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Pakistan Academy of Sciences

Guest Editorial

ANSO-PAS Joint Efforts for Capacity Building in Emerging Pathogens Research

Welcome to the special issue of the Proceedings of Pakistan Academy of Sciences – B. The issue comprises articles submitted by the speakers/ presenters of the ANSO-PAS-MAAP Conference – 2023 entitled "Pathogen transmission beyond borders: Understanding the complexities of cross-species infectious diseases" held at the Pakistan Academy of Sciences. In this event, a total of 35 experts presented their research findings in the form of invited/oral talks, providing insights into different aspects of the conference themes. The conference is part of the ongoing ANSO collaborative Research Project 2022-24.

The project provides a basis for PAS-ANSO strategic planning for epidemic and pandemic preparedness. The project's experimental work contributes to capacity development for the detection of emerging and bat-borne viruses. It is for the first time in Pakistan that we are investigating bat's-virome from bats-guano samples. In addition to the wet-lab experiments, we organized seven events (five training/workshops and two conferences) consisting of practical lessons, theoretical knowledge, poster presentations and brainstorming sessions. In addition, it provides collaboration opportunities to more than 1200 participants/experts from all over the globe. In these events, more than 100 distinguished scholars, both domestically and internationally renowned, converged their collective expertise to deliberate upon the crucial facets of cross-species & cross-border infections, emerging viruses, epidemic & pandemic preparedness, laboratory risk assessment/ management, biological safety and security etc.

As outcome of the current project, we reported the beta-coronaviruses for the first time in bats from Pakistan [1]. In addition, we published conference/workshop proceedings, abstract books and recommendations [2-7]. Moreover, we investigated the bat-virome for detecting Orthomyxoviruses because bats serve as potential reservoir hosts for unique influenza A virus subtypes. The analysis is based on Influenza A viruses' highly conserved region (partial M gene). The phylogenetic analysis of the studied viral isolates (submitted to GenBank under the accession numbers PP151243-PP151250) suggests they are closely related to previously reported Influenza A viruses from diverse species (bats, ducks, and environmental samples) and reference sequences reported from diverse geographic locations, including Korea, Japan, Egypt, and Sweden. Furthermore, we optimized PCR amplification experiments for the detection of bats-associated Flaviviruses and Adenoviruses.

Events organized under ANSO project:

- ANSO-PAS-QAU Workshop "Experimental and ethical considerations in non-human virus research" (August 03-05, 2024). University of Loralai, Balochistan, & Bolan University of Medical & Health Sciences (BUMHS), Quetta, Balochistan.
- ANSO-PAS Workshop "Emerging viral infections: Insights from molecular studies" (May 11-12, 2024). Directorate Health Services Baltistan, and University of Baltistan, Skardu, Pakistan.
- ANSO-PAS-MAAP Conference on "Pathogen transmission beyond borders: Understanding the complexities of cross-species infectious diseases" (October 16-18, 2023). Pakistan Academy of Sciences, Islamabad.
- ANSO-PAS-QAU Workshop "Ensuring Biosafety: Empowering Trainers in Risk Management and Biosecurity" (August 12-14, 2023). Bara Gali Campus, University of Peshawar.
- ANSO-PAS Workshop on Biological Safety and Risk Management (December 23, 2022). Department of Biotechnology, Quaid-i-Azam University Islamabad.
- ANSO-PAS-MAAP conference on Epidemic and Pandemic preparedness (December

5-7, 2022). Pakistan Academy of Sciences, Islamabad.

- MAAP-PAS-ANSO Hybrid Workshop on "Ecosystem Restoration: One-Health and Pandemics" (June 5, 2022). Pakistan Academy of Sciences, Islamabad.

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Pakistan Academy of Sciences

Review Article

Contemporary Strategies for Managing and Controlling Viral Outbreaks: An Overview

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Abstract: Viruses are notorious for causing a significant array of infectious diseases, rendering them a prominent contributor to global morbidity and mortality rates. Throughout history, various regions have experienced outbreaks, epidemics, and pandemics, resulting in significant mortality rates. The Influenza virus gave rise to highly fatal outbreaks that disseminated on a global scale, subsequently resulting in a pandemic during the initial decades of the 20th century. This catastrophic event led to >75 million fatalities, accompanied by a substantial incidence of illnesses. In addition to the development of efficacious treatments for viral diseases, it is imperative to establish and implement various preventive measures to mitigate the transmission of diseases within both local and global populations. Furthermore, it is critical to implement proven conventional and contemporary strategies for managing viral infections, alongside bolstered surveillance systems. Viruses employ diverse modes of transmission, encompassing respiratory, oral-fecal, blood-borne, and vector-borne pathways. Consequently, effective measures to mitigate viral dissemination must be tailored to address each distinct route of transmission. This review discusses the existing strategies employed to mitigate the transmission and containment of viral outbreaks, as well as the dissemination of the disease within a sizable population, intending to reduce their detrimental and fatal impacts on a community.

Keywords: Blood-borne viruses, Epidemic, Pandemic, Respiratory viruses, Vector-borne viruses, Viral outbreaks.

1. INTRODUCTION

Viruses belong to a distinct group of infectious agents having unique and simpler acellular organization and their way of multiplication as compared to bacterial and fungal pathogens [1]. Viruses are mainly composed of nucleocapsids comprising of the nucleic acid genome (DNA/RNA) and protein coat, while some of the viruses have an additional lipid bilayer surrounding the protein coat called the envelope. Despite their simple organization, they are one of the major causes of diseases and can infect all types of cells, including plants, animals, humans, protists, fungi, and bacteria. The International Committee on Taxonomy of Viruses (ICTV) designates about 9110 viral species and classifies them into 224 genera, 189 families, 59 orders, 39 classes, 17 phyla, 10 kingdoms, and 6 realms [2].

The convergence of infectious viral illnesses poses a significant public health obstacle in the 21st century. An emerging virus, contingent upon its capacity for human-to-human transmission has the potential to cause isolated or sporadic instances leading to a localized outbreak that necessitates public health action [3]. In the most severe situations, it may escalate into a widespread epidemic or global pandemic. Epidemics and outbreaks of infectious diseases have killed more humans in history as compared to any other cause [4]. An epidemic is the rapid and instant spread of a particular disease in a large number of individuals in a given population of a specific geographic location within a short period. When an epidemic occurs worldwide, crossing the boundaries of several countries and affecting a large population it is called a pandemic [5]. A viral infection ranges from trivial infections to smallpox and flu that altered the course of history. There have been several different emergence episodes in

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the previous twenty years. The viruses encompass both novel viruses such as the SARS and MERS coronaviruses [6, 7], and well-known reemerged epidemics such as swine- and avian-origin influenza as well as Ebola and Zika viruses [8-10]. Because of the immense variation in viruses' epidemiology, mode of transmission, and pathogenesis, there is no single magic bullet to control viral diseases.

The landmark in combating the viral infection was made in 1798 by Edward Jenner when he inoculated a boy with less virulent cowpox to form immunity against the smallpox virus [11]. For about two centuries after the first vaccination by Jenner, healthcare providers tried to limit the spread of the virus by providing effective vaccines for protection against viral infections and scientists spared no efforts in developing effective vaccines [12]. In recent decades, there has been a surge in various types of viral outbreaks ranging from influenza pandemics to emerging zoonotic diseases and COVID-19 [13, 14]. These outbreaks have required a multifaceted and dynamic response from the scientific and public health sectors. Researchers are exploring the complex mechanisms of viral pathogenesis, host-virus interactions, and the ecological factors that contribute to viral spillover. As a result, new combating strategies have been developed to detect prevent, and reduce the impact of viral outbreaks. Currently, approaches and advances are in progress to confine a virus to the place of its origin and try to stop its spread to a large population. When it comes to combating outbreaks and epidemics, it is crucial to not only focus on effective immunization and therapeutic interventions but also prioritize the implementation of preventive measures. Approaching the prevention and control of infectious diseases requires a scientific approach which involves following strict hygiene practices, maintaining high sanitation standards, implementing vector control measures, and conducting regular screenings for pathogens. These measures are crucial in reducing the occurrence and effects of infectious diseases. The scientific perspectives highlight the importance of using a comprehensive strategy that combines preventive interventions and curative measures to control the spread and impact of harmful outbreaks in different epidemiological settings [15, 16].

Factors contributing to viral emergence have been extensively studied and documented. These factors include population growth, travel, land use changes, dietary shifts, conflicts, social changes, and climate change [17]. These factors contribute to the increased interactions between humans and reservoir hosts, which in turn lead to greater exposure to zoonotic viruses and the transmission of infections to people. Additionally, these factors also facilitate the spread of emerging viruses within human populations. Understanding the intricate connections between virus ecology, host factors, and genetics that contribute to virus emergence is an incredibly intricate task, and if connections are made accurately the viral outbreaks can be controlled by applying respective mitigation strategies [4, 18, 19]. Moreover, virus genomics has been employed for many years to examine and analyze epidemics of viral diseases. The phenomenon is feasible because viruses, especially those with RNA genomes produce genetic diversity at the same rate as virus transmission. This is achieved by a combination of rapid mutation and replication processes. Therefore, it is feasible to deduce the epidemiology and emerging dynamics by analyzing virus genomes that have been sampled and sequenced during short epidemic periods. However, it is challenging to anticipate which virus will trigger the next epidemic. Therefore, it is crucial that our response is based on sound scientific knowledge, resilient strategies, and effective efficiency [20]. This review will delve into the important aspects of current viral epidemic management using contemporary approaches including preventive measures, vaccine research, antiviral treatments, and public health initiatives.

2. VIRAL DISEASES EPIDEMICS AND PANDEMIC

The emergence of microbes that are pathogenic to humans appears to be accelerating every year. Of all the pathogenic microorganisms that have been identified since the 1980s, approximately 60% spread from animal source to human, either due to interaction with vector or carrier (mosquitos, ticks, etc.) due to direct contact with microorganism, respiratory transmission (Influenza), the bite of an infected animal (rabies), or through contact with body fluids like tears, saliva, or blood [21]. Major epidemics and pandemics due to viruses worldwide from 1918 up till now are described in Table 1.

Table 1. Major outbreaks of viral diseases in the recent past (1918-2023).

Year of Outbreak	Outbreak Event	Countries Affected	Pathogens	Number of Deaths	References
1918-1922	Influenza Pandemic	Worldwide	Influenza A/H1N1	50 million +	
1924-1925	Smallpox epidemic	Minnesota, US	Variola virus	500	
1940	Yellow fever epidemic	Sudan	Yellow fever virus	1,627	
1948-1952	Polio epidemic	US	Poliovirus	9000	
1957-1958	Asian flu Pandemic	Worldwide	Influenza A/H2N2	2-4 million	
1974	London Flu	US	Influenza A/H3N2	1,027	
1974	Smallpox epidemic	India	Variola virus	15,000	
1977-78	Soviet Flu	Worldwide	Influenza A/H1N1	10,000-30,000	
1981-present	AIDS	Worldwide	HIV	32,000,000 +	
1998-1999	Nipah epidemic	Malaysia	Nipah virus	105	
2000	Dengue epidemic	Central America	Dengue virus	40+	
2002-2004	SARS epidemic	Worldwide	SARS-CoV	774	
2004	Dengue outbreak	Indonesia	Dengue virus	658	
2004	Ebola outbreak	Sudan	Ebola virus	7	
2005	Dengue outbreak	Singapore	Dengue virus	27	
2006	Dengue epidemic	India	Dengue virus	50+	
2006	Dengue epidemic	Pakistan	Dengue virus	50+	
2007	Ebola epidemic	Demographic Republic of Congo	Ebola virus	187	
2007	Ebola outbreak	Uganda	Ebola virus	37	
2008	Dengue epidemic	Brazil	Dengue virus	67	
2008	Dengue epidemic	Cambodia	Dengue virus	407	
2009	Dengue epidemic	Bolivia	Dengue virus	18	
2009	Hepatitis outbreak	India	Hepatitis B virus	49	
2009-2010	Swine flu pandemic	Worldwide	Influenza A/H1N1	18,449	[13-15, 22]
2010-2014	Measles outbreak	Demographic Republic of Congo	Measles virus	4500+	
2011	Dengue epidemic	Pakistan	Dengue virus	350+	
2012	MERS outbreak	Worldwide	MERS-CoV	935	
2013-2016	Ebola epidemic	Worldwide	Ebola virus	11,323+	
2013-2015	Chikungunya outbreak	America	Chikungunya virus	183	
2015-2016	Zika virus outbreak	Worldwide	Zika virus	53	
2017	Dengue outbreak	Pakistan	Dengue virus	69	
2018	Nipah outbreak	India	Nipah virus	17	
2018-2020	Kivu Ebola epidemic	Uganda and the Demographic Republic of Congo	Ebola virus	2280	
2019-2021	Measles outbreak	Philippines	Measles virus	415	
2019-2021	Dengue epidemic	Latin America, Asia-Pacific	Dengue virus	3930	
2019-2022	COVID-19	Worldwide	SARS-CoV-2	6 million+	
2022-2023	Monkeypox	Worldwide	Monkeypox virus	100+	

3. PREVENTION AND CONTROL STRATEGIES FOR VIRAL OUTBREAKS

For every kind of viral outbreak, the concept is to break the chain of infection to mitigate its spread. The current prevention, control, and management strategies for viral outbreaks involve both pharmaceutical interventions (vaccine and drugs) and non-pharmaceutical interventions like isolation, contact tracing, vector control, hygienic practices, and case management tools that could influence the spread of infection and hence break the chain of infection. Outbreak management and control is a stepwise approach that includes surveillance, determining the reality of an outbreak, case definition and ascertainment by laboratory detection, epidemiological studies, epidemiological curve monitoring, determining the root cause of an outbreak, comparative studies, intervention, control measures, assessment of intervention for their effectiveness, and sharing of the findings with national and international healthcare bodies. Figure 1 illustrates the strategies involved in controlling the viral infection and preventing its transmission to a large population.

4. CURRENT STRATEGIES TO COMBAT RESPIRATORY VIRAL INFECTIONS

Respiratory tract infections caused by viruses are one of the leading causes of morbidity and mortality in the world, representing an enormous health and economic burden [23]. Respiratory viruses replicate and damage the respiratory tract, and shed via respiratory routes to infect other individuals. Three different routes are used by respiratory viruses to transmit from one host to another; droplet, contact (indirect or direct), and aerosol transmission. The transmission of the viruses via these routes depends upon several variables like environmental factors, overcrowding of people, and host cell receptor distribution in the respiratory tract. Respiratory infections caused by viruses or other related pathogenic agents are more common in the winter season, possibly due to the propensity of people to remain inside houses and shelters nearby [24]. The Influenza seasonality is strongly modulated by relative humidity (HR) and temperature as the virus has shown enhanced transmissibility in low temperature and humidity. A study reported the enhanced transmission rate of the virus among guinea pigs at 5 °C while inhibited at 30 °C. Similarly, dry conditions (RH of 20-35%)

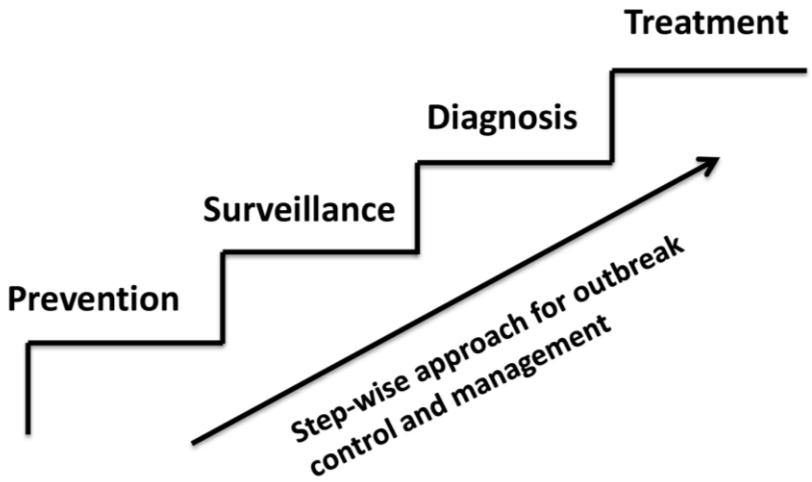

Fig. 1. Illustration of the generalized step-wise approach for the control and management of viral outbreaks. The first step toward the management and control of viral outbreaks is "Prevention" which could be brought about by education, awareness about the diseases, following occupational safety measures, proper sanitation system, and proper hygienic practices. The second step is "Surveillance" which involves the epidemiological investigation, seroprevalence, and characterization of the pathogen in the population. The third step is "Diagnosis" which involves the laboratory detection of pathogen and the last approach in control and management of viral outbreaks is the "Treatment" by using different antivirals and supportive therapies

were observed more favorable in viral transmission compared to intermediate (RH of 50%) or humid (RH of 80%) conditions [25].

Generally, respiratory tract infections are mostly non-lethal, but some people are at risk of developing severe symptoms and are more vulnerable to disease. The individuals who are more vulnerable to respiratory tract infections include old individuals, individuals with pre-existing lung infections, and immune-compromised individuals [26]. The following strategies could be adopted to prevent an outbreak or mitigate the effect of an ongoing epidemic of respiratory viral diseases

4.1. Enhanced Surveillance System

When a cluster of cases is reported in an area, an enhanced surveillance system is required to manage and limit the spread of associated diseases, enabling the public health authorities to effectively monitor and manage the potential risk of disease (Fig. 2). The main objectives of an enhanced surveillance system include the rapid testing, detection, and management of infected and suspected cases, contact tracing, implementation of effective control measures, the impact of the epidemic on healthcare systems, epidemiological trends of pathogenic organisms, and co-circulation of that pathogenic organism with other related pathogens [27]. The comprehensive national action plan for the management of viral respiratory diseases requires the adaptation and enforcement of national systems under the recommendations of the national health regulatory system of the country. Since the world has faced many respiratory viral outbreaks including influenza virus (H1N1, H7N9, and H10N8) [28], human adenovirus, and coronaviruses (SARS-CoV, MERS-CoV, and SARS-CoV-2) [29] various surveillance systems have been developed to monitor the respiratory viral outbreaks [30]. These systems include a web-based system, syndrome identification, and a system that obtains data from healthcare facilities. The Global Influenza Surveillance and Response System called Global Influenza Surveillance Network (GISN) was established in 1952 and is the classical example of global network surveillance of the disease that currently has 6 WHO Collaborating Centers and 138 National Influenza Centers [31]. Figure 2 shows the systems involved in the enhancement of valuable data on disease for the management and control of an outbreak.

The enhanced surveillance system involves the collection of integrated data from primary care centers, hospitals, and diagnostic labs. The data collection of cases from private practitioners and residential facilities like an orphanage, daycare centers, and sports centers also need to be considered in the surveillance. Event-based surveillance and participatory surveillance can be

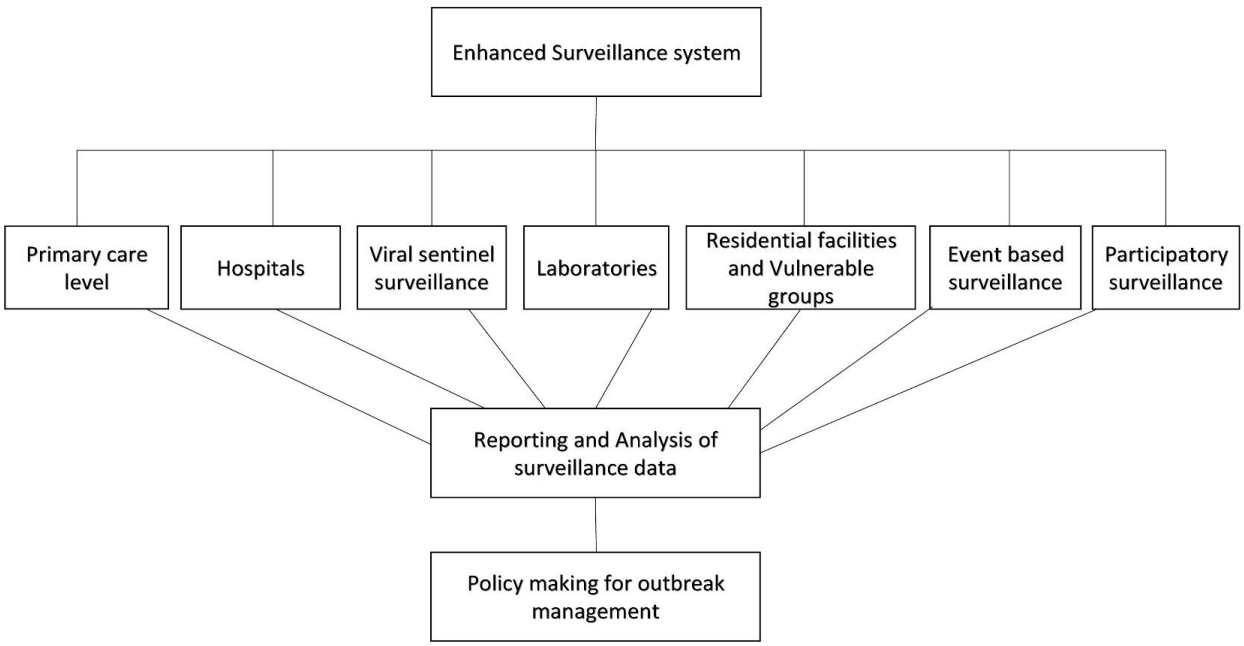

Fig. 2. Enhanced surveillance system for rapid detection and management of viral outbreaks.

included in the enhanced surveillance system to determine the incidence of disease in a particular area. The collective data will then be analyzed and policies to control the outbreak can be made by governments easily, as it can give detailed accounts of the geographic locations and vulnerable groups. The policies should be based on the assessment of existing research, public communication capacity, and community understanding including demographics, socioeconomic status, literacy level, and ethnicity followed by coordination of different related departments. The policymakers then can construct an emergency plan that captures previously reported cases and develop plans to control the newly emerging cases.

Several global surveillance systems have been developed for predicting, monitoring, and determining possible threats of outbreaks to public health. This surveillance system includes Program for Monitoring Emerging Diseases (proMED), the CDC's Global Disease Detection Network (GDD), WHO's Global Outbreak Alert and Response Network (GOARN), U.S Department of Defense's Global Emerging Infections Surveillance and Response System (GEIS), a joint network by WHO, World Organization for Animal Health (OIE) and Food and Agriculture Organization (FAO) of Global Early Warning System for Major Animal Diseases including zoonosis (GLEWS), and USAID's Emerging Pandemic Threat Program (EPT) [31].

4.2. Prompt Isolation after Diagnosis and Social Distancing

If the patient is diagnosed with a respiratory infection caused by viruses, he/she needs to be isolated from other family members or hospital patients. The caretaker of the patients should follow the safety measures. Use of personal protective equipment (PPE) in the patient's room and removal of PPE before leaving the room, and washing hands after providing care to the patients need to be ensured. Other members of the family and staff should monitor themselves daily for symptoms associated with that particular viral infection. It should be made sure other members suffering from the same symptoms are provided with medical care immediately to prevent the further spread of the disease.

The strategy for the isolation can be either centralized (imposition of the lockdown by governments), de-centralized (social distancing by removal of social networks), or hybrid (centralized and decentralized) both strategies. The results of the study conducted by Topîrceanu et al. show that the hybrid strategy (including both centralized and decentralized isolation policy) is the most effective isolation strategy in moderating the speedy spread of respiratory viral diseases (COVID-19) and has the potential to reduce the peak of incidence to <10% of initial values. The study also describes that stronger social distancing (75% cutting of social relations) can reduce the burden of an outbreak by 87% for hybrid (centralized and decentralized) strategy, 75% for centralized, and 33% for decentralized isolation [32]. Wilasang et al. report a drop in the reproduction number of COVID-19 in the countries that employ prompt isolation after case detection policy [33].

Whenever there is an epidemic or outbreak of contagious diseases like COVID-19, Influenza, and other respiratory diseases, maintaining social distancing is a reliable method of controlling the infection. A safe distance should be kept and visits to infected persons should be restricted. A minimum social distance of about 1-2 meters should be maintained during physical contact [34]. Social distancing is one of the major factors in the prevention of transmission of respiratory viruses [35]. For example, if the persons who are asymptomatic carriers of a virus meet a couple of people and sit in the gatherings, they spread the virus more frequently as they sneeze or cough in the gatherings. Khataee et al. reported the effect of mitigating COVID-19 transmission due to social distancing and the imposition of the lockdown [36]. Several studies describe the reduction in the incidence of COVID-19 cases as a "flattening of the curve" due to social distancing [36, 37]. The ultimate goal of social distancing in the respiratory viral outbreak is to mitigate its effect by limiting the spread of the disease. The study conducted in Italy reported that the use of masks and observance of social distancing could reduce the potential transmission of COVID-19 by 1000 times [38]. Physical distancing measures were found to be more effective if a phased return to work is followed; it is predicted to reduce the median number of infections by >92% (IQR 66-97) and 24% (13-90) in mid-

2020 and by the end of 2020 respectively [39].

4.3. Hand Hygienic Practices

In an epidemic of respiratory diseases, the WHO and CDC recommend washing hands regularly and sanitizing to prevent getting infected [40]. The enveloped viruses are highly susceptible to alcohol-based hand sanitizers as alcohol targets the lipids envelope of viruses but their efficacy reduces against non-enveloped viruses. The efficacy of the ethanol solution can be enhanced by adding acids to the ethanol solution against the viruses that are resistant to ethanol solution alone. For any contagious infection, hand washing with soap and water after contact with the potentially infected objects, surfaces or persons can effectively protect one and others from getting infected. The CDC in a recent pandemic of COVID-19 recommended hand-washing with soap and water for at least 20 seconds frequently a day or the use of hand sanitizer having 60% alcohol [41].

4.4. Occupational Safety Measures

Occupational safety measures by using PPE like medical masks, gloves, gowns, and eye protection are necessary to prevent transmission while working with the pathogen or dealing with the infected person. Improper occupational safety measures can result in a large number of hospital-acquired infections. Vaccination of healthcare workers is of utmost priority in reducing hospital-acquired infections and disease burden, as these workers are at higher risk of acquiring infections [42]. Nosocomial infection during an outbreak or epidemic is a major threat to hospital staff and hospitalized patients. Horcajada et al. reported the nosocomial outbreak of the Influenza A virus in a period without an Influenza outbreak concluding that the hospital setting and improper safety measures could spread the infection to other healthy individuals [43]. Several studies reported the transmission of COVID-19 in the hospital setting due to a lack of occupational safety measures [44]. Schwierzeck et al. report about forty-eight COVID-19 cases in its nosocomial outbreak [45].

4.5. Vaccination

Vaccination provides immunity to the individual and is one of the key parameters in the control of viral epidemics. The specific group of the population in a specific season may be targeted depending on the national vaccination program, access to vaccination, and effectiveness of the vaccine [46].

For a respiratory virus like the influenza virus, three types of vaccines are available; live attenuated influenza vaccine, inactivated influenza vaccine, and recombinant vaccine. Conventionally, live attenuated and inactivated influenza vaccines have been produced to protect against three different types of influenza virus (trivalent vaccine). The trivalent vaccine contains one of the two influenza B lineage viruses, Influenza A (H1N1) and Influenza A (H3N2) [47]. As trivalent vaccines contain only one influenza B lineage strain, the seasonal vaccines were improved by including both lineage strains of influenza B. Thus, resulting quadrivalent influenza vaccines respond more effectively in controlling the global influenza epidemiology [48]. Most influenza vaccine elicits an immune response against viral surface proteins including neuraminidase and hemagglutinin but influenza vaccines need to be updated regularly due to antigenic drift in surface proteins [49]. For other respiratory viruses like rhinovirus, effective vaccine development is challenging for scientists, as they have many serotypes and there is little or no cross-protection between their serotypes [50, 51]. The development of a vaccine against the respiratory syncytial virus (RSV) has also faced challenges like the enhancement of vaccine-induced disease in infants. Efforts are being made to develop effective vaccines against RSV that elicit age- age-appropriate immune responses in the target population [52, 53]. Due to the effectiveness of the vaccine like smallpox vaccines and polio vaccines, the world is now relying on the vaccination program to control the recent pandemic of COVID-19 and the struggle for vaccine development started immediately with its spread. The current most important and widely used vaccines to control respiratory viral infections are summarized in Table 2.

4.6. Treatment and Biochemical Prevention

Biochemical prevention is an alternative approach to antiviral drugs and vaccines, used for the control of viral infection when vaccines or drugs cannot be generated or are ineffective for the control of

Table 2. Treatment and vaccination for respiratory diseases caused by viruses.

Pathogen	Vaccine type (Commercial names)	Treatment	Reference
Influenza A virus	Inactivated vaccine (Fluzone Quadrivalent, Vaxigrip Tetra, Afluria Quandrivalent) Quadrivalent Recombinant vaccine (Flublok Quadrivalent)	Oseltamivir, Zinamivir, Baloxavir, Amantadine, and Rimantadine	[59-61]
Respiratory syncytial virus	No approved vaccine	Palivizumab and Ribavirin	[62]
Measles virus	Live attenuated vaccine (Measles-mumps-rubella vaccine)	Ribavirin, Vitamin A, Ibuprofen	[63, 64]
Mumps virus	Live attenuated vaccine (Measles-mumps-rubella vaccine)	No specific antiviral drugs, acetaminophen, and Ibuprofen used to ease symptoms	[65]
Adenovirus	Live oral adenoviral vaccine type 4 and 7 (for military personnel only)	Brincidofovir	[66, 67]
Rhinovirus	No approved vaccine	No specific treatment	[68]
SARS-CoV-2	mRNA-based vaccine (Pfizer-bioNtech, Moderna), Viral vector vaccine (AstraZeneca, Sputnik V), Inactivated vaccine (Sinovac, Sinopharm), Protein subunit vaccine (Novavax)	Remdesivir, Paxlovid, Molnupiravir, Dexamethasone, and other supportive care	[69-71]

viral infections [54]. The most successful approach for treating respiratory syncytial virus infection has been the use of anti-viral antibodies relying on biochemical prevention. In 1996, RespiGam™ globulin (REV-IG) was offered for use in children aged < 2 years and was found to be an effective way to control respiratory diseases caused by the respiratory syncytial virus (RSV) [55]. Similarly, Palivizumab serves as primary medical care for RSV prevention and is shown to reduce the infection risk by 55% in infants [56].

Human rhinovirus causes more than 80% of the common cold infections during the winter season and developing a vaccine against these viruses is unfeasible due to 115 antigenic-ally different serotypes [54]. In rhinovirus infection, around 90% of human rhinovirus serotypes use a receptor called ICAM-1 for the attachment of the virus and their subsequent entry. Administration of soluble monoclonal antibodies against ICAM-1 in clinical trials has shown a decrease in the severity of symptoms of the disease but cannot prevent the occurrence of the disease completely [57]. The randomized control trials of combination therapy of Bamlanivimab and Etesevimab have shown the reduction of viral load of SARS-CoV-2 on the 11th day in patients with mild to moderate COVID-19 infection [58].

5. MEASURES IN MANAGING AND CONTROLLING GASTROINTESTINAL VIRAL OUTBREAKS

The first epidemiological investigation for the disease transmitted via the fecal-oral route was performed by John Snow in 1848-54 and reported the association between drinking water and deaths due to cholera. He compared the mortality rate due to cholera in the Soho district with different water supplies and revealed that the mortality rate was higher among the people who drink water supplied by Southway Company [72]. He concluded that cholera was spread by contaminated water through fecal-oral routes [73].

The gastrointestinal tract is the susceptible organ to infection, which comes in contact with pathogenic microorganisms, mainly via the oral-fecal route. Gastrointestinal infections range from mild to more severe forms of inflammation or may cause direct damage to the epithelial lining of the gastrointestinal tract resulting in nausea, vomiting, and diarrhea. Gastroenteritis is responsible for 2-3 million deaths each year [74]. Children in developing countries and immune-compromised individuals mostly suffer from viral gastroenteritis [75]. The viruses including rotavirus, norovirus, and Hepatitis A virus enter via the fecal-oral route

in contaminated water and food causing a large number of infections worldwide. These viruses after entry, replicate in the cells of the gastrointestinal tract and cause gastroenteritis. Viruses pathogenic to humans had already been described since 1901, but the viral gastroenteritis caused by norovirus was first identified in 1972 in the outbreak of diarrhea in Norwalk, US [76]. After the discovery of Norovirus, several other gastroenteritis-causing viruses were identified and are summarized in Table 1 with their epidemic history.

The prevention of outbreaks of gastrointestinal viruses has been challenging because the outbreaks that start with a single common exposure to contaminated water or food can rapidly spread in a community due to similar food or water sources. Investigating and tracking the outbreak requires the isolation of the virus from secondary cases in which the transmission route might be different from the primary one. Knowledge about the chain of disease transmission to common exposure like contaminated food, water, or oysters can identify the associated virus [77]. Sequencing the virus in a specific epidemic can identify the specific strain of the virus that is linked with the outbreaks, monitoring its evolution and spread could help the public health worker to establish the policy against that particular strain [78]

5.1. Sanitation and Hygienic Practices

Many of the viral outbreaks can be prevented by a proper sanitation system that does not have any source of food or water contamination by human or animal feces. Preventing the secondary spread of the gastrointestinal virus via contaminated environmental surfaces such as cruise ships, hospital wards, canteens, person-to-person contact, etc. can stop the chain of the outbreak. Enforcing public hygienic practices including not allowing ill food handlers to remain on the job until clear the infection and strict personal hygiene for food handlers can prevent the spread of the disease [79]. A study conducted by Belliot et al. demonstrated the *in vitro* inactivation of infectious norovirus by ethanol and isopropanol in mice infected with norovirus [80].

5.2. Laboratory Detection

A rapid detection system by the public health department should be launched as soon as there is a cluster of cases observed in a specific region. Assays that can detect the presence of gastrointestinal viruses in contaminated water and food need to be prepared and adapted for routine screening of water and food [81]. Considerable efforts by governments and healthcare departments need to update the conventional approaches to identify infectious agents and development of methods for the detection, identification, and elimination of viruses from contaminated sources to prevent large epidemics [82].

5.3. Vaccination and Treatment

Effective vaccines against some of the enteric viruses have been developed and their use reduces the large outbreaks and epidemics worldwide. Rotarix vaccine, first licensed in 2004, has effectively prevented the infection from Rotavirus and is used across 123 countries [83]. Poliomyelitis, a crippling disease that results from infection with any of the 3 related poliovirus types can be prevented by using one of the two types of vaccines such as inactivated polio vaccine and oral polio vaccine. The use of the polio vaccine effectively eradicated viruses from all around the world except Afghanistan and Pakistan, where wild-type polio cases are being reported. The treatment and vaccination against enteric viruses are described in Table 3.

6. VECTOR-BORNE VIRUSES

Arthropods are considered the main vector that transmit pathogens from reservoirs to hosts or from one host to another. Arthropods are capable of transmitting the disease in two ways. Firstly by mechanical vectors in which the passive transport of pathogens on the arthropod's body or feet occurs and when the insects make contact with food, capable of transmitting the pathogen to food that another host can consume and become infected. The second method of transmission is biological transmission which is an active process. In biological vectors, the arthropod bites an infected animal or person taking a meal along with a potential pathogen. The pathogen reproduces in the vector's gut and will ultimately migrate to salivary glands. The vector is thus capable of injecting the pathogen in healthy individuals by biting or taking a blood meal [90]. The most endemic and common viral vector-borne diseases include Dengue, Chikungunya, Zika,

Table 3. Treatment and vaccination for gastrointestinal infections caused by viruses.

Pathogen	Vaccine type (Commercial name)	Treatment	References
Rotavirus	Live attenuated vaccine (Rotarix, Rotateq)	Antidiarrheal, Antiemetic, Thiazolides	[84, 85]
Norovirus	No approved vaccine (P particle and virus-like particle-based vaccine under clinical trials)	Interferon alpha	[86]
Hepatitis A	Inactivated vaccine Avaxim, Biovac A, Havrix)	Acetaminophen, Paracetamol	[87, 88]
Poliovirus	Live attenuated oral polio vaccine (OPV, Orimune, Sabin) Inactivated polio vaccine (Salk vaccine)	Pain relievers, Ventilators, and supportive care	[89]

Yellow fever, Japanese encephalitis, Rift Valley fever, tick-borne encephalitis, West Nile, and Crimean Congo hemorrhagic fever. The common vectors for Dengue and Chikungunya are *Aedes aegypti* and *Aedes albopictus* mosquitoes while the *Culex* mosquito transmits the West Nile virus from one individual (infected) to another (healthy) [91]. Rabies is transmitted by an animal host like dogs, raccoons, cats, foxes, and skunks to humans and the Crimean Congo hemorrhagic virus is a tick-borne disease transmitted to humans by Hyalomma tick [92, 93].

6.1. Mechanical Control Measures for Vector-Borne Outbreaks

Mechanical control measures have been adopted and practiced for centuries in several countries since they are cost-effective and easy methods for the control of vectors like mosquitos and ticks. Mechanical control measures involve the removal of unwanted stored water and the covering of water-containing utensils, as these are the primary sites of mosquito breeding [94]. Streets, buildings, and housing units must be properly cleaned. Wearing long-sleeved shirts and trousers and use of arthropod repellents on exposed skin can protect from being bitten by mosquitos, ticks, or sandflies. Installation of window screens at workplaces and homes can keep mosquitos outside, therefore reducing the risk of exposure. In tick-infested areas luggage, clothing, and other belongings should be examined carefully to remove any ticks. If tick-infested on the skin, it should be removed from the skin using tweezers, and applying disinfectant at the surface can reduce the risk of transmission of pathogens [95].

6.2. Chemical Control Measures for Vector-Borne Outbreaks

Chemical control measures include the use of chemicals like organochloride, pyrethroids, thiacloprid, and organophosphorus that primarily target the nervous system of the vector [96]. The use of fogging and spraying with insecticides has been adopted in outdoor environments to control the vector's population. Repellents like N, and N-Diethyl-meta-toluamide (DEET) need to be used in households to prevent mosquito irritation at home. Rodriguez et al. compared the efficacy of different mosquito repellent sprays and reported the highest efficacy of DEET and p-methane-3,8-diol against mosquitos [97]. A similar study reported that insect growth regulators (IGRs) like Pyriproxyfen are effective in reducing the immature *Aedes* population [98]. An essential approach for managing arbovirus outbreaks, such as dengue involves the utilization of synthetic pesticides that rapidly eliminate adult vectors through space spraying [99]. Most of the insecticides that are advised belong to the pyrethroid chemical class. However, this poses difficulties in preventing the selection of mosquito populations that are susceptible to these insecticides, as well as controlling mosquitoes that are resistant to pyrethroids [100]. When it comes to managing the population of arbovirus vectors, particularly *A. aegypti*, controlling the larvae has been suggested and put into practice as the main technique which involves using chemical and microbial larvicides, and IRGs.

Besides traditional repellents, spatial repellents are specifically created to emit volatile chemicals into a given area, to alter insect behavior to minimize interaction between insects and humans,

therefore reducing the transmission of pathogens [101]. The spatial repellent product category is now under Stage 3 of the Vector Control Advisory Group (VCAG) assessment method, where it is being evaluated for proof-of-principle efficacy through clinical trials [102].

6.3. Biological Control Measures for Vector-Borne Outbreaks

The alternative approaches of chemical use against mosquitoes have been exploited by using biocontrol agents like bacteria, fungi, and plants against the growth and propagation of the mosquito population. In 1976, *Bacillus thuringiensis* (Bt) was isolated and characterized to be toxic against mosquito larvae. Since then, Bt-based insecticides have spread to the global market. Bt-based insecticides are target-specific and produce specific delta-endotoxin by the time of sporulation that is toxic to mosquito larvae and other related flies [103]. Sterile insect techniques and incompatible insect techniques can be used to control the population of mosquitos. Sterile insect techniques involve the release of sterile mosquitos in a wild environment, these sterile mosquitos compete with other wild mosquitos to mate with females. The mating of sterile males and females does not produce offspring, reducing the population of the next generation. The incompatible insect technique involves the mating of *Wolbachia*-infected males and wild-type females that cannot produce offspring [104]. Incompatible insect techniques using intracellular bacteria *Wolbachia* have also been used as bio-pesticides to control the population of mosquitoes [105]. The *Wolbachia* method is an innovative and self-sustaining strategy for the biological management of Aedes-borne diseases. It involves genetically modifying Kales aegypti mosquitoes by introducing the Wolbachia bacterium into their cells, resulting in a decreased ability to transmit diseases [106]. Pinto et al. reported that the intervention of Wolbachia (wMel strain) was related to a reduction of 37%, 56%, and 69% in the incidence of Zika virus, chikungunya virus, and dengue virus, respectively [107].

Moreover, biological mosquito larvae control also involves strategies to augment the effectiveness of natural adversaries by adding bio-control agents such as fishes (*Gambusia spp*)and copepods, or by facilitating the colonization of isolated water bodies by natural predators through the excavation of connecting ditches [108]. The predatory native copepods (*Macrocyclops albidus)* can be cultivated and transferred into artificial containers. Once introduced, they proliferate and effectively decrease the population of mosquito larvae and have proven strong efficacy against *Aedes* mosquitoes, but only moderate efficacy against *Culex* species [109]. Similarly, fungal species like *Beauveria bassiana* and *Metarhizium anisopliae* have biocontrol properties against mosquitoes [110].

The treatment and vaccination strategies have also been developed for vector-borne diseases due to viruses and are listed in Table 4. The live attenuated vaccine called Dengvaxia and the Yellow fever virus vaccine have been approved and are used worldwide to prevent the spread of Dengue and YFV infections respectively [111,

Table 4. Treatment and vaccination for common vector-borne diseases caused by viruses.

Pathogen	Vaccine type (Commercial name)	Treatment	References
Dengue Virus	Live attenuated vaccine (Dengvaxia)	Chloroquine, Corticosteroids, and Iminosugars	[111, 114, 115]
West Nile Virus	No approved vaccine (Live attenuated chimeric vaccine 'ChimeriVax-WN02" under clinical trials)	Interferon alpha and Favipiravir	[116, 117]
Yellow fever Virus	Live attenuated yellow fever virus vaccine (YF-VAX®)	Ribavirin, Pyrazoline compounds, Tiazofurin, Interferon, and Carboxamide,	[112, 118]
Chikungunya Virus	No approved vaccine	Ribavirin, Anti-rheumatic drugs (DMARDs) and Non-steroid anti-inflammatory drugs (NSAIDs)	[113, 119]
Nipah Virus	No approved vaccine	Ribavirin, Supportive care	[120]

112]. The treatment strategies to prevent the Chikungunya virus (CHIKV) and Dengue virus (DENV) infections involve fluid balance along with other supportive care like NSAIDs for CHIKV and corticosteroids respectively [113, 114].

7. APPROACHES IN CONTROL OF BLOOD-BORNE VIRAL OUTBREAKS

Blood-borne viruses such as Human Immunodeficiency Virus (HIV), Hepatitis B virus, and Hepatitis C virus are considered a major public health threat, responsible for millions of deaths annually. It was estimated that in 2014, about 1.2 million infected individuals died of HIV with about ~40 million individuals being infected chronically worldwide [121]. Infections caused by HBV, HCV, or HEV can cause hepatitis that subsequently leads to liver cirrhosis, fibrosis, and hepatocellular carcinoma. It is estimated that 240 million people are chronically infected with HBV with ~780,000 annual deaths. The risk of developing chronic infections varies with the age of the individual infected. About 90% of toddlers and 25-50% of children aged 1-5 years can remain chronically infected with the Hepatitis B virus, however, approximately 95% of adults can eliminate the viral infection [122]. HCV infections account for 58 million people with chronic infections and 1.5 million new cases per annum worldwide [123]. The major route of transmission of blood-borne viruses involves the parenteral routes via blood and other body fluids.

Prime prevention strives to avoid the occurrence of infection by decreasing the risk factor in uninfected individuals. It involves the strategies that are useful before the disease or infection. Primary interventions or prevention involve a reduction in viral exposure, prophylaxis, and vaccination, awareness about the disease, disinfection, safety of blood products, and good hygienic practices. Secondary prevention strategies involve the surveillance and identification of infected individuals and the treatment of infected individuals with appropriate medical drugs

7.1. Awareness of Disease

Successful prevention of viral diseases requires the awareness of infected people about the transmission and spread of the disease as well as complete awareness among society and healthcare experts. Public campaigns and educating people about the disease can reduce the risk of the spread of the disease if they start adopting the prevention strategies or guidelines by health care professionals [124]. Wide-ranging and frequent education of healthcare professionals is the precondition and increasing awareness about care, prevention, and treatment on World Disease Day like 28th July as World Hepatitis Day, and 1st December as World AIDS can enhance the knowledge and expertise of social and medical professionals. The updated guidelines by the World Health Organization (WHO) and the Center for Disease Control and Prevention (CDC) need to be followed and implemented by every country that is at risk of acquiring the epidemic or outbreak of a particular viral infection [125, 126].

7.2. Screening of Blood and Blood Products

Since the last decade, the screening of blood products has significantly reduced the burden of blood-borne diseases around the globe. Nucleic acid amplification tests such as polymerase chain reaction (PCR), enzyme-linked immunosorbent assay ELISA, and rapid diagnostic tests such as lateral flow assay have been successfully developed for the detection of HBV, HCV, and HIV, thus reducing the risk of transmission in transfusion of blood and blood products to healthy individuals [127].

7.3. Biosafety and Hygienic Practices

Good microbiological practices, hygienic practices, and disinfection of possible contaminated sources can help in reducing viral transmission. To prevent occupational transmission, prevention, and control measures involve the implementation of standard primary barriers including personal protective equipment (gloves, eyewear, gowns, etc.), minimal manipulation of sharp instruments (needles, surgical blades), and discarding of sharp instruments into the proper leak-proof container after experiments. Furthermore, the sterilization of dental and medical equipment in addition to the disinfection of contaminated surfaces can reduce the spread of disease. The non-sterile setting and contaminated instruments in tattoo and piercing can increase the risk of transmission of the virus [128].

Sexual transmission can be a key risk factor and preventive measures have been applied to reduce the spread of viruses. Behavioral interpolations like the use of condoms have revealed a reduction in the incidence of HBV, HCV, and HIV [129]. Moreover, medical male circumcision has shown a reduction in transmission of HIV by the venereal route and new interference including rectal and vaginal microbiocides are under development to reduce the risk of transmission [130, 131]. Intravenous drug use is another major risk factor for the transmission of blood-borne diseases, due to sharing of virus-contaminated syringes and their preparation environment. The use of harm reduction measures like opiate substitution therapy and needle/syringe programs (NSP) effectively reduce the risk of viral transmission and hence prevent further spread [132].

7.4. Prophylaxis

In some cases, prophylaxis can be achieved either due to pre-exposure (PrEP) or post-exposure (PEP) to the virus. Pre and post-exposure prophylaxis have shown promising results in controlling viral transmission in certain risk groups. Pre-exposure prophylaxis is the administration of antivirals to the possible viral exposures of an individual. Pre-exposure prophylaxis has shown a reduction in the occurrence of HIV infections in individuals which is also recommended by CDC [133]. Post-exposure prophylaxis (PEP) is the immediate intervention with antivirals after exposure to the potential pathogen to prevent the infection. It is recommended to use post-exposure prophylaxis immediately following high-risk practices like sex with HIV-infected individuals, needle stick injury during processing of infected blood, and intravenous drug use. For HIV, post-exposure prophylaxis should be no later than 48-72 hours [134]. Several studies have reported the use of antiretroviral therapy in anticipation of potential infection by HIV either as pre-exposure prophylaxis in high-risk factor exposure or as post-exposure prophylaxis [135]. Oral pre-exposure prophylaxis like tenofovir disproxil fumarate/ emtricitabine has shown great potential against HIV infections [136]. The administration of zidovudine has been reported to be effective prophylaxis in mothers and newborns, resulting in the reduction of perinatal transmission of HIV [137]. Similarly, acyclovir is used to prevent the dissemination of the Herpes simplex virus and Varicella zoster virus in immunocompromised and immunologically normal individuals [138, 139]. Oral valganciclovir is an effective oral regimen used as universal prophylaxis to prevent cytomegaloviral infections after lung transplantation [140]. Ganciclovir also serves as a prophylactic antiviral that could effectively suppress the dissemination of CMV after engraftment [141]. Similarly, post-exposure prophylaxis is also significant in preventing lethal infections. WHO recommends the administration of two doses of immunoglobulin and three doses of HBV vaccine as PEP [142].

7.5. Vaccination

Effective vaccination programs for the public contribute efficiently to the eradication and prevention of viral outbreaks. The safe and effective vaccine against the Hepatitis B virus has been available since late 1982 and vaccination programs have successfully been implemented in 47 European countries with good results [143]. The Recombivax vaccine is highly effective in preventing HBV infection in individuals who are frequently exposed to blood and other body fluids such as health care professionals and patients receiving dialysis or multiple transfusions [144]. The enforcement of stringent vaccination programs has resulted in a global decline in the occurrence of this disease. However, this may have also triggered the creation of viral variants that are capable of evading the immunity provided by the hepatitis B surface antibody [145]. Due to the increase in escape mutagens, the concern for transmission of these variants is on the rise even in vaccinated individuals [146]. Therefore, there is a need for an active surveillance system and the introduction of novel vaccination strategies such as combination vaccines (multi-epitope/ subunit) and therapeutic vaccination [147, 148].

For other blood-borne viruses like HCV, HDV, HEV, and HIV, the vaccine is not available and is in the process of developing to reduce the disease burden of blood-borne infection worldwide.

7.6. Treatment

Antiviral therapeutics prevent transmission by either reducing the viral load or by decreasing the number

of infected persons in the population. Combination therapy is used to treat AIDS patients by using highly active antiretroviral therapy (HAART), in which a cocktail of drugs like nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase strand transfer inhibitors (INSTIs), Fusion inhibitors (FIs), and chemokine receptor antagonists (CCR5 antagonist) were used for limiting multiplication of HIV [149]. Some of the notorious viruses transmitted via blood or other body fluids with their treatments and vaccination are listed in Table 5.

8. ZOONOTIC VIRAL DISEASES AND STRATEGIES TO CONTROL VIRAL OUTBREAKS

Zoonotic diseases are naturally transmitted from animals to humans, with or without an intermediate arthropod host. It is reported that approximately 75% of the newly emerged human infectious diseases are zoonotic [112]. Due to the increased interdependence of humans on animals and their products and the close association between them, the newly emerged and reemerged zoonotic viral diseases have increased over the last three decades. Zoonoses are thus considered one of the critical risk factors for human health and infectious disease. The eradication programs for the control of zoonotic-borne viral diseases are majorly focused on breaking the transmission chain in which three controlling factors are involved including neutralization of reservoirs, decreasing the potential contact between reservoirs and susceptible hosts, and increasing the host resistance [154].

The neutralization of the reservoir involves preventing the spread of viral infections by removing the infected or susceptible animals from the reservoir or by manipulating the habitat of reservoirs. The removal of infected individuals can be achieved by employing vaccination, mass therapy, or the testing and slaughter of infected animals. For example, arboviral infections are endemic in wildlife reservoirs, the control strategies should be based on vector control, limiting direct animal contact, and mass vaccination of people and domestic animals [155]. The cross-species transmission of viral diseases from animals to humans is common as seen in different cases like cross-species transmission of H7N9 influenza virus from poultry, rabies virus from infected cats, dogs, or wild animals, potential transmission of COVID-19 from wildlife, swine flu from infected pigs, and Nipah virus from fruit bats [156]. The decrease in potential contact between reservoirs and susceptible hosts by prohibiting the eating and transport of wildlife animals can prevent these transmissions. Increasing host resistance can also reduce the burden of zoonotic-borne viral diseases and it could be achieved by herd immunization, such as that which happened with rabies immunization of dogs.

Besides the close contact between humans and animals, other factors also contribute to cross-species transmission of infectious agents. So, it is critical to establish effective mechanisms for collaboration and coordination between animal, human, and environmental health sectors before the emergence of another threat like COVID-19 by bringing different sectors together to cope with endemic zoonotic viral diseases of public health concerns. For the prevention and control of zoonotic diseases, researchers and international organizations have adopted a concept called the One Health concept. The concept describes the

Table 5. Treatment and vaccination for blood-borne diseases caused by viruses.

Pathogen	Vaccination (Commercial name)	Treatment	References
Hepatitis B virus	Subunit vaccine (Recombivax, Engerix-B)	Pegylated interferon, Nucleotide analogs (Tenofovir, Adefovir) and Nucleoside analogues (Entecavir, Lamivudine, Telbivudine,)	[150, 151]
Hepatitis C virus	No vaccine available	Protease inhibitors (Boceprevir, Telaprevir, Daclatasvir), Nucleotide analog (Sofosbuvir), Ribavirin, and Pegylated interferon	[152]
HIV	No vaccine available	Highly active antiretroviral therapy (HAART)	[153]

relationship among animals, humans, and the environment. It encourages collaborations among veterinarians, microbiologists, wildlife biologists, biomedical engineers, physicians, ecologists, and agriculturists to cope with global health challenges and to ensure good health for humans, animals, and environment [157]. The multi-sector partnerships should start with the identification of priority zoonotic-borne viral diseases, improvements in surveillance and data sharing among these sectors, enhancement of laboratory diagnosis and outbreak response capacities, and research for the preparation of vaccines and medicine for the management of the disease [154].

9. APPRAISAL AND FUTURE PERSPECTIVES

A combination of traditional and latest strategies to combat the challenge of viral epidemics and associated risks to human life has helped reduce the global burden of viral diseases, nevertheless, still far from achieving significant success. Diagnostic capabilities, surveillance, and monitoring systems, as well as forecasting systems, are poor in many developing countries, while the availability of adequate healthcare facilities including vaccination remains a serious challenge in such communities. An infrastructure improvement has been witnessed in the case of COVID-19, nevertheless, they still need to go a long way.

Standard hygienic practices may control water and foodborne viral illnesses. Developed countries have been successful in reducing the burden of viral enteric illnesses by improving sanitary and hygienic conditions. Little success has been achieved in developing countries where the provision of clean water is one of the biggest challenges. Improved sanitary and hygiene standards, as well as clean drinking water, may greatly reduce the morbidity and mortality of viral diseases. The mass media campaign has been witnessed in the response to COVID-19 across the globe with fruitful outcomes. Public awareness about viral diseases and preparedness to respond to epidemic situations remain better established in the industrialized world as compared to poor nations. Future efforts need to be directed at public awareness at the individual level in electronic and print media, schools, social media, etc. Social distancing and the use of appropriate personal protective equipment should be encouraged to be practiced in public places, schools, workplaces, etc.

Vaccines have saved millions of lives from the most significant viral diseases of humans such as smallpox, polio, measles, hepatitis B, rotavirus, influenza virus infections, yellow fever, etc. The current COVID-19 pandemic has reiterated the dire need for technological advancements in vaccine development capabilities to rapidly respond to such global threats that humans may face at any time. Traditional methods of vaccine development are safer and more effective but are time-consuming and require prolonged protocols that may be further improved by incorporating the cutting-edge techniques of molecular medicine and computational biology. Currently used vaccines against COVID-19 are employing both classic techniques of vaccine development as well as modern vaccines like recombinant protein-based vaccines, adenovirus-based vaccines, and mRNA-based vaccines. Mass vaccination with COVID-19 vaccines has helped reduce the prevalence of cases. Nevertheless, continuous surveillance and monitoring of their safety and efficacy are required. To date, 10 vaccines developed by different companies have been approved by WHO.

Similarly, the management of zoonotic disease may also require the vaccination of animals, which is a serious economic challenge, especially when it comes to vaccinating wild animals or small animals like bats. Unfortunately, vaccinating masses of such animals as bats by spraying or aerosols is not possible. Nevertheless, improved vaccination strategies for humans, animals, and poultry may be considered by both developing and developed countries. Tremendous efforts have been made to develop antiviral drugs and a number of antiviral drugs are available in the market. Nonetheless, the treatment of viral diseases using antiviral drugs has been a challenge. So, advances in the research of antiviral drugs are required to treat the reemerged and emerging viral diseases.

There is a need for the establishment of a global consortium to combat such global threats that may emerge in any part of the world. No country can fight this war alone without utilizing the experiences of other institutions across the world. Despite massive efforts by global stakeholders in the past, eradication of viral diseases has always

been a nightmare. Still, many viral diseases including COVID-19, AIDS, dengue fever, yellow fever, Crieamen Congo hemorrhagic fever, hepatitis, influenza, measles, etc. cost millions of lives globally. Global, integrated networks need to be established/ strengthened with the ultimate goal of control and prevention of such diseases. Lessons learned in different countries may be utilized by people working in other countries.

Moreover, besides traditional approaches, certain modern media tools like digital twins can play a significant role in combating viral outbreaks by improving monitoring, reaction capabilities, and predictions [158]. Digital twins have the capability to replicate the transmission of epidemics through the creation of virtual representations of the impacted population. These models take into account several elements including mobility, interactions, and healthcare infrastructure [159]. Digital twins have the ability to accurately reproduce and continuously monitor the current condition of healthcare facilities, equipment, and resources in real time. This aids in the identification of regions that may experience overwhelming conditions or require supplementary assistance during an outbreak. These solutions offer a comprehensive perspective on the changing situation by combining data from several sources, including wearable devices, IoT sensors, and healthcare records. Predictive analytics, enabled by digital twins helps in predicting the path of an epidemic, allowing authorities to make well-informed decisions on the allocation of resources and implementation of preventive measures. Furthermore, digital twins can also amalgamate data from many sources including social media, healthcare records, and geographic information systems (GIS) to offer a holistic perspective on the effect and dissemination of the pandemic [160].

10. CONCLUSIONS

Pandemics are the consequence of viral outbreaks and epidemics that have expanded across international borders. It is necessary to implement effective preventive measures to restrict the disease's spread to a greater number of communities to reduce morbidity and mortality, as well as the community's social and economic burden. Besides medical interventions, the primary strategy involved in controlling an outbreak is to break the chain of infection by implementing control measures against the risk factors. The route of transmission of viruses is important in developing preventive measures. For instance, the transmission of respiratory viruses could be prevented by isolation, social distancing, and hand hygiene practices. Similarly, vector-borne viral infection could be prevented by using effective strategies to control the spread of vectors like mosquitos, ticks, and mites. Gastrointestinal infection and blood-borne infections caused by viruses could be prevented by proper hygienic practices and screening of blood products before transfusion respectively. There is an urgent need for the implementation of these kinds of strategies to minimize the lethal effects of viral outbreaks, especially in developing countries where the emergence and re-emergence of viral load are high. To provide a more thorough understanding of issues and potential solutions, the complexity of health and environmental difficulties needs to be assessed in an integrated and holistic manner. It's crucial to make coordinated efforts in the paradigm shift away from silo-based health systems and toward the One Health concept. The One Health approach should be used by decision-makers responsible for disease prevention and control in order to plan for and prevent illness, hospitalization, death, and the financial burden associated with disease epidemics. An early warning system to prevent epidemics is typically put into place right away in any public health disaster. Response networks cooperate with institutions and networks to pool their technical and human resources to combat outbreaks, such as the Global Outbreak Alert and Response Network. In a quickly changing environment with little or biased information available, the crucial choice to launch a disease response is frequently reactive and urgently required. The data in traditional surveillance systems are updated often. However, these systems are retrospective and delayed by nature, which limits their usefulness for rapid response to epidemics.

11. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Review Article

Quadripartite Vision and Mission of Dog-Mediated Rabies Elimination: Cross Border Transmission Threats

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Abstract: Dog-mediated rabies is an evident global health problem due to the transmission of bite injuries inflicted by free-roaming dogs in developing countries. More than 95% of human rabies cases are happening due to free-roaming dogs. The World Organization for Animal Health, World Health Organization, Global Alliance for the Control of Rabies, and the Food and Agriculture Organization of the United Nations jointly signed the agenda and mission to eliminate dog-mediated human rabies by the end of 2030. The vision has been set to combat this oldest recorded infectious disease of mankind, but it seeks serious commitment, planning, and investment in the developing countries of Asia and Africa where the legislative involvement and overhead support of their respective government are already deficient. Variable challenges to attain this vision in diverse countries exist, and one of the major issues is the cross-border transmission risks of circulating strains of rabies viruses through animal reservoirs and trades. The present review article has been designed to investigate the global purposes and motives of various activities and working groups of the quadripartite and the United Against Rabies (UAR) forum with a special focus on developing countries and the existing One Health approach. Moreover, various strains and risk factors of rabies virus at cross-regional borders have also been highlighted by searching articles through various online databases such as Google Scholar, PubMed, ScienceDirect, and Google. A well-designed surveillance system and support from the governments may foster this challenging mission of the quadripartite.

Keywords: Cross-border, Dog-mediated rabies, One Health, Quadripartite, Risk factors.

1. INTRODUCTION

The rabies virus (RABV), a severe infectious disease of public health importance, causes the contagious zoonotic disease in humans by the bite of a dog which is commonly known as dog-mediated rabies [1]. The most frequent way that dog-mediated rabies spreads and causes injuries to humans and animals is mostly by free-roaming dogs and infrequently by wild animals in Asia and Africa [1, 2]. Rabies can be prevented before symptoms appear, but it is nearly difficult once the clinical symptoms appear. Therefore, raising public awareness of dog-bite injuries to humans is crucial. The World Health Organization (WHO) classifies rabies as one of the neglected tropical diseases [3]. Despite having a disease that is curable by vaccine, the most recent estimate of annual human rabies deaths from a 2015 study is as high as 59,000 across 150 countries [4]. Dog-bite injuries account for more than 95% of human rabies infections, making the elimination of dog-mediated rabies a

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global priority [5]. In order to completely eradicate dog-mediated rabies by the year 2030, the WHO, World Organization for Animal Health (WOAH), the Food and Agriculture Organization (FAO) of the United Nations, and the Global Alliance for Rabies Control (GARC) have jointly proposed the One Health framework [3].

By bringing together these four essential stakeholders, the quadripartite approach leverages their respective capabilities to create a more comprehensive and effective strategy for dog-mediated rabies elimination. Collaboration enables the pooling of resources, knowledge, and experience to tackle this complex challenge [6]. Dog-mediated rabies elimination requires a multifaceted approach that involves the solutions present in human health, animal health, and environmental health sectors at national levels of the countries. The quadripartite approach ensures that experts from various fields work together to address the complexity of dog-mediated rabies transmission [7]. Rabies knows no borders, and cross-border transmission threats are a significant concern for molecular epidemiologists and phylogenetic studies. The quadripartite approach acknowledges the need for international cooperation to prevent the spread of dog-mediated rabies, making it a bold global issue [8].

To protect both humans and animals from dog-mediated rabies, the One Health approach's strategy is crucial [9]. One Health is a fusion of three distinct fields: environmental sciences, veterinary medicine, and public health [9]. This approach enables cooperation among the major global organizations which are strong advocates of human, and animal health and the environmental ecosystem. To protect both humans and animals from dog-mediated rabies, this strategy's primary goal is to raise people's understanding regarding disease control, prevention, and treatment [10].

According to the vision of the quadripartite organizations, [11] insightful information on the economics of dog-mediated rabies is important to consider while planning disease control programs and these organizations also provided crucial advice for the program orientation. Similarly, these organizations updated their assessment of the dog-mediated rabies burden in Asia and Africa and provided new insight into the disease's effects on these continents [12]. A case study on rabies in the Serengeti environment was also carried out to study the dog-mediated rabies reservoirs, which explored reservoir dynamics and advanced our knowledge of the disease's intricacies [13]. In this article, various credible research studies were examined to investigate the visionary guidelines of the quadripartite and how cross-border transmission of dog-mediated rabies has influenced this vision and mission. The extracted studies pertaining to the objectives of the articles were categorized into various subheadings to discuss and elaborate the mission of the quadripartite. Moreover, the cross-border transmission of dog-mediated rabies virus also threatens this mission and few logical studies also describe details regarding the border-based transmission pattern of the rabies virus strains.

Various keywords and Boolean operators were used to include credible research papers from the journals that align with the objective of the paper, while predatory and web-based resources were excluded. Moreover, articles in languages other than English were also excluded.

2. RABIES THREATENS GLOBAL HEALTH

The impact of dog-mediated rabies is worldwide and most commonly it is present in Asia and Africa. The RABV affects thousands of people and animals in underdeveloped and marginalized countries Figure 1. Besides these, the RABV is the major

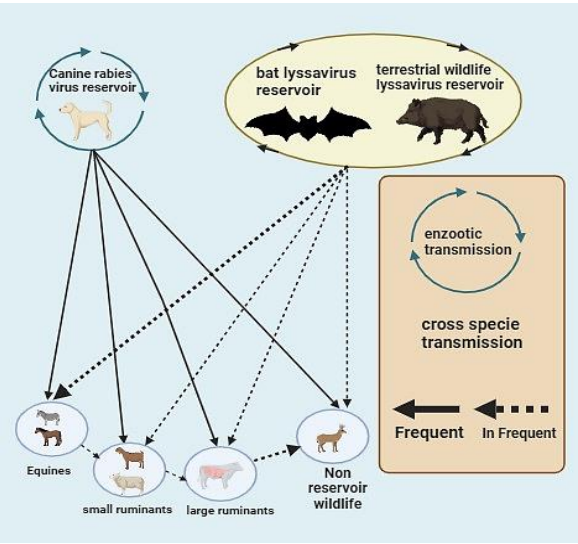

Fig. 1. Diagram showing various food and wild animal reservoirs that frequently and infrequently facilitate the cross-species transmission of RABV that potentially play a role in the cross-border transmission of the RABV.

cause of animal deaths across the world [14]. Therefore, we can't be able to help and grow the livestock sector because we are spending the budget on zoonotic diseases treatment and prevention [15].

Several zoonotic diseases clearly show the dire need for a One Health approach to solve the problems infecting humans, animals, and the environment [16]. Dog-mediated rabies is also one of these deadly diseases that demands collaboration like quadripartite to control disease or prevent disease at the human-animal interface due to the complex nature of the disease dynamics [17]. Similarly, the changes in environmental conditions sometimes may cause changes in morbidity, mortality, and disability adjusted life year score (DALYs) among humans and animals of various geographical regions in the developing world [18].

To successfully manage and prevent zoonotic illnesses like rabies, the links between human, animal, and environmental health must be understood and addressed [19]. We can enhance global health outcomes and defend both human and animal populations by putting One Health concepts into practice including coordinated surveillance, early identification, and quick response to disease outbreaks [20].

3. THE NEED OF QUADRIPARTITE

The quadripartite purpose is to realize that the 'Rabies 0 by 30' vision, supports the Sustainable Developmental Goals (SDGs) such as 'Good Health and Well-Being', Reduce Poverty, and 'Life on Land' [21]. The quadripartite approach acknowledges the need for international cooperation to prevent the spread of dog-mediated rabies, making it a global issue [22]. By eliminating dog-mediated rabies, the quadripartite initiative contributes to the improvement of public health. It reduces the burden on healthcare systems, saves lives, and enhances the well-being of communities affected by rabies [23]. Dog-mediated rabies has ecological implications as well. The vaccination and control of rabies in dogs help protect wildlife and preserve biodiversity, aligning with the goal of sustainable land use and conservation [24].

The quadripartite method, with its emphasis on dog-mediated rabies elimination, not only helps to save human lives, but also advances animal welfare, environmental protection, and public health [25]. It will result in better ecosystems, healthier communities, and a critical step towards reaching sustainability and global health goals [26]. The quadripartite vision and mission of dog-mediated rabies elimination represents a united effort to combat rabies. By bringing together government health agencies, veterinary authorities, and international partners, this collaborative approach aims to eliminate rabies and make the world safer for both humans and animals [27]. This initiative holds great promise for the future, aligning with sustainable developmental goals and benefiting the global community [23].

4. UNITED AGAINST RABIES (UAR) FORUM

The United Against Rabies (UAR) forum stands as a vital global initiative aimed at rallying stakeholders from a diverse array of sectors to collectively combat rabies [28]. Established with a common vision for the global elimination of this deadly disease, the UAR forum serves as a platform that fosters collaboration and coordination to accelerate progress toward a rabies-free world [26]. One of the primary strengths of the UAR forum is its inclusivity. It brings together stakeholders from a wide range of sectors, including public health, veterinary medicine, government agencies, international organizations, non-governmental organizations (NGOs), and private industries of the developing world. This diverse coalition of partners ensures that various perspectives and expertise contribute to the fight against rabies [25].

The cornerstone of the UAR forum is a shared vision: the global elimination of dog-mediated rabies. This common goal unites participants and provides a clear direction for their collective efforts [25]. The UAR forum operates under the overarching objective of achieving '0 by 30' which aims to eliminate human deaths from dog-mediated rabies by the year 2030. This ambitious target is a testament to the commitment of all stakeholders involved [29]. To effectively address the multifaceted challenges associated with dog-mediated rabies, the UAR forum establishes topic-specific working groups. These groups focus on various aspects of rabies control and elimination, including vaccination strategies, surveillance, community engagement, policy, and research. By concentrating efforts in these specific areas, the UAR forum can make significant progress in

a coordinated manner and facilitate the 0 by 30 mission of the quadripartite [30].

One of the core missions of the UAR forum is to support and enhance the progress towards the '0 by 30' vision. This involves developing and implementing comprehensive strategies to eliminate dog-mediated rabies [25]. The UAR forum provides a platform for sharing best practices, coordinating resources, and advocating for the importance of rabies elimination on a global scale [31]. The health priorities of the G20, particularly those highlighted during the recent India-G20 summit, are closely connected to the goals of the UAR forum [32]. One of the priorities emphasized during the summit is 'Building resilient systems for health emergency prevention, preparedness, and response [33]. The UAR forum's efforts to eliminate rabies align with this priority by strengthening health systems and emergency preparedness, as rabies is indeed a significant health emergency that can be prevented through effective mass dog vaccination programs and control programs [34].

The UAR forum represents a collaborative global effort to eliminate dog-mediated rabies, uniting stakeholders from diverse sectors with a common vision [31]. Through the establishment of working groups in this forum, support for the '0 by 30' initiative, and alignment with G20 health priorities, have been justified to benefit the health of communities worldwide [35].

5. MASS DOG VACCINATION: A MISSION OF QUADRIPARTITE

The most important and crucial aspect in preventing dog-mediated rabies and other zoonotic diseases is the vaccination of animals and humans [18]. Because vaccinating animals breaks the transmission bond between animals and humans [36]. Similarly, the vaccination of health care workers (especially veterinary doctors and vaccination team workers) helps us to protect their lives. Therefore, it is very important to work together in preventing the spread of dog-mediated rabies and make sure the vaccination of animals and humans have been well planned [37].

In addition to rabies, implementing preventive measures and management techniques is crucial to control dog-mediated rabies. Due to the public's ignorance regarding knowledge of dog-mediated rabies, organizing awareness programs and seminars is also crucial [18]. The diagnosis of dog-mediated rabies is the other crucial element in this regard. The early detection of the illness enables us to stop new cases from occurring in the future [38]. As a result, keeping an effective surveillance network on affected animals and people on a regular basis aids in the further contact tracing of the diseased animals [39].

We can make all these immunization campaigns and one-health prevention initiatives successful if veterinary healthcare experts and human health professionals collaborate well on identified gray areas [40]. Specialists in veterinary medicine have key responsibilities in the diagnosis, treatment, and prevention of animal cases, and their close collaboration with medical health experts helps to prevent human infection [41]. We can expand this cooperation by establishing programs for data sharing, and the One Health approaches to rabies control will lead to more effective coordination and communication at regional, national, and global levels [10].

6. CHALLENGES AND ROADBLOCKS IN 0 BY 30 MISSION

An increasingly serious threat is being presented by dog-mediated rabies. Unfortunately, it has imparted negative effects on the social and economic conditions of entire nations, in addition to the animal species, the effects of this deadly disease's spread are catastrophic, putting a heavy financial load on both governments and communities Figure 2. Budgets for healthcare are already severely constrained, and the expenses associated with vaccination campaigns, post-exposure prophylaxis, and animal control activities are extremely burdensome [42].

Additionally, the death toll from dog-mediated rabies has had a significant impact on our livestock business, particularly in rural areas where people rely largely on these animals for their livelihoods [43].

The One Health strategy must be implemented in all countries, despite numerous obstacles. For instance, it can be extremely challenging at times to vaccinate all the animals due to financial and social issues [44]. The inability to vaccinate every animal poses a limitation and a resource shortage as well

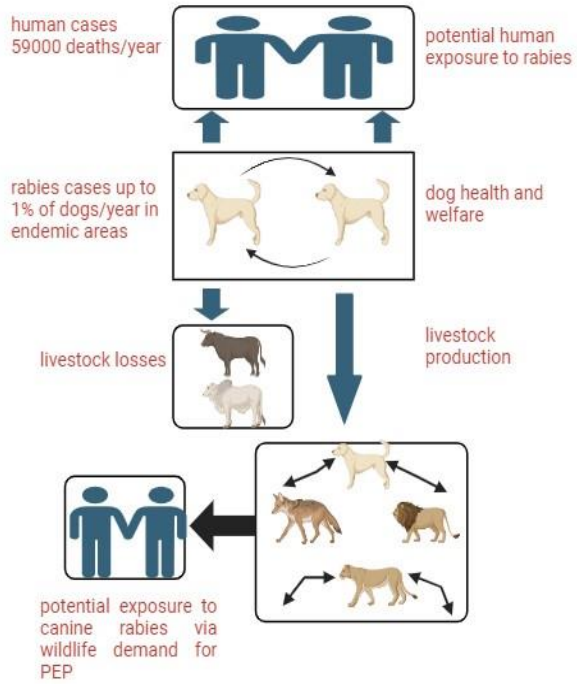

Fig. 2. Impact of dog-mediated rabies on dog, food animals, and human deaths that occur due to suspected dog bite injuries. The dog-mediated rabies can also be transmitted to wild animals which again infect humans

[6]. In addition, cultural practices and beliefs that prevent people from immunizing their pets are a significant barrier [15]. The issue at hand is how to get past these obstacles. By increasing social funding for these people, we can remove these obstacles and inform them about the horrifying clinical pictures of forthcoming events due to dog-mediated rabies.

7. CROSS-BORDER TRANSMISSION THREATS OF DOG-MEDIATED RABIES

Rabies is a viral disease that knows no borders, and its elimination requires global efforts [45]. Cross-border transmission threats play a significant role in perpetuating the disease, making collaborative strategies essential. Several factors contribute to these threats, each with its unique challenges and implications[46]. Illegal cross-border trade in animals, particularly dogs, can introduce dog-mediated rabies into non-endemic regions [47]. This challenge emphasizes the importance of stringent border controls and regulations to prevent the illegal movement of animals[48]. Travelers visiting rabies-endemic regions may be at risk of exposure. Promoting rabies awareness and pre-travel vaccination is crucial for travelers to mitigate this risk [49]. Disparities in vaccination coverage between neighboring countries can create pockets of susceptibility near borders. Collaboration and harmonization of vaccination programs across borders are essential to achieve consistent protection [50].

Wildlife reservoirs can contribute to the perpetuation of dog-mediated rabies, especially in areas with extensive land borders [51]. Enhanced surveillance and control measures targeting wildlife populations are needed to prevent spillover to domestic animals and humans [52]. Difficulties in accessing and sharing canine vaccination data and dog bite registries across national borders hinder effective disease monitoring and response [53]. Improved data-sharing mechanisms and international cooperation are essential to track and control dog-mediated rabies [54].

Discrepancies in dog population size estimates or censuses across borders can complicate vaccination planning. Standardized methodologies for estimating dog populations and data sharing are critical for effective vaccination campaigns [55]. The trade-in of dogs and cats for meat can facilitate the movement of potentially rabid animals. Strict regulations and enforcement regarding the trade of these animals are necessary to reduce transmission risks[56].

The pet trade, particularly unregulated and informal breeding and sales activities, can introduce rabid animals across borders. Strengthening regulations and promoting responsible pet ownership can mitigate this threat [56]. Cross-border migrants may face challenges in accessing post-exposure prophylaxis (PEP) in a timely manner [46]. Ensuring equitable access to PEP for all individuals, including migrants, is crucial[45]. The absence of robust surveillance and monitoring systems can impede early detection and response to rabies cases [57]. Investment in surveillance infrastructure and capacity-building is vital for effective control [58].

Gaps in the endeavor to control dog-mediated rabies can be caused by variations in response techniques across borders [59]. For a united approach, coordinating epidemic responses and harmonizing response methods are crucial [60].

The need for global cooperation in tackling these concerns of cross-border transmission cannot be overstated. To achieve the global aim of eradicating dog-mediated rabies by 2030, cooperative efforts, standardized practices, and information exchange are essential [61].

8. QUADRIPARTITE AT THE POLICY LEVEL

Effective lobbying for the One Health approach at the policy level is required to include factors for the health of people, animals, and the environment in complete policies. By adopting One Health laws and regulations, policymakers can promote collaboration amongst many sectors and enhance efforts at disease surveillance, prevention, and response. This approach has gained popularity since it is effective in addressing challenging health concerns [62]. By highlighting the interdependence of human, animal, and environmental health in policy frameworks, resources can be allocated more successfully and disease patterns can be understood better [63]. Through this advocacy, decision-makers can promote all-encompassing and long-lasting solutions to public health problems, such as the control of zoonotic diseases like rabies.

It is essential to translate One Health advocacy into real rules and regulations to address global health challenges and prevent zoonotic disease outbreaks successfully. Governments, public health agencies, veterinary organizations, and environmental departments must work together to implement the One Health policy framework through legislative ordinance [64]. Such policies can include coordinated disease control strategies, cross-sectoral coordination mechanisms, and combined surveillance and reporting systems. Countries can promote the flow of important information and data sharing by harmonizing legislative frameworks and standardizing methods [65]. Utilizing technology and innovation to improve infectious disease monitoring and response skills is another aspect of putting One Health policy into practice [66].

Adequate financing and resources are needed to support research, surveillance systems, and capacity-building efforts to sustain the One Health project. Stakeholders can promote the application of One Health policies by arguing for additional financial assistance from governments, international organizations, and the corporate sector [67]. Innovations in disease prevention, diagnostics, and control strategies can result from securing funding for cross-sectoral collaborations and collaborative research [68]. Additionally, pushing for specialized financing sources for One Health initiatives can guarantee sustained commitment to the cause [69]. Advocates can get the required funding to effectively tackle rising health dangers by convincing politicians of the cost-effectiveness and societal advantages of One Health solutions.

Governments, non-governmental organizations, academia, and international organizations must work together effectively on a global scale for One Health projects to be successful [70]. Collaborative tools can make it easier to share knowledge, best practices, and insights from fruitful One Health initiatives [71]. Stakeholders can access a wide range of expertise, pool resources, and work together to address global health concerns by developing international partnerships [72]. As early warning systems are supported by international cooperation, prospective disease outbreaks can be addressed quickly [73]. Additionally, collaborations with the business sector and industry stakeholders can spur advancements in disease prevention strategies, surveillance technology, and vaccine research [74].

9. FACILITATIONS IN ONE HEALTH COMPETENCY SUSTAIN THE MISSIONS OF QUADRIPARTITE

When utilizing the One Health idea, interdisciplinary research is crucial to address complicated health concerns like rabies prevention and control. By bringing together experts from several fields, such as human and veterinary medicine, epidemiology, ecology, and environmental scientists, the researchers can get comprehensive insights into the dynamics of dog-mediated rabies [75]. Cooperative research initiatives have aided the creation of innovative solutions and the understanding of transmission routes [76]. Those nations who have relatively controlled dog-mediated rabies such as India, Sri Lanka, Philippine, and Thailand have an understanding of the importance of the connections between human, animal, and environmental health. These connections have been improved by this interdisciplinary study which also makes it simpler to develop evidence-based preventative strategies for zoonotic diseases like rabies [77].

Collaboration across many stakeholders,

including public health agencies, veterinary services, wildlife conservationists, and local populations is essential for effective rabies prevention and control [78]. These many organizations can pool their knowledge, resources, and experience through collaborative studies to develop comprehensive methods that are adapted to certain situations [63]. These may include vaccination efforts for both domestic animals and wildlife, as well as community outreach initiatives to encourage responsible pet ownership and raise knowledge of rabies transmission [79]. Together, these parties can develop sustainable and comprehensive rabies management strategies, thereby easing the burden of this grave global health concern.

Powerful techniques for enhancing rabies surveillance and monitoring activities have been made available through technological improvement [80]. Maps of high-risk locations and probable disease hotspots can be created using remote sensing, Geographic Information Systems, artificial intelligence, and big data analytics [81]. Furthermore, mobile applications and real-time reporting platforms can improve disease surveillance in both human and animal populations, helping the early identification of suspected cases in animals and humans. The ability to quickly and accurately identify rabies strains is also made possible by developments in molecular diagnostics and viral genomes, aiding in epidemic investigations and tracing disease transmission pathways [82]. Public health officials and researchers can improve their capacity to spontaneously predict and react to new rabies outbreaks by utilizing technology-based disease detection tools [83].

The effectiveness of One Health's efforts in rabies prevention depends on fostering a culture of knowledge-sharing and cooperation [84]. Platforms to exchange research results, best practices, and lessons learned to encourage collaboration among stakeholders at the local, national, and international levels need to be explored and implemented [85]. Researchers, practitioners, and policymakers can communicate on shared initiatives including international conferences, workshops, and online networks [86]. By developing a common knowledge base, the international community can take advantage of one another's failures and triumphs to enhance rabies prevention and control methods throughout time which will get nations one step closer to reliable success.

10. ENGAGING THE COMMUNITY: ONE HEALTH EDUCATION AND EMPOWERMENT

We can spread correct information about illness prevention and the significance of ethical pet keeping by supporting public awareness initiatives and educational programs. Communities are better able to protect themselves and their cherished animals when they are informed about the dangers of rabies transmission and the advantages of vaccination. Various platforms may be used to carry out public awareness campaigns, ensuring that information reaches a variety of groups and distant locations where the disease burden may be high. The prevention and management of dog-mediated rabies depend greatly on public education. Communities must be informed about the dangers of rabies transmission, the value of responsible pet ownership, and the advantages of vaccination if they are to adopt a proactive approach to disease prevention [87]. To spread accurate and understandable information about rabies, public health campaigns can make use of a variety of communication platforms, including social media, radio, and community workshops [88]. By raising public knowledge of rabies prevention, communities may decide for themselves whether to report probable rabies cases, participate in vaccination programs, and seek prompt medical attention for animal attacks.

Successful One Health projects require strong community participation and engagement. The success and viability of such programs are improved when communities are given the tools they need to actively participate in zoonotic disease surveillance, prevention, and control activities [89]. One Health project such as the UAR forum progress and vision of the quadripartite can target unique community needs and personalize treatments by incorporating local community leaders, healthcare practitioners, veterinarians, and environmentalists in decision-making processes [90]. Through collaborative initiatives that promote ownership and accountability within the community and capacity-building seminars, training sessions, and projects, empowerment may be attained [91]. Participating in One Health projects helps communities become more resilient to disease outbreaks and fosters a feeling of shared accountability for preserving the health of people, animals, and the environment.

It is essential to develop the upcoming generation of activists and experts as One Health continues to develop. The interdependence of human, animal, and environmental health may be effectively promoted by educational institutions [92]. Future professionals can develop a multidisciplinary perspective by incorporating One Health principles into academic curricula, veterinary and medical training, and environmental research [93]. Students may also be motivated to pursue jobs in sectors connected to One Health through research opportunities and mentoring programs [93].

11. CONCLUSIONS

In the context of zoonotic illnesses like rabies, the idea of One Health has emerged as a potent and crucial strategy for solving complicated health concerns. One Health enables us to take a comprehensive approach to the prevention and control of animal-originated diseases and epidemics. Group efforts and collaborations are essential for assuring a safer and healthier future as we become more alert of how closely bordered countries and interrelated human, animal, and environmental health are at risk of contracting an emerging or remerging disease from nowhere.

Rabies continues to be a serious worldwide health issue, especially in areas with poor resources and access to healthcare. We all have a responsibility to actively contribute to rabies prevention initiatives, both as an individual and as a community. Furthermore, promoting the adoption of One Health techniques at the policy level can lead to a considerable shift in disease surveillance, preventive, and control methods. For the One Health goal to be advanced and emerging illnesses like rabies to be properly combated, ongoing research and innovation are essential. For identifying gaps and creating evidence-based solutions, scientific research on disease transmission, monitoring, and control techniques is crucial. We can improve disease surveillance, provide effective diagnostic tools, and track disease outbreaks in real time by using technology and transdisciplinary research. Additionally, funding studies to learn more about how disease transmission is impacted by climate change may help us to proactively address changing health concerns. We improve our readiness and response capacities via ongoing research and innovation, creating a more robust global health system.

One Health stands for a uniting force that cuts beyond distinctions and unites people, communities, and organizations in the quest for the health and well-being of all and the quadripartite is a real-time example in front of us. We have the chance to build a future that is safer, healthier, and more sustainable for both people and animals if we embrace the One Health principles, then active participation, public awareness, and dedication to research and innovation are all requirements of this approach that should be carried out in rabies endemic countries. Together, we can protect the well-being of our neighborhoods and maintain the precarious equilibrium between people, animals, and the environment for future generations.

12. CONFLICT OF INTERESTS

The authors declared no conflict of interest.

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Research Article

Genomic Variation in Dengue Virus Non-Structural Protein 1 (NS1)

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Abstract: To understand in an improved way how the dengue virus (DENV) spreads, presents, and becomes hazardous, researching its genetic makeup is necessary. The positive sense RNA of DENV encodes three structural proteins and seven non-structural proteins. One of the non-structural proteins that aids in the replication of viral RNA is the non-structural protein 1 (NS1). The objective was to identify the most frequently repeated mutations in the NS1 protein in DENV RNA isolated from dengue patients in the province Punjab, Pakistan. Selection of 120 DENV isolates was done from laboratories of tertiary care hospitals of Punjab for analysis of sequencing of the whole genome. Only 23 samples were sequenced after viral isolation, quantification and cDNA synthesis. A total of 133 different types of mutations were detected along the entire length of NS.1. The most common mutations with the highest frequency were, K324R and K347R (n=7), D278N (n=6), K174R, and F178S (n=4), found at c-terminal of NS.1 protein. Mutations K347R, K174R, and F178S are novel. Future DENV vaccination development research will be especially profited by the mutations found in the current study. During each DENV outbreak in different places, studying genomic variations is crucial for strengthening societal health and developing new policies for future outbreaks.

Keywords: Dengue Virus, Genomic Variation, Mutations, Nonstructural protein 1, Pakistan.

1. INTRODUCTION

Dengue fever, caused by dengue virus (DENV), is a common and grave infectious disease. Humans contract this disease by bites of Aedes female mosquitos, primarily *Aedes aegypti* and *Aedes albopictus* [1]. During the last few decades, dengue has become an epidemic all across the world, affecting 390 million people every year [2].

Dengue fever has been rapidly spreading in Pakistan, with Sindh, Punjab, and Khyber Pakhtunkhwa being the major affected provinces. Dengue fever cases discovered in Pakistan in 2017 were 22,938 as compared to more than 3,200 cases in 2018, 24,547, and 3,442 patients in 2019 and 2020 respectively, according to the National Institute of Health (NIH) Islamabad [3]. For better disease management in the future, molecular characterization of regional extracted DENV during outbreaks for different new mutations which will affect the function of the dengue virus may be helpful.

Dengue infection is brought on by dengue viruses (DENV), which are members of the Flaviviridae family and genus Flavivirus. High fever, muscle and joint pain, vomiting, fatigue, myalgia, skin rash, haemorrhagic episodes, abdominal pain, and circulatory shock are among the signs and symptoms of dengue infection [4]. The

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DENV viruses are encapsulated, single-stranded RNA viruses. There are four DENV serotypes from 1 to 5[5]. All four serotypes have caused outbreaks in Pakistan over the years, but generally, serotypes 2 and 3 have been more common [6]. The DENV genome is an 11-kilobase open reading frame (ORF), which is surrounded by the 5' and 3' untranslated portions and is encrypted. The ORF only encodes one polyprotein. This polyprotein is made up of three structural proteins (C: capsid, M: membrane, and E: envelope) and seven non-structural proteins (NS-1, NS-2A, NS-2B, NS-3, NS-4A, NS4B, and NS-5) [7]. These proteins are essential for the virus's formation and reproduction. While the non-structural proteins have a variety of roles in processes including viral RNA replication and immune evasion, the structural proteins are responsible for creating the viral particle.

The N-linked non-structural glycoprotein 1 (NS1) is 48 kilodaltons of pliability and excellent preservation. It has a membrane-associated form and a soluble, secreted variant. NS.1 is a 352-residue polypeptide with 46 to 55 kDa molecular weight. The soluble NS1 protein is composed of two identical subunits. Each subunit has three separate structural domains as shown in Figure 1. The first, referred to as the "-roll" dimerization domain, is composed of residues 1–29 and consists of two -hairpins (β) stabilized by a disulfide (Cystein4–Cystein15). The four -hairpins intertwine to make a -sheet. Subdomain / (amino acids 38 - 151) has a four-stranded -sheet, two -helices, and a distal tip (residueS.108-128); it is part of the second wing domain (amino acids 30-180) that also contains two glycosylation sites (Asparagine 130, Asparagine 175) and an internal disulfide (C55-C143). The third domain with 18 β-strands (residues 181–352). A conserved tip region of 79 residues (residues278–352) has four β-ladder, three β-sheet, and three disulfides [8]. There is an important role of NS1 protein in DENV replication. NS1 is the protein that can immediately affect the pathogenesis and is related to the severity of the disorder, due to the fact it can additionally cause vascular leaking in the lung, liver, and small intestine. By activating CD4 and CD8 T cells, NS.1 produces drastically excessive stages of each TNF-alpha and IL -6 within side the blood and additionally induces the launch of inflammatory cytokines that can cause vascular leaking resulting in excessive disorder [9]. Vascular leaking is a serious concern of dengue patients as it can cause death [10]. In evaluation to be worried about the pathogenesis of the disorder, it additionally works therapeutically. In addition to small molecule drugs, antibody remedy is nicely favoured due to its specificity towards disorder. Till now, numerous structural proteins targeting antibodies have been fashioned toward DENV infection. These are termed "neutralizing antibodies" and worried about stopping viral attachment with host mobiliary however it's far useless for all 4 DENV serotypes because of the chance of ADE (antibody-based enhancement) [11]. However, in evaluation to neutralizing antibodies, anti-NS.1 Antibodies can offer exceptional healing mechanisms through now no longer simply lowering the viraemic segment but additionally lowering the important segment. Also, it isn't related to the chance of ADE because it isn't a structural protein [9].

2. MATERIALS AND METHODS

During the 2022 dengue epidemic, the data from the Institute of Molecular Biology and Biotechnology, The University of Lahore, Lahore, Pakistan and Tertiary care hospitals of Punjab was collected. Ethical clearance was taken from the institute. Patients were selected from hospitals' dengue wards using a temporal sampling strategy, and their informed permission was documented. Dengue infection was verified by a positive PCR test for DENV, a positive NS1antigen test, or a positive IgM antibody test. Performa contained the patient's complete medical record, including their medical history, physical examination results, and all laboratory and other diagnostic tests. Patients from both genders above the age of 13 who have been diagnosed with dengue fever were included in the study. Patients with dengue shock syndrome, infective and chronic liver disease, and any other infection like typhoid fever, and malaria were excluded.

Within 7 days of the commencement of symptoms, dengue patients' blood was drawn, centrifuged, and preserved. The DENV RNA was extracted and purified from the serum of confirmed patients using a GeneJET viral DNA/RNA purification kit. The virus-containing serum was centrifuged at maximum speed to remove particles and cells, followed by adding binding

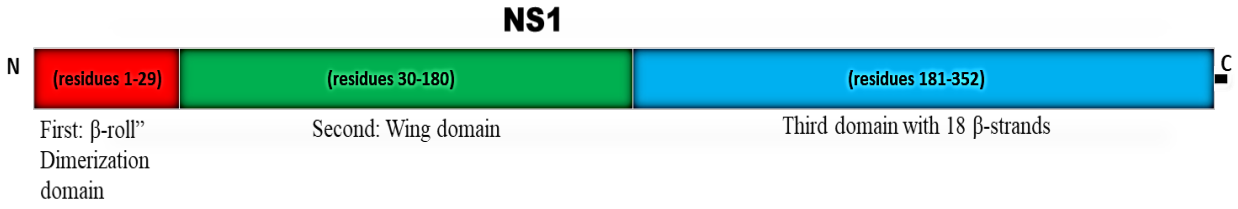

Fig. 1. Domain organization of NS.1 protein of DENV.

solution and 96-100% ethanol to ensure proper lysis. A spin column was then placed in the column, centrifuged, washed, and eluted. The purified viral RNA was then transferred to a new microcentrifuge tube and centrifuged at 6,000 × g for 1 minute to elute the purified viral RNA. Using RTq-PCR and gel electrophoresis, we determined how much RNA had been extracted. Selected samples were sequenced using DENV WGS on an Ion 510 chip. After loading the prepared chip into the Ion XL 5 sequencer, the data was transferred to Torrent Suite Server 4.10. The mutation frequencies were computed and summarised using EpiData Analysis, a software programme created by the WHO. Data quality was ensured by an Excel examination. To isolate RNA, we employed a viral RNA purification kit (K0821, Cat. no.). Quantification of the extracted RNA was performed by polymerase chain reaction and gel electrophoresis. Then, we used the Ion 510 chip to sequence DENV WGS data from a subset of the samples. The data was transferred to Torrent Suite Server 4.10 when the chip was loaded onto the Ion XL 5 sequencer. The mutation frequencies were computed and summarised using EpiData Analysis, a software programme created by the WHO. Data quality was ensured by an Excel examination. Alpha folds 2 and PyMOL tool was used to create a 3-D picture of NS1 protein.

3. RESULTS AND DISCUSSION

The sequencing of the entire genome was performed on a total of 120 serum samples that were chosen for the investigation. RNA from DENV was extracted from them, and after that, they went through the stages of PCR quantification and gel purification. After that, it was determined that just 23 of the samples were adequate to go through the sequencing process. Only 19 of the samples were able to have their complete sequences determined, whereas the remaining 4 samples could not. A total of 133 different types of mutations have been detected along the entire length of NS.1 as depicted in Table 1. The most common mutations with the highest frequency were, K324R and K347R (n=7), D278N (n=6), K174R, and F178S (n=4). The highest frequency mutations were most commonly detected at the C-terminal of NS.1 proteins. K324R and D278N have been reported and three (K347R, K174R, and F178S) are novel.

Nine mutations have been detected in the first β-roll" dimerization domain with a single frequency each. The second domain harbored 74 mutations with various frequencies from 1 to 4. Position D136 has six different amino acid substitutions in different genomic isolates. The third C-terminal domain contains 49 mutations with frequencies from 1 to 7, among which K324R and K347R were the most common (n=7) as shown in Figure 2.

In Table 2, the effect of Different Mutations is shown on the protein function. In DENV1 serotypes, mutation D278N repeated in 6 samples, K122Q in 2 samples, and I124K in 2 samples affects the protein function. Similarly, in DENV2 serotypes Mutation I135T and D136V were repeated in 2 samples each affecting protein function.

We are taking the mutation D278N into further consideration because of its high frequency and its ability to affect NS1 protein function. In this mutation aspartic acid at position 278 is replated by Asparagine as shown in Figures 3 and 4. A domain with an unidentified function's surface contains this mutant residue. In the employed structure, the residue was not discovered to be in touch with any other domains whose function is known. Even so, this mutation may have an impact on interactions with other molecules or domains.

Urgent action is required in Pakistan to improve the examination of Dengue markers, laboratory capacities for improved case management and identification, and warning indicators for sensitization in order to reduce the seasonality of

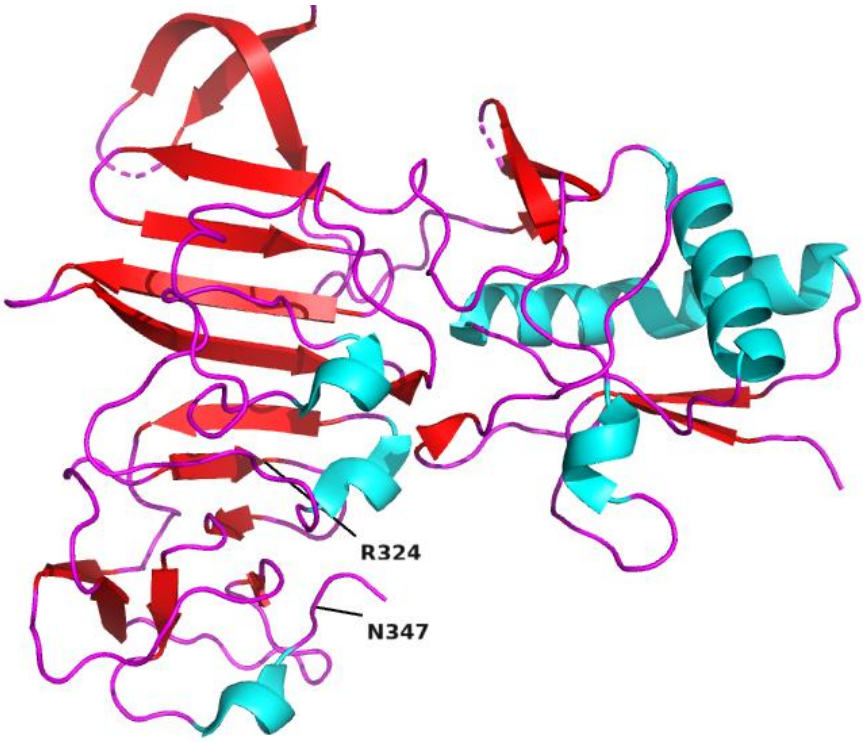

Fig. 2. Structure of DENV NS.1 protein and location of most common mutations at position number 324 and 347.

Fig. 3. Schematic structures of the original Aspartic acid (left) into an Asparagine (right) amino acid at position 278.

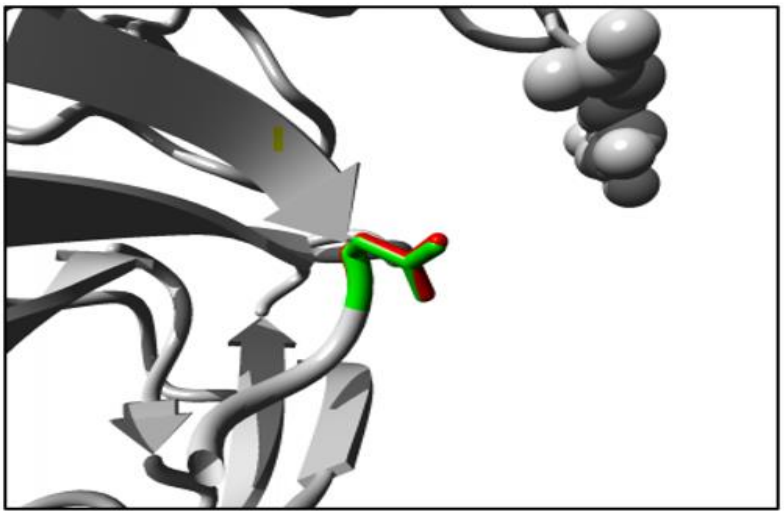

Fig. 4. Close-up of the mutation D278N. The protein is colored grey, and the side chains of both the wild-type and the mutant residue are shown and colored green and red respectively.

Table 1. Mutations in the NS.1 protein and their frequency.

MUT	S.11	S.12	S.13	S.14	S.15	S.16	S.17	S21	S22	S23	S24	S26	S30	S31	Freq
W8G							P								1
W8R				P											1
K9E				P											1
N10K			P												1
N10T			P												1
K11Q			P												1
K11T			P												1
E12G			P												1
E12K			P												1
K33R	P														1
D37N							P								1
R41G				P											1
G47E				P											1
K48R	P														1
C55Y	P														1
M67I				P											1
E74K				P											1
L75F							P								1
H77Y	P			P			P								3
S80T						P									1
N82I				P											1
N82Y							P								1
T87I							P								1
V88A							P								1
M89I						P									1
G91R							P								1
D92E				P		P									2
V93A	P			P			P								3
S94N	P			P			P								3
G95R				P											1
I96V	P			P			P								3
A98T	P			P			P								3
Q99H							P								1
G100E							P								1
K101N							P								1
K101R							P								1
K102E							P								1
K102R							P								1
K102X	P			P			P								3
S.103P						P									1
I104V							P								1
R105W						P									1
P106L				P											1
S.117T				P											1

MUT	S.11	S.12	S.13	S.14	S.15	S.16	S.17	S21	S22	S23	S24	S26	S30	S31	Freq
T117A						P									1
W118G				P											1
G119E				P											1
A121P	P														1
A121T						P									1
K122Q				P			P								2
I123F	P														1
I123T	P														1
M123L						P									1
I124K	P			P											2
I124V	P														1
D127G							P								1
S.128L						P									1
V128R				P											1
V128T	P			P			P								3
N130D							P								1
Q131H						P									1
L134R			P												1
L134V			P												1
I135F			P												1
I135L			P									P			2
I135M			P												1
I135S			P									P			2
I135T			P												1
I135V			P			P									2
D136A			P			P									2
D136E			P												1
D136G			P									P			2
D136H			P			P									2
D136N												P			1
D136V			P												1
G137A			P												1
N139D	P			P			P								3
N146D	P			P			P								3
I162V	P			P			P								3
K174R			P			P						P	P		4
V177I						P						P	P		3
V177T			P												1
F178S			P			P						P	P		4
D190E						P									1
D190G						P									1
D190N						P									1
N191H						P									1
F217L												P			1
N222S			P			P						P			3

MUT	S.11	S.12	S.13	S.14	S.15	S.16	S.17	S21	S22	S23	S24	S26	S30	S31	Freq
S239G								P		P					2
E240G									P						1
I246V								P							1
L247F			P			P						P			3
G248E										P					1
G249R	P														1
I264T			P			P						P			3
T265A			P			P						P			3
G266V	P														1
H269R	P			P											1
G271D										P					1
K272R			P			P						P			3
E274G	P														1
F277L	P					P		P							3
D278I								P							1
D278K								P							1
D278N	P			P			P	P	P	P					6
C280R										P					1
D281E			P			P						P			3
E281G								P		P					2
N293H			P												1
N293K			P												1
N293S			P												1
R294K			P												1
R294S			P												1
V303A							P								1
K306E						P									1
K306X						P									1
T307I	P			P			P	P	P	P					6
I308T				P											1
T309A						P									1
E310K	P														1
C313S								P							1
C313Y	P														1
P319T				P											1
K324R	P	P		P			P	P	P	P					7
G328E						P									1
G328R	P														1
E340D						P									1
E340G						P									1
N344S						P									1
V346A										P					1
K347R	P	P		P			P	P	P	P					7

Table 2. Predictions About Mutations on NS1 protein Function.

Serotype	Mutation	Frequency	Median Sequence Conservation	Sequences Represented At This Position	Protein Function	Prediction Score
DENVI	K324R	7	3.00	43	Tolerated	I .00
	K347R	7	3.00	43	Tolerated	1.00
	D278N	6	3.00	43	Affect protein function	0.02
	T3071	6	3.00	43	Tolerated	0.48
	H77Y	3	3.00	44	Tolerated	I .00
	V93A	3	3.00	44	Tolerated	
	S94N	3	3.00	44	Tolerated	0.39
	196V	3	3.00	44	Tolerated	I .00
	A98T	3	3.00	44	Tolerated	0.28
	V128T	3	3.00	44	Tolerated	0.53
	N139D	3	3.00	44	Tolerated	0.92
	N146D	3	3.00	44	Tolerated	0.56
	1162V	3	3.00	44	Tolerated	I .00
	F277L	3	3.00	44	Tolerated	0.55
	D92E	2	3.00	44	Tolerated	0.21
	K122Q	2	3.00	44	Affect protein function	0.04
	1124K	2	3.00	44	Tolerated	0.45
	S239G	2	3.00	43	Affect protein function	0.00
	E281G	2	3.00	43	Affect protein function	0.01
DENV2	K174R	4	2.97	38	Tolerated	0.23
	F178S	4	2.97	38	Tolerated	0.58
	771	3	2.97	38	Tolerated	0.47
	N222S	3	2.98	37	Tolerated	0.79
	L247F	3	2.98	37	Tolerated	0.06
	1264T	3	2.98	37	Tolerated	I .00
	T265A	2	2.98	37	Tolerated	0.76
	K272R	2	2.98	37	Tolerated	0.34
	D281E		2.98	37	Tolerated	0.34
	1135T	2	2.97	38	Affect protein function	0.00
	D136V	2	2.97	38	Affect protein function	0.01

the occurrence of DENV disease. The province of Punjab, which may be found in the middle of Pakistan's eastern half, is the nation's second-largest in terms of both its population and its land area [12]. It may be beneficial to perform molecular characterization of locally extracted DENV from Punjab during an epidemic in order to conduct genomic surveillance and determine the mutation frequency. This would allow for better disease management in the future. When we have a better understanding of the DENV proteome, we will have a better understanding of the mutational

patterns that are responsible for the high incidence and severity of the disease. Despite recent scientific progress, our understanding of the clinical significance of protein information in DENV infection, and its role in the development of DENV-related diseases, remains incomplete. Mutations at position Lys227Arg were detected in the current study and Thr307Ile is absent in the current study, in the NS.1 has also been reported in earlier studies [13]. Previously it was reported that mutations in NS.1 clinical samples, Val236>Ala or Trp68>stop are associated with low NS.1 protein secretion [14]. In another study, it was shown that Thr164Ser mutation inNS.1 is associated with the disease severity in the Americas [15]. The NS.1 monomer structurally consists of three domains. The initial dimerization domain, known as a "roll" domain (residues 1-29), consists of two -hairpins that are held together by a disulfide (Cystein4-Cystein15). The hairpins combine to create a four-stranded sheet. The second wing domain (amino acids 30-180) has two glycosylation sites (asparagine 130, asparagine 175), an internal disulfide (C55-C143), and two subdomains, one of which, subdomain /, has a four-stranded -sheet, two -helices, and a distal tip (residueS.108-128). The third domain (residues 181-352) has 18 -strands. There are four -ladder residues, three -sheet residues, and three disulfides in a conserved tip area of 79 residues (residues 278-352) [16].

4. CONCLUSIONS

During this analysis, we found that the DENV NS1 protein included 133 different types of mutations along the entire length. The most common mutations with the highest frequency were, K324R and K347R (n=7), D278N (n=6), K174R, and F178S (n=4). The highest frequency mutations were most commonly detected at the c-terminal of NS.1 proteins. K324R and D278N have been reported and three (K347R, K174R, and F178S) are novel. The results of experiments reveal that mutations can have an effect not only on a virus's ability to replicate but also on its severity, its ability to penetrate a host cell, and its ability to disseminate. Based on this genetic heterogeneity, diagnostic procedures and markers can be developed, which may in the future lead to improvements in the treatment of DENV fever.

5. CONFLICT OF INTERST

The authors declared no conflict of interest.

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Rsearch Article

Synthesis and *In vitro* Evaluation of Doxorubicin loaded Polymeric Nanoparticles on Cancer Cells

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Abstract: Cancer is the second biggest mortality rate globally. Most of anti-cancer drugs are hydrophobic and when they are administered in the body, they get clear from the blood. That's why polymeric nanoparticles (NPs) have been used for delivering anti-cancer drugs to targeted sites. Biodegradable and self-assembled nature, PEG-PLGA has been used as a nanocarrier for biomedical applications. We developed PEG-PLGA NP for the doxorubicin (DOX) delivery to cancerous cells. The successful PEG-PLGA synthesis was confirmed by its ^{1}H NMR spectrum. All NPs displayed individual spherical morphology and 100 nm size range with -18.5mV zeta potential. Drug release profile showed DOX had sustained release pattern from DOX@NPs. *In vitro,* MTT assay and apoptosis analysis revealed that low-dose DOX@NPs exhibited more toxic effects on cancerous cells as compared to DOX alone. Overall results demonstrate that polymeric-based nanosystems increase the efficacy of DOX on cancer cells.

Keywords: Cancer, Chemotherapy, Doxorubicin, Drug delivery, PLGA nanoparticles

1. INTRODUCTION

Cancer is a main public health issue globally, with a growing number of cases diagnosed each year [1]. The most significant distinction between healthy and cancerous cells is their uncontrollable reproductive nature. One of the biggest challenges in the treatment of most of the diseases is getting the drug to target infectious site [2]. Cancer can be treated in a variety of ways. Treatment options are dependent on the specific cancer you have and its stage of development. One course of treatment may be sufficient for some cancer patients. However, many patients undergo multiple treatments simultaneously [3].

Chemotherapy is a type of aggressive treatment for cancer in which toxic medications are used to kill fast-dividing cancer cells. It can be used to decrease the tumor size or the tumor cells number in the body, lowering the probability of spreading cancer [4]. Unfortunately, current chemotherapy has a number of limitations, the majority of which are attributable to the lack of target specificity, which results in unsatisfactory clinical outcomes. Chemotherapy kills fast-dividing cells; because normal cells divide quickly, cancerous drugs have toxic effects on normal cells such as bone marrow, hair follicles, macrophages, and digestive tract. [5]. Nanotechnology is concerned with materials that have dimensions of one-hundredth of a millimeter or less [6]. Because of its uses in biotechnology, targeted drug administration, gene delivery, and drug delivery, nanotechnology has grown in popularity in recent years [7]. Nanoparticles (NPs) with smaller sizes (in nm) can easily penetrate various cells, particularly cancer cells. Since nanocarrier has the benefit of the EPR effect in cancer and escape filtration in the spleen while still being sufficiently large to avoid absorption in the liver, a size of 100-200 nm is currently considered the ideal size for drug delivery systems [8].

Polymer nanotechnologies are crucial for overcoming obstacles to drug delivery, such as targeted therapy and the delivery of molecules

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that can't be delivered, like hydrophobic therapeutics, oligonucleotides, or RNA-interfering molecules [9]. Only a few polymers can be used as components of NPs intended to deliver drugs *in vivo*. Consider that an appropriate polymer needs to fulfill several criteria to be used in this application to better understand this [10]. It must be biodegradable or swiftly eliminated from the body so that it can be administered repeatedly without the danger of uncontrolled accumulation. It should not be immunogenic or toxic. If there are any degradation products, they must also be nontoxic and immunogenic. [11]. Polymeric NPs have lots of advantages in drug delivery, including protect therapeutic payload and biological molecules during their journey to the target site and increasing bioavailability and therapeutic index. Because of the continuous polymeric network that forms their structure, drugs can be maintained within or adsorbed onto the nanosphere surface [12]. Because it has been demonstrated that they are safe to use in the clinic. Poly (D, L-lactic-co-glycolic acid) (PLGA) based NPs are considered as the sustained release polymer system for drug delivery applications. PEG-PLGA NPs are mostly wanted because systematic clearance of pegylated polymeric NPs is lower than similar particles without PEG. PEG can enhance the pharmaceutical properties of many FDA-approved drugs. DOX is a highly effective anticancer drug that has been authorized for use against a variety of tumor types. Unfortunately, toxicities associated with anthracycline drugs, the most serious of which is heart failure, limit its long-term clinical use. Encapsulating DOX in PEGylated liposomes reduces DOX-induced cardiomyopathy but maintains anticancer activity against solid tumors [13]. Doxorubicin is a hydrophobic drug and clears in bloodstream very fast [14]. PEG is hydrophilic that's why it is encapsulated in PEG to convert it into a hydrophilic form. Polymeric micelles consist of hydrophilic and hydrophobic monomer units and are primarily amphiphilic co-polymers, an efficient drug delivery system for anticancer medications that are only weakly water-soluble. The blood circulation of the polymeric micelles is prolonged, and they concentrate more at the site of the tumor [15]. In this study, DOX is transformed into a hydrophilic condition by encapsulated in polymeric nanoparticles to improve its anticancer efficacy.

2. MATERIALS AND METHODS

2.1. Synthesis of the Block Co-polymer (PEG-b-PLGA)

Ring-opening polymerization approach was used to synthesize PEG-b-PLGA with little modification in the previous methodology [16]. mPEG-OH (500 mg, 0.09 mmol) was mixed in toluene (60 mL) and was treated with azeotropic distillation for 6 hours using a Dean-Stark apparatus. After cooling under nitrogen to room temperature, lactic acid (LA) (1.0 g, 6.8 mmol), glycolic acid (GA) (0.22, 1.8 mmol), and tin (II) ethoxyhexanoate (20.0 mg) were heated to 130 °C for 8 hours in a glove box (water and oxygen contents 0.1 ppm). In chloroform, the resultant polymer was dissolved then precipitated and dried in cold diethyl ether. The white solid product (PEG-b-PLGA) achieved (900 mg, 68%) was analyzed using ^{1}H-NMR spectroscopy.

2.2. Preparation of Nanoparticles

For DOX@NPs preparation, 10 mg of PEG-b-PLGA and 10 µl of DOX (10 mg/mL in DMSO) were mixed in 1 mL of DMSO under constant stirring. After adding ultra-purified water (5.0 mL), the solution was stirred for another 30 minutes. After that, DMSO was then removed by dialysis membrane method. Newly prepared DOX@NPs solution was dialyzed with membrane MWCO of 14 kDa against distiller water for 24 hours. After that NPs solution was centrifuged to remove the un-encapsulated drug and polymeric debris from the supernatant.

2.3. Characterization of Nanoparticles

Mean diameter and surface charge measured by Dynamic light scattering (DLS). The size distribution profile and morphology of DOX@NPs were analyzed by transmission electron microscopy (TEM).

2.4. DOX Encapsulation Efficiency and Loading Content

For encapsulation efficiency and DOX loading content, newly prepared 10 mg of DOX@NPs were dissolved in DMSO (5 mL). To remove polymeric debris, solution was centrifuged at 16000 rpm,

15 °C for 30 min and washed twice with fresh DMSO. After that pellet was dissolved in 1 ml DMSO and analyzed for DOX content at λ max value of 485 nm by UV spectrophotometer.

The drug loading content (DLC) and encapsulation efficiency (EE) were quantified with belove mentioned equations:
DLC= (DOX weight in the NPs) / (Total NPs weight) × 100%
EE= (encapsulated DOX on NPs / total DOX added to polymer) × 100%

2.5. Drug Release Profile

To determine the release pattern, the dialysis membrane of 14 kDa was used. Drug-loaded nanoparticles (15 mg) were dissolved in phosphate buffer saline (PBS) (1mL) at pH 7.4 and immersed in the dialysis bag in 30 mL of PBS while being stirred at 110 rpm and 37 °C. After preset intervals, 1 mL of PBS was collected from the immersed solution and replaced with fresh PBS. The concentration of released DOX was quantified using a DOX standard curve developed with known concentrations. DOX release curve was drawn by plotting the cumulative % DOX release against preset time interval.

2.6. Cytotoxicity Assay

HepG2 and MDA-MB-231 cells (5000 cells/ well) were plated in 96 well plates respectively and cultured at 37 °C for 24 hours. Afterward, cells were incubated with different concentrations (from 3.15 to 50 g/mL of DOX) of DOX@NPs and free DOX respectively. Cells that had not been treated served as a control. After 24 hours, an MTT assay was carried out on cells cultured in 96 well plates to compare the proliferative potential of different groups on HepG2 and MDA-MB-231 cell lines. After washing the cells with PBS, they were incubated for 2 hours in a 500 µl complete medium containing 25 µl of MTT (10 mg/mL) solution. MTT is converted into purple formazan in alive cells, which is then solubilized with 10% sodium dodecyl sulphate (SDS) and checked the absorbance at 570 nm using a microplate reader. Triplicates were run for each treatment.

This equation calculates the percentage cell viability:

% viability = Mean OD Sample / Mean OD blank × 100.

2.7. Apoptotic evaluation by p53 ELISA

Quantification of apoptotic protein level in the post-treated cells was measured by p53 ELISA (Zokeyo, China) kit according to the manufacturer's protocol. Initially, 50 µL of lysate from different treatment groups was added in triplicate along with standard solution in particular wells. After the addition of HRP-conjugated reagent (100 µL), plate was incubated for at 37 °C 1 hour. The HRP-conjugated reagent was removed by washing with wash buffer three times. After washing, Chromogen A and Chromogen B solutions (50 µL each) were added to all wells and incubated for 15 min at 37 °C. After that 50 µL of stopping solution was added in each well and reading was measured at 450 nm.

3. RESULTS AND DISCUSSION

3.1. Characterization of Polymer

The successful PEG-PLGA synthesis was confirmed by its ^{1}H NMR spectrum (Fig. 1). The *d, l*-lactide peaks appear at 5.21 (m, nH, CH) and 1.5 (m, 3nH, CH_3) and glycolide peaks of PLGA was found at 4.72 (s, 2nH, CH_2). The PEG peak has appeared at 3.2 (m, 4nH, OCH_2CH_2).

3.2. Physicochemical Characterization

Hydrodynamic diameter of newly synthesized NPs was measured by DLS. At pH 7.4, the hydrodynamic diameter of DOX@NPs was approximately ~115nm (Fig. 2A). The zeta potential (mV) of DOX@NPs was found to be -18.5 mV.

DOX@NPs showed size distribution was approximately 100 nm and displayed individual spherical morphology as captured by TEM (Figure 2B). Based on UV spectrometry analyses, the DOX loading content and encapsulation efficiency of NPs were 9.5 ± 0.07 µg/mg and 96.13 ± 4.05 % respectively.

3.3. *In vitro* Release Study

To evaluate drug loading efficiency and drug release profile, standard curve was obtained.

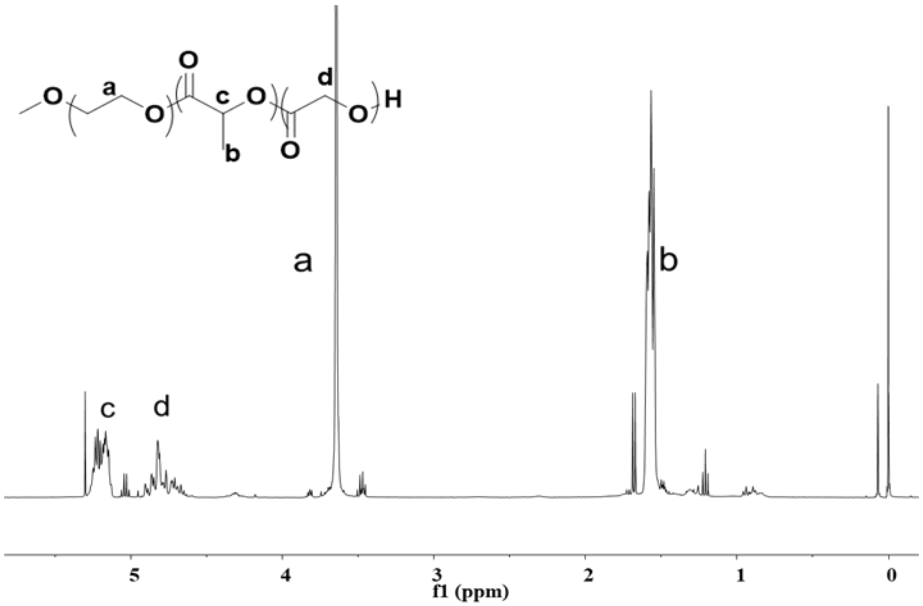

Fig. 1. [1]H NMR spectra of co-polymer (PEG-PLGA). Peak "a" shows a functional group of PEG while peaks "b", "c" and "d" show functional groups of PLGA.

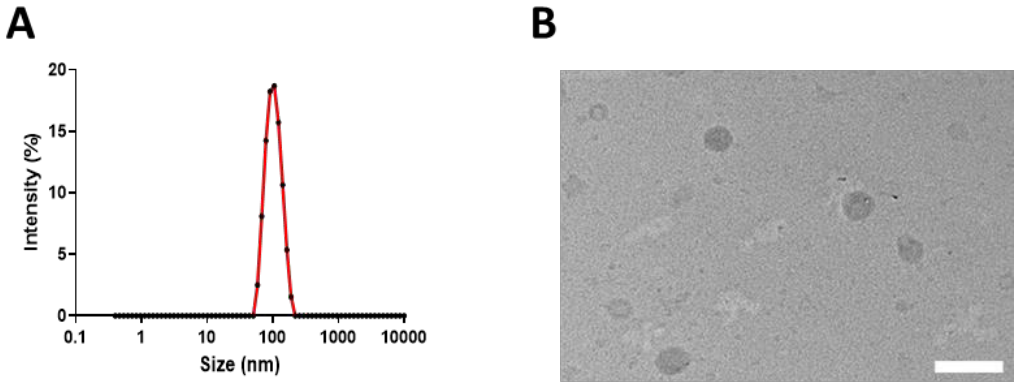

Fig. 2. Physicochemical characterization of the DOX@NPs **(A)**. Hydrodynamic diameter of DOX@NPs at physiological pH 7.4 in PBS solution. **(B)** TEM image of DOX@NPs. The bar size is 200 nm.

Different concentrations of DOX (0.03125µg/ml to 0.00024375µg/ml) were checked using a UV spectrophotometer at 485 nm and a standard curve (Fig. 3A).

The DOX release behavior from NPs was studied shown in Figure 3B. The DOX@NPs had a significantly longer period of sustained release. During the first six hours, about 40% of the drug was released, and in 48 hours, 98% of the DOX was released. As a result, the results show that DOX@NPs have more consistent drug-release and controlled-release properties.

3.4. Cytotoxicity Study of DOX@NPs

HepG2 and MDA-MB-231 cells were utilized to evaluate the *in vitro* cytotoxic effect of DOX@NPs by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Figure 4A and B show the cellular cytotoxicity of free-Dox and DOX@NPs at equivalent concentrations of loaded Dox. The viable cell percentage was measured by an MTT assay. Both Free-Dox and DOX@NPs showed a significant decrease in cell viability in dose dose-dependent manner against both MDA-MB-231 and HepG2 cells. Auspicious results were observed

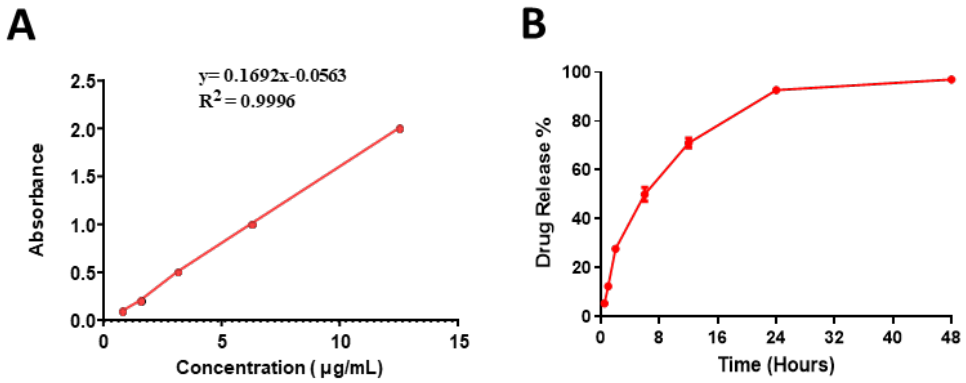

Fig. 3. *In vitro* release study. **(A)** Standard curve for free DOX in DMSO was determined at 485 nm UV wavelength. **(B)** DOX release profile of the DOX@NPs at pH 7.4 at 37 °C. DOX release was observed after regular time intervals.

in MDA-MB-231 cells because of a significant decrease in IC_{50} values of DOX@NPs (12.5 µg/mL) as compared to free DOX (30 µg/mL). At the highest concentration, cell viability was reduced to 10 % for the DOX@NPs while free DOX, which was > 35%. Similar pattern was found in HepG2 cells, IC_{50} of DOX@NPs against HepG2 cells was much lower (10.7 µg/mL) in comparison with free DOX (25 µg/mL). As shown by IC_{50}, the anti-proliferation ability of the formulations followed the order: DOX@NP > DOX. The findings indicate that DOX@NPs appear to have a higher antitumor effect than free DOX.

3.5. Apoptotic Analysis

The apoptosis in post-treatment groups along with the control group was assessed by a p53 ELISA kit. The level of p53 was quite low in the control group and a similar low apoptotic effect was found in PEG-PGLA treated cells which confirmed the biocompatibility of co-polymer. Moreover, the prevalence of apoptosis in DOX@NPs was higher than that of free DOX in both cells which was in line with cytotoxicity MTT results. These findings revealed that the enhanced cytotoxic effect and apoptosis induction of DOX@NPs were accredited to higher internalization and sustained DOX release, which could efficiently inhibit cancer cell proliferation.

4. DISCUSSION

Cancer is a leading cause of death globally, approximately 9.5 million people died due to cancer in 2020. The incidence of cancer is increasing worldwide, due to multiple reasons, such as population aging, changes in lifestyle, and environmental factors [17]. However, the cancer survival rate is also increasing, because of early

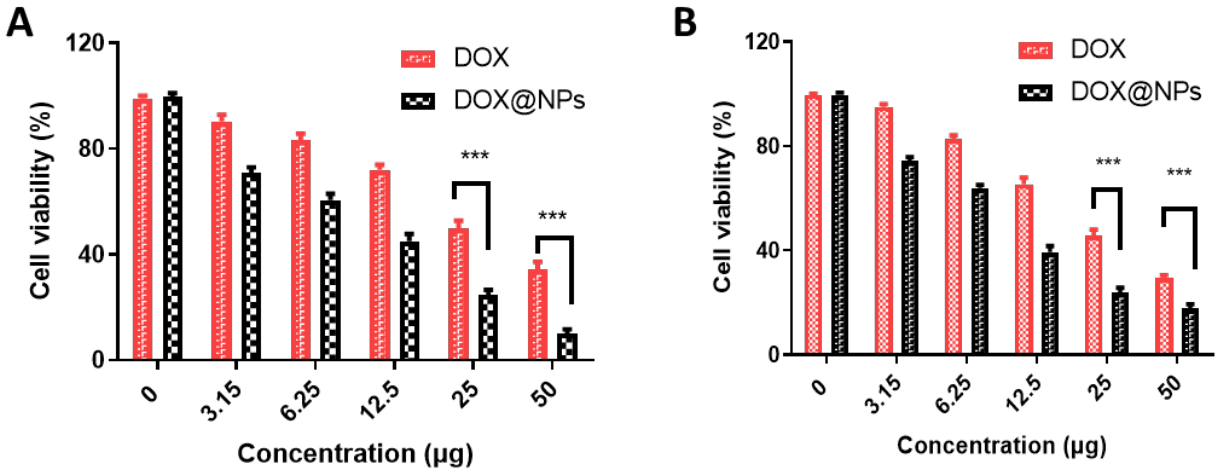

Fig. 4. Cytotoxic effect of DOX@NPs on MDA-MB-231 **(A)** and HepG2 **(B)** cells evaluated by MTT assay.

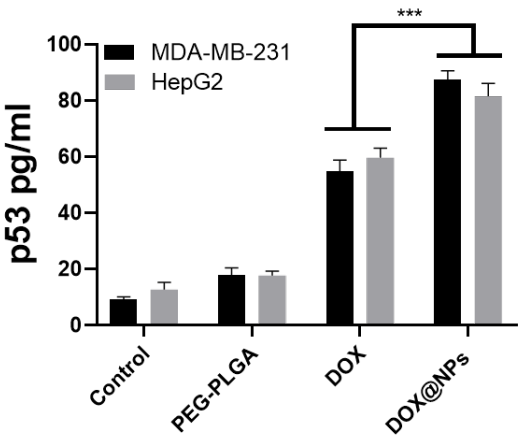

Fig. 5. Apoptosis of cancer cells was analyzed by p53 ELISA kit after DOX@NPs (20 µg/mL) treatment.

diagnosis and advancement in treatments. Multiple important habits can be done to minimize the risk of cancer, including a healthy diet, avoid smoking, getting regular exercise, maintaining a healthy weight, and regularly performing cancer screening tests [18]. Among different cancer treatment approaches, chemotherapy is one the most effective treatment approaches that directly kills the cancer cells by different chemo-therapeutics. [19]. Because of the effectiveness of DOX in a wide range of cancers, the DOX belongs to the class anthracycline is most commonly used in chemotherapy. By intercalating with DNA, doxorubicin suppresses macromolecular production. [20].

Nanoparticles (NPs) show unique features for transportation, biological activity and interaction, optical, thermal, and magnetic properties that cannot be offered at molecular and macroscale levels. The supportive entries can facilitate hydrophobic drug delivery, and protect the drug during blood circulation, controlled release, immune evasion, deep tumor tissue penetration, site-specific activation, and higher cellular uptake [6]. We investigated the delivery of hydrophobic drug by encapsulating the drug into polymeric NPs and checked *in vitro* cytotoxicity and apoptosis induction on cancer cells. The ^{1}H NMR characteristic chemical peak values of our synthesized co-polymer were similar to previous studies [21, 22] confirming the successful synthesis. The polymeric nanoparticles in this study are 100 nm in size with a spherical shape which allows them for longer *in-vivo* circulation half-life and higher intracellular uptake in cancer cells [23]. In the present work, DOX was loaded in the hydrophobic core of PLGA which gives stability to the drug, PEG is advantageous in terms of extending the blood half-life and cell membrane interaction [23].

Slow drug release from nanoparticles which leads to a sustained release of the payload over a period of time is a more effective way to kill cancer cells [24]. The release profile of DOX@NPs demonstrated that our nanosystem has sustained release properties and it was observed that about 90% drug was released in 48 h. Our sustained-release finding is in agreement with the other drug release reports which demonstrate that hydrogen bonding between drug and PLGA matrix and slow degradation rate of polymers [25, 26]. The sustain release drug delivery systems have the potential to improve efficacy, reduce toxicity, and improve patient compliance with chemotherapy [27].

A well-established method in which cancer cell lines are utilized to evaluate nano-formulations biocompatibility and determine their cytotoxicity and pharmacological response to the delivery of loaded drug [28]. Compared by the treatment of free-Dox, equivalent concentration of DOX@ NPs exerted a much stronger cytotoxic effect on both types of cancer cells. These results are in agreement with previous published reports on the anti-cancer toxicity of DOX which showed that DOX@NPs display a higher strength at lower doses than free DOX due to higher cellular uptake of DOX@NPs [29, 30]. Apoptosis is the naturally programmed cell death mechanism, which is one most applied approach for most chemotherapeutic drug and nanomedicine analyses [30]. As shown in Figure 5, the prevalence of apoptosis in cells after treatment with DOX@NPs much higher than free DOX is probably related to the fast internalization kinetics of NP drug that increases the local drug concentration in the cell [29]. Previously, it was reported that DOX caused early activation of p53 in tumor cells which was followed by caspase-3 activation and DNA fragmentation [31]. Overall, these findings indicate that DOX@NPs have considerable anticancer potential against MDA-MB-231 and HepG2 cells and can enhance the therapeutic effects of anti-cancer drugs, making them promising adjuvant therapy for breast and liver cancers.

5. CONCLUSIONS

In this study, we developed an effective nanosystem based on PEG-PLGA block co-polymer and DOX. TEM revealed that nanoparticles were well-dispersed, and spherical shape. The release profile showed that the DOX@NPs, as nanocarriers for DOX, had a good sustained-release effect. Cytotoxicity results showed that anticancer activity of DOX was significantly improved when it was encapsulated in PEG-PLGA NPs. We believe this DOX@NPs system has considerable potential for cancer treatment.

6. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Research Article

Investigating the Burden of *Pseudomonas aeruginosa* Infections in COVID Patients and Resistance Profiles in Abbottabad, Pakistan

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Abstract: Nosocomial infections are a great menace for hospitalized patients and *Pseudomonas aeruginosa* has emerged as one of the most potent nosocomial pathogens due to its continuous emanation of multi-drug resistance. Conjointly, *P. aeruginosa* coinfection is a significant problem in multitudinous infections. The present study aimed to investigate the frequency and antibiotic susceptibility profile of *P. aeruginosa*, from the specimens isolated from patients from Ayub Teaching Hospital in Abbottabad, Pakistan. In this cross-sectional study, eighty strains of *P. aeruginosa* were obtained from 200 patients (urine, wounds, and pus samples), using routine microbiological methods, and antibiotic susceptibility testing was performed using the Kirby Bauer disc diffusion method. The majority of isolates (51.25%) were taken from wounds, followed by pus (38%) and urine (27.27%). Of the 80 isolates, 12 originated from individuals who were also infected with the *Coronavirus* (a 34% coinfection rate). These isolates were sensitive to levofloxacin (80%) and vancomycin (75%) but were resistant to moxifloxacin (80%) and amikacin (69%). *P. aeruginosa* is found in high frequency in clinical specimens from patients in Abbottabad, and these microorganisms are transiently resistant to routinely given antibiotics, making it critical to utilize anti-Pseudomonas medications correctly. It is concluded that the *P. aeruginosa* infections and resistance continued to increase owing to various intrinsic and extrinsic factors.

Keywords: Antimicrobial Resistance, Co-infection, Moxifloxacin, *Pseudomonas aeruginosa*, Vancomycin.

1. INTRODUCTION

Pseudomonas aeruginosa is a gram-negative, rod-shaped bacterium that has become a global health issue and widespread use of antibacterial drugs has increased bacterial resistance levels and severity [1, 2]. *P. aeruginosa* causes an opportunistic human disease that infects only a small percentage of healthy persons. It primarily affects immunocompromised individuals, such as those with HIV, cancer, or cystic fibrosis, where fatality rates can reach 90%. It is highly invasive and associated with both acute and chronic vision problems.

Bacterial coinfection is a significant problem in COVID-19 patients and *P. aeruginosa* is one of the predominant coinfecting bacteria identified [3]. *P. aeruginosa* is a common nosocomial organism that causes fatal chronic infections in immunocompromised people with diseases such as cystic fibrosis, catheter-related infections, and burn wounds [4]. A study was conducted that revealed that 43 (12.46 %) of 340 COVID-19 patients got secondary bacterial infections. The most commonly isolated bacteria included *P. aeruginosa* i.e., 9.30% [5]. Other studies also showed that *P. aeruginosa* is the second most often detected infection in COVID-19 patients. It is a frequent co-infection that causes illness aggravation in people with COVID-19 [6]. Additionally, *H. influenza, S. aureus, K. pneumoniae, Mycoplasma pneumoniae,* and *Streptococcus pneumoniae* are frequent co-infecting microorganisms [3].

Treatment options include broad-spectrum carbapenem, aminoglycosides, fluoroquinolones, and aztreonam, which are the most widely recommended antibiotics for bacterial infections caused by the bacteria *P. aeruginosa* [7]. Eradication of this organism is difficult and complicated due to its inherent resistance to many different families of chemotherapeutic agents and antibiotics [8].

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Antimicrobial resistance develops in *P. aeruginosa* by various mechanisms, including innate resistance, characterized by overexpressed efflux systems and decreased membrane permeability [9]. Resistance to beta-lactam antibiotics, carbapenem antibiotics, aminoglycoside antibiotics, and fluoroquinolone antibiotics can be acquired through acquiring resistance genes or mutations in genes producing porin efflux pumps, penicillin-binding proteins, or chromosomal beta-lactamase [10]. Multidrug-resistant *P. aeruginosa* isolates limit options for treatment and greatly increase morbidity rates [11]. Many antibiotics are ineffective against *P. aeruginosa,* due to the presence of resistance genes such as the chloramphenicol resistance gene catB and the ampicillin resistance gene ampC, which encodes a beta-lactamase enzyme that hydrolyzes ampicillin and confers beta-lactam resistance. *P. aeruginosa* has been identified as a significant concern in bacterial infections [12], especially those isolated from hospitalized patients, particularly those in critical care units [13].

Antibiotic misuse and abuse are a rising public health problem, since they may result in serious adverse effects and the rise of resistant bacteria [14]. As a consequence, developing novel treatment options for P. aeruginosa infections is a high priority that has received significant attention over the last decade. Moreover, as a result of misuse or overuse of antibiotics, the sensitivity profile of P. aeruginosa to different antibiotics is constantly changing regionally, selection antibiotics remains a challenge for the resistant strains. Additionally, no comparable data was previously accessible under this framework. This study was conducted to develop a framework to investigate the prevalence of *P. aeruginosa* in the local population of Abbottabad District, KPK, Pakistan, during the Covid-19 outbreak and its susceptibility pattern. The objectives of the present investigation were to determine the antibiotic susceptibility of *P. aeruginosa* and its prevalence in Covid-19 patients.

2. MATERIALS AND METHODS

2.1. Study Area

This cross-sectional study was conducted at Ayub Medical Complex, Abbottabad, Pakistan. Ayub Teaching Hospital is a public sector, non-profit tertiary level center of academic health sciences located in Abbottabad, Khyber Pakhtunkhwa, Pakistan.

2.2. Sample Collection

The samples were obtained from Ayub Medical Complex (Microbiology Laboratory) in Abbottabad, Pakistan. In this study, 200 urines, wound, and pus samples were isolated from several wards of the hospital, including the OPD ward, the ENT ward, and the emergency ward. 35 out of 200 samples came from coronavirus-infected patients. Samples were collected from urine, wound, and pus from different wards and OPD from the patients by using sterile swab sticks according to the standards of CLSI [15].

2.3. Samples Culturing

All samples were cultured on MacConkey, CLED, and Blood agar and incubated at 37 °C for 24 h. Isolated colonies were picked by using a sterile wire loop and suspended in 2 ml of distilled water in a sterilized tube to make a standard suspension. A sterile swab was dipped into the suspension and streaked on Muller Hinton Agar. *P. aeruginosa* isolates were confirmed by performing standard microbiological procedures such as colony characteristics, gram staining and biochemical assays including catalase, oxidase and citrate tests.

2.4. Antimicrobial Susceptibility

Antimicrobial susceptibility of isolated *P. aeruginosa* strains was performed against thirteen different commonly used antibiotics. To assess susceptibility to antibiotics, *P. aeruginosa* was sub-cultured on Muller Hinton at 37 °C for 24 h and the Kirby Bauer disc diffusion method was used as per CLSI guidelines.

2.5. Antibiotics Used

Levofloxacin 30 µg, Ciprofloxacin 30 µg, Amikacin 30 µg, Piperacillin 30 µg, Aztreonam 30 µg, Cefoperazone 30 µg, Ceftazidime 30 µg, Cefepime 30 µg, Moxifloxacin 30 µg, Tazobactam 30 µg, Imipenem 30 µg, Meropenem 30 µg, and Vancomycin 30 µg were used for screening. A zone size exceeding 22 mm was considered dignified

susceptible, whereas a zone size of more than 21 mm was considered dignified resistant. As per CLSI criteria, isolates were classified as susceptible or resistant [15]. As a control *P. aeruginosa* ATCC 27853 was taken.

2.6. Data Analysis

The data analysis was done using a variety of tools in Microsoft Excel 2016.

3. RESULTS

3.1. Growth Profile and Antibiotic Susceptibi lity of *P. aeruginosa*

The growth rate of *P. aeruginosa* was evaluated using urine samples, 41% wound swab samples, and 38% pus samples (Figure 1). The results showed that the bacteria grew in 80 of the 200 samples, as well as 35 coronavirus patients, 12 of which (34%) were also infected with *P. aeruginosa*. Susceptibility to antibiotics was determined using isolates from patient samples. Antibiotics from multiple categories were used. Total of 80 % of *P. aeruginosa* isolates were sensitive to levofloxacin, 75% to vancomycin, 70% to aztreonam, 68% to ciprofloxacin, 63% to cefepime, and cefoperazone 60%. The majority of the strains are resistant to moxifloxacin 80%, followed by amikacin 69%, ceftazidime 61%, and piperacillin 59%.

3.2. Sensitivity in COVID vs Non-COVID Patients

The sensitivity of *P. aeruginosa* in the COVID and non-COVID patient samples were showed in Figure 2. The levofloxacin showed 82% sensitivity, ciprofloxacin showed 69% sensitivity, piperacillin showed 43% sensitivity, aztreonam showed 72% cefoperazone 62%, ceftazidime 31%, cefepime 62%, moxifloxacin 19%, amikacin 31%, tazobactam 46%, imipenem 46%, meropenem 43%, and vancomycin showed 82% sensitivity in non-covid patients while in covid patients the sensitivity pattern is as follows levofloxacin 67% sensitivity, ciprofloxacin showed 58% sensitivity, piperacillin showed 33% sensitivity, aztreonam showed 58%, cefoperazone 50%, ceftazidime 83%. The sensitivity of *P. aeruginosa* in different age groups is indicated by the Levofloxacin and vancomycin show varying sensitivity profiles in each age group. In the COVID and non-COVID patients, the sensitivity pattern is 68% sensitivity, ciprofloxacin (68%), piperacillin (70%) and aztreonam (62%) with age-specific sensitivity, and 73% sensitivity in the cosmopolitan population.

3.3. Sensitivity According to Gender

The sensitivity of *P. aeruginosa* in male and female were different. The levofloxacin showed 84% and 74% sensitivity, ciprofloxacin showed 69% and

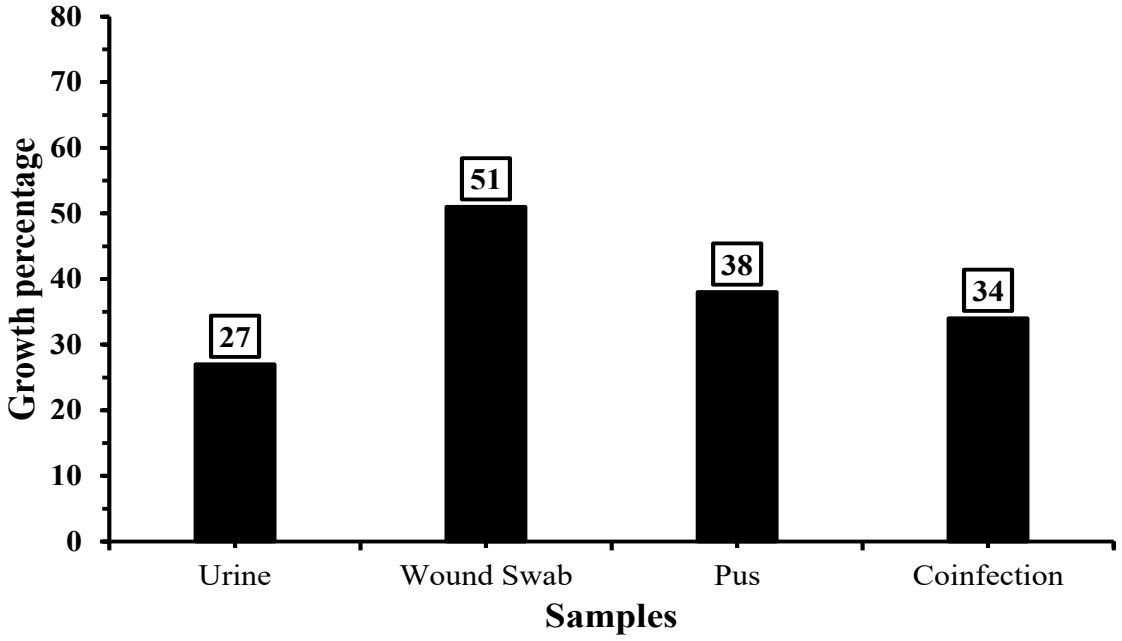

Fig. 1. Growth profile of *Pseudomonas aeruginosa* (n=80).

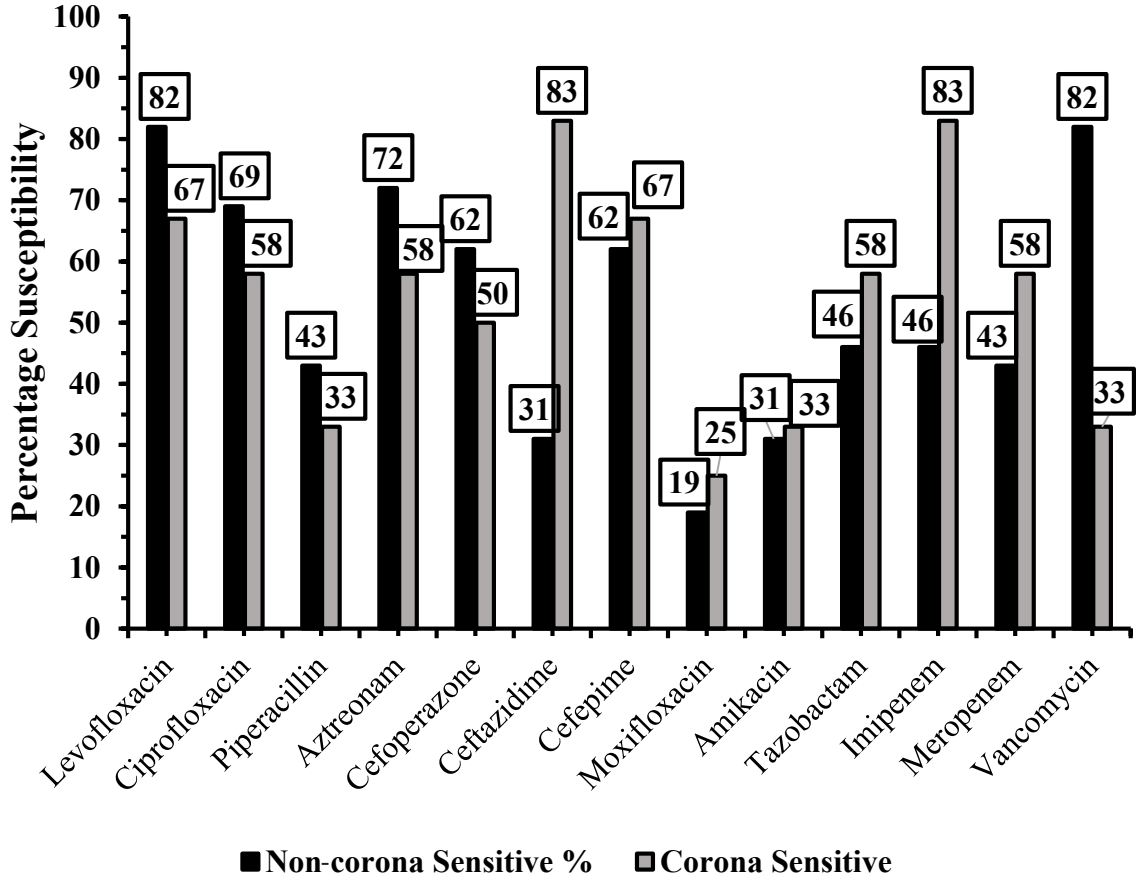

Fig. 2. Comparison of antibiotic susceptibility of *P. aeruginosa* in COVID and non-COVID patients.

66% piperacillin showed 33% and 51% sensitivity, aztreonam showed 67% and 74%, cefoperazone 60%, ceftazidime 38% and 40%, cefepime 64% and 60%, moxifloxacin 22% and 17%, amikacin 33% and 29%, tazobactam 51% and 43%, imipenem 53% and 49%, meropenem 47% and 43% and vancomycin showed 82% and 66% sensitivity pattern. The most important details from the text are the sensitivity patterns of the subjects, as well as the physical and emotional factors that affect the sensitivity.

3.4. Sensitivity in Different Areas

The sensitivity of *P. aeruginosa* in different areas is as follow; the levofloxacin showed 67% sensitivity, ciprofloxacin showed 80% sensitivity, piperacillin showed 33% sensitivity, aztreonam showed 73%, cefoperazone 67%, ceftazidime 33%, cefepime 73%, moxifloxacin 13%, amikacin 20%, tazobactam 40%, imipenem 53%, meropenem 33%, and vancomycin showed 67% sensitivity in patients living in Nawanshahr. Those patients who were living in Cantonment areas their sensitivity pattern was as follows, levofloxacin 92% sensitivity, ciprofloxacin showed 68% sensitivity, piperacillin showed 36% sensitivity, aztreonam showed 72%, cefoperazone 68%, ceftazidime 44%, cefepime 52%, moxifloxacin 20%, amikacin 28 %, tazobactam 52%, imipenem 56%, meropenem 36%, and vancomycin showed 76% sensitivity. Levofloxacin showed 72% sensitivity, ciprofloxacin showed 67% sensitivity, piperacillin showed 44% sensitivity, aztreonam showed 72%, cefoperazone 61%, ceftazidime 30%, cefepime 67%, moxifloxacin 17%, amikacin 44%, tazobactam 50%, imipenem 33%, meropenem 56%, and vancomycin showed 78% sensitivity in patients living in Jhugian. Those patients who are living in Havelian their sensitivity pattern is levofloxacin 82% sensitivity, ciprofloxacin showed 69% sensitivity, piperacillin showed 50% sensitivity, aztreonam showed 64%, cefoperazone 45%, ceftazidime 36%, cefepime For 64%, moxifloxacin 27%, amikacin 32%, tazobactam 45%, imipenem 59%, meropenem 55%, and vancomycin showed 77% sensitivity.

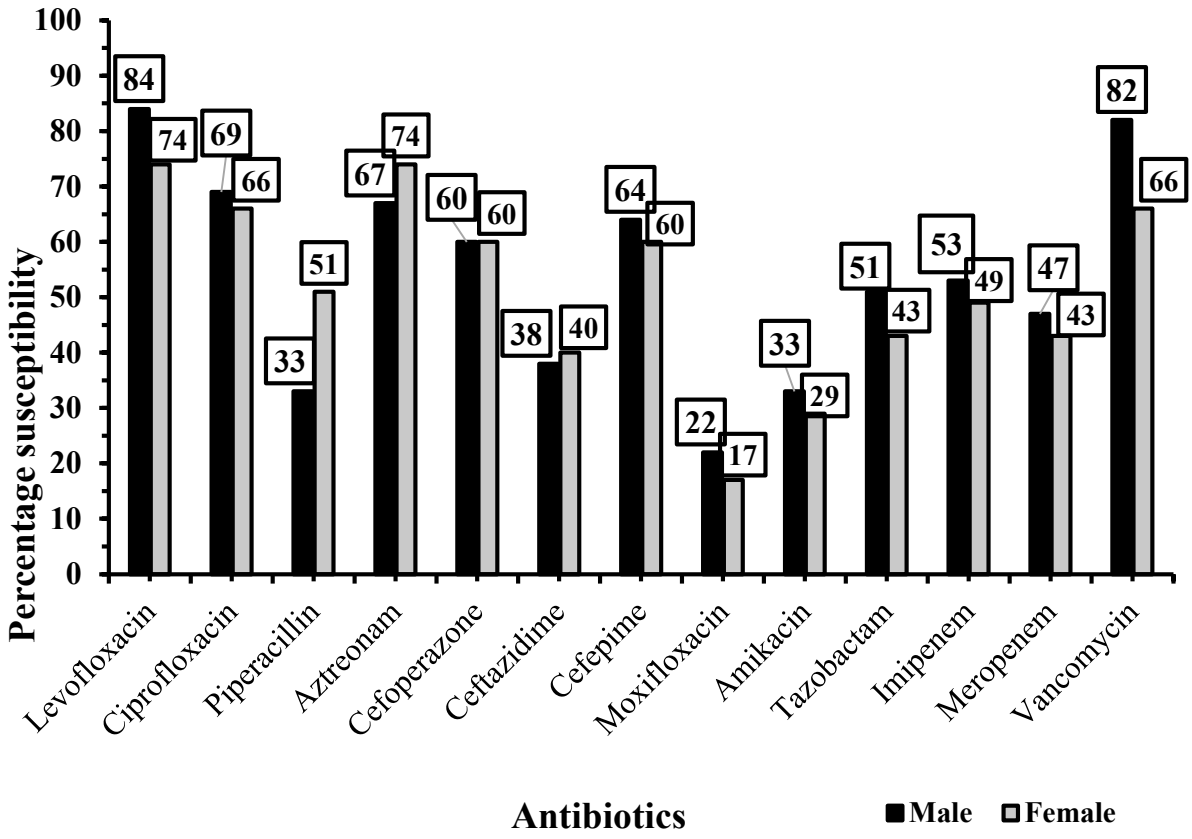

Fig. 3. Susceptibility profile of *P. aeruginosa* in different age group.

4. DISCUSSION

Pseudomonas aeruginosa is the most common infection in hospitals due to its ease of adaptation. The samples were obtained from Ayub Medical Complex (Microbiology Laboratory) in Abbottabad, Pakistan. In this study, 200 urines, wound, and pus samples were isolated from several wards of the hospital, including the OPD ward, the ENT ward, and the emergency ward. In 80 samples, *P. aeruginosa* was isolated. It was most frequently detected in wound samples (51.25%), pus samples (37.9%), and urine samples (27.27%). Previously reported results indicated that more than 70% of *P. aeruginosa* isolates originated from pus, wounds, and tracheal aspirates [16]. Our findings corroborate with [17] and [18], who found that the prevalence of *P. aeruginosa* in urine samples was lowest at 18.7% and 10%, respectively. 35 of the 200 samples came from coronavirus-infected patients. Twelve patients (34%) were co-infected with *P. aeruginosa* from these 35 samples.

Many *P. aeruginosa* infections are challenging to treat because of their drug resistance. The antibiotics levofloxacin, ciprofloxacin, aztreonam, cefoperazone, cefepime, and vancomycin were sensitive to *P. aeruginosa* isolates. Resistance to moxifloxacin, amikacin, ceftazidime, piperacillin, meropenem, tazobactam, and imipenem was identified in decreasing order. Levofloxacin was efficient against *P. aeruginosa* which was multidrug-resistant. *P. aeruginosa* isolates exhibited a 61% resistance to ceftazidime in this study. This result is higher than those reported by Mohamed and Abdelhamid (2020) and Khan and Faiz (2016), who reported resistance rates of 46% and 14%, respectively, while Mahmoud *et al*. (2013) and Pokharel *et al*. (2019) reported resistance rates of 91.2% and 63% respectively [19-22].

The results of 57.9% imipenem resistance opposed the findings of Al-Zaidi (2016), Zahoor *et al*. (2020), and Hasan et al. (2020), who reported 5.5%, 5.5%, and 5% resistance to imipenem, respectively [18, 23]. The findings contradict Feretzakis et al. study, which found resistance to cefepime and levofloxacin to be 56% and 55%, respectively [24].

Ceftazidime and imipenem were the most effective antibiotics in patients with coronavirus illness, with each having an 83% sensitivity, whereas moxifloxacin had a 25% sensitivity. Levofloxacin and vancomycin were the most effective antibiotics in non-Corona patients, with a sensitivity of 82%, while moxifloxacin has a sensitivity of just 19%. Antibiotics are more effective in non-corona patients than in corona patients. This is because of the treatment of infections in corona patients, which has resulted in increased resistance.

In contrast to a study in northern Nigeria [23, 25] and Kirkuk city of Iraq [18] this study indicated a higher prevalence of *P. infections* in male patients than in female patients (56.25 and 43.75%). Levofloxacin was the most effective antibiotic in men, with an 84% sensitivity, followed by vancomycin, with an 82% sensitivity, while ciprofloxacin and aztreonam were the most effective antibiotics in women, with a 74% sensitivity. Moxifloxacin was the most resistant antibiotic, with a sensitivity of 22% and 17%.

According to the findings, the highest frequency of *P. aeruginosa* (53.75 %) was observed in young patients (aged 15 to 35 years), while the lowest incidence was reported in patients aged 35 and older (46.25%). These findings contradict previous research conducted in Ethiopia and Al-Sulaimfania, Iraq by Shewatatek et al., 2014. Their findings indicated a higher prevalence of these bacterial isolates in elderly ill people [26]. On the other hand, our findings were consistent with those of Okon et al. in Nigeria, who reported the highest prevalence (20.7%) in patients aged 29 years [23, 27] and under and Hasan et al., who reported a 45.6% prevalence in young patients (ages 15 to 30 years), while the lowest rate (20.1%) was found in elderly patients aged 45 years and above [18]. This could be explained by the fact that the young-old group is more active and involved in a variety of clinical hygiene activities. Levofloxacin was the most effective antibiotic for adults under the age of 35, whereas vancomycin was the most effective antibiotic for people above the age of 35. Antibiotic resistance, according to our research, increases with age. This could be because older adults use an excessive number of antibiotics.

Ciprofloxacin was the most effective antibiotic in rural areas, with a sensitivity of 76%, followed by aztreonam and vancomycin, both of which have a sensitivity of 73%, whereas levofloxacin was the most effective antibiotic in urban areas, with a sensitivity of 86% and vancomycin having a sensitivity of 77%. European study, nosocomial isolates of *P. aeruginosa* showed 40% resistivity to Amikacin [28]. Amikacin has been used sparingly only in severe forms of the disease owing to high treatment costs and administrated intravenously. Therefore, drug resistance has been slow to emerge in such scenarios [29].

5. CONCLUSIONS

The findings of this research, concluded that antibiotic-resistant bacteria represent a severe public health issue around the globe. The rapid development of resistance by pathogenic bacteria in the environment has led to the rapid development of MDR, XDR, and PDR microorganisms. As a result of the rising prevalence of antibiotic resistance, alternative antimicrobial medications must be investigated as well. During the research, it was noticed that the bacteria *P. aeruginosa* was most sensitive to the antibiotic levofloxacin and the most resistant to the antibiotic moxifloxacin. *P. aeruginosa* is becoming a more dangerous disease due to its increased resistance and capacity to survive in a variety of environments, notably hospitals. Constant monitoring of the development of antimicrobial resistance, the use of suitable antibiotics, the use of combination treatments, and basic measures such as hand washing have all become critical in the management of the organism's growth and spread. *P. aeruginosa* MDR strains would be prevented from developing if the appropriate combination of chemical therapies were used.

6. FUTURE PROSPECTIVE

Antibiotic resistance is a serious problem caused by bacteria adapting to and acquiring resistance to antibiotics, which is a major source of infection. Antibiotic-resistant bacteria are capable of surviving and reproducing in the presence of antibiotics. There is a potential that microorganisms will develop antibiotic resistance with each serving that is consumed. There are many measures to avoid drug-resistant diseases, including immunizations,

proper food preparation, hand washing, and the use of antibiotics only when required. In addition, infection prevention aids in the prevention of the spread of antibiotic-resistant bacteria in the environment. Other measures for lowering antibiotic resistance exist, such as expanding antibiotic-resistant sickness surveillance. Policies and efforts for infection prevention and control should be developed and implemented more effectively. High-quality medications should be used and disposed of in a safe manner, which should be regulated and encouraged. There was evidence of multidrug resistance in several clinical specimens, including pus, wounds, and urine. These findings indicate the necessity for periodic antimicrobial susceptibility testing to monitor the resistance pattern throughout a broad geographic area. This will aid in the preservation of therapeutic efficacy as well as the health of patients. Before administering antibiotics, it is necessary to do a thorough investigation of their effectiveness. Antibiotics should be strictly regulated, and their effectiveness should be closely scrutinized as well. It is not recommended to do an antibiotic susceptibility test until after the antibiotic susceptibility test has been completed.

7. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Research Article

Implementing GRADE-ADOLOPMENT for Clinical Practice Guidelines in Resource-Limited Settings: The AKU Experience

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Abstract: In regions grappling with limited resources to support the formulation of evidence-based Clinical Practice Guidelines (CPGs), GRADE-ADOLOPMENT offers a unique pathway to craft guidelines tailored to the specific needs of areas with sparse CPGs. The Aga Khan University in Karachi, Pakistan, used GRADE-ADOLOPMENT to create guidelines for prevalent diseases in Pakistan. An adaptation of the GRADE-ADOLOPMENT methodology was employed in collaboration with the US GRADE working group. ADOLOPMENT is a combination of de-novo creation, adoption (use as is or with minor changes), or adaptation (modification based on local context) of recommendations. After the selection of a source guideline (SG), the recommendations were either adopted, adapted, or excluded. Adaptations were done using the Evidence-To-Decision table. Contextualized CPGs were developed for Pakistan, covering over 25 medical specialties. The article discusses the wide variety of topics and specialties covered using GRADE-ADOLOPMENT in Pakistan for the first time. The lessons learned from this resource-constrained GRADE-ADOLOPMENT experience provide valuable guidance for teams undertaking projects in similar resource-limited settings. The GRADE-ADOLOPMENT experience at AKU in Pakistan serves as a valuable example, showcasing both the challenges and creative solutions in the context of medical guideline development in resource-limited regions.

Keywords: Adaptation, Clinical Practice Guidelines, De-novo guidelines, Evidence-to-Decision tables, GRADE-ADOLOPMENT, .

1. INTRODUCTION

Clinical practice guidelines (CPGs) play an important role in making healthcare decisions, even though their formation is resource-intensive [1]. The GRADE-ADOLOPMENT methodology offers a cost-effective approach that can provide local CPGs to experts. The process is used to provide a structured assessment of evidence and recommendations that can help healthcare professionals make informed decisions based on the best evidence-based suggestions. It is an extensive approach to guideline development and helps to ensure that recommendations are based on high-quality evidence and are clear about the reliability of those recommendations. This methodology has been proven successful in different regions of the world such as Saudi Arabia [2], Australia [3], the Eastern Mediterranean region [4], the Asia-Pacific region [5], Mexico [6], and the United Kingdom [7], but remains to be underutilized in lower-middle-income countries (LMICs).

Pakistan, an LMIC, has its major share of healthcare funds being utilized for health and access leading to a scarcity of available CPGs catering to local context. GRADE-ADOLOPMENT's transparency and efficiency make it a compelling choice [2]. Since CPGs play a role in patient care and their outcomes, quality-based evidence is essential to validate their recommendation. The development of CPGs is based on three pathways; adoption, creation, and adaptation [6]. Adoption is done when previously recognized CPG is directly included without any modifications or alterations. Creation refers to the de novo creation of a CPG

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with recommendations through an extensive literature review. A PICO (Patient, Intervention, Comparison, and Outcome) question is made and then a systematic review is conducted. After that, an expert panel is involved which ultimately, leads to the creation of a new CPG. Adaptation refers to modifications of already established CPGs to tailor to local context.

While adopting guidelines is an acceptable approach, it is important to keep in mind the limitations associated with this method. The occurrence of different diseases, as well as the ways they are diagnosed and treated, can vary based on regional factors. As a result, CPGs may be influenced by individual physician preferences, which can result in a lack of standardized care. This can lead to a significant number of patients receiving either inadequate or excessive treatment. Hence, our country needs to develop CPGs that are tailored to needs according to local context. This will help ensure consistent and evidence-based healthcare. This paper talks about the Aga Khan University's (AKU) initiative to adolop CPGs for Pakistan by using the GRADE-ADOLOPMENT. We convey our personal experiences and obstacles faced that provide our perspective on developing guidelines in a country with limited resources.

2. METHODS

The adolopment process was initiated within the vicinity of AKU based on GRADE-ADOLOPEMENT. A budget was established for the guideline developing group and was authorized by AKUH's Provost. Finally, the CCBP was created in May 2020. This would help ensure standardized healthcare access in Pakistan which would provide a local perspective. GRADE-ADOLOPMENT is accepted widely for evidence-based CPGs and clinical recommendations. Over 100 organizations have approved this methodology and follow this protocol for quality evidence-based recommendations. In collaboration with the US working group, AKU conducts the process in 8 stages (Table 1).

2.1. Creation of a Center for Clinical Best Practices

AKU introduced CCBP to create a platform for which they can collect the finest clinical practices for Pakistan, with the help of the US GRADE working group as well.

2.2. Compiling a Content List

Section heads and department chairs collaborated with CCBP to compile a content list. In AKU, each section head led their respective specialty or subspecialty. They were given the task of providing a list of diagnoses and diseases that were to be emphasized in the development of CPGs.

2.3. Identification of Source Guidelines

Source guidelines were selected; those that were based on the GRADE methodology were preferred and relevant recommendations were selected. For every CPG on the list, CCBP collaborated with each section head to modify the recommendation(s) according to local relevance. A source guideline is already a pre-existing guideline that is relevant to the topics identified from the content list. These could be national or Society-Based. Original evidence-to-decision tables were made by contacting the committee chair or members who were involved in the CPGs for the source guidelines.

2.4. Table of Recommendations (ToR)

The recommendations were extracted from the source guidelines and were arranged in the form of a table as seen in Table 2. The recommendations were categorized as adopted, adapted, or excluded. Adopted points are the recommendations that are accepted as it is, without any modifications or very minor changes. Adapted points are those points that are modified to tailor local relevance. Excluded recommendations are those that are removed from the final CPGs due to multiple reasons such as lack of availability in Pakistan, if they were not applicable under these settings, or if the recommendations advise advanced and modern technological interventions for a particular purpose that would not be sufficient in the Pakistani population and so on.

2.5. Evidence to Decision (EtD) Table

The adapted recommendations are then further evaluated by the CCBP team to create the Evidence

Table 1. A step-by-step guide for the development of CPGs.

Tasks	Steps
Creation of a Centre for Clinical Best Practices (CCBP)	
Create a centre for clinical CPG development	1. Establishment of Clinical and Translational Research Incubator (CITRIC) 2. Creation of CCBP within CITRIC 3. Formation of a core team comprising a director, manager, and methodologists 4. Collaboration with the US GRADE working group
Compiling a Content List	
Create a 'problem list' to prioritize for CPG creation/adaptation	1. Collaboration with all section heads and department chairs 2. Identification of a list of diagnoses to be prioritized for the CPG manual. 3. Review as well as share the list with the entire section faculty before finalizing
Identification of Source Guidelines	
Identify a source guideline to modify a CPG for local context	1. CCBP collaboration with each section head on one guideline from the list 2. Precise identification of the source guideline(s) 3. Extraction of the original evidence to decision tables via contacting SG's committee chair/members
Creation of Table of Recommendations (ToR)	
Create a table of recommendations for CPG development	1. Selection of all recommendations from each identified source CPG 2. Categorization of each recommendation as adopted, adapted, or excluded. 3. Review by the CCBP team 4. All recommendations marked as "to be adapted" selected for the next stage
Creation of Evidence to Decision Table (EtD)	
Use the GRADEPro software to create the EtD	1. Collaboration between CCBP methodologist and section head to extract all relevant information addressing each domain of the EtD. 2. Inclusion of the 12 domains in the GRADEPro EtD 3. A comprehensive review by the section heads and US GRADE working group. 4. Sharing of finalized tables electronically with the group of experts
Expert panel review	
Review of the EtD by an expert panel to determine the strength of recommendations and finalize EtD	1. Information session with all panelists to comprehensively explain the EtD domains, the questions to be addressed, and the process of utilizing GRADEPro panel voice. 2. Incorporation of all suggestions by expert panels into the EtD 3. A panel meeting to determine the strength of recommendations. 4. A final "consensus" meeting to finalize EtD
Formulation of CPGs	
Formulate the final version of the CPGs for dissemination	1. Formatted CPG document shared with a graphic designer to condense it into a pre-decided template. 2. Approval of the final version by section heads and panel members
Internal and External Boards	
To give their valuable feedback(s) in improving these guidelines	1. These experts were provided with the liberty to assess and modify the guidelines. 2. Changes or suggestions made by the members were considered and incorporated after discussions with the guidelines development team.
9. Dissemination of CPG	
To disseminate the CPGs ensuring their availability and accessibility across Pakistan	1. Dissemination across AKUH health centers all over Pakistan 2. Dissemination at a district and provincial level

Table 2. A template of the table of recommendations (ToR).

S. No.	Recommendations	To Adopt	To Adapt/Modify	To exclude
1				
2				
3				

for Decision (EtD) table to serve as the key tool in the presentation of evidence and corresponding results. These tables were created using GRADEPro software which in turn, summarizes research evidence. For the recommendations that were adapted into the final CPGs, the methodologists in the CCBP department and the respective section heads collected information through local data literature, expert input, and Mini-Systematic Reviews. The EtD tables are carefully evaluated and finalized by the respective section heads.

2.6. Expert Panel Review

An expert panel is established which is led by the section head and consists of a group of experts, who were selected by the section head, in the respective specialty and included AKU faculty and national experts as well from the relevant clinical fields. The GRADEPro Panel voice feature was used to review EtD in real-time and provide necessary feedback. Suggestions and feedback from the panelists were incorporated and then were followed by revision and modification of the final EtD table. The panelists were invited to a final panel consensus meeting to determine the strength of the recommendations. The final decision was based on the comments of all the panel members after which the final EtD was made.

2.7. Formulation of CPGs

After reviewing from the panelists, the recommendations were adapted and then compiled in a formatted CPG document. The final version was then further refined and approved by section heads, department chairs, and panel members.

2.8. Internal and External Boards

The guidelines went through an extensive review process, which involved both internal and external reviewers. The internal reviewers comprised healthcare professionals from AKU itself and external members included healthcare professionals from Pakistan who were affiliated other than AKU. Both these experts were provided with the liberty to assess and modify the guidelines. Any changes or suggestions that were made in the process were carefully considered and incorporated after discussions with the guidelines development group.

2.9. Dissemination

CPGs will be disseminated internally across AKU health centers in Pakistan, and then later followed by dissemination at district and provincial levels. Dissemination planning involves in publication of these CPGs in peer-reviewed journals and amalgamation into AKU's electronic health resource (EHR) for increasing availability.

3. RESULTS

With the collaboration between CCBP and the section heads, several contextualized CPGs for Pakistan were developed. These CPGs covered 25 medical specialties which include the following subjects; Cardiology, Dermatology, Gastroenterology, Internal Medicine, Nephrology, Neurology, Pulmonology, Endocrinology, Rheumatology, Infectious Disease, Breast, Dental, General Surgery, Neurosurgery, Ophthalmology, Orthopedics, ENT, Urology, Vascular Surgery, Gynecology, Emergency Medicine, Family Medicine, Psychiatry, Anesthesia and Palliative Medicine.

4. DISCUSSION

Starting the guideline development by utilizing already existent evidence syntheses, particularly

those that are employed in the guidelines, disqualifies the need to conduct extensive systematic reviews regarding health impacts for many questions. This approach is beneficial as it significantly reduces the resource burden associated with guideline development and also aligns with the vision for better guideline development [8]. One of the few advantages of our practice is that the GRADE-ADOLOPMENT process provides a systemic approach for various healthcare and region-specific settings as it uses already existent evidence-based guidelines while involving local stakeholders to systematically and transparently participate in the modification of the guidelines. The purpose of this methodology is to help organizations and healthcare professionals adapt guidelines and choose what suits their needs best.

The guidelines developed involved a vast area of topics, and with the help of EtDs and tools such as GradePro, the CPGs were modified according to Pakistan's healthcare settings. Policymakers can keep certain factors under consideration such as availability of time, availability of manpower and financial resources, experience in using a specific structure, and the ability to build a group in the framework of interest. EtDs play a role in determining the criteria that may revise the strength or course of a recommendation. By focusing on questions and using EtDs, the process uses an already-existent source guideline and conducts an extensive systematic search for evidence-based recommendations as mentioned in the methodology section. Our searches for patients' values and preferences according to local context as well availability of limited resources and facilities were well received by panelists but provided limited data. The inclusion of local experts and internal and external board members helped modify and identify relevant recommendations for the guidelines. International organizations such as WHO can benefit from this approach as the process can often develop recommendations that may require a local touch and context.

Guideline adaptation gives us an understanding of how different variables such as culture, organization of care, epidemiology, and social values can contribute to evidence-based clinical practice guidelines for clinical, public, and healthcare practices.

5. CONCLUSIONS

AKU developed an adaptation protocol to create evidence-based with regards to local context using the GRADE-ADOLOPMENT methodology. The aim is to ensure the delivery of uniform care to all areas of Pakistan. This methodology gives the impression that this process can be utilized worldwide, especially in resource-limited regions as well as to modify guidelines. Another aim to increase dissemination of the guidelines is by introducing a Guideline Manual Book that every physician can carry with them wherever they go to provide quality patient care. It is also important that CPGs should be reevaluated every few years and adapted whenever needed to keep up with modern medicine and practices.

6. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Research Article

Molecular Epidemiology of Coronavirus Disease and its Effect on the Hematological Profile in District Kech, Balochistan

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Abstract: The coronavirus disease (COVID-19) is a respiratory disease caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). This study aimed to investigate the occurrence of COVID-19 in district Kech, associated risk factors, clinical presentation, and its effect on blood cells. A structured questionnaire was designed to collect the data and the samples were investigated by RT-PCR. In total, 575 suspects were screened, among which 64 (11.16%) had COVID-19 infection, 40.6% were males, and 59.4% were females. An increased positivity of 53.12% was measured in the younger age group (15-30 years) and the lowest (18.75%) was noticed in elders > 50 years. 95.3% of the patients were newly infected, and 7.7% had a contact history with COVID-19-infected patients. The clinical symptoms, such as fever, cough, nausea, vomiting, and diarrhea, were identified in 89.1%, 67.2%, 51.6%, 46.9%, and 12.5%, respectively. The prominent clinical features, like fever, cough, nausea, diarrhea, chest distress, chest pain, and psychological stress were observed. Noticeably, 10.9% of obesity and 7.8% of asthma patients were co-infected with COVID-19 disease including 39.1% of cases showed psychological trauma. Leucocytosis and a decrease in hemoglobin concentration and platelets were determined in COVID-19-infected patients. A significant effect was observed on the hematological profile, however, the effect was severe in the older age group (>50 years). The results suggest that the large population of the study area is infected by SARS-CoV-2, appealing to the need for surveillance on a large scale and the implementation of preventive measures to control further dissemination of the disease.

Keywords: Blood, Coronavirus disease, Epidemiology, Risk factors, SARS-CoV-2

1. INTRODUCTION

The coronavirus disease (COVID-19) was first identified in December 2019 In China, and later on March 12, 2020, declared as a pandemic. The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was involved in causing COVID-19 disease identified in a seafood market in Wuhan, China [1]**.** A quick transmission from human to human was later reported [2] and updated the earlier findings of transmission between animals and humans [3]. Previous research has shown that the SARS-CoV epidemics in 2003 and Middle East Respiratory Syndrome (MERS) coronaviruses in 2015, belonged to the same virus family [4].

By December 2020, WHO [5] announced that most countries were affected by COVID-19 infection, and above 67.5 million confirmed cases and >1.5 million deaths occurred and spread rapidly and hit countries worldwide, including South Asia [6].

The first case in South Asia was reported in Nepal, having a traveling history from China, and the infection was spread to the neighboring countries of Sri Lanka and India, respectively [7]. In Pakistan, till May 10, 2023, the WHO has reported 1,580,631 confirmed cases, among which the mortality rate was 30,656 [6]. The first case in Balochistan was reported on March 10th, 2020, in

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Quetta city and the victim had a travel history from Iran [8].

The rural population is at greater risk of coronavirus disease infection than the urban population, as the people in urban areas are more educated and have basic knowledge about COVID-19 disease and the hygienic conditions are better than those in urban areas. However, combined sources of transport, and not following the proper preventive measures in urban areas are considered primary risk factors in the COVID-19 pandemic [9, 10].

Different studies have determined the epidemiology, clinical features, and transmission patterns of COVID-19 [11]. In general, COVID-19-infected individuals suffer from the symptoms of fever, cough, anosmia, loss of taste, and abdominal pain [12, 1]. In a previous study, it was shown that most of the participants were suffering from anxiety (71.0%) and depression (52.0%) during the COVID-19 pandemic, and about 32.4% of the participants had poor knowledge about the COVID-19 disease [10]. The severity of the clinical features depends on age, gender, and associated infections such as diabetes, obesity, high blood pressure, cardiac disease, and renal disease [13, 14].

Earlier, it was considered that the lungs were the target organs of SARS-CoV-2, but later, it was determined that it can also damage other body organs, such as the intestines, blood vessels, and kidneys [15]. Kidney infection has been seen in several cases, and the lungs were observed as the second most affected organ in COVID-19 infection [16]. Patients with cardiac and metabolic disorders like diabetes mellitus were severely affected by COVID-19 disease, and an increased mortality rate has been observed in diabetic patients [17]. In addition, hematological abnormalities, like leucocytosis, lymphopenia, and thrombocytopenia also reported during COVID-19 infection [18]. The COVID-19 pandemic harmed lives globally and people suffered from various social, physiological, economic, and psychological complications, and psychopathologies [19].

District Kech is situated in the Makran division of Balochistan province which consists of 909,116 individuals according to the 2017 census. To the best of our knowledge, a detailed study of the COVID-19 situation such as epidemiological characteristics, clinical traits, and related risk factors, is not well studied in Kech district. Therefore, we designed this study to evaluate the molecular epidemiology of COVID-19, identify risk factors, demographic characteristics, clinical features, comorbidities, and psychological trauma, and specifically effect on the blood physiology in COVID-19 infected patients.

2. MATERIALS AND METHODS

2.1. Study Design

The study was designed to determine the molecular epidemiology, risk factors, demographic characteristics, clinical features, comorbidities, and psychological trauma of the COVID-19 disease and its effect on the hematological profile of COVID-19-infected patients.

2.2. Study Area

This research was conducted in the areas of Dasht, Ginnah, Sharak, and Tijaban in district Kech. Dasht is located in the west of district Kech, around 50 km away from Turbat city, and has a population of about 65,000 individuals. Ginnah is located about 20 km far from Turbat City and has an estimated population of about 25,000. Sharak and Tijaban are about 60-70 kilometers away from Turbat. Both cities have a population of approximately 15,000 individuals each.

2.3. Study Population

All the participants were willingly allowed to participate in this study. There was no gender or age limitation. Individuals who were facing problems in the sampling, due to surgery, or had allergies from sampling swabs were excluded from the study.

2.4. Questionnaire Design

A pre-structured questionnaire was used to collect the demographic data, clinical features, risk factors, comorbidities, and psychological trauma such as stress, anxiety, and depression.

2.5. Sample Collection and Processing

Ethical approval was obtained from the Ethical Research Committee, University of Turbat. Oral informed consent was taken, and the nasopharyngeal samples were collected by inserting the swab through the nostril and placing it for a few seconds at the posterior wall of the nasopharynx to allow the swab to be well-saturated with the specimen. The samples were placed in a viral transport medium (VTM), and transported immediately to the laboratory for investigation. The blood samples were collected under aseptic conditions and analyzed for a hematological profile at the district headquarters (DHQ) hospital laboratory, Turbat Kech.

2.6. RNA Extraction

RNA was extracted from the SARS-CoV-2 by a nucleic acid extraction kit (Zybio Inc), following the manufacturer's instructions. Briefly, the extraction kit was equilibrated at room temperature for up to 5 minutes. The aluminum film of the 96-well plates was carefully opened, and 15µl Proteinase-K was added to positions A1H1 and A7H7 in order, followed by 200µl sample (B-100: 100µl) in order. The automatic nucleic acid extraction system was turned on, and the extraction process was set according to instructions. The extracted RNA was proceeded for RT-PCR.

2.7. RT-PCR

The COVID-19 RT-PCR kit was equilibrated to room temperature. The components were vortexed to mix properly and prepared according to the instructions of the manufacturer (Sansure Biotech Inc). Briefly, 10µl of the template RNA was added to the ready-to-use master mix to the PCR reaction tube. Two reaction tubes for positive and negative control respectively were included with the test samples, including internal control. A program was set in the RT-PCR system and the equipment was run according to the manufacturer's instructions.

2.8. Haematology Profile

The blood of a COVID-19-positive patient was collected under aseptic conditions, and the effect on the blood physiology was determined by measuring blood parameters with an automatic cell counter according to the instructions of the manufacturer. The blood physiological parameters such as haemoglobin (Hb%), erythrocytes (RBCs), leucocytes (WBCs), and thrombocytes (platelets), and indices such as PCV, MCV, MCH, and MCHC, were analyzed.

3. RESULTS

3.1. Epidemiological and Demographic Features

In total, 575 individuals were screened for COVID-19 infection. Results showed 64 (11.16%) were positive among the total screened individuals for COVID-19 infection. The data showed that the highest positivity rate (53.12%) was realized in the younger age group 15–30 years, followed by the age group 31–50 years, with a positivity rate of 28.12%. Interestingly, the lowest positivity rate (18.75%) was noticed in the age group > 50 years. Among total positive cases, 40.6% were male and 59.6% were female. The highest number of positive patients were from Ginnah (53.1%), followed by Dasht (23.4%), Tijaban (18.8%), and Sharak (4.7%), respectively. 95.3% of patients were newly infected and 7.7% of positive cases had a contact history with COVID-19-infected patients. The results further showed that 82.8% of the COVID-19-positive individuals were unemployed and 67.2% were uneducated. Moreover, 70.4% of individuals were married among the positive, and their socio-economic condition was low on average (Table 1).

3.2. Clinical Features

The common symptoms such as fever and cough were realized in 89.1% and 67.2%, respectively. Nausea and vomiting were observed in 51.6% and 46.9%, respectively. The diarrhea was realized in fewer cases (12.5%), and patients complaining of abdominal pain were 34.4%. The symptoms of chest distress were realized in 48.4%, and chest pain was observed in 43.8%; however, shortness of breath was seen in 28.1%. Other common symptoms like muscle pain, fatigue, runny nose, and sore throat were 56.2%, 78.1%, 25%, and 28.1%, respectively. The less common symptoms of loss of taste and smell were 23.4% and 17.2%, respectively. Noticeably, only 10.9% of the COVID-19 positive patients were involved in smoking (Table 2).

Table 1. Epidemiological and demographic characteristics.

Characteristics	Frequency (%)
Positive	64 (11.16)
Negative	511 (88.84)
Age (Years)	
15-30	34 (53.12)
31-50	18 (28.12)
>50	12 (18.75)
Gender	
Male	26 (40.6)
Female	38 (59.4)
Region	
Ginnah	34 (53.1)
Dasht	15 (23.4)
Tijaban	12 (18.8)
Sharak	3 (4.7)
New infected	59 (95.3)
Contact history	5 (7.7)
Employment status	
Employed	11 (17.2)
Unemployed	53 (82.8)
Education level	
Uneducated	43 (67.2)
Primary	12 (18.8)
Secondary	2 (3.1)
Higher-Secondary	7 (10.9)
Marital status	
Married	45 (70.4)
Unmarried	19 (29.6)
Socio-economic status	
High	3 (4.7)
Moderate	4 (6.2)
Low	57 (89.1)

3.3. Risk Factors Involve

More than half (53.1%) of the infected patients were not vaccinated against SARS-CoV-2. In 59.3% of cases, 3–4 people were living together in close contact. There was no trend of quarantine, and 100% of the patients either didn't isolate themselves or followed social distancing, and 90.6% of the positive individuals were visiting crowded places (Table 3).

Table 2. Clinical features of the COVID-19 positive patients.

Characteristics	Frequency (%)
Fever	
Yes	57 (89.1)
No	7 (10.9)
Cough	
Yes	43 (67.2)
No	21 (32.8)
Nausea	
Yes	33 (51.6)
No	31 (48.4)
Vomiting	
Yes	30 (46.9)
No	34 (53.1)
Diarrhea	
Yes	8 (12.5)
No	56 (87.5)
Abdominal pain	
Yes	22 (34.4)
No	42 (65.6)
Chest distress	
Yes	31 (48.4)
No	33 (51.6)
Chest pain	
Yes	28 (43.8)
No	36 (56.2)
Shortness of breath	
Yes	18 (28.1)
No	46 (71.9)
Muscle pain	
Yes	36 (56.2)
No	28 (43.8)
Bodyache	
Yes	36 (56.2)
No	28 (43.8)
Fatigue	
Yes	50 (78.1)
No	14 (21.9)
Runny nose	
Yes	16 (25.0)
No	48 (75.0)
Sore throat	
Yes	18 (28.1)
No	46 (71.9)

Characteristics	Frequency (%)
Loss of Smell	
Yes	11 (17.2)
No	53 (82.8)
Loss of Taste	
Yes	15 (23.4)
No	49 (76.6)
Smoking	
Yes	7 (10.9)
No	57 (89.1)

Table 3. Risk factors involved in causing COVID-19 infection.

Characteristics	Frequency (%)
Vaccination	
Yes	30 (46.9)
No	34 (53.1)
No individuals/room	
1-2/room	18 (28.2)
3-4/room	38 (59.3)
6-7/room	9 (9.3)
8-9/room	2 (3.2)
Social distancing	
Yes	00
No	64 (100)
Quarantining	
Yes	00
No	64 (100)
Close contact setting	
Yes	54 (84.4)
No	10 (15.6)
Indoor ventilation	
Yes	4 (6.2)
No	60 (93.8)
Use face mask	
Yes	11 (17.2)
No	53 (82.8)
Hand washing	
Yes	13 (20.3)
No	51 (79.7)
Handshaking	
Yes	56 (87.5)
No	8 (12.5)
Visit to crowded places	
Yes	58 (90.6)
No	6 (9.4)

Most (84.4%) of the positive patients were noticed to live in close contact settings and be deficient in indoor ventilation (93.8%). The majority of the COVID-19 patients (82.8%) were not using face masks, frequently (87.5%) involved in handshaking, and not washing 79.7% or desensitizing their hands after handshaking (Table 3).

3.4. Comorbidities

Results showed that (1.6%) of cardiac patients, (3.1%) diabetic, and (1.6%) chronic lung disease had COVID-19 infection. Noticeably, (10.9%)

Table 4. Comorbidities in relation to COVID-19 infection.

Characteristics	Frequency (%)
Cardiac disease	
Yes	1 (1.6)
No	63 (98.4)
Diabetes	
Yes	2 (3.1)
No	62 (96.9)
Obesity	
Yes	7 (10.9)
No	57 (89.1)
Chronic Lung disease	
Yes	1 (1.6)
No	63 (98.4)
Asthma	
Yes	5 (7.8)
No	59 (92.2)

Table 5. Psychological stress.

Characteristics	Frequency (%)
Psychological trauma	
Yes	25 (39.1)
No	39 (60.9)
Stress	
Yes	28 (45.4)
No	35 (54.6)
Anxiety	
Yes	17 (26.6)
No	47 (73.4)
Depression	
Yes	8 (12.5)
No	56 (87.5)

were obese, and (7.8%) of asthma patients had COVID-19 disease (Table 4).

3.5. Psychological Trauma

Analysis showed 12.5% depression, 26.6% anxiety, and 45.4% stress in COVID diseased patients. Overall, psychological trauma was realized in 39.1% of cases (Table 5).

3.6. Haematological Profile

To analyze the effect of COVID-19 infection on haematological parameters, patients were grouped into 15–30 years, 31–50 years, and >50 years. An increase, but within the normal range of leucocyte count was realized in the age group 31–50 years; however, a significant increase was observed in the older age group (>50 years) (Fig 1A). The age group 15–30 years showed 64.4% neutrophils, followed by 68.31% in the age group 31–50 years, and the highest neutrophil count (77.69%) was realized in the age group >50 years. The lymphopenia (18.13%) was realized in the age group above 50 years. The midcells, which consist of monocytes, eosinophils, and basophils, showed no significant effect on COVID-19 infection (Fig 1B).

The results further showed a significant effect of the SARS-CoV-2 infection on haemoglobin concentration. The effect was realized in all three age groups, showing Hb% of 9.4 g/dl, 9.2 g/dl, and 8.02 g/dl in age groups 15–30 years, 31–50 years, and above 50 years, respectively. However, this effect was more prominent in the elderly (>50 years) (Fig. 2A). A significant impact was seen on the red blood cell count ($3.481x10^{12}$/l) specifically in the elder age group (>50 years) (Fig. 2B). However, this effect was non-significant on patients in the age groups of 15–30 years and 31–50 years.

The result showed an impact on the indices of RBCs such as hematocrit or packed cell volume (Fig 2C). The PCV was significantly reduced in all three age groups. However, the effect was more prominent in the age group >50 years. Similarly, reduced MCV was observed in all three age groups in COVID-19-diseased patients. This ultimately led to a reduced value for MCH. while a mild effect on the MCHC levels was realized. Results showed platelet counts of $230.8x10^9$ /l, $170.3x10^9$ /l, and $128.5x10^9$ /l in the age groups of 15–30 years, 31–50 years, and >50 years, respectively (Fig. 2D).

4. DISCUSSION

During the pandemic, several COVID-19 disease waves affected the Makran region. The current research revealed the molecular epidemiology of the COVID-19 infection and identified the risk factors that contributed to facilitating the development of the disease in humans. A higher positivity rate (11.16%) correlates with the associated risk factors, which are linked with COVID-19-infected patients before, during, and after infection, which ultimately leads to the spread of the disease in the community. It is interesting to mention that in this study, female participants were more infected than males,

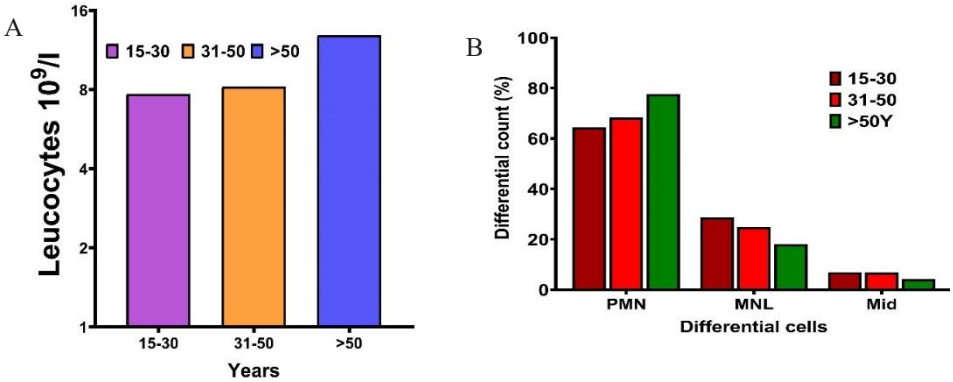

Fig. 1 (A & B). Effect of COVID-19 infection on leucocytes and differential count The blood samples of the COVID-19 positive patients were investigated by a hematology analyzer and the results were analyzed in the Graph Pad, for statistical analysis. (A) shows the effect of COVID-19 infection on leucocytes and (B) shows the effect on the differential count of blood.

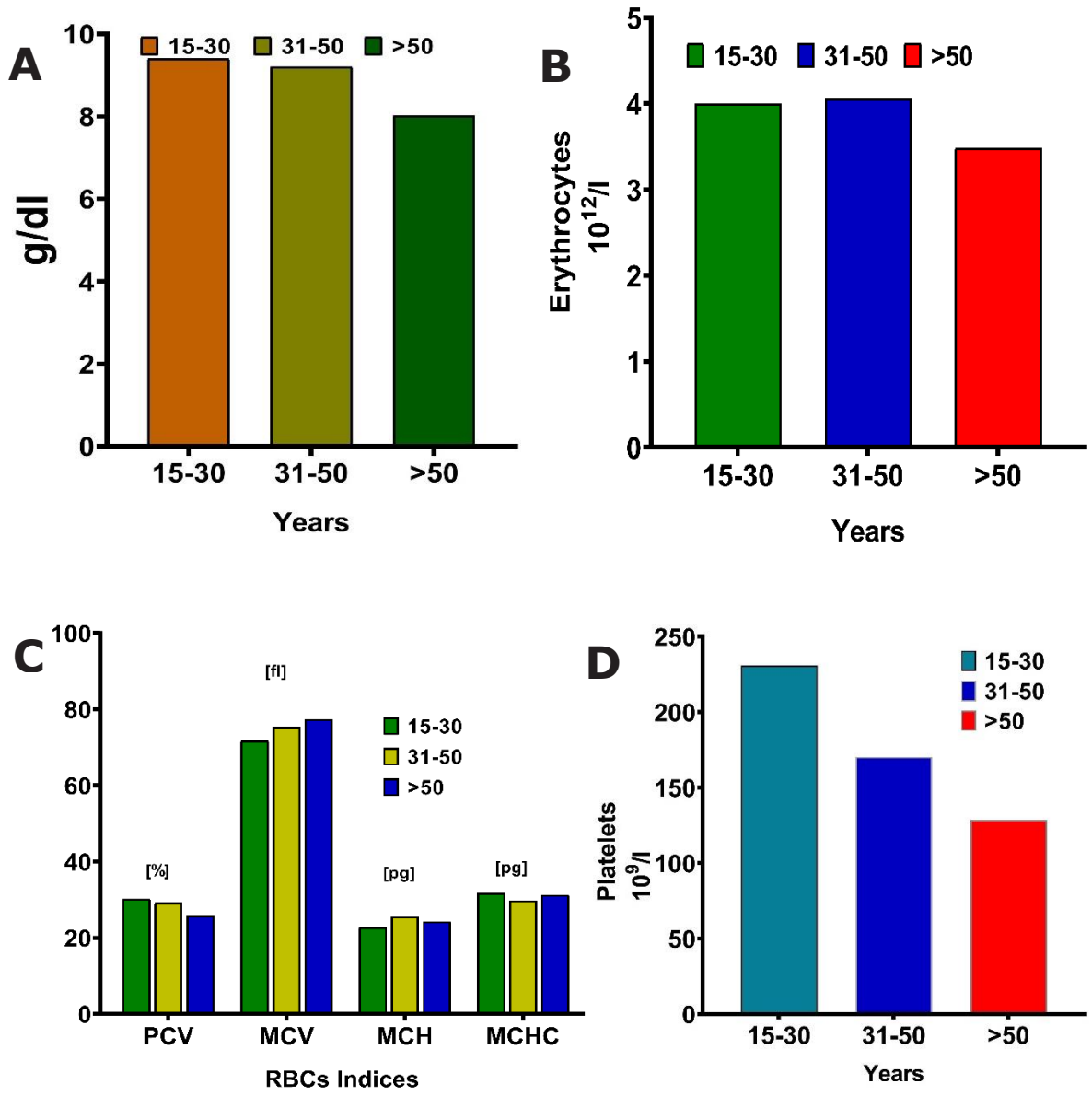

Fig. 2 (A-D). Effect of SARS-CoV-2 infection on hematological profile.
The effect of coronavirus disease was statistically analyzed in the Graph Pad Prism software. The effect of SARS-CoV-2 on Haemoglobin (A), Erythrocytes (B), RBC Indices (C), and Platelets (D) are shown here.

suggesting that females are more vulnerable to infection. This is most probably due to the changes in daily life activities. Because in the Mekran region, the females are also involved in small-scale businesses, they are in contact with the public and get infected by COVID-19 from other individuals. However, the results of this study are in contrast to other studies [20-25], which determined that males are more susceptible to the virus and are more affected by COVID-19 infection than females. It is assumed that due to poor hygienic conditions, nutritional deficiency, and a weak immune system, the females are more vulnerable to the diseases. However, it is determined that gender-specific hormones also play a role in immunity against viral disease [26].

In contrast to previous studies, our study showed a higher prevalence rate of COVID-19 infection in younger individuals as compared to older people. The possible reason for this could be that individuals of this age are in close contact with their educational institutes and classrooms, which puts them at greater risk of getting infections. Additionally, individuals in this age group are more responsible and struggle to earn for their

families, due to which they are more in contact with the community and are at greater risk of getting infection. Another possible assumption is that the prevalent variants of SARS-CoV-2 in the region could be more transmissible to children and infectious.

Ginnah which is located close to Turbat city, is more populated and crowded. The people daily visit Turbat city for work and daily requirements which ultimately communicate and in contact with each other. This close contact with undiagnosed COVID-19 patients may cause or spread the infection, due to which a higher positivity rate was seen in this study. This suggests that the prevalent serotype may be more contagious, infectious, and harmful. This research demands that a study be designed to investigate the available variants of SARS-CoV-2 that are involved in causing COVID-19 disease. This is in agreement with the study of Zhang and Atkinson (2008), who determined that urbanization promotes the transmission of infectious diseases due to increased contact rates [27]. In contrast to Ginnah, the areas of Dasht, Tijaban, and Sharak are far from Turbat City and less populated and scattered, due to which COVID-19 infection was less prevalent. Our study findings are in concordance with previous studies, where an increase in COVID-19 infection was determined in urban areas as described above. Factors such as access to healthcare in urban areas and inadequate surveillance and monitoring in rural areas also affect the data on COVID-19 infection [28].

Overall, data showed that the majority of the positive patients were not following the standard operating procedure (SOPs). An increase in the positivity rate of the COVID-19 infection is related to the non-vaccination against SARS-CoV-2. In addition, an incomplete vaccination is also a possible reason not to protect an individual against the infection. Individuals are more likely to get infections when they smoke or consume alcohol. However, our data determined that about 10% of the positive patients were involved in smoking. Therefore, at some point, smoking could not be considered the only significant factor in inducing COVID-19 infection. Data further showed that the majority of the patients had a history of visiting crowded places before and after infection. Visiting crowded places such as bazaars, marriage ceremonies, and social gatherings was identified as a major risk factor for causing infection.

This study showed that social distancing was not followed due to the cultural trend of social interactions at the workplace and other social occasions that make individuals more susceptible to infection. This correlates with another study [29] carried out previously. While not following quarantine and living in close living conditions and improper indoor ventilation are also considered risk factors involved in causing infection in this study. Another risk factor involved is the trend of joint family systems in the study area. Large families live together within one boundary in a close-contact setting, which places the individuals at risk and causes the spreading of infection among the community. Hand washing or the use of hand sanitizer was not in practice specifically after handshaking**.**

Patients with coinfections such as asthma and chronic obstructive pulmonary disease (COPD) showed critical health conditions and increased risk of hospital admission [30], which suggests that SARS-CoV-2 most likely infects comorbid patients [13]. This correlates with our study, which revealed that patients with chronic lung disease and asthma were prone to COVID-19 infection. Chronic blood sugars result in a weakened immune system, with a possible link to angiotensin-converting enzyme 2 (ACE2) expressions in cardiac tissues [31]. Patients with renal disease and those on dialysis were reported to have increased mortality when infected with the SARS-CoV-2 virus [32]. ACE2 expression in kidney cells is a unique target for SARS-CoV-2, causing tubular and glomerular damage [33].

Fever and cough, which are considered to be the most common symptoms of an infection, were realized in this study. The appearance of these symptoms shows the organ's response to infections [34] and is considered the most common clinical manifestation [35, 1]. The results of this study showed clinical symptoms of vomiting, nausea, and diarrhea. These symptoms have been reported previously in other studies in COVID-19-positive patients [36, 37]. This indicates that cell types in the GIT are potentially susceptible to SARS-CoV-2 infection, and the study indicates that diarrhea is

associated with COVID-19 infection [38].

Moreover, the association between chest distress and shortness of breath with COVID-19 infection is in agreement with previous studies [39, 40]. Additionally, the clinical manifestations of body aches, fatigue, and loss of smell and taste were also realized in the COVID-19-infected patients. Including damage to the human body, stress, and fear seriously disturbing the mental health of the people during the pandemic [14, 15, 41]. The COVID-19 pandemic has shown a negative effect on the economy, social life, and human health globally [42], and among health issues, the mental health of people has been seriously disturbed. Psychological traumas like stress, anxiety, and depression have been realized in this study, which correlates to previous studies carried out in different regions globally [43, 44].

The haematological abnormalities are associated with the severity and type of disease and are used to facilitate the early diagnosis or prognosis and disease severity [45]. Haematological abnormalities in COVID-19-positive patients are a major cause of disease progression, severity, and mortality. Thrombocytopenia, an abnormal coagulation profile, and lymphopenia are associated with disease progression, severity, and risk of mortality [18]. The leucocytosis was realized at an older age compared to a younger age. The increase in leucocytes is according to the previously published study [13], however, leucopenia is also reported in a study [46]. A decrease in lymphocyte count (16.9%) was previously reported [47]. Lymphopenia occurs in viral disease, which is associated with the cytopathic effect [48]. Another explanation of lymphopenia is an increased inflammatory response of the granulocytes and apoptosis of the lymphocytes [49].

Patients infected with COVID-19 infection had reduced haemoglobin concentrations in all three age groups, and this effect was more prominent in the older (>50 years) age. The low haemoglobin concentration is associated with the severity of the disease [50]; however, this was also linked with older age, at which the immune system becomes weakened and the individuals become more prone to infections. Moreover, the reduced count of RBCs due to the effect of coronavirus disease is also studied elsewhere [51]. Analysis showed that the effect of COVID-19 infection on platelets becomes more severe with age, due to weakened immune systems. This ultimately disrupts the blood cells, such as leucocytes, red blood cells, and platelets. It was determined that SARS-CoV-2 has an impact on megakaryocyte maturation. The COVID-19 infection increases platelet aggregation, which leads to platelet consumption in the microcirculation and damages lung tissue. Additionally, the novel coronavirus inhibits erythropoiesis in the bone marrow, which consequently causes thrombocytopenia by reducing platelet production [52]. Previous studies also reported thrombocytopenia in COVID-19-positive patients and considered as biomarkers that facilitate the diagnosis and severity of the disease [15].

5. CONCLUSIONS

The molecular analysis revealed the occurrence of coronavirus disease in the study area. The high-risk factors involved in spreading the SARS-CoV-2 virus include lack of social distancing, visits to crowded places, living in close contact settings, and not using face masks. The common clinical features, such as fever, cough, nausea, and diarrhea, and the less common symptoms, like chest distress, shortness of breath, and psychological stress, were observed. Noticeably, coronavirus disease was mainly associated with obesity and asthma, as well as diabetes and cardiovascular disease, and showed a significant effect on hematological profile. The study suggests that the large population of the study area is infected by SARS-CoV-2, appealing to the need for surveillance on a large scale and the implementation of preventive measures described in this study to control further dissemination of the disease.

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7. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Research Article

Antibiotic Resistance in Pediatric Pneumonia Patients: Assessing Prevalence and Prescribing Practices

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Corresponding author: Abstract: Antimicrobial resistance (AMR) has become a significant worldwide health issue, endangering public health through diminished opportunities for treatments, an increasing incidence of morbidity and mortality, and increasing healthcare costs. This study investigates the prevalence of antibiotic resistance, prescribing patterns and antibiotic responsiveness in pediatric pneumonia with an effort to highlight the urgent need to address this issue. This study assessed the antibiotic prescriptions, hospital longevity and treatment outcomes in 73 pneumonia patients. A total of 506 drugs were prescribed, with an average of 6.9 drugs per prescription, surpassing the recommended WHO standard of 1.6-1.8 drugs. Antibiotics accounted for 38.7% of the prescribed drugs (n=197). Injectable drugs, generic names, and drugs from the Essential Drug List (EDL) comprised 58.5%, 12.84%, and 95% of the prescriptions, respectively. The most common antibiotic combination was ampicillin-cloxacillin, prescribed to 33% of the patients, of which 84.5% of patients did not responded and switched to another drug combination. Ampicillin-cloxacillin-ceftazidime combination was prescribed to 14.6% of patients, however this combination received a non-response rate of 7.1%. Patients unresponsive to these regimens were switched to more efficacious antibiotics such as linezolid, vancomycin, amikacin, ceftriaxone, and cefotaxime. This study highlights the rapid emergence of antibiotic resistance in children with pneumonia, which is associated with their vulnerability to infections due to a compromised immune system. Empirical antibiotic therapy practices, which are frequently utilized in pediatric patients, have led to a rise in resistance to first-line antibiotics (amoxicillin-cloxacillin) used to treat pneumonia. In order to address this critical issue, collaborative efforts from healthcare professionals, policymakers, and the community will be required to raise awareness and promote appropriate antibiotic usage and combat the emergence of AMR in Pakistan.

Keywords: Antimicrobial resistance (AMR), Antibiotic responsiveness, Amoxicillin-cloxacillin, Pediatric pneumonia

1. INTRODUCTION

Pneumonia is a significant cause of mortality among children worldwide, with an estimated 120 million cases in children under the age of five each year, resulting in 1.3 million deaths [1]. It is characterized by lower respiratory tract infection accompanied by fever, respiratory symptoms, and evidence of lung involvement as observed through physical examination or chest radiography [2]. The term "walking pneumonia" is often applied to school-aged children and young people who have radiographic and clinical indications of pneumonia but have minor respiratory symptoms that do not impede with daily activities. *Mycoplasma pneumoniae* is commonly associated with walking pneumonia [3]. Community-acquired pneumonia

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(CAP) and is influenced by factors such as malnutrition, the use of charcoal for cooking, lack of exclusive breastfeeding in the first year of life, and overcrowding. Hospital-acquired pneumonia develops in children within 48 hours of hospital admission and tends to be more severe [4, 5].

On a global scale, pneumonia is one of the main reasons for death in children specifically under the age of five. In 2015, about 700,000 children under five years died from the pneumonia worldwide, with the highest incidence recorded in the African and South-East Asian regions [6]. The World Health Organization (WHO) estimates a clinical pneumonia incidence of 0.37 episodes per kid per year, with India accounting for 36% of the overall WHO South, East Asia regional burden [7]. In Pakistan the incidence is estimated to be 0.26 per child 2 year contributing to 28% of all childhood deaths [8]. Pneumonia risk factors include a lack of exclusive breastfeeding, low birth weight, undernutrition, indoor air pollution, crowded living environments, and a lack of measles vaccination [7]. According to a study conducted in Khyber Pakhtunkhwa, Pakistan, that focused on the prevalence of pneumonia, where 61.8% were children and 38.2% were babies. Male to female ratios were 31.4 and 68.6 percent, respectively. The percentage of economic loss among poor families was significant, at 60%, whereas it was 30% and 10% for middle and high economic status families, respectively. The percentages of rural and urban occurrences were around 51 and 49 percent, respectively [9].

Antibiotics have revolutionized medicine since Alexander Fleming's discovery of penicillin in the late 1920s, reducing pain and suffering, saving millions of lives each year, and even being used prophylactically to prevent infectious diseases. However, we are currently facing a situation where many medications are ineffective against even the less severe diseases [10]. The initial therapy for pneumonia should be effective against *Streptococcus pneumoniae* (the commonest cause of pneumonia) and *Streptococcus aureus*. High dose penicillin and ampicillin, amoxicillin-clavulanic acid, or a 2nd and 3rd generation cephalosporin (ceftriaxone and cefuroxime) are mostly used intravenously. However in areas where there is high prevalence of Methicillin resistance *S aureus*, it is advisable to add vancomycin along with first line agent [11]. The duration of intravenous antibiotic therapy is a topic of debate, but oral antibiotic therapy should be initiated as soon as possible [12].

The rising antimicrobial resistance (AMR) is considered one of the most threatening challenges to global public health, increasing morbidity, mortality, and costs, as well as limiting the choice of antimicrobials for potential treatment [13]. In response, global health organizations, including WHO, have introduced a Global Action Plan (GAP) in 2015 in response to complaints about AMR and its effects in 2016 assembly strengthening the GAP [14]. All nations supported GAP, including Pakistan, which is currently the sixth-most populated nation in the world and is projected to overtake fourth rank by 2050. AMR increases medical costs, prolong hospital stays, and elevates mortality. The world must quickly modify the way antibiotics are given and utilized [15]. Addressing AMR is crucial to prevent a post-antibiotic era marked by increased medical costs, prolonged hospital stays, and elevated mortality. Considering the growing threat of AMR in pediatric pneumonia, the current study aims to document the prescribing patterns of antibiotics and assess the prevalence of AMR and antibiotics resistance in pediatric pneumonia patients in a tertiary care hospital in Mardan, Khyber Pakhtunkhwa, Pakistan.

2. METHODOLOGY

2.1. Data Collection

The study included a total of 73 patients, and their case histories were collected with the official permission of the chief pharmacist and ward in-charge. The data collector received proper education and ethical guidance from the university prior to data collection. The data collection process was based on consent among physicians, nurses, and the patients themselves.

2.2. Study Setting and Design

This study was conducted as a retrospective study at a tertiary care teaching hospital in Mardan, Pakistan. The study specifically focused on the hospital's pediatric ward, which serves as a referral center for patients from different regions such as

Mardan, Dargai, Skhakot and Swabi.

2.3. Excluding and Including Criteria

Specific criteria were used to determine which patients were included or excluded from the study. Patients taking single medications, hospitalized for less than 1 day, had inadequate medical records, and were bedridden were excluded. However, the study also included patients who had already been discharged from the hospital and had complete medical records.

2.4. WHO Core Indicators

WHO has set the standard values for drugs per encounter which are known as core indicators. The core indicators dictate that how many drugs should be there in prescription, number of injectable, number of antibiotics, drugs from essential drug list and drugs prescribed by their generic name to ensure appropriate and rational use of medicines [16, 17]. WHO has also developed essential drug list to ensure the availability of drugs to all for safe and cost effective use [18]. Every WHO core indicator, including the total number of items per prescription, the proportion of drugs with generic names, the average number of antibiotics prescribed per prescription, the proportion of injectable prescribed per prescription, and the EDL of 2018, were carefully examined for each prescription. These measures were developed for any healthcare facility based on an analysis of clinical sessions with patients. A patient interaction is generally understood to be "the time spent interacting with the healthcare provider [19], and "Taking a patient's history, making a diagnosis, choosing between pharmaceutical and non-pharmacological therapy, and prescribing (and sometimes distributing) medication are all components of the ideal consultation, monitoring the patient, informing them about the treatment's side effects, and preventing future issues. Drug Regulatory of Pakistan (DRAP), a division of the Ministry of Health Services of Pakistan, has determined which drugs from the 2018 list of essential drugs are necessary [20].

2.5. Hospital Stay and Antibiotic Responsiveness

The study also considered the length of hospital stays and changes in antibiotic regimens among in-patients as indicators of antibiotic responsiveness. Hospital longevity and antibiotics switching indicates the declination of antibiotic responsiveness. So, along with CSTs, which is not mostly followed in pediatrics, especially in pneumonia infections, hospital longevity was followed for assessing antibiotics resistance. Switching-over from one class of antibiotics to another assist us in prevalence of AMR among pediatrics pneumonia. Date of admission, date of discharge, and antibiotic therapies schedule were regularly followed for antimicrobial therapy and associated resistance. CSTs report were not found throughout the study, as it is expensive process and take almost 48-72 hours Pneumonia in children is fatal, Prescribers can't wait for CST reports that's why empirical treatment was practiced.

2.6. Assessment of Antibiotic Resistance from Patient's Hospital Stay

Pneumonia hospitalized patients receive different types of antibiotics during their stay at the hospital [21]. After starting an antibiotic regimen, some patients indicate responsiveness to the antibiotics resulting in early discharge of patients from the hospital within 2-5 days [22]. However, some patients tend to show no responsiveness to that specific antibiotic regimen with results in antibiotic switching-over to another agents or class of antibiotics [23]. This switching-over increases the hospital stays and longevity of pneumonia patients [24]. This can be considered as an important factor for assessing the antibiotic resistance within pneumonia patients receiving therapy.

2.7. Data Analysis

Data analysis was performed using Microsoft Word while Graphpad prism (version 8.0.2) was used for tabulation and graphical presentation of the data. Pharma guide and pharmapedia were utilized as references for generic drug names.

3. RESULTS

3.1. Gender Wise Distribution of Patients

In this study, a total of n=73 prescriptions were collected and evaluated. The percentage of male

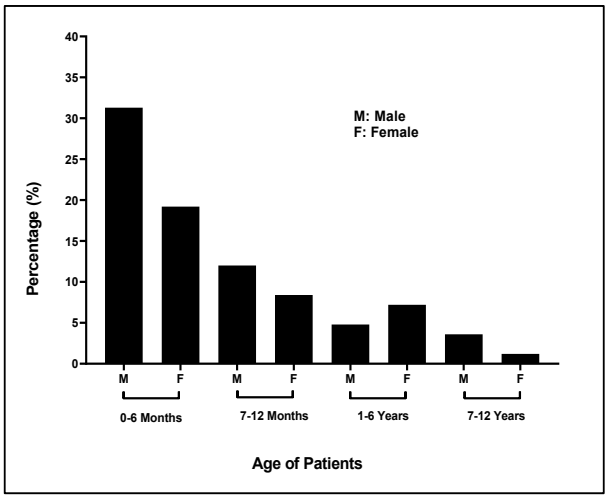

Figure 1. Gender and Age-wise distribution of patients.

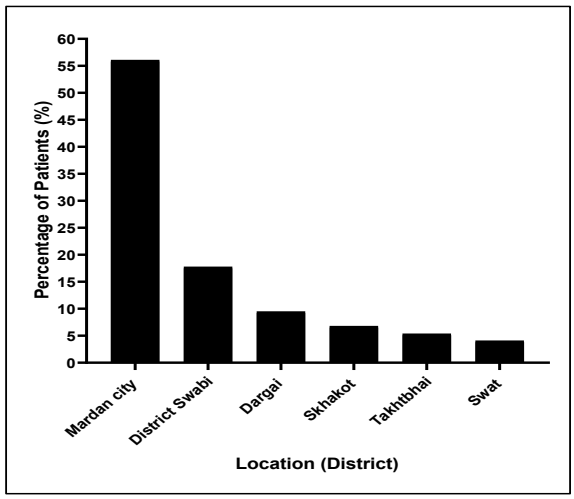

Figure 2. Geographical distribution of patients participated in the study.

patients was 58.9% (n=43) while the percentage of female patients was 41.1% (n=30).

3.2. Age Wise Distribution of Patients

Age-wise distribution showed 1 month and 12 years as minimum and maximum age of the admitted children's patients. The age groups were categorized in 04 groups that included 1-6 months (n=42, 57.5%), 7-12months (n=17, 23.2%,), 1-6 years (n=10, 13.7%) and 7-12years (n=4, 5.4%) years respectively. It shows that the prevalence of pneumonia is very common at age of 1 to 6 months as maximum n=42 patients among the 73 patients. The attack is less common at age 7 to 12 years as minimum n=4 patients reported among the total cases. Gender and aged based distribution of patients is shown in (Figure 1).

3.3. Geographical Distribution

The patients admitted belonged to six (06) different locations that included n=41 patients from Mardan city with a percentage of 56.1%, from district Swabi n=13 with a percentage of 17.8%, from Dargai n=7 with a percentage of 9.5%, from Skhakot n=5 with a percentage of 6.8%, from Takhtbhai n=4 with a percentage of 5.4%, the number of patients from Swat n=3 with a percentage of 4.1%, respectively (Figure 2).

3.4. WHO Core Indicators

In the current study, a total of n=73 prescriptions were evaluated, encompassing a total of n=506 drugs. The evaluation of drug prescribing practices was conducted using the WHO core indicators, as presented in (Table 1). The observed indicators were compared to the WHO proposed and standard core indicators to assess the appropriateness of prescribing practices.

3.5. Frequency and Percentage of the Prescri bed Antibiotics

In this study, a total of 197 antibiotics were prescribed with an average of 2.7 or 3 antibiotics per prescription. The most frequently prescribed antibiotics were ampicillin-cloxacillin, cefotaxime, ceftazidime, ceftriaxone, linezolid, vancomycin, amikacin, and clarithromycin as shown in (Figure 3). These antibiotics were commonly used in the treatment of pneumonia among the pediatric patients included in the study. The prescription patterns indicate the preferences of healthcare providers in selecting antibiotics for managing pneumonia cases in this particular setting. The most prescribed antibiotic ampicillin-cloxacillin accounted for n=65 (33%), cefotaxime n=46 (23.3%), linezolid n=32 (16.2%), vancomycin n=23(11.6%), ceftazidime n=17 (8.62%), ceftriaxone n=9 (4.45%), amikacin, meropenem and clarithromycin accounting for n=6 (3%) each having 1%, respectively.

3.6. Hospital Stay and Longevity

The average hospital stay of male and female patients was 3.79 and 3.2 days, respectively. Average hospital stay of male patients was found to be higher than the average hospital stays of female

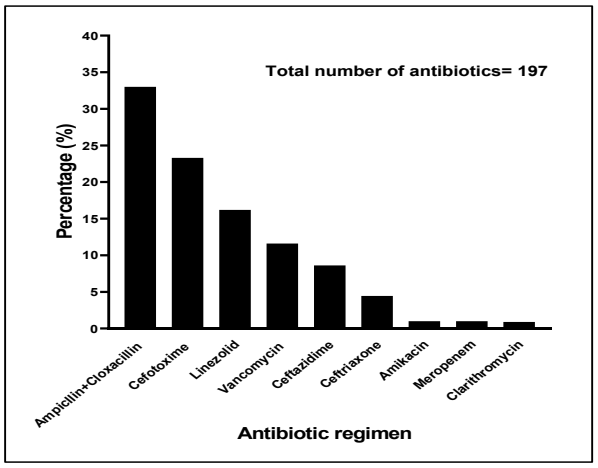

Figure 3. Percentage of the prescribed antibiotics.

patients (Figure 4).

3.7. Observed Antibiotics Resistance during the Study

In this study, the entire medical records of 73 patients were assessed to identify possible antibiotic resistance. The patients' antibiotic therapy was analyzed based on their hospital stay duration. Antibiotic sensitivity was thought to exist in patients who received their discharge quickly after the antibiotics regimen without switching-over to other class of antibiotics. Antibiotics switched-over is a sign of non-responsiveness and possibly prevalence of AMR.

A total of n=65 (79.2%) patients received the ampicillin-cloxacillin combination. However, out of 65 patients, n=55 (84.6%) of patients doesn't responded to ampicillin-cloxacillin combination and the antibiotic regimen was changed to linezolid, vancomycin, or 3rd generation cephalosporins including ceftazidime, ceftriaxone, and cefotaxime. Furthermore, some patients were found to have shown responsiveness to combination therapy having ampicillin-cloxacillin in combination with ceftazidime, ceftriaxone, and cefotaxime.

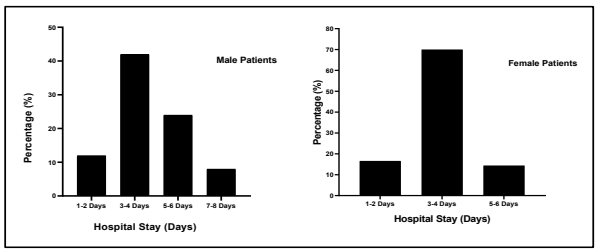

Figure 4. Hospital stay and longevity (Left: Male Patients, Right: Female Patients).

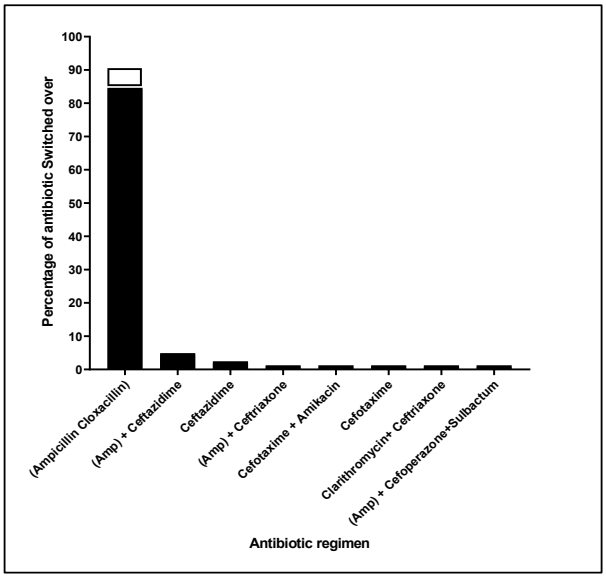

Figure 5. Antibiotic switching-over observed during the study.

Moreover, combination therapy resulted in patient discharge without further switching over. In conclusion, these results suggest that ampicillin-cloxacillin combination have lost its efficacy due to the onset of AMR strains in pediatric pneumonia patients. The antibiotic switching-over observed during the study is presented in (Figure 5). (Figure 6a and 6b) presents the overall consumption of antibiotics by individual patients.

4. DISCUSSION

The current research was carried out at Pediatric wards of a tertiary care hospital at District Mardan, Khyber Pakhtunkhwa, Pakistan. In this study, the percentage of male patients was 58.9% (n=43) while the percentage of female patients was 41.1% (n=30). Age-wise distribution showed 1 month and 12 years as minimum and maximum age of the admitted patients. The age groups were characterized into 04 groups that included infants age: 1-6 months (n=42, 57.5%), neonates age: 7-12months (n=17, 23.2%,), children age: 1-6 years (n=10, 13.7%) and grade-schooler kids: 7-12years of age (n=4, 5.4%) years respectively. The prevalence of pneumonia was found to be more common at age of 1 to 6 months as maximum n=42 patients among the 73 patients. The prevalence of pneumonia was found to be less common at the age of 7 to 12 years as minimum n=4 patients were reported among the total cases.

During evaluation prescription trends were

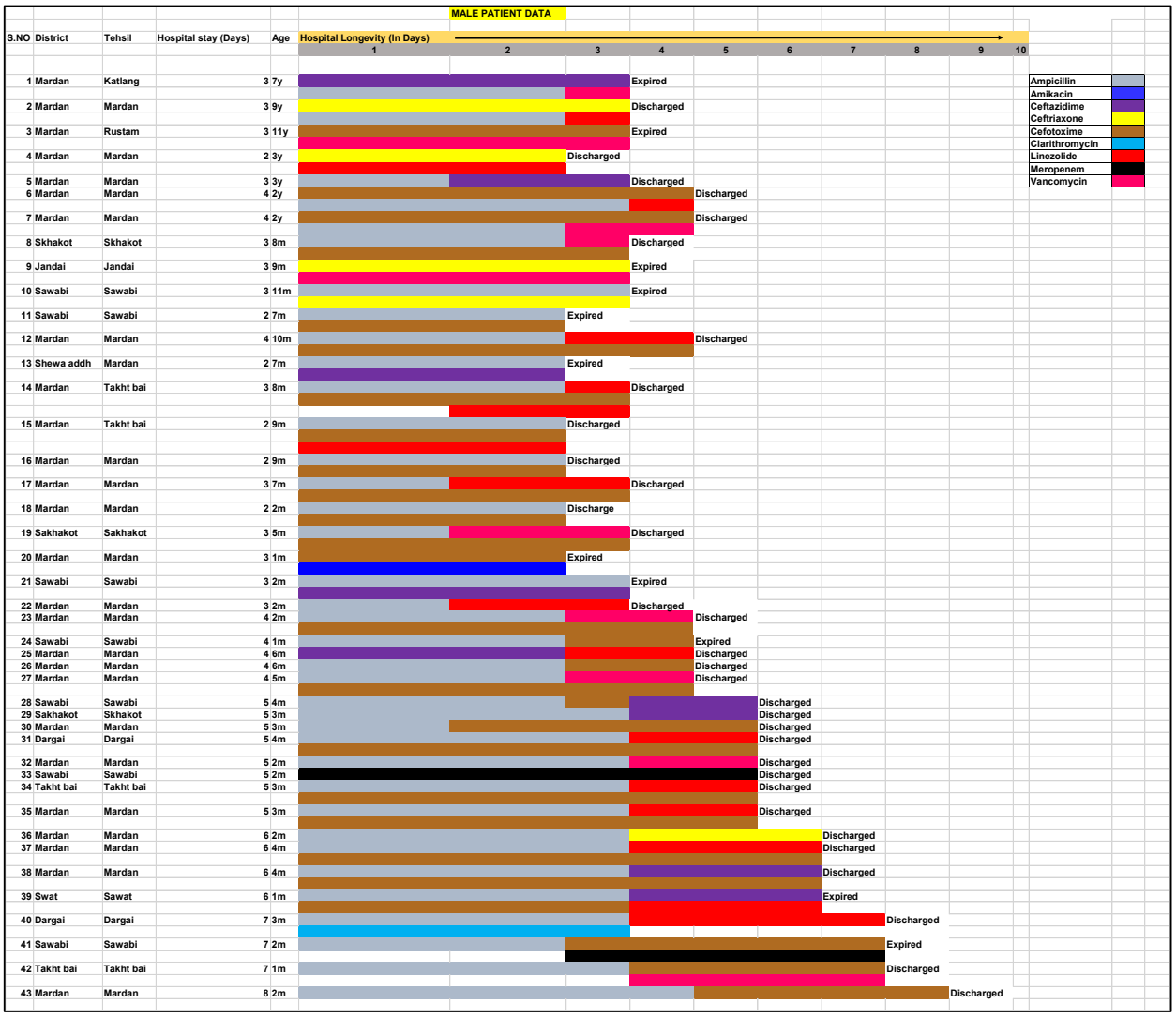

Figure 6a. Antibiotic consumption chart (Male Patients).
Note: The colored bar lines denote antibiotic regimen, while termination/change of colour denotes that antibiotic is switched-over to other antibiotic agent/class as a result of non-responsiveness as evident from patient condition during the study.

taken into consideration in a particular scenario and assessed multiple prescription drug-related factors. The average number of drugs prescribed per encounter was calculated as 6.9, which is higher than the value recommended by WHO (1.6-1.8). A similar nature study was conducted in 2 private and 2 government hospitals in Pakistan, the average number of drugs prescribed per encounter was 4.7 [25]. Comparing our findings with other studies conducted in Pakistan, another study carried out in Bahawalpur Pakistan, the average number of prescribed drugs per prescription was 3.4 [26]. In contrast to our findings, the average number of drugs prescribed per encounter was lower in Ethiopia where an average of 2.1 drugs were prescribed per encounter while in Nepal an average of 3.2 drugs were prescribed per encounter [27, 28].

The WHO (World Health Organization) has mentioned that the average number of drugs prescribed per encounter must be in the range of 1.6-1.8. Polypharmacy, defined regular use of at least 05 medications. During the study, we observed polypharmacy in the pediatric ward [29]. There are many reasons which causing polypharmacy including lack of proper medical knowledge, improper training, poor diagnosis, patient non-compliance, improper counselling and the shortage of therapeutically correct drugs. Polypharmacy leads to poor therapeutic response and many

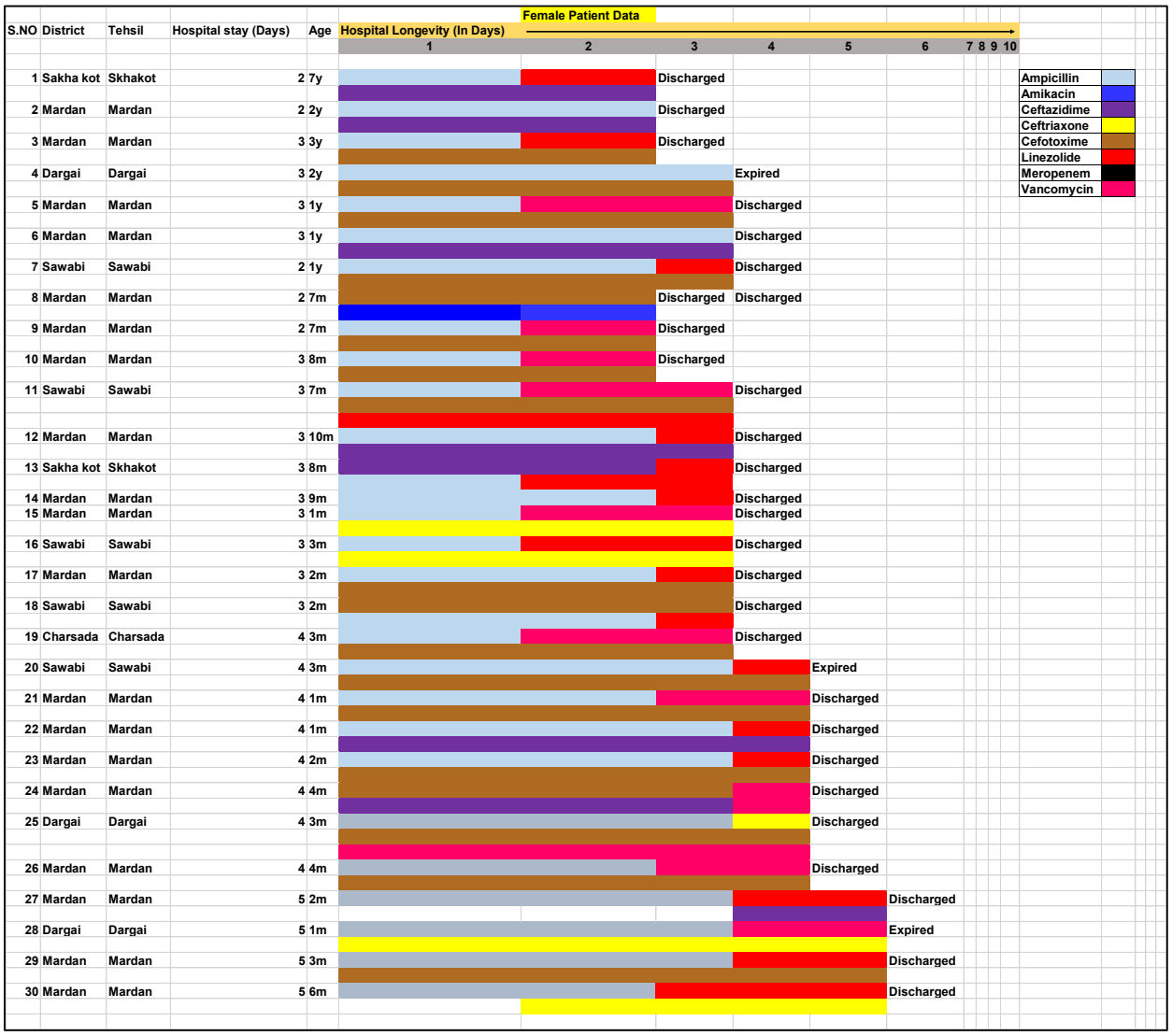

Figure 6b. Antibiotic consumption chart (Female Patients).
Note: The colored bar lines denote antibiotic regimen, while termination/change of colour denotes that antibiotic is switched-over to other antibiotic agent/class as a result of non-responsiveness as evident from patient condition during the study.

chances of drug-drug interactions [30]. According to WHO, 100% of drugs should be prescribed by generic names. In our study 12.84 % of drugs were prescribed by generic name. A study was conducted in Kenya, the prescription of drugs by generic name was 27.7% which very much high than our findings. Another study was conducted in the teaching hospitals of Punjab, 39.5% of drugs were prescribed by generic names [31, 32]. In contrast, a study conducted in Bangladesh reported that no drugs were prescribed using generic names [33].

Most pharmaceutical businesses in Pakistan employ sales personnel who pressure doctors to recommend their product in exchange for rewards, gifts, samples, and other inducements. Pharmaceutical representatives have a considerable impact on prescribing practices and are prejudiced in favor of name-brand drugs, which fosters an unfavorable perception of generic drugs. The benefit of prescribing medications by their generic names is that it is more affordable and patient access is made easier because they are not needed to search for a particular drug with a brand name [28]. According to WHO all drugs should be prescribed from EDL (Essential Drug List). Each country has developed its own NEDL (National Essential Drug List) based on the availability drugs and its cost understand the supervision on WHO. In our study total 506 drugs were prescribed in 82 prescriptions. 482 (95%)

drugs out of 506 were from EDL (essential drug list). Another study conducted in Tertiary care hospital Punjab, Pakistan the percentage of drugs prescribed from National Essential Drug List 2008 Pakistan was 70.37 %. In Nepal, 21.3% of the medications on the essential drug list were prescribed while in Ethiopia 83.0% of the drugs were prescribed from essential drug list [27, 28].

The lack of awareness about necessary medications or the pharmaceutical company's marketing of newer compounds might be the cause for not prescribing from the essential drug list. Prescriptions for pharmaceuticals from the essential drug list prevent adverse drug reactions and drug reactions, and they also increase the patient's financial and therapeutic benefit since essential medicines are safe, high-quality, and cost-effective [28]. The percentage of antibiotics prescribed in this study was 39.32%, which is significantly higher than the recommended value of 20-20.6% by the WHO. This high rate of antibiotic prescription raises concerns about antibiotic overuse and the associated risk of recurrent hospitalization. A study conducted in Tanzania reported that 51.9% of prescriptions included antibiotics, while in the Amhara regional state of Ethiopia, the percentage was even higher at 69.6% [34]. Due to the lack of culture and sensitivity tests (CSTs) in this study, clinicians had to treat patients on an empirical basis and give them broad-spectrum antibiotics, which ultimate results in antibiotic resistance.

The most frequently prescribed antibiotics were ampicillin-cloxacillin, cefotaxime, ceftazidime, ceftriaxone, linezolid, vancomycin, amikacin, and clarithromycin as shown in Figure 3. These antibiotics were commonly used in the treatment of pneumonia among the pediatric patients included in the study. The prescription patterns indicate the preferences of healthcare providers in selecting antibiotics for managing pneumonia cases in this particular setting. The most prescribed antibiotic ampicillin-cloxacillin accounted for n=65 (33%), cefotaxime n=46 (23.3%), linezolid n=32 (16.2%), vancomycin n=23(11.6%), ceftazidime n=17 (8.62%), ceftriaxone n=9 (4.45%), amikacin, meropenem and clarithromycin accounting for n=6 (3%) each having 1%, respectively. During this study, out of 65 patients, n=55 (84.6%) patients were found non-responsive to the first line of therapy ampicillin-cloxacillin combination for pneumonia, as a result the antibiotic regimen was switched-over to a more powerful antibiotic regimen such as linezolid, vancomycin or 3rd generation cephalosporins. In addition, resistance developed to the ampicillin-cloxacillin-ceftazidime combination administered to 14.6% of the patients, and 7.1% of the patients did not responded. Furthermore, some patients were found to have shown responsiveness to combination therapy having ampicillin-cloxacillin in combination with ceftazidime, ceftriaxone, and cefotaxime. Unsuitable antibiotic medication has been linked in the past to poor patient outcomes and a rise in AMR, according to research. According to Sano et al., improper early antibiotic therapy is substantially linked to emergence of AMR. Furthermore, when the detected bacteria are susceptible to narrow-spectrum antibiotics, usage of broad-spectrum antibiotics may increase mortality. Thus, it is critical to identify people at risk of AMR at the time of diagnosis [35]. A significant issue with hospital-acquired infections is biofilm-related multi-drug resistance, which raises patient morbidity and death rates as well as financial expenditures from high medical expenses and extended hospital stays [36].

Lastly, the current study highlights the urgent need for antibiotic stewardship programs in pediatric settings to ensure the rational use of antibiotics. In order to prevent the emergence of AMR, healthcare providers should receive education on the proper selection and administration of antibiotics. Furthermore, there is a strong need for policy making and implementation of guidelines regarding the empirical therapy in pediatric pneumonia which will reduce the onset of AMR. Additionally, there is a need for public education and proper surveillance to monitor the onset of resistant strains in the region and community.

As this study was confined to a single tertiary care hospital in District Mardan, Khyber Pakhtunkhwa, Pakistan, there remains a room for additional research studies on the mentioned topic to assess the prevalence of AMR in pediatric pneumonia patients on a national and global scale. Longitudinal studies are needed to evaluate the long-term impact of antibiotic therapy on pediatric patients with pneumonia, including the development of antibiotic resistance, recurrence of

infections, and clinical outcomes. Future research should explore non-antibiotic interventions, such as vaccination strategies, respiratory hygiene measures, and supportive therapies, to reduce the burden of pneumonia and minimize the reliance on antibiotics.

5. CONCLUSIONS

Antibiotics are slowly and gradually losing their efficiency due to their unintentional use in children for minor infections. During the current study, we came across a significant proportion of pediatric pneumonia drug-resistant cases. In particular, frequently prescribed therapy consisting of ampicillin-cloxacillin, 84.6% of patients doesn't responded to this combination while ampicillin-cloxacillin and cefotaxime (57.3% of cases) was unsuccessful in 26.82% of patients, necessitating the administration of different antibiotics. In addition, resistance developed to the ampicillin, cloxacillin, and ceftazidime combination administered to 14.6% of the patients, and 4.9% of the patients did not respond.

In conclusion, the current study showed an alarming increase in antibiotic resistance to the first line therapy (ampicillin-cloxacillin) in combination with 3rd generation Cephalosporins namely; cefotaxime and ceftazidime for pneumonia among children. Despite the fact that this study only focused on one hospital, it nonetheless highlights the urgent need for further investigation in order to completely comprehend the scope of this issue as well as develop effective AMR management strategies. In order to address this serious issue, collaborative efforts from healthcare professionals, policymakers, and the community will be required to raise awareness and promote appropriate antibiotic usage and combat the emergence of AMR.

6. ACKNOWLEDGEMENTS

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7. CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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Research Article

Antimicrobial Resistance, Pathogen Transmission and Cross-Infections across Regions and Borders

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Abstract: Antimicrobial resistance is now considered a global dilemma and threat to many antibiotics running in clinical practices. In Pakistan, its worst consequences appear and rise day by day, especially in hospital and community-acquired healthcare settings. In Khyber Pakhtunkhwa (KP) especially in Peshawar, most of the Tertiary Care Hospitals, have a big flow of in and outpatients throughout the country as well across the border from Afghanistan, Iran, and Middle Eastern countries. So, the opportunities for cross infections, and transmission of Multidrug resistance/superbug strains are higher up, which needs to be assisted and evaluated. The current study was a combined prospective and retrospective nature study carried out in the endocrinology ward, in a tertiary care hospital in, Peshawar, KP, Pakistan. To ensure a thorough evaluation of treatment outcomes and the validity of the findings, the study attempted a combination of prospective monitoring and retrospective analysis to assess AMR, CSTs, and hospital stay longevity among 97 DFU inpatients. DFU was most prominent in the age range of 51-60 years, followed by 41-50 and patients above 60 years of age, respectively. Various antibiotics were prescribed to DFU patients. Among antibiotics, cefoperazone-Salbactam combination was highly prescribed (31.8%) followed by Linezolid (18.8%), and Ciprofloxacin and Meropenem (13.6% each). Patients having an age limit of 10-40 years were mostly resistant to antibiotic regimens including Ampicillin-clavulanic acid, cefipime, and cefoperazone-sulbactam. Similarly, patients in the age limit 41-50 were resistant to cefotaxime-sodium and ceftazidime. Furthermore, patients in the ages ranging 51-60 were resistant to co-trimaxazole, levofloxacin and moxifloxacin. In contrast, patients above the range of 61 years were found to be resistant to Ampicillin-clavulanic acid, cefipime, cefotaxime, ceftriaxone, co-trimaxazole, and levofloxacin. In conclusion, there is a strong need for comprehensive studies considering pathogen transmission, and cross-border infections in Pakistan to prevent the growing issue of AMR.

Keywords: Antimicrobial Resistance, Beyond Borders, Cross-Infections, Multidrug resistance, Superbugs.

1. INTRODUCTION

Pakistan has shared international borders with Afghanistan, Iran, China, Tajikistan and India. Diversities in regional, socio-cultural, and religious attributes and shared various norms, traditions, relations, business exchanges, health concerns, sports, education, import and exports across borders and gate-passes. Afghanistan shares a 2670 Km, shared border with Pakistan, they have the highest exchanges of human across-boarders (Chaman border, Baluchistan and Torkham border, Khyber Pakhtunkhwa (KP), as compared to other neighbouring countries. Most of the Afghan

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people come to Pakistan for business, jobs, sports, and health-treatment concerns. They are seeking treatment, especially in the public and private sector hospitals like Hayatabad Medical Complex (HMC), Khyber Teaching Hospital (KTH), Lady Reading Hospital (LRH), North-West General Hospital and Research Center (NWGH-RC), Rahman Medical Institute (RMI) and many more in private sectors. Each day thousands of Afghan people come to Peshawar for various ailments treatment. So, there may be chances that they may bring or go back with MDR pathogens. (Figure 1), depicts the human traffic and mobility across borders for various purposes, but the most associated are health concerns. The spread of MDR has increased due to human traffic and mobility in cross-border regions throughout the globe, which may be considered to increase AMR, especially in underdeveloped countries, where there are no strategies to overcome excessive misuse of antibiotics. So, regional infection prevalences are one of the key factors in AMR/MDR prevalences, which are mostly ignored in Asian countries like Afghanistan, Iran, Pakistan, India, Nepal, Sri Lanka and most of the Arabic countries.

Diabetes mellitus (DM) is a global dilemma, which are highly prevailed in Asian countries. The complications associated with DM are life-threatening and lead to permanent organ damage and disabilities [1]. Uncontrolled Diabetes mellitus may extend into various complications, among which one of the common complications is diabetic foot or diabetic foot ulcer (DFU) [2]. It may be due to poor glycemic control, vascular diseases, neuropathies and poor foot care by diabetic patients. In 15% of diabetic patients, a sore or wound at the bottom of the foot may commonly occur [3].

Global prevalence of DFU among diabetic patients is about 6.3%, whereas only in Asia, where its prevalence is about 5.5%. The highest number of DFU patients was reported in Belgium at 16.6%, where the least accounted for in Australia which is about 1.5%. The highest number of DFU patients was reported in Belgium at 16.6%, where the least accounted for in Australia is about 1.5%. Though, DFU may be associated with the global burden of diabetes, which estimated in 2019, that 9.3%, almost 463 million people have diabetes mellitus. It will be raised to 10.2% (578 million) in 2030, while in 2045, it may reach 10.9% (700 million) [4, 5].

As in Pakistan, we do not have that much prospective research, that's why we don't have exact prevalences, especially in each province. Though very few and limited publications were founded on DFU patients in Pakistan. It has been found that the prevalence of DFU patients in Pakistan ranges from 2.1 to 50.9%, which may be considered an alarming situation [6]. If the infections in DFU are uncontrolled it may lead to limb loss [7]. The uncontrolled infections may be associated with antibiotic non-responsiveness, which is considered as an AMR/MDR or extended drug resistance (XDR). A limb is lost every 3 h in Australia as a result of uncontrolled infections in DFU. It is estimated that approximately 50%-70% of all lower limb amputations are due to DFU [8]. In addition, it is reported that every 30 s one leg is amputated due to DFU worldwide [9]. Most of the diabetes related-deaths are associated with DFU, which attributes up to 8% among other complications [10].

Antibiotics are effective against pathogenic bacteria, but excessive consumption and misuse may lead to the development of genetic mutations extended to resistance [11]. Overcrowded inpatient hospital wards and hospital-acquired cross infections may enhance the global burden of AMR/MDR infections. Overconsumption, decreased responsiveness, and long hospital stays may indicate AMR. As we know, mostly in Pakistan and many other Asian countries, antibiotics are prescribed without CSTs and rely on empirical therapy which may be due to high burdens of infections, limited hospital and healthcare facilities, and longer hospital stay which increase the healthcare costs.

The current study is an approach, to determine the cross-border infection demographics, antibiotic consumption, responsiveness and hospital stay longevity specifically in DFU inpatients in a tertiary care hospital in Peshawar. It is an attempt that evaluate the antibiotic excessive use, switching over from one class to another class of antibiotics, and the hospital stay prolongation in DFU inpatients.

2. MATERIALS AND METHODS

2.1. Study Universe

The current study was a combined prospective and retrospective nature study carried out in the endocrinology ward, in a tertiary care hospital of,

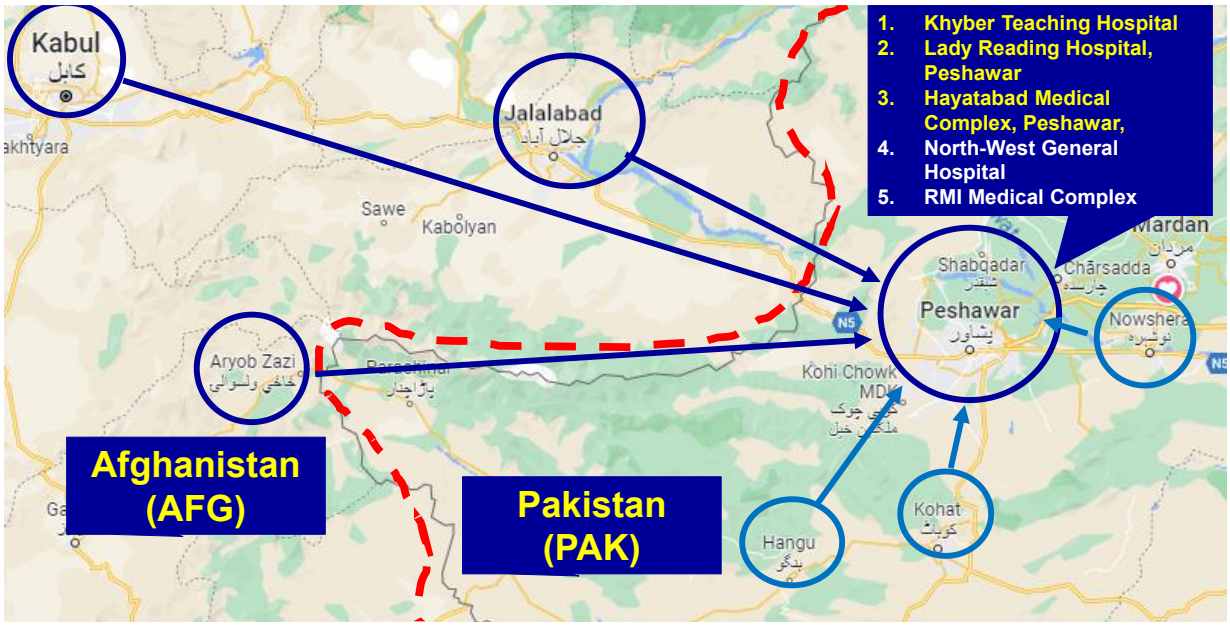


Fig. 1. Pakistan-Afghanistan border, periphery cities and hospitals (Source Google map with modification).

Peshawar, Khyber Pakhtunkhwa (KP), Pakistan. To ensure a thorough evaluation of treatment outcomes and the validity of the findings, the study attempted a combination of prospective monitoring and retrospective analysis to assess AMR, CSTs, and hospital stay longevity among DFU inpatients. By combining previous information from patient records with real-time observations of antibiotic usage, our method ensured a thorough evaluation of treatment outcomes in DFU patients.

The hospital comprised more than 33 Departments, 1797 beds, above than 1350 physicians and surgeons, with 450 other technical staff. The hospital provides both medical and surgical services to both inpatients, n= 139693 and outpatients (OPD), n= 535912, emergency patients n= 1061690, in the last year, 2022 (https://www.lrh.edu.pk/, retrieved on 26-Septmeber-2023).

2.2. Including/Excluding Criteria

Only those DFU patients (included across borders) were included in the study who were hospitalized (in-patients) for their severe sepsis, ulcers and infections. Outpatients and all DFU patients were excluded from this study who visited the hospital for their routine checkups, and dressings or had incomplete medical records.

2.3. Patient Data Collection Procedure

Both prospective and retrospective procedures and methodology used by Haleema et al., 2023 were adopted with a little modification for data collection [12]. The regional vicinity and cross-border DFU inpatients were specifically sorted out and were noted down for AMR, identified through CSTs. The Patient antibiotic consumption records were properly checked for DM and DFU diagnosis. Patient ID, name, age, sex, locality, date of Admission (DOA), and date of discharge (DOD) for hospital longevity/stay were specifically noted. Antimicrobial therapy and antibiotic consumption charts, dose and regimen schedules were properly evaluated for responsiveness and sensitivity. Empirical therapy and culture sensitivity tests (CSTs) were properly followed and noted down. An Excel sheet was properly maintained for the said parameters.

2.4. DFU Inpatient Demographics

DFU inpatient demographics were identified through age group ranges (including across borders). We classify the patient demographics into various age ranges, which start from 10 years and extend up to 60 and above years. The patient's demographic (regional vicinity and across borders) were further sorted for male and female DFU inpatients' which were separately tabulated, according to the following age groups (**Table 1**).

2.5. Culture Sensitivity Tests (CSTs)

As we know in Pakistan, mostly we have empirical therapies for antibiotic prescriptions, which means,

Table 1. DFU in patients' demographics (age ranges).

Age ranges (years)	DFU inpatients from Pakistan (n=x)	DFU inpatients Across borders (n=y)
10-29 Years		
30-40		
41-50		
51-60		
61-above		

that the physicians/clinicians prescribe antibiotics without CSTs. So, we also collected information for empirical and CST therapies, especially in DFU inpatients. CSTs were also noted down from the patient medication history profile.

2.6. Hospital Stay and Longevity

The hospital stays and longevity (H-S/L) were calculated from the date of admission and date of discharge in all DFU inpatients including across. The date of admission and discharge were all mentioned on the patient medication chart history.

2.7. Antibiotic Consumption and Responsiveness

During the hospital stay, antibiotic consumptions were properly monitored and evaluated in DFU inpatients in both regional and across-border inpatients. The sensitivity and responsiveness were properly monitored and followed for antimicrobial resistance and sensitivity. During the hospital longevity, switching over from the initial antibiotic therapy to another antibiotic treatment was also noted down. Age range-wise antibiotic consumption and responsiveness were also evaluated for AMR. Empirical therapy and CSTs were also properly observed for each patient [12].

2.8. Statistical Analysis

The co-relation of hospital stay and antibiotic was evaluated through regression correlation and t-test multiple while using GraphPad Prism. Another mode, the mean median and percentages were calculated through MS Word Excel.

3. RESULTS

The age range of male and female DFU inpatients' demographics information is mentioned in (Figure 2). DFU was most prominent in the age range of 51-60 years, followed by 41-50 and patients above 60 years of age, respectively. A total of n= 97 DFU inpatients, where the majority of them were male patients of almost all age ranges. Similarly, among these DFU inpatients, in some cases, some patients belonged to Afghanistan, which is a neighbour country to Pakistan. A total of n=8 cases were reported from Afghanistan (male=3, female =5) (Figure 3).

Some of the cases of AMR were reported both from within the regional vicinity of Pakistan as well as from across the border. (Figure 4a) is an

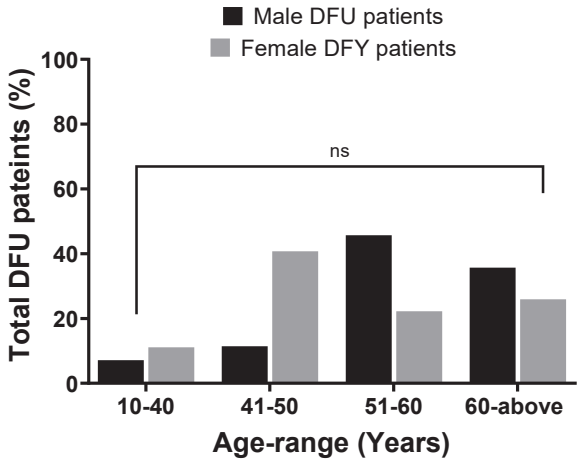


Fig. 2. DFU inpatients age-range demographic information's in tertiary care hospital of Peshawar during (January-March, 2023).

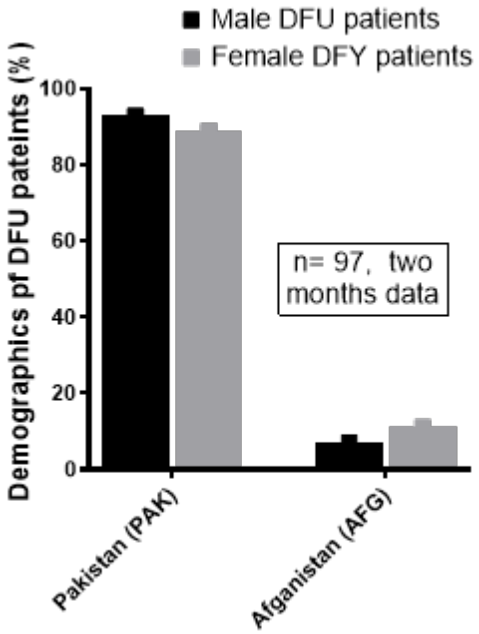


Fig. 3. DFU inpatients demographics including cases reported from Afghanistan (a cross-border), in tertiary care hospital of Peshawar during (January-March, 2023).

across-border AMR case reported of a female (40 Years) from Afghanistan, where Figure 4b is a CST, where the most commonly used antibiotics were highlighted. The CST report of the DFU patient revealed that the strain is MDR showcasing resistance to 12 tested antibiotics including; Penicillin (amoxicillin and amoxicillin+clavulanic acid combination), Cephalosporins (cefixime, cefixime, cefotaxime, ceftazidime, ceftriaxone), Fluoroquinolone (ciprofloxacin, moxifloxacin and levofloxacin), Imipenem, Meropenem and piperacillin+tazobactam combination therapy, respectively (Figure 4). Likewise, (Figures 5a & 5b) show AMR cases and their CSTs reported from the regional vicinity of Pakistan. Moreover, the CST report of the DFU patient from Pakistan (regional vicinity) showed that the strain is MDR showcasing resistance to 08 tested antibiotics including; Penicillin (amoxicillin+clavulanic acid), Cephalosporins (cefipime, cefotaxime, ceftazidime, ceftriaxone), Fluoroquinolone (ciprofloxacin, and levofloxacin), and doxycycline antibiotic therapy, respectively (Figure 5).

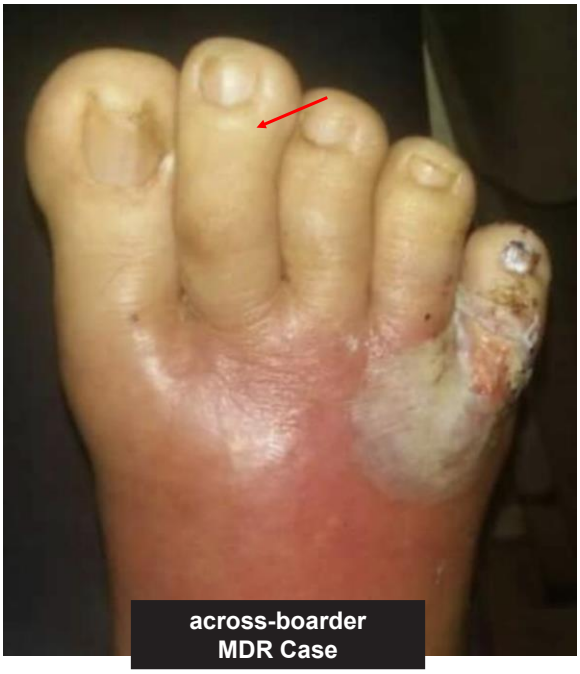
Figure. 6 shows the percentage of empirical therapy and CSTs in the current prospective and retrospective study of DFU inpatients.

Various antibiotics were prescribed to DFU patients during the study as shown in (Figure 7). Among antibiotics, cefoperazone-Salbactam combination was highly prescribed (31.8%) followed by Linezolid (18.8%), and Ciprofloxacin and Meropenem (13.6% each). Additionally, clindamycin, cefixime, cefotaxime, moxifloxacin, and clarithromycin were also prescribed to some patients.

The AMR percentage among all clinically sensitive antibiotics in DFU patients is depicted in (Figure 8). Patients having an age limit of 10-40 years were mostly resistant to antibiotic regimens including Ampicillin-clavulanic acid, cefipime, and cefoperazone-sulbactam. In addition, patients in the age limit 41-50 were resistant to cefotaxime-sodium and ceftazidime. Furthermore, patients in the age range of 51-60 were resistant to co-trimaxazole, levofloxacin and moxifloxacin. Moreover, patients above the range of 61 years were found to be resistant to Ampicillin-clavulanic acid, cefipime, cefotaxime, ceftriaxone, co-trimaxazole, and levofloxacin.

Lastly, hospital stay and longevity were also assessed for DFU patients as shown in (Figure 9). Mostly, patient stay in the hospital was revealed to be between 6-15 days due to non-responsiveness of antibiotics during antibiotic therapy. This increase in hospital stay can be attributed to AMR as

A



B

Antibiotics	AMR
Amoxicillin	
Amoxicillin-Clavulanic Acid	R
Cefepime	R
Cefixime	R
Cefoperazone-Sulbactam	
Cefotaxime Sodium	R
Ceftazidime	R
Ceftriaxone	R
Ciprofloxacin	R
Clarithromycin	
Clindamycin	
Colistin	
Co-trimoxazole	
Doxycycline	
Erythromycin	
Fusidic acid	
Imipenem	R
Levofaloxacin	R
Linezolid	
Meropenem	R
Moxifloxacin	R
Polymyxin	
Piperacillin-Tazobactam	R
Vancomycin	

Fig. 4. Across border DFU MDR case reported from Afghanistan, Female, age 40 Years, in Peshawar, KP, Pakistan, A) DFU ulceration B) Culture sensitivity tests.

A

B

Antibiotics	AMR
Amoxicillin	
Amoxicillin-Clavulanic Acid	R
Cefepime	R
Cefixime	
Cefoperazone-Sulbactam	
Cefotaxime Sodium	R
Ceftazidime	R
Ceftriaxone	R
Ciprofloxacin	R
Clarithromycin	
Clindamycin	
Colistin	
Co-trimoxazole	
Doxycycline	R
Erythromycin	
Fusidic acid	
Imipenem	
Levofaloxacin	R
Linezolid	
Meropenem	
Moxifloxacin	
Polymyxin	
Piperacillin-Tazobactam	
Vancomycin	

Fig. 5. Regional vicinity (Pakistan) DFU MDR case reported, Male, age 44 Years, in Peshawar, KP, Pakistan, A) DFU ulceration B) Culture sensitivity tests.

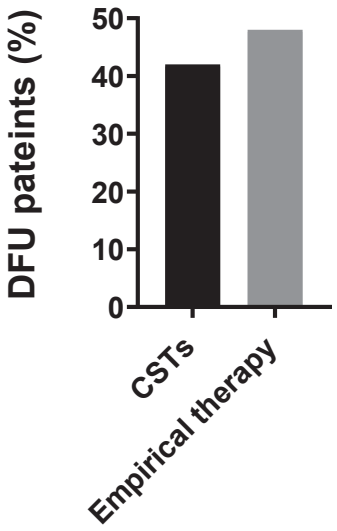


Fig. 6. The percentage of empirical therapies and CSTs performed for DFU inpatients.

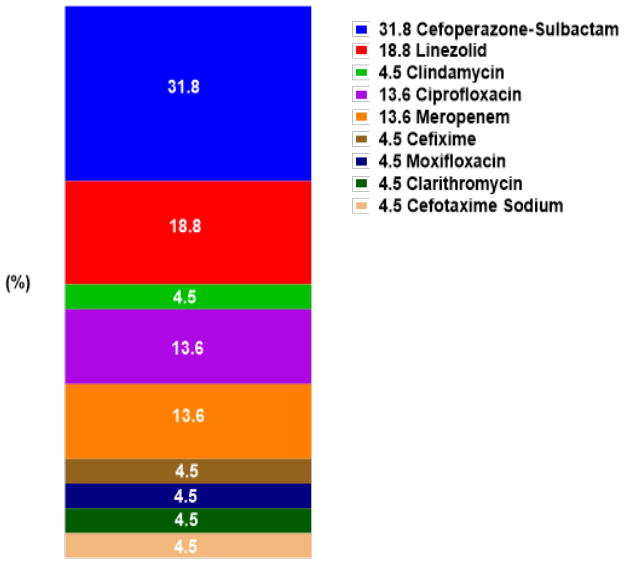


Fig. 7. Antibiotic consumption in DFU.

antibiotics are not working on DFU patients.

4. DISCUSSION

The current study is an attempt to find out the cross-border AMR infections in DFU patients. The study included 97 DFU patients from Pakistan as well as Afghanistan. All the medical records of these patients were evaluated for antibiotic consumption. Additionally, the CST reports were also taken into consideration for figuring out the susceptibility and resistance pattern of bacteria that caused these infections. The ratio of DFU patients was found to be higher in the age ranges 51-60, followed by 41-50 and above 60 years, respectively. It was noticed that patients in the age range of 51-60 years or above presented with comorbidities such as hypertension. Due to its contribution to peripheral vascular disease and delayed wound healing, hypertension is linked to an increased risk of diabetic complications,

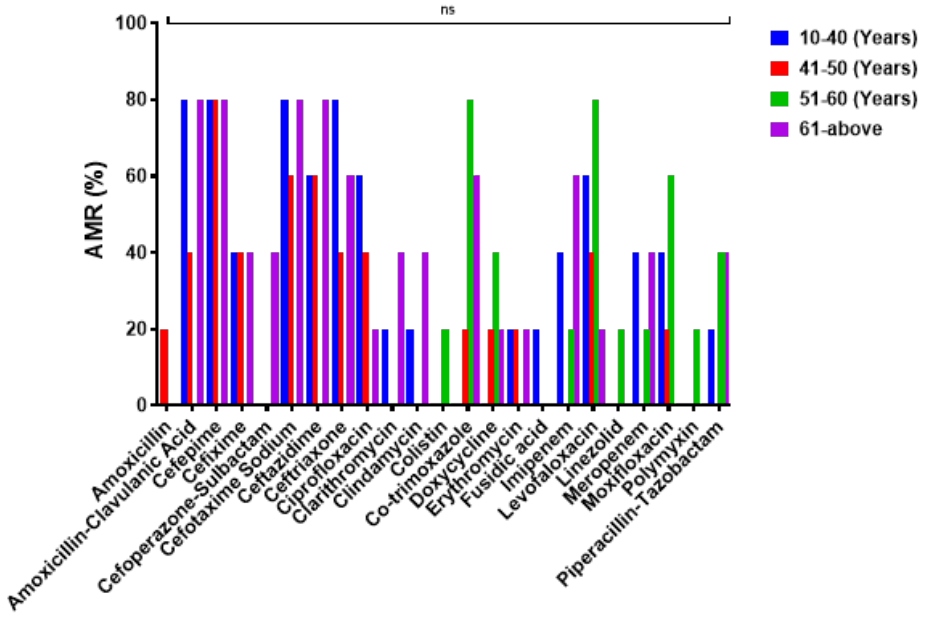


Fig. 8. Antimicrobial resistance percentage among all clinically sensitive antibiotics in DFU in-patients.

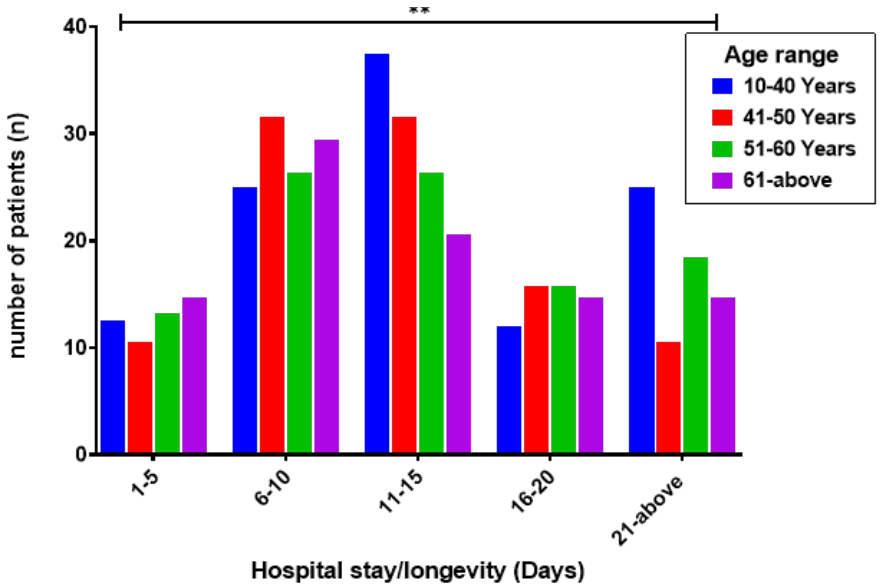


Fig. 9. Hospital longevity of DFU in-patients.

including DFUs [13]. For DFU patients, the existence of hypertension as a comorbidity in this age range emphasizes the significance of proactive measures, including blood pressure control [14], to maximize treatment success and lower the risk of consequences including infection and amputation [8].

During the current study, we came across two special and highly resistant (MDR/superbug) cases of DFU patients. CST report of the DFU patient from Afghanistan revealed that the strain is MDR showcasing resistance to 12 tested antibiotics including; Penicillin (amoxicillin and amoxicillin+clavulanic acid combination), Cephalosporins (cefipime, cefixime, cefotaxime, ceftazidime, ceftriaxone), Fluoroquinolone (ciprofloxacin, moxifloxacin and levofloxacin), Imipenem, Meropenem and piperacillin+tazobactam combination therapy, respectively (Figure 4a). Similarly, the CST report of the DFU patient from Pakistan (regional vicinity) showed that the strain is MDR showcasing resistance to 08 tested antibiotics including; Penicillin (amoxicillin+clavulanic acid),

Cephalosporins (cefipime, cefotaxime, ceftazidime, ceftriaxone), Fluoroquinolone (ciprofloxacin, and levofloxacin), and doxycycline antibiotic therapy, respectively (Figure 4b). In hospital settings, there are higher chances of hospital-acquired infections and cross-infection incidents [15]. For this purpose, the cross-border pathogens and MDR cases need to be reported scrutinized, and treated in controlled, highly hygienic hospital setups to safeguard public health [16].

Various antibiotics were prescribed to DFU patients, such as a cefoperazone-sulbactam combination (31.8%) followed by linezolid (18.8%), and ciprofloxacin and meropenem (13.6% each). Furthermore, clindamycin, cefixime, cefotaxime, moxifloxacin, and clarithromycin were also prescribed to DFU patients with a percentage of 4.5% each. MDR cases were reported from Pakistan as well and some cases were reported from Afghanistan. Furthermore, MDR cases from Afghanistan were resistant to Ampicillin-clavulanic acid, cefotaxime, ceftazidime, ceftriaxone, Meropenem and piperacillin-tazobactam combination. Hospital longevity of the majority of patients was between 6-15 days which is an ample time for cross infections to occur, which may result in the onset of hospital-acquired infections. Specifically, MDR strains can transmit from one patient to another during a hospital stay [17].

During the study, we noticed a shift of resistance towards broad-spectrum antibiotics with an increase in patients' age. Patients having an age limit of 10-40 years were mostly resistant to narrow-spectrum antibiotic regimens including Penicillin and first and 2nd generation Cephalosporins. In addition, patients in the age limit 41-50 were resistant to 3rd generation Cephalosporins. Furthermore, patients in the age range of 51-60 were resistant to broad-spectrum antibiotics such as co-trimaxazole and Fluoroquinolones including levofloxacin and moxifloxacin, respectively. Moreover, patients above the range of 61 years were found to be resistant to penicillins (Ampicillin-clavulanic acid), Cephalosporins (cefipime, cefotaxime, ceftriaxone) and Fluoroquinolones (moxifloxacin and levofloxacin), and co-trimaxazole. This may be linked to antibiotic overuse/misuse, comorbidities in old age, and a weakened immune system to fight off pathogens [18]. Healthcare-associated antimicrobial resistance places a substantial burden on patients [19]. If these scenarios were not taken into consideration, there may be the issues of cross infections, AMR transmission and chances of acquiring MDR infections.

Access to sanitation, proper utilization and purchasing of effective antibiotics are lower in poor nations and refugees which increases the purchasing of low-grade antibiotics, and empirical therapies, which fuels the onset of AMR [20]. Moreover, AMR cases in DFU are now becoming more frequent due to poor sanitation of infected areas of patients as a result of poverty and lack of patient education. During the study, various patients were presented with severe ulcers on their limbs and feet. Additionally, patients also received empirical therapies for their DFU conditions. Merely depending on empirical therapy compromises effective treatment approaches, leaving patients with extended hospital stays and heightened susceptibility to resistant infections [21]. These empirical therapies may result in the prevalence of AMR and can shape the onset of MDR, XDR and superbugs [22]. These resistant infections are responsible for increasing hospital longevity and overall healthcare costs [23]. As evident from the results of this study, the majority of the patients stayed in the hospital for more than one week. This increase in hospital longevity may be associated with infections caused due to drug-resistant strains [24].

There are several limitations to relying solely on hospital data when evaluating cross-infections and AMR. These include the possibility of underreporting cases resulting from mild or asymptomatic infections [25], differences in diagnostic procedures between healthcare facilities that could cause differences in reported AMR rates [26], and the impact of previous antibiotic use on resistance patterns. It is essential to apply established processes for data collecting and analysis alongside other monitoring techniques, such as community- and laboratory-based surveillance, to overcome these constraints with hospital data [27]. Furthermore, to effectively combat AMR in healthcare settings, efforts must be made to improve reporting procedures [28], expand diagnostic capabilities, and implement antimicrobial stewardship programs [29].

The results of the study highlight how cross-border healthcare significantly affects antibiotic resistance. AMR and cross-infection patterns found in Khyber Pakhtunkhwa tertiary care hospitals have the potential to significantly affect regional and

global health outcomes, even outside of the local context. AMR strains are becoming more prevalent, which not only puts the local people at risk but also has the potential to spread resistant diseases throughout the entire globe [30]. The spread of AMR strains from KP hospitals to nearby regions and beyond is an urgent concern considering the international movement of individuals and the interconnection of healthcare systems. This might cause multidrug-resistant (MDR) pathogens to spread, which would make common infections more difficult to treat [31]. Concerns over the spread of drug-resistant bacteria are raised by the influx of people seeking medical care in Pakistan from neighbouring countries such as Afghanistan, particularly with regard to DFU. The spread of superbugs and MDR infections is facilitated by these cross-border migrations, which increases the global AMR threat [32].

A multimodal approach is necessary to effectively reduce the spread of cross-infections and antimicrobial resistance (AMR) in the context of the study region. First of all, healthcare facilities may optimize antibiotic use and stop the spread of resistant bacteria by putting antimicrobial stewardship policies into place. For the purpose of reducing transmission within hospital settings, enhanced infection control measures such as isolation protocols and hand hygiene procedures are essential [33]. Enhancing the capacity for antimicrobial susceptibility testing (AST) diagnostics can also direct focused antibiotic treatment and minimize the inappropriate use of broad-spectrum antibiotics [34]. Since pathogens are borderless and rapidly travel across individuals, collaborations with neighbouring countries might encourage cross-border collaboration in the fight against antimicrobial resistance [35]. Working together can help strengthen surveillance systems and track the regional and worldwide spread of resistant infections by facilitating the interchange of information, resources, and credentials [36]. Furthermore, public health education initiatives can increase awareness about safe antibiotic usage and infection prevention, while strong surveillance and monitoring systems are essential for tracking resistance trends and early epidemic detection. Lastly, there is a strong need for global collaboration, one health approach and antibiotic stewardship programs to be implemented [37].

Several factors, such as resource restrictions, infrastructure limitations, and stakeholder participation, affect the feasibility and scalability of proposed interventions to minimize cross-infections and AMR in the context of the current study. In environments with limited resources, antimicrobial stewardship programs may be difficult to establish because they need initial investment in infrastructure, employees, and educational programs [38, 39]. However, by decreasing antibiotic overuse and AMR-related healthcare expenditures, these programs promise long-term cost reductions [40].

5. CONCLUSIONS

The migration of patients seeking medical attention from Afghanistan to Pakistan is a major factor in the spread of drug-resistant infections, which in turn increases the burden of AMR worldwide. Furthermore, considering the high prescription rates of certain antibiotics, the widespread use of empirical antibiotic therapy without culture sensitivity testing in the treatment of DFU patients promotes antibiotic misuse and the development of resistant strains. This highlights the critical need for improved diagnostic techniques and appropriate prescription practices, particularly for the treatment of DFU patients. To address these issues, global collaboration is required. This collaboration includes improving monitoring systems, setting antimicrobial stewardship programs into place, as well as developing antibiotic consumption regulations that consider health disparities and increase access to high-quality healthcare. Lastly, there is a strong need for comprehensive studies considering pathogen transmission, and cross-border infections in Pakistan to prevent the growing issue of AMR.

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7. CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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Research Article

Molecular and *Insilico* Characterization of Antimicrobial Resistant *Escherichia fergusonii* and *Morganella morganii* causing Urinary Tract Infections

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Abstract: Urinary tract infections (UTIs) are a major public health concern causing mortality and morbidity worldwide. There are approximately 13,000 deaths that are linked to UTIs every year. Bacterial antibiotic resistance is increasing globally, making it difficult to treat urinary tract infections. Biochemical tests have resulted in inaccurate identification, due to which wrong empirical treatment of antibiotics is prescribed. The present research work aims to identify rare bacterial isolates along with the assessment of antibiotic resistance patterns. A total of 339 samples were examined for urine cultures. Based on biochemical testing, three positive samples were selected. Culture sensitivity results revealed two multidrug-resistant and one extensively drug-resistant bacteria. MDR strains showed sensitivity to meropenem, gentamicin, cefoperazone-sulbactam, nitrofurantoin, tigecycline and imipenem, and co-amoxiclav while XDR bacteria showed sensitivity to nitrofurantoin and tigecycline. The 16S rRNA sequencing results revealed two *Escherichia fergusonii* strains and one *Morganella morganii* strain. Our findings revealed the identification of rare and opportunistic bacteria less reported from Pakistan. In addition, it is the first report of *E. fergusonii* from Pakistan. In conclusion, using molecular identification of rare and neglected bacteria via PCR is more sensitive and highly specific than other identification methods. In the future, this approach will help in accurate diagnosis and will help in minimizing antibiotic resistance.

Keywords: Urinary Tract Infections (UTIs), *Escherichia fergusonii*, *Morganella morganii*, Antimicrobial Resistant, Antibiotics

1. INTRODUCTION

The word “urinary tract infection” (UTI) is a common term used extensively, indicating an infection across the urinary tract from the urethral meatus to the perinephric fascia, including ureters, bladder, renal pelvis, urethra, and parenchyma [1]. Various microbial species are the main source that gives rise to UTIs. Still, most of the infections in both inpatient and outpatient populations are caused by gram-negative, uropathogenic, and facultative anaerobic bacteria. *Escherichia coli* is the major and supreme cause of UTIs, a study revealed that 80% of UTIs are caused by uropathogenic *E. coli* in healthy women aged 18 to 39 years [2].

UTIs are the fifth most frequent kind of medical care-associated infection, with approximately 62,700 total UTIs in critical-care hospitals in 2015. In addition, UTI reports are higher than 9.5% of infections reported by critical-care-health centers [3]. Nearly all UTIs (health center-linked) are caused by urinary tract instruments. Relatively, 12 to 16% of hospitalized patients will possess an indwelling urinary catheter throughout their span of hospitalization. The duration of the catheter remained for more than 2 days which leads to a 3 to 7% risk of catheter-acquired urinary tract infection to the patient (CAUTI) [4,5]. Around 0.34% of manifestations occur due to contagious agents causing cystitis, which proliferates and multiplies and further mounts to the kidney through the ureters, where these agents infect calyces, cortex, and pelvis, ultimately leading to the symptoms and

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signs of pyelonephritis [6]. Approximately 27% of the clinical manifestations can be allocated to urinary isolates in patients visiting the emergency department while suffering from sepsis and thus are entitled to urosepsis. In clinical settings, the percentage of sepsis due to UTIs surged to 42% [7,8].

Antimicrobial resistance (AMR) arises because various pathogenic organisms including bacteria, fungi, viruses, and parasites, progress so that drugs of multiple classes do not alter them. Because of resistance, it is complicated or occasionally difficult to cure infections, leading to a high morbidity and mortality rate. There is a global threat due to multi, pan, and extensively drug-resistant bacteria. The resistant bacteria are called superbugs because they carry resistance to current and previous classes of antibiotics [9]. MDR bacteria are insusceptible to multiple regimens, while pan-drug-resistant bacteria are insusceptible to all previously existing antimicrobial drugs.

Similarly, XDR bacteria resist most crucial regimens while sensitive to several regimens [10]. Resistance in bacteria is a major challenge due to less naturally presenting drugs. In contrast, many drugs are synthetic, and it is hard to synthesize novel regimens as opposed to bacteria previously resistant to such antibiotics. This disaster is a consequence of overuse and misuse of these regimens and insufficient doses throughout the recovery course of infection [11].

Numerous antibiotics have been utilized as a wide-range regimen to fight against UTIs, *i.e.,* community and hospital-acquired. However, the persistent antibiotic resistance and the researcher's regard for the function of dependent representative of the host micro-flora have emphasized the crucial demand for regimens or strategies that can particularly recover UTIs without any modifications and changing the gut structure and vaginal micro-flora. In the digestive tract, utilization of antibiotics escalates inflammation, weakens the immune surrounding of the host, and further progresses pathogen proliferation by escalating substrate accessibility for instance, *E. coli* proliferation by escalated nitrate accessibility while survival in the digestive tract is additionally linked with a high risk of recurrent UTI [12,13,14]. Recovery with an antibacterial regimen may hinder the vaginal micro-flora (that protects the surface from pathogens) by disrupting colonization with *lactobacillus spp, w*hich produces peroxides that suppress ascension and colonization [15,16]. Consequently, antibacterial drugs can also be a risk factor for recurrent UTIs due to the contagious effect on vaginal and gut micro-flora [12,17,18]. The study aimed to isolate and identify rare, opportunistic, and neglected XDR and MDR bacteria via biochemical testing and molecular characterization.

2. MATERIALS AND METHODS

2.1. Design and Setting

Patients with UTIs were examined at Khyber Teaching Hospital (KTH), Peshawar. A total of 339 urine culture samples were assessed from July 2021 to October 2021. Morphological analysis, biochemical testing, gram staining, and culture sensitivity of the selected isolates were performed in the microbiology section of KTH. At the same time, a molecular study was subsequently conducted at the Department of Biotechnology Quaid-i-Azam University, Islamabad.

2.2. Inclusion Criteria

Specimens with urease and/or mannose-positive bacterial isolates (n=3) were included in the study.

2.3. Exclusion Criteria

Specimens without urease and/or mannose-positive bacterial strains were excluded from the study.

2.4. Sample Collection and Primary Screening

Urine samples were collected from the patients admitted to various wards and diagnosed with UTIs. Samples were processed, and cultured in the Microbiology laboratory.

2.5. Isolation of Bacteria

A drop of urine from each sample was taken and placed on freshly prepared MacConkey agar plates. The streaking was done using a sterile inoculating loop 3 to 4 times. The plates were labeled and covered to prevent any contamination. The plates

were then incubated at 37 ℃ for 24 hours until the growth of bacterial colonies. The isolated strains were labeled as QAU. 1011, QAU. 1012 and QAU. 1013 respectively.

2.6. Gram Staining

Isolated bacteria were stained to identify the type of bacteria. Initially, a drop of normal saline was added to the microscopic slide. A pure colony was picked, mixed with normal saline, and smeared. The gram staining procedure was then performed. Finally, the slide was observed under the microscope at 1000 X magnification power.

2.7. Biochemical Tests

Biochemical classification was executed following standard protocols. Urea agar, Simmon's citrate agar, triple sugar iron (TSI) agar, and one phenol red mannose broth were prepared. For the urease test, *Proteus vulgaris* (ATCC 13315) and *Escherichia coli* (ATCC 25922) were used as positive and negative controls. Agar slants and broth containing pure bacterial isolates were then incubated at 37 ℃ for 24 h to examine test results.

2.8. Antibiotic Sensitivity Test

Culture sensitivity testing was done to assess the activity of various antibiotics. For this purpose, Muller Hinton agar media was prepared. Pure bacterial isolates were initially streaked on MHA plates. A total of 12 antibiotic discs with various concentrations were used (Table 1) and placed on the agar plates containing pure bacterial isolates. The plates were then incubated at 37 ℃ for 24 h. The next day, plates were examined for antibiotic resistance following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) and Antimicrobial Susceptibility (AST).

2.9. Molecular Characterization

For molecular identification of bacteria, genomic DNA from the bacterial isolates was extracted via the plain boiling method as defined previously [19] with slight modifications.

2.9.1. PCR Amplification

The extracted DNA was amplified with 16S rRNA gene universal primers, i.e., 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (TACGGCTACCTTGTTACGACTT) as described previously [20]. The reaction mixture consisted of 3 µl of template DNA, 1.5 µl forward primer, 1.5 µl reverse primer 10 µl of 2X GoTaq® Green Master Mix, and 4 µl PCR water (total reaction volume = 20 µl). PCR (peqSTAR 96x gradient-VWR) amplification conditions were: 95 ˚C for 3 min; 35 cycles of 95 ˚C for 30 s, 54 ˚C for 30 s, and 72 ˚C for 60 s; and a final extension of 72 ˚C for 15 min before holding at 8 ˚C.

After the PCR reaction, the amplified DNA products were tested on 1.5% gel. A ladder of 1kb was used to compare the product sizes (GeneRuler

Table 1. Concentrations of various antibiotics utilized for culture sensitivity testing.

S. No.	Antibiotics discs	Concentrations (µg)
1.	Meropenem (MEM)	10
2.	Ceftazidime (CFM)	5
3.	Azithromycin (ATM)	30
4.	Ciprofloxacin (CIP)	5
5.	Cefepime (FEP)	30
6.	Co-trimoxazole (CTX)	30
7.	Imipenem (IPM)	25
8.	Gentamicin (GN)	120
9.	Cefoperazone-sulbactam (SCF)	105
10.	Nitrofurantoin (NA)	30
11.	Tigecycline (TGC)	15
12.	Co-amoxiclav	25

DNA ladder 1kb, Fermentas). The amplified products were then eluted and were checked on 1.5% agarose gel to confirm the amplification before sequencing.

2.10. DNA Sequencing

The amplified products were sequenced via Sanger sequencing (Macrogen Inc., Korea).

2.11. Bioinformatics Analysis

Consequently, the sequences obtained after Sanger sequencing were analyzed using BLAST (Basic Local Alignment Search Tool) from the National Centre for Biotechnology Institute (NCBI) (www.ncbi.nlm.nih.gov/BLAST). Similarly, sequences were aligned using the clustal W program, phylogenetic tree construction was done via the Neighbor-Joining method of MEGA-X with a bootstrap value of 0.010 [21], and were submitted to the NCBI gene bank.

3. RESULTS

In this research study, three urine samples were collected from various patients with UTI from care units of KTH Peshawar, based on gender distribution. Of the three samples, 2 were females, and one was male (Table 2).

Table 2. Patient's Attributions.

S. No.	Isolate name	Age	Gender
1.	QAU. 1011 (*Escherichia fergusonii*)	8 months	Girl (child)
2.	QAU. 1012 (*Escherichia fergusonii*)	10 months	Girl (child)
3.	QAU. 1013 (*Moranella morganii*)	19 years	Male

3.1. Morphology and Gram Staining

After the incubation period, the MacConkey agar plates were checked in a sterile environment. All the bacterial isolates appeared colorless on media plates which revealed the isolates as non-lactose fermenting bacteria (Figure 1 a,b,c). Similarly, gram staining results of isolated bacterial strains under microscopic with resolution (1000X) depicted that all isolates were gram-negative bacteria as the colonies for all the isolates appeared pink in color (Figure 1d).

3.2. Biochemical Tests

After gram staining, the morphology observation on MacConkey agar plates, bacterial morphology was identified through various biochemical tests to differentiate desired bacteria from other gram-negative rods and bacilli bacteria. Two bacterial strains were urease negative as no color change was observed after incubation, indicating the bacteria were within the *Escherichia* family. At the same time, one isolate was urease positive as a positive reaction was observed after incubation (pink color indication) depicting the non-*Escherichia* family. The results were compared with the positive and negative controls. Similarly, the TSI test for two isolates revealed a change in the slant color depicted sugar fermentation with color change.

In contrast, no change occurred in the control (TSI without inoculated colony). At the same time, 1 sample revealed no sugar fermentation, as no color change was observed after incubation for both the inoculated colony and control. Citrate for all the isolated bacterial strains was negative, as no color change was observed after incubation. The mannose fermentation test revealed one isolated mannose fermented bacteria depicting *Morganella* species as the culture changes its color after incubation. In contrast, 2 strains showed mannose non-fermented bacteria indicating *Escherichia* species with no color change in the medium (Table 3).

3.3. Antibiotic Sensitivity

Out of 3 specimens, 2 species (*E. fergusonii* and *Morganella morganii*) were found to be multidrug-resistant (MDR), while 1 sample (*E. fergusonii*) was extensively drug-resistant (XDR) bacteria. Of the 2 MDR strains, the first showed sensitivity to meropenem, gentamicin, cefoperazone-sulbactam, nitrofurantoin, tigecycline, and imipenem while resistant to all other regimens. Similarly, the second revealed sensitivity to meropenem, nitrofurantoin, tigecycline, co-amoxiclav, and imipenem, while the XDR strain revealed sensitivity to nitrofurantoin and tigecycline as detailed in (Figure 2 and Table 4).

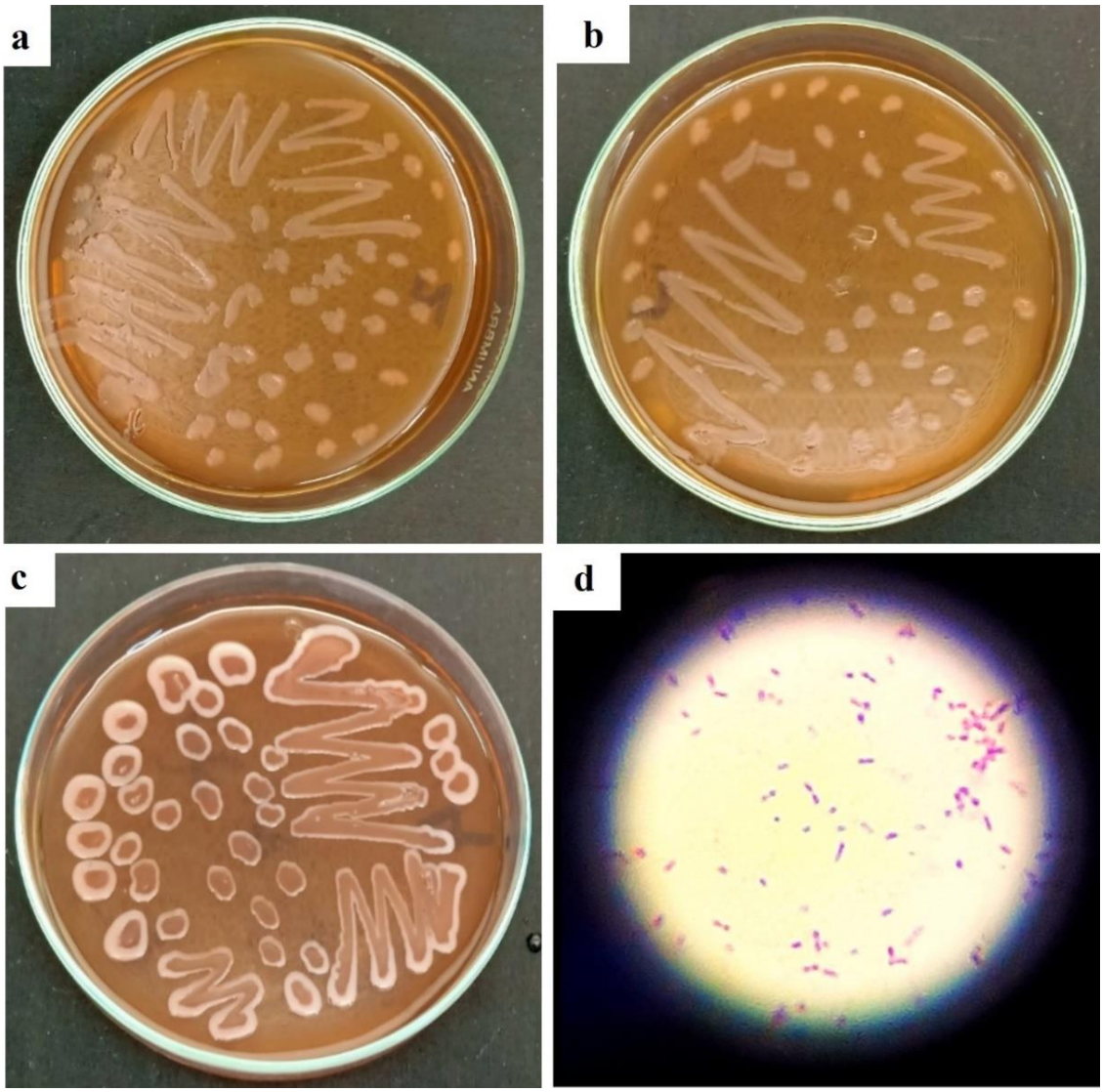

Fig. 1a. Image of pure bacterial isolate QAU.1011 of the urine sample. **b.** Pure bacterial isolate QAU.1012. **c.** Isolate QAU.1013. The colorless colonies of bacterial isolates indicate non-lactose fermenting bacteria. **d.** Small rod-shaped, pink-colored, and gram-negative bacteria under the light microscope (with a resolution of 1000X).

Table 3. Biochemical tests indicating positive and negative results.

S. No.	Biochemical tests	Results		
		Escherichia fergusonii **(QAU. 1011)**	***Escherichia fergusonii*** **(QAU. 1012)**	***Morganella morganii*** **(QAU. 1013)**
1.	Triple sugar iron (TSI)	+	+	-
2.	Urease	-	-	+
3.	Citrate	-	-	-
4.	Swarming	-	-	-
5.	Mannose	-	-	+

3.4. Molecular identification

The amplified product size was approximately 1500bp for all the samples, as shown in (Figure 3). After PCR product purification and sequencing, the isolate QAU. 1011 and isolate QAU. 1012 were identified as *E. fergusonii* with accession No. ON076882 and OP810623, and the isolate QAU.

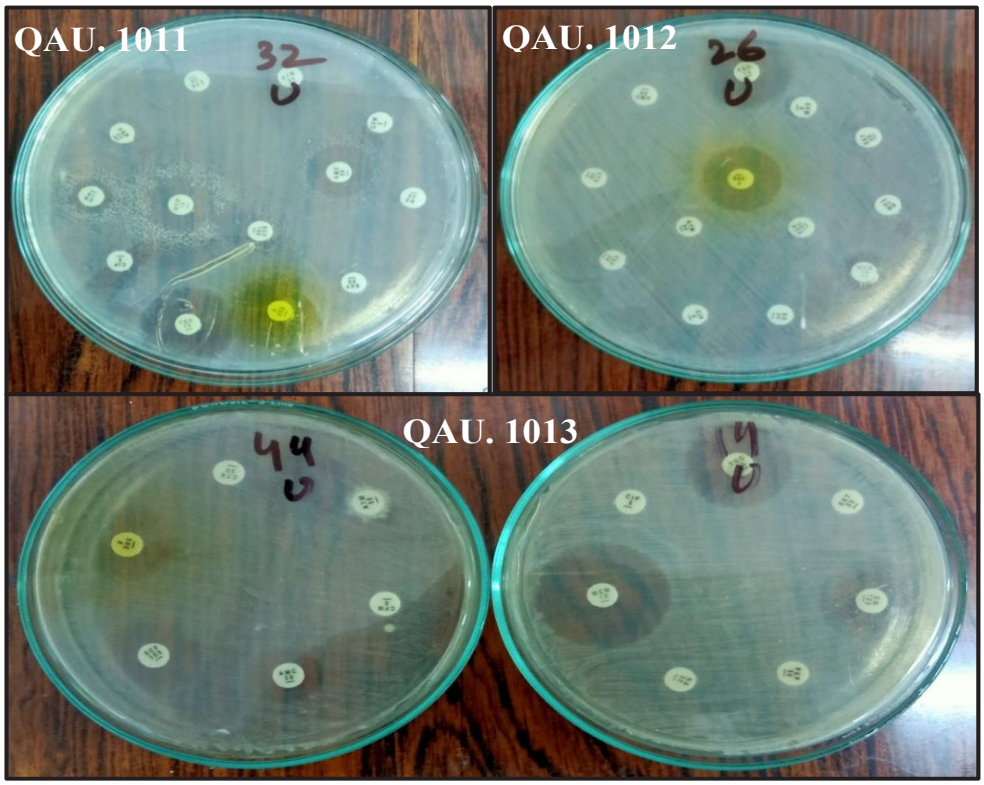

Fig. 2. Culture sensitivity test results of bacterial isolates from patients depicting sensitivity to some while resistance to several antibiotics. QAU. 1011 and 1012 are *E. fergusonii,* while QAU. 1013 is *Morganella morganii*.

Table 4. Drug profile of isolates showing resistance and sensitivity pattern.

S. No.	Antibiotics	Isolates		
		QAU. 1011 (*Escherichia fergusonii*)	**QAU. 1012** (*Escherichia fergusonii*)	**QAU. 1013** (*Morganella morganii*)
1.	Meropenem (MEM)	S	R	S
2.	Ceftazidime (CFM)	R	R	R
3.	Azithromycin (ATM)	R	R	R
4.	Ciprofloxacin (CIP)	R	R	R
5.	Cefepime (FEP)	R	R	R
6.	Co-trimoxazole (CTX)	R	R	R
7.	Gentamicin (GN)	R	R	S
8.	Cefoperazone-sulbactam (SCF)	R	R	S
9.	Nitrofurantoin (NA)	S	S	S
10.	Tigecycline (TGC)	S	S	S
11.	Co-amoxiclav	S	R	R
12.	Imipenem (IPM)	S	R	S

Abbreviations: S: Sensitive, R: Resistant.

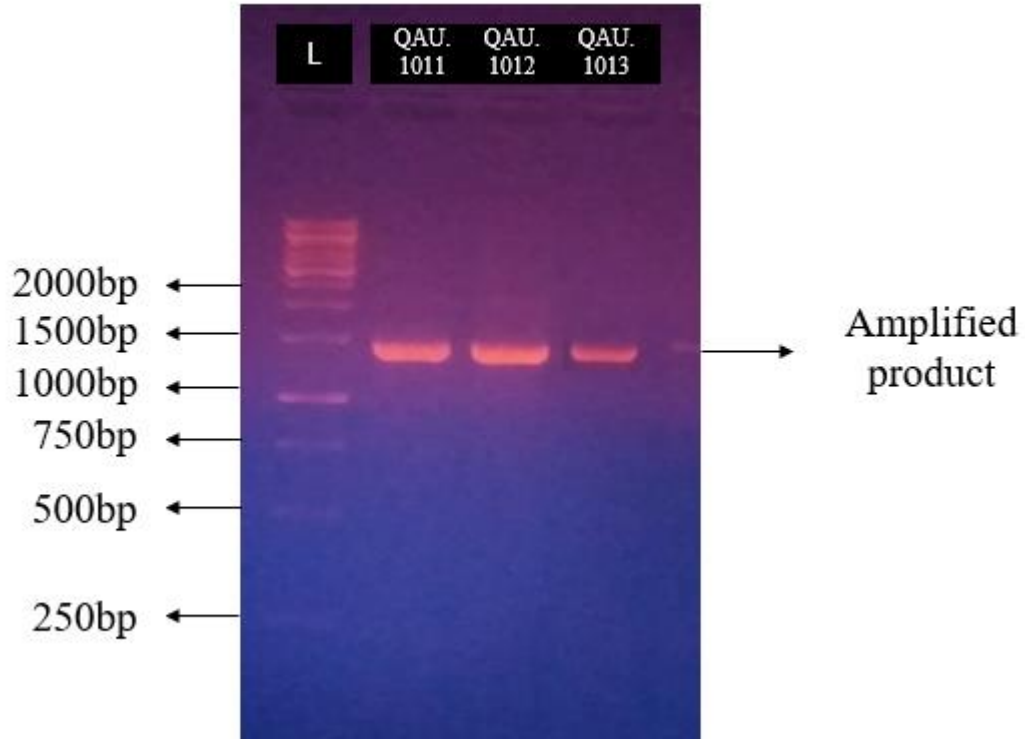

Fig. 3. Agarose gel electrophoresis (1.5% agarose) of Amplified PCR products via universal primer sets. Lane 1-3 depicts the sample strains *(Escherichia fergusonii* and *Morganella morganii). Lane L illustrates the DNA ladder of 1kb.*

1013 was identified as *M. morganii* with accession No. OP810942 after submitted to NCBI gene bank.

3.5. Phylogenetic Analysis

Partial sequences (16S rDNA) genes of the genus were recognized. The obtained sequences were aligned with the genera i.e., *Escherichia, Shigella, Salmonella, Kosakonia, Citrobacter, Morganella, Providencia,* and *Proteus*. Based on BLAST results, isolate QAU. 1011 and QAU. 1012 were closely related to *Escherichia species* with 99% similarity. The genus *Escherichia* formed a distinct clade with the highest sequence similarity found among *E. fergusonii*. In contrast, QAU. 1013 was closely associated with *Morganella species* with a similarity index of 97%. The genus *Morganella* arose from the similar clade of *M. morganii* depicting higher similarity with the strain of Japan. Based on phylogenetic analysis, the isolate QAU. 1011 (*E.scherichia fergusonii* with accession No. ON076882) and QAU. 1012 (*E. fergusonii* with accession No. OP810623) were genetically related to *E. fergusonii* strain ATCC 35469 of Canada (Accession No. NR 027549) with 98% maximum identity. The sublines were determined in the *Escherichia* clade, both included *E. fergusonii* (Figure 4). Similarly, isolate QAU. 1013 (*M. morganii* with accession No. OP810942) was closely related to *Morganella morganii* strain JCM1672 (Accession No. NR112191) belonging to Japan. The subline was determined in *Morganella* clade included *M. morganii*. The clade depicts the evolutionary bacteria associated with the strain of *M. morganii* with 86% maximum identity (Figure 5).

4. DISCUSSION

UTIs are one of the extreme and usual causes of bacterial infections, influencing approximately 150 million individuals worldwide yearly and establishing greater than six billion dollars in immediate medical management costs [22]. Resistance against antibiotics has emerged with destructive effects on useful microflora of the host via antibiotic use, revealing a deficiency in the treatment for UTIs [23]. The existing impact of bacteria causing urinary tract infections is a major challenge to evaluate due to the misdiagnosis of bacteria with other gram-negative bacterial isolates. At the same time, identification can be done via

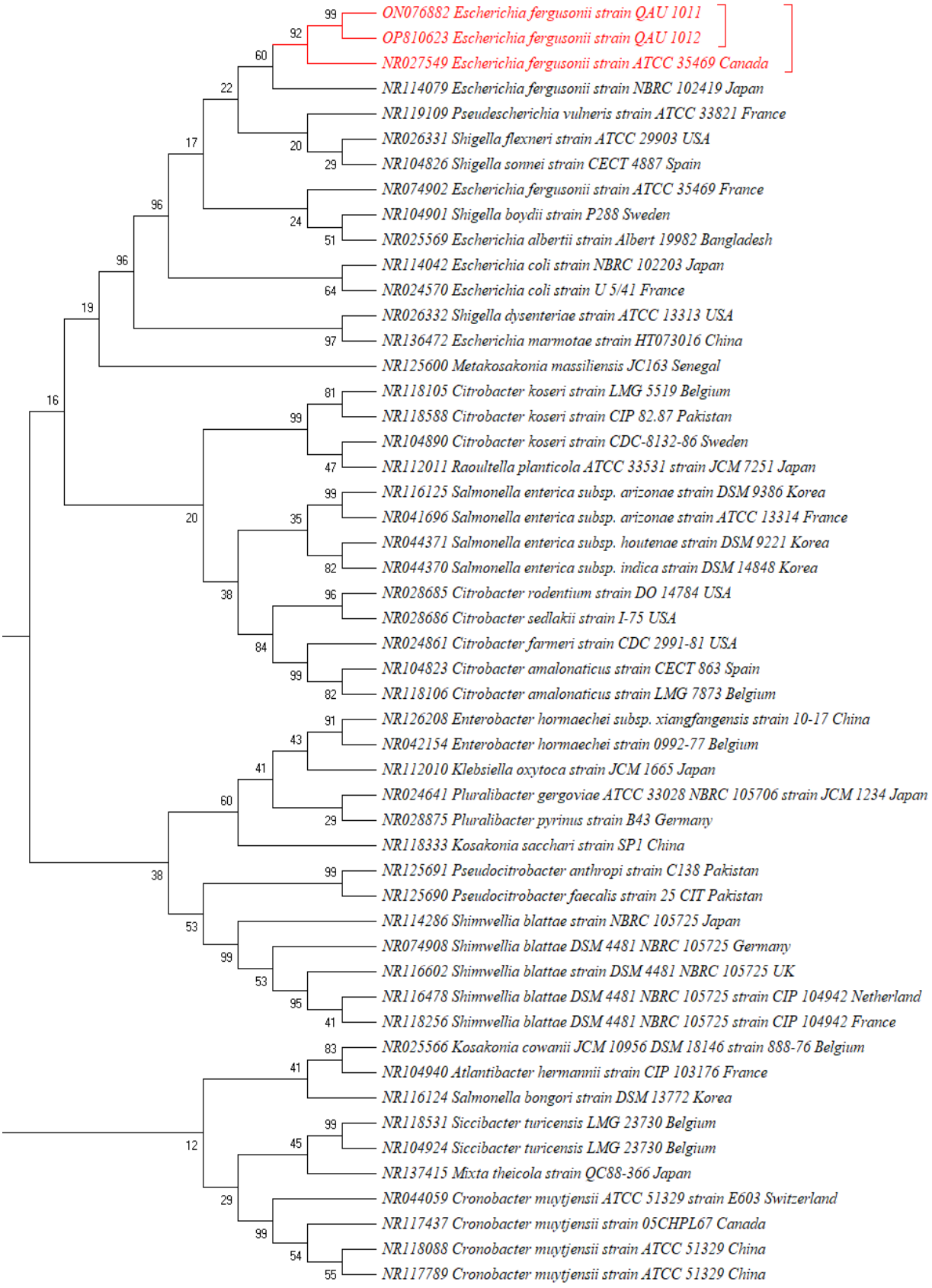

Fig. 4. Phylogenetic relationship of isolate QAU. 1011 and QAU 1012 with the closely related strains of Escherichia fergusonii with a bootstrap value of 0.010.

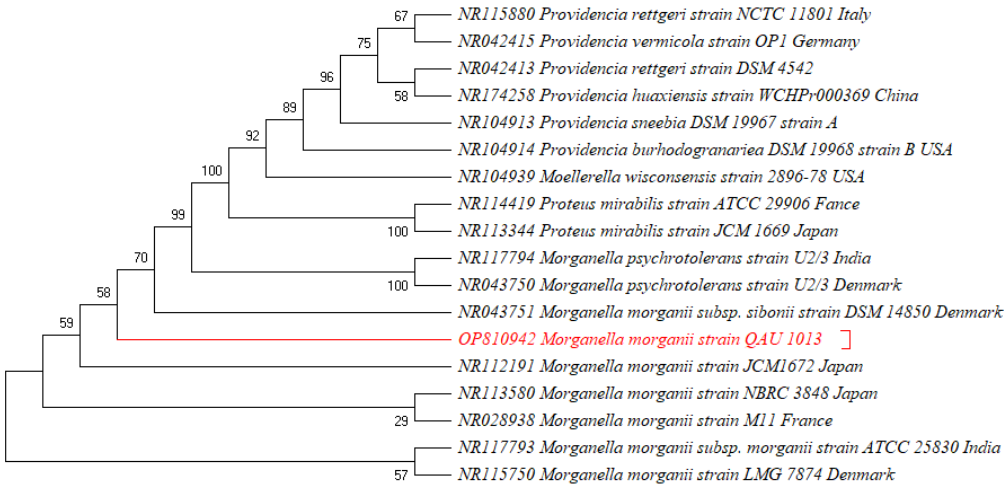

Fig. 5. Phylogenetic relationship of isolate QAU. 1013 with the closely related strains of *Morganella morganii* with a bootstrap value of 0.010.

biochemical and molecular detection using PCR assay [24].

The PCR-based assay is highly specific and sensitive for the detection of isolates compared to biochemical testing. Moreover, phenotypic identification using various automatized equipment or biochemical tests may lead to false identification [25]. The molecular method for the identification of bacteria remains the best and most appropriate approach. *E. coli* with respected representatives, i.e., *E. Alberti* and *E. fergusonii* were accurately distinguished from *E. coli* based on specific primers to certain genes in both strains. PCR results with specific primers revealed accurate identification of both strains. Similarly, beta-lactam genes, i.e., antibiotic resistance strains, can be identified accurately via molecular approach as the previous study demonstrated that various isolates of bacteria producing extended-spectrum beta-lactamase (ESBL) were detected using 16S rRNA gene sequencing [26, 27]. In contrast to these studies, our experiment revealed false identification of the strains via biochemical tests, while results of 16S rRNA gene sequencing and phylogenetic analysis of all the isolates revealed the strains as *E. fergusonii* and *M. morganii* indicating PCR-based assay (16S rDNA) as highly specific and sensitive.

Resistance to antibiotics and drug regimens is progressive and constantly emerging in healthcare settings [28]. UTI-causing bacterial isolates, specifically *E. coli* contribute to total infections of 80% with resistance to first-line antibiotics and other regimens such as nitrofurantoin, ciprofloxacin, trimethoprim and trimethoprim-sulfamethoxazole (TMP-SMX) in both community and hospital-acquired clinical-care settings. Similarly, urease-positive bacterial isolates previously showed resistance against nitrofurantoin, nalidixic acid, ceftriaxone, and cefotaxime while revealing sensitivity to gentamycin, norfloxacin, ciprofloxacin, vancomycin, amikacin, ciprofloxacin, and imipenem [29,30]. Another study demonstrated the susceptibility pattern of XDR and MDR gram-negative isolates in kidney transplant patients with UTI. Results revealed a total of 88 events of gram-negative XDR and MDR bacteria. All MDR and XDR isolated bacteria resisted first-generation and second-generation beta-lactam (monocyclic) and cephalosporin. The sensitivity pattern of all isolated bacteria was observed for amikacin, tigecycline, and meropenem. Among them, 12 XDR bacteria showed meropenem resistance, while tigecycline resistance was up to 25%. Among all the XDR isolated strains *E. coli* and *Acinetobacter baumannii* were tigecycline sensitive [31]. Our findings revealed all the isolates as urease and mannose-positive bacteria, in which 2 were MDR strains while one isolate was XDR. MDR strains showed sensitivity to meropenem, gentamicin, cefoperazone-sulbactam, nitrofurantoin, tigecycline, imipenem, and co-amoxiclav while resistant to ceftazidine, azithromycin, ciprofloxacin, cefepime and co-trimoxazole. Similarly, the XDR strain showed sensitivity to nitrofurantoin and tigecycline while

resistant to meropenem, gentamicin, cefoperazone-sulbactam, imipenem, co-amoxiclav, ceftazidine, azithromycin, ciprofloxacin, cefepime and co-trimoxazole respectively.

PCR-based assays and phylogenetic analysis previously revealed that UTI-causing bacteria are characterized by the genus *Escherichia, Streptococci, Enterobacter, proteus, and Morganella* [32]. The phylogenetic analysis previously revealed 98% similarity with *E. fergusonii* when isolated from dairy cattle causing diarrhea [33]. It was depicted in *Escherichia* clade as it was similar to *E.coli*. In contrast to the study, our finding revealed that the clades depicted *Escherichia* while the sublines indicated *E. fergusonii*. Similarly, another MDR strain revealed 98% similarity with *Morganella* and the subline depicted *M. morganii*. To the best of our knowledge, it is the first study that reported *E. fergusonii* as a UTI-causing bacteria in Pakistan with a high ratio of antibiotic resistance.

5. CONCLUSIONS

Escherichia fergusonii and *Morganella morganii* are rare bacteria with a high resistance rate. It is the first study that reported *E. fergusonii* as a UTI-causing bacteria for the first time from Pakistan. Our study concluded that these neglected and rare bacteria can pose major challenges at the lateral stage. Similarly, molecular diagnostic approaches are mandatory to overcome the problem of misdiagnosis. Further, rare antibiotic-resistant organisms should not be overlooked since these bacterial infections can lead to several morbidities.

6. ACKNOWLEDGMENTS

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7. CONFLICT OF INTEREST

The authors declared no conflict of interest.

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Research Article

Expression Profiling of Genes Associated with the Pathogenesis of Recurrent Laryngeal Papillomatosis

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Abstract: Recurrent Laryngeal Papillomatosis (RLP) affects the aero-digestive intersection with a predilection for the glottis. It is predominantly a juvenile-onset disease. The main infectious agents are type 6 and 11 low-risk human papillomaviruses. Understanding the genetic changes associated with the pathogenesis of RLP might prove helpful to mark the severity and aggression of the disease and lead to better clinical management. Clinically diagnosed RLP children below the age of 12 years along with a control sample of healthy tissue from age and gender-matched children undergoing tonsillectomy and thyroidectomy were collected. All the samples were processed for total RNA extraction followed by first strand cDNA synthesis. Real-time PCR was done to determine the relative gene expression of EGFR, ER-α, CXCL12, CXCR4, GLUT-1, IGF-1, HIF-1α, VEGF, ERK1/2, PI3K and AKT genes along with GAPDH as gene of reference. An increase in the transcriptional level expression of the genes CXCL12/CXCR4, GLUT-1, EGFR, ER-α, and IGF-I was observed in the cases in comparison to the controls. The expression of HIF-1α, VEGF, PI3K, and AKT genes was not noticeably elevated. The gene expression analysis may open the avenues for possible strategies that can be employed to treat RLP more effectively.

Keywords: Gene Expression Profiling, Human papillomavirus (HPV), Laryngeal Papillomatosis, Transoral Laser Microsurgery (TLM)

1. INTRODUCTION

Patients suffering from Recurrent Laryngeal Papillomatosis (RLP) develop multiple benign papilloma of the respiratory tract that have a particular predilection for the true vocal cord. RLP is the commonest form of benign mesenchymal neoplasm of the larynx categorized by hyperplastic stratified squamous epithelium with fibrovascular core in aerodigestive tract of kids. Moreover, it is the toughest to treat histologic conditions because of high recurrence and its spread in the surrounding respiratory tract [1]. Malignant progression is rare but can be found in papilloma harboring high-risk (HR) human papillomavirus subtypes. The course of human papillomavirus (HPV)-induced recurrent laryngeal papillomatosis is variable and unpredictable. Some patients experience spontaneous remission after one or two surgical procedures while others suffer from recurrent aggressive growths with dire consequences. RLP is a multifaceted ailment and presents with substantially discrete outcomes. Administration through ancillary-assisted therapies has shown remarkable treatment outcomes that reportedly help eradicate or reduce the lesion size and less frequent surgeries [5].

The contagious nature of the disease has only been documented through contact with laser smoke on the laser surgeons and supporting staff at the time of the patient's surgery. HBV 6 and 11 had been found adequately on the gel foams drenched in the operation theater of RLP. However, the virulent viral load was way smaller to cause infectious transmission. The precautionary measures are in line with the SOPs of the surgical area while handling laryngeal papilloma [2].

The modes of transmission for the juvenile-onset include; at the time of childbirth, primigravida, recently acquired genital warts, and longer stages

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of the mother's labor during first baby birth. While in the adult onset of the disease, the speculated transmission mode is oral foreplay and sex [3].

The incidence of laryngeal papillomatosis has been reported as 4.3 and 1.8 per 100,000 in children and adults respectively [4]. Zumaeta-Saavedra et al. reported a case of a 13-year-old male with laryngeal papillomatosis right from the age of 2 years. The hallmark manifestations presented for the disease included respiratory distress and multiple stenosis nodules in the larynx and trachea along with pulmonary cysts. A single dose administration of bevacizumab 400mg and respiratory therapies followed by excision of papillomatous and tracheostomy; yielded promising treatment outcomes without disease relapse in follow-up [6]. On a malignancy comparative evaluation between laryngeal squamous cell carcinoma (LSCC) as compared to Laryngeal Papillomatosis; it was seen that the intensity of nuclear staining of TLR4 was significantly lower in LSCC as compared to Laryngeal Papillomatosis that did not undergo malignant transformation [7]. Another study revealed that HPV-6 viral load in adult-onset laryngeal papillomatosis decreased gradually to zero following several surgeries and intralesional cidofovir therapy. This provides evidence that relapses can be avoided if latent laryngeal HPV reservoirs are eradicated [8]. In RLP, spontaneous molecular changes may be involved in neoplasia. Therefore, underlying genetic mutations contribute to the disease initiation and progression. A mutation from C to T at codon 273 of the P53 gene at CpG dinucleotide was reported for integrating HPV-11 in histologically classified malignant lesions in a 28-year-old symptomatic papillomatosis patient [9].

It is pertinent to mention that there is very little gene expression data available for RLP; though targeted cancer drugs have been used to lessen the papillomatous growth. As a consequence of the HPV infection, different signaling pathways are deregulated.

The present work aims to investigate the deregulated genetic signaling mechanisms underlying the clinically diagnosed RLP children of age less than 12 years. The study focuses on the signaling pathways associated with cell survival and proliferation. Various cell signaling pathways give growth advantage to the rapidly proliferating cells. In accordance with this concept; EGFR, ER-α, CXCL12, CXCR4, GLUT-1, IGF-1, HIF-1α, VEGF, ERK1/2, PI3K and AKT in RLP patients were investigated. This likely deregulation in these gene expressions will help to understand if the overexpression or underexpression of target genes modulates the disease and its severity.

2. MATERIALS AND METHODS

2.1. Subjects

The study was approved for use of tissue biopsy samples of the patient and normal tissue specimens by the ethical review committee (Reference ERC-19/03/02), KRL Hospital, Islamabad, Pakistan. All the study subjects provided the informed consent to participate in the study.

The inclusion criteria comprised of children less than 12 years of age either presenting for the first time or recurrent laryngeal papillomatosis to the ENT department with symptoms of respiratory distress, airway obstruction, and hoarseness of voice who on flexible nasendoscopy had papillomatous growths on the surface of the vocal cords and other laryngeal sites were included in the study. The patients without any clinical hallmark of papillomatous growth on the larynx or vocal cords were excluded from the study.

Biopsies of the laryngeal papilloma of the 19 RLP patients undergoing CO_2 laser evaporation (Figure 1, showing a representation of various stages of surgery from complete obstruction to normal laryngeal inlet) were collected. The control tissue samples, however, were obtained from the larynges of the of age and gender-matched individuals undergoing surgeries like thyroidectomy or tonsillectomy. The samples were stored at -80°C till further use.

2.2. Isolation of RNA And First Strand cDNA Preparation

Complete RNA of the patients' and normal tissue biopsies was isolated by GeneJET RNA isolation kit (Thermo Fisher Scientific, USA) as per the manufacturer's instruction. RNA quantification

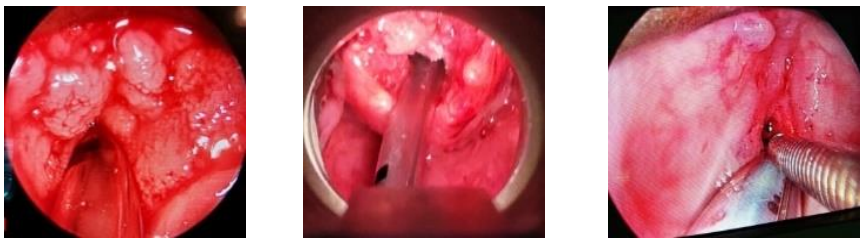

Fig. 1. (A) Severe respiratory obstruction caused by papillomas. (B) During CO_2 laser-assisted evaporation. (C) View at the end of surgery.

was done and First-strand cDNA was synthesized from the extracted RNA by the RevertAid First Strand cDNA Synthesis Kit (K1621; Thermo Fisher Scientific, USA).

2.3. Expression Profiling of Cell Survival Genes

Transcription level relative expression profiling was carried out for target genes EGFR, ER-α, CXCL12, CXCR4, GLUT-1, IGF-1, HIF-1α, VEGF, ERK1/2, PIK3, and AKT along with GAPDH as housekeeping gene. First-strand cDNA was employed for relative gene quantification in RT-PCR kit Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Lithuania) using gene-specific primers according to the standard cycling program described in the user manual of the kit. The RT PCR was carried out on an SLAN96 RT-PCR Machine (Sansure, China).

2.4. Expression Level and Statistical Calculations

The change in the expression of target genes in terms of "fold change" for disease samples relative to normal samples was calculated. The calculation was done by adjusting the GAPDH expression level (as housekeeping) for the target gene expression in disease and normal tissues using the double delta cT method [10]. The positive value indicates an increase in the expression of gene(s) and a negative fold change indicates a decrease in the expression. The fold change value for cases and controls was represented in actual (Figure 2-5) and in logarithmic scale (base 2) log2 (Figure 6). Chi-square was calculated to determine the significance of differential expression in patients as compared to controls. A *p-value* <0.05 was taken as significant for differential expression.

3. RESULTS AND DISCUSSION

Laryngeal obstruction due to extensive growth in the larynx and in some cases, trachea and hypopharynx caused mainly respiratory distress and dysphonia and only occasionally led to swallowing difficulty. The RLP obstruction was removed with transoral CO_2 laser microsurgery under general anesthesia. Expression profiling for EGFR, ER-α, CXCL12, CXCR4, GLUT-1, IGF-1, HIF-1α, VEGF, ERK1/2, PI3K and AKT genes along with the housekeeping gene was quantitated. The relative gene expression analysis between the disease and normal samples showed discrete transcriptional level gene expression changes as presented below:

3.1. Epidermal Growth Factor Receptor (EGFR) and Estrogen Receptor Alpha (ER-α)

Aggressive overexpression of EGFR in 64% and ER-α in 67% of patients was observed in the present study (Figure 2). Association of EGFR has been well-established in many tumors. Likewise, EGFR translational availability has been implicated in patients of this study. EGFR inhibition in Recurrent Respiratory Papillomatosis (RRP) with EGFR inhibitors as an adjuvant therapy has been shown to lower RRP operative frequency and improve the modified Derkay scores and general disease [11].

Estrogen receptors are hormone-activated transcription factors with an important role in carcinogenesis [12]. ERα-positive cases are not only responsive to endocrine therapies but also sensitive to CDK4/6 inhibitors [13-14]. Whereas, ERα-negative tumors are more aggressive and metastatic [15]. Treatment choices of ER-positive cases by reported evidence for other cancers or malignancies can be opted for laryngeal papillomatosis.

3.2. Chemokine Ligand and Its Receptor (CXCL12/CXCR4)

Overexpression of the CXCL12/CXCR4 signaling axis was observed (Figure 3) This favors virus

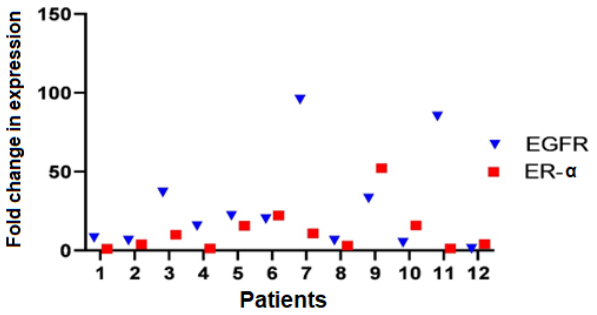

Fig. 2. Relative gene expression of EGFR and ER-α.

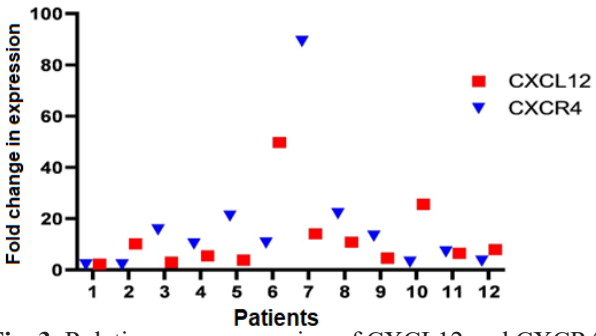

Fig. 3. Relative gene expression of CXCL12 and CXCR4.

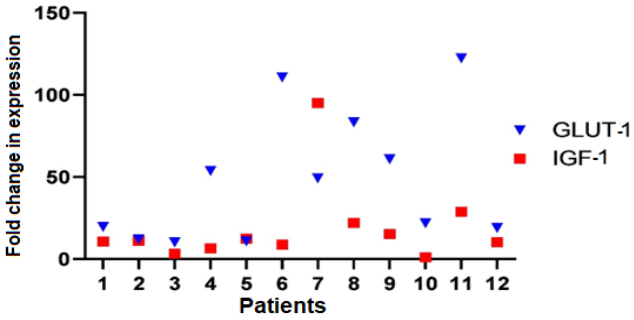

Fig. 4. Relative gene expression of GLUT-1 and IGF-1.

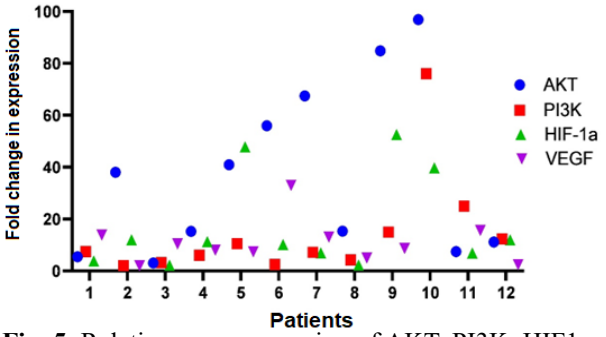

Fig. 5. Relative gene expression of AKT, PI3K, HIF1-α and VEGF.

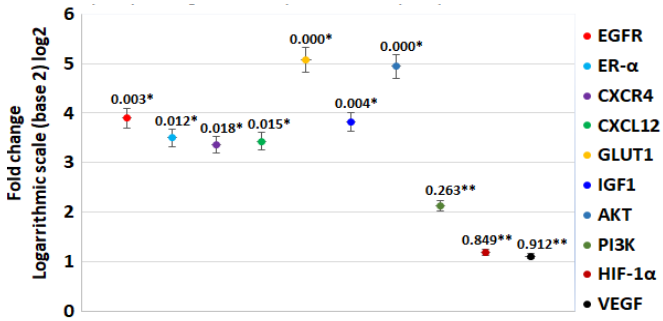

Fig. 6. Comparison of the studied genes in RLP patients and controls.

production and HPV-induced proliferation. This axis has been verified by the gain of function mutation in CXCR4. CXCR4 promotes HPV oncoproteins stabilization; derailing the development and propagation of the host cell cycle, mechanistically knitted with viral genes expression responsible for virus survival [16].

3.3. Insulin-Like Growth Factor 1 (IGF-1) And Glucose Transporter 1 (GLUT-1)

High-grade neoplastic activity was observed in the majority of RLP cases in the present study. An increased metabolite uptake by the proliferating cells is demanded, and tumor cells require the grasping of the nutrients from the environment/ serum. The critical threshold step might be regulated by glucose uptake during the neoplasm and cancer cells metabolic activity for growth. Glucose cannot simply pass the cell membrane; protein channels in the cell membrane namely Sodium-Glucose linked transporters (SGLT), Sugars will eventually be exported transporters (SWEET) and GLUT-1 were identified in mammals. [17-20]. Interestingly the gradient-dependent glucose transport in the absence of ATP hydrolysis is enabled by GLUT-1 only [21]. The current work identified an increased expression of the GLUT-1 gene in 90 percent of patients. GLUT-1 overexpression can be considered as a strategy to increase glucose uptake for unprecedented cell

growth like other cancer cells' hallmark features. A parallel increase in IGF-I has been seen (Figure 4). An evident increase in the transportation of glucose in response to IGF-I-linked stimulation of IGF1R and translocation of GLUT1 has been reported [22].

3.4. Hypoxia-inducible Factor (HIF-1α) – Vascular Endothelial Growth Factor (VEGF), Extracellular Signal-Regulated Protein Kinase 1/2 (ERK1/2) and Phosphatidylinositol-4 (PI3K), 5-Bisphosphate 3-Kinase-Protein Kinase B (AKT)

Enhanced angiogenesis through HIF-1α dependent VEGF expression is the hallmark of tumor vascularization and growth. In the context of the high-risk HPV type 16; E6 and E7 oncoproteins induced an increase in accumulation of HIF-1α protein and subsequently, HIF-1α triggered the expression of VEGF by ERK1/2 and PIK3/AKT. These were suggestive findings observed for increased HIF-1α and VEGF expression in tumor growth of cervical cancer [23]. E6 and E7 oncogenes also inhibit tumor suppressor p53 and Rb [24]. The RLP patient's expression profiling presented relatively surprising data in the case of HIF-1α, VEGF, AKT, and PI3K in the present study (Figure 5). Only in about 2 percent of patients, the said genes were overexpressed while the rest of the cases did not show any aberrant expression.

In RLP, the papilloma causes airway obstruction, which leads to a fall in oxygen saturation levels in spontaneously breathing patients. In this event, patient also retains CO_2 so that their blood CO_2 concentration rises. Hypoxia (low oxygen) and hypercapnia (high carbon dioxide) are concurrently present in the tissue microenvironment in a variety of pathophysiological conditions due to respiratory diseases e.g., obstructive sleep apnea syndrome, pneumonia and chronic obstructive pulmonary disease (COPD) [25-26]. The hypercapnia has been well studied in vivo and in vitro to counter-regulate and suppress hypoxia-induced HIF-1α pathway activation. The mechanism involves CO_2-dependent pH reduction which assists in non-canonical lysosomal degradation of HIF-1α protein (27). This can be the best hypothetical model for the pathophysiology of a current study where HIF-1α and its target genes expression were not overexpressed in the presence of high CO_2 retention in airway-obstructed patients.

3.5. Comparison of Studied Genes in RLP with Controls

The relative gene expression of the oncogenes targeted in the present study revealed concerning disturbances in the genetic transcription levels in RLP in comparison to controls. Expression levels of GLUT1, AKT, EGFR, ER- α, CXCL12/CXCR4, and IGF-1 were found to be upregulated (Figure 6). The overexpression of these genes in laryngeal papillomatosis in the present study suggests that they work to enhance cell growth, survival, and proliferation. However, the expression profiles of HIF-1α, VEGF, and PI3K were not found to be different from the controls. Hence there is a need for controlling the upregulation of specifically GLUT1, AKT, EGFR, ER-α, CXCL 12/ CXCR4, and IGF-1.

4. CONCLUSİONS

This study highlights the important molecular-level mechanisms involved in the progression of RLP. In-depth knowledge of these may prove helpful in limiting the disease. The key targets as understood in the genetic expression profiling include the inhibition of chemokine receptors through targeted therapy, adjuvant therapy by EGFR inhibitors, and ER-α antagonist agents (like Tamoxifen), which are structurally similar to estrogen and used for patients with ER-α overexpression. While the study of expression profiling for cell proliferation and oncogenes in RLP patients revealed the molecular targets to treat, further insight at the level of protein analysis is required to achieve new treatment regimens in the management of this disease condition.

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6. CONFLICT OF INTEREST

The authors declared no conflict of interest associated with this article.

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Conference Recommendations

ANSO-PAS-MAPP Conference 2023: Recommendations & Media Coverage

Muhammad Ali & Zabta Khan Shinwari

1. CONFERENCE RECOMMENDATIONS

The conference participants and resource persons performed brainstorming sessions under group activities and compiled a list of recommendations, which will help tackle cross-species and cross-border transmission of pathogens.

1.1. Establishment of a Global Pathogen Monitoring Network

To enhance global preparedness and response to transnational disease transmission and implement advanced data-sharing technologies and robust analytics for real-time surveillance. The proposed global network should integrate state-of-the-art pathogen detection systems that identify potential outbreaks early. By leveraging big data analytics and artificial intelligence, the network can swiftly analyze trends, detect anomalies, and predict potential hotspots. Moreover, establishing standardized protocols for data sharing and a central repository for global pathogen data would facilitate collaborative approaches to understanding and countering the threats posed by infectious diseases.

1.2. Global Health Certification for International Travelers

It is ensuring the health security of international travelers and preventing disease spread across borders to formulate standardized health certification protocols accepted globally. A comprehensive global health certification framework should include vaccination records and mandatory health screenings for international travelers. Developing an interconnected digital platform to store and share travelers' health information securely is imperative while ensuring data privacy and confidentiality. This system would enable authorities at international entry points to efficiently verify travelers' health statuses and promptly respond to potential health risks.

1.3. Creation of a Global Research Fund for Emerging Pathogens

Accelerating research and development for effective prevention and control strategies to emphasize collaboration between public and private sectors for sustainable funding. A dedicated global research fund can catalyze interdisciplinary research efforts, fostering innovation and the development of novel solutions to combat emerging pathogens. By encouraging public-private partnerships, the fund can leverage both governmental resources and private sector expertise, promoting the translation of scientific discoveries into practical applications. Furthermore, the fund should prioritize long-term sustainability to ensure continuous support for critical research initiatives.

1.4. Standardization of Sanitary Procedures

Promoting consistent and effective pathogen management to prevent cross-border transmission. Facilitate comprehensive training programs for border control officials on best practices. Moreover, to ensure the uniform implementation of sanitary measures, it is essential to establish standardized guidelines and protocols for pathogen control at international borders. Specialized training programs should be conducted regularly to equip border control personnel with the necessary knowledge and skills to handle potential health threats effectively. Additionally, fostering international collaborations for information exchange and capacity-building workshops can strengthen the enforcement of these standardized sanitary procedures.

1.5. Annual Vaccination Programs for Endemic Diseases

Safeguarding animal populations and reducing the risk of pathogen transmission to humans encourage research into developing cost-effective and durable vaccines. Annual vaccination programs should be supported by extensive research and development initiatives to produce affordable and long-lasting vaccines. By investing in advanced vaccine technologies, such as DNA-based vaccines and nanoparticle delivery systems, we can ensure sustained immunity among animal populations. Collaborative efforts between veterinary authorities and research institutions can streamline the integration of these cutting-edge technologies into routine vaccination programs, bolstering overall disease prevention strategies.

1.6. Investigation and Mitigation of Avian-Mediated Pathogens

Understanding and controlling the spread of tick-borne illnesses facilitated by avian populations. Fostering international collaborations in avian ecology research and surveillance to conduct in-depth investigations into the transmission dynamics of avian-mediated pathogens is crucial for designing effective intervention strategies. By promoting international partnerships among research institutions and wildlife conservation organizations, we can gather comprehensive data on avian migration patterns and their potential role in pathogen dissemination. Integrating advanced tracking technologies and remote sensing techniques can provide valuable insights into the complex interactions between avian populations and disease vectors, guiding the development of targeted surveillance and mitigation measures.

1.7. Stringent Safety Standards for zoonotic diseases

Preventing global dissemination of zoonotic diseases through international trade of wild animals and/or their products. Integrate technology for tracking and monitoring product origins and handling, and enforcing stringent safety standards for the trade of animal products necessitates the implementation of robust regulatory frameworks and inspection protocols. Utilizing blockchain technology and digital tagging systems can enable real-time monitoring of product origins and supply chain processes, ensuring transparency and accountability. Moreover, establishing international certification mechanisms for high-quality animal products can foster trust and confidence among trading partners, thereby reducing the risks associated with the cross-border transmission of zoonotic diseases.

1.8. Regulations for Wildlife Markets and to prevent Deforestation

Mitigating the potential spread of diseases in areas of human-wildlife interaction. Advocating for community engagement in sustainable conservation practices, regulating wildlife markets, and addressing deforestation requires a multifaceted approach involving the active participation of local communities and stakeholders. Implementing stringent regulatory frameworks for wildlife trade, coupled with community-driven conservation initiatives, can effectively reduce the risk of disease transmission at the human-wildlife interface. Promoting sustainable land management practices and reforestation efforts can further restore ecological balance and minimize the likelihood of infectious disease emergence in these vulnerable ecosystems.

1.9. Comprehensive Diagnostic Facilities at Key Entry Points

Facilitating early detection and containment of potential pathogen transmission. Establishing comprehensive diagnostic facilities at critical entry points demands robust infrastructure and collaborative partnerships among global health authorities and diagnostic technology providers. Equipping these facilities with state-of-the-art laboratory equipment, rapid diagnostic tests, and skilled personnel can significantly enhance the capacity for timely pathogen detection and containment. Furthermore, fostering international collaborations and resource-sharing initiatives can promote equitable access to diagnostic capabilities, particularly in regions with limited healthcare infrastructure.

1.10. Strengthening Animal Quarantine Facilities at Ports

Preventing the spread of infectious diseases through enhanced quarantine measures. Implement rapid response protocols for emergent infectious outbreaks. Strengthening the capabilities of animal quarantine facilities at ports requires integrating advanced biosecurity measures and efficient response mechanisms. Investing in cutting-edge disinfection technologies and quarantine protocols can minimize the risk of pathogen transmission through imported livestock and animal products. Establishing collaborative frameworks for information exchange and joint response strategies among port authorities and veterinary agencies can enable swift and coordinated actions in the event of potential disease outbreaks, thereby safeguarding global public health.

1.11. Promotion of On-Farm Biosecurity Practices

Minimizing pathogen dissemination across boundaries through farm-level measures. Foster community-based initiatives for knowledge sharing and education. Promoting on-farm biosecurity practices necessitates actively engaging farming communities and agricultural extension services. Farmers can adopt proactive steps to prevent the introduction and spread of infectious diseases among livestock and agricultural produce by providing comprehensive training and educational resources on biosecurity protocols. Encouraging the establishment of community-based biosecurity networks and knowledge-sharing platforms can facilitate the dissemination of best practices and the adoption of innovative biosecurity technologies, bolstering overall disease control efforts at the grassroots level.

1.12. Integration of interdisciplinary approaches

Understanding and combatting multi-species viral diseases through a holistic approach to support interdisciplinary research and training programs in Health (concerning plants, animals, humans, and the environment). One example could be integrating the One Health philosophy into global health policies requires concerted efforts to bridge the gap between human, animal, and environmental health disciplines. It can promote a comprehensive understanding of the interconnectedness between ecosystem health and disease dynamics by fostering interdisciplinary collaborations and knowledge exchange platforms. Establishing specialized training programs and research fellowships in this direction can cultivate a skilled workforce capable of addressing complex health challenges at the human-animal-environment interface, thereby fostering sustainable and integrated disease management strategies.

1.13. Climate-Resilient Measures for Disease Control

Addressing the impact of climate change on disease transmission dynamics. Developing climate-resilient measures for disease control requires proactive planning and strategic collaborations between public health authorities and environmental agencies. Implementing adaptive healthcare infrastructure and early warning systems can mitigate the risks posed by climate-induced shifts in disease distribution and prevalence. By fostering partnerships for climate adaptation research and resource allocation, we can develop targeted interventions that enhance community resilience and reduce the vulnerability of ecosystems to emerging infectious diseases, ultimately fostering sustainable and adaptive disease management practices.

1.14. Accelerated Development of New Vaccines

Promoting equitable access to innovative vaccines for emerging zoonosis/infections. Accelerating the development of new vaccines for emerging diseases demands collaborative efforts and investment in cutting-edge vaccine technologies. Establishing international consortia for vaccine research and development can facilitate the pooling of resources and expertise, expediting the discovery and production of effective vaccines. Promoting regulatory harmonization and transparent vaccine distribution frameworks can ensure equitable access to life-saving vaccines globally, reducing health disparities and promoting global health security in the face of evolving infectious disease threats.

1.15. Implementation of Stricter Regulations Against Illicit Wildlife Trade

Mitigating the risks associated with the illegal trade of wildlife and animal products. Strengthen international enforcement and intelligence-sharing mechanisms. Implementing stricter regulations against illicit wildlife trade demands robust enforcement mechanisms and international cooperation to combat transnational criminal networks. Enhancing border surveillance and intelligence-sharing networks can facilitate the identification and apprehension of wildlife traffickers, thereby reducing the potential risks of disease transmission associated with the illegal wildlife trade. Fostering partnerships between law enforcement agencies and conservation organizations can further amplify efforts to dismantle illicit wildlife trade networks and promote the sustainable conservation of biodiversity and ecosystems.

1.16. Monitoring and Control Protocols for Antibiotic Use

Mitigating the emergence of antibiotic-resistant diseases through responsible antibiotic use and promoting public awareness campaigns on the prudent use of antibiotics. Establishing monitoring and control protocols for antibiotic use requires comprehensive regulatory frameworks and public awareness initiatives to promote responsible antimicrobial stewardship. Implementing stringent prescription policies and antimicrobial resistance surveillance programs can effectively regulate the use of antibiotics and minimize the risks of antimicrobial resistance development. Educating the public and healthcare professionals on the importance of judicious antibiotic use through targeted awareness campaigns can foster a culture of responsible antibiotic management, safeguarding public health from the threat of antibiotic-resistant pathogens.

1.17. Implementation of Comprehensive Antimicrobial Stewardship Programs

Mitigating the escalation of antimicrobial resistance and promoting the prudent use of antibiotics in healthcare, animal farming, and agriculture. Establish robust surveillance systems to monitor antimicrobial resistance patterns globally. Implement educational campaigns for healthcare providers, veterinarians, and farmers to raise awareness about responsible antibiotic usage. Foster research and development for novel antimicrobial agents and alternative therapies to combat resistant pathogens effectively. Invest in developing diagnostic tools for rapidly and accurately identifying resistant pathogens across different ecosystems.

1.18. Development of Plant-Pathogen Surveillance and Early Warning Systems

Enhancing global capacity to detect and respond to emerging plant diseases that threaten food security and agricultural productivity. Implement remote sensing technologies and satellite imagery for real-time monitoring of crop health and disease outbreaks. Strengthen international partnerships for information-sharing and capacity-building initiatives in plant pathology research. Promote the adoption of integrated pest management strategies and disease-resistant crop varieties to mitigate the impact of plant pathogens on global food production.

1.19. Investment in Research and Development of Broad-Spectrum Antiviral Therapies

Addressing the challenges of viral infections by developing effective and versatile antiviral treatments. Support collaborative research programs focusing on discovering and characterizing broad-spectrum antiviral agents. Encourage the exploration of innovative therapeutic approaches, such as nanotechnology-based drug delivery systems and gene-editing technologies. Foster public-private partnerships to expedite the translation of research findings into clinically viable antiviral therapies.

1.20. Integration of Genetic Sequencing and Bioinformatics in Pathogen Surveillance

Enhancing the identification and characterization of emerging infectious agents through advanced genomic analysis and data-driven approaches. Invest in the development of high-throughput sequencing technologies and bioinformatics tools for real-time pathogen identification and tracking. Establish global databases for genetic sequences

of known pathogens to facilitate comparative genomics and evolutionary studies. Provide training programs and capacity-building initiatives for healthcare professionals and researchers in the application of genomic surveillance for infectious disease control.

1.21. Promotion of Sustainable Agriculture Practices for Disease Control

Encouraging the adoption of environmentally friendly and sustainable farming methods to reduce the incidence and spread of agricultural pathogens. Advocate for implementing organic farming techniques and agroecological principles to minimize the use of chemical pesticides and fertilizers. Support research and innovation in developing bio-based alternatives for plant disease management. Facilitate knowledge-sharing platforms and farmer education programs on integrated disease management strategies and soil health preservation.

1.22.Strengthening Biosafety and Biosecurity Measures in Research Laboratories

Ensuring the safe handling and containment of hazardous pathogens to prevent accidental releases and laboratory-acquired infections. Establish stringent regulatory frameworks and compliance standards for biosafety practices in biomedical and agricultural research facilities. Conduct regular inspections and risk assessments to identify potential biosecurity vulnerabilities and gaps in containment protocols. Provide specialized training and certification programs for laboratory personnel on biosafety protocols and emergency response procedures.

2. ACKNOWLEDGEMENTS

We are thankful to all the guests, speakers, participants, facilitators, repertoires, and organizers for contributing to the event. We also extend our heartfelt gratitude towards the students of the Infectious Diseases and Molecular Pathology Lab (IDMPL) and Molecular Systematics and Applied Ethnobotany Lab (MoSAEL). We are also grateful to the Alliance of International Science Organizations (ANSO) and the Pakistan Academy of Science (PAS) for their support and financial assistance.

3. MEDIA COVERAGE

ANSO-PAS-MAAP conference on Pathogen transmission beyond borders: Understanding the complexities of cross-species infectious diseases got huge media coverage.

Some of the links are as follows.

1. https://dailyfrontierstar.com/abdulla-malik-ampampamp-chairman-hec-dr-mukhtar-inaugurate-anso-pas-maap-conference-on-pathogen-transmission-beyond-borders-understanding-the-complexities-of-cross-species-infectious-disease/
2. https://en.wenews.pk/pakistan-minister-hec-chairman-inaugurate-pathogen-conference/
3. https://sabahnews.net/english/news/abdulla-malik-chairman-hec-dr-mukhtar-inaugurate-anso-pas-maap-conference-on-pathogen-transmission-beyond-borders-understanding-the-complexities-of-cross-species-infectious-diseases/
4. https://leadpakistan.com.pk/news/minister-hec-chairman-inaugurate-pathogen-conference/
5. https://www.technologytimes.pk/2023/10/17/intl-conference-on-pathogen-transmission-beyond-borders-held-in-pakistan/
6. https://gwadarpro.pk/1714167679105728514/conference-on-cross-border-pathogen-transmission-inaugurated
7. https://islamabadpost.com.pk/minister-hec-chairman-inaugurate-pathogen-conference/#:~:text=Mukhtar
8. https://mnfsr.gov.pk/NewsDetail/
9. MTgyNjBjMDEtZjlmYS00MjU0LWE1YmYtZTRjYzg4YTIzOGRm

Instructions for Authors

Manuscript Writing

The manuscript may contain a Title, Abstract, Keywords, INTRODUCTION, MATERIALS AND METHODS, RESULTS, DISCUSSION (or RESULTS AND DISCUSSION), CONCLUSIONS, ETHICAL STATEMENT (if applicable), ACKNOWLEDGEMENTS, CONFLICT OF INTEREST and REFERENCES, *and any other information that the author(s) may consider necessary*.

Title (Bold and font size 16): The title should be expressive, concise, and informative to the entire readership of the journal. It may include common terms, to make it more identifiable when people search online. Please avoid the use of long pervasive terms and non-standard or obscure abbreviations, acronyms, or symbols.

Abstract (font size 10, max 250 words)**:** Must be self-explanatory**,** stating the rationale, objective(s), methodology, main results, and conclusions of the study. Abbreviations, if used, must be defined on the first mention in the Abstract as well as in the main text. Abstracts of review articles may have a variable format.

Keywords (font size 10)**:** Provide five to eight keywords consisting of words and phrases that are closely associated with the topic depicting the article.

INTRODUCTION (font size 11)**:** Provide a clear and concise statement of the problem, citing relevant recent literature, and objectives of the investigation. Cite references in the text by number in square brackets**,** the reference must be cited in a proper English sentence [1]. or "... as previously described [3, 6–8]". For a single author: Bednorz [2] investigated the environmental pollution ... When there are only two authors: Bednorz and Allan [2] investigated the environmental pollution ... and for three or more authors: Bednorz *et al.* [2] investigated the environmental pollution ..; and list them in the REFERENCES section, in the order of citation in the text.

MATERIALS AND METHODS (font size 11)**:** Provide an adequate account of the procedures or experimental details, including statistical tests (if any), concisely but sufficiently enough to replicate the study. Relevant references to methodology must be cited.

RESULTS (font size 11)**:** Be clear and concise with the help of appropriate Tables, Figures, and other illustrations. Data should not be repeated in Tables and Figures but must be supported with statistics. The data presented in Tables and Figures must be elaborated in the main text.

DISCUSSION (font size 11)**:** Provide interpretation of the RESULTS in the light of previous relevant studies, citing published references.

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2. A. Fayyaz, N. Ali, Z.A. Umar, H. Asghar, M. Waqas, R. Ahmed, R. Ali, and M.A. Baig. CF-LIBS based elemental analysis of Saussurea simpsoniana medicinal plant: a study on roots, seeds, and leaves. *Analytical Sciences* 40(3): 413-427 (2024).

3. W. Bialek and S. Setayeshgar. Cooperative sensitivity and noise in biochemical signaling. *Physical Review Letters* 100: 258–263 (2008).

b. Books

4. W.R. Luellen (Ed.). Fine-Tuning Your Writing. *Wise Owl Publishing Company, Madison, WI, USA* (2001).

5. U. Alon and D.N. Wegner (Eds.). An Introduction to Systems Biology: Design Principles of

Biological Circuits. *Chapman & Hall/CRC, Boca Raton, FL, USA* (2006).

c. Book Chapters

6. M.S. Sarnthein, J.E. Smolen, and J.D. Stanford. Basal sauropodomorpha: historical and recent phylogenetic developments. In: The Northern North Atlantic: A Changing Environment. P.R. Schafer and W. Schluter (Eds.). *Springer, Berlin, Germany* pp. 365–410 (2000).

7. S. Brown and L.A. Boxer. Functions of Europhiles. In: Hematology, (4^{th} ed). W.J. Williams, E. Butler, and M.A. Litchman (Eds.). *McGraw Hill, New York, USA* pp. 103–110 (1991).

d. Reports

8. M.D. Sobsey and F.K. Pfaender. Evaluation of the H_2S method for Detection of Fecal Contamination of Drinking Water. Report No.-WHO/SDE/WSH/02.08. *Water Sanitation and Health Programme, WHO, Geneva, Switzerland* (2002).

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10. L.M. Highland and P. Bobrowsky. The landslide handbook–A guide to understanding landslides. Circular 1325. *US Geological Survey, Reston, Virginia* (2008).

    https://pubs.usgs.gov/circ/1325/pdf/C1325_508.pdf

f. Conference Proceedings

11. M. Khalid, A.B. Majid, F. Mansour, and C.R. Smith. Word Representations with Recursive Neural Networks for Morphology. *27^{th} European Conference on Signal Processing, (2^{nd} - 6^{th} September 2021), Madrid, Spain* (2021).

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12. M. Afzal. Investigation of structural and magnetic properties of nanometallic Fe-Mn Alloys. Ph.D. Thesis. *Quaid-i-Azam University, Islamabad, Pakistan* (2023).

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