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Next-generation COVID-19 vaccines: Opportunities for vaccine development and challenges in tackling COVID-19

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SUMMARY The ongoing COVID-19 pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become a global threat. Although non-pharmaceutical interventions have been rigorously and widely implemented, living conditions caused by the pandemic will last until highly effective vaccines are successfully improved and globally administered. Several first-generation COVID-19 vaccines were approved at the end of 2020. However, the COVID-19 pandemic is persisting worldwide. To be clear, the efficiency and the coverage of current vaccines are insufficient, but newly emerging and rapidly spreading variants are the most pressing concern. A second-generation COVID-19 vaccine worth mentioning, NVX-CoV2373, has demonstrated 90% overall efficacy as well as a high level of efficacy against circulating variants in Phase 3 clinical trials. Currently, NVX-CoV2373 is the only vaccine that has proven successful against variants during Phase 3/4 trials. Therefore, developing the next generation of vaccines is a promising strategy to ultimately prevail against SARS-CoV-2. This review provides up-to-date information on COVID-19 vaccines in terms of their efficacy and new platforms and the progression of COVID-19 vaccination. Moreover, this review also summarizes the efficacy of approved COVID-19 vaccines against variants. Lastly, this review highlights the global challenges for COVID-19 vaccines in development and vaccination, and it discusses opportunities for development of future COVID-19 vaccines and vaccination coverage.

Keywords COVID-19, SARS-CoV-2, vaccines, NVX-CoV2373, vaccination, distribution

1. Introduction

As of June 25, 2021, more than 17 million confirmed cases of COVID-19 and 3,840,223 deaths have been reported by the WHO (*1*). The COVID-19 pandemic has posed a serious crisis to both health care systems and economies worldwide. Since there are no effective treatments for COVID-19, the chances of controlling the COVID-19 pandemic depend mainly on two main factors: *i*) public health interventions and *ii*) development and administration of safer and more effective vaccines (*2*).

Public health interventions such as non-pharmaceutical measures were obviously effective in reducing the spread of COVID-19 (*3*). Governmental measures including travel restrictions, border restrictions, quarantine of travelers, confirmed cases, and contacts, orders to avoid confined spaces and large gatherings, social distancing, compulsory mask wear, school closures, and establishment of designated hospitals were useful at preventing the spread of COVID-19. Individual interventions, such as use of protective equipment by healthcare workers and attention to personal hygiene, were also effective in tackling COVID-19. Nevertheless, sporadic cases of COVID-19 continue to emerge even in countries with strict controls compared to countries with less stringent interventions (*4,5*).

More importantly, a number of newly emerged mutations have accelerated the rapid spread of SARS-CoV-2. There are 4 known major variants: the B.1.1.7 lineage (called the Alpha variant) that was first identified in the United Kingdom, the B.1.351 lineage (called the Beta variant) that was identified in South Africa, the P.1 lineage that was identified in Brazil (called the Gamma variant), and the B.1.617 lineage (called the Delta variant) that was verified in India. These variants have been labeled variants of concern (VOC) by the WHO (*6*). At the current point in time, the B.1.617 variant has been blamed for the current surge of COVID-19 in India (*7*). The variants have increased transmissibility or increased virulence compared to the original virus. Changes in those variants cause worse disease presentation and negatively impact COVID-19 epidemiology and public health measures. Therefore, strict control measures

alone are not effective enough to stop the COVID-19 pandemic, more efficacious vaccines need to be quickly developed to prevent the COVID-19 pandemic.

2. Strategies for the development of COVID-19 vaccines

As of June 25, 2021, a total of 574 vaccines have been developed, including 103 vaccines in clinical trials, and 184 vaccines in pre-clinical studies. Of the 103 vaccines in clinical trial, 23 are in Phase 3 or 4. Multiple platforms have been used to develop the 103 vaccines. Eighteen are inactivated and live attenuated vaccines, including BBIBP-CorV (COVILO) from Sinopharm (Beijing and Wuhan) and CoronaVac from Sinovac (*8*). Thirty-three are recombinant protein vaccines, or recombinant subunit vaccines, such as NVX-COV2373 from Novavax and ZF2001 from Zhifei Biology. Twenty-six are nucleoid vaccines, such as BNT-162b2 from BioNTech and mRNA-1273 from Moderna. Twenty-one are viral vector vaccines, such as ChAdOx1-nCoV-19 from AstraZeneca, Ad5-nCoV from CanSino, Ad26.COV2.S from Janssen Pharmaceuticals, and Sputnik V from Gamaleya (*9*).

Vaccines developed using different platforms have pros and cons because of the different techniques used (*10,11*). *i*) Inactivated vaccines and live attenuated vaccines are quick to prepare and easy to produce but do not readily induce T-cell immunity and usually need to be administered 2-3 times to enhance immunity. In addition, the long-lasting immune response to inactivated vaccines relies more on adjuvants than other vaccines, and live attenuated vaccines against complex pathogens are challenging (*12-13*). *ii*) Nucleoid vaccines are easy to rapidly mass produce and confer long-term host immunogenicity but have potential genetic risks since mRNA is unstable and they remain ineffective when administered to humans with compromised immunity (*14-15*). *iii*) Viral vector vaccines can induce strong humoral and cellular immunity but are not able to induce immunogenicity in the face of preexisting immunity (*16-18*). *iv*) Recombinant vaccines such as NVX-COV2373 from Novavax and ZF2001 from Zhifei Biology have very clear advantages such as a distinct composition and a high level of safety and stability but also have weak immunogenicity and require adjuvants (*19*). (Table 1)

3. Progress of COVID-19 vaccination

Widespread vaccination is also required to end the COVID-19 pandemic. In mid-December 2020, the first reports of a COVID-19 vaccine outside of clinical trials were published in the UK, thus sparking a race for vaccine development. Waves of vaccination subsequently occurred daily around the world. With the successful development of the first-generation vaccine, the COVID-19 vaccination rate increased in a wide range of countries from January to May 2021 (*20*).

Thanks to the tremendous breakthrough in COVID-19 vaccine development, the WHO is working tirelessly with partners to manufacture vaccines and to promote their safety and effectiveness. As of June 25, 2021, a total of 471,786,361 persons were fully vaccinated and 1,031,602,050 persons were vaccinated with at least one dose, accounting for 22.4% of the world's population (*1,21*). Two-point-eight billion doses have been administered, and 40.8 million doses are administered each day (*21*). However, the vaccination rate (> and = 2nd dose) among people in low-income countries was less than 1%, which implies that the ongoing COVID-19 vaccination faces great challenges.

4. Challenges with COVID-19 vaccines in development and vaccination

4.1. Issues with the efficacy of COVID-19 vaccines against variants

SARS-CoV-2 is an enveloped virus with a positive-polarity single-stranded RNA genome that contains four major structural proteins: the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins. The trimeric S protein consists of a receptor-binding subunit (S1) and a membrane-fusion subunit (S2). The S1 subunit consists of the N-terminal domain (NTD), the receptor-binding domain (RBD), and two small subdomains. The role of the S protein is to mediate SARS-CoV-2's entry into host cells *via* surface receptor angiotensin-converting enzyme 2 (ACE2). The S1 subunit is involved in cell entry, and the RBD domain is responsible for direct binding. Thus, the S, S1, and RBD proteins are 3 major targets for vaccine development (*22-24*).

However, mutations in those target proteins represent a great challenge to the efficacy of COVID-19 vaccines (*25*). As reported, variants of concern (VOC) are associated with increased transmissibility and virulence due to notable mutations in key proteins. For example, mutations in the B.1.1.7 lineage involve multiple sites, such as a N501Y substitution in the RBD region, H69/V70 deletion in the N-terminal region, and P681H mutation adjacent to the furin cleavage site in the S protein. Mutations in the B.1.351 variant (20H/501Y.V2) include K417N, E484K, and N501Y. In the P.1 variant (B.1.1.28.1), mutations involve K417T, E484K, and N501Y substitutions in the RBD domain. B.1.617.2 is defined by more mutations in the S protein, including T19R, DEL157/158, T478K, and D950N. In addition, these variants share the D614G mutation, which has been found to increase the rapid spread of the virus. Therefore, inadequate public healthcare measures and vaccination coverage have accelerated the emergence of variants. Moreover, the rapid spread of variants has increased the global demand for more effective vaccines. Whether the 6 prototype vaccines in Phase III/IV trials will remain effective against variants

Table 1. Comparison of Novavax and other COVID-19 vaccines on the market (8)

Developer	Representative	Type	Platforms	Advantages and disadvantages	Approval	Vaccine Efficacy in clincal trails
Pfizer/BioNTech	BNT 162b2	3 LNP-mRNAs	Nucleoid vaccines	1). Easy to rapidly mass produce and confer long-term host immunogenicity; 2). but have potential genetic risks since mRNA is unstable and and remain ineffective when administered to humans with compromised immunity	EUA	95% effective against COVID-19 beginning 28 days after the first dose; efficacy in adults over 65 years of age was over 94%
Moderna mRNA-1273	mRNA-1273	LNP-encapsulated mRNA			EUA	94.1% efficacy at preventing Covid-19 illness
AstraZeneca	ChAdOx1-nCoV-19 (AZD1222)	ChAdOx1-S	Viral vector vaccine	1). Can induce strong humoral and cellular immunity; 2). but are not able to induce immunogenicity in the face of preexisting immunity	EUA	90·0% effective after the 2nd dose. Overall vaccine efficacy across both groups was 70·4%
Johnson & Johnson	Ad26.COV2.S	Adenovirus Type 26 vector (Non-Replicating Viral Vector)			EUA	52.0- 81.7% efficacy
CanSino Biologics	Ad5-nCoV	Adenovirus Type 5 Vector (Non-Replicating Viral Vector)			EUA	65.7% efficacy
Gamaleya Research Institute Gam-COVID-Vac	Sputnik V	Adeno-based (rAd26-S+rAd5-S) (Non-Replicating Viral Vector)			On the market (Russian)	vaccine efficacy is 91.6%
Sinovac CoronaVac	Corona Vac	Inactivated	Inactivated vaccines and live attenuated vaccines	1). Quick to prepare and easy to produce; does not readily induce T-cell immunity, needs to be administered 2-3 times to enhance immunity; 2). The long-lasting immune response to inactivated vaccines relies more on adjuvants than other vaccines; live attenuated vaccines against complex pathogens are challenging.	On the market (China)	50.4-60.7% efficacy
Wuhan Institute of Biological Products/ Sinopharm	Sinopharm	Inactivated			On the market (China)	72.5% efficacy
Sinopharm (Beijing)	BBIBP-CorV	Inactivated			On the market (China)	78.1% efficacy
Bharat Biotech	BBV152	Whole-Virion Inactivated			Emergency use (India)	77.8% efficacy
Novavax	NVX-CoV2373	Full length recombinant SARS CoV-2 glycoprotein nanoparticle vaccine adjuvanted with Matrix M	Recombinant vaccines	Have a distinct composition and a high level of safety and stability, but weak immunogenicity and require adjuvants.	Phase 3	overall 95.6% efficacy

Source: *https://www.who.int/emergencies/diseases/novel-coronavirus-2019/covid-19-vaccines*

Table 2. Vaccine efficacy against variants of concern (5)

WHO label	Lineage	First discovery place	Date of confirmation	Mutations	Vaccine Efficacy aginast variants
Alpha	B.1.1.7	United Kingdom	18-12-2020	N501Y substitution in the RBD region, H69/V70 deletion in the N-terminal region, and P681H mutation adjacent to the furin cleavage site in the S protein. D614G mutation	NVX-CoV2373 (85.6%)
Beta	B.1.351	South Africa	18-12-2020	K417N, E484K, N501Y, and D614G mutation	NVX-CoV2373 (49.4%) ChAdOx1 nCoV-19 (10%)
Gamma	P.1	Brizal	11-01-2021	K417T, E484K, and N501Y substitution in the RBD domain, and D614G mutation	unknown
Delta	B.1.617.2	India	11-05-2021 labled as VOC	more mutations in the spike protein, namely T19R, DEL157/158, T478K and D950N, D614G mutation	unknown

Source: *https://www.ctvnews.ca/health/coronavirus/b-1-617-variant-first-identified-in-india-classified-as-variant-of-global-concern-1.5421363*

is also a great concern (*26*). (Table 2)

4.2. The challenges of globally promoting and distributing vaccines

As of June 25, 2021, 60% of people are fully vaccinated against COVID-19 in Israel, and only 4.3% were partly vaccinated. A high level of vaccination coverage was noted in China (51% had received the 2nd dose, 13% had received the 1st), the United Kingdom (47% had received the 2nd dose, 17% had received the 1st), and the United States (45% had received the 2nd dose, 8.1% had received the 1st). The cumulative number of doses administered per 100 people was 123.44 in Israel. In some areas, however, such as South Africa countries, the total number of vaccinated per 100 people was less than 1 (*21*). The large differences in the rate vaccination coverage between countries imply significant inequality in vaccine distribution, which may be caused by different national economics, difficulties with logistics and storage, lower rates of vaccination, the extent to which vaccination has been promoted by the government (*27*).

Moreover, different countries have taken markedly different approaches to vaccination. European Union countries like the United Kingdom have adopt a 'first dose first' approach. This vaccination strategy priority promotes wider coverage with the first dose while delaying administration of the second dose. Other countries, such as Israel, the United States, and China taken the approach of fully vaccinating smaller populations first. Different approaches also led to different rates of vaccination coverage among different populations worldwide (*20,28-31*). The rapid development of COVID-19 vaccines within one year has been a great breakthrough in scientific and economic cooperation among countries. However, now that vaccines are being developed, the question is whether the global distribution of vaccines can match the speed of the COVID-19 pandemic, *i.e*. whether those vaccines can be administered quickly and widely distributed around the world (*32*).

5. Strategies for development of future COVID-19 vaccines and vaccination coverage

5.1 Optimizing the vaccine platform, learning from Novavax

Thus far, only a few clinical trials have investigated the effects of COVID-19 vaccines on variants. A phase 3 trial was conducted in South Africa to assess the efficacy of a single dose of the Ad26.COV2.S vaccine (Johnson & Johnson/Janssen). Efficacy was reported to be 52% at 14 days and 64% at 28 days after the first dose of Ad26.COV2.S vaccine. The trial was contemporaneous with 95% of subjects being infected with the B.1.351 variant, but no vaccine is reported to be effective against the B.1.351 variant (*33,34*). Another phase 2 trial was also conducted in South Africa to evaluate the efficacy of the ChAdOx1 nCoV-19 vaccine (AstraZeneca). However, overall efficacy was only 22% and efficacy against the B.1.351 variant was only 10% (*35*).

Most recently, the efficacy of the NVX-CoV2373 vaccine against variants was examined in a phase III clinical trial. Results indicated that the efficacy of the NVX-CoV2373 vaccine against SARS-CoV-2 was 95.6%, that against the UK variant was 85.6%, and that against the South African variant was 49.4%. NVX-CoV2373 is the only vaccine effective against COVID-19 variants, but the next generation of vaccines will presumably be developed worldwide. A point worth noting is that the platform for NVX-CoV2373 used recombinant nanoparticle technology to generate an antigen from the spike (S) protein. The patented saponin-based Matrix-M™ adjuvant in Novavax was designed to promote a humoral and cellular immune response (*35*). (Table 2)

The efficacy of NVX-CoV2373 in clinical trials implies that the next generation of vaccines, and especially protein vaccines, need to optimize their

protein targets to enhance immunogenicity and to cover emerging and potential mutation sites. Moreover, appropriate adjuvants are useful at enhancing the immune response, and especially at inducing high levels of humoral immunity. As SARS-CoV-2 variants emerge, their surveillance, detection of neutralization antibodies, and the immune response to them in clinical trials are also crucial.

5.2. Optimizing vaccination strategies combined with non-pharmaceutical interventions

In rich countries such as Israel, promising vaccines are easier to develop and vaccination strategies are easier to optimize, and the country has done remarkably well with its vaccination efforts. In developing countries, however, vaccines are uncertain, so non-pharmaceutical interventions to combat COVID-19 was useful at limiting the spread of the virus and its variants. Under such conditions, the best strategies are social distancing, attention to personal hygiene, frequently handwashing, and mask wear while awaiting vaccines and affordable drugs (*2*).

Overall, vaccination strategies should be optimized in combination with non-pharmaceutical interventions to control the epidemic. Non-pharmaceutical interventions should be implemented as much as possible in developing or low-income countries. In the war against viruses, the faster the public health interventions and the more optimal the vaccine options, the more likely we are to win.

6. Discussion

Given problems with the efficacy of COVID-19 vaccines against variants, NVX-CoV2373 was a great scientific breakthrough, marking the first human victory in the battle against the virus and its variants. However, countries cannot rely on vaccines alone. Appropriate vaccination strategies and sufficient vaccination coverage, combined with non-pharmaceutical interventions, are key steps in controlling the global COVID-19 pandemic.

For a more complete and rapid future public health response to a disease like COVID-19, several aspects should be taken into account. First, the design of and clinical trials on next-generation vaccines have to take emerging variants into account. Second, countries where vaccines are lacking should to adopt a more flexible vaccination strategy since "administering a second dose of another vaccine is better than not administering one at all". As an example, further evidence is need to verify which types of vaccines can be mixed and which vaccines have sufficient safety and efficacy to cope with a temporary shortage of a given vaccine (*36,37*). Third, special populations, including the elderly, infants, and immunocompromised patients such as those with AIDS or cancer, are not yet fully covered in clinical trials on COVID-19 vaccines and should be considered in the future (*35,38*).

Once COVID-19 vaccines are developed, widespread vaccine distribution and adequate vaccination coverage are also crucial steps. Thus, a plan for widespread vaccine distribution is needed based on different demographics, logistics, and acceptance and with the cooperation of various levels of government (*39,40*). Lastly, non-pharmaceutical interventions should be implemented more rigorously, and especially in countries with insufficient COVID-19 vaccination coverage.

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Mini-Review

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Antimicrobial resistance and COVID-19 syndemic: Impact on public health

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SUMMARY The COVID -19 pandemic has had a catastrophic impact on the global economy and the healthcare industry. Unfortunately, the scientific community still hasn't discovered a definite cure for this virus. Also, owing to the unscrupulous use of antibiotics in wake of the current situation, another ongoing pandemic of antimicrobial resistance (AMR) has been entirely eclipsed. However, increased compliance to infection control measures like hand hygiene (both at hospital and community level), and restricted travel might be favorable. It is evident that the AMR strategies will be impacted disproportionately varying with the respective policies followed by the countries and hospitals to deal with the pandemic. The vaccination drive initiated globally has provided a glimmer of hope. In this article, the possible reciprocity between the two contemporaneous pandemics has been addressed. The world needs to be vigilant to punctuate the symphony between these lethal threats to global health. The restraint to combat against AMR will be boosted as our discernment of the problem also changes with the epidemiological interplay becoming more apparent in near future.

Keywords COVID-19, antimicrobial resistance (AMR), MDR, pandemic

1. Introduction

It has been more than a year since the COVID-19 pandemic has had a catastrophic impact on the global economy and the healthcare industry. The world going into a complete lockdown was beyond imagination. According to the World Health Organization (WHO), even 173,331,478 confirmed cases and 3,735,571 deaths later (as on 8th June 2021 as per WHO COVID-19 dashboard) (*1*), and despite scientists and doctors working tirelessly, the world still hasn't managed to discover a definite cure for this virus. However, in our desperation to deal with the current situation, another ongoing pandemic of antimicrobial resistance (AMR) has been overshadowed. With an increased compliance to infection control measures like hand hygiene (both at hospital and community level), but also a documented rise in antimicrobial use, a debate is pertinent as to whether the COVID-19 pandemic has been a boon or bane for the menace of AMR (*2-4*). The recent vaccination drive across the world might bring an end to the pandemic, but the repercussions on the AMR program will subsequently be observed over a long period. The authors would discuss the same through this review.

2. How big is the problem?

AMR was certainly one of the biggest concerns of microbiologists and clinicians throughout the world in the pre-COVID era. High resistance rates have been reported for organisms like *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecium*, *Pseudomonas spp*., *Acinetobacter baumanii*, *etc*., causing common bacterial infections like urinary tract infections, respiratory tract infections, skin and soft tissue infections subsequently leading to life-threatening events. These organisms have developed resistance to even the last-resort antibiotics including carbapenems, polymyxins, and glycopeptides. Approximately, 700,000 deaths/year are attributed to AMR worldwide. At this rate, the number is expected to rise to an estimated ten million deaths by 2050 (*5,6*). India has been hit even worse with the challenge of AMR. Increasing rates of multidrug resistant pathogens have been reported across national surveys. As per available data, while in 2008, about 29% of *Staphylococcus aureus* isolates were methicillin resistant, by 2014, the percentage had risen to 47%. A multi-centric study carried across seven cities in India reported Extended Spectrum β-Lactamase (ESBL)

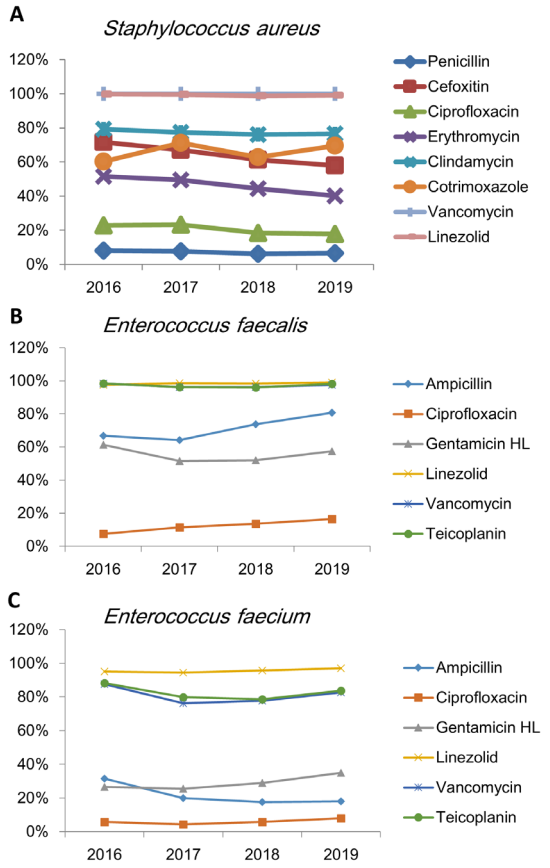

Figure 1. Susceptibility trends of commonly isolated Gram positive organisms. (A) *Staphylococcus aureus*, **(B)** *Enterococcus faecalis*, **(C)** *Enterococcus faecium*. The figure is prepared by the authors based on the published susceptibility data of commonly isolated Gram positive organisms in the Annual reports (2016-2019) of Antimicrobial Resistance Surveillance and Research Network, an initiative of Indian Council of Medical Research (ICMR).

production in 61% *E. coli* and carbapenem resistance in 31-51% *Klebsiella spp*. Amongst *Pseudomonas spp*., 65% were found to be resistant to ceftazidime and 42% to imipenem (*7*). The current susceptibility trends of commonly isolated Gram-positive (GP) and Gram-negative (GN) bacteria have been depicted in Figures 1 and 2 respectively (*8*). Along with being a public health problem, AMR is also a contributor to the economic loss for the country due to increased hospital stays and a requirement of repeat surgical interventions on account of complications as a result of infections.

3. What steps were being taken?

Fortunately, the governments across the world realized this issue in time and had started working on the menace of AMR far before the COVID pandemic doomed us. A multi-faceted approach needs to be followed to control the resistance development and transmission. Various stakeholders need to collaborate and work on altering human behavior, animal rearing practices, agricultural practices and the environment as a whole such that it results in good health for all. Excess use of antimicrobials ultimately leading to AMR might be dealt with surveillance of all these aspects under the One health approach (*9,10*). Interventions to emphasize rational use of antibiotics must address important issues like restricting antimicrobial use without impacting their availability for patients especially in low and middle-income countries (LMIC). The said response may be achieved by a two-tier approach, one targeting the AMR specifically and antimicrobial

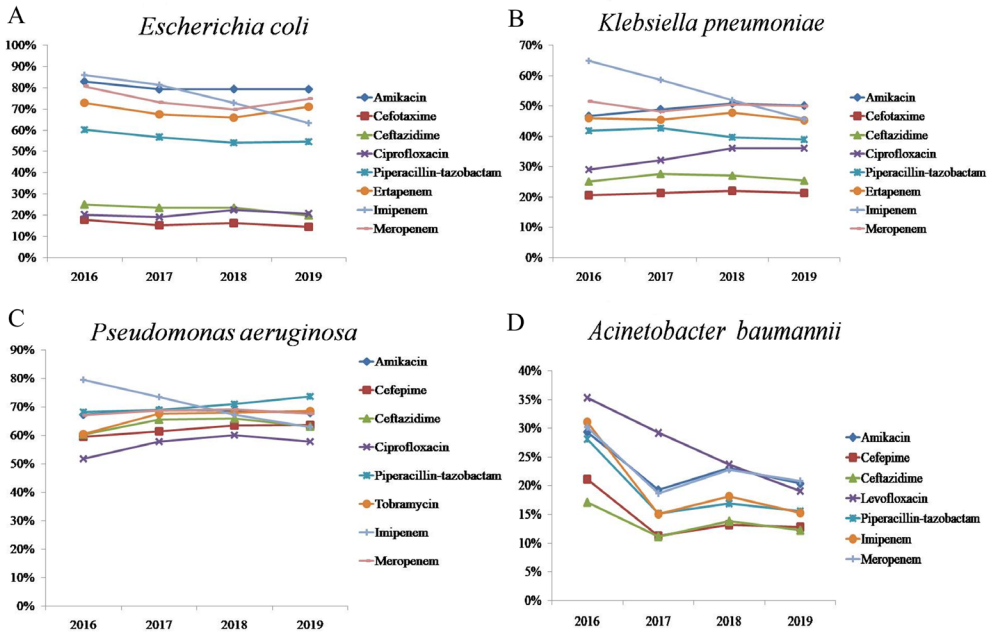

Figure 2. Susceptibility trends of commonly isolated Gram negative organisms. (A) *Escherichia coli*, **(B)** *Klebsiella pneumoniae*, **(C)** *Pseudomonas aeruginosa*, **(D)** *Acinetobacter baumannii*. The figure is prepared by the authors based on the published susceptibility data of commonly isolated Gram negative organisms in the Annual reports (2016-2019) of Antimicrobial Resistance Surveillance and Research Network, an initiative of Indian Council of Medical Research (ICMR).

usage and the other dealing with the aspects like improved infection control practices, sanitation, and hygiene in general. Globally, the United Nations (UN), Food and Agriculture Organization (FAO), the World Organization for Animal Health (OIE), and the World Health Organization (WHO), formalized in 2010 as the Tripartite, have been the guiding forces to tackle AMR by coordinating and sharing relevant information amongst the stakeholders (*11*).

The 68th World Health Assembly (WHA), in May 2015, endorsed the Global Action Plan on Antimicrobial Resistance (GAP-AMR). Antibiotic resistance was included as being one of the most urgent drug trends. All member states were urged to align their National Action Plan on AMR with GAP-AMR by May 2017. The Government of India in collaboration with the World Health Organization (WHO) organized an international conference in February 2016 pertaining to "Combating Antimicrobial Resistance: A Public Health Challenge and Priority", which was attended by more than 350 participants. The UN General Assembly adopted the Political Declaration on Antimicrobial Resistance in September 2016. This marked the formalization of a major step towards strengthening the AMR response by collaborative efforts of various nations (*7*). The National Health Policy in India released in 2017 identified AMR as a menace and called for effective action to address it. India got enrolled in the Global Antimicrobial Resistance Surveillance System (GLASS) network in 2017. Globally, various hospitals and institutions have been sharing their data with the WHO to enumerate the exact situation of AMR for years so that an effective strategy might be worked out. To fortify and re-enforce AMR surveillance in India, the Indian Association of Medical Microbiologists (IAMM) is collaborating with the WHO to establish a supplementary AMR surveillance network called the WHO-IAMM Network for Surveillance of Antimicrobial Resistance (WINSAR). It shall serve as a common dynamic platform to share and assess dependable AMR data from hospitals/laboratories (both government and private) across India.

Hence, immense work was being done across the world on the line of AMR. However, it got entirely eclipsed in the COVID era.

4. Is COVID a blessing in disguise: A boon from AMR's perspective?

AMR is associated with numerous factors like an indiscriminate antibiotic use, suboptimal dosing, antagonistic combination prescription, *etc*. However, the global resistance rates are influenced by the spread of the organism as much as they depend on the irrational antimicrobial usage (*4*). Since time immemorial, microbiologists have been advocating the importance of hand hygiene on multiple platforms. But it took a massive pandemic for the public to understand the same. The COVID-19 pandemic has fortunately paved the way for improved infection control practices, both at the level of healthcare facilities as well as the community per se. Most resistant organisms like methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE) and carbapenem-resistant *Enterobacterales* (CRE) have been reported to get transmitted through person to person contact. People have now been washing hands more frequently, thus possibly limiting the spread of resistant micro-organisms (*4,12*). Another argument in favor of this pandemic is the immense reductions it brought in travel which may negatively impact the spread of AMR. There has been indisputable evidence for the transmission of AMR through the key genes (*13*). These genes are harbored and transferred by the organisms present not only in the contaminated food and vegetables but also those colonizing the animals and humans (*12,14*). For instance, as published by Shwartz *et al*., more than 60% of travelers to India were later found to be colonized with an ESBL organism while approximately 0.4% with CRE (*15*). The detection and global dissemination of carbapenem resistance through the *NDM-1* gene and colistin resistance through the *mcr-1* gene has been widely reported (*16,17*). And hence, the COVID pandemic might have proven to be a blessing in disguise from the AMR perspective.

5. Will it curtail our progress and worsen the challenge of AMR: A bane?

While we may acknowledge that the COVID-19 pandemic might have been a boon from the AMR perspective, there are various arguments against this belief. Firstly, although it is understandable that owing to the lockdown and a general fear of visiting hospitals, the patient attendance in outpatient departments of hospitals might have decreased. However, the probability of patients being administered empirical broad-spectrum antibiotics is quite high. Additionally, the risk factors predisposing for AMR infections like diabetes, chronic systemic illnesses, old age, malignancies, *etc*. are also the co-morbidities associated with worse outcomes in COVID-19. The prolonged stays in intensive care units, with increased morbidity and mortality amongst patients, along with clinicians' concern and probable inability to diagnose secondary bacterial infections due to lack of infrastructure, has not only augmented the prescribing of antimicrobials but also has indirectly contributed to the rise of AMR. A study done by Chen *et al*. reported that 15% and 71% of COVID-19 patients were administered anti-fungal and antibiotic treatments, respectively. Out of these 25% received a single antibiotic while 45% received combination therapy. The spectrum of antimicrobials used included cephalosporins, fluoroquinolones, carbapenems, tigecycline, and linezolid (*18*).

Randomized trials from China evaluating remdesivir and lopinavir/ritonavir reported that antimicrobials were prescribed in approximately 90% of patients (*2,19,20*). An eye-opener meta-analysis published recently by Langford *et al*. on the bacterial co-infections and secondary infections in COVID-19 patients, distinctly mentioned that although the bacterial infection was found to be ranging from 5.9-8.1%, the patients receiving antibiotics were almost 70% including mainly fluoroquinolones and third-generation cephalosporins (*21*). Another review from Asia with similar results reported the prescription of antimicrobials in around 70% of patients in spite of the bacterial or fungal co-infection rates reported to be less than 10% (*22*). Such non-judicious use of antimicrobials will negatively impact the antimicrobial stewardship program. Also, crowded living spaces like prisons, psychiatric hospitals, and nursing homes, *etc*. have been associated with a majority of their inhabitants getting infected and admitted with COVID-19 (*23*). Patients from these facilities especially nursing homes and hospitals may harbor highly resistant microbes including carbapenemase-producing organisms (CPO), MRSA, VRE, and fungi like *Candida* (*3*). Understanding transmission in such settlements would be challenging. Another worrisome consequence of this pandemic was the inadvertent use of broad-spectrum antimicrobials like azithromycin with/without hydroxychloroquine and doxycycline due to random reports claiming a possible therapy option (*24,25*) with inadequate evidence (*26*). While data is still scarce, the widespread prescription of these drugs has negatively impacted the AMR measures. Secondly, some of the countries worst hit by the pandemic including China, India, Italy, Spain, USA have already been AMR hotspots dealing with various multidrug-resistant (MDR) and pan drug resistant (PDR) bugs (*3,27,28*). Further, the LMICs, with improper sanitation, poor infrastructure, and quality of healthcare as well as restricted preparedness for outbreaks are prospective epicenters for AMR spread. Thirdly, the use of sanitizers, antimicrobial soaps, and disinfectant cleaners has exponentially increased, both in the hospitals as a part of the infection control protocols as well as in the community due to the sensitization of public in general. It can now be recognized as an individual habit that might prove instrumental in reducing the spread of AMR. However, the possibility of these products containing biocides, *i.e*, antimicrobials in disinfectants and cleaners cannot be ruled out (*29-33*). Their increased usage during the COVID-19 pandemic and beyond will lead to increased levels of biocides in wastewater treatment plants and the environment in general which will not only be a health hazard for the exposed individuals but also a public health concern due to its anticipated contribution towards AMR (*12*). Fourthly, the pandemic has not only caused an overloading of the healthcare capacities and laboratory systems but also contributed towards disruptions in working of various industries including research and development (pharmaceuticals), vaccine production, and agricultural industry. The environmental surveillance activities and screening cultures of patients for resistant organisms in hospitals have been affected as most of the workforce has been assigned towards COVID-19. The entire research industry has been redirected and obsessed with the development of new diagnostic tests, medicines, and vaccines for COVID-19. All work for other diseases like influenza, HIV, dengue, malaria, TB, *etc*. has suffered. Moreover, due to the lockdown, vaccine production and delivery systems have been affected. The catastrophic consequences due to shortages in vaccines for influenza, TB, measles, pneumococcal pneumonia, and other infectious diseases can be anticipated (*34*). The agricultural industry has also been hit thus endangering the food supply chains. Elevated usage of antibiotics for food production is associated with the risk of AMR by the development of resistance within the microbiome of exposed animals as well as by environmental leakage in excreta and wastewaters (*34*). Subsequently, the risk of circulation and dissemination of novel resistance genes will also rise once the travel resumes.

6. What might be done?

6.1. At the level of the laboratory

The clinical microbiologists, today have the cardinal responsibility of conquering antimicrobial resistance. Their work should not just be limited to reporting results in the laboratory but to discuss those reports with the respective clinicians. The clinicians need to be made aware of the advancements in the field of microbiology, the upcoming antibiotics, and the spectrum and uses of the existing ones. Regular training sessions should be held across specialties to discuss their concerns regarding the same. The antibiotic policies may be made not only considering the antibiogram of the hospital or ward but also the specialty involved. The microbiologists may be made a part of the clinical rounds so that they can participate and suggest with due consideration to infection control practices as well the antibiotic use. Escalation or de-escalation of the antibiotics may be suggested on the rounds as per the culture grown in the lab. Also, a very important step towards rationalizing the use of antibiotics is the strict compliance to graded reporting of antimicrobials as suggested by the Clinical and Laboratory Standards Institute (CLSI), *i.e*., the sensitivity pattern for higher second-line drugs might be held and not reported in case the first line is sensitive. In case the pattern for the second-line drug is specifically required, the clinician might discuss and inform.

6.2. At the level of the clinicians

The prime task of rational antimicrobial prescription lies at the hands of the clinicians. The right antimicrobial therapy depends on a lot of factors. The first and foremost is diagnosing the right site of infection with detailed history taking and adequate investigations, and prescribing targeted therapy for the same. This should be followed by the choice of the right antibiotic, right dose, and the right route. They need to take into account the pharmacokinetics-phamacodynamics (PK/PD) of the drug that they prescribe. Due consideration should be given to drug interactions. Also, renal or hepatic drug dose modifications need to be considered. The timing for sending culture samples has to be strictly monitored. The hospital should have an Antimicrobial Stewardship Program (AMSP) which should be instrumental in devising the antibiogram and antibiotic policy, guiding the empirical antimicrobial therapy, and monitor the compliance to policy, usage of restricted antimicrobials as well as escalation/de-escalation based on culture results and clinical scenario. The clinicians should actively participate in the policy-making of Hospital Infection Control (HIC) protocols and abide by the same.

6.3. At the level of Government

The government may help by incorporating AMR policies in the national programs. Data capturing might be made mandatory at a national level. Personnel may be held responsible at national and state levels to coordinate the data delivery and submission. All such data should be shared and analyzed at an international level by a single agency. Another step that might be significant is stopping the Over-The-Counter (OTC) sale of antimicrobials. They should be sold only on prescription by registered medical professionals so that indiscriminate use may be put to an end.

7. Conclusion

As is evident from the above review, the probability of the COVID-19 pandemic negatively impacting our fight against AMR far outweighs that of it having a positive outcome. However, reports are still scarce and the likelihood of the data being potentially underestimated and undocumented cannot be excluded. It can be well anticipated that the impact of the current pandemic, from AMR perspective might be disproportionate, varying with geographical regions and even different hospitals/areas within the same region. It will depend on the respective strategies followed by the countries and hospitals to deal with the pandemic. It still needs to be seen whether the vaccination drive started globally will be able to vanquish the pandemic or the world is still about to witness another wave of new mutated COVID strains. Nevertheless, with the current usage of antimicrobials, several of which have been classified as restricted and the last resort drugs, the implications of AMR on the human and animal health and per se environment will be unimaginable and should be dealt with caution.

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Review

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Management of dengue with co-infections: an updated narrative review

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SUMMARY Dengue is a life-threatening mosquito borne viral disease. We are still in the era of supportive treatment where morbidity and mortality are a major concern. Dengue infection in presence of other co-infections makes this scenario rather worse. Timely recognition and raising alarm to be intensive is the need of the hour for primary care physicians practicing in the community and indoors. This review provides a comprehensive knowledge about the recent trends of coinfection in dengue as well as their management consideration which will be particularly helpful for physicians practicing in rural and remote areas of India.

Keywords Dengue, co-infection, management, approach, atypical Dengue

1. Introduction

Dengue infection has rapidly emerged as the most important mosquito-borne viral disease in the last few decades. According to the World Health Organization (WHO) estimates, the disease is endemic in more than 100 countries, with about 390 million new dengue infections occurring annually, and there has been a steady increase in these numbers (*1*). Dengue fever typically presents as a self-limiting disease, with a mortality rate of less than 1%. Severe dengue accounting for 3-4% of dengue fever cases, has a mortality rate of 2-5% when adequately treated, but if not treated, the mortality rate rises to as high as 50%. Dengue patients having co-infections with other microorganisms are associated with a greater risk of severe disease and mortality (*2*). Adding to this picture is the risk of morbidity and mortality associated with coinfections. The aim of this review was to give a comprehensive picture about the different clinical presentation and management issues of dengue with different coinfection particularly in respect to Indian setting.

2. Dengue serotypes and association with virulence

The dengue virus has four antigenic groups (serotypes) and each serotype is further divided into one to six genetic groups (genotypes) as shown in Table 1. The four serotypes dengue virus 1-4 (DENV 1-4) of the virus share almost 65-70% sequence homology with each other. These viruses have the highest mutation rate among the flaviviruses, leading to the generation of different genotypes and lineages within each serotype. More recently, a fifth serotype has been discovered in 2013 from the forests of Sarawak, Malaysia.

Infection with any of the four dengue serotypes can progress to severe dengue infection. However, DENV-2 is found to be most virulent amongst the four strains having association with most of the severe cases. With the advent of newer technologies for the genetic analyses of viruses, studies from the Western hemisphere have deciphered that severe dengue is mostly associated with infection with specific genotypes of dengue virus within each serotype (*3*). The initial descriptions of differences in DENV virulence came from epidemiologic and entomologic studies conducted in the South Pacific by Gubler *et al*. (*4*). Some outbreaks in this region had very few or no cases of severe dengue, and the transmitted viruses were considered to be of lower virulence as compared to other outbreaks that had many cases of severe dengue, after primary infection and thus considering the latter viruses to be more virulent (*5*).

3. When to suspect co-infections?

Differentiating patients of dengue fever from patients with other co-infections such as malaria, chikungunya, enteric fever, scrub typhus, leptospirosis on clinical grounds alone is difficult on many occasions. The clinical presentations may be similar in few cases of co-infection, but majority of them present with more severe manifestations and complications. Hence having a high index of clinical suspicion for co-infection is essential in order to make a timely diagnosis and administration of the specific treatment required. Symptoms and clinical findings that may suggest co-infections are depicted in Table 2.

Enteric fever can manifest with a wide variety of symptoms and is essentially a clinical diagnosis. However, much of the knowledge on the clinical manifestations of the same disease as a coinfection in dengue are largely based on case reports. In patients with dengue, enteric fever should be suspected when there is persistence of fever beyond first week of illness with prominent gastrointestinal symptoms (*5,6*).

Patients can have urinary tract infection (UTI) with gram negative bacilli during the course of hospital stay. The symptoms of superadded UTI are usually prolonged fever beyond first week with or without lower urinary tract symptoms (LUTS) like dysuria, increased frequency, urgency (*6*). Appropriate culture based antibiotics is usually adequate for treatment of UTI in dengue (Table 3).

Dengue when presents with any coinfection will have additional laboratory abnormalities apart from those caused by DENV depending on the co-infecting pathogen. Table 4 represents prominent laboratory features of some of the common infections that can occur during dengue season in India.

Table 1. Different dengue virus serotypes

Serotype	Number of genotypes
DENV 1	5
DENV 2	6
DENV 3	4
DENV 4	4
DENV 5	Sylvatic

4. Reinfection by dengue serotype

Several epidemiologic studies have shown that prior dengue exposure is significantly associated with

Table 2. Clinical features distinguishing different aetiologies of co-infection in dengue

Dengue with	Fever more than 5-7 days	Cough	AKI	Jaundice	GI	Spleen	Eschar	HRS	LN	Altered mental status, seizures	Rash	Red eyes	Myalgia	Joint pain
Enteric fever	√				√	√				√	+/-			
UTI	√													
Leptospirosis	√		√					√				√	√	
Malaria	√		√							√				
Scrub	√	+/-	√			√	√	√	√	√	√			
Chikungunya										+/-	√			√
Zika											√	√	√	√
COVID 19		√	+/-	+/-	Diarrhoea								√	

AKI, acute kidney injury; GI, gastrointestinal; HRS, hepato renal syndrome; LN, lymph node; UTI, urinary tract infection; COVID, corona virus disease.

Table 3. The diagnostic tests for co-infection

Diseases	Tests	Sample
Dengue	NS1 antigen ELISA or RT PCR: For < 5 days of illness IgM capture ELISA (MAC-ELISA): For > 5 days of illness	Blood/Serum
Chikungunya	Early disease: RT PCR After first week of illness: IgM capture ELISA	Blood/Serum
COVID 19	Acute phase: RT PCR	Nasopharyngeal/ Oropharyngeal swab
Malaria	RDT (bi-valent both Pf/Pv detection) Quality microscopy for slide positivity confirmation	Blood Blood
Leptospirosis	In endemic areas: IgM ELISA and MAT tests Non-endemic areas: IgM ELISA followed by MAT test for confirmation	Serum Serum
Scrub Typhus	Detection of IgM antibodies by Weil-Felix Test (WFT) Enzyme linked Immunosorbent assay (ELISA)	Serum Serum

Table 4. Prominent laboratory features of different mono-infections

Items	Leucocytosis	Leucopenia	Anemia	TCP	Transaminitis	Raised bilirubin	Raised cre-atinine	Coagulopathy
Enteric fever	√	Lymphopenia						
Chikungunya		Lymphopenia		√				
UTI	√						√	
Malaria			√	√	√	√	√	√
Leptospirosis	√Neutrophilia		√	√	√	√	√	√
Scrub	√				√		√	√
Zika	Eosinopenia			+/-				
COVID 19		√			√			√

TCP- thrombocytopenia

greater risk of severe disease when encountered with a secondary dengue virus infection, than during the primary infection. This phenomenon can be explained by the Halstead theory of antibody mediated immune enhancement and has been supported by several observations. Cuba experienced an outbreak of DENV-2 in 1981, which was preceded by DENV-1 in 1977. The outbreak of 1981 affected 45% of the nation's population, with 98% of severe cases occurring in children and adults being associated with secondary infections (*7,8*). A prospective study conducted in Bangkok in 1980 showed that hospitalization was not required in any of the 47 children with primary dengue infection as compared to the children with secondary infections, of whom 7 of 56 required hospitalization (*9*).

5. Different serotypes

Dengue infection can occur with various serotypes at the same time in any permutation and combination. Depending on infection with specific combination of serotypes the severity also differs. According to a meta-analysis by Soo *et al*., primary infection with DENV2 followed by a secondary infection with DENV 2, DENV3 and DENV4 were associated with a more severe outcome in south east Asian region (*3*).

6. Malaria

Malaria, another mosquito-borne disease, is a common co-infection with dengue fever. It is widely endemic in the countries where dengue also causes a menace, including India. The transmission period of malaria also coincides with dengue infection. Exclusion of the diagnosis of malaria should be done as early as possible as malaria treatment requires administration of artemisinin combined treatment (ACT), which yields very good results. There have been recent reports of complicated vivax malaria being on rise, with a large number of the patients presenting with fever and thrombocytopenia. In endemic areas for dengue and malaria, jaundice (in dengue patients) and spontaneous bleeding (in malaria patients) should raise the suspicion of co-infection. Testing of blood with rapid diagnostic test (RDT) kit for malaria should be done at the first presentation in all the patients suspected with malaria.

Antimalarial treatment should be begun at the earliest in order to prevent complications and ensure better outcome in case of co-infection. In a bi-variate analysis study conducted in French Guiana from 2004 to 2010 amongst 104 hospitalized patients with dengue co-infection and 208 controls with dengue and malaria mono-infections, the results revealed that the clinical picture of dengue and malaria co-infection was more severe than mono-infections, and the co-infected patients were at a greater risk of severe thrombocytopenia and anaemia (*10*). A cross-sectional study was conducted from 2009 to 2011 in Brazilian Amazon, among the 1,578 hospitalized patients, of which, 176 (11.1%) presented with *P. vivax* malaria mono-infection, 584 (37%) had dengue fever mono-infection, and 44 (2.8%) of them were co-infected. It was observed that the co-infected patients had a higher incidence of presenting with severe disease (*vs*. dengue mono-infected), with features such as severe bleeding (*vs*. *P. vivax* mono-infected), and hepatic manifestations such as hepatomegaly, and jaundice (*vs*. dengue mono-infected) (*11*). The below mentioned Table 5 shows different studies worldwide on dengue and malaria co-infection.

7. Enteric fever

Incidence of water borne diseases such as enteric fever and gastroenteritis also rises during the monsoon season, when the dengue infection is also reported in large numbers. There is scarcity of data on dengue-typhoid co-infection in medical literature from both developed and developing countries. There are many features common to both the diseases, including leucopenia and high grade fever. Early administration of antibiotics is essential in cases of dengue-typhoid co-infection in order to prevent the development of complications. In patients with high index of suspicion of enteric fever and dengue co-infection (*i.e*. with fever > 5 days), early blood cultures should be sent for detection of typhoid fever and empirical antibiotics need to be started (*12-14*). The list of studies in dengue-typhoid coinfection is depicted in Table 6.

In a study conducted by Capeding *et al*., in 2013 in 5 Asian countries, out of 71 patients positive for dengue,

Table 5. Studies on malaria and dengue coinfection

S.N	Author	Title	Journal	Year	Place	Outcome /Conclusion	Finding/Significance (*p* value)
1	Magalhaes BM, *et al*., 2014 (*11*)	*P. vivax* malaria and dengue fever co-infection: a cross-sectional study in the Brazilian Amazon.	PLoS Negl Trop Dis	2014	Brazil	Jaundice in dengue and spontaneous bleeding in malaria should raise the suspicion of coinfection.	
2	Epelboin L, *et al*., 2013 (*12*)	Discriminating malaria from dengue fever in endemic areas	Plos Negl Trop Dis	2013	French Guiana	CRP > 5 mg/dL independently associated with malaria compared to dengue.	< 0.001
3	Kotepui M, *et al*.,2017 (*13*)	Differentiating between dengue fever and malaria using hematological parameters in endemic areas of Thailand.	Infect Dis Poverty	2017	Thailand	Decision tree model starting with node as neutrophil count and ending in leaf as dengue or malaria.	
4	Barua A, *et al*., 2016 (*14*)	A comparative study of concurrent dengue and malaria infection with their mono infection in a teaching hospital in Mumbai.	JAPI	2016	India	Epigastric discomfort, anemia, low haematocrit and transaminitis in coinfection group	< 0.001

Table 6. Dengue Typhoid co-infection

S.N	Author	Title	Year	Place	Journal	Finding
1	SharmaY, *et al*., 2014 (*16*)	Dengue and Typhoid Co-infection–Study from a Government Hospital in North Delhi	2014	New Delhi, India	J Clin Diagn Res	7.8% of dengue cases coinfected with enteric fever
2	See KC, *et al*., 2013 (*17*)	Identification of concurrent bacterial infection in adult patients with dengue	2013	Singapore	Am J Trop Med Hyg	4.65% culture proven enteric fever coinfection on dengue cases
3	Thein TL, *et al*., 2017 (*18*)	Risk factors for concurrent bacteremia in adult patients with dengue	2017	Singapore	J Microbiol Immunol Infect	17.2% of enteric fever among bacteremia detected in dengue cases

17 of them had co-infection with enteric fever (*15*). In an observational study from North India, the prevalence of dengue and enteric fever coinfection was found in 7.8% of proven dengue cases (*16*). In another study from Singapore, the prevalence of culture proven enteric fever was found to be 4.65% (*17*). A similar study from Singapore in 2017 showed 17.2% prevalence of enteric fever among bacteremia detected in dengue cases (*18*). Co-infections with enteric fever if present can modify the clinical presentation of dengue, thus resulting in missed or delayed diagnosis and management of dengue shock. The management should include administration of ceftriaxone and/or azithromycin at the earliest suspicion to avoid complications.

8. Chikungunya

Chikungunya and dengue fever have been reported to be prevalent at the same time from many geographical areas. Halstead *et al*. in 1969 reported the first cases of dengue-chikungunya co-infection in Thailand. They detected four co-infected cases among 150 patients diagnosed with either dengue or chikungunya virus (CHIKV) (2.6%) (19). Acute severe complications have sometimes been reported in cases of chikungunya co-infection with dengue fever. Predominant joint involvement along with prodromal symptoms and occurrence of similar acute onset arthritis in the neighbourhood or a proven chikungunya outbreak in an area should raise the suspicion of chikungunya fever. Chikungunya joint pain requires stronger analgesics (nonsteroidal anti-inflammatory drugs (NSAIDs)) in acute stage, which could result in fatal bleeding inpatients co-infected with dengue. Furuya-Kanamori *et al*. in 2016 published a meta-analysis about dengue-chikungunya co-infection (*20*). They searched three biomedical databases (PubMed, Scopus and Web of Science) right from their inception until May 2015, for studies reporting co-infection of chikungunya and dengue viruses from the same patient.

In addition to that, data from WHO, CDC and Health map alerts were also extracted, in order to create up-to-date global distribution maps for both the diseases. The meta-analysis greatly emphasises on the likelihood of misdiagnosis of chikungunya infections in

Table 7. Dengue and chikungunya coinfection

S.N	Author	Title	Journal	Year	Place	Finding
1	Halstead SB, *et al*., 1969 (*19*)	Dengue d chikungunya virus infection in man in Thailand, 1962-1964. II Observations on disease in outpatients	American journal of tropical medicine and hygiene	1969	Thailand	Ten fold risk of progression of dengue to severe dengue in cases misdiagnosed as Chikungunya
2	Furuya-Kanamori L, *et al*., 2016 (*20*)	Co-distribution and co-infection of chikungunya and dengue viruses	BMC Infectious Dis-eases	2016	Africa and south-east Asian countries	Widespread geographical distribution of coinfec-tion
3	Kaur M, *et al*., 2018 (*21*)	Coinfection of chikungunya and dengue viruses: a sero-logical survey from North Western region of Punjab, India	J Lab Physicians	2018	India	9.54% coinfection. Arthralgia and thrombocytopenia significantly higher in co-infected cases
4	Mukherjee S, *et al*., 2017 (*22*)	Evidence of dengue and chikungunya virus co-infection and circulation of multiple dengue serotypes in a recent Indian outbreak	Eur J Clin Microbiol Infect Dis	2017	Kolkata, India	23% coinfection
5	Ramachandran VG, *et al*., 2016 (*23*)	Chikungunya: a reemerging infection spreading during 2010 dengue fever outbreak in National Capital Region of India	Virus Disease	2016	Delhi, India	9.91 % positivity for Chikungunya IgM among suspected dengue sera negative for dengue IgM
6	Kaur N, *et al*., 2017 (*24*)	Chikungunya outbreak in Delhi, India, 2016: report on coinfection status and comorbid conditions in patients	New Microbes New Infect	2016	Delhi, India	25.33% Coinfection
7	Edwards T, *et al*., 2016 (*25*)	Co-infections with Chikungunya and Dengue Viruses, Guatemala, 2015	Emerg Infect Dis.	2016	Guatemala	32% coinfection
8	Londhey V, *et al*., 2016 (*26*)	Dengue and Chikungunya Virus Co-infections: The Inside Story	JAPI	2016	Maharashtra, India	10% coinfection detected by PCR

the background of dengue transmission and vice versa (*20*). Misdiagnosis of dengue fever as chikungunya or missing a dengue infection when co-infection exists carries the risks of delayed or disrupted dengue-specific intensive supportive treatment, having a consequent ten-fold likelihood of progression from dengue fever to severe disease. It is also associated with the risks of inappropriate prescription of arthralgia alleviating non-steroidal anti-inflammatory drugs, usually used to treat chikungunya patients, which could result in severe bleeding in patients with thrombocytopenia or severe dengue. The other potentially more likely scenario where chikungunya infection could be misdiagnosed as dengue or even missed in a co-infected-individual would result in masking of the true geographical extent of CHIKV and the population at risk of infection. A similar scenario resulted in the unsolved issue, when the increased fatality rate reported in chikungunya diagnosed patients in post 2004 epidemic resulted from a mutated CHIKV. Whether it was otherwise attributable to deaths due to a dengue-like illness resulting from increased awareness of chikungunya during the outbreak is still a matter of debate.

Management of both viral disease is supportive treatment and use of safer antipyretic/analgesic like acetaminophen to avoid potential risk of complications associated with usage of NSAIDs. The prevalence of coinfection in India varies depending on geographical location as per published studies. A list of studies on dengue and chikungunya co-infection is depicted in Table 7. Mukherjee *et al*. reported 23% from Kolkata in 2017, Ramachandra and Kaur *et al*. reported 9.91% and 25.33% respectively in the year 2016 from Delhi by PCR based assay from Maharashtra in the same year (*21-23*). There are few other studies which suggest similar incidences. The vast difference in prevalence rates may be a result of cross reactivity or could be a result of varying rates of co-circulation of the viruses in specific geographical areas which depends on many host as well as environmental factors.

9. Scrub typhus

The onset of scrub typhus could be either insidious with headache, anorexia, and malaise, or abrupt with fever and chills. Some important clinical and laboratory findings consistent with scrub typhus include thrombocytopenia, normal or low leukocyte counts, mild to moderate elevations of hepatic aminotransferases. These features are seen as well in cases of dengue fever and hence pose

Table 8. Dengue and scrub typhus coinfection

S.N	Author	Title	Journal	Year	Place	Finding
1	Shelke YP, 2017 (*27*)	Spectrum of infections in acute febrile illness in central India	Indian J Med Microbiol	2017	India	47% prevalence of scrub typhus in acute undifferentiated febrile illness (AUFI) cases
2	Behera B, *et al*., 2019 (*28*)	Clinico-epidemiological analysis of scrub typhus in hospitalised patients pre-senting with acute undifferentiated febrile illness: A hospital-based study from Eastern India	Indian J Med Microbiol	2019	Odissa, India	26.3% scrub typhus prevalence among AUFI cases
3	Basheer A, *et al*., 2016 (*29*)	Clinical and Laboratory Characteristics of Dengue-Orientiatsutsugamushi co-Infection from a Tertiary Care Center in South India	Mediterr J He-matolInfec Dis	2016	India	6 cases of scrub-dengue coinfection from 2010 to 2014

a difficulty in differentiating dengue from scrub typhus. Rash is seen in almost half of the patients with scrub typhus, which is characteristically non-pruritic, macular or maculopapular rash. The rash typically begins on the abdomen and spreads to involve the extremities and the face. Petechial rash may rarely develop. The case-fatality rate in untreated classic cases is about 7-30%. Some patients may develop a localized necrotic skin lesion, the eschar, at the site of the bite of the infected chigger. The eschar may precede the onset of systemic symptoms, and may be found in multiple sites (*24-26*).

Treatment of scrub typhus includes a course of doxycycline or azithromycin for a period of 3-5 days. As dengue has no specific treatment, and scrub typhus responds dramatically to appropriate antibiotics, offering treatment for scrub typhus in suspected cases of co-infection timely can be of great value. Three studies from India are shown in Table 8. In a study from central India, the prevalence was found to be 47% among acute undifferentiated febrile illnesses and an additional cases were found positive by enzyme linked immunosorbent assay (ELISA) among those negative by an initial screening rapid diagnostic test kit (*27*). Another recent study from Odissa, India had reported 26.3% prevalence of scrub typhus among acute febrile illness cases (*28*). Clinical features like regional tender lymphadenopathy, eschars at hidden body parts (axilla, back *etc*.), early hepatic enzymes and creatinine elevation, near normal leukocyte counts, early drop in the platelet counts and hypoalbuminemia in a patient of dengue fever should create suspicion in the clinician and should order additional investigations including a sensitive test for scrub typhus, either IgM detection or PCR if available.

Timely detection and appropriate management of co-infection with O. tsutsugamushi with antibiotic has significant implications as it is likely to reduce the duration of hospital stay of the patient and the cost of therapy. In a study from South India by Basheer *et al*., six cases of dengue co-infected with scrub typhus was reported over a four years period from January 2010 to July 2014. In this study, they observed that co-infection in most cases was characterized by a lower nadir platelet count as compared to scrub typhus, and was associated with a lesser time to nadir platelet count and they had a longer duration of hospital stay as compared to those with either isolated dengue or scrub typhus (*29*).

10. Leptospirosis

There has been increased reports of dengue and leptospirosis coinfection from different parts of India especially the south Indian states and Maharashtra. Every year during rainfall and floods, these states witness a sharp spike in cases of these infections particularly because of stagnant water which acts as breeding ground for mosquitoes and increased rat population feeding on garbage brought to localities by flood water thereby transmitting leptospira in their urine. For the same reasons coinfections are also common during the same time. Clinical features of mild cases of dengue and leptospirosis can be similar. However, severe cases of these two infections have some striking differences which makes diagnosis easier. Dengue usually presents with fever, retro-orbital pain, headache and varying degrees of myalgia.

Myalgia is very pronounced in leptospirosis. Conjunctival haemorrhage and jaundice is very common in leptospirosis compared to dengue. Liver enzymes are moderately elevated, usually above 500 IU/mL whereas in dengue there is mild to moderate transaminitis. Lung and renal involvement is very common in severe cases of leptospirosis known as Weils's syndrome. Severe dengue usually presents with bleeding manifestations and plasma leakage compared to leptospirosis. Various studies across world are shown in Table 9. In a study published from Malaysia in 2018, myalgia, arthralgia, diarrhoea and jaundice were significantly associated with coinfection compared to mono infections (*30*). In a study published from south India in 2018 the prevalence of dengue and leptospirosis coinfection was found to be 3.4% (*31*). In another pilot study published from Malaysia, shock at presentation and male gender were significant predictors of coinfection in multiple logistic regression analysis (*32*).

Table 9. Dengue and leptospirosis coinfection

S.N	Author	Title	Journal	Year	Place	Outcome /Conclusion
1	Hishamshah M, *et al*., 2018 (*30*)	Demographic, clin-ical and laboratory features of lepto-spirosis and dengue co-infection in Malaysia.	J Med Microbiology	2018	Malaygia	Arthralgia, myalgia, diarrhoea, jaundice more in coinfection ($p < 0.05$)
2	Sachu A, *et al*., 2018 (*31*)	Prevalence of dengue and lepto-spirosis co-infection in a tertiary care hospi-tal in south India.	Iran J Microbiology	2018	India	Prevalence of dengue and leptospirosis coinfection was 3.4%
3	Suppiah J, *et al*., 2017 (*32*)	Clinical predictors of dengue fever co-infected with leptospirosis among patients admitted for dengue fever - a pilot study	J Biomed Sci	2017	Malaysia	Shock and male gender are significant predictors of coinfection ($p < 0.03$)

Table 10. Dengue and Zika co-infection

S.N	Author	Title	Journal	Year	Place	Outcome /Conclusion
1	Siqueira C, *et al*., 2020 (*33*)	Six cases of Zika/dengue coinfec-tion in a Brazilian cohort, 2015-2019	Viruses	2020	Brazil	Early rash, conjunctival hy-peremia, joint swelling and low grade fever favours Zika virus infection
2	Vogels CBF, *et al*., 2019 (*34*)	Arbovirus coinfection and co-transmission: A neglected public health concern?	PLoS Biology	2019	USA	Co-transmission in vectors is proportional to co-transmission in population which can con-tribute to huge number of co-infected cases compared to vectors with sequential trans-mission
3	Joob Bet *et al*., 2020 (*35*)	Clinical relevance of Zika symptoms in the context of a Zika Dengue epidemic	J Infect Public Health	2020	Cuba	Arthralgia, asthenia, diarrhoea favoured Zika virus infection during dengue zika epidemic (OR- 1.38, 1.33, 1.54 respectively)

Diagnosis of leptospirosis includes detection of IgM antibody by ELISA which is highly sensitive and specific and becomes positive as early as 2 days into illness. Management of dengue is symptomatic whereas leptospirosis needs immediate administration of either penicillin of ceftriaxone or doxycycline in order to prevent complications.

11. Zika virus

Zika virus causes a self-limiting illness in most cases which is indistinguishable from mild cases of dengue. Coinfection in the population has public health implications. The implications are especially grave in pregnancy because of its potential to cause severe congenital malformations. Table 10 shows studies on dengue-Zika coinfection. In a report of six cases from Brazil in 2020, low grade fever, early rash within first day of illness, conjunctival hyperaemia and joint swelling favoured Zika virus disease (*33*).

Vogels *et al*. reported that co-transmission of both viruses among vector population is directly proportional to transmission among human population (*34*). In a study from Cuba, arthralgia of small joints, asthenia and diarrhoea favoured a diagnosis of Zika virus disease during co-circulation of both the arboviral diseases (*35*). As far as management is concerned, there is no specific antiviral for both the disease. Coinfection if presents with severe dengue predominant features, the management would be similar to severe dengue.

12. Coronavirus disease-19 (COVID-19)

The course of illness in dengue and COVID-19 coinfection is similar to mono-infected patients. Of concern, is the management consideration especially because treatment of one infection adversely affects the outcome of the other. We have our first Indian national guideline on the management of COVID-19 with seasonal infections (*36*). Steroid is the cornerstone of therapy in moderate and severe COVID-19 cases whereas there is no concrete evidence for use in dengue infected patients and might also lead to severe disease. Anticoagulation is widely used to prevent complications related to microvascular thrombosis in COVID-19. But anticoagulation in dengue can be life threatening especially in settings of thrombocytopenia.

So in areas where both the diseases are co-circulating, clinical discretion is of paramount importance. Hydration in initial phase of both the illness is of paramount importance and with proven distinct benefit except for fluid restriction indicated because of some existing co-

morbidity. While COVID-19 requires good amount of fluid through the illness, same is not true for severe dengue management in second half of illness because of reabsorption of fluid leaked into interstitium. Nevertheless, data on this coinfection is still evolving.

13. Conclusion

The incidence of dengue has risen dramatically in recent times with about half of the world population at risk of this infection. The vaccines available have variable efficacy and are not in widespread use in tropical countries. Although the percentage of case fatality in severe dengue is low, the absolute numbers would still be very high especially in countries like India. The severity of dengue infection rises proportionately in presence of coinfections which are co-circulated during dengue season. The management of dengue is mainly conservative with intravenous fluids and platelet transfusion but the coinfections prevalent in tropical and subtropical climates are treatable which has direct implications on positive outcome especially if recognised early with prompt initiation of specific therapy. This review provides a comprehensive knowledge on management of dengue fever with coinfections.

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Applying the silkworm model for the search of immunosuppressants

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SUMMARY Various stresses (high temperature, starvation, or sublethal *Cryptococcal* infection) increased the susceptibility of silkworms to bacterial infection by up to 100-fold, confirming the stress-induced immunosuppression reported in a range of species. When the silkworm was injected with a steroidal drug, betamethasone (1 mg/larva), the susceptibility of the silkworm to bacterial infection increased about 100-fold. This indicates that the immune function of the silkworm can be suppressed by a known compound that shows immunosuppressive effects in humans. We further tested the immunosuppressive effect of the culture supernatants (acetone extracts) of soil bacteria, and 24 out of 193 isolates showed the immunosuppressive activity. These results suggest that it is possible to search for immunosuppressive agents targeting innate immunity by using a silkworm bacterial infection model as a screening system, and that there may be candidate compounds for immunosuppressive agents among the substances produced by soil bacteria.

Keywords Silkworm model, immunosuppressants, screening system, natural products

1. Introduction

Immunosuppressive drugs are used in various inflammatory diseases and organ transplantation to suppress excessive immune responses (*1*). However, patients suffering from inflammatory diseases are still in need of new immunosuppressive compounds to improve their therapeutic efficacy. Currently used immunosuppressive agents can be classified into three categories: those that suppress the function of immunocompetent cells, those that suppress the production of cytokines, and those that exhibit cytotoxicity (*1,2*). In recent years, cyclosporine has been identified through *in vitro* screening of soil bacteria and is mainly administered during organ transplantation (*3*). In addition, antibody-based drugs targeting molecules expressed by immunocompetent cells are also being developed. However, since these screenings are mainly conducted *in vitro*, it is difficult to consider the pharmacokinetics and toxicity (ADMET) of the candidate compounds at the early stage of the screening, which poses a challenge for the research and development of immunosuppressive agents (*1*).

To solve the above problems, we have proposed a screening system for drug candidates by using silkworms (*4-9*). Screening of antimicrobial agents using a silkworm infection model has led to the identification of a new antimicrobial compound, Lysocin E, which has shown good therapeutic results in vertebrates (*10,11*). Furthermore, by using the silkworm model of hyperglycemia, we have discovered functional lactic acid bacteria that improves the blood glucose homeostasis (*12-14*). These findings indicate the usefulness of constructing a disease model in silkworm and conducting a large-scale exploration. In this study, we attempted to construct a model of immunosuppression in the silkworm with the aim of screening for new immunosuppressive agents.

2. Materials and Methods

2.1. Silkworms and injection technique

Silkworms were reared and injected with samples into the hemocoel by the method reported previously (*15-17*). Silkworm used in the experiment were 5th instar larvae, fed during day 1 (on the day of molt) and 2 (1 g/larva). Silkworms were utilized for experiments on day 2 (5th instar) unless otherwise specified.

2.2. Thermal stress and *Staphylococcus aureus* infection

Silkworms were injected with different doses of *S. aureus* MSSA1 strain and kept at 27ºC (normal condition) or 37ºC (high temperature condition). The silkworms were not fed during the infection experiment. The number

of surviving silkworms at 48 hours after infection was counted, and the LD_{50} value of MSSA1 strain for silkworms was estimated from the dose-action curve (see 'Estimation of LD_{50} values' section).

2.3. Starvation stress and *S. aureus* infection

In the control group (no starvation stress), the silkworm was fed food (1 g/larva) on day 1 of the 5th instar, and the infection experiment of *S. aureus* (MSSA1 strain) was conducted using the silkworm on day 2. In the starvation-stressed groups, the silkworms were not fed after day 2 of the 5th instar, and the same infection experiments as above were conducted either on day 3 (1 day of starvation), on day 4 (2 days of starvation), or on day 5 (3 days of starvation). The number of surviving silkworms at 48 hours after infection was counted, and the LD_{50} value of MSSA1 strain for silkworms was estimated from the dose-action curve (see 'Estimation of LD_{50} values' section) for each group.

2.4. Sublethal *Cryptococcal* infection and *S. aureus* infection

A group of silkworms (*Cryptococcal* infection stress group) was injected with a 4-fold concentrated culture of *C. neoformans* (50 μL/larva). The other group of silkworms (control group) was injected with the same volume of saline. Then silkworms of each group were injected with different doses of *S. aureus* MSSA1 strain and kept at 27ºC. The number of surviving silkworms at 48 hours after infection was counted, and the LD_{50} value of MSSA1 strain for silkworms was estimated from the dose-action curve (see 'Estimation of LD_{50} values' section) for each group.

2.5. Betamethasone treatment and *S. aureus* infection

Silkworms were injected with 10% DMSO (control group) or Betamethasone (betamethasone treated group) dissolved at a concentration of 20 mg/mL, and immediately afterwards injected with different doses of *S. aureus* MSSA1 strain and kept without food at 27ºC. The number of surviving silkworms at 48 hours after infection was counted, and the LD_{50} value of MSSA1 strain for silkworms was estimated from the dose-action curve (see 'Estimation of LD_{50} values' section) for each group.

2.6. Estimation of LD_{50} values

The statistical programming language R (version 3.6.1) was used to estimate the LD_{50} values. The function 'drm' implemented in the package 'drc' (*https://cran.r-project.org/web/packages/drc/drc.pdf*) was used, and LL.3 was applied to the argument fct. From the results of the obtained model, LD_{50} estimates and their standard errors were read are described in the main text. The infected cell number was estimated from the injected volume: when overnight culture of MSSA1 contains 1×10^{10} CFU/mL (*i.e.*, 1×10^{7} CFU/μL) of live cells, then the infected dose for injecting 50 μL of the culture will be 5×10^{8} CFU/larva.

2.7. Brief search for immunosuppressive agents from a soil bacterial library

Fifty microliters of culture supernatant samples (acetone extracts) of 193 soil bacterial isolates were injected into the silkworm, and immediately afterwards, 50 μL of a 1000-fold diluted *S. aureus* (MSSA1 strain) overnight culture was injected into the silkworm. There were three batches of bacterial isolates. Batch #1: 70 randomly isolated strains, Batch #2: 52 strains that formed an inhibition circle against *S. aureus* (HH *et al.*, unpublished results), and Batch #3: 71 strains that were previously found in our laboratory to exacerbate the *Staphylococcal* infection in silkworms (AM *et al.*, unpublished results)). To prepare acetone extracts, 20 mL of 4-day culture (YME broth) was mixed with 20 mL of acetone, then the supernatant was dried and dissolved in 2 mL of water. In the control group, the silkworm was injected with saline (50 μL/larva) and injected with 50 μL of a 1000-fold diluted *S. aureus* (MSSA1 strain) overnight culture. The number of surviving silkworms was measured 48 hours after the injection, and those whose survival rate was lower than that of the control group were judged to have immunosuppressive effect. The samples that showed immunosuppressive effects were further examined to see if they killed the silkworm *per se*, and if true those samples were excluded from the hit samples because it may contain toxic substances that is not suitable for further preclinical development.

3. Results

3.1. Starvation stress increases susceptibility of silkworm to infection

The susceptibility of starvation-stressed silkworms to *S. aureus* infection increased with the duration of starvation, and the LD_{50} value of *S. aureus* MSSA1 strain was up to 20-fold lower (*i.e.*, silkworms being more susceptible) for 3-day starvation conditions than control group (Figure 1a. Estimated LD_{50} values from logistic regression: Control, 0.74 ± 0.13 [$\times 10^{7}$ CFU/larva]; 1-day starvation, 0.34 ± 0.03 [$\times 10^{7}$ CFU/larva]; 2-day starvation, 0.072 ± 0.033 [$\times 10^{7}$ CFU/larva]; 3-day starvation, 0.043 ± 0.004 [$\times 10^{7}$ CFU/larva]).

3.2. *Cryptococcal* infection increases susceptibility of silkworm to *S. aureus* infection

The LD_{50} value of *S. aureus* MSSA1 strain was

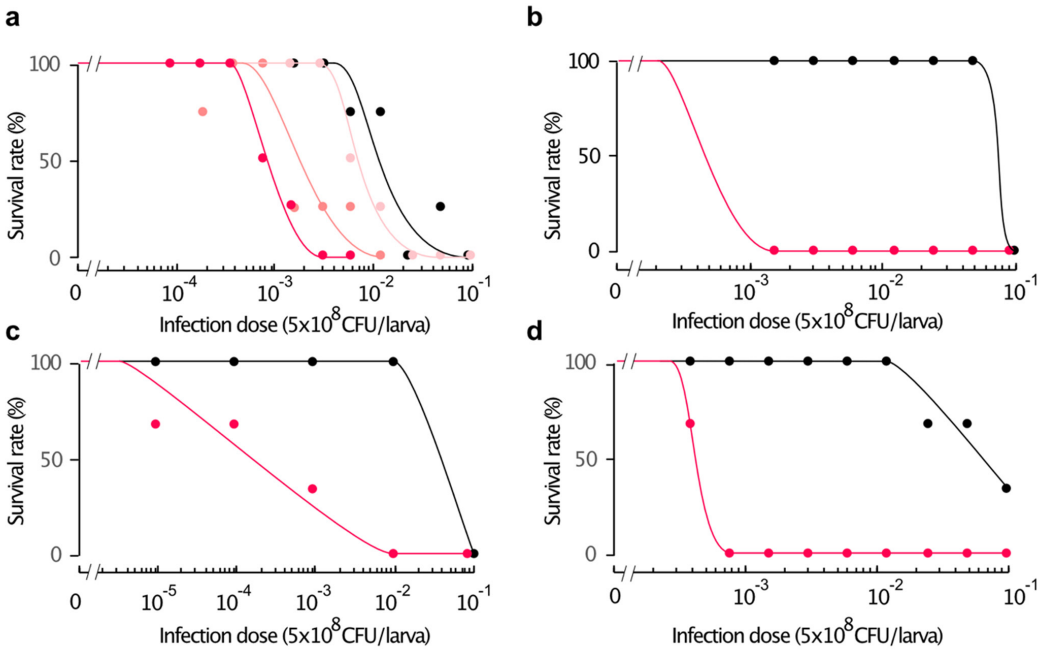

Figure 1. Increased susceptibility to bacterial infections due to high temperature, starvation, *cryptococcal* infection stress, and immunosuppressive steroids. Dose-response curves of *S. aureus* infection are shown in the panels. In all four panels (a-d), the x-axis represents the infected dose (× 50•10^8 CFU per larva) and the y-axis represents the survival rate of silkworms 2 days after MSSA1 infection. **(a)** Starvation stress. Black circles indicate the untreated silkworm group. Red circles indicate the starvation groups: 1-day starvation (lightest red), 2-day starvation (middle red), and 3-day starvation (darkest red). **(b)** *Cryptococcus* infection stress. Black circles indicate the untreated silkworm group. Red circles indicate groups infected with sub-lethal doses of *Cryptococcus* prior to MSSA1 infection. **(c)** Temperature stress. Black circles indicate the group infected at 27°C. Red circles indicate the group infected at 37°C. **(d)** Effect of immunosuppressive steroid (betamethasone). Black circles indicate the untreated (vehicle injection) silkworm group. Red circles indicate the betamethasone treated group.

more than 45 times smaller in silkworms infected with a sublethal dose of *C. neoformans* than control silkworms (Figure 1b. Estimated LD_{50} values from logistic regression: Control, 3.5 ± 0.1 [×10^7 CFU/larva]; *Cryptococcus* pre-infected, < 0.078 [×10^7 CFU/larva]).

3.3. Thermal stress increases susceptibility of silkworm to *S. aureus* infection

The LD_{50} value of *S. aureus* MSSA1 strain in the silkworm reared at high temperature (37ºC) was about 100 times smaller than that in the silkworm reared at normal temperature (27ºC) (Figure 1c. Estimated LD_{50} values from logistic regression: 27ºC infection, 1.6 ± 0.1 [×10^7 CFU/larva]; 37ºC infection, 0.012 ± 0.013 [×10^7 CFU/larva]).

3.4. Increased susceptibility to infection in silkworms treated with betamethasone

The LD_{50} value of *S. aureus* MSSA1 strain for silkworms treated with betamethasone were about 100 times smaller than that for silkworms in the control group (10% DMSO-injected group) (Figure 1d. Estimated LD_{50} values from logistic regression: untreated group, 3.0 ± 0.4 [×10^7 CFU/larva]; betamethasone treated group, 0.021 ± 0.00001 [×10^7 CFU/larva]).

3.5. Brief search for of immunosuppressive agents from a soil bacterial library

Among 193 soil bacterial strains, 29 strains showed immunosuppressive effects, and the culture supernatants of 24 of these strains were not toxic to silkworms when administered alone. The above 24 soil bacterial strains were each from batch 1 (6 out of 70 strains), batch 2: (8 out of 52 strains), or batch 3 (10 out of 71 strains). The detailed information for each batch is described in the Methods section.

4. Discussion

In the present study, we showed that (1) high temperature stress, (2) starvation stress, (3) *Cryptococcal* infection, and (4) administration of betamethasone, an anti-inflammatory steroid used in humans, increased the susceptibility of silkworms to infection by *S. aureus*. These results suggest that the various stressors suppress the immune function of the silkworm, which is consistent with the immunosuppressive responses to stressors that are found in a range of species (*18*). The susceptibility of silkworm as measured by the LD_{50} value of *S. aureus* MSSA1 strain was increased by between 20- and 100-fold by those stressors. Further investigation is needed to determine by what molecular

mechanism the four stresses suppress the immune function of the silkworm.

Since betamethasone, which shows immunosuppressive effects in human, also showed immunosuppressive effects in silkworm, it may be possible to use silkworm for screening of immunosuppressive agents. The screening of immunosuppressive agents using silkworms has the advantage that the pharmacokinetics and toxicity (ADMET) of candidate compounds can be considered in the early stage of screening, unlike *in vitro* screening systems. As demonstrated in this paper, 24 out of 193 soil bacterial samples showed immunosuppressive effects in the silkworm and are a good candidate for further development. The molecular properties, such that the active substance was extracted with 50% acetone, facilitate further investigation for the 24 candidates; purification and structural analyses for each substance is the next step of this study. Also, in future, the development of a method to remove or inactivate immunosuppressive agents from soil bacterial samples will improve the efficiency of antibiotics screening using the silkworm system.

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Original Article

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Use of an electrophysiological technique for stepwise detection of trace agonist constituents of Hochuekkito in *Xenopus* oocytes injected with serotonin 2C receptor mRNA

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SUMMARY An electrophysiological bioassay was used to isolate the active compound from Hochuekkito (HET), which the current authors previously described as having potent agonist action against serotonin 2C receptors (5-HT2CR). Synthetic *5-HT2CR mRNA* was injected into *Xenopus* oocytes to specifically express these receptors. Crude extracts and purified products were subjected to an electrophysiological bioassay using the voltage clamp method. HET stimulated a 5-HT2CR-induced current response, whereas Juzentaohoto (JTT), which has anti-depressive action similar to that of HET, did not. Current responses were not observed with an extract mixed with five types of herbal medicines common to HET and JTT but were detected with an extract with the five types of herbal medicines found in HET alone (Hoc5). When the responses to each of the five types of Hoc5 were examined, current responses were noted with *Cimicifugae rhizoma* (CR) and *Citrus unshiu Markovich* extracts. Since efficacy and the EC_{50} value were higher for CR, its constituents were separated using three-dimensional high-performance liquid chromatography and the current response at each of the isolated peaks was examined. One constituent displayed a strong response and was identified as a single substance with a molecular weight of 283.1393 based on liquid chromatography/mass spectrometry. These results will contribute to the isolation of 5-HT2CR-stimulating constituents in HET and the identification of trace constituents with agonist action.

Keywords Kampo medicine, current, Xenopus oocyte, mRNA, 3D-HPLC, LC/MS

1. Introduction

Kampo medicine is a Japanese variant of Chinese traditional medicine that involves the extensive use of herbs. Since Kampo medicines are prescribed depending on the symptoms of a patient and differences in the individual's condition, they are considered to be a forerunner in "tailor-made medicine". Studies on the mechanisms of action of each Kampo prescription are based on certain theories, and not on an analytical method that prioritizes determination of its structure, so those findings will contribute to the discovery of novel research concepts and ultra-low-dose therapeutics (*1*).

The current authors and others have reported that many antidepressants act as blockers of the serotonin 2C receptor subtype (5-HT2CR) (*2*). Many Kampo prescriptions affect "mood", such as "Hochuekkito (HET): Bu Zhong Yi Qi Tang" and "Juzentaihoto (JTT): Shih Quan Da Bu Tang". Accordingly, the current authors previously examined the effects of HET and found that it has antidepressant action on behavioral pharmacology (*3*) and specifically the 5-HT2CR response. Contrary to expectations, results indicated that HET potently activated 5-HT2CR (*4*). Activation of the digestive system is also important as a therapeutic action of HET. The serotonin receptor system is also known to perform important functions in the digestive system. To the extent known, no study of the 10 crude drugs in HET has identified a constituent that stimulates a serotonin receptor. These facts lead to 3 questions: 1) which crude drug in HET is responsible for stimulating 5-HT2CR, 2) what are its constituents, and 3) what are the functional

differences between HET and other Kampo medicine prescriptions that have antidepressant action? Therefore, the current study is the first to attempt to identify this active ingredient *via* bio-assays.

To date, studies on development of new drugs from Kampo medicines have been based on analytical methods from modern science; they have resulted in significant advances and they have provided extensive evidence of the effectiveness of each Kampo prescription. The main approach is to analyze and identify the constituents of an effective crude drug, to examine its physiological activity, and to consider the result to be the action of the crude drug. However, the effectiveness of a herbal medicine is sometimes markedly reduced or its activity is lost during the process of fractionation. A trace constituent may have activity that may be overlooked when prioritizing the identification of constituents. Fortunately, electrophysiological techniques are very sensitive and suitable for high-fidelity bioassays. In addition, the response of a targeted agonist is generally observed even at a concentration lower than that of an inhibitor/blocker, and a transient stimulus is sufficient.

In light of these facts, the current study used an electrophysiological technique to bioassay HET for a potent 5-HT2CR-activating constituent. This study also briefly discusses the specific concepts of Kampo medicine.

2. Materials and Methods

2.1. Extraction of Kampo prescriptions and galenicals

Approximately 50 g of each prescription or galenical was added to 900 mL of boiling water for 60 min. Galenicals were removed while the solution was still hot, and the extract was frozen using liquid nitrogen. The frozen extract obtained was freeze-dried.

All galenicals were purchased from Tochimoto Tenkai-do (Osaka, Japan). The contents of extracted prescriptions and galenicals and the lot number of each galenical and extracted yields are shown in Table 1. The weight of each galenical in a prescription is listed on the left side of the table.

HET and JTT are combinations of 10 galenicals, with five out of the 10 galenicals being common to both (Com5). Com5 and the 5 other galenicals in HET (Hoc5) were extracted and freeze-dried. The gross galenical weight of the extract of HET, JTT, Com5, and Hoc5 was 49, 57, 46.5, and 45 g, respectively. The weight of Com5 was assessed based on its constituent ratio in HET.

Before the extract was administered to the oocytes that expressed 5-HT2CR on their surface, the freeze-dried extract was resolved using the buffer for the electrophysiological experiment and then centrifuged to remove insoluble matter.

2.2. Three-dimensional high-performance liquid chromatography (3D-HPLC) analysis of extracts

Each extract solution (10 mg/mL) of the supernatant obtained by centrifugation followed by filtration through a 0.22-μm membrane filter was subjected to 3D-HPLC analysis (20 μL). The HPLC apparatus (Hitachi Ltd., Japan) consisted of a pump (L-2130) with analysis system software (Elite LaChrom), a photodiode array detector (UV 230-400 nm, L-2455), a system controller, an auto injector (L-2200), and a column oven (L-2300). HPLC conditions were as follows: column: LaChrom

Table 1. List of galenicals in the Kampo prescription used in the present study

g	g	Name	Lot. No	Yield (%)	Production Site	
2		*Citrus unshiu* Markovich (CM)*	007807004	34.5	Japan	Shikoku
2		Zizyphi Fructus (ZF)	007108004	56.0	China	
2		Zingiberis Rhizoma (ZR)	005808001	10.5		
2		Bupleuri Radix (BR)	004208001	12.8	Japan	Nara
1		Cimicifugae Rhizoma (CR)	006007001	26.1		
		(all 5 of the above (Hoc5))		25.2		
4	3	Ginseng Radix	008607037	20.0	Korea	
4	3	Atractylodes Rhizoma	009307015	37.8	China	
3	3	Astragali Radix	001007006	22.8	Japan	
3	3	Angelicae Radix	008007016	41.3	Japan	Yamato
1.5	1.5	Glycyrrhiza Radix	002007034	28.6	China	Northwest
		(all 5 of the above (Com5))		33.3		
	3	Poria	009508003		Korea	
	3	Rehmanniae Radix	005007023			
	3	Cnidii Rhizoma	006207005			
	3	Paeoniae Radix	005308002		Japan	
	3	Cinnamomi Cortex	002807017		Vietnam	
Hochuekkito (HET)				27.9		
	Juzentaohoto (JTT)			25.2		

Lot numbers for the site of production and source as well as recovery rates are listed. All extract concentrations are shown in herbal equivalents based on these recovery rates. Individual extraction of the five herbal medicines specific to JTT was not performed. Citrus unshiu Markovich (CM) is a mixture of CM and Citrus reticulate Bianoco. This table will also facilitate an understanding of the abbreviations used in the text.

Ultra C18 (5 μm, lot no. 21D5-011; Hitachi Ltd., Japan) with 150 × 4.6 mm I.D.; eluant: (A) H_2O containing 0.1% formic acid and (B) CH_3CN containing 0.1% formic acid. A linear gradient was used from '95% A and 5% B' to '30% A and 70% B' for 90 min. The temperature of the column was controlled at 20°C. The flow rate was 0.2 mL/min.

2.3. Synthesis of *5-HT2CR mRNA* and injection into *Xenopus* oocytes.

pBluescript II KS(-) vectors (Stratagene, La Jolla, CA, USA) with a rat 5-HT2CR cDNA insert (approximately 3.0 kb) were used as a template to make *in vitro* synthesized mRNA (*5*). The vector was transformed into DH5α *Escherichia coli* (Nippon Gene, Toyama, Japan) to increase the amount of mRNA *via* estimation. The vector obtained with 5-HT2CR cDNA was linearized with the restriction enzyme XhoI (Nippon Gene, Toyama, Japan) at 37°C for 60 min. The linearized vector (250 ng) was incubated with T7 RNA polymerase and the mCAP analog in the reaction buffer of the transcription kit (Stratagene, La Jolla, CA, USA) to make *5-HT2CR mRNA in vitro*. Products were extracted with a phenol/chloroform solution (Nippon Gene, Toyama, Japan) and precipitated in ethanol and sodium acetate.

Synthesized *5-HT2CR mRNA* (100 ng) was injected into *Xenopus* oocytes isolated from female *Xenopus laevis. X. laevis* (Hamamatsu Seibutsu Kyozai, Shizuoka, Japan) were anesthetized in ice water, and a lobe of the ovary was dissected and placed in sterile modified Barth's solution (MBS: 88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, and 7.5 mM HEPES-NaOH, pH 7.6). Oocytes were then isolated manually and defolliculated by incubation in 1.5 mg/mL collagenase (type IA; Sigma, St. Louis, MO, USA) at 20°C in calcium-free MBS solution. Synthetic mRNA was injected into oocytes using a microinjector (Drummond, Broomall, PA, USA), and oocytes were then incubated in MBS containing 2.5 units/mL penicillin and 2.5 μg/mL streptomycin at 18°C.

Xenopus oocytes do not naturally express 5-HT2CR, ion channels, or many receptors (*6*). Muscarinic receptors are only expressed in the follicular cells and if the follicle cannot be removed, oocytes react to acetylcholine (*7*). In addition, *Xenopus* oocytes are traditionally used for cloning and functional analysis of ion channels and receptors because they efficiently translate injected mRNA (*8*). In the case of 5-HT2CR expression, responsiveness to 5-HT is evident about 18 h after injection of mRNA, and that responsiveness usually continues for 3 to 4 d (*6*).

Since Xenopus is a poikilotherm, it is completely anesthetized when soaked in ice water. After the reflex reaction was confirmed to have disappeared, the base of the foot was opened about 1-2 cm and oocytes were removed. The opening was then sewn shut with suture and surgical adhesive was used to waterproof the wound. Xenopus awakens when placed in ice-free water overnight as the water temperature gradually returns to room temperature. After confirming that Xenopus behaved properly, it was returned to the aquarium. One Xenopus can be used several times. Based on the above facts, the burden of surgery on Xenopus is minimal, and normal behavior resumes immediately after surgery.

2.4. Electrophysiological recordings

Responses to 5-HT were recorded using a two-electrode voltage-clamp amplifier (Nippon Kohden, Tokyo, Japan) at a holding potential of − 60 mV. Oocytes were positioned in a 50-μL chamber and continuously perfused with MBS solution at approximately 1 mL/min at room temperature (less than 25°C). For extracts of the Kampo prescription or galenicals, drugs were administered by changing the perfusing solution to a buffer containing the given drug. When an isolated compound was administered, one drop of the MBS solution (approximately 20 μL) was directly dropped from a micropipette into the chamber. Data were recorded and digitized for analysis (MacLab, AD Instruments, Castle Hill, NSW, Australia).

2.5. Liquid chromatography/mass spectrometry (LC-MS) analyses

LC-MS analyses were performed using a new type of mass spectrometer that combines ion trap and time-of-flight mass spectrometry and that is equipped with an electrospray ionization (ESI) interface (Shimazu, Kyoto, Japan). The following ESI parameters were used: source voltage: 3.5 kV (negative mode), capillary temperature: 200ºC, and nebulizer gas: 1.5 L/min. The mass spectrometer was operated in negative ion mode by scanning the m/z range from 100 to 2,000. A Waters Atlantis dC18 column (2.0 mm *i.d.* × 150 mm) (Waters Corp, MA, USA) was used and the column temperature was maintained at 40ºC. The mobile phase was a binary eluent of (A) 5 mM ammonium acetate solution and (B) CH_3CN under the following gradient conditions: 0-30 min linear gradient from 10 to 100% B and 30-40 min isocratic gradient at 100% B. The flow rate was 0.2 mL/min.

3. Results

The current authors previously reported that many types of antidepressants inhibit 5-HT2CR (*2*). Based on those findings, Kampo medicines that exhibit anti-depressive action, such as HET (*3*), are anticipated to exhibit similar inhibitory activity. Contrary to expectations, HET exhibited significant stimulating rather than inhibitory action (*4*). Similarly, in the current experiment, HET at 3 mg/mL potently activated *Xenopus* oocytes expressing

5-HT2CR (Figure 1). HET consists of 10 types of herbal medicines, 5 of which are common to JTT. The five types specific to HET (Hoc5) had potent 5-HT2CR-stimulating action while Com5 and JTT did not (Figure 1). The stimulatory action of Hoc5 was concentration-dependent (Figure 1).

Since Hoc5 had potent 5-HT2CR-stimulating action, the activity of each of the five herbal medicines was examined. Figure 2 shows the current responses of each herbal medicine at 3 mg/mL. Cimicifugae rhizoma (CR) had potent stimulating action, with an EC50 value of 0.9 mg/mL and a confidence interval (CI) value of 0.48-1.65 mg/mL. Stimulating action was also observed with Citrus unshiu Markovich (CM), but it was weaker, with an EC50 value of 4.0 mg/mL and a CI value of 1.73-9.29 mg/mL. Significant activation was not observed with the three other crude extracts.

Since CR activated 5-HT2CR, its constituents were separated using 3D-HPLC and their activity was examined *via* direct administration to oocytes. The 3D-HPLC chart for CR is shown in Figure 3A, and that

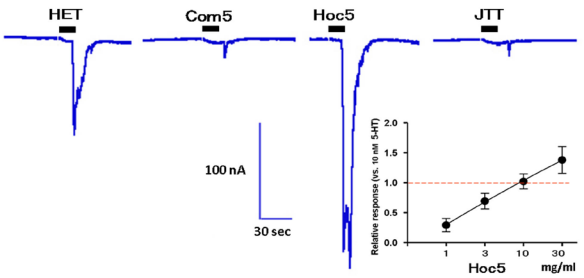

Figure 1. Effects of HET, JTT, and constituent crude drugs, Com5 and Hoc5, on 5-HT2CR-induced current responses in *Xenopus* oocytes injected with synthetic *5-HT2CR mRNA*. *Xenopus* oocytes were injected with *5-HT2CR mRNA* and the voltage was clamped at – 60 mV. Each extract was added to the perfusing solution. The concentration of each extract was 3 mg/mL (estimated galenical weight). The inset shows the concentration dependency of the Hoc5 application relative to current intensity induced by 10 nM 5-HT. Refer to Table 1 for each abbreviation.

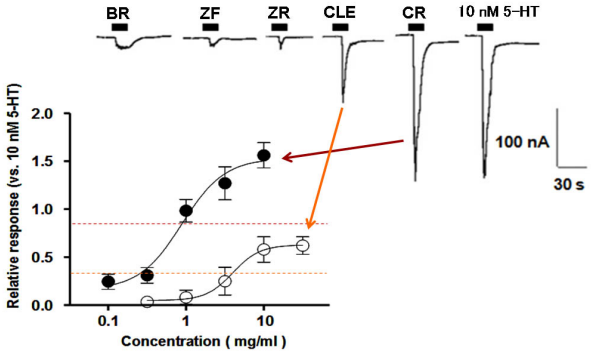

Figure 2. Effects of each extract of Hoc5 constituent crude drugs on 5-HT current responses in Xenopus oocytes injected with synthetic *5-HT2CR mRNA*. *Xenopus* oocytes were injected with *5-HT2CR mRNA* and the voltage was clamped at – 60 mV. Each extract was added to perfusing solution. The concentration of each extract was 3 mg/mL (estimated galenical weight) in the current response figure. The lower figure shows the concentration dependency of CR and CM relative to the current intensity induced by 10 nM 5-HT. The EC50 value was 0.9 mg/mL for CR and 4.0 mg/mL for CM. Refer to Table 1 for each abbreviation.

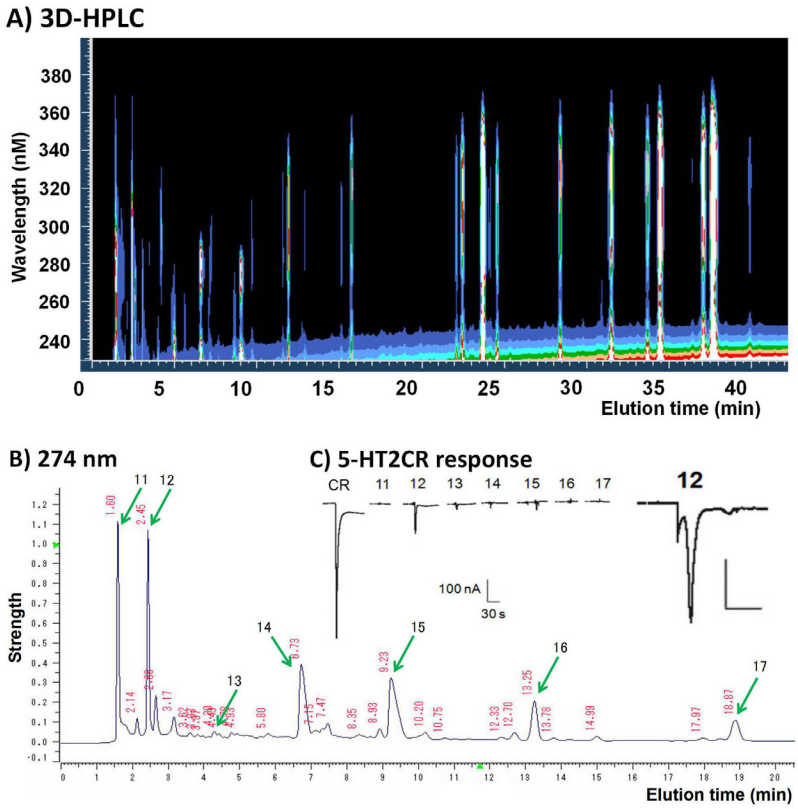

Figure 3. Constituent analysis and fractionation of the CR extract by 3D-HPLC and 5-HT2CR current responses by each fraction. A) 3D-HPLC chart using the *Elite LaChrom* system (Hitachi, Japan); a change in color indicates the height of the signal at that wavelength and outflow time. **B)** An isolated chart at 274 nm from 3D-HPLC and **C)** electrophysiological experiments for each isolated fraction using the same system described in Figures 1 and 2.

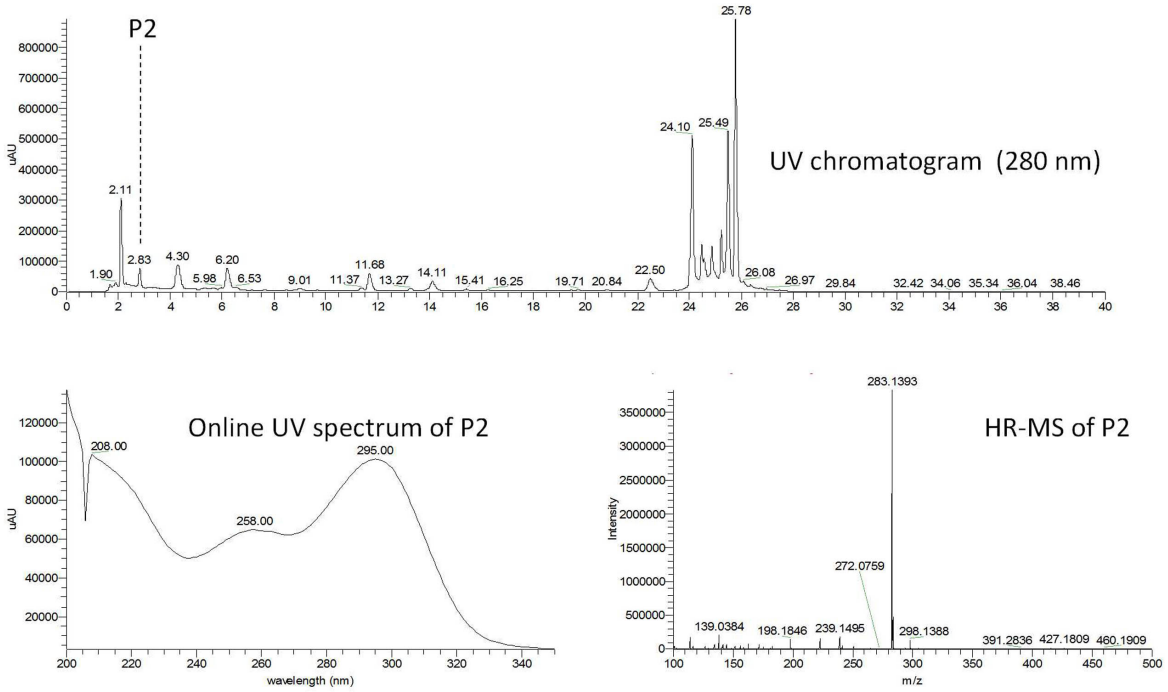

Figure 4. LC-MS analyses of peak No. 12 in Figure 3. A: The chromatogram at 280 for CR; the UV spectrum of peak P2 in Figure 4A was equivalent to peak No. 12 in Figure 3. **B**: The UV spectrum of peak P2. **C**: An MS analysis of the molecular weight of peak P2.

recorded at 274 nm is shown in Figure 3B. The fraction producing large peaks under these HPLC conditions was purified. After the HPLC solvent was removed by evaporation, CR was dissolved in a small amount of HBS buffer and administered to cells. Therefore, its exact concentration could not be quantified. Potent activation by fraction no. 12 was observed. There was no change in the large peak after an elution time of 20 min (data not shown).

The molecular weight of the substance producing peak no. 12 was assessed using LC/MS. The LC results for CR at 280 nm are shown in Figure 4A. The absorption wavelength of peak P2 in Figure 4A is shown in Figure 4B, and it was similar to the spectrum in Figure 3. The molecular weight of the substance was estimated to be 283.1393; however, the substance could not be identified based on that value. Identification using nuclear magnetic resonance (NMR) was attempted, but it was unsuccessful because the required amount with sufficient purity was not obtained.

4. Discussion

The active constituents responsible for the 5-HT2CR-stimulating action of HET were examined, and a trace amount of a constituent with a molecular weight of 283.1393 was obtained. Since the constituent was obtained in a very limited amount, its structure could not be determined with NMR.

The effectiveness of herbal extracts has been successfully assessed to date by considering the main constituents to be active and contributing factors. However, the current results demonstrated that, similar to aromatic constituents, some agonist constituents may exhibit action even when present in only a small amount. While limited evidence is currently available to support this contention, some studies have indicated that the aroma emitted during the extraction of a Kampo medicine is also effective as a treatment. An assessment of electrophysiological activity is a highly sensitive method that may be used to identify active and stimulating trace constituents in the future. When an electrophysiological technique is used to evaluate active ingredients, it can detect both agonist and antagonist action; it is effective at evaluating agonists because it reveals a response by a constituent even in small amounts. In many cases, however, a large amount is needed to examine antagonist action. An electrophysiological technique is highly sensitive and is characterized by a dynamic reaction, so it is suitable for bioassay-like-purification and detection of agonists. In the future, agonist constituents will presumably be detected and analyzed using this technique.

The primary aim of the current study was to identify the active constituents in HET with anti-depressive action on 5-HT2CR. Anti-depressive action could not be directly attributed to a certain "trace constituent", but an active constituent that produced a strong response even when present in a small amount was successfully identified.

The active constituent seems to directly affect the function of the digestive system and not the brain and

appears to be related to another action of HET, its enhancement of digestive function. Among the anti-depressive Kampo medicines, Rikkunshito (RKT): Hsiang Sha Lu Chun Tzu Tong is known to act on the digestive system (*9,10*). HET and RKT have similar herbal medicine compositions, but RKT does not contain CR. The stimulation of digestive function by RKT involves ghrelin and 5-HT2CR function (*10-12*). A point worth noting is the possibility that the trace constituent identified in the current study may involve the function of ghrelin *via* a mechanism unlike that of RKT. Further studies will be conducted to determine the constituent's structure and to subsequently synthesize that constituent.

In Kampo medicine, HET is an effective treatment for mild depression, and JTT is more effective than HET when mood is further depressed. JTT, which is considered to have potent anti-depressive action, did not affect 5-HT2CR in the current experimental system. HET and JTT consist of 10 herbal crude medicines, 5 of which are common to both. The 5 shared herbal medicines (Com5) do not have prescription names and are also included in "Kihito: Gui Pi Tang". Kihito is known to act on the brain and to have the potential to serve as an anti-dementia drug (*13*). Com5 may contain some constituents that are active in the brain and that reach their target site through absorption and metabolism. While the underlying mechanisms could not be fully elucidated in the current study, the current authors previously reported that the level of BNIP-3 mRNA expression increased as a result HET in another experimental system using cultured neuronal cells (*14,15*). This finding is currently being examined more intensively by the authors' group, including identification of the site of expression in the brains of small animals using MRI (*16*).

The current study used an electrophysiological bioassay to identify the constituents responsible for the 5-HT2CR-stimulating action of HET, and, as a result, it discovered an unknown constituent with a molecular weight of 283.1393. CM also acts on 5-HT2CR, so this constituent alone is unlikely to be solely responsible for the activation of 5-HT2CR by HET, but the current results indicate that ultra-trace constituents and not the main constituents of HET are involved in the responses generated by the Kampo medicine. The current study indicated that ultra-trace constituents of Kampo medicines also have potent "biofunctional action" and play a role in the specificity of those prescriptions. In the future, the identification of these "ultra-trace active ingredients" will promote research on Kampo and Wakan-yaku medicines.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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Original Article

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Association between anaphylaxis and anti-influenza drug use: An analysis of the Japanese Adverse Drug Event Report database

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SUMMARY We aimed to investigate the association between anaphylaxis and anti-influenza drug use using the Japanese Adverse Drug Event Report (JADER) database, a national spontaneous reporting database in Japan. We surveyed registered cases from the JADER database between April 2004 and November 2019. The target drugs were five anti-influenza drugs, namely oseltamivir, zanamivir, peramivir, laninamivir, and baloxavir. Adverse events associated with anaphylaxis, "anaphylactic reaction," "anaphylactic shock," "anaphylactoid reaction," and "anaphylactoid shock," were evaluated. The association between anaphylaxis and anti-influenza drug use was assessed by calculating the reporting odds ratio (ROR) and information component (IC) as a measure of disproportionality. Signals were considered positive if the lower limit of the 95% confidence interval (CI) of ROR was > 1, and that of IC was > 0. The number of anaphylaxis cases associated with anti-influenza drug use was 199 (0.9%). Signals were detected for inhaled laninamivir (ROR: 4.24 [95% CI: 3.06-5.88], IC: 1.83 [1.35-2.30]), intravenous peramivir (ROR: 2.97 [2.11-4.17], IC: 1.40 [0.90-1.89]), and oral baloxavir (ROR: 3.05 [2.22-4.18], IC: 1.44 [0.98-1.90]). Conversely, signals were not detected for oral oseltamivir or inhaled zanamivir. Although zanamivir and laninamivir were used as dry powder inhalers containing lactose as an additive, they differed in terms of signal detection. Our analysis indicated that the signal of anaphylaxis may varies based on the main component or dosage form of each anti-influenza drug. Appropriate use of these drugs is essential to prevent anaphylaxis and improve health status.

Keywords Anti-influenza drug, anaphylaxis, Japanese Adverse Drug Event Report database, laninamivir, peramivir, baloxavir

1. Introduction

Influenza is one of the most common viral respiratory infections, associated with high morbidity and mortality. According to the United States Centers for Disease Control and Prevention, the annual number of influenza-related deaths ranges from 291,000 to 646,000 worldwide (*1*). Neuraminidase and cap-dependent endonuclease inhibitors are commonly used to treat and prevent influenza. Japan is one of the largest consumers of anti-influenza drugs worldwide (*2*). Currently, five anti-influenza drugs are mainly used in Japan, and their route of administration and dosage form vary based on the main component (oral formulation: oseltamivir capsule/dry syrup, baloxavir tablet/granules; inhaled formulation: zanamivir and laninamivir dry powder inhalants; intravenous formulation: peramivir intravenous) (Table 1). The supply of anti-influenza drugs to healthcare facilities in Japan between 2018 and 2019 accounted for 13.72 million individuals, consisting of oseltamivir, 4.64 million; zanamivir, 590,000; laninamivir, 2.89 million; peramivir, 320,000; and baloxavir, 5.28 million (*3*).

Drug-induced anaphylaxis is a well-known life-threatening adverse effect associated with several drug classes (antimicrobials (especially beta-lactams), nonsteroidal anti-inflammatory drugs, opiates, and local anesthetics) (*4,5*). Recently, a study using data from the Food and Drug Administration (FDA) Adverse Event Reporting System (FAERS) database identified the top 50 drugs associated with reports of drug-induced anaphylaxis and drugs with more than 20 reported deaths following anaphylaxis (*6*). Although anti-influenza drugs were not included in this list, anti-influenza drug-induced anaphylaxis has been reported in clinical trials and post-marketing settings and the corresponding warning has been issued in package inserts. Nevertheless, to date, there is limited literature on anaphylaxis associated with neuraminidase inhibitors, and hence the relevant information is lacking. Another study using data from the FAERS database reported that signals associated

Table 1. Dosage form and administration route of anti-influenza drugs

Generic name	Dosage form	Administration route
Oseltamivir	Capsule, dry syrup	Oral
Zanamivir	Dry powder inhalant	Inhaled
Peramivir	Intravenous	Intravenous
Laninamivir	Dry powder inhalant	Inhaled
Baloxavir	Tablet, granules	Oral

with anaphylaxis were detected for baloxavir (*7*). Although most drug-induced anaphylaxis episodes are caused by the main drug components, additives have also been reported to cause anaphylaxis. A case of anaphylaxis attributable to a miniscule amount of milk protein contained in lactose used as an additive has been reported (*8*). In addition, a case of anaphylactic shock caused by benzyl alcohol in an intravenous formulation, after a change in the route of administration from oral formulation, has also been reported (*9*). Intravenous administration is associated with an increased rate of anaphylactic shock (*10*). Therefore, as anti-influenza drugs contain various ingredients and vary in dosage forms and administration routes, it is important to consider these differences when evaluating their association with anaphylaxis. In addition, anti-influenza drugs are often used in outpatient settings, except for peramivir, which is an intravenous formulation, and the dosage form is often selected based on the patient's age and respiratory function. Therefore, clarifying the association between anaphylaxis and the use of each anti-influenza drug will help in improved drug selection.

In recent years, studies have utilized data from the Japanese Adverse Drug Event Report (JADER) database, a national spontaneous reporting database in Japan (*11-13*), to investigate the association between adverse events and drugs. Therefore, in this study, data from the JADER database were used to analyze the association between anaphylaxis and anti-influenza drug use.

2. Materials and Methods

2.1. Data source

Data recorded in the JADER database between April 2004 and November 2019 were obtained from the Pharmaceuticals and Medical Devices Agency (PMDA) website (*http://www.info.pmda.go.jp/fukusayoudb/CsvDownload.jsp*). The data structure consists of four sets, namely, patient demographic information (demo), drug information (drug), adverse event (reac), and medical history (hist). The adverse events in reac are based on the medical terminology as preferred terms (PT) in the Medical Dictionary for Regulatory Activities/ Japanese version (MedDRA/J).

The target drugs were four neuraminidase inhibitors (oseltamivir, zanamivir, peramivir, and laninamivir) and a cap-dependent endonuclease inhibitor (baloxavir). Adverse events were associated with anaphylaxis, "anaphylactic reaction (PT code: 10002198)," "anaphylactic shock (PT code: 10002199)," "anaphylactoid reaction (PT code: 10002216)," and "anaphylactoid shock (PT code: 10063119)" in MedDRA/J version 21.0. Cases with both target drugs and adverse events were extracted from all cases recorded in the JADER database, and sex, age, drug involvement, and clinical outcome were surveyed. In the JADER database, patient age is rounded to every 10 years (*e.g.*, 10's, 20's, and 30's). In some cases, age is registered as a particular category ("newborn," "infant," "child," "adolescent," "adult," or "elderly"). In this study, age was categorized into four groups (< 20 years, 20-59 years, ≥ 60 years, and unknown/other) for tabulation; cases belonging to the categories of "newborn," "infant," "child," and "adolescent" were categorized as < 20 years, "adult" as 20-59 years, and "elderly" as ≥ 60 years. Drug involvement in the JADER database is classified into three categories, namely "suspected drugs," "drug interactions," and "concomitant drugs." Cases in which anti-influenza drugs were reported as "suspected drugs" were included in the analysis. The clinical outcomes of anaphylaxis cases were categorized into either "poor outcomes," that included "unrecovered," "death," or "sequelae," or "good outcomes," that included "recovery" or "remission." The missing data were categorized as "unknown."

2.2. Disproportionality analysis

The reporting odds ratio (ROR) (*14*), as a frequency-based method, and information component (IC) (*15*), as a Bayesian method, were used to evaluate the association between drugs and adverse events based on the case/non-case method. We compiled a cross-tabulation table based on the presence or absence of adverse events associated with anaphylaxis and anti-influenza drugs, and calculated the ROR and IC. We defined the signals as positive when the lower limit of the 95% confidence interval (CI) of ROR was > 1, and that of IC was > 0.

3. Results

The total number of cases recorded in the JADER database between April 2004 and November 2019 was 622,289, and the number of cases with anaphylaxis was 23,016. Among the cases with anaphylaxis, 199 (0.9%) cases that included anti-influenza drugs as suspected drugs were identified – oseltamivir in 57 cases, zanamivir in 23 cases, peramivir in 37 cases, laninamivir in 42 cases, and baloxavir in 43 cases (Table 2). In three of these cases, two drugs, peramivir and laninamivir, were together reported as suspected drugs (based on the individual number of cases specified for each drug, the total number of cases summed up to 202 (57 + 23 + 37 +

Table 2. Background data (sex and age) of cases with anti-influenza drug as the suspected drug among all the anaphylaxis cases (*n* = 23,016)

	No. of cases (%)					
	Oseltamivir *n* = 57	Zanamivir *n* = 23	Peramivir *n* = 37[a]	Laninamivir *n* = 42[a]	Baloxavir *n* = 43	Total *n* = 199
Sex						
Male	15 (26.3)	10 (43.5)	19 (51.4)	23 (54.8)	15 (34.9)	79 (39.7)
Female	40 (70.2)	13 (56.5)	17 (45.9)	19 (45.2)	26 (60.5)	115 (57.8)
Unknown	2 (3.5)	0 (0.0)	1 (2.7)	0 (0)	2 (4.7)	5 (2.5)
Age						
< 20 years	14 (24.6)	10 (43.5)	13 (35.1)	23 (54.8)	9 (20.9)	66 (33.2)
20-59 years	36 (63.2)	9 (39.1)	11 (29.7)	13 (31.0)	30 (69.8)	99 (49.7)
≥ 60 years	7 (12.3)	1 (4.3)	12 (32.4)	6 (14.3)	4 (9.3)	30 (15.1)
Unknown/Others	0 (0.0)	3 (13.0)	1 (2.7)	0 (0.0)	0 (0.0)	4 (2.0)

[a]As there were three cases in which both peramivir and laninamivir were considered suspected drugs, the total number of cases listed in the column for all individual suspected drugs did not add up to 199.

Table 3. List of drugs reported as suspected drug along with anti-influenza drugs

Suspected drug	No. of cases
Acetaminophen	21
Levofloxacin	6
Cefcapene pivoxil hydrochloride, loxoprofen sodium	5 per drug
Lysozyme hydrochloride, maoto (herbal medicine), moxifloxacin hydrochloride, piperacillin sodium	2 per drug
Ambroxol hydrochloride, ampicillin sodium, aspirin-dihydroxyaluminum aminoacetate-magnesium carbonate combination, azithromycin, benproperine phosphate, cefazolin sodium, cefditoren pivoxil, ceftriaxone sodium, clofedanol hydrochloride, codeine phosphate, dimemorfan phosphate, garenoxacin mesylate, L-carbocysteine, meropenem, methylprednisolone sodium succinate, nafamostat mesylate, pranoprofen, prednisolone sodium succinate, tipepidine hibenzate, tosufloxacin tosylate	1 per drug

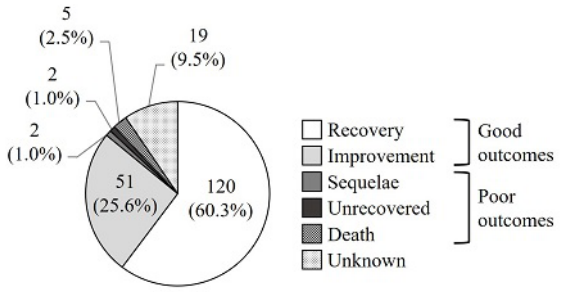

Figure 1. Clinical outcomes of anaphylaxis associated with the use of anti-influenza drugs. The clinical outcomes of anaphylaxis cases were categorized into either "poor outcomes," that included "unrecovered," "death," or "sequelae," or "good outcomes," that included "recovery" or "remission." Nine patients (4.5%) had a poor clinical outcome, of which five (2.5%) died.

42 + 43 = 202)).

In anaphylaxis cases associated with anti-influenza drug use, females and patients aged 20-59 years accounted for a large proportion in each category (57.8% and 49.7%, respectively). In cases in which oral oseltamivir or baloxavir was the suspected drug, the proportion of females and patients aged 20-59 years exceeded 60% in each category. In cases in which inhaled zanamivir or laninamivir was the suspected drug, patients aged < 20 years accounted for a large proportion (43.5% and 54.8%, respectively). In cases in which intravenous peramivir was the suspected drug, minor differences were found in the proportion of reports among the age groups (Table 2). Acetaminophen was the most common drug reported as a suspected drug along with anti-influenza drugs in 21 cases, followed by levofloxacin in 6 cases, cefcapene pivoxil hydrochloride in 5 cases, and loxoprofen sodium in 5 cases (Table 3). Nine patients (4.5%) had a poor clinical outcome, of which five (2.5%) died (Figure 1).

The results of the disproportionality analysis are shown in Table 4. A signal for anaphylaxis was detected for all five anti-influenza drugs combined (ROR: 1.26 [95% CI: 1.09-1.45], IC: 0.31 [0.10-0.52]), whereas no signal was detected for the four neuraminidase inhibitors combined (ROR: 1.08 [0.92-1.27], IC: 0.11 [-0.13 to 0.34]), and a signal was detected for the inhaled neuraminidase inhibitors (ROR: 1.50 [1.17-1.93], IC: 0.54 [0.18-0.91]). In the analysis of each neuraminidase inhibitor, signals were detected for laninamivir (ROR: 4.24 [3.06-5.88], IC: 1.83 [1.35-2.30]) and peramivir (ROR: 2.97 [2.11-4.17], IC: 1.40 [0.90-1.89]). Further, a signal was detected for baloxavir (ROR: 3.05 [2.22-4.18], IC: 1.44 [0.98-1.90]). For the three drugs, laninamivir, peramivir, and baloxavir, wherein anaphylaxis signals were detected, further analysis based on different age groups was conducted, and the results are shown in Table 5. For laninamivir and peramivir, signals were detected in all age groups, with the highest ROR and IC in patients aged < 20 years for laninamivir and patients

Table 4. Signal index of each anti-influenza drug

Suspected drug	Cases	Non-case	ROR [95% CI]	IC [95% CI]
All	199	4123	1.26 [1.09-1.45]	0.31 [0.10-0.52]
Neuraminidase inhibitors	156	3760	1.08 [0.92-1.27]	0.11 [-0.13 to 0.34]
<Oral>				
Oseltamivir	57	2318	0.64 [0.49-0.83]	-0.62 [-1.00 to -0.23]
<Inhaled>	65	1130	1.50 [1.17-1.93]	0.54 [0.18-0.91]
Zanamivir	23	873	0.69 [0.45-1.04]	-0.51 [-1.11 to 0.09]
Laninamivir	42	258	4.24 [3.06-5.88]	1.83 [1.35-2.30]
<Intravenous>				
Peramivir	37	325	2.97 [2.11-4.17]	1.40 [0.90-1.89]
Cap-dependent endonuclease inhibitor				
Baloxavir	43	368	3.05 [2.22-4.18]	1.44 [0.98-1.90]

ROR: reporting odds ratio; IC: information component; 95% CI: 95% confidence interval

Table 5. Signal index by age for the three anti-influenza drugs, peramivir, laninamivir, and baloxavir, for which signals were detected in Table 4

	Cases	Non-case	ROR [95% CI]	IC [95% CI]
Peramivir				
< 20 years	13	81	2.61 [1.45-4.70]	1.10 [0.27-1.93]
20-59 years	11	66	3.19 [1.69-6.05]	1.30 [0.40-2.20]
≥ 60 years	12	174	2.16 [1.20-3.87]	0.93 [0.11-1.76]
Laninamivir				
< 20 years	23	102	3.68 [2.34-5.80]	1.53 [0.88-2.17]
20-59 years	13	97	2.57 [1.44-4.58]	1.10 [0.29-1.92]
≥ 60 years	6	58	3.35 [1.45-7.77]	1.25 [0.10-2.40]
Baloxavir				
< 20 years	9	88	1.66 [0.84-3.30]	0.58 [-0.38 to 1.54]
20-59 years	30	123	4.68 [3.14-6.98]	1.84 [1.27-2.41]
≥ 60 years	4	154	0.84 [0.31-2.27]	-0.20 [-1.51 to 1.11]

ROR: reporting odds ratio; IC: information component; 95% CI: 95% confidence interval

aged 20-59 years for peramivir. A signal for baloxavir was detected only in patients aged 20-59 years.

4. Discussion

In this study, we evaluated the association between anaphylaxis and anti-influenza drug use by analyzing data available from the JADER database. Our results suggest that inhaled laninamivir, intravenous peramivir, and oral baloxavir are associated with anaphylaxis.

There have been several case reports of anaphylaxis associated with neuraminidase inhibitor use (*8,16,17*), but to date, the status in the real world and the relevance have not been clarified. Recently, an analysis of the adverse effects associated with neuraminidase inhibitors using data from the FAERS database was conducted (*18*); however, anaphylaxis was not the focus of that study. Meanwhile, another analysis using data from the FAERS database detected a signal associated with anaphylaxis following baloxavir use, a cap-dependent endonuclease selective inhibitor (*7*). Anti-influenza drugs are often used in outpatient settings, except for peramivir, which is an intravenous formulation, and the dosage form is often selected based on the patient's age and respiratory function. Therefore, clarifying the association between anaphylaxis and the use of each anti-influenza drug will help in drug selection.

Among all anaphylaxis cases registered in the JADER database, anti-influenza drugs were reported as suspected drugs in 199 (0.9%) cases. Generally, drug-induced anaphylaxis occurs more frequently in females/women than in males/men (4,19), and a similar trend was observed for anti-influenza drugs. Furthermore, for individual drugs, the percentage of females was higher for oral oseltamivir and baloxavir than for other drugs, whereas for inhaled zanamivir, laninamivir, and intravenous peramivir, minor difference in sex was observed. Anaphylaxis associated with anti-influenza drug use was reported more frequently in patients aged 20-59 years, accounting for approximately half of all cases. For oral oseltamivir and baloxavir, most cases were patients aged 20-59 years (63.2% and 69.8%, respectively), whereas the percentage of patients aged < 20 years was relatively higher for inhaled zanamivir and laninamivir (43.5% and 54.8%, respectively). For intravenous peramivir, a minor difference was observed in the percentage of patients by age, whereas elderly patients accounted for a higher percentage than that observed with other drugs (dosage forms). The percentage of anaphylaxis cases by dosage form differed

by age, and this may be attributed to the differences in dosage forms selected based on age.

A signal for anaphylaxis was detected for five anti-influenza drugs combined, but disappeared when the patients were limited to neuraminidase inhibitors. A signal was also detected for baloxavir; hence, the effect of baloxavir on the association between anaphylaxis and anti-influenza drug use was large. Further analysis by age showed that the baloxavir signal was detected only in patients aged 20-59 years. Therefore, careful observation is necessary for this age group.

Morikawa *et al.* reported a case of anaphylaxis caused by laninamivir dry powder inhaler, and attributed the anaphylaxis to β-lactoglobulin and its sugar adducts contained in the lactose used as an additive (*8*). Lactose is widely used as an excipient in pharmaceutical products, and its allergenicity has not yet been characterized. However, it has been confirmed that a trace amount of milk protein is present as a contaminant, even in high-purity lactose (*20*). The lungs can better absorb macromolecular compounds, such as proteins, than gastrointestinal tract (*21*); therefore, pulmonary administration may increase the absorption of allergens (*8*). The dosage forms of zanamivir and laninamivir are dry powder inhalants, both of which contain lactose as an additive; thus, the package insert of these drugs cautions against their use in patients with milk allergy. In this study, a signal associated with anaphylaxis was detected in case of inhaled neuraminidase inhibitors (zanamivir and laninamivir) combined. However, when analyzed by individual drugs, a signal was detected only for laninamivir, but not for zanamivir. This difference is attributed to the purity of lactose used as an excipient, the amount of lactose inhaled at one time, and the allergenicity of the main ingredients.

As mentioned earlier, anaphylaxis associated with inhaled neuraminidase inhibitor use occurred mostly in patients aged < 20 years. Furthermore, for laninamivir, a signal associated with anaphylaxis was detected in all age groups, with the highest ROR and IC in patients aged < 20 years. Previous studies have shown that the cause of anaphylaxis varies by age, with food being the most common cause in children, and drugs in adolescents and adults (*4,22,23*). In this study, we could not determine whether the main component or the additive was the cause of anaphylaxis associated with inhaled neuraminidase inhibitor use. The possibility that the additive lactose may be the cause of anaphylaxis can explain the high proportion of reports in children. Several patients with anaphylaxis caused by inhaled neuraminidase inhibitor use had a history of food allergy (data not shown). On September 4, 2019, laninamivir in inhalation suspension as a new formulation was listed in the National Health Insurance, and is currently available for clinical use. This formulation does not contain lactose as an additive, and can be administered by spontaneous breathing in patients with difficulty using the existing dry powder inhalers. In the future, it may be possible to compare the incidence of anaphylaxis between two commercially available inhaled laninamivir formulations, and clarify the effect of lactose, as an additive, on anaphylaxis development.

For intravenous peramivir also, a signal associated with anaphylaxis was detected. As intravenous administration delivers the drug directly into the blood stream, it has a relatively high potential to induce anaphylaxis. In an analysis using data from the Italian pharmacovigilance database, intravenous formulations were reported to have a high rate of anaphylactic shock (*10*), which is in line with our results. Furthermore, for peramivir, as signals were detected in all age groups with minor differences in the percentage of reports, caution must be exercised in patients of all ages.

In this study, we focused on the use of anti-influenza drugs, and ignored the effects of co-administered drugs. When we surveyed the suspected drugs besides anti-influenza drugs in each case, acetaminophen was the most common co-administered drug (21 cases), followed by levofloxacin (6 cases), cefcapene pivoxil hydrochloride (5 cases), and loxoprofen sodium (5 cases). Of these drugs, acetaminophen and levofloxacin were among the top 50 drugs associated with anaphylaxis in a survey using data from the FAERS database (6). Therefore, analysis considering the presence or absence of these drugs may be necessary in the future.

Drugs, as anaphylaxis inducers, are associated with the severity level, including fatal, of anaphylaxis (*19,24*). It has also been reported that drugs are the most common cause of fatal anaphylaxis, accounting for 58.8% of anaphylaxis-related deaths (*24*). In this study, 4.5% of anaphylaxis cases associated with anti-influenza drug use presented poor clinical outcomes, whereas 2.5% of patients died. It may be possible to reduce anaphylaxis-associated fatality by effectively selecting drugs based on individual patient characteristics.

This study had some limitations. As this study was a disproportionality analysis using data from a spontaneous report database, the ratio of adverse event onset to each target drug user was not calculated. Additionally, differences in the drugs prescribed based on patients' background, such as age and presence or absence of predisposition to allergy were not considered. Therefore, caution should be exercised when interpreting these results.

In conclusion, a signal associated with anaphylaxis was detected for inhaled laninamivir. As dry powder-type inhalants often contain lactose as an additive, caution must be exercised when administering them to patients with milk allergy. Signals were also detected with intravenous peramivir and oral baloxavir. Proper use of these drugs is necessary to prevent anaphylaxis and improve health status. Further extensive studied are needed to validate these results.

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Original Article

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Clinical predictors of long COVID-19 and phenotypes of mild COVID-19 at a tertiary care centre in India

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SUMMARY A variable proportion of patients develop persistent/prolonged symptoms of Coronavirus Disease 2019 (COVID-19) infection (long COVID). We aimed to study the clinical predictors of persistent symptoms in patients with mild COVID-19 at 30 days post discharge (long COVID-19). We also tried to identify symptom clusters among mild COVID-19 patients. Fifty-seven patients admitted at a tertiary care centre after a positive RT-PCR report over a period of 2 months, were enrolled in the study. Details of presentation, history of illness, laboratory investigations and disease outcomes were recorded from documented medical records and discharge slip. The patients were contacted (telephonically) at 30 days after discharge and enquired regarding persistent symptoms, if any. Follow up data at 30 days post-discharge was available for 53 patients. Among them, the most common persistent symptom was fatigue (22.6%), followed by cough (9.4%) and myalgias (7.5%). There was a significant association of persistent symptoms with diarrhoea at presentation [OR 14.26 (95% CI 2.30-142.47; p = 0.009] and gap between symptom onset and admission [OR 1.40 (95% CI 1.08-1.93; p = 0.020] on multivariate logistic regression analysis. On cluster analysis, three phenotypes of mild COVID-19 were identified, which may have implications on monitoring and management. There appears to be a positive association of diarrhoea as a presenting manifestation and gap between symptom onset and admission with the persistence of symptoms classified as long COVID-19, even in mild illness. We also identified multiple phenotypes of mild COVID-19 illness, which warrant further exploration.

Keywords COVID-19, long COVID, phenotypes, predictors

1. Introduction

The Coronavirus Disease 2019 (COVID-19) has caused significant global health loss, undermining economy and destabilising society (*1*). COVID-19 manifests as a broad clinical spectrum, ranging from asymptomatic illness to a flu-like illness to severe viral pneumonia, culminating in Acute Respiratory Distress Syndrome (ARDS) (*2*). The most common symptom is fever (92.0%) followed by symptoms such as cough (53.0%), shortness of breath (40.8%) and fatigue (39.9%) (*3*), with some studies claiming digestive symptoms to be present in about 50.5% patients including loss of appetite, diarrhoea, vomiting, abdominal pain, *etc.* (*1*).

Clinically, SARS-CoV-2 infection can be categorised into mild, moderate, and severe based on parameters such as respiratory rate (RR) and saturation (SpO_2). As per the clinical management guidelines by Ministry of Health & Family Welfare ,India (MoHFW) a patient with RR < 24/min and room air $SpO_2 \geq 94\%$ is categorised as mild COVID, while a RR ≥ 24/min and room air $SpO_2 < 94\%$, as moderate COVID, and RR ≥ 30/min and $SpO_2 < 90\%$ as severe COVID. The classification is simple and clinically based, easily applicable even in resource limited setting, with management being mainly observation and supportive care. Moderate and severe cases should receive oxygen supplementation along with steroids and anti-coagulation.

The most prominent laboratory abnormalities seen are lymphopenia (47.9%), decreased eosinophils (58.4%), elevated C-reactive protein (CRP) (73.6%), followed by hypoalbuminemia (62.9%), elevated erythrocyte sedimentation rate (ESR) (61.2%), increased interleukin-6 (IL-6) (53.1%), and lactate dehydrogenase (LDH) (46.2%) (*4*). Data regarding difference between laboratory parameters suggests that cases with severe

disease had higher leukocyte and neutrophil counts, lymphopenia, higher neutrophil-to-lymphocyte ratio (NLR). Inflammatory markers in the severe cases as compared to the non-severe cases demonstrated raised serum biomarkers, including procalcitonin, ferritin, CRP and IL-6 (*5*).

"Long covid" is a term used for illness in people who have either recovered from COVID-19 but still report lasting symptoms of the infection or have had the usual symptoms for a duration far longer than expected (*6*). While there are no agreed upon definitions, Greenhalgh *et al.* defined "post-acute COVID-19" as illness extending beyond 3 weeks and "chronic COVID-19", beyond 12 weeks from symptom onset (*7*). The reason for prolonged illness in some patients is largely unknown. It may be due to prolonged viremia, immune responses or mental health of the patient.

There exists a paucity of literature on long term follow up among patients with COVID 19 after discharge, especially in the mild illness subset, from a resource-limited setting, such as ours. It was our aim to follow up patients with mild COVID-19 at a tertiary care centre, for persistent symptoms at 30 days after discharge and to study clinical predictors for the same.

2. Materials and Methods

2.1. Study setting

The study was conducted in a designated COVID ward of a tertiary care centre in India. Individuals included were healthcare workers and their direct dependents who had mild symptoms. Fifty seven patients admitted after a positive reverse transcription polymerase chain reaction (RT-PCR) for severe acute respiratory coronavirus-2 (SARS-CoV2) over a period of 2 months were enrolled in the study. Details of presentation at admission, history of illness along with duration and progression, laboratory investigations and outcome of disease were recorded from documented medical records and discharge slip. Case definitions of severity were as provided by the MoHFW. The patients were contacted (telephonically) at 30 days after discharge using the details provided in medical records and enquired regarding persistent symptoms, if any. The study was approved by the Institute's ethics committee.

2.2. Tools and definitions

Long COVID-19 has been defined as symptoms of COVID-19 extending beyond 4 weeks.

2.3. Statistical analysis

Baseline characteristics of the study subjects were summarized. Shapiro-Wilk test of normality was used to assess the variable for data distribution. Continuous variables were reported as mean (± standard deviation) if they were normally distributed, otherwise reported as median (Inter Quartile Range). Categorical variables were reported as counts (percentages). To observe the difference, with assumed normality, two categories student's *t* test was performed; if otherwise, Wilcoxon-Rank sum test was used. To establish the association between categorical variables Pearson Chi square test/Fisher's exact test was used. Correlation between continuous variables was performed using Pearson's correlation or Spearman's rank correlation test (for non-normal distribution) depending upon the normality of bivariate joint distribution. Logistic regression was performed to find the risk of the standardized parameters (with penalisation, shown in **Appendix A**). Effect sizes were labelled according to Chen's (2010) recommendations. A value of $p \leq 0.05$ was considered statistically significant. The statistical analysis was done using R software version 3.5.2.

3. Results

3.1. Baseline characteristics

In this study, a total of 57 patients were enrolled. Of these, 50 (87.7%) were healthcare workers while 7 (12.3%) were their direct dependents (spouses/children). The mean age for our patients was 34.90 ± 12.09 years. The baseline characteristics were as summarised in the Table 1. The most common comorbid conditions were hypertension and hypothyroidism (10.5% each) followed by diabetes (7%), heart disease (5.3%) and respiratory disease (1.7%). Apart from these, a small minority of patients (5.3%) had arthropathies, transient ischemic attacks (TIA) and dyslipidaemia.

3.2. Clinical presentation and course

The commonest presenting symptom was fever (63.2%), followed by myalgias (50.9%), cough (49.1%), sore throat (49.1%), diarrhoea (21.1%), *etc.*, as summarised in Table 2. Two patients (3.5%) were asymptomatic at presentation and throughout hospital stay. Only 31.6% patients gave a history of contact with a confirmed COVID-19 case. 26.3% patients were working at a COVID-19 area at the time of exposure. 11 patients had taken weekly pre-exposure prophylaxis with hydroxychloroquine (HCQ) for a mean duration of 6.4 ± 1.4 weeks.

93% patients were managed as mild COVID-19 while 4 patients (7%) worsened during hospital admission and were managed as moderate illness. Two patients had to be transferred to a step-up facility with the institution. Laboratory profile of the patients was as summarised in Table 3. Distribution of laboratory parameters by disease severity has been depicted in Table 1.

Table 1. Baseline demographic characteristics and comorbidities

Characteristic	*N* (%)
Sex	
Male	30 (52.6)
Female	27 (47.4)
Co-morbidities	
Diabetes	4 (7.0)
Hypertension	6 (10.5)
Hypothyroidism	6 (10.5)
Respiratory Disease (Asthma/COPD)	1 (1.7)
Heart Disease (CAD, RHD)	3 (5.3)
Others	3 (5.3)
Symptoms at presentation	
Fever	36 (63.2)
Myalgia	29 (50.9)
Cough	28 (49.1)
Sore throat	28 (49.1)
Diarrhoea	12 (21.1)
Rhinitis	12 (21.1)
Dyspnea	9 (15.8)
Fatigue	8 (14.0)
Others	9 (15.8)
Contact History	
Contact with a confirmed COVID-19 case	18 (31.6)
Healthcare worker at a COVID-19 area	15 (26.3)
Healthcare worker at a non-COVID-19 area	26 (45.6)
Residing at or visiting a hotspot	15 (26.3)
History of Hydroxychloroquine (HCQS) prophylaxis	
Patients taking HCQS pre-exposure prophylaxis	11 (19.2)
Severity of COVID-19	
Mild	53 (93.0)
Moderate	4 (7.0)
Severe	0 (0.0)
Treatment	
HCQS	44 (77.2)
Doxycycline	20 (35.1)
Ivermectin	13 (22.8)
Outcome	
Discharged	55 (96.5)
Transferred	2 (3.5)

Table 2. Baseline laboratory profile

Laboratory Parameter	Median [IQR]
Total Leukocyte Count (per cu mm)	5875 [4887.5-6940.0]
Neutrophils (%)	52.9 [45.4-60.5]
Lymphocytes (%)	33.5 [28.5-42.7]
Neutrophil-Lymphocyte Ratio (NLR)	1.70 [1.04-1.96]
CRP (mg/dL)	0.20 [0.10-0.69]
CPK (mg/dL)	95.0 [59.0-95.0]
Procalcitonin (ng/mL)	0.02 [0.01-0.04]
Ferritin (ng/mL)	78.10 [28.30-156.40]
D dimer (ng/mL)	0.36 [0.12-0.51]
Aspartate Transaminase (AST)	25.0 [21.0-34.0]
Alanine Transaminase (ALT)	26.0 [18.8-40.5]
Alkaline Phosphatase (ALP)	195.5 [176.8-214.8]

Table 3. Laboratory profile distribution by severity of disease

Laboratory Parameter	Mild disease (*N* = 53) [Median Value]	Moderate disease (*N* = 4) [Median Value]
Ferritin	80.00	57.65
CRP	0.28	2.12
CPK	105.00	94.50
Procalcitonin	0.02	0.01
NLR	1.69	2.55

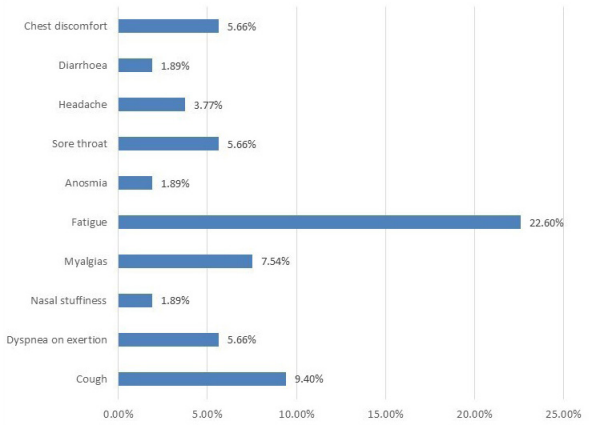

Figure 1. Persistent symptoms at 30 days (*n* = 53).

3.3. Persistent symptoms at 30-day follow up

After discharge, all patients were followed up telephonically at 30 days from date of discharge, for persistent symptoms, if any. While 2 patients could not be reached, 2 refused to participate in the study. A total of 53 patients were included in the analysis. 25 patients (47.17%) reported persistent symptoms at 30 days post discharge. The most common symptom reported was fatigue (22.60%), followed by cough (9.60%), myalgias (7.54%), chest discomfort, sore throat, dyspnea on exertion (5.66% each) and diarrhoea, anosmia, nasal stuffiness (1.89% each) (Figure 1). There was a significant association of persistent symptoms among patients who had diarrhoea at presentation [χ^2 (1, N = 53) = 6.687; p = 0.010 by chi square analysis]. There was no significant association with other symptoms at presentation or comorbidities. For multivariate logistic regression analysis, we fitted a logistic model (estimated using maximum likelihood) to predict long COVID (persistent symptoms at 30 days post discharge) with age, female gender, fever, dyspnea and diarrhoea at presentation, gap between symptom onset and admission (Figure 2). The model's explanatory power is substantial (Tjur's R^2 = 0.36). The model's intercept is at -2.83 (standard error (SE) = 1.35, 95% confidence interval (CI) [-5.77, -0.40], $p < 0.05$). Within this model, the individual components have been shown in the Table 4 given below. Therefore, diarrhoea at presentation and gap between symptom onset and admission were positively correlated with persistent symptoms at 30 days post discharge.

3.4. Symptom clusters: COVID-19 phenotypes

We performed K nearest neighbour cluster and

exploratory factor analysis. We found 3 clusters. 2 Dimensions (principal component 1 and principal component 2 explained up to 48% of variability addition of other dimensions had minimal incremental effect). Hence, this principal component analysis (PCA)-biplot was made showing effect of dimensions, component and contribution of symptom variable to various dimensions of our factor analysis. Dimension 1 and 2 denote the principal component (1 and 2 of exploratory factor analysis). Narrow angle of vector and longer vector length indicate closer association and effect size to a dimension. Colours denote clusters based on K nearest neighbour classification.

Cluster analysis revealed three possible phenotypes of mild COVID illness: Cluster 1 (diarrhoea, rhinitis, myalgias, and sore throat, majorly upper respiratory tract involvement), cluster 2 (fever and fatigue, comprising constitutional symptoms), and cluster 3 (dyspnea and cough, majorly lower respiratory tract involvement) (Figure 3). On exploratory factor analysis, most vectors follow their clusters except for rhinitis that may not be aligning with its cluster due to the small sample size in our study.

4. Discussion

Our study recruited 57 Health care workers (or their dependents) admitted with mild COVID-19 of which 53

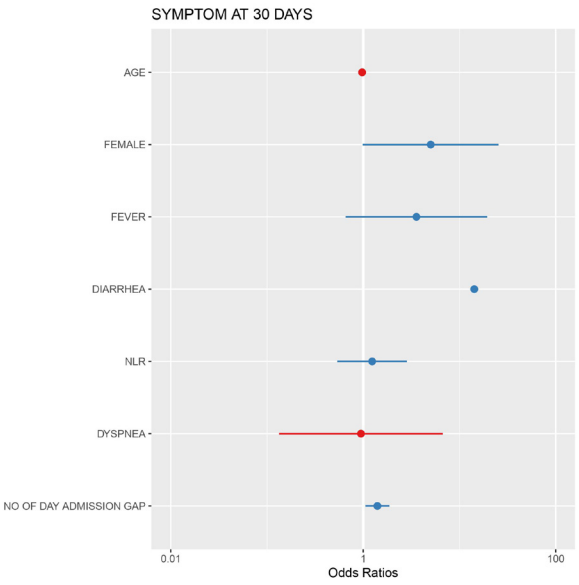

Figure 2. Forest plot for multivariate regression analysis for persistent symptoms. [Covariates: Age, sex, fever, dyspnea and diarrhea at presentation, neutrophil lymphocyte ratio and gap between symptom onset and admission; all except age and dyspnea (marked in red), showing varied association with long COVID-19].

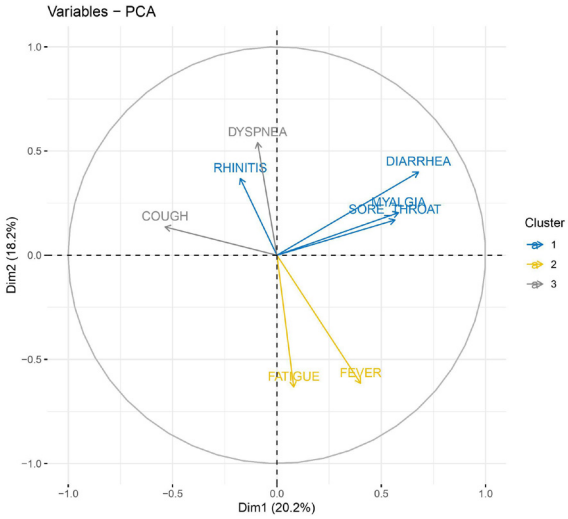

Figure 3. Symptom cluster analysis: 3 phenotypes of mild COVID-19. Narrow angle of vector and longer vector length indicate closer association and effect size to a dimension. Three phenotypic clusters identified: cluster 1 (diarrhea, rhinitis, myalgia, sore throat); cluster 2 (dyspnea and cough); cluster 3 (fever and fatigue). PCA, principal component analysis; Dim 1, Dimension 1; Dim 2, Dimension 2.

Table 4. Analysis for persistent symptoms at 30 days

Characteristic	Persistent symptoms absent ($N = 28$)	Persistent symptoms present ($N = 25$)	Odds Ratio (univariable)	Odds Ratio (multivariable)
Age (years), Mean (SD)	32.2 (12.74)	37.28 (11.37)	1.04 (0.99-1.09; $P = .139$)	0.97 (0.90-1.05; $P = .484$)
Sex				
Male (%)	18 (64.3)	10 (40.0)	2.70 (0.90-8.48; $P = .080$)	5.02 (1.07-29.63; $P = .049$)
Female (%)	10 (35.7)	15 (60.0)		
Days between symptom onset and admission, Mean (SD)	2.96 (1.89)	4.64 (3.59)	1.24 (1.01-1.58; $P = .051$)	1.40 (1.08-1.93; $P = .020$)
Neutrophil-Lymphocyte Ratio (NLR), Mean (SD)	1.6 (0.6)	2.0 (1.3)	1.54 (0.87-3.13; $P = .175$)	1.24 (0.57-3.17; $P = .614$)
Fever at admission				
Absent	13 (46.4)	8 (32.0)	1.84 (0.61-5.83; $P = .286$)	3.57 (0.72-23.07; $P = .141$)
Present	15 (53.6)	17 (68.0)		
Dyspnea at admission				
Absent	25 (89.3)	19 (76.0)	2.63 (0.61-13.76; $P = .209$)	0.95 (0.13-7.38; $P = .957$)
Present	3 (10.7)	6 (24.0)		
Diarrhoea at admission				
Absent	26 (92.9)	16 (64.0)	7.31 (1.63-52.14; $P = .018$)	14.26 (2.30-142.47; $P = .009$)
Present	2 (7.1)	9 (36.0)		

patients were included in the final analysis for persistent symptoms at 30-day follow up. The most common persistent symptom reported by far, was fatigue, followed by cough and myalgia. There was a significant association of persistent symptoms with diarrhoea at presentation, and gap between symptom onset and admission on multivariate logistic regression analysis. On cluster analysis, three phenotypes of mild COVID-19 were identified, which may have implications on monitoring and management.

Tenforde *et al.* followed up 292 COVID-19 outpatients in the US, telephonically for up to 14-21 days from symptom onset (median 16 days) (*8*). They reported the persistence of cough, fatigue and breathlessness in 43%, 35%, and 29% patients, respectively, at 14-21 days from symptom onset. These findings would suggest that post-COVID-19 is an entity with implications even in mildly affected individuals.

Carfi *et al.* followed up 143 patients admitted with COVID-19 at a hospital in Italy at 60 days after onset of first symptom, for persistent symptoms, if any (*9*). The mean age of the study participants was 56.5 ± 14.6 years and 53 patients (37%) were women. Only 18% patients were found to be asymptomatic at follow up, while 55% had at least 3 symptoms. The symptoms reported most often was fatigue (53.1%), as seen in our study. Other common symptoms were shortness of breath (43.4%), arthralgia (27.3%) and chest pain (21.7%). The overall proportion of patients with persistent symptoms was lower (47.17%) in our population, probably because we included a major proportion of mild (93.0%) and moderate (7.0%) cases, and no severe COVID cases. On the contrary, Carfi *et al.* recruited 21 patients (15.0%) who had been on non-invasive ventilation and 5 patients (7.0%) who had been on invasive ventilation during hospital stay, which may explain the higher prevalence of persistent symptoms. Around 44.1% participants reported a worsened quality of life at follow up, as per the EuroQoL questionnaire.

There is a paucity of data on predictors of persistent symptoms in COVID-19 infection. Chen *et al.* reported significant impairment in physical and psychological health among 361 COVID-19 patients at one-month post discharge, using the HRQoL questionnaire (*10*). It was seen that the more severe the condition of patients, the worse the impact on physical, emotional as well as mental health. There was a significant association between higher body-mass index (BMI) and poorer physical health outcomes.

Our study revealed a possible association of persistent symptoms with diarrhoea at presentation. A retrospective single-centre analysis by Wei *et al.* reported longer duration of illness (fever and dyspnea) among patients presenting with diarrhoea than those without (10.5 ± 4.7 days *vs.* 7.6 ± 3.4 days, $p < 0.005$; 8.1 ± 3.2 days *vs.* 4.7 ± 2.3 days, $p < 0.002$, respectively) (*11*). The hospital stay duration was longer in diarrhoea group than in non-diarrhoea group (16.5 ± 5.2 days *vs.* 11.8 ± 5.6 days; $p < 0.001$). It was also seen that patients with diarrhoea were more likely to complain of headache, myalgia or fatigue, cough, nausea and vomiting than those without diarrhoea.

Shang *et al.* retrospectively studied the implications of diarrhoea among 564 hospitalised COVID-19 patients in China (*12*). They categorised the 157 diarrhoea patients among two groups: 38 patients with diarrhoea alone (group A) and 119 with both diarrhoea and respiratory symptoms (group B). They reported worse clinical outcomes among group B patients than group A patients and group C (respiratory symptoms alone) in terms of higher levels of inflammatory activity (higher ferritin and CRP levels), longer hospital stay (27.5 *vs.* 23.0 *vs.* 22.0 days, $p < 0.029$). Group B patients also had an odds ratio of mortality of 3.2 compared to group A patients. This was attributed to possible higher viral loads and in turn, a stronger inflammatory response in patients with both respiratory and gastrointestinal involvement, though the exact mechanism is not known. Group A patients had a longer time from onset of symptoms to admission (14.5 *vs.* 11.0 days, $p < 0.04$) but an overall milder illness.

As per the report of the WHO-China Joint Mission on COVID-19, diarrhoea is an important manifestation of COVID-19 with public health implications due to prolonged faecal shedding of the virus, creating a potential for faecal-oral transmission (though not proven to be significant so far). It has been shown that angiotensin-converting enzyme-2 (ACE 2) receptors are present not only in the lung, but also in the gastrointestinal tract (stratified cells of the oesophagus and enterocytes in the ileum and colon) (*13*). SARS-CoV2 may directly damage the epithelial barrier leading to diarrhoea, and mucosal inflammation and cytokine release. This may even promote translocation of pathogens. Moreover, intestinal mucosal barrier injury could affect the mucosal immune response of the lung, thereby, worsening the pneumonia and may give rise to a cytokine storm through the 'gut-lung axis' (*14*).

Therefore, our study brings forth certain important observations. It is important to consider gastrointestinal symptoms as an important presentation of COVID-19. As demonstrated in our cluster analysis, these symptoms may occur in the absence of typical symptoms of fever or cough. Therefore, a high suspicion for COVID-19 is paramount in patients with diarrhoea. This is highlighted by the fact that diarrhoea at presentation may portend worse clinical outcomes in the form of prolonged symptoms, such as fatigue, myalgias, chest discomfort, *etc.* This is an outcome with important implications and should prompt further research into the matter. It is also of concern to note that patients with a longer gap between symptom onset and admission are more likely to complain of persistent symptoms at follow up. Therefore, it is important to recognise symptoms of

COVID-19 (respiratory and gastrointestinal), especially in mild illness, so that patients are brought to medical attention at the earliest. Prolonged duration of symptoms also indicates a need for "post-COVID" rehabilitation services (yoga, meditation, breathing exercises, adequate nutrition, *etc.*) (*7*) to ensure speedy return to usual state of health. We also identified specific phenotypic clusters which could possibly lead us to further observation in large population and then developing as a tool for prognosis.

There are certain limitations to our study. Firstly, the sample size was small and definite conclusions about predictors of clinical outcomes could not be formed. Secondly, most patients had mild COVID-19 illness, which may introduce a bias in our findings.

5. Conclusion

It can be concluded that there may be a positive association of diarrhoea as a presenting manifestation and gap between symptom onset and admission with the persistence of symptoms classified as long COVID, even in mild illness. There also appear to be multiple phenotypes of mild COVID-19 illness, which warrant further exploration.

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Appendix A

Logistic regression showing the risk of the standardized parameters

Items	Coefficient	S.E.	Wald Z	Pr (> | Z |)
Intercept	-2.1520	1.1038	-1.95	0.0512
AGE	-0.0045	0.0280	-0.16	0.8733
Female = 1	0.8461	0.5628	1.50	0.1327
Fever = 1	0.5983	0.5754	1.04	0.2984
Diarrhoea = 1	1.3170	0.6272	2.10	0.0358
NLR	0.2509	0.3209	0.78	0.4342
Dyspnea = 1	0.1133	0.6587	0.17	0.8634
Number of days between admission & symptom onset	0.2144	0.1075	1.99	0.0462

Brief Report

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The clinical significance of cytokeratin 20 staining pattern in Merkel cell carcinoma

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SUMMARY In the present study, to identify the clinical significance of the cytokeratin (CK) 20 staining pattern in Merkel cell carcinoma (MCC), we retrospectively analyzed the major clinicopathological and immunohistochemical characteristics of 12 cases of MCC. Typical dot-like pattern was seen in eight of our patients, while four patients showed peripheral staining pattern. Interestingly, all cases of MCC with dot-like CK20 tumor cells occurred in the head and neck region, while those with peripheral CK20 pattern tended to be located in other lesions (forearm, knee, or buttock): The difference of frequency in the head and neck regions was statistically significant. Dot-like CK20 staining pattern may therefore be resulted from ultraviolet exposure. Additionally, although without significance, metastasis was more frequent in those with dot-like CK20 than in peripheral CK20 staining: All patients with peripheral CK20 pattern had complete remission by surgical excision with or without radiation therapy. CK20 staining pattern may be a novel predictor of prognosis.

Keywords Immunohistochemistry, skin, CK20

1. Introduction

Merkel cell carcinoma (MCC) is a rare and aggressive epithelial cutaneous neoplasm with neuroendocrine differentiation. It typically appears as solitary red-violaceous tumors in sun-exposed head and neck lesions of elderly people (*1*); it grows rapidly without pain. Males are more susceptible, and the mean age of patients is reported to be 70 years (*2*). Merkel cell polyomavirus is detected in 80% of the lesions. Histopathologically, the tumor typically consists of a dense population of small, round, and uniform cells in the dermis and subcutaneous tissue without change to the overlying epidermis. Differential diagnosis includes the "small round blue cell" tumor group, *e.g.*, metastatic small cell carcinoma of the lung. Immunohistochemical analysis of cytokeratin (CK)20, epithelial membrane antigen, neuron-specific enolase, synaptophysin, chromogranin A, thyroid transcription factor (TTF)-1, S100 protein, leucocyte common antigen, and CD56 has been shown to be useful for the diagnosis (*1*).

CK20 staining of tumor cells shows perinuclear dot-like patterns in the majority of patients with MCC. Meanwhile, we recently noticed peripheral CK20 staining pattern in a patient subset. In this study, to identify the clinical significance of CK20 staining pattern, we retrospectively analyzed the major clinicopathological and immunohistochemical characteristics of patients with MCC.

2. Materials and Methods

2.1. Clinical assessment and patient material

The present study includes 12 patients with MCC (6 men and 6 women) who visited Wakayama Medical University Hospital between 2010 and 2020. All patients were diagnosed based on clinical manifestation and immunohistopathological findings. Patient data (age, gender, location, diameter of MCC, immunohistological findings, and prognosis) were collected retrospectively (Table 1).

2.2. CK20 immunoreactivity

Skin samples were paraffin-embedded, and sections were dewaxed in xylene and rehydrated in graded alcohols. Hematoxylin and eosin staining was performed as described previously (*3*).

For immunostaining, anti-cytokeratin 20 rabbit

monoclonal primary antibody (Roche Diagnostics, Indiana, USA) was optimized for use with VENTANA OptiView DAB IHC Detection Kit (Ventana medical system, Arizona, USA) on automated VENTANA BenchMark ULTRA platform (Roche Diagnostics). CK20-positive cells were randomly observed in five different 800-fold magnification fields under the inverted microscope. Dominant staining patterns were recorded.

2.3. Statistical analysis

Statistical analysis was carried out with Fisher's exact test to compare frequency. P values < 0.05 were considered significant.

3. Results and Discussion

3.1. Clinical and histopathological features of MCC patients

We collected the clinical data of 12 patients with MCC for the present study. Detail of one of the patients (case No.3) was previously published as case report focusing on reconstruction surgery (*4*).

As shown in Table 1, the clinical characteristics of 12 lesions were as follows: mean age was 82.1 years (age range: 75-89 years), and male:female ratio was 6:6. Distribution of the tumors was 3 lesions in the cheeks, 2 in the eyelids, 1 in the external canthus, 3 in the earlobes, 1 in the forearm, 1 in the lower leg, and 1 in the buttock. The mean diameter of the tumors was 2.6 cm (range: 1-5 cm). Regarding the immunoreactivity of the tumor cells, staining of CK20 was positive in all cases. CD56 was also positive in all seven tested cases. Positive staining of synaptophysin or chromogranin A, the most common neuroendocrine markers, was also observed in eight out of the nine tested cases. None of the cases tested were positive for CK7 or TTF-1 (0/7 and 0/9, respectively). Merkel cell polyomavirus was detected in three cases.

These clinical and immunohistopathological features are generally consistent with previous notions (*2*). We noticed, however, that there are two different CK20 patterns of tumor cell immunoreactivity: typical dot-like pattern, a perinuclear globular aggregation, was seen in eight cases, while four cases showed peripheral staining patterns. Representative clinical and histopathological findings of cases with dot-like pattern and those with peripheral pattern are shown in Figures 1 and 2, respectively.

3.2. Clinical significance of different CK20 immunoreactivity of tumor cells

We then attempted to identify the clinical significance of different CK20 immunoreactivity of tumor cells. There

Table 1. Summary of clinical and histopathological features of our patients

Case	Age/Gender	location	Diameter (cm)	CK20	CK7	Synap	CgA	TTF1	CD56	MCV	prognosis
1	89/M	external canthus	2	dot-like	ND	+	+	-	+	+	metastasis after surgical excision
2	79/F	earlobe	3	dot-like	-	ND	ND	ND	ND	ND	suspected metastasis after radiation
3	79/F	eyelid	1	dot-like	ND	+	+	-	ND	ND	CR by surgical excision
4	80/M	cheek	2	dot-like	-	+	-	-	+	ND	CR by radiation
5	75/F	cheek	5	dot-like	ND	+	+	ND	ND	+	CR by surgical excision
6	79/F	cheek	2.5	dot-like	-	-	+	ND	+	ND	metastasis after surgical excision and radiation
7	87/M	earlobe	1.5	dot-like	ND	+	+	-	+	ND	CR by surgical excision
8	84/F	earlobe	1.5	dot-like	-	+	+	-	+	ND	metastasis after surgical excision, radiation, and avelumab
9	89/F	forearm	2	peri	-	ND	ND	-	ND	ND	CR by surgical excision
10	89/M	knee	3	peri	ND	+	+	-	+	+	CR by surgical excision and radiation
11	78/M	eyelid	3	peri	-	+	+	-	+	ND	CR by surgical excision
12	77/M	buttock	5	peri	-	ND	ND	-	ND	ND	spontaneously regressed and CR by surgical excision

peri: peripheral pattern; ND: not determined; Synap, Synaptophysin; CgA, Chromogranin A; TTF1, thyroid transcription factor; MCV, Merkel cell polyomavirus; CR: complete remission.

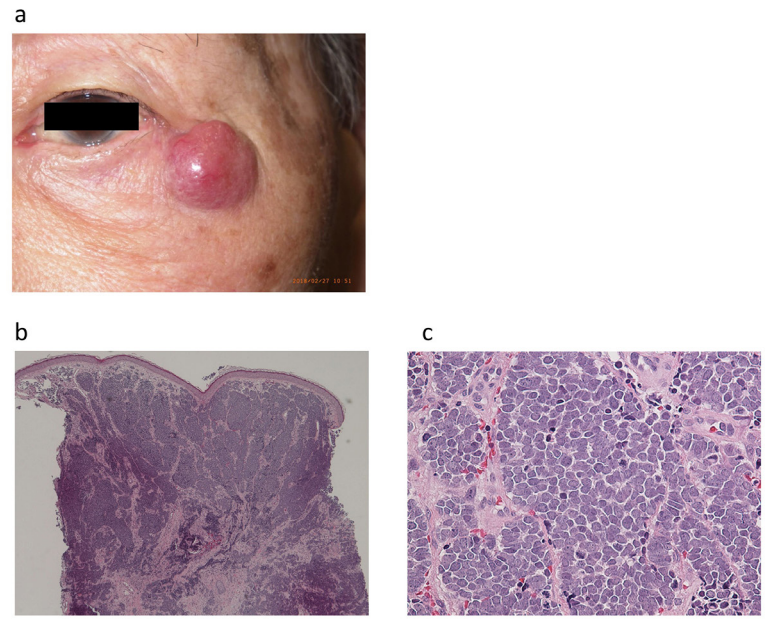

Figure 1. Representative clinical pictures and histopathological findings of a patient with dot-like perinuclear CK20 (patient No.1). **(a)** Clinical presentation of the tumor on the external canthus. **(b)** Hematoxylin and eosin staining showing dermal proliferation of small round blue cells (magnification: × 40). **(c)** The tumor cells were round and basophilic with high nuclear:cytoplasmic ratio, hyperchromatic nuclei, and finely stippled salt and pepper chromatin pattern. Mitotic figures were also found (magnification: × 400). **(d)** The tumor cells were positive for CK20 (magnification: × 40). **(e)** CK20 staining showed perinuclear dot-like pattern (magnification: × 800).

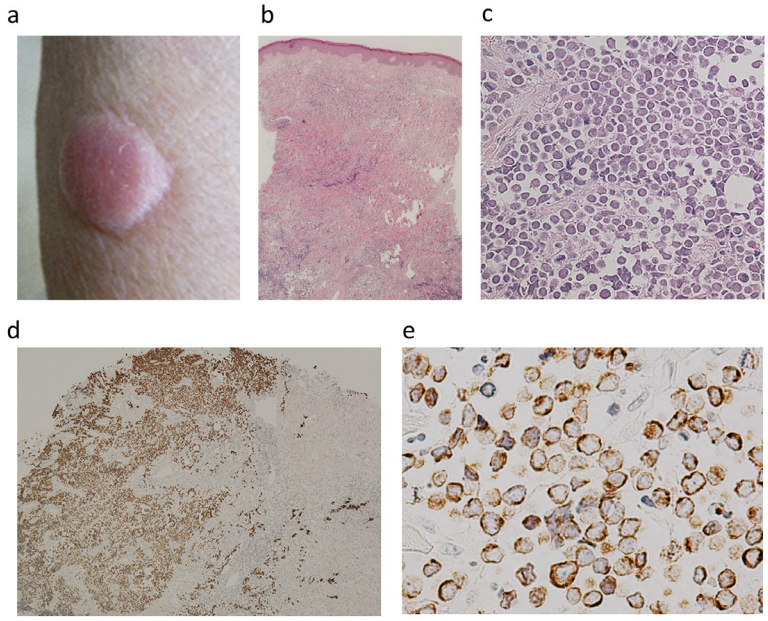

Figure 2. Representative clinical pictures and histopathological findings of a patient with peripheral CK20 (patient No.9). **(a)** Clinical presentation of a tumor on the forearm. **(b)** Hematoxylin and eosin staining showing dermal proliferation of small round blue cells (magnification: × 40). **(c)** The tumor cells were round and basophilic with high nuclear:cytoplasmic ratio and finely stippled salt and pepper chromatin pattern (magnification: × 400). **(d)** The tumor cells were positive for CK20 (magnification: × 40). **(e)** CK20 staining showed peripheral nuclear pattern (magnification: × 800).

was no statistically significant correlation with age, gender, location, or diameter of the tumors. However, all of the MCCs with dot-like CK20 tumor cells occurred in the head and neck region, while those with peripheral CK20 pattern tended to be located in other lesions (forearm, knee, or buttock), and the difference of frequency in head and neck lesions was statistically significant (100.0% *vs*. 25.0%, $p = 0.0182$ by Fisher Exact test).

In addition, although insignificant, metastasis was more frequent in those with dot-like CK20 (50.0% *vs*. 0.0%, $p = 0.21$) than peripheral CK20 staining; all patients with peripheral CK20 pattern resulted in complete remission by surgical excision with or

without radiation therapy. There was no difference in the positivity of other immunostaining between the two groups.

CK20 is a type I cytokeratin, which is a major cellular protein of mature enterocytes and goblet cells. CK20 expression is usually therefore observed in normal gastric and intestinal mucosa (*5*), and CK20 staining is used to identify a range of adenocarcinoma arising from CK20-positive epithelia, including colorectal cancer. In addition, tumor cells of MCC are well known to express CK20, but are absent in metastatic lung cancer.

CK20 staining pattern of enterocytes is usually diffuse, while dot-like perinuclear pattern was characteristic of the tumor cells of MCC (*6,7*). To our knowledge, the mechanism of dot-like CK20 staining, however, remains unknown. Furthermore, we noticed peripheral staining pattern of CK20 in a subset of MCC patients. We found that all MCCs with dot-like CK20 tumor cells occurred in the head and neck region, while those with peripheral pattern CK20 tended to be located in the trunk and extremities, and the difference was statistically significant. Established risk factors for MCC are immunosuppression and extensive sun exposure: increased frequency of MCCs in patients with acquired immune deficiency syndrome and in those receiving immunosuppressive therapy has been reported (*7*). The incidence rate of cutaneous MCC is also increased by sun exposure (*7*). Here, all MCCs with dot-like CK20 tumor cells occurred in the head and neck region, so dot-like CK20 staining pattern may be the result of ultraviolet exposure. There has been quite a few report to focus on the staining pattern of CK20 in patients with MCC.

Metastasis was more frequent in patients with dot-like CK20 than in peripheral CK20 staining, whereas all patients with peripheral CK20 pattern resulted in complete remission by surgical excision with or without radiation. The rates of local and regional recurrence were reported to be 29% and 59%, respectively, in MCC patients (*7*). In addition, the estimated mortality rate for all patients with MCC is between 25% and 35% (*8*). Previous studies reported poor prognostic factors to be male sex, tumor size > 5 mm or > 20 mm, location (in the buttock/thigh/trunk or in the head), advanced clinical stage, small cell size, high mitotic index, diffuse growth pattern, as well as p53 positivity (*9*). CK20 staining pattern may also be a novel predictor of prognosis in MCC. Detailed research with a larger number of samples is necessary to prove clinical significance of CK20 staining pattern.

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Commentary

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Aducanumab: The first targeted Alzheimer's therapy

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SUMMARY Alzheimer's disease (AD) is an irreversible brain disorder associated with severe progressive dementia and is characterized by deposits of amyloid plaques in the brain. Over the past 20 years, the mortality of strokes and heart disease has decreased, but deaths from AD have increased. The four drugs used clinically to treat AD can only relieve symptoms but cannot slow the progression of the disease. Aducanumab, a human monoclonal antibody that preferentially binds to aggregated amyloid-β to reduce the number of amyloid plaques and slow disease progression, was approved to treat AD by the US Food and Drug Administration on June 7, 2021. It is the first disease-modifying therapy for AD, but there is considerable controversy regarding the drug's approval. Aducanumab offers hope for millions of patients.

Keywords Alzheimer's disease, aducanumab, amyloid-β, clinical trials

Alzheimer's disease (AD) is a progressive neurodegenerative disorder with memory loss and decline in cognitive function. It is the most common cause of dementia and accounts for 60-70% of dementia cases (*1*). In the US, approximately 6.2 million people suffer from AD, and the number is predicted to reach 13.8 million by 2060. From 2000 to 2019, deaths from AD increased more than 145%, so AD has become the sixth-leading cause of death in the US (*2*). However, current treatments do not meet clinical need. By June this year, just four drugs were approved by the US Food and Drug Administration (FDA) for the treatment of cognitive impairment and dysfunction in symptomatic AD, including three cholinesterase inhibitors (donepezil, rivastigmine, and galanthamine) and a glutamate regulator memantine. However, these drugs can only help lessen symptoms, such as memory loss and confusion (*3*).

On June 7, 2021, the FDA announced that aducanumab was approved to treat patients with AD. Aducanumab is the first novel therapy approved for AD since 2003. More significantly, it is the first treatment directed at the underlying pathophysiology of AD (*4*). The key pathological changes that are observed in AD brain tissue are increased levels of both the amyloid-β (Aβ) peptide and hyperphosphorylated tau (p-tau). Aβ is a tiny protein fragment that forms and accumulates in the brain as a plaque. These plaques disrupt communication between nerve cells and may also activate immune system cells, triggering inflammation and phagocytosis of damaged nerve cells (*5*). The amyloid hypothesis holds that Aβ-related toxicity is the main cause of synaptic dysfunction and subsequent neurodegeneration, which is the basis of AD progression (*6*). Although scientists are not sure what causes cell death and tissue loss in the process of AD, amyloid plaques are one of the potential factors. Aducanumab is a type of human monoclonal antibody that can selectively interact with Aβ aggregates, including soluble oligomers and insoluble fibrils, and that then clears Aβ. Aducanumab is the first therapy to prove that removing Aβ leads to better clinical outcomes (*7*).

Thirty-seven years have passed since when Aβ was first proposed as the pathogenesis of AD (*8*) and when aducanumab was approved. Over the past 20 years, several drugs that decrease the production of Aβ (mainly γ-secretase and β-secretase inhibitors) or increase Aβ brain clearance (anti-Aβ monoclonal or polyclonal antibodies and Aβ antigens) have been identified. Unfortunately, in all clinical trials to date, these treatments have failed to improve cognitive outcomes despite reducing brain Aβ (*9*). Aducanumab's approval was also a long and arduous journey. Biogen and Eisai conducted two global Phase 3 trials on aducanumab in 2015 – ENGAGE and EMERGE. Both trials were terminated in March 2019 due to previous invalid analyses using smaller datasets. Afterwards, the researchers obtained a large dataset and analyzed it again. Results of the EMERGE trial indicated that the cognitive ability of patients treated with high-

dose aducanumab improved significantly (*10*). On October 22, 2019, they announced their plan to seek regulatory approval for aducanumab. On July 8, 2020, Biogen announced the completion of its application to the FDA for a Biologics License for aducanumab for the treatment of AD. On November 6, 2020, the FDA Peripheral and Central Nervous System Drugs Advisory Committee voted against the proposal (*11*). Later, three members of the committee, G. Caleb Alexander, Scott Emerson, and Aaron S. Kesselheim, expressed their opposition to the drug in an article in the Journal of the American Medical Association (*12*). Their paper focuses on the contradictory results of the two trials and aducanumab's potential safety hazards. On June 7, 2021, the FDA approved aducanumab as the first and only drug to reduce Aβ plaques in the brain to solve the pathological problems of dementia plaques. As part of the accelerated approval, Biogen will conduct a controlled trial to verify the clinical benefits of aducanumab in patients with AD (*4*).

According to statistics, in 2021, 126 drugs for the treatment of AD are undergoing clinical trials. One hundred and four drugs (82.5%) target the pathophysiology of AD, with 17 (13.5%) targeting Aβ and 11 (8.7%) targeting the tau protein. A point worth noting is that there are 19 drugs for inflammation/infection/immunity and 17 drugs for synaptic plasticity/neuroprotection in the clinical stage; these 2 groups represent the largest proportion (13). Reviews of clinical trials indicate that there is progressive emphasis on non-amyloid targets, such as synaptic plasticity, inflammation, metabolism, and proteostasis (*13-15*). Over the past few years, researchers have found new targets for AD. Wang *et al*. confirmed that an imbalance in intestinal microbiota promotes the infiltration of peripheral immune cells in a mouse model of amyloidosis, which is related to behavioral and AD-related pathological changes. A drug to address this mechanism, GV-971, is in a phase 3 clinical trial in China (*16*). APOE4 accelerates blood brain barrier (BBB) decomposition and neurodegeneration in AD mice via the cyclophilin A pathway in pericytes, which is crucial to the pathogenesis and treatment of vascular and neurodegenerative disorders in AD (*17*). Lau *et al*. found that an IL-33-PU.1 axis is involved in transcriptional regulation and that it promotes beneficial microglial functions in AD (*18*). Their findings provided important insight into the therapeutic potential of targeting glial- and endothelial-specific pathways to restore brain homeostasis in AD (*19*). Long-term delivery of antibodies blocking CD22 in the central nervous system reprograms microglia into stable transcription and improves cognitive function in older mice (*20*). TREM2 enables the microglial response in AD by maintaining cell energy and biosynthesis (*21*). Reducing the time between finding targets and developing effective drugs may accelerate the approval of new drugs for AD.

There are other reasons for the difficulty of developing drugs for AD. AD develops well before patients develop the symptoms associated with Alzheimer's dementia. In fact, research suggests that brain changes associated with the disease may begin 20 or more years before symptoms appear (*22*). Therefore, early screening and prevention of AD is vital. Guidelines on preventing AD based on meta-analyses were published in 2018 (*23*). The guidelines suggest that a combination of evidence-based clinical recommendations may be the best choice for the prevention of AD. That said, a comprehensive and individually tailored strategy to prevent and treat AD should also be formulated. Therefore, personalized prevention and treatment should be developed for high-risk groups.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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Communication

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The percutaneous tandem drainage technique for radical treatment of intractable hepaticojejunostomy leakage

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SUMMARY The principal concept of the percutaneous tandem drainage procedure for an intractable hepaticojejunostomy (HJ) leakage is to decrease the amount of fluid and divide the fluid-filled cavity into several small cavities, which can then be drained individually. Percutaneous abscess drainage (PAD) has a role in drainage of the fluid cavity, whereas percutaneous trans-anastomotic jejunum drainage (PTAJD) has a role in drainage to reduce the bile fluid and digestive juices. A decrease in fluid induces effective drainage of the fluid cavity by PAD. A negative pressure suction drain accelerates reduction of the fluid cavity. PAD is removed when the localized fluid cavity has collapsed. PTAJD is finally removed after a clamping test is performed. Since 2020, we performed the percutaneous tandem drainage for two patients, and an intractable HJ leakage was gently resolved within 3 months without any adverse event. The percutaneous tandem drainage technique is safe for steady drain management of an intractable HJ leakage.

Keywords Anastomotic leakage, hepatectomy, drainage

A hepaticojejunostomy (HJ) leakage post-hepatectomy can sometime cause intractable leakage and require long-term drainage or re-anastomosis of the HJ; percutaneous abscess drainage (PAD) is the gold standard therapy for well-localized fluid collections (*1,2*). The principal strategy for a major leakage is to decrease the amount of fluid and divide the fluid-filled cavity into several small cavities, which can then be drained individually (*3*). At the time of cavity was not shrinkage and leakage continued even though PAD worked appropriately, we performed the percutaneous tandem drainage technique for radical intense treatment of intractable HJ leakage as additional therapeutic step. The details of the percutaneous tandem drainage procedure are shown in Figure 1. CT-guided PAD was performed on intra-abdominal fluid cavities with low-pressure contrast radiography. HJ leakage is usually detected 1-3 weeks after PAD, depending on the effectiveness of PAD and the size of the fluid cavity. At this timing, percutaneous trans-anastomotic jejunum drainage (PTAJD) is implanted into the jejunum *via* the HJ leakage point from the same entry site of the skin as the PAD in a tandem manner (Figure 2a). Namely, two guidewires were inserted into the abscess cavity from PAD. And then one of the guidewire placed in the abscess cavity was inserted into the jejunum *via* the HJ leakage point. One catheter was inserted over the wire which was advanced into the jejunum, due to decompressed jejunum and blocked the HJ leakage. Another drainage catheter was placed in the abscess cavity. PTAJD easily reaches the leakage site with PAD guidance. The PAD tip is maintained 1-2 cm away from the leakage site. PAD has a role in drainage of the fluid cavity, whereas PTAJD has a role in drainage to reduce the bile fluid and digestive juices. A decrease in fluid induces effective drainage of the fluid cavity by PAD. The PAD tip is gradually maintained 2-3 cm away from the leakage site, then the fluid cavity is divided into localized fluid and peri-HJ cavities (Figure 2b). A negative pressure suction drain accelerates reduction of the fluid cavity. PAD is removed when the localized fluid cavity has collapsed. PTAJD is finally removed after a clamping test is performed.

We performed hepatectomy with HJ in 24 patients who had hepatobiliary disease between 2019 and 2020. Even though external trans-anastomotic bile drainage stent was routinely placed during hepatectomy with HJ, HJ leakage was developed in three patients who had biliary tract cancer. Since 2020, we performed the percutaneous tandem drainage for two patients who had

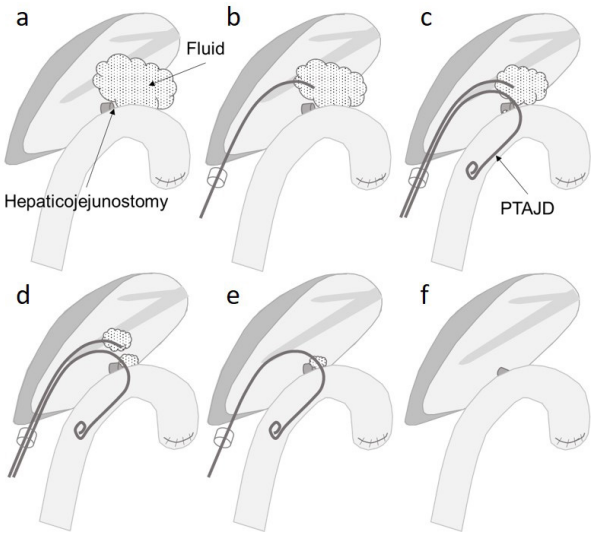

Figure 1. Procedure of percutaneous tandem drainage for an intractable leakage of hepaticojejunostomy. At the time of undrainaged fluid (thick dot) was confirmed **(a)**, CT guided percutaneous abscess drainage (PAD) was performed **(b)**. If major leakage of hepaticojejunostomy (HJ) was confirmed, percutaneous trans anastomotic jejunum drainage (PTAJD) was added from same entry site of skin tandem with PAD **(c)**. Fluid cavity would be divided into localized abscess cavity and peri-HJ cavity **(d)**. PAD was removed whenever localized abscess cavity disappeared **(e)**, then PTAJD was removed **(f)**.

intractable HJ leakage. We preferred using soft type guide wire (Radifocus®) and its sheath for every drain management, and PAD guided tandem insertion of PTAJD was simply performed without any technically failure. Because a drainage tube for PTAJD was soft and highly trackable, we have never experienced tear of HJ leakage point. Consequently, HJ leakage was gently resolved within 3 months without any adverse event. The percutaneous tandem drainage technique is simple and safe for steady drain management of an intractable HJ leakage.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

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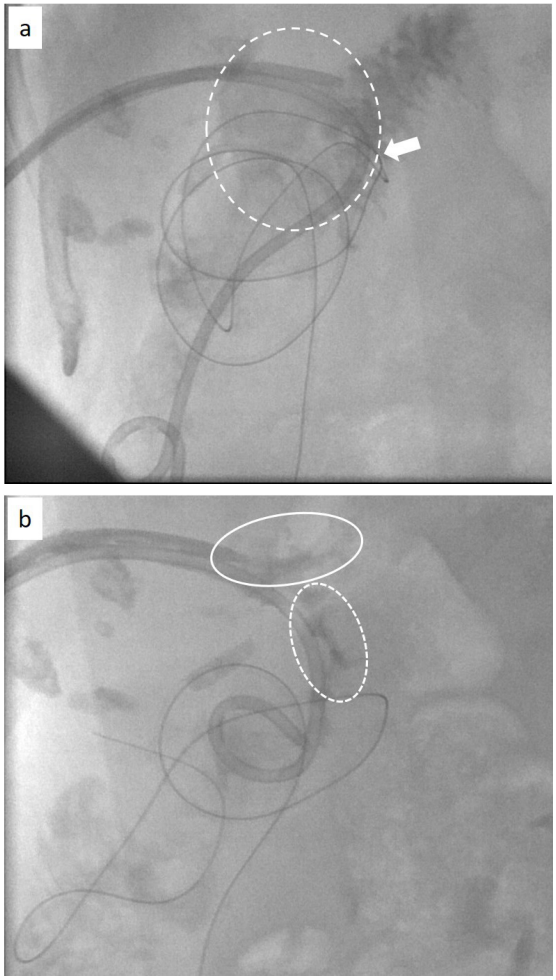

Figure 2. Percutaneous tandem drainage divided abscess cavity into two of separated small cavity. Percutaneous abscess drainage tandem with percutaneous trans anastomotic jejunum drainage was displayed. White allow indicated hepaticojejunostomy, and white dot circle indicated fluid cavity of major leakage of hepaticojejunostomy **(a)**. 2 months later **(b)**, fluid cavity was divided into localized abscess cavity (white circle) and peri-hepaticojejunostomy cavity (white dot circle).

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