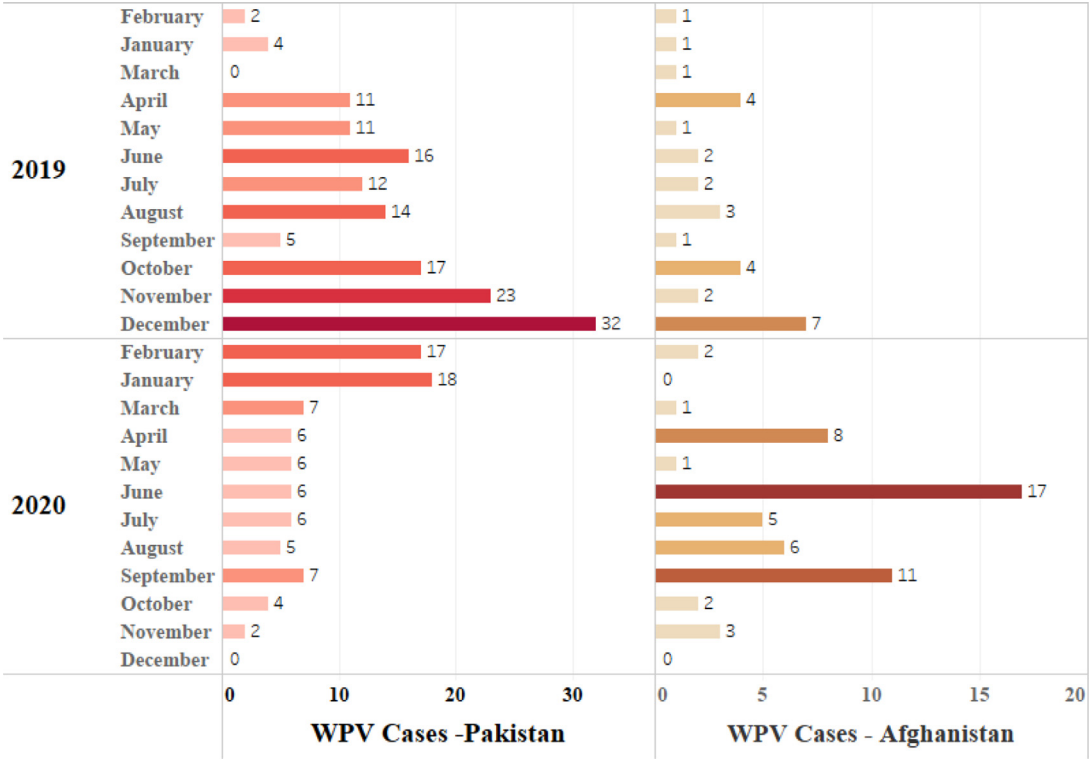

Fig. 2. Number of wild polio cases reported in each month for both years 2019 and 2020. Data trend revealed a higher number of polio cases in Afghanistan in 2020 compared to 2019, which is an alarming situation.

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Declaration of Competing Interest

We declare no conflict of interest.

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Usman Ayub Awan[1]
Department of Medical Laboratory Technology, The University of Haripur, Haripur, Khyber Pakhtunkhwa, Pakistan

Muhammad Wasif Malik[1]
Field Epidemiology & Disease Surveillance Division, National Institute of Health (NIH), Islamabad, Pakistan

Haroon Ahmed
Department of Biosciences, COMSATS University, Islamabad, Pakistan

Usman Hassan
Department of Pathology, Shaukat Khanum Memorial Cancer Hospital and Research Centre, Pakistan

Abdul Sami Khan
Technical Support Officer, Department of Health, Emergency Operation Center, Balochistan, Pakistan

Shaarif Bashir
Department of Pathology, Shaukat Khanum Memorial Cancer Hospital and Research Centre, Pakistan

Muhammad Sohail Afzal*
Department of Life Sciences, School of Science, University of Management and Technology (UMT), Lahore, Pakistan

*Corresponding author.
E-mail address: sohail.ncvi@gmail.com (M.S. Afzal)

[1] These authors contributed equally.

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Genomic analysis of maternal carriage of group B *Streptococcus* and transmission routes of neonatal sepsis

Dear Editor,

Group B *Streptococcus* (GBS) remains a major cause of invasive infection in neonates and young infants, leading to a significant mortality and morbidity. [1] We read with great interest the article by Takata et al. who reported that GBS remained to be the most common Gram-positive organism causing bacteremia in neonatal units in UK from 2005 to 2018.[2] Neonatal GBS disease, according to onset timing, can be further divided to early-onset disease (EOD; onset from birth to 6 days) and late-onset disease (LOD; onset beyond 7 days).[3] The classification is attributed to the possible different transmission modes. The pathogenesis of EOD has been well characterized and is acquired in utero or during passage through the vagina, namely vertical transmission. [4] On the other hand, the understanding on the transmission of LOD is still limited. It is believed that LOD can be acquired via two routes: (1) vertical transmission and colonization at the time of birth, with evolution into later infection and (2) horizontal acquisition from hospital environments or community. However, it is difficult to determine the transmission route of LOS by clinical investigations alone.

GBS can be classified into 10 serotypes (Ia, Ib, and II to IX) based on its capsular polysaccharide (CPS). The most common serotypes of maternal GBS colonization are VI, followed by III, V, Ia and I; however, neonatal invasive GBS infection are mainly caused by GBS Ia, Ib, and III.[5,6] In addition, previous reports showed that some women were colonized by multiple strains of GBS in the genital tract, an issue that is easily overlooked.[7,8] In studies of GBS serotype distribution, most only performed single colony testing, so that co-colonization could be missed. To further understand the mechanism of GBS colonization and transmission, we performed serial experiments including multiple colonies testing and paired GBS analysis to explore the GBS transmission routes.

During June 2014 and June 2016, we prospectively collected infants' GBS isolates from blood and/or cerebrospinal fluid (CSF). For the multiple GBS colonies testing, we picked 8 positive blood cultures from diseased neonates and another 8 positive vaginal swab cultures from pregnant women for further experiments. LIM broth (BD Diagnostics, Sparks, MD) of the positive vaginal swab culture was sub-cultured on a 5% sheep blood agar plate, which was then incubated at 37 °C for 24 h. For each sample, 130 colonies of GBS were harvested from the plate and each colony was transferred to a microcentrifuge tube for DNA extraction. Then pooled DNA from the 130 colonies were subjected to multiplex polymerase chain reaction (PCR) for molecular serotyping. For neonatal invasive isolates, the positive blood cultures were also sub-cultured onto blood agar plate with incubation overnight. DNA of 130 colonies from each positive culture were extracted and pooled for PCR testing for serotyping as mentioned above. In paired GBS analysis, we col-

Table 1
Clinical information and genomic characteristics of group B *Streptococcus* isolated from paired diseased infants and their mothers.

Pair	Brief history	GBS screen and IAP	Infant'sGBS (A)	Mother'sGBS (B)	GBS inLIM broth (C)	SNV[a](A & B)	SNV[b](B & C)
1			III/ST17	III/ST17		4	48
	GA 39 weeks via VD, female	Unknown			GBS (80.4%)		
	LOD GBS at 119 days of age(sepsis)						
2			III/ST17	III/ST17		4	12
	GA 38 weeks via VD, male	Uknown			GBS (74.8%)		
	LOD GBS at 71 days of age(sepsis)						
3			Ib/ST12	Ib/ST12		4	118
	GA 39 weeks via VD, female	Negative GBS screen			GBS (44.3%)		
	EOD GBS at 6 days of age						
	(sepsis)						
4			III/ST17	III/ST17		2	3
	GA 38 weeks via VD, male	Positive GBS screen			GBS (100%)		
	EOD GBS at 2 days of age	IAP with cefazolin > 4 h					
	(sepsis and meningitis)						
5		No screen and no IAP	III/ST17	Ia/ST23		11,812	4
	GA35 weeks via CS, female				GBS (90.3%)		
	LOD GBS at 47 days of age (sepsis)						

Abbreviations: EOD: early-onset disease; LOD: late-onset disease; GA: gestational age; GBS: group B *Streptococcus*; VD: vaginal delivery; CS: Cesarean section; IAP: intrapartum antibiotics prophylaxis; SNV: single nucleotide variations.
[a] Difference in the number of SNV between the genomes of infant's GBS (A) and mother's GBS (B).
[b] Difference in the number of SNV between the genome of mother's GBS (B) and metagenome in LIM broth (C).

lected the vaginal swabs of the matched mothers from the diseased infants for LIM broth culture at the diagnosis of neonatal GBS disease. PCR and multi-locus sequence typing (MLST) were performed on single GBS colony from paired GBS to characterize the infants' and mothers' GBS isolates. Metagenome of LIM broth from mothers collected at the diagnosis of neonatal GBS disease was sequenced to investigate mothers' vaginal microbiome, and to unveil the possible existence of multiple GBS serotypes in the vaginal microbiome in the diseased infants' mothers. Whole-genome sequencing (WGS) was performed to determine whether the infants' GBS and mothers' GBS were identical. The detailed methods of PCR, MLST, WGS and metagenome sequencing were described in the Supplementary Methods.

In the multiple colonies testing, the serotypes of maternal carriage isolates examined were four GBS VI, and the other four GBS Ia, Ib, II and III each. Among neonatal invasive GBS isolates, three were GBS Ia, two GBS Ib, one GBS III, and two GBS VI. Multiplex PCR on a total of 16 pooled DNA samples, each from multiple colonies, showed no evidence of multiple serotypes carriage in the mothers as well as infection in the infants.

Five GBS pairs were matched successfully between mothers and neonates. The clinical information and molecular characteristics of the five pairs of GBS isolates were summarized in Table 1. Among the infants, two had EOD and three LOD. Serotype III with sequence type 17 (GBS III/ST17) accounted for 4 of the 5 infants' GBS isolates. The other one was GBS Ib/ST12.

Except for pair 5, all other 4 mothers' GBS showed the same molecular characteristics as infants' GBS. Infant in pair 5 was a female late preterm twin baby born by Cesarean section at 35 weeks of gestational age, and her mother did not receive GBS screening before delivery. The infant developed GBS LOD by GBS III/ST17 at 47 days of age. However, the vaginal swab culture of her mother yielded GBS Ia/ST23. Her twin sister did not develop GBS disease.

We sequenced the genomes of all paired GBS as well as the mothers' vaginal LIM broth microbiome. First, we compared the paired single colony and microbiome from the mothers and identified few single nucleotide variations (SNVs), suggesting that in these womens' vaginal microbiome, there was a predominant GBS strain instead of mixed strains. Next, we compared the genomes of the mothers' GBS and the infants' GBS. For all subjects except pair 5, the SNV distances between the paired genomes were smaller than 5 SNVs, indicating the mothers' vaginal GBS as the source of the infection in the infants. In pair 5, such a pairwise concordance was not observed; thus, there was other environmental source responsible for the infant's infection.

The mother in pair 3 had a negative antenatal GBS screening, but the newborn still developed GBS EOD by GBS Ib/ST12 at 6 days of age. The mother in pair 4 had a positive GBS screening and received appropriate IAP with cefazolin, but the infant still developed GBS EOD with sepsis and meningitis by GBS III/ST17. Metagenome of maternal LIM broth showed that 100% of the reads belonged to GBS colonizing in the mother's vagina. The other four mothers' vaginal LIM broth microbiome showed multiple bacteria. Although LIM broth selectively enriched the growth of GBS, metagenome analysis still identified other bacterial colonization in maternal genital tract, including *Enterococcus avium, Enterococcus faecalis, Staphylococcus aureus, Staphylococcus lugdunensis* and *Streptococcus anginosus* (Supplementary Table 1).

We investigated the diseased infants' GBS isolates, mothers' GBS carriage isolates and mothers' vaginal microbiome to explore the source of infants' GBS infection and the possibility of multiple GBS carriage. Although previous studies reported that 6.6% of GBS-positive mothers may carry multiple strains,[7] there was no evidence of co-colonization found in this study. When analyzing GBS from paired mothers and infants, we found that infants' GBS, except pair 5, were identical to their mothers' GBS. In pair 5, although GBS was also cultured from the mother's vagina, the serotype and genotype of the isolate were different from the infant's GBS. After comparing the DNA of mother's GBS and vaginal microbiome, the possible existence of multiple GBS strains colonizing in the mother's vagina was excluded. Therefore, the infant's GBS in pair 5 was undoubtedly acquired through horizontal transmission from the environment.

This study identified limitation of current IAP policy. The latest GBS prevention strategy by American College of Obstetricians and Gynecologists (ACOG) in 2019 suggested that pregnant women

should receive IAP more than 4 h before delivery for a positive GBS culture, and a negative GBS culture be valid only for 5 weeks.[9] In our study, the mother in pair 3 had negative GBS culture which was obtained less than 5 weeks before delivery, but the infant still developed EOD with vertical acquisition of GBS from mother, suggesting false negative result of maternal GBS screening. The mother in pair 4 had GBS colonization in the vagina and received IAP for more than 4 h before delivery. However, the newborn developed EOD with sepsis and meningitis. Moreover, the metagenome analysis revealed that the mother's vaginal microbiome was composed of mainly GBS, indicating an extremely high GBS burden colonizing in the vagina. Indeed, previous studies have demonstrated that vaginal colonization with a high burden ($>10^5$ colony-forming units/mL) of GBS during pregnancy increases the risk of vertical transmission.[10] Therefore, IAP no later than 4 h before delivery may not be enough for mothers with such extremely high GBS colonization. Another possibility for this case was that IAP might inhibit other bacterial growth in maternal vagina and LIM broth further selectively enriched GBS growth, thus causing 100% GBS identification in LIM broth in the study.

Although only a small number of bacterial isolates were examined, the strength of this study lies in the use of different technologies, including PCR, MLST, WGS and metagenome sequencing to determine the route of GBS transmission to the diseased infants. Our study provides evidence at the bacterial strain level for both modes, the vertical and horizontal acquisition of GBS in newborn infants, and direction for future prevention.

Ethics statement

This study was approved by the Institutional Review Board of Chang Gung Memorial Hospital, Taiwan (103-2479B and 104-9360B).

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Supplementary materials

Supplementary material associated with this article can be found, in online version, at doi: XXXXX.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Supplementary materials

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Chien-Chung Lee
Division of Neonatology, Department of Pediatrics, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Taoyuan, Taiwan

Ye Feng
Sir Run Run Shaw Hospital, Institute for Translational Medicine, Zhejiang University School of Medicine, Hangzhou, People's Republic of China

Chyi-Liang Chen
Molecular Infectious Disease Research Center, Chang Gung Memorial Hospital, Taoyuan, Taiwan

Yuan-Ming Yeh
Genomic Medicine Core Laboratory, Chang Gung Memorial Hospital, Taoyuan, Taiwan

Mei-Hua Hsu
Molecular Infectious Disease Research Center, Chang Gung Memorial Hospital, Taoyuan, Taiwan

Wan-Ting Liao
Taiwan Food and Drug Administration, Ministry of Health and Welfare, Taipei, Taiwan

Ying-Li Zhou
Molecular Infectious Disease Research Center, Chang Gung Memorial Hospital, Taoyuan, Taiwan

Petrus Tang
Department of Parasitology, Graduate Institute of Biomedical Sciences, Chang Gung University College of Medicine, Taoyuan, Taiwan

Cheng-Hsun Chiu*
Molecular Infectious Disease Research Center, Chang Gung Memorial Hospital, Taoyuan, Taiwan
Division of Pediatric Infectious Diseases, Department of Pediatrics, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, No. 5, Fu-Hsin Street, Kweishan 333, Taoyuan, Taiwan

*Corresponding author at: Division of Pediatric Infectious Diseases, Department of Pediatrics, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, No. 5, Fu-Hsin Street, Kweishan 333, Taoyuan, Taiwan.
E-mail address: chchiu@adm.cgmh.org.tw (C.-H. Chiu)

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Real-time whole genome sequencing direct diagnosis of *Streptococcus pneumoniae* meningitis: A case report

Dear Editor,

As reported in this Journal recently, bacterial meningitis is a life-threatening invasive infection that could progress to mortality within 24 h in the absence of an appropriate treatment, causing annually more than 50% deaths from all-cause meningitis and leaving one in five people recovering with chronic neurological disorders.[1] Current rapid point-of-care (POC) diagnosis of community acquired bacterial meningitis is based on PCR-based multiplex assays detecting pathogen-specific sequences in the cerebrospinal fluid (CSF). [2] These assays however do not provide all the pieces of information desirable for the optimal medical management of the patient clinically diagnosed with community-acquired meningitis. Antibiotic susceptibility to finely tune the antibiotic treatment and genotyping to guide source tracing. Real-time whole genome sequencing is an alternative approach recently developed for detecting and genotyping pathogens directly from clinical samples, and to determine antibiotic susceptibility profile, in real time. [3]

Here, we reported a 69-year-old woman was admitted to the emergency department with a 38.5 °C fever which had lasted a day, repeated dizzy spells, and vomiting. Clinical examination disclosed neck stiffness, headache, and photophobia, without rash or purpura. Biological testing found an inflammatory syndrome with C-reactive protein at 350 mg/L. CSF analyses yielded hyperproteinorrhachia at 1.16 g/L and hypoglycorrhachia at 0.11 mmol/L, a leukocyte count of 4 cells/mm^3 and erythrocytes at 50 cell/mm^3. Gram staining showed Gram-positive diplococci identified as *S. pneumoniae* using a real-time multiplex PCR assay (bioFire FilmArray® Meningitis/Encephalitis panel, bioMérieux, Marcy-l'Etoile, France) at the POC laboratory.[2] The cultured *S. pneumoniae* was categorized *in vitro* susceptible to penicillin G, ceftriaxone, erythromycin, doxycycline, and chloramphenicol by Mueller-Hinton culture antibiogram after two days. The patient was treated with ceftriaxone, 6 g/day for 14 days and dexamethasone, 40 mg/day for five days. Further evaluations found hyperproteinemia at 110 g/L and a plasmatic monoclonal peak of IgG kappa quantified at 39 g/L (Capillarys, Sebia, Evry, France), which led to a subsequent diagnosis of multiple myeloma.

Alongside the bioFire FilmArray® investigation, total DNA was extracted from 200 µL of cerebrospinal fluid using the EZ1 DNA Kit and an EZ1 automaton (Qiagen, Courtaboeuf, France) following 20 min incubation with proteinase K at 56 °C and eluted in a 50 µL final volume. Next-generation sequencing was performed using a MinION device (Oxford Nanopore Technology, Oxford, UK), as previously described,[3] using 47 µL of the extracted DNA for the MinION library preparation (Appendix 1). The final library was recovered in 15 µL volume and was further diluted in 75 µL of flow cell loading mix and sequenced for 20 min on a MinION instrument. Sequencing data were analyzed in real-time using the EPI2ME online software (version 2019.11.11-2920621). In a second analysis, we assembled and mapped the generated reads using CLC Genomics Workbench software version 7.5.0 (Qiagen). Furthermore, 1 ng of DNA was used for NGS sequencing using the Illumina Nextera XT library preparation and the paired-end protocol (Illumina, San Diego, USA), as previously described,[4] on an iSeq 100 instrument (Illumina) and sequences were analyzed using the CLC Genomics Workbench software (Qiagen).[5]

A twenty-minute MinION sequencing run generated 61,150 reads, including 11,659 *S. pneumoniae* reads (Fig. 1). Assembly process generated 92.5% genome coverage and further blast analy-

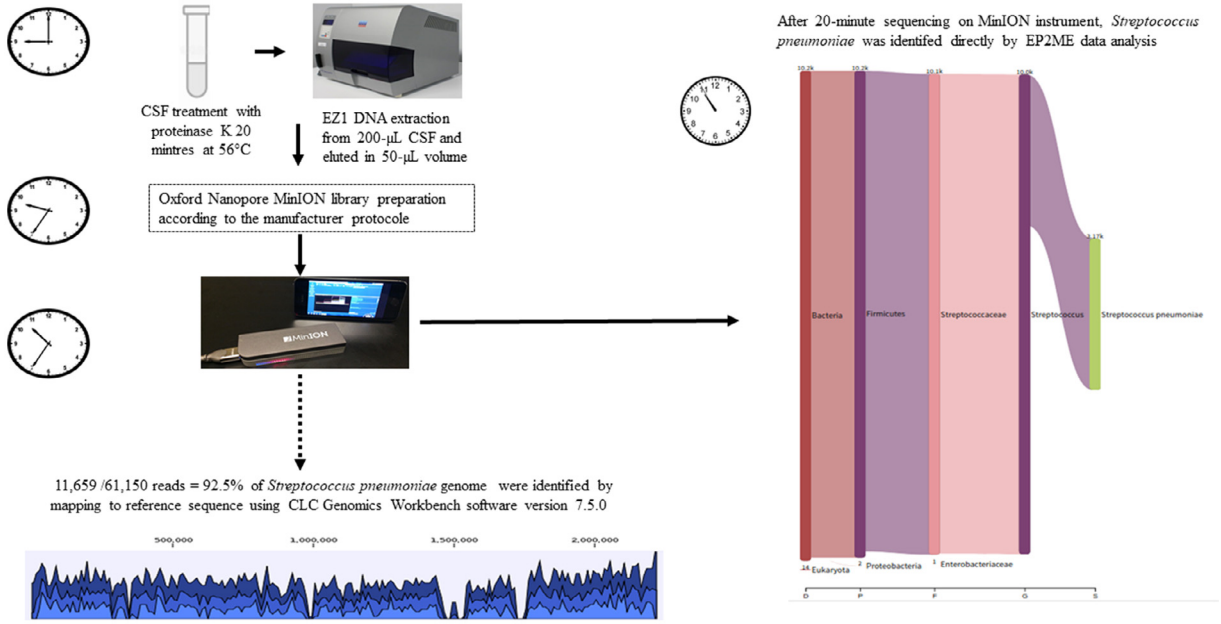

Fig. 1. . Real-time whole genome sequencing of *Streptococcus pneumoniae* (*S. pneumoniae*). Total handling time was less than two hours, including 35 min for cerebrospinal fluid (CSF) processing and EZ1 DNA extraction, 60 min for the Oxford Nanopore library preparation and 20 min for MinION library sequencing. The MinION sequencing result was directly analyzed by EP2ME software, then the generated reads were mapped to the reference genome by CLC software. In this case, the *S. pneumoniae* genome was detected after a 20 min sequencing run, and the full data analysis yielded 92.5% genome coverage directly recovered from CSF sample.

sis yielded *S. pneumoniae* with 98.97% sequence identity with the reference *S. pneumoniae* strain A6-10, a serotype 10-A (GenBank accession no. CP053210.1). The clinical strain was *in silico* susceptible to all routinely used antibiotics as determined using the ResFinder bio-tool (http://cge.cbs.dtu.dk/services/ResFinder/) (Appendix 2) (Fig. 1). MinION sequencing data were confirmed by Illumina iSeq sequencing, generating 503,192 reads. Blast comparison of the 73,597-bp longest contig identified a best match with *S. pneumoniae* strain A6-10 with 98.97% sequence similarity. A total of 338,879/503,192 (67.35%) reads mapped to the reference sequence, identifying 90% of the *S. pneumoniae* genome. Further online analysis using Multilocus Sequence Typing (MLST), revealed a perfect match with the *S. pneumoniae* reference genome, sharing common characteristics with the 128 isolates disponible in the GenBank database, including 104 isolates from European countries of which 58 were serotype 10A, all of them being susceptible to all antibiotics as confirmed by routine bacterial culture (Appendix 2).

S. pneumoniae meningitis is a life-threatening invasive infection warranting its rapid complete diagnosis.[6,7] Diagnosis methods currently available at the POC laboratory, do not provide pieces of information regarding typing and antibiotic susceptibility of the detected *S. pneumoniae*.[2] Here, we successfully applied direct near whole genome real-time sequencing diagnosis of community-acquired *Streptococcus pneumoniae* meningitis using MinION technology, providing in less than two-hour workflow including only 20 min sequencing time, the detection, identification, typing and *in-silico* antibiogram; after we developed a specific sequencing procedure as here reported (Fig. 1). The fact that this procedure is time-competitive with that of the real-time PCR multiplex assay, providing valuable additional pieces of information, makes real-time whole genome sequencing a suitable approach for the diagnosis of community-acquired meningitis at the POC laboratory.

Ethical statement

All data were generated as part of routine work at the Assistance Publique-Hôpitaux de Marseille (Marseille University hospitals), and this study is the result of routine clinical management. This study was approved by our Institute's Ethics Committee under number (2021-004). No specific clinical sampling was performed in this study.

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Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Supplementary materials

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M. Morsli
IHU Méditerranée Infection, Marseille, France
Aix-Marseille University, IRD, MEPHI, Institut Hospitalo-Universitaire Méditerranée Infection, 19-21 Boulevard Jean Moulin, Marseille 13385 Cedex 05, France

Q. Kerharo
IHU Méditerranée Infection, Marseille, France
Laboratoire de Microbiologie, Assistance Publique-Hôpitaux de Marseille, Marseille, France

S. Amrane, P. Parola
IHU Méditerranée Infection, Marseille, France
Aix-Marseille University, IRD, SSA, VITROME, Marseille, France

P.E. Fournier, M. Drancourt*
IHU Méditerranée Infection, Marseille, France
Aix-Marseille University, IRD, MEPHI, Institut Hospitalo-Universitaire Méditerranée Infection, 19-21 Boulevard Jean Moulin, Marseille 13385 Cedex 05, France
Laboratoire de Microbiologie, Assistance Publique-Hôpitaux de Marseille, Marseille, France

*Corresponding author at: Aix-Marseille University, IRD, MEPHI, Institut Hospitalo-Universitaire Méditerranée Infection, 19-21 Boulevard Jean Moulin, Marseille 13385 Cedex 05, France.
E-mail address: michel.drancourt@univ-amu.fr (M. Drancourt)

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Multicentric evaluation of a 3D-printed simulator for COVID- 19 nasopharyngeal swab collection in testing centers

Dear Editor,

In 2021, reliable knowledge of the COVID status of individuals became a global public health issue, from an epidemiological point of view - to contain the pandemic - but also from an economical one, as repeated testings represent a significant financial burden. COVID-19 diagnosis replies on RT-qPCR from nasopharyngeal swab (NPS) sample, which is considered as the gold standard for testing both asymptomatic and symptomatic persons, especially as recent studies described up to 46% of false positivity of antigen tests.[1–3] If NPS were performed by medical staff at the beginning of the pandemic, they were rapidly passed on to other providers, sometime without any specific training or past experience. This enabled widescale upscaling with the retrieval of larger number of samples, but the absence of specific training for samplers raised the issue of sample quality and patients' acceptance and comfort during swabbing. Incorrectly performed, NPS can result in false negatives, therefore missing positive patients and detrimentally impacting on the pandemic,[4] Furthermore, several oto-rhino-laryngological injuries have been reported, including osteo-meningeal breaches with cerebrospinal fluid leaks.[5] Thus, poorly implemented swabbing and insufficient anatomical knowledge could have prejudicial consequences for patients and public health.[6] Therefore, all studies and initiatives aimed at improving the efficiency of tests, making them more fluid and limiting the number of false negative/positive cases are more than welcome. For instance, Li et al. described a very interesting Integrated Mobile Container PCR Laboratory (IMCPL), aimed at reducing unnecessary rapid antigen testing.[7] Their IMPCL is a "standard PCR laboratory established inside a walk-in container and can be quickly set up at any convenient locations to carry out the large-scale diagnostic testing". Similarly, our own initiative in this field is a nasopharyngeal sample collection simulator (NP-SC), which we designed using a 3D printer, to optimize sample quality and decrease procedure-related discomfort. It was created to train professional samplers to safely perform NPS with the least discomfort for patients, aiming at an optimal swabbing of the area with the highest viral concentration. The aim of this study was to solicit opinions on our simulator from professional samplers from different in testing centers, regarding the realism and utility of this simulator for learning and perfecting NPS.

This nasopharyngeal swab collection (NP-SC) simulator was designed by BONE 3D (Paris, France). A presentation video is freely available online: https://landing.bone3d.com/np-swab-simulator, as well as comprehensive 3D files and full instructions for manufacturing models.[8] A swabbing target on the posterior wall of the nasopharynx colors the swab tip if correctly inserted. The simulators were distributed to different laboratories (one per center). Experts professionals samplers from each laboratory, who had carried out >50 NPS, tested the device and provided a written evaluation through a questionnaire, divided into three parts:

- Their swabbing experience and their opinion regarding the utility of the simulator.
- The simulator modeling, relevance and use.
- Experience acquired with the use of such simulator.

All data collected were processed anonymously. The questionnaire included a form in which participants confirmed that they had no objection to data collection. The study was registered with the French National Commission for Information Technology and Civil Liberties (CNIL No. 2221408).

This multicentric study was performed from October to December 2020 in 40 certified COVID-19-sampling laboratories. The NP-SC simulator was widely appreciated by its users, with a good overall rating of 7.9/10: 95% of users (38/40) found it easy to use and 85% (n = 34/40) thought it was realistic. Testers considered that this simulator had genuine utility for educational purposes (87.5%, 35/40) and 73.7% (28/38) thought that use of this simulator could improve their sampling technique. Additionally, 95% (38/40) reported that the device made the nasopharyngeal ducts anatomy easier to understand and 82% (32/39) estimated that the simulator, providing an in-real time visualization of the swab progression through the nasal cavity fossa, enabled a better understanding of how it was possible to go "off track", therefore improving patients' safety during sampling. The coloring of the swab tip upon correct insertion provided users with in-real-time visual feedback regarding their sampling quality. Therefore, 52.5% (21/40) thought the

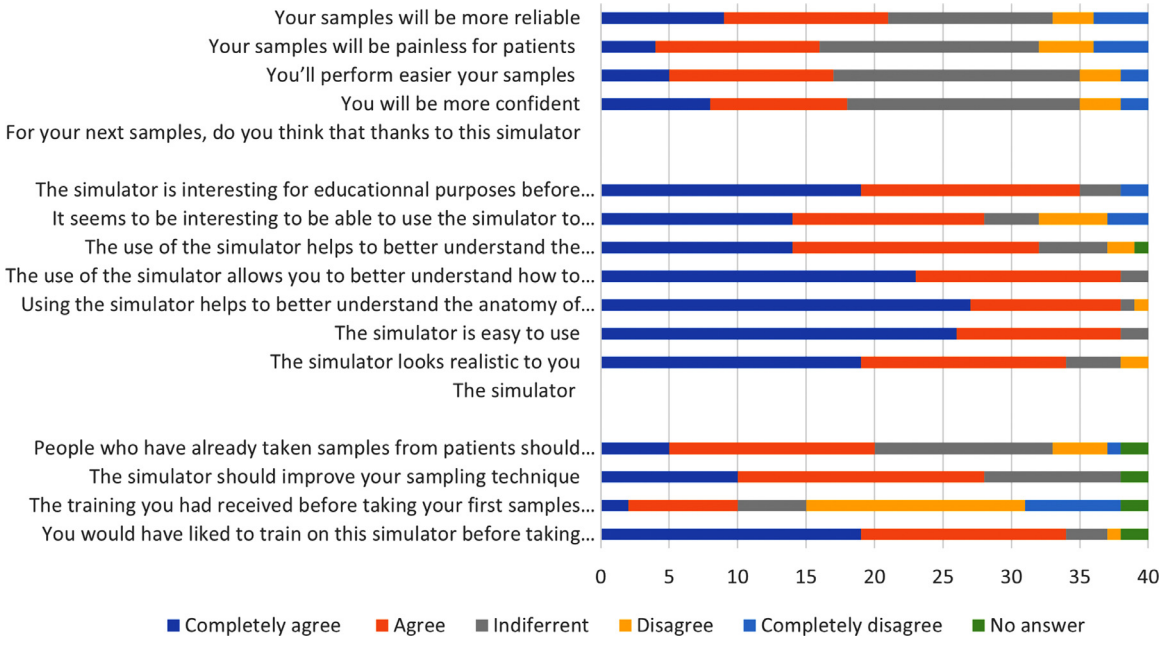

Fig. 1. Assessment of simulator realism and utility in the total study population (n = 40)

simulator improved their sampling technique and that their samples would now be more reliable (Fig. 1). Despite having previous swabbing experience (> 50 NPS), nearly half of the testers (18/40) felt more confident after training with the simulator.

With increasing numbers of COVID-19 cases worldwide, NPS sampling has now become a routine procedure, with more than 1 million RT-qPCR tests carried out monthly in the US. Correct sampling is essential to limit false-negative cases, mostly related to poorly-performed NPS. In addition to being a financial burden, NPS are also not risk-free, especially for unexperienced samplers. Francesca et al.,[9] highlighted the need for NPS-training on mannequins, resulting in improved swabbing technique and increased comfort for patients. Therefore, we developed a 3D-printed nasopharyngeal sample simulator, to train professional samplers perform NPS under the safest possible conditions, with the least discomfort for patients aiming at an optimal swabbing of the area with the highest viral concentration. The simulator, available free-of-charge online, is made of a single rigid material, which enables highly-realistic and easy printing at low costs by NPS-performers all around the world. Experts professional samplers, performing dozens of tests day, widely acclaimed the device, judging it easy-to use (95%), realistic (85%) and anatomically-accurate (95%). It also had genuine utility for educational purposes, making the anatomy of the nasopharyngeal ducts easier to understand for novice samplers and illustrates the possibilities of going “off track”, which is known to be at risk of causing pain/discomfort and may cause various injuries.[4]

In a similar way as several others initiatives (e.g., the IMCPL) our simulator aims at improving the cost-effectiveness ratio of tests, by reducing NPS-related morbidity as well as the number of false positives/negatives cases through improved practice. Judged realistic and easy-to-use by testers, this simulator, available online for free, can be manufactured on a non-professional 3D printer and appears as a very promising educational tool, especially for novice samplers.

Declaration of Competing Interest

None.

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Lise Lecointre*
Obstetrics and Gynecology Department, Strasbourg University Hospital, 1 avenue Molière, Strasbourg 67200, France
I-Cube UMR 7357, Engineering, Information Processing and Image Sciences Lab, Strasbourg University, Strasbourg, France
IHU-Strasbourg (Institut Hospitalo-Universitaire), Strasbourg, France

Aïna Venkatasamy
IHU-Strasbourg (Institut Hospitalo-Universitaire), Strasbourg, France
Streinth Lab (Stress Response and Innovative Therapies), Inserm UMR_S 1113 IRFAC, Interface Recherche Fondamental et Appliquée à la Cancérologie, 3 avenue Molière, Strasbourg, France

Mégane Wehr, Antoine Koch, Axel Sananes
Obstetrics and Gynecology Department, Strasbourg University Hospital, 1 avenue Molière, Strasbourg 67200, France

Christian Debry
Streinth Lab (Stress Response and Innovative Therapies), Inserm UMR_S 1113 IRFAC, Interface Recherche Fondamental et Appliquée à la Cancérologie, 3 avenue Molière, Strasbourg, France
ENT Department, Strasbourg University Hospital, Strasbourg, France

Massimo Lodi
Obstetrics and Gynecology Department, Strasbourg University Hospital, 1 avenue Molière, Strasbourg 67200, France

Nicolas Sananes
Obstetrics and Gynecology Department, Strasbourg University Hospital, 1 avenue Molière, Strasbourg 67200, France
ENT Department, Strasbourg University Hospital, Strasbourg, France
INSERM UMR-S 1121 ‘Biomaterials and Bioengineering’, Strasbourg University, Strasbourg, France

*Corresponding author at: Obstetrics and Gynecology Department, Strasbourg University Hospital, 1 avenue Molière, Strasbourg 67200, France.
E-mail address: lise.lecointre@chru-strasbourg.fr (L. Lecointre)

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Self-collected mid-nasal swabs and saliva specimens, compared with nasopharyngeal swabs, for SARS-CoV-2 detection in mild COVID-19 patients

Dear Editor,

The use of self-collected specimens for the screening of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has gained interest as they may facilitate massive screening campaigns. Various authors have reported that mid-nasal swabs[1,2] and saliva[3–6] are reliable specimens, alternative to nasopharyngeal

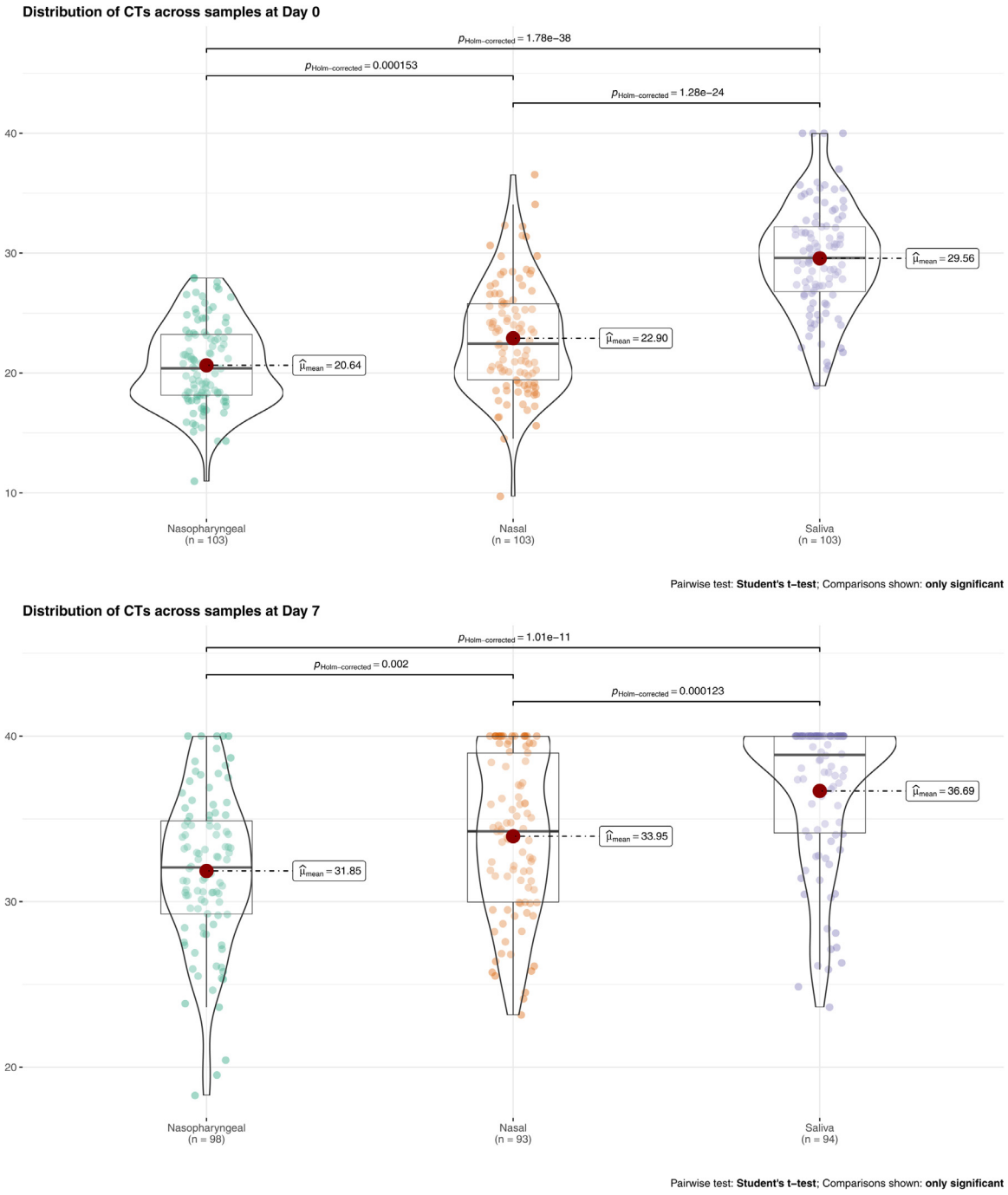

Fig. 1. Distribution of RT-qPCR cycle threshold (Ct) of nasopharyngeal swabs, nasal swabs, and saliva specimens collected at baseline (A) and day 7 (B). Cts correspond to the viral RNA-dependent RNA polymerase (RdRP) gene.

swabs, to detect SARS-CoV-2 infections by RT-qPCR, irrespective of the age group tested.[7] Despite drawing consistent conclusions, studies reported heterogeneous results regarding the performance of each type of sample, particularly sensitivity, which strongly depends on the viral load distribution of the investigated population and sample collection protocols. In the case of saliva, discrepancies regarding sensitivity might be even higher due to optimized protocols for RNA extraction adapted to the rheological properties of saliva.[6,8] Therefore, there is a need for better characterizing-also from a quantitative perspective-the performance of self-collected specimens before using them as an alternative to nasopharyngeal swabs for SARS-CoV-2 screening.

In the context of a randomized clinical trial targeting mild COVID-19 patients (NCT04621123), we enrolled 130 adults in a substudy to directly compare self-collected mid-nasal swabs and saliva specimens for SARS-CoV-2 detection by RT-qPCR, using nasopharyngeal swabs collected by the study nurses as a reference. Included patients had a mean age of 59 (SD 8.5) and a median of 4 days (95% IC 3–5) from symptoms onset; 43.2% were females. Patients received written instructions to self-collect a mid-nasal swab from both nostrils by introducing the swab 2,3 cm and rotating during 5 s, and 1 mL of saliva by spitting inside the funnel of a collection device (DANASALIVA^{TM} sample collection kit). Participants were advised to avoid eating, drinking, smoking, and

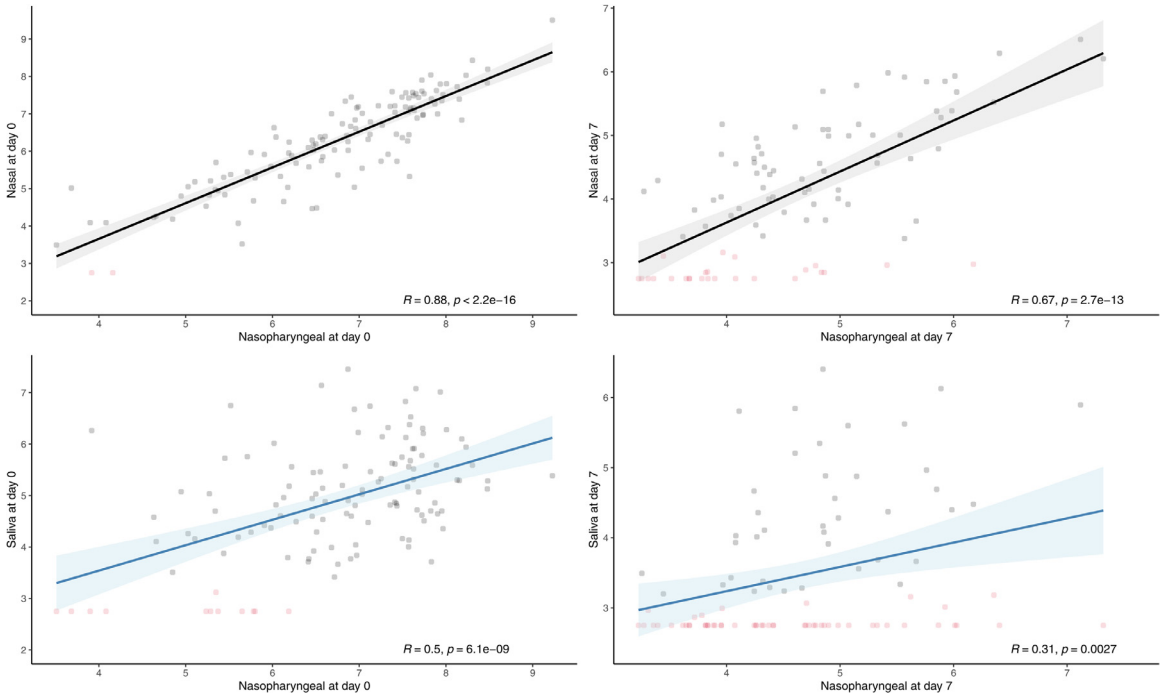

Fig. 2. Correlation of the viral load (VL) of nasopharyngeal swabs with that of nasal swabs and saliva specimens at baseline (A) and day 7 (B). Viral loads were estimated using a calibration line obtained with serial dilutions of SARS-CoV-2 control RNA, run in parallel to a set of samples covering all thermal cycles used in the analysis. Negative samples (in red) were assigned to a Ct of 40 (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

brushing their teeth within 30 min prior to sample collection. Self-collection was done in the presence of a study nurse, although they did not intervene during the collection process. Next, the study nurse collected a nasopharyngeal swab from both nostrils. Swab specimens were placed into sterile tubes containing viral transport media (DeltaSwab Virus). Saliva was mixed with 1 mL of saliva preservation solution in the collection device, following manufacturer's instructions. No additional pre-treatment step potentially increasing saliva sensitivity[6,8] was used before RNA extraction. All three specimens were transported to the Microbiology laboratory of *Hospital Germans Trias i Pujol* and stored at 2–8 °C for up to 24 h before RT-qPCR. Paired samples were collected at baseline and 7 days after enrollment.

RNA was extracted using the STAR Mag reagent (Seegen) for the Microlab Starlet IV or Nimbus platforms (Hamilton life Science Robotics, USA), according to the manufacturer's instructions. PCR amplification was conducted according to the recommendations of the 2019-nCoV RT-qPCR Diagnostic Panel of the Centers for Disease Control and Prevention (CDC) using the Allplex™ 2019-nCoV assay (Seegene, South Korea) on the CFX96 (Bio-Rad, USA) according to manufacturer's instruction.

The three paired samples were successfully obtained in 129 of 130 patients, with 120 (92.3%) showing a positive nasopharyngeal swab at baseline. Compared to nasopharyngeal swabs, self-collected mid-nasal swabs and saliva samples showed a sensitivity of 99.2% (119/120) and 90.0% (108/120), respectively. This result is in line with a recently published head-to-head comparison, which included 38 positive COVID-19 cases.[8] Of the nine participants with nasopharyngeal swabs testing negative, two had a positive saliva specimen and one a positive nasal swab. Given the successful internal PCR controls and the high cycle threshold (Ct) values (all above 30; days from symptom onset ranging from 4 to 6), we presume these patients were approaching the recovered state, with viral loads close to negativity.

In addition to the qualitative and semi-quantitative analyses conducted in previous works, we estimated the viral load of each specimen and correlated the values observed in self-collected samples with those of nasopharyngeal swabs. The RT-qPCR Ct of positive specimens was, in mean (SD), 20.64 (3.43) for nasopharyngeal swabs, 22.90 (4.60) for mid-nasal swabs, and 29.56 (4.50) for saliva specimens (Fig. 1A). The Pearson lineal correlation between viral loads obtained from nasopharyngeal swabs and self-collected specimens revealed a strong correlation for mid-nasal swabs ($R = 0.88$; $p < 0.001$) and a fair correlation for saliva specimens ($R = 0.50$; $p < 0.001$) (Fig. 2A).

Of the 120 nasopharyngeal swabs obtained on day 7 (i.e., a mean of 11 days [range 10–12] from symptom onset), 28 tested negative and 92 positive. Mean Ct values were 31.85 (4.63), 33.95 (4.75), and 36.69 (4.42) for positive nasopharyngeal, mid-nasal and saliva specimens, respectively (Fig. 1B). As expected, the mean viral load was lower at day 7 than at baseline ($p < 0.001$). Compared to nasopharyngeal swabs, self-collected mid-nasal swabs and saliva specimens showed a sensitivity of 72.8% (67/92) and 42.4% (39/92), respectively, suggesting poorer performance at low viral loads. Of note, most negative self-collected samples with a positive paired nasopharyngeal swab yielded Ct values above 30 (92% and 52% of the nasal and saliva specimens, respectively). The viral load correlation with nasopharyngeal swab was poor for saliva specimens ($R = 0.3$; $p = 0.003$) and moderate for mid-nasal swabs ($R = 0.67$; $p < 0.001$) (Fig. 2B).

In summary, our findings show that self-collected mid-nasal swabs have better performance than saliva for detecting SARS-CoV-2 when compared with the gold-standard nasopharyngeal swabs. Of note, the sensitivity of saliva was remarkably high in samples with higher viral load, despite not using any of the RNA extraction protocols adapted to the rheological properties of this sample. Considering that respiratory specimens with Ct above 33,34 are unlikely to be contagious,[9] our finding indicates that saliva would be

sensitive enough to identify individuals at risk of transmission. Furthermore, the enhanced sensitivity achieved with adapted protocols for RNA extraction from saliva suggests that this might be the sample of choice for systematic screenings in settings in which a specific laboratory pathway can be implemented (e.g., school children).

Taken together, the existing literature and the results provided in our analysis encourage the use of self-collected specimens (midnasal when possible and saliva in vulnerable populations such as children) for massive screenings of SARS-CoV-2.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics

The study was conducted according to the Helsinki Declaration of the World Medical Association. The study protocol was approved by the Ethics Committee at Hospital Germans Trias i Pujol (number PI 20–313) and the institutional review boards of participating centers. All patients provided written informed consent before enrolling the study, which was supervised by an independent data and safety monitoring board.

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Andrea Alemany[1]
Fight AIDS and Infectious Diseases Foundation, Badalona, Spain
Hospital Universitari Germans Trias i Pujol, Barcelona Institute for Global Health, Rosselló, 132, Badalona 08036, Spain

Pere Millat-Martinez[1]
ISGlobal, Hospital Clínic, Universitat de Barcelona, Barcelona, Spain

Dan Ouchi, Marc Corbacho-Monné
Fight AIDS and Infectious Diseases Foundation, Badalona, Spain

Antoni E. Bordoy, Cristina Esteban, Águeda Hernández, Cristina Casañ, Victoria Gonzalez
Hospital Universitari Germans Trias i Pujol, Barcelona Institute for Global Health, Rosselló, 132, Badalona 08036, Spain

Gèlia Costes, Mar Capdevila-Jáuregui, Pamela Torrano-Soler, Alba San José
Fight AIDS and Infectious Diseases Foundation, Badalona, Spain

Jordi Ara
Hospital Universitari Germans Trias i Pujol, Barcelona Institute for Global Health, Rosselló, 132, Badalona 08036, Spain
Gerència Territorial Metropolitana Nord, Institut Català de la Salut, Barcelona, Spain

Núria Prat
Gerència Territorial Metropolitana Nord, Institut Català de la Salut, Barcelona, Spain

Bonaventura Clotet
Fight AIDS and Infectious Diseases Foundation, Badalona, Spain
Hospital Universitari Germans Trias i Pujol, Barcelona Institute for Global Health, Rosselló, 132, Badalona 08036, Spain
Universitat de Vic-Universitat Central de Catalunya (UVIC-UCC), Vic, Spain

Quique Bassat
ISGlobal, Hospital Clínic, Universitat de Barcelona, Barcelona, Spain
Centro de Investigação em Saúde de Manhiça (CISM), Maputo, Mozambique
ICREA, Pg. Lluís Companys 23, Barcelona 08010, Spain
Pediatrics Department, Hospital Sant Joan de Déu (University of Barcelona), Barcelona, Spain
Consorcio de Investigación Biomédica en Red de Epidemiología y Salud Pública (CIBERESP), Madrid, Spain

Montserrat Gimenez
Hospital Universitari Germans Trias i Pujol, Barcelona Institute for Global Health, Rosselló, 132, Badalona 08036, Spain

Ignacio Blanco
Hospital Universitari Germans Trias i Pujol, Barcelona Institute for Global Health, Rosselló, 132, Badalona 08036, Spain
Gerència Territorial Metropolitana Nord, Institut Català de la Salut, Barcelona, Spain

Bàrbara Baro*
ISGlobal, Hospital Clínic, Universitat de Barcelona, Barcelona, Spain

Oriol Mitjà
Fight AIDS and Infectious Diseases Foundation, Badalona, Spain
Hospital Universitari Germans Trias i Pujol, Barcelona Institute for Global Health, Rosselló, 132, Badalona 08036, Spain
Lihir Medical Centre, International SOS Clinic, Lihir Island, Papua New Guinea

*Corresponding author.
E-mail address: barbara.baro@isglobal.org (B. Baro)

[1] These authors contributed equally to this work.

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Letter in response to article in journal of infection: "The microbiology of chronic osteomyelitis: Changes over ten years"

Dear Editor,

We have read with interest the paper of Dudareva et al. about the microbiological etiology of chronic osteomyelitis[1]. They report a lowered rate of methicillin-resistant *Staphylococcus aureus* (MRSA) infections in a cohort between 2013 and 2017 compared to 2001–2004 with a proportion of infections caused by multi-drug resistant bacteria of 15.2% and 17.2%.

Fracture-related infection (FRI) has been defined as a distinct category of osteomyelitis a few years ago. Hereby, FRIs can be differentiated as early (< 2 weeks), delayed (3–10 weeks) and late (> 10 weeks) depending on the biofilm maturation[2]. Treatment strategies are often extrapolated from periprosthetic joint infection (PJI)[3] although, in contrast to PJI, detailed analysis of pathogens is missing for FRI.

Therefore, we have performed a retrospective study to identify the microbial epidemiology of fracture-related infections and to determine whether isolated pathogens in FRI patients differ between early, delayed and late onset of infection.

The study was approved by the institutional ethics committee of the University Medical Center Regensburg and included FRI patients treated in a level 1 trauma center in Germany between January 1, 2013 to December 31, 2020. FRI was confirmed by the consensus diagnostic criteria[4]. Patients were classified regarding the onset of infection and grouped as early (0–2 weeks), delayed (3–10 weeks) and late (>10 weeks)[2]. Bacteria were identified from deep tissue samples obtained intraoperatively by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF MS) using a Microflex LT mass spectrometer and BioTyper software (Bruker Daltonik, Bremen, Germany) after 14 days of incubation. FRI were classified as difficult-to-treat (DTT) when at least one of the causing microorganisms was resistant to biofilm-active antibiotics including rifampicin-resistant staphylococci, enterococci, fluoroquinolone-resistant gram-negative bacteria and fungi.

Descriptive and statistical data analysis was performed using the IBM SPSS Statistics software (version 24.0, IBM Corp, Armonk, USA). Chi-square test was used for comparison of categorical variables. Significance was set at $p < 0.05$.

The final cohort comprised 117 patients (73% male, 27% female, mean age 55.5 ± 16.8 years). Patients had comorbidities with a mean Charlson Comorbidity Index (CCI) of 1 (range 0–6) and a mean ASA score of 2 (range 1–4). FRI mainly occurred at the tibia (39.3%) followed by infections of the ankle (18.8%) and femur (14.5%). The cohort was grouped into 19 patients (16.2%) with early onset of infection, 60 patients (51.3%) with delayed onset of infection and 38 patients (32.5%) with late onset of infection. The subgroups did not differ significant in gender ($p = 0.8$), age ($p = 0.738$), ASA score ($p = 0.929$), CCI ($p = 0.590$), BMI ($p = 0.885$) or fracture site ($p = 0.301$).

Overall, in 11 cases (9.4%) the infection was culture-negative, whereby nine cases occurred in the delayed onset group and two in the late onset group. Infection was polymicrobial in ten cases (8.6%). In 12 cases (10.3%) at least one of the causing microorganisms was classified as difficult-to-treat, defined as resistant to biofilm-active antibiotics. A resistance to a vancomycin / meropenem combination treatment was present in 2 cases (1.7%).

Methicillin-sensitive *Staphylococcus aureus* was the most frequently detected pathogen (39.7%), followed by *Staphylococcus epidermidis* (17.2%) and gram-negative bacteria (16.4%) (Table 1). In one case *Staphylococcus aureus* was methicillin-resistant (0.9%). In patients with delayed onset of infection, more gram-negative bacteria than *Staphylococcus epidermidis* were prevalent (23.2% vs. 16.1%). A chi-square test was used to compare pathogen distribution between the subgroups, resulting in no statistically significant difference ($\chi^2 = 7.022$, $p = 0.99$, $\varphi = 0.174$).

The reported number of culture negative infections is comparable with other previously published rates of 9–10%[5] [6]. In our cohort, the amount of polymicrobial infections was relatively low. In the literature, the rate of polymicrobial infection has been reported as 14.3% in infected nonunion[7]. Meanwhile, higher rates of polymicrobial infections in FRI have been described comparing different sampling protocols (25% and 36%, respectively)[8]. This emphasizes the importance that laboratory protocols and diagnostic criteria become standardized to ensure comparability between studies.

Staphylococcus aureus was the most frequently detected pathogen in total, as well as in each subgroup, which is in line with other findings[7] [9]. The onset of infection symptoms has been associated with specific pathogens, however strong evidence is lacking[9] [10]. Here, no statistically significant difference in pathogen distribution between the subgroups could be found.

Percentage of FRIs caused by *S.aureus* were higher compared to the study by Dudareva and colleagues, whereby the rate of methicillin-resistant *S.aureus* was lower (0.9% vs. 4.3% in the cohort 2013–2017). In addition, resistance to a vancomycin / meropenem combination treatment was less (1.7% vs. 2.2% in the cohort 2013–2017). Finally, Dudareva et al. reported a proportion of multi-drug resistant pathogens of 15.2% and 17.2%[1]. Here, 10.3% of the infections were classified as difficult-to-treat.

This study has some limitations. First, data analysis of only one orthopedic center may lead to a local epidemiological bias. Further, the retrospective file analysis did not consistently allow identification of antibiotic pretreatment and its effect on the detection of infection-causing pathogens. Also, subgroup analysis regarding the

Table 1
Isolated microorganisms.

Pathogen	All (n = 116)	Early (n = 22)	Delayed (n = 56)	Late (n = 38)
Staphylococcus aureus (MSSA)	46 (39.7%)	9 (40.9%)	22 (39.3%)	15 (39.51%)
Staphylococcus aureus (MRSA)	1 (0.9%)			1 (2.6%)
Staphylococcus epidermidis	20 (17.2%)	4 (18.2%)	9 (16.1%)	7 (18.4%)
Other *Staphylococcus* species	11 (9.5%)	3 (13.6%)	4 (7.1%)	4 (10.5%)
Streptococcus species	7 (6.0%)	1 (4.6%)	3 (5.4%)	3 (7.9%)
Enterococcus species	6 (5.2%)	2 (9.0%)	3 (5.4%)	1 (2.6%)
Gram-negative bacteria	19 (16.4%)	1 (4.6%)	13 (23.2%)	5 (13.2%)
Other	6 (5.2%)	2 (9.0%)	2 (3.6%)	2 (5.3%)

relevance of distinct anatomical localization would have been underpowered due to the low numbers of participants.

In conclusion, microbiological pattern for the causative microorganism between early, delayed, and late FRI are comparable. Further, methicillin-resistant *S. aureus* seems not to play a crucial role in FRI while difficult to treat pathogens should be considered a relevant clinical problem in the treatment of FRI. Therefore, establishment of alternative treatment strategies is warranted.

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Nike Walter, Susanne Baertl, Ulrike Engelstaedter
Department for Trauma Surgery, University Medical Center Regensburg, Franz-Josef-Strauß-Allee 11, 93053 Regensburg, Germany

Martin Ehrenschwender
Institute of Clinical Microbiology and Hygiene, University Medical Center Regensburg, Franz-Josef-Strauß-Allee 11, 93053 Regensburg, Germany

Florian Hitzenbichler
Department of Infection Prevention and Infectious Diseases, University Medical Center Regensburg, Franz-Josef-Strauß-Allee 11, 93053 Regensburg, Germany

Volker Alt, Markus Rupp*
Department for Trauma Surgery, University Medical Center Regensburg, Franz-Josef-Strauß-Allee 11, 93053 Regensburg, Germany

*Corresponding author.
E-mail address: markus.rupp@ukr.de (M. Rupp)

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The high expression of SARS-CoV-2 cell receptors might lead to higher COVID-19 infection rates in cancer patients[1]

Dear Editor,

We read with interest the recently published research in *Journal of Infection* by Dr. Cai et al., who observed the expression levels of SARS-CoV-2 receptor *ACE2* and host cell protease *TMPRSS2* in pan-cancer. Dr. Cai et al. suggested that the high expression of *ACE2* and *TMPRSS2* in cancer patients would increase the risk of the receptor binding to SARS-CoV-2, and Uterine corpus endometrial carcinoma (UCEC) patients might be the most susceptible to COVID-19[1]. However, the distribution of *ACE2* expression in human organs and tissues is not completely related to the organ tropism of SARS-COV-2, especially in the whole respiratory tract and a variety of immune cells, the expression of *ACE2* is negative.[2–4] Recent evidence highlights the possible presence of receptors other than *ACE2* that mediates virus entry into host cells in different tissues, including membrane proteins *LDLRAD3, TMEM30A, CLEC4G* and *AXL*, and lysosomal protein *TMEM106B*[4, 5]. In addition, *ACE2*-dependent co-receptor *NRP1* was also ignored by Dr. Cai et al.[1, 4] These might be potential reasons why Dr. Cai et al. 's results were inconsistent with previous observational studies.[6]

In this study, we investigated the impact of all known potential SARS-COV-2 receptors in the four clinically observed tumor types most susceptible to COVID-19, including lung cancer, esophageal carcinoma, B-cell lymphoma and leukemia.[6] *NRP1*, the *ACE2*-dependent co-receptor, and *TMEM106B*, a proviral host factor for SARS-CoV-2 were differentially expressed in all the four tu-

[1] These authors contributed equally to this work.

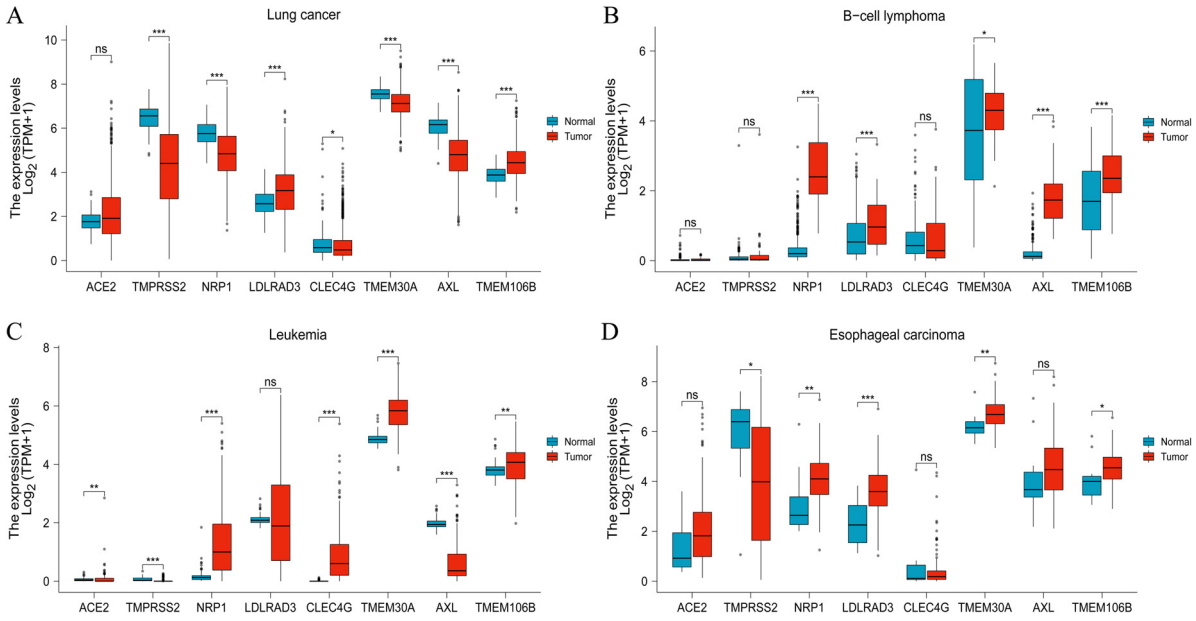

Fig. 1. The expression levels of all known SARS-COV-2 receptors in four types of tumor patients that might more likely be infected by COVID-19. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (A) Lung cancer; (B) B-cell lymphoma; (C) Leukemia; (D) Esophageal carcinoma.

mors.[4, 5] Among the three potential SARS-COV-2 receptors identified by Zhu et al, *LDLRAD3* was significantly up-regulated in lung cancer, esophageal carcinoma and B-cell lymphoma, *CLEC4G* was significantly up-regulated in leukemia, and *TMEM30A* was significantly up-regulated in esophageal carcinoma, B-cell lymphoma and leukemia.[4] Interestingly, whether in normal tissues or tumor tissues, the level of *ACE2* expression was quite low. We believed that the SARS-COV-2 receptor that made cancer patients more susceptible to infection was unlikely to be *ACE2*.

In summary, we explained the potential reasons why patients with four malignant tumors were more susceptible to COVID-19, namely the upregulation of SARS-COV-2 receptors in tumor tissues made individuals more likely to be infected by SARS-COV-2. We suggested *NRP1, TMEM106B, LDLRAD3, TMEM30A* and *CLEC4G* as a potential genetic shared genes for tumors and COVID-19, but *ACE2* might not be included (Fig. 1).

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Shizheng Qiu*
School of Life Science and Technology, Harbin Institute of Technology, N o. 2, Yikuang Street, Harbin, Nangang District 150001, China

Yu Zhang
Department of Neurosurgery, Heilongjiang Province Land Reclamation Headquarters General Hospital, China

Donghua Wang
Department of General Surgery, Heilongjiang Province Land Reclamation Headquarters General Hospital, China

Yang Hu*
School of Life Science and Technology, Harbin Institute of Technology, China

*Corresponding author.
E-mail address: huyang@hit.edu.cn (Y. Hu)

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Dengue and Chikungunya seroprevalence in waste pickers from the largest Latin American open-air dump

Dear Editor,

We read with great interest the manuscript entitled "Emergence of co-infection of COVID-19 and dengue: A serious public health threat" by Saddique et al.[1] The authors examine a group of Paquistani patients, who showed a confirmed SARS-CoV-2 and Dengue virus (DENV) coinfection, related to high mortality. In endemic for DENV countries (including Brazil) the co-circulation of both viral agents, especially during the rainy period, may trigger additional overloading of the healthcare system. It may require specific treatment protocols due to the differential nature of both infections and the possiblity of rapid evolution of the co-infection to DENV shock. Some marginalized populations like waste pickers populations may be more severely impacted by such a co-infection. No study which examines the seroprevalence to arboviral diseases in this population exists and this may be of significant importance, especially when evaluation the risk of severe DENV infection. Our objective, therefore, was to estimate the seroprevalence of DENV and CHIKV among waste pickers in the largest open dump in the world located in the Federal District of Brazil (the Structural City Open Dump). In this place more than 2000 individuals work scavenging waste. The open-air dump borders the Brasília National Park, encompassing 423.6 km^2 conservation area including the Structural City, and Cabeceira do Valo river, where smallholder vegetable production exists (Fig. 1). Additionally, a large environmentally degraded area surrounds the dump as a consequence of the precarious housing construction without sanitation.[2] Based on the environmental risks, the individuals inhabiting the area are highly susceptible to vector-borne diseases due to the favorable conditions for proliferation of synanthropic mosquitos.[3]

In this study, 1200 waste pickers were eligible for sample collection between June -October 2017. An epidemiology calculator (https://www.openepi.com/) was used to determine the sample size which was based on assumed seroprevalence of 50%. A minimum sample size of 292 samples needed to be tested to achieve a confidence interval (CI) of 95% and a desired precision of ± 5%. We enrolled a sample size of 404 participants. Ethics approval was obtained from the Foundation for Teaching and Research in Health Sciences (FEPECS, protocol N° 55,754,216.5.0000.5553). Samples that were positive for anti-DENV IgM and Anti-CHIKV IgM (recent infection) were also tested by specific RT-PCR. The

Fig. 1. *Map of Brasilia, the Structural City, and the Open-air dump*; The Open-air dump borders to the Brasilia National Park, the Structural City and the Cabeceira do Valo river (Google Earth, 2021).

Table 1
Studied population and arboviruses markers prevalence.

Gender	Male	Female	Total	Estimated true prevalence (CI: 95%) **
n	129 (31.9%)	275 (68.1%)	404 (100%)	———-
Age mean (±SD)	40.6 (±11.8)	40.6 (±10.8)	40.6 (±11.1)	———-
DENV IgM+	28 (21.7%)	48 (17.5%)	76 (18.8%; 15.7 to 21.9%) *	17.2% (14.2 to 20.2%)
DENV IgG+	101 (78.3%)	193 (70.2%)	294 (72.8%; 69.2 to 76.3%) *	72.8% (69.2 to 76.3%)
DENV IgM+ and IgG+	14 (10.9%)	18 (6.5%)	32 (7.92%; 5.8 to 10.1%) *	———-
Overall DENV Seroprevalence	129 (100%) [a]	241 (87.6%) [a]	370 (91.6%; 89.4 to 93.8%) *	———-
DENV RNA and IgM+	0 (0.0%)	0 (0.0%)	0 (0.0%) *	0 (0.0 to 0.7%)
CHIKV IgM +	5 (3.9%%)	9 (3.3%)	14 (3.5%; 2.0 to 4.9%) *	3.3% (1.9 to 4.7%)
CHIKV IgG +	10 (7,8%)	14 (5.1%)	24 (5.9%; 4.1 to 7.8%) *	5.9% (4.1 to 7.8%)
CHIKV IgM+ and IgG+	1 (0.8%)	1 (0.4%)	02 (0.5%; 0.0 to 1.1%) *	———-
Overall CHIKV Seroprevalence	15 (11.6%)	23 (8.4%)	38 (9.4%; 7.1 to 11.7%) *	———-
CHIKV RNA and IgM+	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0 to 0.7%)

SD: Standard deviation; CI: confidence interval.
* Apparent prevalence observed in this study (CI:95%).
** estimated true prevalence considering 1200 waste pickers.
[a] p-value <0.0001.

tool http://www.winepi.net was used to estimate the true serological markers prevalence in the waste picker population (considering the specificity and a sensitivity of each serological kit and 95% CI). We used the Chi-square test with level of significance of $p<$ 0.05 for evaluation of the difference of the overall DENV and CHIKV seroprevalence.

Serological and molecular results are shown in Table 1. Of the samples tested, 18.8% (n = 76/404) were positive anti-DENV IgM. Fifty-four samples (n = 54/404; 13.4%) showed undetermined result for anti-DENV IgM (CI: 95%; 10.7 to 16.1%) (data not shown). The anti-DENV IgG testing showed a seroprevalence of 72.8% (n = 294/404). Undetermined anti-DENV IgG results were obtained in 1.2% (5/404) of the cases (CI: 95%; 0.36–2.12%) (data not shown). Concomitant presence of anti-DENV IgM/IgG was observed in 7.92% (n = 32/404) of the samples. The overall DENV seroprevalence was 91.6%. Anti-CHIKV IgM was found in 3.5% (n = 14/404) of the tested waste pickers. One sample (n = 1/404; 0.2%) was undetermined (CI: 95%; 0.0–0.6%) (data not shown). Anti-CHIKV IgG was positive in 5.9% (n = 24/404) of the cases. Two samples showed a concomitant presence of anti-CHIKV IgM/IgG (n = 2/404; 0.5%). The overall CHIKV seroprevalence was 9.4%. All of the positive for anti-DENV IgM or anti-CHIKV IgM samples showed a negative result for viral RNA. Comparing the demographic data and the obtained results showed that DENV and CHIKV seroprevalence was higher in males than females, but only the overall DENV seroprevalence showed significant difference.

The results on the anti-DENV seroprevalence in our study are comparable with findings observed in hyperendemic for DENV areas in Brazil. For instance, a study performed in the city of Salvador showed anti-DENV IgG seroprevalence of 75.7%.[4] The same DENV seroprevalence (74.6%) was obtained in an impoverished neighbourhood in the city of São José do Rio Preto located in Southeast Brazil and in a region with high DENV endemicity.[5] A seroprevalence between 74.3 and 91.1% was observed in low socioeconomic statues areas of the Brazilian cities of Recife and Rio de Janeiro which are also highly endemic for DENV.[6,7] We therefore believe that similar to our study, in addition to endemicity, the socioeconomic factors play a pivotal role for DENV acquirement and in consequence to higher seroprevalence rates in specific urban populations, including waste-pickers. This high DENV seroprevalence may contribute to unfavorable clinical outcome especially during DENV reinfections which could be further aggravated by the theoretic ease of SARS-CoV-2 acquirement among this population.[8] Worldwide seroprevalence to CHIKV can vary between 10 and 75%.[9,10] The difference obtained in our study might be due to specifications of the studied populations and sample timing which coincides with increased viral circulation. In this respect, the CHIKV dissemination in the Federal District might have been lower thus leading to lower seroprevalence in the studied population (overall seroprevalence of 9.4%). As this is the first study which evaluates the anti-CHIKV IgG prevalence in the Federal District of Brazil, we are unaware of its distribution in other population settings. Anyway, due to the unsanitary conditions to which waste pickers are subject, anti-CHIKV seroprevalence in this population is expected to be higher than in the nearby general population. Indeed, data from healthy blood donors have shown a seroprevalence around 5 times lower (unpublished).

This study, which is unique in its sample types shows high DENV seroprevalence and to a lesser extent to CHIKV, in a population of waste pickers. We can relate the observed findings to the low socioeconomic conditions in which these individuals live, the geographic location (close to national parks), and the environmental characteristics originating from the dumpsite. This study, aims also to attract the attention of the authorities for the urgent need for public health measures aiming to improve the life quality of vulnerable populations, such as waste pickers, not only in regards of arboviral diseases but also due to the ongoing SARS-CoV-2 pandemic which can further impact in the most vulnerable populations.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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Évelin Mota Cassemiro
Center for Tropical Medicine, University of Brasília, Brasília, Brazil.

Daiani Cristina Cilião-Alves
Euro-American University Centre-UNIEURO, Brasilia, Federal District, Brazil

Drielly Rodrigues Moura
Center for Tropical Medicine, University of Brasília, Brasília, Brazil.

Svetoslav Nanev Slavov
Blood Center of Ribeirão Preto, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil
Butantan Institute, São Paulo, São Paulo, Brazil

Tatyane de Souza Cardoso Quintão
Center for Tropical Medicine, University of Brasília, Brasília, Brazil.

James Alexander Scott
Division of Occupational and Environmental Health, Dalla Lana School of Public Health, University of Toronto, Ontario, Canada

Carla Pintas Marques
Faculty of Ceilândia, University of Brasilia, Brasilia, Federal District, Brazil.

Marcos Takashi Obara, Wildo Navegantes de Araújo
Center for Tropical Medicine, University of Brasília, Brasília, Brazil.
Faculty of Ceilândia, University of Brasilia, Brasilia, Federal District, Brazil.

Vanessa Resende Nogueira Cruvinel
Faculty of Ceilândia, University of Brasilia, Brasilia, Federal District, Brazil.

Rodrigo Haddad*
Center for Tropical Medicine, University of Brasília, Brasília, Brazil.
Faculty of Ceilândia, University of Brasilia, Brasilia, Federal District, Brazil.

*Corresponding author at: Faculdade de Ceilândia, Universidade de Brasília. Centro Metropolitano, Campus Universitário, Ceilândia Sul, Brasília-DF, CEP: 72220-275, Brazil.
E-mail address: haddad@unb.br (R. Haddad)

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Why do clinicians treat, or not treat, a patient for sepsis? Using the theoretical domains framework to elicit barriers and enablers to performing the Sepsis Six in UK hospitals ☆☆

Dear Editor,

We read with interest recent correspondence from Tidswell et al. in response to earlier papers in the Journal of Infection.[1]

Inaccurate diagnosis and coding against the Sepsis-3 definition is a key challenge facing multiple aspects of sepsis policy, from correct treatment of patients to managing public health campaigns.[1] The impact of this on the increasingly polarised 'sepsis hysteria' debate is well documented.[2,3] The crux of this debate is the impact on under or overtreatment of patients diagnosed as septic. In order to understand this it is necessary to understand the factors affecting why patients do – or do not – receive treatment for sepsis.

The simple care bundle 'Sepsis Six' is a cornerstone of UK national sepsis guidelines and targets, but implementation of this has been shown to be highly variable.[4,5] The barriers and enablers which affect performance of this bundle have been studied previously on a small scale using behavioral science frameworks (the Theoretical Domains Framework (TDF)) (Table 1).[6,7] We applied the TDF to explore barriers and enablers to performing the Sepsis Six in septic patients across a multi-centre sample of UK hospitals, with the aim of identifying targets for efficient and effective behavior change intervention.

Questionnaires containing 54 belief statements (for example 'I have a time-based goal for Sepsis Six completion') covering all TDF domains (e.g. Knowledge, Skills) were circulated to nurses, consultants, and doctors-in-training in medical/surgical/emergency departments in ten UK hospitals (Table 2). Likert scales scored statements for importance (to differentiate important from unimportant belief statements) and agreement (to differentiate barriers and enabler belief statements). Results were analyzed across groups, hospitals, and overall. Logistic regression identified differences between groups and beliefs important to all.

616 responses were received from participants in ten hospitals. The most important barriers to implementation of the Sepsis Six fell within the TDF domain Environment/Context/Resources, for example, insufficient staffing, time or bed availability. Most important enablers related to the domains *Knowledge* and *Skills*, for example knowing what was in the Sepsis Six, and how and when to perform it, *Social and Professional Role*, for example believing it was the participant's role to deliver the Sepsis Six, and *Beliefs In Consequences*, for example beliefs that the Sepsis Six improves outcomes, and should be delivered quickly. The majority of belief statements from these domains were consistently rated as important across all hospitals and participant groups, with no significant difference between them. However, there was often significant variation in agreement scores, with some hospitals rating an important belief statement as a barrier (I do not have sufficient staff) and others as an enabler (I do have sufficient staff) (Fig. 1).

Our findings highlight consistent targets for intervention to improve implementation of the Sepsis Six. National interventions aiming to increase Sepsis Six performance should focus on addressing resource shortages, maintaining skills and knowledge, maintaining beliefs of beneficial effect, and maintaining 'it is my

☆☆ Patients and/or the public were not involved in the design, conduct, reporting or dissemination plans of our research. The lead author affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained. Dr. Guy Hooper

Table 1
Theoretical Domains Framework.

Domain	Content	Sample belief statement
Knowledge	An awareness of something	I am aware of what the Sepsis Six involves
Skills	Ability or proficiency acquired through practice	I have the necessary skills to perform the Sepsis Six
Social/Professional Role	Set of behaviors and qualities of an individual in social or work setting	It is part of my role to perform the tasks in the Sepsis Six
Beliefs about Capabilities	Views about one's ability/talent/capability to perform the target behavior(s)	I am confident performing the Sepsis Six
Optimism	Confidence that things will happen for the best or that desired goals will be attained	Sepsis Six performance will improve at this hospital
Beliefs about Consequences	Acceptance of the truth, reality or validity about outcomes of a behavior in a given situation	Performing the Sepsis Six improves patient outcomes
Reinforcement	Increasing the likelihood of a behavior being performed by establishing an association between performing a behavior and a given stimulus or cue	I get rewarded for good Sepsis Six performance
Intentions	Conscious decision to perform a behavior or resolve to act in a certain way	I will prioritize performing the Sepsis Six over other tasks
Goals	Mental representation of outcomes or states that an individual wants to achieve	I have a time-based goal for completing the Sepsis Six
Memory/Attention/Decisions	The ability to retain information, focus selectively on aspects of the environment and choose between two or more alternatives	It is easy to remember the steps in the Sepsis Six
Environment/Context/Resources	Circumstances of a person's situation/environment that affect behavior	I have sufficient time to perform the Sepsis Six
Social Influences	Interpersonal processes that can cause individuals to change thoughts/feelings/behaviors	My colleagues believe the Sepsis Six to be of benefit
Emotions	Complex reaction pattern by which individual attempts to deal with a personally significant matter or event	I feel bad if I do not perform the Sepsis Six
Behavioral Regulation	Anything aimed at managing or changing objectively observed or measured actions	I get sufficient feedback on my performance of the Sepsis Six

Table 2
Stakeholder group responses, by hospital.

Hospital	Location			Role			Number invited from hospital	Responses from hospital	Hospital Response Rate (%)
	ED	MAU	SAU	Cons	DiT	Nurse			
1	25	30	49	10	69	25	361	104	28.8
2	3	4	20	3	14	10	Unknown	27	Unknown
3	30	32	28	11	36	43	Unknown	90	Unknown
4	29	25	17	17	28	26	Unknown	71	Unknown
5	23	3	3	3	8	18	Unknown	29	Unknown
6	10	14	2	4	3	19	229	26	11.4%
7	39	34	28	14	26	61	201	101	50.3%
8	38	45	32	29	34	52	255	115	45.1%
9	15	12	7	8	7	19	446	34	7.62%
10	4	15	0	0	19	0	Unknown	19	Unknown
Total	216	214	186	99	244	273		616	

role to do this' as a core belief. Implementation science based methodology could now be used to match the relevant TDF domains to behavior change techniques and improve implementation on a large scale. Knowing when to perform the Sepsis Six, and being able to diagnose sepsis, were both important factors in whether treatment was initiated or not. This study fits with broad themes identified in previous single center studies, which highlighted 'what to do and why', 'working together', 'risks and benefits' and 'staffing levels' as key factors affecting performance of the Sepsis Six.[6–8]

Our large study, alongside previous small studies, highlights multifactorial reasoning for performing the Sepsis Six, but in particular the vital role that *Knowledge, Skills* and *Beliefs In Consequences* play in defining clinician behavior. Ensuring that all relevant stakeholder staff have the knowledge and skills to diagnose patients correctly according to Sepsis-3 criteria, and are well informed about the consequences of the Sepsis Six bundle, would seem like an important step to minimize undertreatment of those with sepsis and overtreatment of those without.

Declaration of Competing Interest

There was no other involvement of outside agencies and no influence on any aspect of the study.

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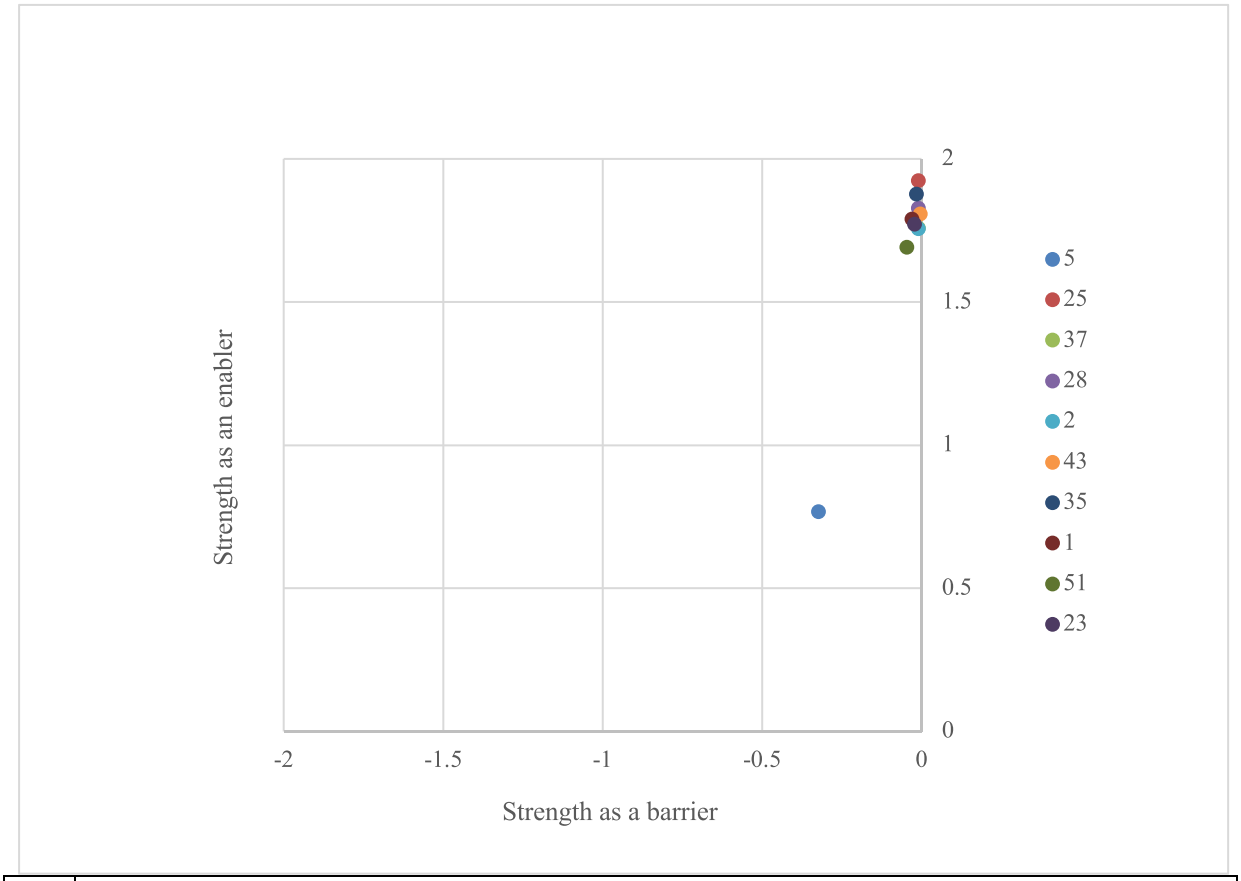
Data availability

Data can be made available on request in accordance with data protection rules.

Ethical approval

Ethical approval requirement was waived as this was part of a service improvement project.



5	Whether there are sufficient staff to perform the Sepsis Six
25	Whether it is part of my role to identify septic patients
37	Whether performing the Sepsis Six improves patient outcomes or not
28	Whether delivering the Sepsis Six quickly increases its benefit
2	Whether I have the required skills to perform the Sepsis Six
43	Whether early and regular reassessment of septic patients has an effect on outcomes or not
35	Whether I intend to continue to perform the Sepsis Six on septic patients
1	Whether I'm aware of what the Sepsis Six involves
51	Whether I have a time-based goal for Sepsis Six completion or not
23	Whether I feel able to escalate if I'm concerned about a patient I think needs the Sepsis Six

Fig. 1. Graph showing strength of agreement (as barrier and/or enabler) for top ten most important belief statements.

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Dr Guy Hooper, Dr Neil Roberts
Specialist Registrar
Departments of Intensive Care and Anaesthesia, Royal Cornwall Hospital, Cornwall, United Kingdom

Dr Fabiana Lorencatto
Research Lead for Behaviour Change
University College London, London, United Kingdom

Dr Adam Pollard
Innovation Development Manager
Organisational Development, Royal Cornwall Hospital, Truro, Cornwall, United Kingdom

Dr Michael Spivey
Consultant in Intensive Care Medicine and Anaesthesia
Royal Cornwall Hospital, Truro, Cornwall, United Kingdom

Dr Wendell Storr
Specialist Registrar
Department of Anaesthesia, Royal Victoria Infirmary, Newcastle, United Kingdom

Dr Deborah Webster
Consultant in Intensive Care Medicine and Anaesthesia
University Hospitals Plymouth, Plymouth, Devon, United Kingdom

Mr Alan Eccles
Specialist Registrar
Department of Radiology, University Hospitals Plymouth, Plymouth, Devon, United Kingdom

Mr George Ramsay
Specialist Registrar and Clinical Lecture
Department of Surgery, Raigmore Hospital, Inverness

Dr Jan Jansen
Associate Professor and Director of Research
Division of Acute Care Surgery, University of Alabama at Birmingham, Birmingham, AL, United States

Dr Ruth Thomas
Consultant in Intensive Care Medicine and Anaesthesia
Dorset County Hospital, Dorchester, Dorset, United Kingdom

Dr Helen Bounds, Dr Michael Eddie
Senior House Officer
Department of Intensive Care, Dorset County Hospital, Dorchester, Dorset, United Kingdom

Dr Stephen Knight
Specialist Registrar Department of General Surgery
Monklands District General Hospital, Airdrie, Lanarkshire, United Kingdom

Dr Robert Pearson, Dr James Chean Khun Ng
Foundation Doctor
Department of General Surgery, Monklands District General Hospital, Airdrie, Lanarkshire, United Kingdom

Ms Theresa Weldring
Programme Manager
Project Monitoring Group, Kingston Hospital NHS Foundation Trust, Kingston Upon Thames, Surrey, United Kingdom

Ms Sandra Courtiour, Ms Laura Smith
Lead AKI/Sepsis Nurse Specialist
Poole Hospital NHS Foundation Trust, Poole, Dorset, United Kingdom

Ms Nicola DeVeaux
Clinical Lead for Sepsis
Great Western Hospitals NHS Foundation Trust, Swindon, Wiltshire, United Kingdom

Dr Biju Cherian
Consultant Emergency Physician
Hull Royal Infirmary, Hull, Yorkshire, United Kingdom

Toby Flack, Stephen Rogen, Nadia Mekki, Hope Ward, Sian Edwards, Nigel Booth, Heather Clarke, Samantha Rowe
University of Plymouth Hospitals Trust, United Kingdom

Kate Adams, Donna Gotts
Hull Royal Infirmary, United Kingdom

*Corresponding author.
E-mail addresses: guy.hooper@nhs.net (D.G. Hooper), neil.roberts8@nhs.net (D.N. Roberts), f.lorencatto@ucl.ac.uk (D.F. Lorencatto), adampollard@nhs.net (D.A. Pollard), michaelspivey@nhs.net (D.M. Spivey), wendell.storr@nhs.net (D.W. Storr), dwebster2@nhs.net (D.D. Webster), eorge.ramsay@nhs.net (M.G. Ramsay), jjansen@uabmc.edu (D.J. Jansen), ruth.thomas@dchft.nhs.uk (D.R. Thomas), helen.bounds@doctors.org.uk (D.H. Bounds), michael.eddie@doctors.org.uk (D.M. Eddie), stephenknight@doctors.org.uk (D.S. Knight), theresa.weldring1@nhs.net (M.T. Weldring), sandra.courtiour@poole.nhs.uk (M.S. Courtiour), laura.smith@poole.nhs.uk (M.L. Smith), n.deveaux@nhs.net (M.N. DeVeaux), biju.cherian1@nhs.net (D.B. Cherian)

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Antibiotic resistance genes in Bacillus cereus isolated from wild Père David's deer (*Elaphurus davidianus*)

Dear Editor,

Antibiotics are the most commonly prescribed therapy to human and animals, however with a high proportion and misuse emergence the antibiotic resistance in animal husbandry. Antimicrobial resistance profile is highly concerned with humans and animal health globally.[1,2] The natural environment provides a large library of drug-resistant genes for microorganisms. The antimicrobial resistance process is accelerated by the circulation of substances in nature (such as air and water) and biological activities (human activities and animal's abundance or insect's diversity) that may contribute to the dynamic evolution of bacterial flora and evolving a variety of unknown drug-resistant genes to adapt the changes in the external environment.[3,4] It is worth mentioning that whether the wild animals contain the drug-resistant genes for microbes.

E. davidianus (Père David's deer) is herbivorous mammal, is considered one of the world's rare species; and regarded as an endangered and protected species originating from China. Recently, severe diarrhea was observed in several deer from the Shishou *E. davidianus* Preserve (Hubei, China) regions.[5] We isolated a strain of *B. cereus* from the lung, hydrothorax, pericardial fluid, and joint fluid of sick wild *E. davidianus*. The genomic DNA of *B. cereus* was prepared and sequenced. We integrated several approaches, databases, and specific software to annotate the assembled sequences. In this study the comprehensive antibiotic research database (CARD) was used predict the antibiotic resistance ontology (ARO). The results showed that the isolate carried tetB(P), otr(A), rpoB, rphB, rphA, cat, mprF, ileS, fabI, vanYM, vanSF, vanSM, FosB, murA, parC, gyrA, mfd, EF-Tu, dfrE, BcII, mecC, mecB, mecA, BLA1 and BcI drug-resistance genes, which considered to resistant the tetracycline, rifamycin, phenicol, peptide antibiotics, mupirocin, isoniazid, triclosan, glycopeptide antibiotics, fosfomycin, fluoroquinolones, elfamycin, kirromycin, diaminopyrimidine and beta-lactam, respectively. Meanwhile, some drug-resistanc genes perform the functions of drug efflux pump complex or subunit conferring antibiotic resistance, like tet (42), TaeA, sav1866, lmrD, lmrB, cdeA, bmr, blt, emeA and bcrA (Table 1).

Emergence of resistance from important bacteria is one of the public threats worldwide. Antibiotics are produced by microbial metabolites of resistant pathogens, that can selectively disrupt or inhibit the growth of pathogens.[3, 4] Various antibiotics have different functions, such as the interfering the synthesis of cell wall, plasma membrane, inhibiting protein synthesis etc., such as quinolones and rifamycin can inhibit the nucleic

Table 1
Annotation of antibiotic resistance genes.

ARG_name	Antimicrobial	ARO_category
tetB(P) and otr(A)	tetracycline	antibiotic target protection protein
rpoB	rifamycin	antibiotic resistant gene variant or mutant
rphB and rphA	rifamycin	antibiotic inactivation enzyme
cat	phenicol	antibiotic inactivation enzyme
mprF	peptide antibiotics	antibiotic target modifying enzyme
ileS	mupirocin	antibiotic resistant gene variant or mutant
fabI	isoniazid, triclosan	antibiotic resistant gene variant or mutant
vanYM	glycopeptide antibiotics	antibiotic resistance gene cluster, cassette, or operon; gene conferring antibiotic resistance *via* molecular bypass
vanSF and vanSM	glycopeptide antibiotics	antibiotic resistance gene cluster, cassette, or operon; gene conferring antibiotic resistance *via* molecular bypass
FosB	fosfomycin	antibiotic inactivation enzyme
murA	fosfomycin	antibiotic resistant gene variant or mutant
parC	fluoroquinolones	antibiotic resistant gene variant or mutant
gyrA	fluoroquinolones	antibiotic resistant gene variant or mutant
mfd	fluoroquinolones	antibiotic target protection protein
EF-Tu	elfamycin; kirromycin	antibiotic resistant gene variant or mutant
dfrE	diaminopyrimidine	antibiotic target replacement protein
BcII	beta-lactam	antibiotic inactivation enzyme
mecC, mecB and mecA	beta-lactam	antibiotic resistance gene cluster, cassette, or operon; antibiotic target replacement protein
BLA1 and BcI	beta-lactam	antibiotic inactivation enzyme
Tet (42), TaeA, sav1866, lmrD, lmrB, cdeA, bmr, blt, emeA, bcrA		efflux pump complex or subunit conferring antibiotic resistance

ARG: Antibiotic Resistance Genes; ARO: Antibiotic Resistance Ontology.

acid replication and transcription, beta-lactam inhibits the synthesis of bacterial cell walls, macrolides inhibit synthesis of bacterial proteins. Meanwhile, efflux pump is capable of directly drain antibiotics, heavy metals or other toxic molecules from bacterial cells.[4,6]

Previous study showed that approximately 90% bacteria have been reported isolated from voles contained resistance to the antibiotic in UK, compared with almost 0% in Finland.[7] In the present study, our finding indicates a large number of drug-resistant genes are present in *B. cereus* in wild *E. davidianus*, despite the absence of human intervention, and where these genes came from? A plausible explanation is that antibiotic resistance genes has high mobility, mainly through the horizontalgene transfer mechanism, namely with the help of some mobile genetic factor or elements in the genome, such as plasmid, integrons, transposons and insertion sequences.[4,6] The drug-resistant genes can transfer among different microorganisms *via* transformation and joint between bacterial cells, or *via* lysogenic phages transduction to integrate into another the genome of the recipient bacteria. At the same time, misuse and overuse in animal production, aquaculture as well as in human's treatment usually through the genetic changes also contributed antimicrobial resistance. The resulting of drug-resistant genes of antibiotics will be removed from the body to excreta, and further spread to humans and other animals directly and indirectly. The environments (microbiota merging), the interactions among bacterial species or clones are being connected in genetic exchange communities, therefore the genetic exchanges, gene fusion, other hierarchical levels and probably microbiome merging etc. plays major role in emergence, spread, and maintenance of antibiotic resistance.

The key to control and combat the antibiotic resistance is to prevent the spread of drug-resistant strains and drug-resistant genes. This spread of channel is very extensive, including humans, insects, birds, water, agriculture, etc.[8] We should establish a monitoring network for agriculture, health, and the environment, and conduct comprehensive monitoring and reporting of antibiotic residues in animal-derived food, antibiotic use in hospitals, and antibiotic pollution in soil and water environments.[2,8]

In summary, the local genetic exchange communities promote the antibiotic resistance among the bacterial species. Therefore, the ecological compatibility species and sharing same environment and niches establish the exchange possibilities. Generally, One Health approach to antibiotic resistance opens a door for understanding in communication, among microbiotas, clones and holistic image of the problem of antibiotic resistance. At the same time, the new biochemical, microbiological, ecological, bioinformatics technologies required to control the problem of antibiotic resistance on a planetary scale.

Declaration of Competing Interest

None of the authors have any conflict of interest.

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Ying Li
Specialist Registrar
College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, China

Khalid Mehmood
Specialist Registrar

Faculty of Veterinary & Animal Sciences, The Islamia University of Bahawalpur, Bahawalpur 63100, Pakistan

Yung-Fu Chang
Research Lead for Behaviour Change
College of Veterinary Medicine, Cornell University, Ithaca, NY, United States

Rui Guo
Innovation Development Manager
Animal Husbandry and Veterinary Institute, Hubei Academy of Agricultural Science, Wuhan 430064, China

Peng Shang*
Consultant in Intensive Care Medicine and Anaesthesia
College of Animal Science, Tibet Agriculture and Animal Husbandry College, Linzhi, Tibet, China

Hui Zhang*
Specialist Registrar
College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, China

*Corresponding authors.
E-mail addresses: nemoshpmh@126.com (P. Shang), hz236@scau.edu.cn (H. Zhang)

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