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ETIOLOGY, CLINICAL MANIFESTATIONS, AND AGE CHARACTERISTICS OF MIXED VIRAL RESPIRATORY INFECTIONS WITHIN 2024-2025

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ABSTRACT

Introduction: This study aims to investigate mixed viral respiratory infections, examining their causes, age patterns, and the involvement of co-pathogens in the lower respiratory tract infections (LRTIs).

Materials and methods: A total of 2825 nasopharyngeal samples were collected from April 2024 to March 2025. Multiplex real-time PCR assays were used to test for 13 respiratory viruses, while capillary electrophoresis was used to determine viral load.

Results*:* This study identified 180 (6.4%) cases of coinfections with respiratory viruses, predominantly involving bocaviruses (BoVs) (51%), rhinoviruses (RVs) (38.8%), and influenza A viruses (34.4%). Children under 5 had the highest mixed infection rate (13.5%), especially involving respiratory-syncytial virus (RSV), rhinoviruses (RVs), adenoviruses (AdV), and bocaviruses (BoVs). The co-infection rate in patients with bronchiolitis was significantly higher than that in patients with infections restricted to the nose, sinuses, and pharynx (p=0.0006). RSV was most commonly associated with bronchiolitis cases (54%). Children under 2 years with mixed infections faced a greater risk of developing lower (58%) versus upper respiratory tract infections (URTIs) (39%) (p = 0.0276). Additionally, in co-infected patients with lower respiratory tract complications (LRTCs), SARS-CoV-2 had higher viral loads compared to those of other co-pathogens.

Conclusion: This study found a relatively low rate of mixed respiratory viral infections, with BoVs RVs, and influenza A viruses being the main causative agents. Children under two years of age are particularly vulnerable and are at higher risk of severe complications such as bronchiolitis. Understanding the interactions of these viruses is essential for developing effective treatments and prevention strategies for this at-risk group.

Keywords*:* acute respiratory infections, respiratory viruses, co-pathogens, lower respiratory tract infections

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1. INTRODUCTION

Acute respiratory infections (ARI) are a significant public health problem, as they are a common cause of doctor visits, hospitalization, and mortality. Studies have shown that age 65+ and the presence of comorbidities may be risk factors for severe disease or death [1]. Influenza viruses cause epidemics every year and affect patients of all ages. The elderly, children under 5 years, as well as persons with concomitant chronic diseases or weakened immune system are particularly vulnerable to influenza infections, and annual revaccination is recommended for these risk groups [2]. With its emergence in 2019, SARS-CoV-2 caused a pandemic of unprecedented scale, affecting millions of people worldwide. This pandemic is historically comparable to the Spanish Flu pandemic of 1918 in terms of its lethality [3]. Like influenza infections, a risk factor for developing severe COVID-19 is age over 65 years in combination with comorbidities [4], Other respiratory viruses, such as respiratory syncytial virus (RSV), rhinoviruses (RVs), human metapneumovirus (hMPV), and adenovirus (AdV), can cause serious complications such as bronchiolitis and pneumonia, especially in children under 5 years of age, often leading to hospitalization [5,6,7,8]. RSV is of particular concern because it is the

leading cause of death in this age group [9]. RVs not only affect the upper respiratory tract (URT) , but also cause severe LRTCs such as acute bronchitis, bronchiolitis, or pneumonia[10]. Children infected with hMPV can develop severe bronchiolitis and pneumonia, which require hospitalization, admission to the intensive care unit, and intubation [11]. In addition, hMPV has been associated with serious health problems in people with asthma and chronic obstructive pulmonary disease (COPD) [12]. On December 29, 2024, Chinese health authorities reported a surge of hMPV cases in the country's northern provinces in the previous weeks. During this major outbreak, the virus was shown to be capable of causing severe respiratory disease, with children under 14 years of age carrying large amounts of infectious virus [13] (https://www.who.int/emergencies/disease-outbreak-news/item/2025-DON550.

A large number of respiratory pathogens (viral, bacterial, fungal) cause similar clinical manifestations, necessitating the use of modern molecular multiplex diagnostic tests to differentiate between different pathogens accurately [14] (Goka, 2015). The application of multiplex diagnostic methods increasingly reveals the possibility to detect more than one pathogen in the same clinical sample [15]. The occurrence of secondary bacterial infections has been described in the literature, often after a previous infection with an influenza virus or SARS-CoV-2 [16]. There are three types of interactions between viruses in cases of mixed infections: homologous interactions, which involve at least two viruses belonging to the same family (e.g. RSV and hMPV); interactions that involve two viruses of the same genus, known as heterotypic interactions (e.g. H1N1 and H3N2 influenza viruses), and interactions between viruses of different families, known as heterologous interactions (e.g. SARS-CoV-2 and influenza virus) [17]. The proportion of mixed bacterial-viral coinfections is reported to be 46%, while that of viral-viral coinfections is 35% of all respiratory infections [18,19].

There is evidence that some viruses cause more common coinfections than others, such as BoVs, AdVs, and RVs [20]. In contrast, influenza A and B, as well as SARS-CoV-2, have been shown to co-infect with other respiratory viruses at rates ranging from 14% - 20% [21,22], and 3% - 8%, respectively [23,24,25,26]. Despite the low rate of confirmed mixed infections with influenza and SARS-CoV-2, co-infections involving these two pathogens can aggravate the course of the disease [27]. In our previous study from 2022, we supported the thesis that patients over 65 years of age with mixed infections have a higher risk of severe COVID-19 and death compared to monoinfected patients from the same age group [28].

The influence of age on the clinical severity and frequency of coinfections has not been completely clarified. The effects of viral coinfections on clinical symptoms and disease progression remain poorly understood. It is of utmost importance to gain a deeper understanding of how these viral coinfections influence disease severity. This study aims to analyze the prevalence, and etiology of viral respiratory infections occurring during 2024-2025. Furthermore, it seeks to determine how the presence of an additional co-pathogen may contribute to the development of LRTCs.

2. MATERIALS AND METHODS

2.1. Population Survey and Sampling

Between April 2024 and March 2025, a total of 2,825 patients with ARI were included in the study. All samples were collected in the course of monitoring the spread of influenza and other respiratory viruses across Bulgaria. The study group comprised outpatients from 217 sentinel sites, as well as hospitalized patients from all 28 districts throughout the country. Nasopharyngeal swabs were collected using viral transport media and transported to the National Laboratory "Influenza and ARI" under refrigerated conditions at 4°C. Before shipping, the samples were stored at 4°C for a maximum of 72 hours. Upon arrival at the laboratory, samples were processed on the same day; if that was not possible, they were stored at -80°C until analysis.

Exclusion criteria

All submitted samples that did not adhere to the respiratory sample collection and transport conditions described above were excluded from the study. Additionally, patients who did not show symptoms of respiratory infection were also excluded.

Nucleic acid extraction and reverse transcription polymerase chain reaction (RT-PCR)

Viral DNA and RNA were extracted by an automated extraction system using the ExiPrep Dx Viral DNA/RNA kit (Bioneer, Daejeon, Republic of Korea) according to the manufacturer's instructions. The QuantStudio™ 5 real-time PCR system, 96-well (ThermoFisher

Scientific), was utilized for amplification.

Screening for influenza and non-influenza respiratory viruses was performed using Multiplex real-time RT-PCR assays with the TaqPath 1-Step Multiplex Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) for the detection of common respiratory viruses: SARS-CoV-2, influenza viruses A and B, RSV, hMPV, parainfluenza viruses (PIV) types 1,2,3, RV, AdV, and BoV. A combination of primers and probes marked with different fluorescent labels was used to prepare four master mixes for the simultaneous detection of 13 respiratory viruses. The preparation and usage of these primers and probes were detailed in our previous publication [29].

Amplification was carried out using a thermal cycler CFX96 (Bio-Rad Laboratories Inc. in Hercules, CA, USA). When it was smaller than < 38, the value of the cycle threshold (Ct) was deemed positive.

Quantitative methods

Viral load of detected viral pathogens in patients with LRTCs and co-infections was determined by an indirect viral load determination method, quantitative capillary electrophoresis. The QIAxcel Advanced capillary system was used to implement the method. The method was developed to amplify genomic targets of individual respiratory viruses using the SuperScript III Platinum One-Step qRT-PCR system (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and appropriate primers. To determine the amplicon concentration (ng/µl) and the length of the amplified region of the viral genome [base pairs (bp)], we used capillary electrophoresis. A formula was used to convert the concentration of viral amplicons to viral copies, as reported in our previous publication [28].

3. RESULTS

Characteristics of studied patients

During the study period, a total of 2825 patients were tested for the presence of SARS-CoV-2, influenza A and B, and eight other respiratory viruses. Among these patients, 1059 (37.4%) were outpatients, while 1766 (62.5%) were hospitalized. The age of the patients ranged from 10 days to 98 years, with a median age of 9 years. In terms of gender, 1395 (49.3%) patients were male, and 1430 (50.7%) were female.

Etiology of detected co-infections.

Out of all tested patients, 1559 (55.2%) were positive for at least one respiratory virus. The breakdown of positive results was as follows: 882 (31.2%) samples tested positive for influenza A and B viruses, namely 204 (7.2%) for A(H1N1)pdm09, 358 (12.7%) for A(H3N2), and 320 (11.3%) for B/Victoria; 253 (8.9%) for RV, 174 (6.2%) for BoV, 162 (5.7%) for RSV, 106 (3.8%) for SARS-CoV-2, 82 (2.9%) for AdV, 38 (1.4%) for PIV-1, 31 (1.1%) for hMPV, 18 for PIV2 (0.6%), and 9 (0.3%) for PIV3. Additionally, 180 (6.4%) cases of co-infections with respiratory viruses were detected, involving combinations of two, three, and four viruses, affecting 168 patients, 11 patients, and 1 patient, respectively. The most frequently detected viruses in coinfections were BoVs (n=92; 51%), followed by RVs (n=70; 38.8%), influenza A (n=62; 34.4%), RSV (n=42; 23.3%), AdV (n=36; 20%), and influenza B (n=32; 17.7%). Unlike them, SARS-CoV-2 (n=22; 12.2%), PIV1 (n=6; 3.3%), PIV2 (n=7; 3.8%), PIV3 (n=3; 1.6%), and hMPV (n=1; 0.5%) were infrequently involved in coinfections. The distribution of individual co-infections is shown in Figure 1. The most frequently detected combinations were A(H3N2)+BoV, B/Victoria+BoV, RV+AdV and RV+BoV.

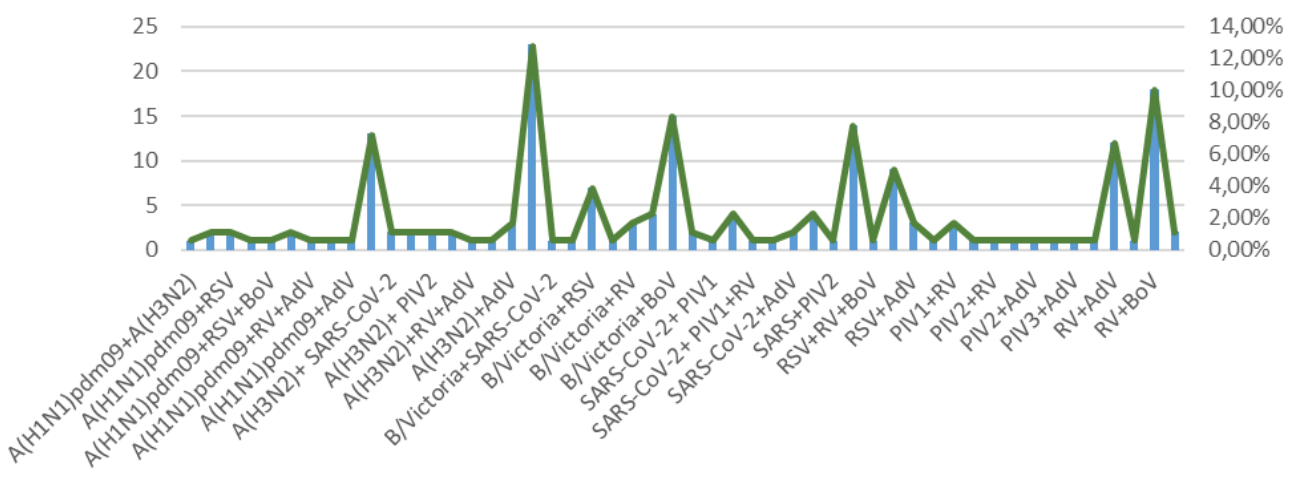


Figure 1. Number (%) of proven mixed infections involving different respiratory viruses.

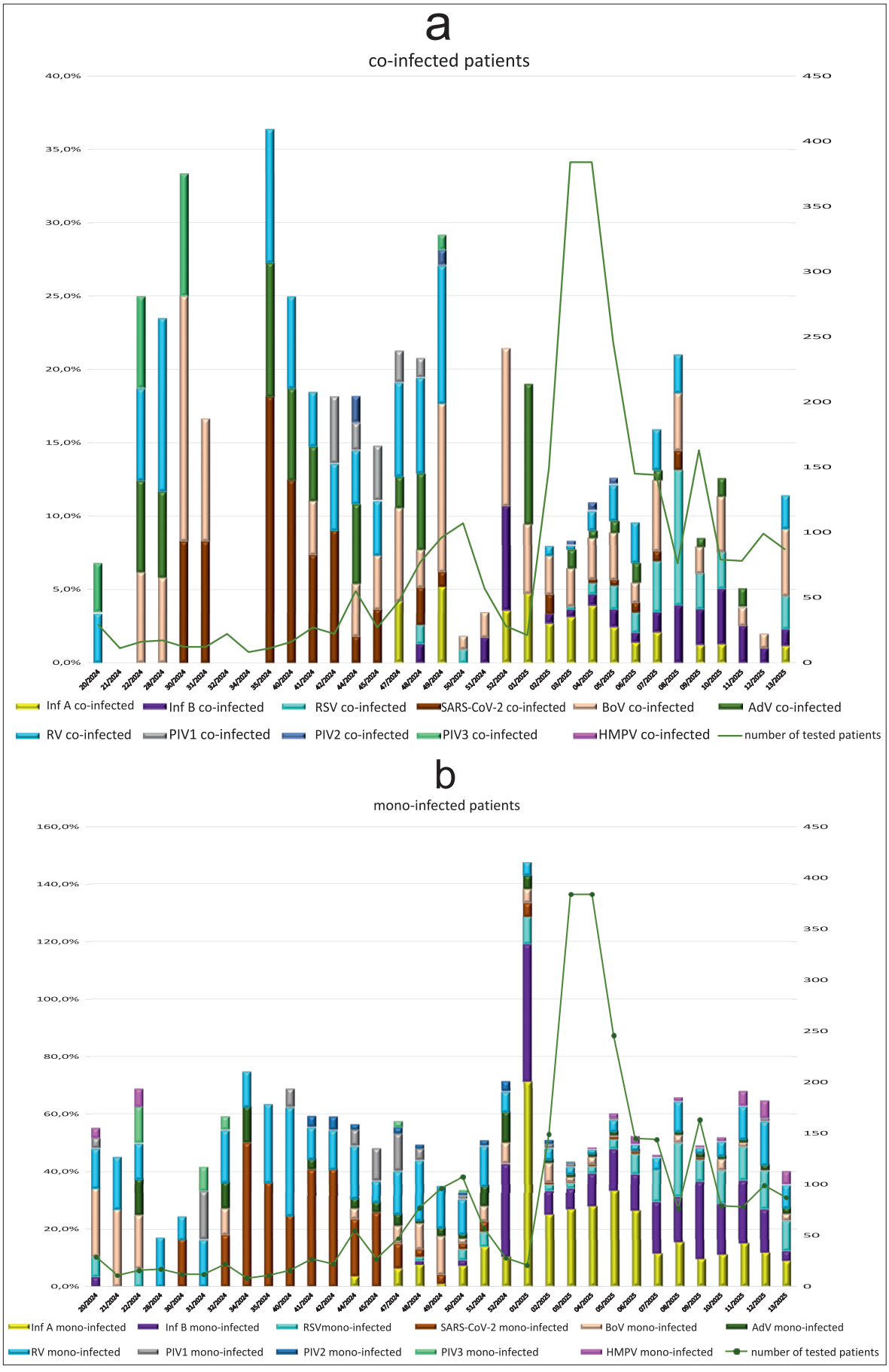


Figure 2. Weekly distribution of detected respiratory infections for the period April 2024 - March 2025.
a) Distribution of confirmed respiratory viral co-pathogens in co-infected patients.
b) Distribution of confirmed respiratory viruses in mono-infected patients.

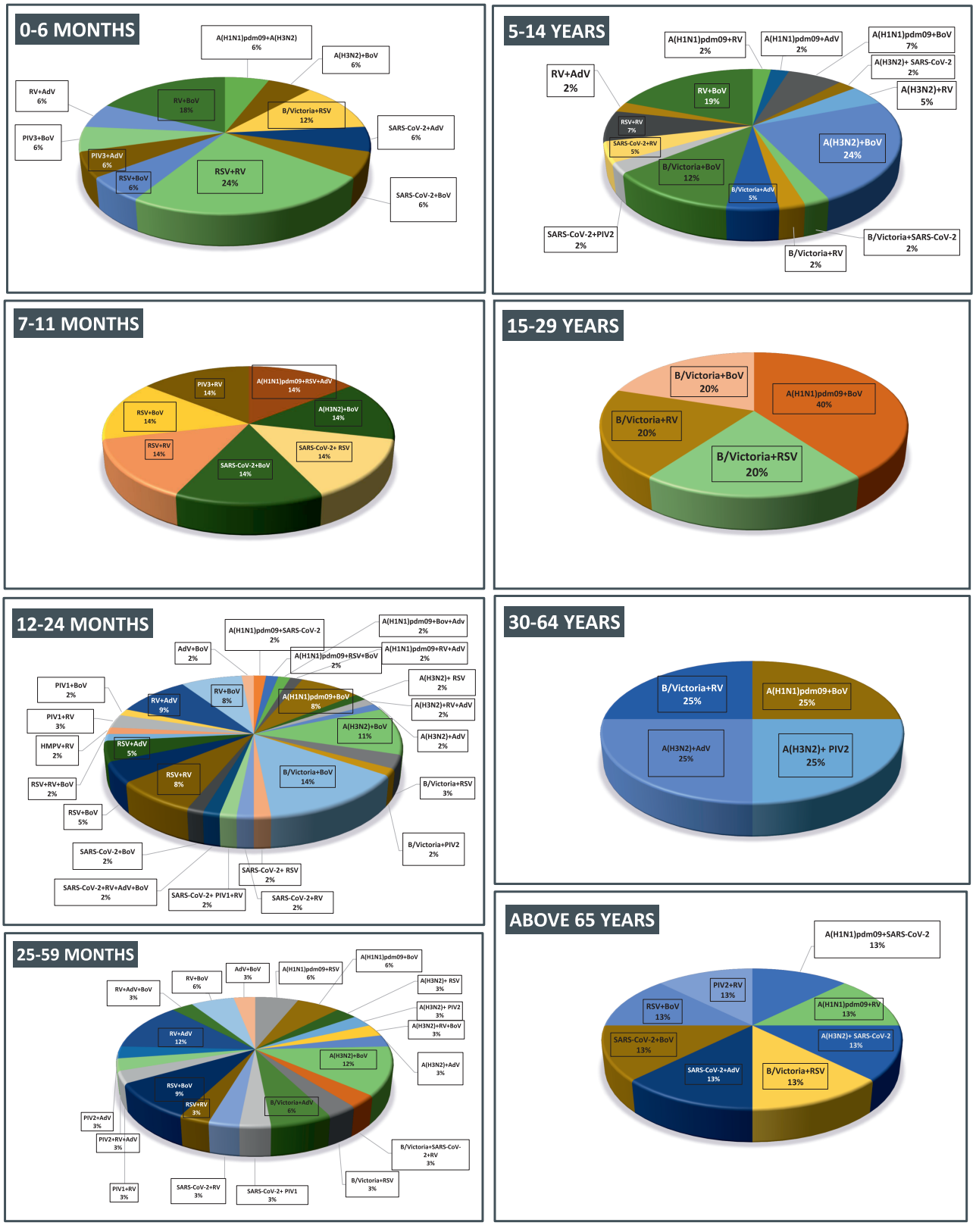


Figure 3. Distribution of identified coinfections across eight age groups: 0-6 months, 7-11 months, 12-24 months, 25-59 months, 5-14 years, 15-29 years, 30-64 years, and 65 years and older.

Seasonal distribution of detected coinfections

The highest proportion of co-infections with respiratory viruses was observed between week 52 of 2024 and the first week of January 2025. This spike correlated with a rise in confirmed influenza and RSV infections. Another peak in the number of co-infections occurred in the fall of 2024, specifically in weeks 40 - 41. This increase was driven by a rise in confirmed cases of SARS-CoV-2 and RV infections. From Figures 2a and 2b it can be seen that coinfections with influ-

enza A, RSV, PIV1, and RV were observed at least one week after the initial appearance of monoinfections with these pathogens. In contrast, for influenza B, SARS-CoV-2, AdV, and RSV, we found monoinfections occurring within the same week as the onset of coinfections. Furthermore, we noted a coincidence in the peaks of evidence for both coinfections and monoinfections among all eight identified respiratory viruses, including SARS-CoV-2, influenza A, and influenza B.

Age distribution of detected co-infections

Children under 5 years of age had the highest rate (13.5%) of mixed infections, compared to older patients , who had a rate of 3.5% ($p < 0.0001$). The highest rate (17%) of proven co-infections occurred in the age group 12 - 24 months. A statistically significant difference was observed compared to other age groups, except for babies aged 0 - 6 months, who had a rate of 12.4% ($p < 0.05$). The co-infection rates for different age groups were as follows: 7.7 % for those aged 7 - 11 months, 11% for those aged 25 - 59 months, 4.5% for those aged 5 - 14 years, 2.6% for those aged 15 - 29 years, 1.6% for those aged 30 - 64 years, and 2.2% for those aged 65 and older. Coinfections with RSV, RV, AdV, and BoV were more common in children under 5 years of age as compared with older children (1.9% vs. 0.4% for RSV, 2.8% vs. 1.3% for RV, 1.7% vs. 0.3% for AdV, and 4% vs. 1.8% for BoV, with $p < 0.05$). Among these, coinfections with RSV were th most prevalent in the youngest children, especially those aged 0-6 months, where 5.8% of 137 infants had coinfection with RSV. In contrast, children aged 12–24 months had the highest proportion of mixed infections involving RV, AdV, and BoV, at 6.8%, 4.1%, and 9.8%, respectively, among the 366 children examined in this age group. The distribution of different combinations of coinfections in the 8 age groups

Clinical significance of mixed respiratory-viral infections

Out of 2,825 patients tested, 1,266 (44.8%) were diagnosed with ARI of the URT, and 105 (3.7%) of them developed complications, such as laryngitis and laryngotracheitis. Furthermore, 656 (23.2%) patients experienced complications affecting the lower respiratory tract, and 51 (1.8%) patients had central nervous system (CNS) symptoms. Among those diagnosed with ARI, 75 (5.9%) individuals had confirmed co-infections with respiratory viruses (see Table 1). The incidence of complications among patients with mixed respiratory viral infections was as follows: laryngitis occurred in 7 (6.7%) patients, bronchitis in 4 (7.4%), bronchiolitis in 24 (13.7%), pneumonia in 21 (4.9%), and encephalitis in 1 (8.3%).

Notably, the share of patients who developed bronchiolitis and had confirmed mixed respiratory viral infection was significantly higher than that of patients diagnosed with ARI of the URT (nose, sinuses,

Table.1 Distribution of co-pathogens among patients with acute respiratory infection (ARI) affecting the lower and upper respiratory tracts (URT), as well as the central nervous system (CNS).

	Number (%) of co-infected patients					
	Upper Respiratory Tract Infection (URTI)		**Lower Respiratory Tract Infection (LRTI)**			**CNS infections**
	Upper respiratory tract, including the nose, sinuses, pharynx,	Upper respiratory tract, including the larynx or trachea	Pneumonia	Bronchitis	Bronchiolitis	Encephalitis
Number of co-infected patients with URTI or LRTI	75	7	21	4	24	1
Respiratory viruses, involved in cases of co-infections						
RSV	14(19)	2(29)	5(24)	0(0)	13(54)	0(0)
AdV	12(16)	2(29)	5(24)	0(0)	5(21)	0(0)
BoV	39(52)	4(57)	12(57)	2(50)	11(46)	1(100)
hMPV	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
RV	20(27)	1(14)	8(38)	3(75)	9(38)	1(100)
PIV1	0(0)	1(14)	1(5)	0(0)	0(0)	0(0)
PIV2	2(3)	1(14)	1(5)	1(25)	1(4)	0(0)
PIV3	1(1)	0(0)	0(0)	0(0)	0(0)	0(0)
A(H1N1)pdm09	16(21)	0(0)	5(24)	0(0)	1(4)	0(0)
A(H3N2)	22(29)	1(14)	2(10)	1(25)	3(13)	0(0)
B/Victoria	22(29)	0(0)	4(19)	0(0)	3(13)	0(0)
SARS-CoV-2	5(7)	1(14)	2(10)	0(0)	1(4)	0(0)

Table. 2. Age distribution of co-infected patients with acute respiratory infection (ARI) affecting the lower and upper respiratory tract (URT), as well as the central nervous system (CNS).

Age of patients	Number (%) of co-infected patients with		
	*URTI n=128	*LRTI n=50	*CNSC n=1
0-6 months	4 (4.8)	5 (10)	0 (0)
7-11 months	3 (3.6)	3 (6)	0 (0)
12-24 months	25 (30.4)	21 (42)	0 (0)
25-59 months	19(23.1)	8 (16)	0 (0)
5-14 yeurs	21 (25.6)	10 (20)	1 (100)
15-29 yeurs	4 (4.8)	1 (2)	0 (0)
30-64 yeurs	5 (6)	0 (0)	0 (0)
<65 yeurs	1(1.2)	2 (4)	0 (0)

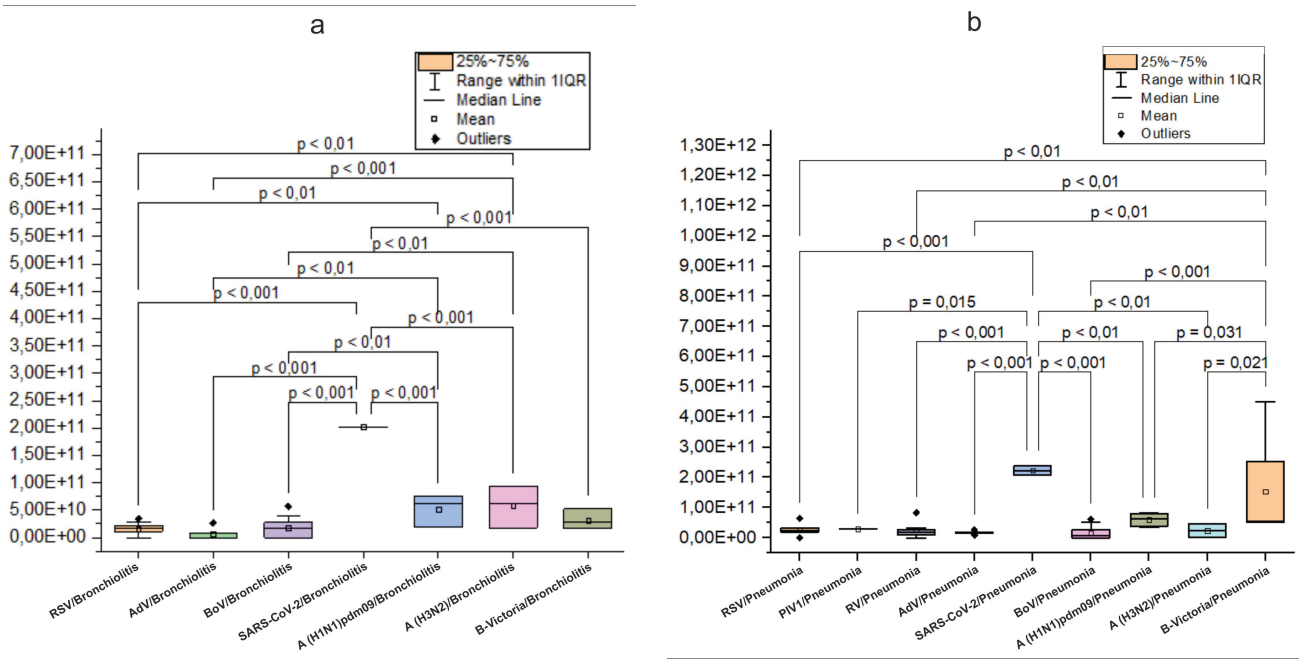
* abbreviations: lower respiratory tract infection (LRTI); upper respiratory tract infection (URTI); central nervous system complication (CNSC)

and pharynx), with a significance level of p=0.0006. Furthermore, the incidence of co-infections among patients with bronchiolitis was higher than that in patients with pneumonia, with a significance level of p=0.0005. Among the various co-pathogens, RSV was the most frequently identified in patients with bronchiolitis (54%), compared to those diagnosed with ARI of URT (19%), with a significance level of p=0.0013.

Children under the age of 2 who had confirmed mixed infections involving two or more respiratory viruses were significantly more likely to develop LRTIs at a rate of 58%, compared to only 39% of those who had URTIs. This difference was statistically significant (p= 0.0474). No similar trends were noted in other age groups (see Table 2). Additionally, only one 5-year-old child with a mixed infection involving RV and BoV experienced a CNS complication.

Viral load of patients with overt concomitant infections and LRTCs

Of the 177 patients identified with coinfections, 49 (27.7%) had LRTCs. Viral load analysis for viruses involved in coinfections was performed on 45 of these patients. We compared viral loads for nine confirmed co-pathogens: RSV, PIV1, RV, AdV, BoV, SARS-CoV-2, A(H1N1)pdm09, A(H3N2), and B/Victoria. Figures 4 (a) and 4 (b) show that in co-infected patients with bronchiolitis, SARS-CoV-2 had significantly higher vi-



Figures 4. Distribution of viral load in co-infected patients: a) patients with bronchiolitis and co-infected with respiratory syncytial virus RSV, adenovirus AdV, bocavirus BoV severe acute respiratory syndrome coronavirus-2 SARS-CoV-2,influenza A(H1N1)pdm09, influenza A(H3N2) and influenza B/Victoria; b) patients with pneumonia and co-infected with respiratory syncytial virus RSV, parainfluenza virus 1 PIV1, rhinovirus RV, adenovirus AdV, severe acute respiratory syndrome coronavirus-2 SARS-CoV-2,), bocavirus BoV, influenza A(H1N1)pdm09, influenza A(H3N2) and influenza B/Victoria. Viral load was determined using capillary electrophoresis and a formula for converting concentrations to viral copies per milliliter. Mean values of viral copies per milliliter are represented by the sign (•). Mean values of viral copies per milliliter (▫), median (—), and range 25–75%, and distribution of viral copies (|) are shown. Values were calculated using the Mann-Whitney U-test.

ral loads as compared to other co-pathogens such as AdV, RSV, and BoV ($p<0.05$). Influenza A viruses A(H1N1)pdm09 and A(H3N2) found in mixed infections had significantly higher mean viral loads as compared to other co-pathogens, such as AdV, RSV, influenza B-Victoria, and BoV ($p<0.05$). We did not observe statistically significant differences between the mean viral loads, measured in viral copies per milliliter, for other combinations of respiratory viruses. A similar trend was observed in patients with pneumonia; the viral load of SARS-CoV-2 was significantly higher than that of all other co-infecting viruses, except for influenza B/Victoria ($p<0.05$). We found significantly higher viral loads for influenza B/Victoria compared to influenza A(H1N1)pdm09 and A(H3N2) viruses, as well as RSV, RV, AdV, and BoV in patients with pneumonia and confirmed mixed infections with these co-pathogens.

4. DISCUSSION

The COVID-19 pandemic has accelerated the development of molecular diagnostics of respiratory viruses and has encouraged the use of a multiplex approach for the simultaneous detection of multiple respiratory pathogens [30,31,32]. This approach allows us to assess the impact of a wider range of respiratory pathogens that cause upper and LRTIs. The current study offers valuable information on the mechanisms of occurrence and spread of mixed respiratory viral infections and assesses their association with respiratory and CNS complications. Researchers from Slovenia conducted a study using a multiplex approach for the detection of respiratory viruses, reporting a 76% detection rate of respiratory infections, with a 16% occurrence of mixed infections [33]. In contrast, our study found a lower detection rate of respiratory viruses, 55%, with only 6.4% of cases classified as mixed infections. A separate 10-year study conducted in Germany reported a similar rate of mixed infections of 7.3% [34].

This study identified influenza A, RV, and BoV as the most commonly detected pathogens in both mixed and monoinfections. A study in China conducted between 2023 and 2024 showed that RV was the most common respiratory co-pathogen, accounting for 30.8% of all confirmed mixed infections [35]. Other investigators have also indicated that BoV is a common cause of mixed infections [36,37]. Although some authors have found that influenza viruses are not frequently associated with mixed infections [21], our findings rank influenza A as the leading cause of coinfections with other respiratory viruses. This discrepancy may be due to the high incidence of reported influenza A virus infections. Therefore, we can infer a correlation between the increased detection of a given respiratory virus and the higher incidence of mixed infections involving that virus.

The monthly distribution of detected respiratory mono- and co-pathogens in this study is consistent with established epidemiological knowledge that respiratory viruses tend to peak during colder months [38]. Understanding these trends is critical for effective preparedness and intervention during periods of high transmission [39].

Age-specific patterns show a strong link to mixed infections, indicating that children under five years old are frequently co-infected with multiple respiratory pathogens [18]. This increased susceptibility may be explained by their less mature immune systems and undeveloped hygiene habits. Furthermore, it is important to consider the role of closed groups of children, such as those in kindergartens, as a significant factor in the spread of different respiratory pathogens, contributing to these mixed infections [40]. In children aged 0 - 6 months, RSV is the most common cause of infection and is a significant contributor to infant mortality in this age group [41,42]. Our finding of a high rate of mixed RSV infections in these infants is alarming and concerning. This is particularly important given the potential for increased disease severity in vulnerable populations like infants and young children [43]. Mixed infections are more challenging to diagnose and treat, leading to longer hospital stays and a higher risk of complications [44]. Studies have shown that BoV is a common pathogen in children between 12 and 24 months of age [45]. For AdV and RV, there is evidence that infections are frequently detected in children over one year of age [46,47]. These observations are consistent with our findings of increased rate of coinfections involving BoV, AdV, and RV in children aged 1 - 2 years. We can conclude that the high rates of respiratory viral infections observed in certain age groups follow certain age patterns and correlate with an increased incidence of coinfections involving these pathogens.

The results of this study suggest that mixed respiratory viral infections are more closely associated with the development of bronchiolitis than with pneumo-

nia. This association may be explained by the higher incidence of mixed infections in young children and the fact that bronchiolitis is the most common complication resulting from respiratory infections in infants and children under 2 years of age [48]. Recent studies have shown that mixed infections may significantly increase the risk of LRTIs and hospitalizations among pediatric patients [49,50]. However, earlier studies have contradicted these findings, reporting no significant association between mixed infections and the severity of respiratory illness in children [51,52]. Notably, this particular study highlights that children under the age of two, especially those infected with RSV along with an additional respiratory co-pathogen, are at a heightened risk of developing bronchiolitis [53] (https://www.cdc.gov/rsv/about/index.html). Although RSV is the main cause of bronchiolitis, respiratory viruses such as RV and hMPV can also cause bronchiolitis. In addition, coinfections, in which multiple viruses are present simultaneously, are relatively common in patients with bronchiolitis [54,55]. The implications of these coinfections remain an area of active investigation, as researchers strive to better understand how they may influence clinical outcomes.

To further investigate the impact of individual co-pathogens on LRTCs, our study demonstrates that high viral loads of influenza viruses and SARS-CoV-2 were more commonly linked to serious syndromes as bronchiolitis and pneumonia. Additional studies on viral load confirmed that SARS-CoV-2 and influenza viruses typically present with higher viral loads as compared to other co-pathogens involved in mixed infections [28,29]. Studies investigating BoV infections, and in particular the ability of the virus to persist in human body for extended periods, have shown that patients with monoinfections tend to exhibit higher mean BoV viral loads compared to those experiencing co-infections with other pathogens [49]. This observation is further supported by findings that reveal a correlation between lower mean BoV viral loads and the incidence of LRTCsin patients. Specifically, individuals exhibiting significant respiratory problems had lower viral titers, suggesting potentially persistent BoV infection.

This study has several highlighted strengths, but it also has important limitations. One significant limitation is its focus on a single year, which limits the possibility to observe seasonal patterns in the incidence of mixed infections. Additionally, we did not collect more detailed clinical and laboratory data, which could have enhanced the clinical analysis.

CONCLUSION

In comparison to other studies, we discovered a lower rate of mixed infections with respiratory viruses. We also identified three predominant viral agents involved in these mixed infections: BoVs, RVs, and influenza A viruses. Importantly, our findings indicate that children under two years of age are the most vulnerable age group facing an increased risk of these coinfections, which can lead to severe complications such as bronchiolitis, a potentially serious respiratory illness. A deeper understanding of the interaction of viruses involved in mixed infections is crucial for developing effective treatment strategies and adapting preventive measures to protect the most vulnerable pediatric populations.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contributions

IT and DP conceptualized, designed the study, and wrote the manuscript; PV, II, DI, TV and TT were responsible for patient enrolment, assessment, and selection; IT, DP, and NK performed the experiments; IT, DP, NK, DI, PV, TV, II and TT analysed the work results; NK IC reviewed the final manuscript. All authors read and approved the final manuscript.

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MULTIORGAN FAILURES IN COVID-19 PATIENTS AND THEIR ASSOCIATION WITH ADVERSE OUTCOMES

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ABSTRACT

Objectives: Organ and kidney failures often occur in patients with severe COVID-19. This study set out to assess organ and kidney failures in COVID-19 patients and their correlation with poor outcomes.

Methods: This retrospective analytical study involved 311 unvaccinated COVID-19 patients admitted at hospital between April and August 2021. Patients' clinical and laboratory information were statistically analyzed. Severity of organ dysfunction was examined using the sequential organ failure assessment (SOFA) score, and kidney dysfunction was assessed using renal parameters and Kidney Disease: Improving Global Outcomes (KDIGO) criteria.

Results: Of the COVID-19 patients, 20.6% (n=64) had kidney dysfunction with common signs of albuminuria [68 (21.8%)] and hematuria [56 (18%)]. Older age, comorbidities, need of mechanical ventilation, chronic kidney disorders, higher SOFA scores, hypoxemia, lymphopenia, albuminuria, and hematuria all associated with COVID-19 severity (P<0.05). The mortality rate was 10%, noting a higher mortality risk in patients with severe infection. The mentioned factors, especially older age, chronic liver/biliary disease, higher SOFA, and lower PaO_2/FiO_2 ratio were independently related to high risk of mortality (P<0.05). However, the zero mortality rate in non-severe group indirectly highlights the dominant role of infection severity for patients' outcomes and the link between the survival and organ failures.

Conclusions: Organ and kidney failures were key indicators of severe COVID-19 and risk of death. However, the severity of COVID-19 remained the paramount factor influencing both survival status and its association with organ and kidney dysfunction. Monitoring these factors can help the sorting and management of patients according to risk.

Keywords: COVID-19 Severity; Kidney Failure; Organ Failure

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1. INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (*SARS-CoV-2*), the cause of COVID-19 pandemic, has dramatically affected global health, resulting in organ failure and negative consequences [1, 2]. Entering cells through ACE2 receptor, the virus induces cell damage, hyperinflammation, endothelial dysfunction, microvascular thrombosis, and tissue hypoxia, impacting several organs simultaneously. Particularly in patients with pre-existing conditions, systemic inflammation can cause multi-organ failure resulting from oxidative stress, coagulation and metabolic abnormalities [2-4]. Activation of the renin-angiotensin-aldosterone system adds to vasoconstriction and renal ischemia, thus worsening organ failure [5–7]. The Sequential Organ Failure Assessment (SOFA) score, which includes renal function, is useful for predicting patient outcomes [8].

Given the major impacts of *SARS-CoV-2* on various organs, this study aimed to examine organ and kidney dysfunction in COVID-19 patients and its correlation with negative outcomes and laboratory results. The results can help us better understand the pathophysiology of COVID-19 and find predictors of serious complications, enabling more efficient management, improving clinical outcomes, and lower the mortality rate.

2. METHODS

2.1. Study design and population

Data taken from the medical records of COVID-19 patients who visited the Emergency Department of Sina

Hospital in Ahvaz, Iran, between April 19, 2021, and August 20, 2021, were used in this retrospective, single-center cohort study. The study was authorized by the ethics committee of university and Sina Hospital in Ahvaz, and adhered to the guidelines set forth in the 2018 Declaration of Helsinki and its subsequent revisions (1401.1.20).

2.2. Inclusion and exclusion criteria

The study included unvaccinated patients with confirmed *SARS-CoV-2* infection, determined by positive results on the real-time reverse transcription polymerase chain reaction (RT-PCR) test and chest CT scan, and typical COVID-19 symptoms. Patients with incomplete medical records or those transferred to other hospitals were excluded from the study.

2.3. Study approach and measured parameters

Simple random sampling was applied using the lottery method whereby the main investigator assigned each patient's medical record a unique code to minimize bias. Those codes were placed in a box, and samples were drawn randomly. Investigators gathered patients' information daily for statistical analysis, including demographics, comorbidities, clinical/vital signs, and laboratory results upon admission.

All COVID-19 patients were categorized into two groups: critically ill patients and non-severe cases, based on the World Health Organization (WHO) severity criteria. Those criteria included: severe involvement of lung lobes or a CT severity score greater than 4, acute respiratory distress syndrome (PaO2/FiO2 ≤100 mmHg), multiple organ failure, prolonged hospitalization lasting more than seven days, significantly elevated WBC and neutrophil counts, lymphopenia, cytokine storm, severe dyspnea, fatigue, and, in some cases, diarrhea and anorexia [9].

Acute kidney injury (AKI) was described using the Kidney Disease Improving Global Outcomes (KDIGO) clinical practice guidelines as an increase in serum creatinine of ≥0.3 mg/dL within 48 hours or serum creatinine elevation to ≥1.5 times baseline within seven days or urine output less than 0.5 mL/kg/hour for six hours for all patients. The diagnosis of AKI in instances where urine output information was unavailable depended solely on serum creatinine alterations [10].

The Sequential Organ Failure Assessment (SOFA) score evaluates six organ systems: respiratory (PaO2/FiO2), coagulation (Platelets), liver (Bilirubin), cardiovascular, renal (Creatinine/Urine Output), and neurological system. The score ranges from 0 to 24, with a higher score indicating more severe organ failure. A SOFA score above 18 is indicative of multi-organ failure [11, 12].

2.4. Main and secondary outcomes

The main outcomes were the evaluation of organ and kidney dysfunction, the relationship between the degree of organ dysfunction (SOFA score) and survival, as well as assessment of the significant predictors of mortality related to COVID-19. The secondary objective was to explore the relationship of medical markers with the disease severity which enables assessment of disease progression.

2.5. Sample size calculation

Given the proportion of acute kidney injury (AKI: 37.2%) in a recent analogous study by Milani et al [13], a margin of error (d) equal to 0.08 with α= 0.05, N≈ 141 patients here calculated with the following formula: $\mathbf{N = ((Z_{1-}\alpha/_2)^2 \times P(1- P))/ d^2}$

Where: Z1−α/2=1.96 (for 95% confidence), P=0.372 (prevalence of AKI)

Yet, to maximize the validity of the results, all eligible patients who presented to the hospital were included, giving a final sample of 311 participants.

2.6. Statistical analysis

Statistical analysis was conducted using SPSS 26 (SPSS, Inc., IL, USA). Variables were presented as mean and standard deviation (SD) or frequency. The chi-square test for categorical or t-test for numerical parameters were employed to assess differences between subgroups. Univariate and multivariate logistic regression analyses were utilized to explore the associations between variables. Additionally, univariate and multivariable Cox models were applied to identify independent prognostic factors of survival. A p-value of less than 0.05 was considered statistically significant.

3. RESULTS

Out of the total 426 patients who presented with COVID-19 symptoms to the Emergency Department of Razi Hospital, 311 unvaccinated patients met the inclusion criteria for clinical and laboratory evaluation.

3.1 Demographics and baseline clinical and laboratory characteristics

Among 311 patients, 44.7% (n=172) were male and 55.3% (n=139) were female, 62.7% (n=195) had comorbidities, 28% (n=87) had severe infection, while

Table 1. Baseline clinical and laboratory characteristics of COVID-19 patients and initial comparative analyses between subgroups.

Variables	All patients (n=311)	Min_Max range	Non-Severe Group (n=224)	Severe Group (n=87)	P-value1	Alive Group (n=280)	Deceased Group (n=31)	P-value2
	Mean ± SD / Frequency (%)		Mean ± SD /Frequency (%)			Mean ± SD /Frequency (%)		
Gender: Male	172 (55.3)	_	121 (54.0)	51 (58.6)	0.545	152 (54.3)	20 (64.5)	0.37
Female	139 (44.7)	_	103 (46.0)	36 (41.4)	_	_	_	_
Age	55.33± 16.54	15_95	52.19 ± 15.70	63.44 ± 15.95	0.0001*	53.73 ± 15.72	69.81 ± 16.97	0.0001*
Comorbidities: Hypertension	195 (62.7) 103 (33.2)	_	117 (52.2) 59 (26.3)	78 (89.7) 44 (50.6)	0.0001* 0.001*	169 (60.4) 77 (27.5)	26 (83.9) 26 (25.2)	0.018 0.7
Cardiovascular diseases	94 (30.3)	_	40 (17.9)	54 (62.1)	0.0001*	74 (26.4)	20 (64.5)	0.001*
Diabetes	73 (23.6)	_	30 (13.4)	43 (49.4)	0.0001*	55 (19.6)	18 (58)	0.0001*
Chronic kidney disorders	64 (20.6)	_	24 (10.7)	40 (46.0)	0.0001*	45 (16.1)	19 (61.3)	0.0001*
Neurological Disorders (Migraine headaches, Encephalitis, Epilepsy and Seizures, Stroke)	61 (19.5)	_	20 (8.9)	41 (47.1)	0.0001*	50 (17.9)	11 (35.5)	0.0001*
Chronic liver and biliary disease	20 (6.4)	_	10 (4.5)	10 (11.5)	0.05	13 (4.6)	7 (22.6)	0.0001*
Asthma	15 (4.7)	_	4 (1.78)	11 (12.6)	0.001*	10 (3.6)	5 (16.1)	0.005
COPD	4 (1.3)	_	0 (0)	4 (4.6)	0.03*	0 (0)	4 (13)	0.001*
Cancer	3 (≈1)	_	0 (0)	3 (≈3.5)	0.04*	1 (0.35)	2 (6.45)	0.02*
Severity of Disease: Severe Non severe	 87 (28) 224 (72)	_	_	_	_	57 (20.35)	30 (96.77)	0.0001*
Mechanical ventilation	107 (34.4)	_	20 (8.9)	87 (100.0)	0.0001*	77 (27.5)	30 (96.8)	0.0001*
Albuminuria No Moderately increased Severely increased	 243 (78.1) 44 (14.1) 24 (7.7)	_	32 (14.3)	36 (41.4)	0.0001*	53 (18.9)	15 (48.4)	0.0001*
Hematuria non Microscopic hematuria Gross hematuria	 255 (82) 47 (15.1) 9 (2.9)	_	16 (7.1)	40 (46.0)	0.0001*	37 (13.2)	19 (61.3)	0.0001*
Mortality rate	31 (10)	_	1 (0.45)	30 (34.50)	< 0.0001*	_	_	_
Hospital Stay (Day)	6.44± 4.02	1_22	6.16 ± 3.72	7.18 ± 4.64	0.067	6.65 ± 4.09	4.55 ± 2.72	0.0001*
GCS	14.39 ±1.087	11_15	14.92 ± 0.27	13.06 ± 1.24	0.0001*	14.67 ± 0.70	11.94 ± 0.81	0.0001*
SOFA Score: Minimal organ dysfunction Mild organ dysfunction Moderate organ dysfunction	3.33± 2.24 283 (91) 18 (5.8 %) 10 (3.2 %)	1_12	2.44 ± 1.29	5.64 ± 2.51	0.0001*	2.82 ± 1.55	8.00 ± 2.14	0.0001*
eGFR	81.19± 23.52	15_103	88.01 ± 13.42	63.63 ± 33.10	0.0001*	84.17 ± 20.21	54.32 ± 33.07	0.0001*
PaO2: 75 to 100 mm Hg	69± 7.64	50_85	72.40 ± 5.21	60.24 ± 5.70	0.0001*	70.64 ± 6.00	54.23 ± 4.30	0.0001*
FiO2	0.25± 0.066	0.21_0.55	0.23 ± 0.02	0.33 ± 0.08	0.0001*	0.24 ± 0.03	0.41 ± 0.09	0.0001*
PaO2/FiO2 ratio	286.88± 75.49	90.91_404.76	321.99 ± 48.64	196.51 ± 54.21	0.0001*	303.14 ± 58.80	140.07 ± 44.77	0.0001*
WBC (10*3µL)	8± 4.80	1.30_50.50	7.11 ± 3.48	10.32 ± 6.65	0.0001*	7.72 ± 4.03	10.61 ± 8.90	0.084
RBC (µL)	4.28± 0.67	2.21_6.29	4.36 ± 0.63	4.07 ± 0.75	0.001*	4.31 ± 0.67	4.04 ± 0.72	0.058
Neutrophiles (%)	69.05± 11.97	26.30_93.80	66.34 ± 11.14	76.08 ± 11.14	0.0001*	68.45 ± 11.76	74.60 ± 12.41	0.012*
Lymphocytes (%)	24.33± 11.45	1.80_67.90	27.15 ± 10.80	17.07 ± 9.79	0.0001*	25.05 ± 11.23	17.83 ± 11.51	0.002*
Hb (g/dL)	12.25± 1.99	7.30_17.90	12.52 ± 1.94	11.58 ± 1.98	0.0001*	12.34 ± 1.96	11.48 ± 2.10	0.037*
Hct (%)	35.41± 5.56	20.60_48.80	36.07 ± 5.35	33.73 ± 5.76	0.001*	35.60 ± 5.51	33.72 ± 5.82	0.095
MCV (Fl)	83.07± 7.2	56.60_112.80	82.86 ± 7.04	83.64 ± 7.62	0.407	82.98 ± 7.24	83.95 ± 6.87	0.464
PLT (10*3/uL)	203.96± 90.38	33_614	206.99 ± 91.31	196.20 ± 88.00	0.338	207.34 ± 90.63	173.48 ± 83.45	0.04*
ESR (Ml/heart beat)	43.68± 25.83	5_140	40.68 ± 24.87	51.41 ± 26.79	0.002*	43.43 ± 25.64	45.97 ± 27.82	0.631
AST	43.76± 29.88	10_261	41.86 ± 29.60	48.66 ± 30.23	0.076	42.87 ± 29.58	51.84 ± 31.89	0.143
ALT	30.15± 37.23	6_430	30.17 ± 38.90	30.13 ± 32.77	0.993	30.77 ± 38.90	24.55 ± 14.56	0.079
Direct bilirubin	0.31± 0.42	0.08_5.20	0.26 ± 0.20	0.46 ± 0.72	0.012*	0.28 ± 0.25	0.65 ± 1.09	0.067
Total Bilirubin (TSB) mg/dl	1.08± 0.56	0.10_3.90	1.04 ± 0.52	1.20 ± 0.67	0.046*	1.07 ± 0.55	1.17 ± 0.74	0.471
BUN, serum (mg/dl)	25.41± 22.56	5_151	21.32 ± 17.78	35.95 ± 29.31	0.0001*	23.18 ± 19.57	45.61 ± 35.07	0.001*
Creatinine (mg/dl)	1.19± 0.82	0.30_9.50	1.06 ± 0.50	1.53 ± 1.27	0.001*	1.11 ± 0.58	1.94 ± 1.78	0.016*

* P-value <0.05 is significant. eGFR: estimated Glomerular Filtration Rate; GCS: Glasgow Coma Scale; Sequential Organ Failure Assessment (SOFA) Score. COPD: Chronic Obstructive Pulmonary Disease

P-value1 signifies comparison of variables between severe and non-severe COVID-19 patients using t-test or chi-square tests.

P-value2 signifies comparison of variables between alive and deceased patients using t-test or chi-square tests.

Some patients had more than one underlying condition.

72% (n=224) had non-severe infection. The most common comorbidities in COVID-19 patients were hypertension, cardiovascular disease, diabetes, chronic kidney disease, and neurological disorders , and these diseases were more evident in patients with severe infection (p<0.05). Also, 34.4% (n=107) required mechanical ventilation, and 20.6% (n=64) had kidney dysfunction. The majority of patients [283 (91%)] had minimal organ dysfunction, indicating mild or no significant organ failure, while a smaller proportion [18 (5.8%)] experienced mild dysfunction and 3.2% (n=10) had moderate organ dysfunction. Albuminuria was observed in 21.8% (n=68) and hematuria in 18% (n=56) of patients. Thirty patients [31 (≈10%)] died. The average length of hospital stay was 6.44 ± 4.02 days. (Table 1).

3.2 Gender-based differences in clinical profile and outcomes

There was no significant difference in mean age between the two sexes (p = 0.915). Overall, 58.6 % (n=51) of males and 41.4 % (n=36) of females had severe infection without any significant difference (p= 0.526). The rate of comorbid conditions was significantly higher in females [97 (≈70%)] than males [98 (57%); p=0.025, OR (95% CI):1.744 (1.088_2.794)]. However, there was no significant difference in the rates of kidney disorders (p= 0.999), liver biliary disease (p= 0.361), mechanical ventilation (p=0.9), and Glasgow Coma Scale (GCS) score (p = 0.428) between the two sexes. At the same time, the mean SOFA score was significantly higher in males (3.66 ± 2.33) compared to females (2.93 ± 2.07; p= 0.004). There was no significant differences in the rates of albuminuria (p= 0.95) and hematuria (p= 0.784) between the two sexes. The mortality rate was higher in males (11%) as compared to females (8%), but without significance (p= 0.441). There was no significant difference in the length of hospitalization between the two sexes, either (p= 0.604).

3.3 Indicators of disease severity

In order to determine the significant variables associated with COVID-19 severity and mortality, the status of variables was first examined between subgroups using initial comparative analyses (e.g., t-test; chi-square; Table 1). Then, a set of variables for inclusion in the logistic and Cox regression models was chosen based on a few criteria: significance in the primary comparison (p< 0.05), no significant multicollinearity (VIF < 5), clinical relevance or importance, and possibility for interpretation. When some multiple variables were correlated to one another, only the most clinically meaningful variable was selected in order to minimize redundancy and enhance the clarity of the model.

Based on the univariate logistic regression analyses, several factors were associated with disease severity including: older age, comorbidities, use of mechanical ventilation, chronic kidney disorders (CKD), higher SOFA Score, hypoxemia, lymphopenia, albuminuria, and hematuria. However, multivariate logistic regression analyses revealed that only comorbidities (OR [95% CI]: 12.0 [4.16 – 34.82], p <0.0001), use of mechanical ventilation (OR [95% CI]: 129.0 [25.8 – 645.8], p= 0.0001), higher SOFA Score (OR [95% CI]:

Table 2. Univariate and multivariate Cox regression analyses for identifying factors associated with overall survival in COVID-19 patients.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	Adjusted HR	95% CI	P-value
Age, yr	1.06	1.03 – 1.08	< 0.0001*	1.01	0.99 – 1.02	0.344
Comorbidities (any)	2.81	1.08 – 7.33	0.034*	0.94	0.51 – 1.70	0.829
Severity of Disease	71.39	9.73 – 523.86	< 0.0001*	19.152	2.261_162.254	0.007*
Chronic kidney disorders	6.12	2.96 – 12.64	< 0.0001*	1.13	0.55 – 2.33	0.748
Chronic liver/biliary disease	6.51	2.78 – 15.21	< 0.0001*	3.06	1.26 – 7.44	0.014*
Mechanical ventilation	45.61	6.21 – 335.27	< 0.0001*	1.34	0.69 – 2.59	0.387
SOFA Score (per point)	1.80	1.60 – 2.02	< 0.0001*	1.25	1.11 – 1.40	0.0001*
PaO_2/FiO_2 ratio	0.95	0.94 – 0.97	< 0.0001*	0.99	0.99 – 1.00	0.0001*
Hematuria	7.23	3.50 – 14.95	< 0.0001*	1.19	0.56 – 2.53	0.644
Albuminuria	3.42	1.68 – 6.94	< 0.0001*	0.86	0.46 – 1.63	0.648
Lymphocyte % (per %)	0.94	0.91 – 0.98	0.0035*	1.00	0.98 – 1.02	0.916
Hb (g/dL)	0.846	0.707_1.012	0.067	_	_	_
PLT (10*3/uL)	0.995	0.990_1	0.053			
Serum Creatinine (mg/dL)	1.72	1.39 – 2.11	< 0.0001*	1.05	0.83 – 1.34	0.672
BUN (mg/dL)	1.02	1.01 – 1.03	< 0.0001*	1.00	0.99 – 1.02	0.562

* P-value <0.05 is significant.

The wide CI reflects the fact that one group (the non-exposed group to specific variable) has no events (deaths) or only a few, which leads to a very large HR and a wide confidence interval. This is a typical issue in survival analysis when a group has no events.

2.03 [1.21 – 3.40], p= 0.007), and lymphopenia (OR [95% CI]: 0.86 [0.79 – 0.94], p= 0.001) were independently associated with disease severity.

3.4 Predictors of mortality

Overall, 30 patients died during hospitalization. The majority of deaths occurred between days 5 and 15 of the hospital stay (Figure 1). Cox regression analyses showed that in addition to disease severity, advanced age, chronic liver/biliary disease, a greater SOFA score, and lower PaO_2/FiO_2 ratio were strongly associated with a higher risk of mortality (Table 2). At the same time, the limited number of deceased cases in non-severe group indirectly highlighted the importance of disease severity as a dominant factor influencing survival.

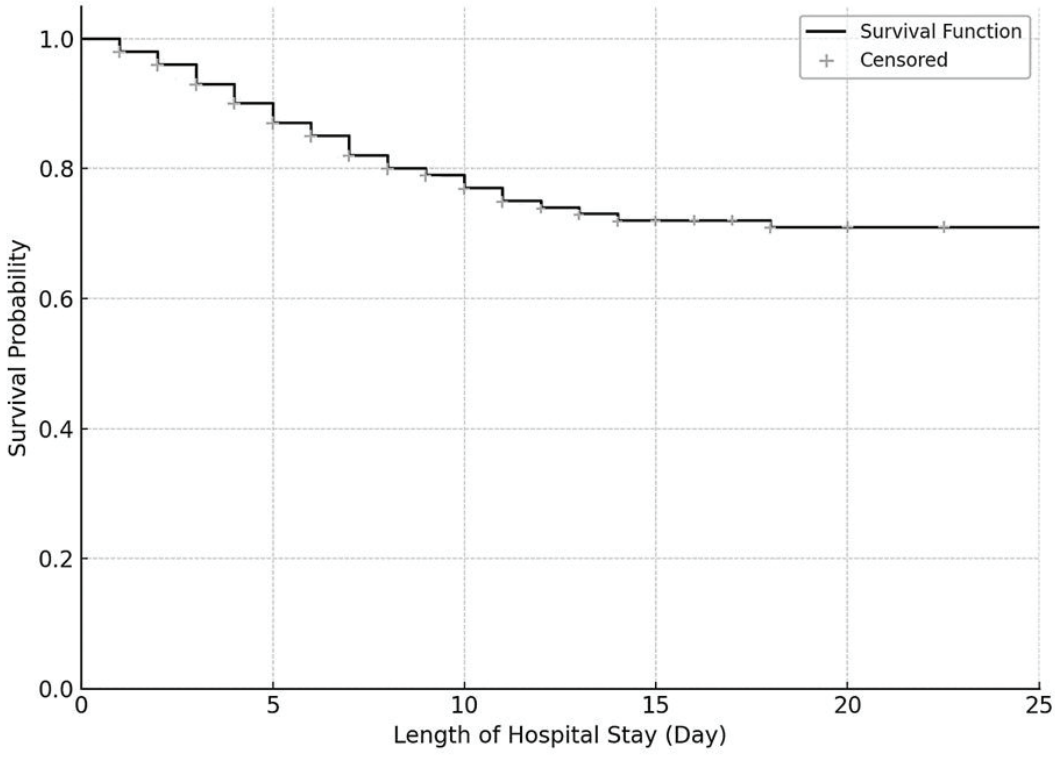

Figure 1. Probability of survival of COVID-19 patients according to time.
Cum Survival: Cumulative survival.

4. DISCUSSION

This research revealed a high incidence of kidney dysfunction in COVID-19 patients (20.6%), as indicated by albuminuria and hematuria. Nine percent of them also had mild to moderate organ failure. Older age, comorbidities, mechanical ventilation, kidney problems, a higher SOFA score, hypoxemia, and laboratory abnormalities, especially hematuria and albuminuria were linked to severe illness. Importantly, only one death reported among non-severe cases indirectly emphasizes that disease severity is the dominant factor influencing survival. Other medical risk factors tend to be present in critically ill patients, rather than independently predicting death. Nonetheless, early identification of kidney and organ dysfunction in COVID-19 patients could serve as a valuable tool for risk stratification. Although many studies have looked at organ or kidney failure in COVID-19, our study is concentrated on a well-defined hospitalized cohort and uses standard, validated criteria such the SOFA score and KDIGO criteria to accurately assess multi-organ and acute renal injury. Furthermore, we examined particular renal markers including hematuria and albuminuria, which are less reported in the current literature. The relatively large sample size and strict statistical techniques help to better characterize organ dysfunction, and may be useful for risk stratification and medical management in comparable groups.

Chen et al. (2021) reported renal impairment in 15–30% of COVID-19 patients, particularly based on serum creatinine and urine protein levels [14]. Our study confirms these results, emphasizing the strong association between kidney dysfunction and disease severity. While Chen et al. focused on serum creati-

nine and AKI, our study emphasized on both KDIGO and albuminuria/hematuria, which offers earlier insights into kidney dysfunction and may better predict poor outcomes. Hematuria (18%) was higher in our patients'cohort than in Chen et al.'s study, possibly because of differences in the sampled population. Our cohort was from one hospital, and therefore potentially included more severe cases, while Chen's cohort recruited a more general sample from multiple hospitals. Although both studies found that older age and comorbidities were linked to disease severity and poor prognosis, our findings identified the dominant role of disease severity for survival.

Similarly to us, Schnabel et al. found out that kidney dysfunction was common among COVID-19 patients and related to severe disease and higher mortality [15]. Both studies concluded that age, comorbidities, and mechanical ventilation were the key risk factors for kidney dysfunction. Furthermore, while both studies agreed that disease severity and kidney dysfunction were significant factors influencing mortality, our results suggested that disease severity played a superior role in patients' outcomes.

Our findings align with Fukui et al. and Karras et al.'s reports [16, 17] with the observation of high rates of AKI in COVID-19 patients, with AKI being related to poor prognosis. All studies pointed out the role of pre-existing diseases in forecasting adverse outcomes; however our findings showed that the link between these impairments and mortality was actually affected by the severity of disease. While Karras et al. conclusions depended on serum creatinine, our research also employed albuminuria and hematuria for early identification of kidney damage. Furthermore, Karras et al. strongly emphasized on chronic kidney disease (CKD) as a key comorbidity worsening clinical outcome, while our study indicated that chronic liver/biliary disease was more significantly related to mortality.

Study strengths and limitations

Our research stands out for its rigorous data gathering, the usage of often overlooked albuminuria and hematuria markers, and its emphasis on both renal and multiple organ failure. Employing the SOFA score aids to a more objective risk assessment in a moderate-sized population. At the same time, the study is limited and possibly biased by the lack of post-discharge long-term outcome analysis, and the single-center retrospective approach.

CONCLUSION

Our study revealed that kidney and organ dysfunction, along with advanced age, comorbidities, hypoxemia, and greater SOFA score, are significantly correlated with COVID-19 severity and mortality. Disease severity plays the dominant role in survival and its association with other risk factors. Nonetheless, further large-scale, multicenter studies are needed to validate these results and explore the long-term effects of kidney dysfunction among coronavirus survivors.

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PREVALENCE OF SEROLOGICAL MARKERS FOR HEPATITIS B AND HEPATITIS C AMONG PRACTICING DENTAL HEALTHCARE PROFESSIONALS

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ABSTRACT

Background: Dental healthcare professionals (DHPs) are exposed to a significant risk of infection with blood-borne pathogens, including hepatitis B virus (HBV) and hepatitis C virus (HCV). The present study aimed to estimate the prevalence of serological markers for hepatitis B and hepatitis C among practicing dental healthcare professionals.

Material and methods: A cross-sectional survey was conducted between 1 June and 31 October 2024 to evaluate the presence of serological markers for hepatitis B and C among DHPs (N = 133). The detection of serological markers for hepatitis B and C was performed using enzyme-linked immunosorbent assay (ELISA) and chemiluminescent microparticle immunoassay (CMIA). The differences between proportions of interest were assessed. Continuous data were expressed as median with interquartile range. Numbers and percentages (n, %) were used to present qualitative variables. A z-test was conducted to evaluate disparities between proportions.

Results: The median age of the enrolled DHPs was 43 years with women outnumbering men almost fivefold. Professional accidents were self-reported by 17% of the participants as 78% were vaccinated against HBV. Dentists were the most affected. The presence of protective HBsAb was detected in 49% of the DHPs who were self-reported as vaccinated. In 37% of the enrolled DHPs, HBsAb were detected, and in 10%, concomitant HBcAb were detected, indicating a past HBV infection.

Conclusions: The findings of this study suggest a necessity for regular screening for viral hepatitis among dental professionals.

Keywords: Dental healthcare professionals, HBV, HCV

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INTRODUCTION

Dental healthcare professionals (DHPs) are at increased risk of viral infections transmitted by both airborne droplets and blood such as hepatitis B (HBV) and C (HCV) viruses. The generation of splatter and aerosols is a result of the use of ultrasonic scalers, high-speed air rotors, air-water syringes, and air polishing. At the same time, the risk of injury to both the patient and the dentist is increased due to the small operating field, frequent patient movements, and the variety of sharp instruments used in dental procedures - burs, scalers, scalpels and endodontic files [1]. Hepatitis viruses (A, B and C) can be detected in oral fluids including whole saliva and gingival crevicular fluid [2]. Patients with chronic HCV infection exhibit more severe periodontitis, including gingival bleeding, increased pocket depth and attachment loss, when compared to healthy controls [3]. Studies on the epidemiology of HCV have demonstrated its low infectivity in saliva [4]. Also, in drops generated during dental procedures, HCV can survive up to 6 weeks, but in low titers [5].

HBV infection can present with extrahepatic manifestations including the oral cavity. The infection can affect the salivary glands, leading to xerostomia and sialadenitis [6]. The gingival sulcus has been identified as the intraoral location with the greatest concentration of HBV [7]. Hepatitis B surface antigen (HBsAg) was detected in the gingival crevicular fluid and saliva samples of 90% of HBV infected patients [8]. It was demonstrated that HBV DNA detection in saliva samples is detected more often in patients with detectable HBsAg and hepatitis B e-antigen (HBeAg) in serum [9]. Also, HBV is characterized with prolonged environmental stability, which was confirmed by animal infection model where positive for HBV human

plasma dried for 1 week and inoculated in chimpanzees resulted in an active infection [10].

Despite the paucity of data, Bulgaria is characterised by a low prevalence of hepatitis C, with an overall anti-HCV prevalence of 1.3%, ranging from 0.7% to 1.6% [11]. The country also exhibits intermediate prevalence of hepatitis B, with a crude rate of 3.9% for HBsAg [12]. According to the latest epidemiological data from the National Center for Infectious and Parasitic Diseases (NCIPD), the incidence of acute viral hepatitis B in 2024 was 4.06 per 100 000, while the incidence of viral hepatitis C was found 1.78 per 100 000. The relative share of the two infections was determined to be 0.49 and 0.22, respectively [13; access date: 27/08/2025]. The screening of healthcare workers (HCWs) for hepatitis B is carried out following Regulation No. 15 from May 2005 on the implementation of vaccination in the Republic of Bulgaria. According to the stipulations of the Terms and Conditions for Recommended Immunizations, it is strongly recommended that medical and non-medical professionals, including service personnel operating within medical and healthcare facilities, undergo immunization against viral hepatitis B through the administration of a recombinant hepatitis B vaccine. This recommendation is particularly applicable to medical and dental students enrolled in higher medical educational institutions, provided they are negative for HBsAg and do not possess any laboratory-confirmed data substantiating naturally acquired or post-vaccination immunity to HBV. This recommendation is further reinforced by Regulation No. 4 of 2002, which stipulates the protection of workers from risks associated with exposure to biological agents at the workplace.

Therefore, dental professionals need special consideration for hepatitis B and hepatitis C screening, and HBV vaccination. Despite the implementation of effective HBV vaccination programs, which have reduced the risk of HBV transmission among HCWs, particularly among DHPs, the evaluation of hepatitis B surface antibody (HBsAb) post-vaccination response remains crucial. The objective of the present study was to estimate the prevalence of serological markers for hepatitis B and hepatitis C among practicing dental healthcare professionals.

MATERIALS AND METHODS

2.1. Design

A cross-sectional survey was conducted to evaluate the presence of serological markers for hepatitis B and C among dental healthcare professionals. The study was conducted between 1 June and 31 October 2024 as a part of a larger sero-epidemiological study on the prevalence of hepatitis B and hepatitis C serological markers among HCWs aunder the National Program for Prevention and Control of Viral Hepatitis in the Republic of Bulgaria 2021–2025. Prior to participation, all subjects provided a written informed consent. The survey protocol was approved by the Expert Advisory Council on Viral Hepatitis at the Bulgarian Ministry of Health (MH).

2.2. Sampling

The calculation of the quota samples for the 28 administrative regions in Bulgaria was based on statistical data for registered HCWs, DHPs included, as published by the National Statistical Institute (NSI) of Bulgaria for 2023 [14]. Due to the significantly smaller number of registered DHPs, they were grouped together with HCWs, and the quota sample for each Regional Health Inspectorate (RHI) was determined

Table 1. Distribution of recruited DHPs participants by RHIs

Regional Health Inspectorate	Calculated quota based on the total number of HCWs	Actual number of recruited DHPs
Blagoevgrad	70	0
Burgas	80	0
Varna	110	29
Veliko Tarnovo	70	10
Vidin	60	7
Vratsa	60	5
Gabrovo	60	10
Dobrich	60	0
Kardzhali	60	10
Kyustendil	60	4
Lovech	60	1
Montana	60	0
Pazardzhik	60	0
Pernik	60	0
Pleven	80	0
Plovdiv	180	11
Razgrad	60	1
Ruse	60	0
Silistra	60	0
Sliven	60	10
Smolyan	60	10
Sofia Province	60	0
Sofia Capital	215	0
Stara Zagora	80	0
Targovishte	50	4
Haskovo	60	18
Shumen	60	3
Yambol	50	0

based on the total number. At the commencement of the study, each RHI was provided with all the requisite documentation, including an official letter from the MH of the Republic of Bulgaria for conducting a seroepidemiological study, a list of quota samples, an informed consent form, a questionnaire for participants, and instructions for sample collection and transport. The decision regarding the collection of samples was delegated to the RHIs. Following the distribution of an invitation letter, the following options for the collection of samples were made available: 1) at the RHI site, or 2) at the hospital/medical centre site. During the sampling, participants self-completed a questionnaire including: socio-demographic data, professional background, history of hepatitis testing, and HBV vaccination status. The voluntary nature of the study resulted in a final number of 133 collected samples . The distribution of participants by RHIs is presented in Table 1.

2.3. Detection of serological markers for hepatitis B and hepatitis C

A qualitative enzyme immunossay was used to determine the presence of antibodies against HCV (HCV Ab v.4 ELISA; DIAPRO, Italy), and HBV surface antigen (HBsAg one v. Ultra ELISA; DIAPRO, Italy). For both tests, samples with a serological index (the ratio between the sample's optical density at a wavelength of 450 nm and the Cutt-Off value) of ≥ 1.1 were designated as positive. A qualitative chemiluminescent microparticle immunoassay (CMIA) was used for the detection of antibodies against hepatitis B core antigen (INNODX HBcAb CMIA; Xiamen Innodx Biotech Co, P.R. of China). The analysis was based on the sandwich principle to detect antibodies to HBV core antigen in human serum and plasma. All samples with the calculated index > 1.0 were considered positive. For the detection of antibodies against HBsAg a quantitative enzyme immunoassay was performed (HBsAb; DIAPRO, Italy). The calculation of HBsAb concentration was performed using a calibration curve derived from five calibrators at 0 IU/ml, 10 mIU/ml, 50 mIU/ml, 100 mIU/ml and 250 mIU/ml. HBsAb titer of >10 mIU/ml was considered positive.

2.4. Statistical analysis

Continuous data were expressed as median with interquartile range (IQR – 25^{th}; 75^{th}). Numbers and percentages (n, %) were used to present qualitative variables. To evaluate disparities between proportions, a z-test was conducted, comparing the predominant proportion with the remaining proportions within the corresponding group. A 2-sided p-value of <0.05 was considered statistically significant. Statistical analyses were performed using the online Epitools Epidemiological Calculators – Ausvet [15] and SPSS Statistics for Windows, v.25 (SPSS Inc., Chicago, Ill., USA).

RESULTS

3.1. Socio-demographic characteristics of the study population.

The study population of DHPs included 133 participants, from whom 55% were dentists, 14% were dental assistants and nurses (p=0.0018), 8% were dental technicians, laboratory technicians, and sanitarians (p = 0.0036), and 23% were administrators and participants

Table 2. Main characteristics of the DHPs population

	N	%	*z-value*	*p-value*
occupation (N=133)*				
dentists	73	55		
dental assistants, nurses	18	14	3.1	.0018
dental technicians, lab.technicians, sanitarians	11	8	2.9	.0036
administrators, others	31	23	3.0	.0027
age [decades] (N=133)				
<30	29	22	0.9	
30-39	29	22	0.9	
40-49	28	21	0.9	NS
50-59	25	19	0.8	
60-69	17	13	0.6	
≥70	5	4		
sex (N=133)				
male	23	17	6.4	< .0001
female	110	83		
year of entry into the healthcare system (N=104)*				
<=1990	20	19	0.3	
1991-2000	22	21	0.2	
2001-2010	22	21	0.2	NS
2011-2020	24	23		
>2020	16	15	0.6	
HBsAg and HCV Ab presence (N=133)				
HBsAg (+)	0	0	--	NA
HCV Ab (+)	0	0	--	

Legend: *Denominator totals vary due to incomplete responses; NS = not significant; A comparison was made between the predominant proportion and the remaining proportions within each respective analyzed feature. Significance level 0.05

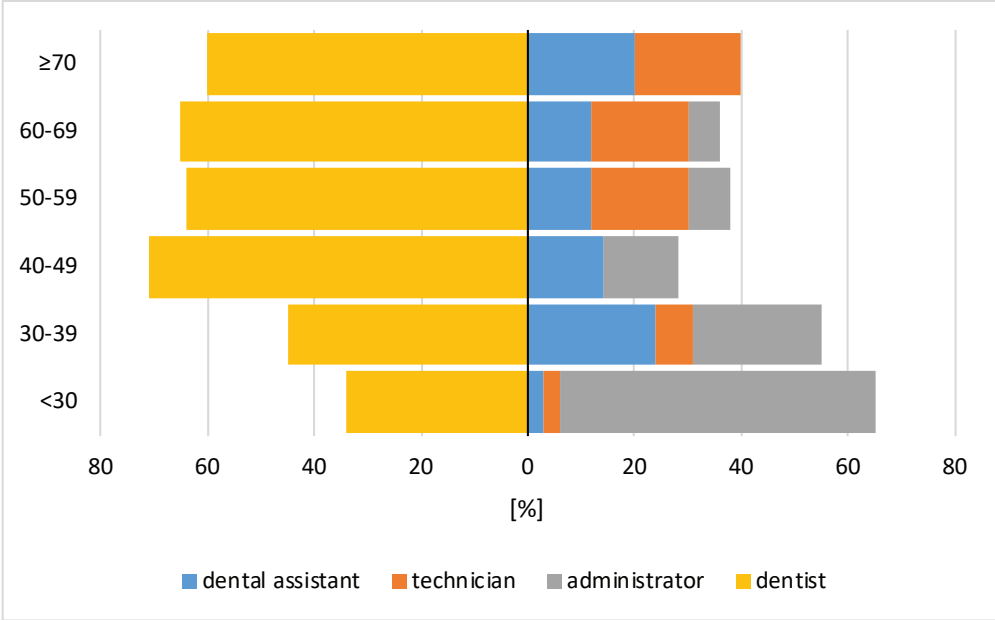

Figure 1. Age distribution of DHPs according their occupation

who self-reported as others (p=0.0027) (Table 2). The age of the enrolled DHPs was (median, IQR) 43 (30.5; 54) years. ranging from 20 to 73 The demographic composition of the study population was almost equally represented across all age groups (ranging from younger than 30 years to 60 - 69 age group), with the exception of the oldest participants (aged ≥70), who constituted 4% of the total population. However, for the dentist the highest percent of participants was in age group 40-49 years (71%), for dental assistants – in age group 30-39 (24%), for administrators - in age group <30 (59%), and for technicians the most represented age group was ≥70 years –with 20% (Figure 1).

Women outnumber men almost fivefold , 83% vs.17%, respectively (p < .0001). The longest presence in the healthcare system was recorded since 1988, and the shortest - since 2024. None of the participants tested positive for HBsAg or HCV Ab.

3.2. Professional accidents among dental healthcare professionals.

Of 133 enrolled DHPs 132 answered the question about professional accident (Table 3). The confirmatory answers were 17% (p <.0001). Dentists were the most affected, with a statistically significant difference ascompared to the group of dental assistants and nurses (74% vs 17%, p=0.0335). From 23 DHPs, who reported a professional accident, 78% (p=.0195) were vaccinated against HBV. On the other hand, 52% of DHPs, who self-reported a history professional accident, denied having undergone a previous hepatitis testing. At the same time, the duration of profession-

Table 3. Correlation between professional accidents and occupied position, HBV vaccination status, previous hepatitis testing and profesional experience duration

	N	%	*z-value*	*p-value*
professional accident, total (N=132)				
Yes	23	17	6.4	< .0001
No	109	83		
occupation (N=23)				
dentist	17	74		
dental assistant, nurse	4	17	2.1	.0335
dental technician, lab technician, sanitarian	1	4	--	NA
administrator, others	1	4	--	
HBV vaccination status among DHPs who suffered professional accident (N=23)				
Yes	18	78	2.3	.0195
No	5	22		
previous hepatitis testing in DHPs self-reported professional accident (N=23)				
Yes	11	48	0.2	NS
No	12	52		
year of entry into the healthcare system (N=23)				
<=1990	5	22	--	NA
1991-2000	5	22	--	
2001-2010	5	22	--	
2011-2020	5	22	--	
>2020	3	13	0.3	NS

Legend: DHPs=dental healthcare professionals; NA = not applicable; NS = not significant. A comparison was made between the predominant proportion and the remaining proportions within each respective analyzed feature. Significance level 0.05

al experience exhibited no correlation with the occurrence of professional accidents.

3.3. HBV vaccinated status in dental healthcare professionals

A total of 132 DHPs provided information regarding their HBV vaccination status (Table 4). The proportion of vaccinated participants was not significantly different from the proportion of non-vaccinated participants (49% vs. 51%, respectively). The most recent vaccination was reported one year prior by 5% of the participants. The majority of respondents (79%) reported receiving the vaccination more than a decade ago. The presence of protective HBsAb was detected in 49% of the DHPs who self-reported as vaccinated, as the time when they were vaccinated against hepatitis B was predominantly more than two years ago. The presence of HBsAb was detected in 25% of the non-vaccinated participants.

Table 4. Vaccination status of the DHPs population

	N	%	*z-value*	*p-value*
HBV vaccine, total (N=132)				
Yes	65	49	0.2	NS
No	67	51		
Time of vaccination (N=63)*				
The current year	0	0	--	--
1 year ago	3	5	2.9	.0041
2 years ago	0	0	--	--
> 2 years ago	4	6	3.2	.0014
> 5 years ago	6	10	3.5	.0004
> 10 years ago	50	79		
detection of HBsAb in vaccinated DHPs (N=65)				
HBsAb (+)	32	49	0.2	NS
HBsAb (-)	33	51		
time of vaccination of HBsAb positive DHPs (N=32)				
the current year	0	0	--	--
1 year ago	2	6		
before 2 years	0	0	--	--
> 2 years	3	9	2.3	.0212
> 5 years	3	9		
> 10 years	24	75		
detection of HBsAb in non-vaccinated DHPs (N=67)				
HBsAb (+)	17	25	3.7	.0002
HBsAb (-)	50	75		

Legend: *Denominator totals vary due to incomplete responses; DHPs=dental healthcare professionals; NS = not significant. A comparison was made between the predominant proportion and the remaining proportions within each respective analyzed feature. Significance level 0.05

3.4. Measured HBsAb and HBcAb status of dental healthcare professionals

The actual HBsAb status of 133 DHPs was determined (Table 5). For 37% of the participants, positive results were established (z=2.9, p=.0037). In 49% of the cases, the titer of HBsAb was found to be greater than 100 mIU/ml. In a subset of 10% of all HBsAb-positive DHPs, the presence of HBcAb was detected (z=4.4, p<.0001). Of these subjects, 20% reported being vaccinated against HBV. Dentists demonstrated the highest prevalence of past HBV infection, with 80% of the samples exhibiting positive results.

Table 5. HBsAb and HBcAb status of dental healthcare professionals

	N	%	*z-value*	*p-value*
detection of HBsAb, total (N=133)				
HBsAb (+)	49	37	2.9	.0037
HBsAb (-)	84	63		
HBsAb titer [mIU/ml] (N=49)				
< 100	25	51	0.1	NS
> 100	24	49		
detection of HBcAb in HBsAb positive DHPs (N=49)				
HBsAb(+)/HBcAb(+)	5	10	4.4	<.0001
HBsAb(+)/HBcAb(-)	44	90		
vaccination status of the HBsAb(+)/HBcAb(+) DHPs (N=5)				
vaccinated	1	20	--	NA
not vaccinated	4	80		
occupation by the HBsAb(+)/HBcAb(+) DHPs (N=5)				
dentist	4	80	--	NA
dental assistant, nurse	0	0		
dental technician, lab technician, sanitarian	1	20		
administrator, others	0	0		

Legend: DHPs=dental healthcare professionals; NA = not applicable; NS = not significant. A comparison was made between the predominant proportion and the remaining proportions within each respective analyzed feature. Significance level 0.05

Discussion

The recent study complements other publications on hepatitis B and C in the context of dentistry in Bulgaria. While others have focused on the oral health of patients with chronic viral hepatitis [7, 16], or on the knowledge regarding viral hepatitis [17], a recent analysis assessed serological markers for viral hepatitis B and C among enrolled practicing dentists. The

present sero-epidemiological study clearly demonstrates an uneven distribution of dental healthcare professionals by gender, with women outnumbering men by almost fivefold. A general absence of statistically significant differences between the age groups is indicated. However, a predominance of dental practitioners in the age groups over 40 years was observed. According to the survey results, 17% of the participants reported having experienced a professional accident, with dentists exhibiting a higher prevalence of such incidents. With respect to the serological markers of hepatitis, the presence of antibodies against HCV was not detected. In the survey, 49% of the DHPs reported a vaccination for hepatitis B. Among those who received the vaccination, approximately 50% exhibited protective antibodies, as over 70% reported receiving vaccination within the past decade. The serological patterns of past HBV infection were identified in 10% of the study participants. Notably, only 20% of these DHPs reported a vaccination against HBV.

This study was the result of collaborative efforts by various participants, including the Ministry of Health, the NRL of Hepatitis viruses and 28th RHIs from across the country. As a result, the sampling stage was subject to variation. Despite the efforts made by each RHIs, the number of participants who voluntarily enrolled in the study was considerably low, with a total of 133 DHPs (who reported their occupation in the dental field and/or place of work in a dental centre). A key challenge in conducting seroepidemiological studies is the inadequate number of participants. For instance, in a pilot study on the prevalence of HCV among the general population, active participation reached 21.6% [11]. Also, the participation in surveys was found to be dependent not only on itheir design but on the sociodemographic characteristics of the participants, including age, sex, country of origin, education, and labor market attachment [18]. In the present cross-sectional survey, dentists constituted the most prevalent professional category among the enrolled participants. The oldest age group (>70 years) was the least represented, which can be attributed to the reduced time presence of this age group at the workplace.

Due to the nature of their work, DHPs are subject to an elevated risk of sharp injuries. It was established that despitethe implementation of safety prevention measures, sharp injuries related to dental explorers and dental injection needles remain a common type of injury [19]. A meta-analysis of global data revealed the prevalence of needle-stick injuries among dentists to be between 27.5% and 69.2%, with a pooled prevalence of 59.1%, which was the highest among healthcare workers based on their job type [20]. Similarly, recent cross-sectional survey has revealed that the prevalence of self-reported professional accidents was the highest among dentists (74%), followed by dental technicians and nurses. Notably, approximately half of affected DHPs reported not having undergone prior hepatitis testing.

Both HBV and HCV can be detected in saliva, despite the fact that oral transmission of HCV has not been confirmed [21]. This finding may provide a rationale for the negative results observed for HCV antibodies in all the tested samples from the resent survey. The transmission of HBV through saliva has been confirmed in an animal infectious model [22]. Furthermore, the presence of HBV DNA is detected in 80% of patients with occult HBV infection [23], thereby underscoring the potential risk for dentists to contract HBV. This correlation was also confirmed in the present study, as dentists had the highest percentage of samples positive for HBcAb, which is a marker for past HBV infection.

The most effective strategy to prevent HBV infection is vaccination. The protection provided by three or four doses of hepatitis B vaccine can persist for a period of at least two decades [24] and the HBsAb levels ≥ 10 mIU/ml are considered protective [25]. The present study revealed that 49% of the DHPs reported receiving a vaccine for hepatitis B. However, only half of these participants had detectable HBsAb. At the same time, over 70% of subjects reported having received the vaccination for a period more than 10 years prior. A study of healthcare workers has established that the cumulative persistence of HBsAb 18 years after vaccination was 76.5% for high responders (1 month after complete vaccination HBsAb > 1000 mIU/ml), 35.4% for medium responders (100-999 mIU/ml) and 23.5% for low responders (10-99 mIU/ml) [26]. This can explain the decline in HBsAb to undetectable levels. At the same time, the present study identified that 25% of DHPs, who reported not having been vaccinated had protective antibodies. These antibodies can be attributed to the inaccurate assessment of vaccination status by the participants. According to the European recommendations for the

management of healthcare workers occupationally exposed to the hepatitis B virus, the presence of post-vaccinal HBsAb levels of at least 10 mIU/ml is indicative of responders [25]. More recent publications have demonstrated that an HBsAb titer above 100 IU/L is indicative of protective immunity [27]. In the recent study, only half of the enrolled DHPs had an antibody titer ≥ 100 mIU/ml. At the same time, in the cohort of DHPs for which HBsAb had been detected, 10% were found to be cumulatively positive for HBcAb. The results suggest a history of HBV infection. Comparable percentages of HBcAb positivity have been documented among dental healthcare workers and by other authors, as the value range from 4.97% [28] to 12.1% [29]. As demonstrated in the study conducted in Japan, the positive rate of HBcAb exhibited an age-related increase, from 2.9% among individuals aged 30-39 to 5.6% among those aged 40-49, 29.4% among those aged 50-59, and 85.7% among those aged 60-69, as 25% was not vaccinated [29].

In summary, to our knowledge, this is the first study conducted to provide a comprehensive overview of the prevalence of serological markers for hepatitis B and C among practicing dental healthcare professionals. Despite the limited number of participants enrolled in the study, the approach adopted was efficacious in estimating the prevalence of serological markersf for hepatitis B and C among practicing DHPs. The findings of this study suggest an enhanced screening for viral hepatitis among dental professionals as well as the development of technical guidelines for the prevention of hepatitis B and C infection among dental professionals.

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Declaration of interests

The authors declare no Conflicts of interest.

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PHENOTYPIC AND METABOLIC FEATURES OF CD4 AND CD8 T CELLS OF AN ELITE CONTROLLER – A CASE REPORT

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Background: People living with HIV (PLHIV) who are capable of long-term viral suppression in the absence of antiretroviral therapy (ART) are defined as elite controllers (EC). Although there is evidence of phenotypic and functional alterations of T-lymphocytes in EC, neither the mechanisms of viral control, nor the necessity and timing of ART in this particular population have been elucidated.

Case presentation: We report the case of untreated HIV+ patient with undetectable viral load (VL), and preserved CD4 T cell absolute count (AC) and CD4/CD8 ratio since diagnosis in 2018. The detailed phenotype of EC CD4 and CD8 T cell pools including differentiation, effector, activation, immunosenescence and exhaustion markers was distinct from sex- and age-matched ART+PLHIV with immune recovery, and close to HIV(-) healthy controls (HC), except for a temporary increase of CD57+ CD8 T cells in 2023. The only changing biomarkers were T cell mitochondrial mass (MM), slightly increased at the level of CD8 T cells in 2023, and drastically increased in both CD4 and CD8 T in 2025, together with the mitochondrial membrane potential (MMP), as compared to ART+ PLHIV and HIV (-) HC.

Conclusions: CD4 and CD8 T cells of EC experience intensive oxidative stress and functional burden, possibly linked to the continuous effective HIV-specific immune response. CD4 and CD8 T cell MM may serve as a monitoring marker preceding the irreversible changes of immune profile and loss of viral control, and possibly predict the time for start of ART.

Key words: HIV, ART, elite controllers, mitochondrial mass, mitochondrial membrane potential

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BACKGROUND

Human Immunodeficiency Virus (HIV) specifically binds CD4 antigens expressed mostly by the helper/inducer and regulatory T cell subset responsible for the orchestration of adaptive immune responses [1]. Immune response to HIV can only partially and temporarily control viral replication but is unable to eliminate HIV from the body. Therefore, untreated HIV infection leads to a gradual decline of CD4 AC, acquired immune deficiency syndrome, and fatal outcome. After the infection and the initial immune response, plasma HIV VL stabilizes at an individually specific level - the virological set point. The latter determines how long the gradual decline of CD4AC will last in the absence of ART. This progression usually occurs several years after the initial HIV infection [2]. Only few exceptional individuals are able to prevent HIV replication relying on their own immune system, in the absence of ART, and have become a focus of interest as a model for a potential functional therapeutic cure. HIV controllers are genetically and immunologically a rather heterogeneous population.

Controller cohorts differ regarding the level of residual HIV viremia, duration of HIV control, level of immunological control, and the time to reach controller status [3]. Long-term non-progressors (LTNP) who comprise approximately 5% of all chronically HIV infected individuals maintain stable CD4 AC above 500 cell/μl, and stable low but detectable HIV VL for more than 7 years. EC are a further restricted population (about 0.3% of all HIV-infected), defined by stable CD4 AC (irrespective of the threshold), and persistently undetectable HIV VL (below 50 copies/

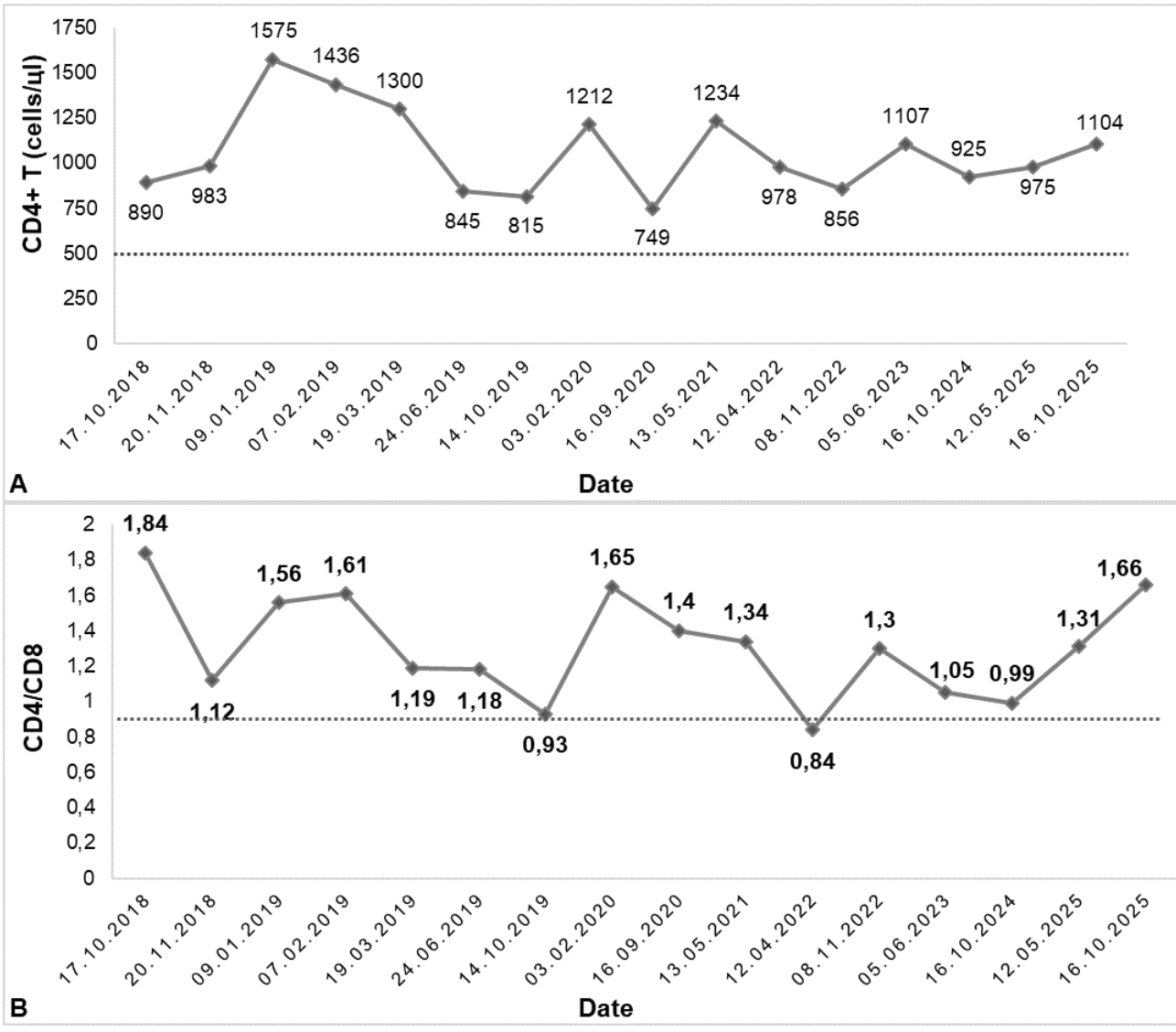

Fig.1. Dynamics of CD4AC (A) and CD4/CD8 ratio (B) of EC. Data from regular monitoring visits of EC are presented. Dotted lines correspond to the respective lower reference values for HIV (-) controls

ml) for more than 12 months [4, 5]. Some individuals showing both features of LTNP and VL control are defined as "elite LTNP" [6]. According to Genovese et al., long term controllers of HIV-1 infection progression are a heterogeneous group relying on different mechanisms of resistance [7]. The most widely accepted definition of an EC could be "an HIV-infected person followed-up for over 1 year, ART-naïve, with over 90% of his plasma HIV-RNA measurements below 50 copies/ml" [3].

The mechanisms of exceptional viral control comprise a number of virological, genetic, and immunity-related factors [8-11]. A relevant role of innate immunity in the long-term control of HIV has been proposed, including NKG2C-memory-like NK cells, $CD16^{dim}CD56^{dim}$ NK subset and TCRγδ2+ cells. [10, 11]. As to adaptive immunity, ECs were characterized by increased CD4 naïve, Th1/17 and CD8 TEMRA cells , at the expense of Th17, Tfh cells and memory CD4 T [12]. However, the mechanisms controlling HIV replication in the absence of ART are still not fully established [13].

At the same time, an EC may eventually lose control on HIV infection, posing the problem of ART timing, and the requirement of routine virologic and immune monitoring. Although a number of studies have delineated cellular and soluble biomarkers possibly associated with loss of viral control, no marker has been accepted for routine laboratory application [11, 14-16].

We describe a case of an EC with undetectable HIV VL who maintained stable CD4AC and CD4/CD8 ratio since diagnosis in 2018. As compared to ART+ PLHIV with successful viral suppression and restored CD4AC

and CD4/CD8 ratio, the detailed T cell immunophenotype of EC revealed neither evidence of chronic immune inflammation, nor a tendency towards immune exhaustion or senescence. At the same time, a significant increase of both CD8 and CD4 T cell mitochondrial mass (MM) and mitochondrial membrane potential (MMP) were registered as probable first signs of intensive oxidative stress and functional exhaustion.

Case presentation

A 48 years old male was diagnosed with HIV infection at the National Center of Infectious and Parasitic Diseases, Sofia, after successive positive results in a rapid test, ELISA and Western blot (11.09.2018), confirmed by a second blood sample on 29.09.2018. He was registered at the Infectious Diseases Clinic at the University Hospital „Georgi Stranski , Pleven in October 2018, and was followed for 7 years thereafter. His HIV VL was <40 copies/ml at registration (17.10.2018) and has remained undetectable since then (last determination on 16.10.2025).

CD4 AC was within the reference range for HIV (-) controls at all test points, mean (min-max) 1041 (749 -1575) cells/µL (**Fig.1A**). The same was valid for the CD4/CD8 ratio: 1.3 (0.84 -1.84). Only once (on 12.04.2022), a suboptimal value of 0.84 was registered due to an increased CD8 AC (1164 cells/µL), **Fig.1B.**

Based on the undetectable HIV VL and steady CD4 AC, the patient was defined as a long-term EC. ART was not recommended, and control examinations and laboratory monitoring were performed at 6-month intervals. The complete blood count (CBC) and basic biochemical parameters, including CRP, blood glucose, total cholesterol, triglycerides (TG) creatinine, urea, AST, ALT did not show important deviations from the accepted reference ranges during the follow-up (Table 1). Importantly, CRP never exceeded 2,9 mg/L. The tests for co-infections (HBsAg, anti-HCV, HAV and HEV antibodies, and *C. albicans* in faeces) were consistently negative.

In 2023 and 2025, a detailed phenotyping analysis of EC's T cell pool was performed, including the shares of naïve (N, CD45RA+CCR7+), central memory (CM, CD45RA-CCR7+), effector-memory (EM, CD45RA-CCR7-) and terminal effector (CD45RA+CCR7-) CD4 and CD8 T, EM1 (CD27+CD28+), EM2 (CD27+CD28-) and EM3 (CD27-CD28-) CD8 T, exhausted/senescent (TIGIT+/CD57+) CD8 T, regulatory ($CD25^{hi}CD127^{low}$) CD4 T (Treg) as well as the number of CD38 molecules (CD38 antibody-binding sites, ABS) on CD4 and CD8 T cell as an indicator of chronic activation. EC phenotype was compared to age- and sex-matched HIV+ART+ patients with undetectable HIV VL and

Table 1. Basic laboratory parameters of EC in 2023 and 2025

Parameter	Unit	EC			Reference range
		2023	2025	min-max for all tests	
Hb	g/l	**152**	**162**	145-166	135 - 175
Er	x10^12/L	**5,09**	**5,67**	4.7-5.7	4.2 - 6.2
Hct	L/L	**0,45**	**0,485**	0.41-0.48	0.37 - 0.55
Leu	g/L	**6,5**	**7,5**	5.9 - 8.3	3.5 - 10.5
Neut	%	**50**	**55,4**	48 - 69	44 -76
Ly	%	**41**	**36,9**	33 - 44	20 - 40
Mo	%	**9**	**7,7**	6-11%	3 - 13
Plt	x10^9/L	**246**	**259**	241-337	130 - 440
CRP	<10.0 mg/L	**1,28**	**1,13**	0.7-2.74	<5
Total Cholesterol	(mmol/L)	**6,2**	**6,58**	5.8-6.6	3.5-5.2
TG	(mmol/L)	**1,62**	**1,74**	1.4-3.7	0.3-1.7
ALT	U/L	**26**	**27**	24-46	<50
AST	U/L	**16**	**18,36**	15-21	<50
Glucose	mmol/L	**5,95**	**6,0**	4.8-6.5	3.6 - 6.1
Creatinine	µmol/l	**91**	**94**	85-99	62 - 106
Urea	mmol/L	**6,3**	**5,32**	4.7-8.1	2.14 - 7.14

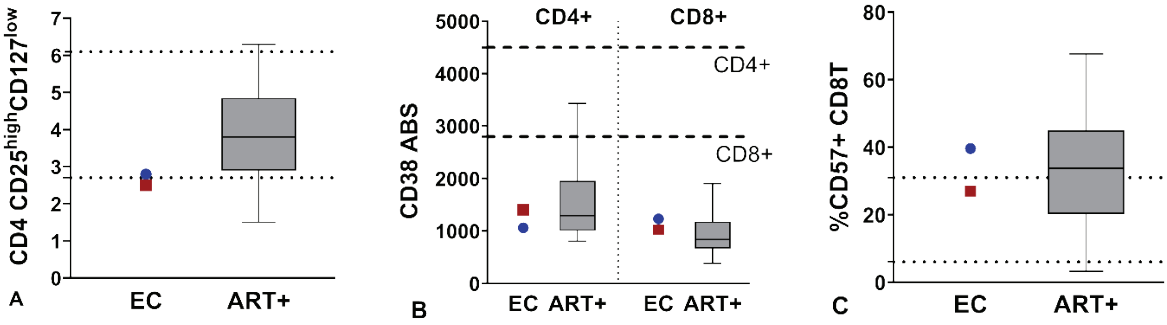

Fig.2 Chronic T cell activation and senescence markers in EC: the share of Treg (A); the number of CD38 molecules (ABS) on CD4 and CD8 T (B), and the expression of CD57 on CD8 T cells (D) were determined in 2023 (blue circle) and 2025 (red square) in comparison to ART+HIV (mean, min-max; box-and whiskers). Dotted lines correspond to Treg and CD57+CD8 reference ranges for HIV (-) HC. Thick lines correspond to the upper limit for CD38ABS on CD4 and CD8 T cells of HIV (-) HC, as designated.

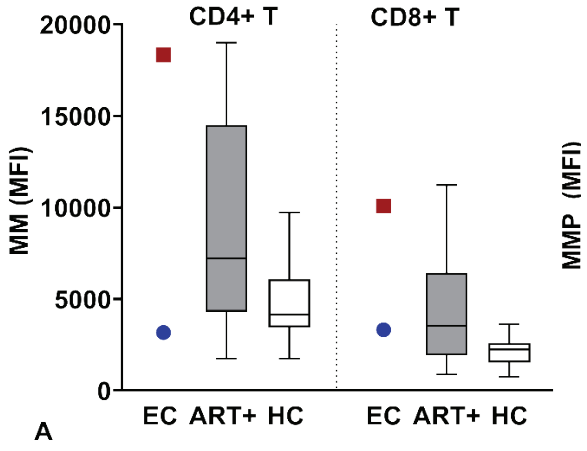

Fig.3 Dynamics of EM CD8 T cell pool. The co-expression of TIGIT and CD57 exhaustion markers in 2023 (blue) and 2025 (red) was analyzed within EM1 (A), EM2 (B) and EM3 (C) CD8 T cell subsets.

CD4 AC > 500 (n=39) (age 45 (34 – 51)), as well as to HIV- healthy controls (n=33) (46 (38 – 53)). (**Table 2 and Fig.2**).

The shares of naïve CD4 and CD8 T of EC were consistently higher as compared to ART+PLHIV: 55% and 46% vs. 34% (25.3% – 41.78%); 37% and 31% vs 24% (17.63% – 33.7%), and within the reference ranges for HIV (-) HC, (25% - 61%) and (25% -70%), respectively. Regulatory T cells (Treg) were consistently lower as compared to ART+PLHIV, 2.8% and 2.5% vs. (2.5% – 4.7%), and comparable to HIV (-) HC: (2.7% – 6.1%), **Fig.2A**. The measured CD38ABS on CD4 and CD8 T cells of EC, as well as those of ART+PLHIV: 1066, 1417 and 1291 (1002 -1957); 1230, 963 and 836 (666 -1174), were within the reference ranges for HIV (-) HC: (600 – 4500) and (510 – 2800) respectively, indicating absence of on-going immune activation **(Fig.2B, C)**. Noteworthy, in 2023 the share of CD57+ CD8 T was significantly increased (39.6%), and similar to ART+PLHIV (20% – 45%) but two years later it has returned within the HIV (-) HC reference range (29% vs. 6% - 31%), **Fig.2D**.

To further characterize the differentiation of the CD8 T cell effector pool, we analyzed the co-expression of CD28 and CD27 co-stimulatory molecules, together with exhaustion and senescence-related TIGIT and CD57. As shown in Table 2 and Fig.3, between 2023 and 2025 the share of EM3 (CD27-CD28-) significantly decreased at the expense of the less differentiated EM1 and EM2 stages. Thus, in 2025 EM1 (69%), EM2 (8.3%) and EM3(21%) CD8 T cells of EC fell within the ranges for HIV(-) controls, (53%-84%); (3.1%-10.7%), and (9% - 33.5%), respectively.

The decrease of CD57 observed between 2023 and 2025 was mostly at the expense of terminally differ-

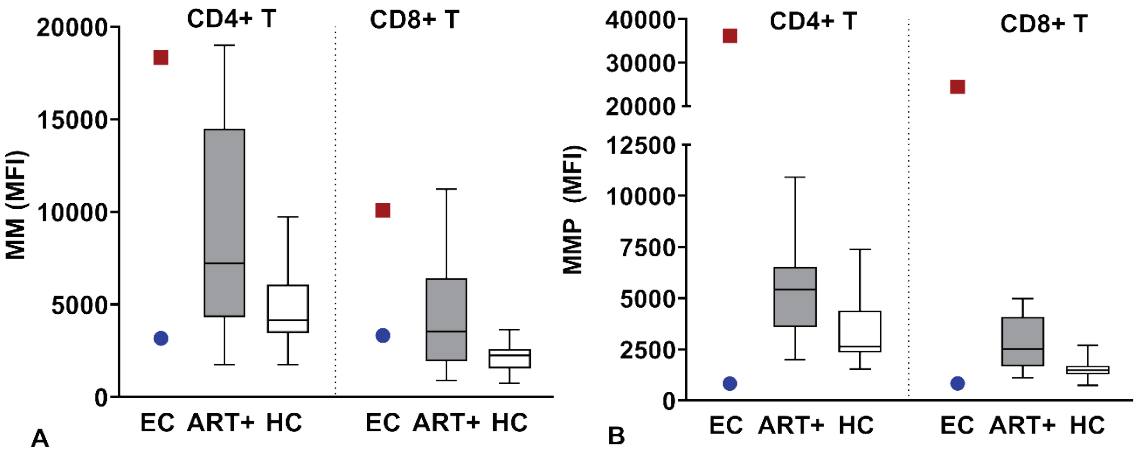

Fig.4 Significant increase of CD4 and CD8 T cell MM (A) of EC between 2023 and 2025 accompanied by an increase of MMP (B). MM and MMP of CD4 and CD T cells were determined in 2023 (blue circle) and 2025 (red square) according to the mean intensity of fluorescence (MFI) in comparison to ART+PLHIV (box and whiskers, gray) and HIV (-) HC (box-and-whiskers, open)

entiated (CD57+TIGIT-) EM3, Fig.3C. Also, the intermediate EM2 which is supposed to contain HIV-specific effector cells, was repopulated with functional TIGIT-CD57-/CD57+ cells (Fig.3B).

It is well established that mitochondrial dysfunction precedes T cell exhaustion. Both MM and MMP are closely related to the functional state of mitochondria. Increased MM is a sign of mitochondrial disruption, while hyperpolarization is associated with lymphocyte activation and imminent apoptotic events. To measure MM and MMP, freshly isolated peripheral blood mononuclear cells (PBMC) were stained with CD45, CD3 and CD8 mAb, followed by Mitotracker Green FM (Thermofisher) and Mitotracker Red FM (ThermoFisher) as previously described [17]. In 2023, EC CD4 T MM was comparable to HIV(-) HC: MFI 3175 vs. 4163 (3462-6073). Although CD8 T MM slightly exceeded the upper level for HIV(-) HC, it was within the range for ART+ PLHIV with undetectable HIV VL: 3323 vs. 2252(1541-2583) vs. 3541(1926-6443). At the same time, CD4 and CD8 MMP were low as compared to both HIV(-) HC and ART+PLHIV **(Fig.4 A,B and Table 2).**

Two years later, while no significant changes were observed in most phenotypic subsets a striking increase was noted for both CD4 and CD8 T cell MM: (MFI) 18340 vs. 3175 and 10068 vs. 3323, respectively. At that point, the MMP of both subsets has also increased to extreme values as compared to ART+PLHIV and HIV(-)HC: 36132 vs. 5430 (3600-6534) vs. 2634 (2352-4388), and 24454 vs. 2527 (1675-4080) vs. 1477 (1280-1691). Therefore, despite the steady CD4 AC, CD4/CD8 ratio, and undetectable HIV VL, EC T cells likely experienced importantly increased metabolic demands probably reflecting intensive viral stimulation, and preceding apoptosis.

DISCUSSION

EC represent the closest natural model to functional cure of HIV infection, offering an opportunity to study the features of protective HIV immunity, as well as to unravel early biomarkers predicting loss of HIV immune control.

A recent study highlighted multiple relevant trajectories among ECs: progress to immune deficiency despite undetectable viral loads (nonviremic progressors); loss of viral and immunologic control in 5 or more years; or rebound in HIV viremia followed by spontaneous viral resuppression (recontrollers) [18]. Due to this heterogeneity, there is no current consensus whether preventive treatment should be started and when. [19]. Therefore, early and reliable predictive markers of viral reactivation and imminent immune control failure are needed.

To this end, we performed detailed phenotypic analysis of an EC T lymphocyte pool and mitochondria at two time points in the settings of over 7 years undetectable HIV VL and stable CD4AC, and propose that the earliest biomarkers predicting loss of control are associated with mitochondrial function.

Not unexpectedly, the detailed immunophenotypic profile of EC resembled much more to a HIV (-) HC,

Table 2. Immunophenotypic analysis* of T cell subsets of EC in 2023 and 2025 in comparison to ART+PLHIV and HIV (-) HC

Subsets	Unit	Phenotype	EC 2023	EC 2025	ART+HIV+ (IQR)	HIV(-)HC (IQR)
CD8 T cells						
N	%	CD45RA+CCR7+	37	31	17 - 30	25 - 70
CM	%	CD45RA-CCR7+	5	8	6 - 18	5 - 14
EM	%	CD45RA-CCR7-	27	24	28 - 46	11 - 29
TEMRA	%	CD45RA+CCR7-	32	36	17 - 31	13 - 47
EM1	%	CD27+CD28+	47	69	38 - 62	53 - 84
EM2	%	CD27+CD28-	2	8	3.2- 9.1	3.1 - 10.7
EM3	%	CD27-CD28-	45	21	29 - 55	9 - 34
Senescent	%	CD57+	39.6	27	20 - 45	6 - 31
Exhausted	%	TIGIT+	43.9	42. 5	43 - 61	32 - 56
Activated	ABS	CD38+	1230	963	666 - 1174	510 - 2800
MM	MFI	-	3323	10068	1926 - 6443	1541 - 2583
MMP	MFI	-	-	24454	1675 - 4080	1280 -1691
CD4 T cells						
N	%	CD45RA+CCR7+	55	46	24.7 - 41.5	25 - 61
CM	%	CD45RA-CCR7+	25	27	31.7 - 47.6	33 - 44
EM	%	CD45RA-CCR7-	13	19	13.7 - 24.6	18 - 30
TEMRA	%	CD45RA+CCR7-	7	8	2.1 - 8.8	1.6 - 4
EM1	%	CD27+CD28+	76	88	73 - 87	73 - 90
EM2	%	CD27-CD28+CD4+	14	5	6.6 - 10.4	2.4 -10.4
EM3	%	CD27-CD28-CD4+	10	6	3.5 - 17.2	2.2 - 17.3
Senescent	%	CD57+CD4	6	4	2.1 - 7.4	3.1 - 11.8
Exhausted	%	TIGIT+CD4	18	17	18.7 - 30.5	17.4 - 27.2
Regulatory	%	$CD25^{hi}CD127$	2.80	2,50	2.5 - 4.7	2.7 - 6.1
Activated	ABS	CD38+	1066	1417	1002 -1957	600 - 4500
MM	MFI	-	3175	18340	4304 -14504	3462 - 6073
MMP	MFI	-	-	36132	3600 - 6534	2352- 4388

*The following mAbs were used in the multicolor flow cytometry panel: anti-h CD3 AmCyan (cat# 339186), anti-h CD4 (PE cat# 565999), anti-h CD25 (APC-Cy7 cat# 557753), anti-hCD45RA (FITC cat# 555488), anti-h CCR7 (PE-Cy cat# 560765), anti-h CD38 (PE cat# 2117530), anti-h CD8 (APC cat# 340659), anti-h CD27 (AF700 cat# 356416), anti-h CD127 (PcpCy5.5 cat# 353220), anti-h CD8 (V450, cat# E-AB-F1110Q), anti-h CD57 (FITC cat# E-AV-F-1067C), anti-h CD45 (FITC cat# 2522025), anti-h CD45 (PerCP cat# E-AB-F1137F), anti-h CD28 (APC cat#377610), anti-h TIGIT (BV421 cat#2463550). T-lymphocyte activation was evaluated by the number of CD38 molecules expressed on CD4+ and CD8+ T cells (CD38 antibody-binding sites, ABS) that were quantified using the Quantibrite PE CD38 calibration flow cytometry kit (cat# 340495, BD Bioscience) according to manufacturer's instructions. Samples were analysed using fresh whole blood.

Abbreviations

AC – absolute count
ART – antiretroviral therapy
CBC - complete blood count
EC – elite controller
LTNP – long term non-progressors
MFI - mean fluorescent intensity
MM – mitochondrial mass
MMP – mitochondrial membrane potential
PLHIV – people living with HIV
PMNC – peripheral blood mononuclear cells
TG - triglycerides
Treg – T regulatory cells
VL – viral load

than to successfully treated HIV+ patients. At the same time, the CD4 and CD8 T cell pools of EC were distinguished by the prevalence of naïve over CM T, and increased TEMRA subset, especially among CD8 T. This particular differentiation profile implies a robust antiviral response, driving a constant repopulation with recent thymic emigrants, and their quick differentiation to the terminal effector stage. A low proliferation of naïve T-cells combined with high proliferation of terminally differentiated effector T-cells has been already associated with a better virus control [11]. Recent deep immunophenotyping studies confirmed a number of specific T cell homeostasis alterations in EC including increased shares of naïve and CM CD4 T, as well as functional effector CD8 T cells as biomarkers of potent anti-HIV response [15]. Although in 2015, Bansal et al. concluded that elite controllers with preserved CD4T cells (EC) can control HIV-driven activation and CD4 percentage could be employed to determine the need for ART, further studies proposed that a "normal" CD4 count does not guarantee immune control [16].

The typical immunophenotypic changes in ART+ PLHIV with restored CD4 AC are a complex result of previous HIV-driven damage, ART-specific side effects plus on-going low level immune activation. Therefore, activation, exhaustion and senescence markers have been largely employed to characterize immune damage and/or restoration of the T cell pool. The effective inflammation control in EC has been previously demonstrated by similar levels of CD38/HLA-DR , CD57/CD28 defined T cell subpopulations, and PD-1 expression in EC and HIV (-) HC [16, 20] . Further on, inefficient viral control has been associated with an increase of CD8 T-cell activation and exhaustion including the presence of PD-1-expressing CD8+ T cells [9], a change from Th1 to Th2 cytokine profile [21], low Gag-specific T-cell polyfunctionality, and high proinflammatory cytokine levels [14]. In our EC case, effective inflammation control was associated with absence of CD38 ABS elevation in the absence of Treg increase.

Interestingly, a significant but transient increase of the terminally differentiated CD57+ CD8 T cell subset was registered which did not precede any deterioration of CD4AC or HIVVL increase. In fact, our observation corroborates with other authors' data defining CD57 rather as a marker of increased cytotoxicity than of T cell senescence and imminent apoptosis [22]. We have already proposed [17] that increased CD57+ CD8 T subset could notify a microbial or non-infectious stimulation , driving the terminal differentiation of a limited number of clones, and not necessarily - a loss of HIV control. In support, the expression of TIGIT by CD8 and CD4 T cell pool of EC did not increase between 2023 and 2025, while TIGIT expression was shown to correlate with HIV disease progression, even in PLHIV with antiretroviral control [24].

Mitochondria are essential for the intensive metabolism of immune cells. We and others have shown that both HIV-infection and ART contribute to mitochondrial damage, and therefore - to accelerated senescence in PLHIV [17, 24]. We reasoned that eventual loss of HIV control in EC would lead to vigorous viral replication, lymphocyte activation and, finally, exhaustion that might be preceded by signs of accelerated mitochondrial function.

A minor MM elevation only at the level of CD8 T cells in 2023 might be associated with a non-HIV-mediated activation, as commented for the transient CD57+CD8 elevation. However, the dramatic increase of MM in both CD4 and CD8 T, accompanied by MMP elevation deserves further close monitoring. Increased MM of CD4 and CD8 was reported as a sign of higher metabolic activity in virally stimulated immune cells of ART-naïve PLHIV [25]. Research data in EC reveal MM similar to HC and lower as compared to their ART+ counterparts [26], and viremic PLHW [24]. Data about loss of mitochondrial "fitness" in EC are limited. A study in EC reported increased MM as a sign of uncontrolled HIV infection [27]. In line with our results, a recent study demonstrated that unlike EC and HIV (-) HC, CD8 T cells from PLHIV, regardless of ART, are enriched in PD-1^{hi}EOMES^{hi}T-bet^{low}TIGIT+ exhausted CD8 T cells, also characterized by high expression of the glucose transporter, Glut-1, and impaired mitochondrial function. Consequently, mitochondrial antioxidant treatment was proposed for combined reconstitution therapies in HIV-1 infection [8].

MMP is essential for cellular respiration and ATP synthesis, and changes in MMP in the settings of HIV infection are associated with apoptosis. Chronic HIV infection leads to metabolic dysregulations of immune cells including mitochondrial damage, higher reactive oxygen species (ROS) production and reduction in glucose uptake [28-30]. In a previous study, we reported that elevated CD4 T cell MMP of PLHIV could further increase in the settings of ART, possi-

bly suggesting reactivation of HIV reservoirs [17]. In the case of EC, abrupt CD4 and CD8 MMP increase accompanied the increase of MM.

Studies in EC have demonstrated superior mitochondrial fitness as compared to ART-suppressed and HIV+ viremic individuals. In particular, the TCF1 transcription factor associated with the expansion capacity of HIV-specific CD8+ T-cells was overexpressed in HIV-specific CD8 T of EC, as compared to ART-suppressed and HIV+ viremic individuals [31, 32]. High TCF1 expression was shown to continuously maintain T cell mitochondrial fitness [33].

Indeed, most studies have linked mitochondrial disruption and reduced respiratory activity to MMP^{lo} / MM^{hi} phenotype [34, 35]. However, a recent study showed that just before activation-induced apoptosis, lymphocytes underwent hyperpolarization of mitochondrial membrane [36].

CONCLUSIONS

EC constitute a heterogeneous group with yet unpredictable loss of HIV VL, and undefined needs and timing of ART. In the settings of efficient anti-HIV immune response, more sensitive prognostic markers than CD4 AC are needed. In our case report phenotypic markers of CD4 and CD8 T cell differentiation, activation, exhaustion and senescence did not differentiate reliably between EC and HIV (-) HC. The registered significant increase of MM and MMP in the settings of undetectable HIV VL and stable immune parameters warrants further close monitoring. MM and MMP may be easily employed as biomarkers of mitochondrial fitness, sensing increased metabolic needs and predicting the loss of HIV control.

Limitations

Some limitations of this case report should be acknowledged. The stark increase of MM and MMP observed between 2023 and 2025 could be the only sign of undetected subclinical co-infection, an effect of developing metabolic syndrome (justified by the elevated cholesterol and triglyceride values) or other unknown factors. The speculation that elevated MM and MMP constitute a very early sign of viral reactivation, a follow-up monitoring would bring more clarity. Finally, the changes observed in a single EC may not be universal due to the presumably different mechanisms of viral control in this heterogeneous group of patients.

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BREAKING BACTERIAL CODE: QUORUM SENSING DISRUPTION AS A NEXT-GENERATION ANTIMICROBIAL APPROACH

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ABSTRACT:

Quorum sensing (QS) is a technique of cell-to-cell communication used by bacterial pathogens to control virulence, biofilm production, and antibiotic tolerance and thus contributes to long-standing and intractable infections. Studies on QS pathways are important in development of new therapeutic interventions against the backdrop of growing antimicrobial resistance. This review recounts the molecular QS phenomenon in the Gram- positive and Gram- negative bacteria and dwells upon the heterogeneity of autoinducers, receptors, chassis and regulatory networks. There is a critical examination of the pathogenic importance of QS, particularly in the propagation of biofilm-associated infections and multidrug-resistant infections. Potential solutions in the form of strategies to interfere with bacterial communication, or quorum quench (QQ) are described to include enzymatic degradation of the signals, inhibitors of signal biosynthesis, utilisation of signal receptor antagonists and natural product quorum sensing Inhibitors (QSIs).

The promising emerging directions of therapy are hyper-specific anti-virulence strategies, the development of nanotechnology, and the combination with traditional antibiotics. The study also involves future directions of CRISPR-based editing of QS genes, multi-omics tools to discover pathways, and non-medical applications of CRISPR as biotechnology and agriculture. Relating knowledge of mechanism and therapeutic research, with the help of this review one could see the possibility of destroying the networks involved in bacterial communication as a new method of treating infectious diseases and reducing antibiotic resistance.

Keywords: Quorum Sensing, Quorum Quenching, CRISPR, Autoinducers, AHL, AIP, LuxR

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INTRODUCTION

Pathogenesis of bacteria:

Bear in mind that the pathogenicity, or capacity of a microbe to produce disease, is a balance between four large factors: the predisposition of the host, i.e., its stored immune system; the character of the visitor that enters in, or becomes congested within us; the genetic map that the pathogen brings with it; and the particular program that it executes while infecting. It starts with a molecular movement from the host to the microbe. From genetic and molecular data, we can determine which traits are linked with virulence or defence mechanisms that enable a germ to persist. Even with this understanding, the exact processes by which bacteria become established in the host are not always very well understood; some of these processes may be unique to a particular bacterial genus that infects humans, others may be more general across the microbial world [1].

Microbes are all around and they are unavoidable, some of them can cause illness, others are protective or beneficial to the human body, with the most common bacterial infections being spread by direct contact, contaminated water, air, food or by living vectors like insects and animals. For instance, when the causative bacteria from the hospitals go outside and get transmitted around, it can result in higher morbidity and mortality among the weakened bodies [2].

Quorum Sensing Concept and Discovery:

Quorum sensing (QS) refers to the cell-to-cell communication; typically employed by bacterial patho-

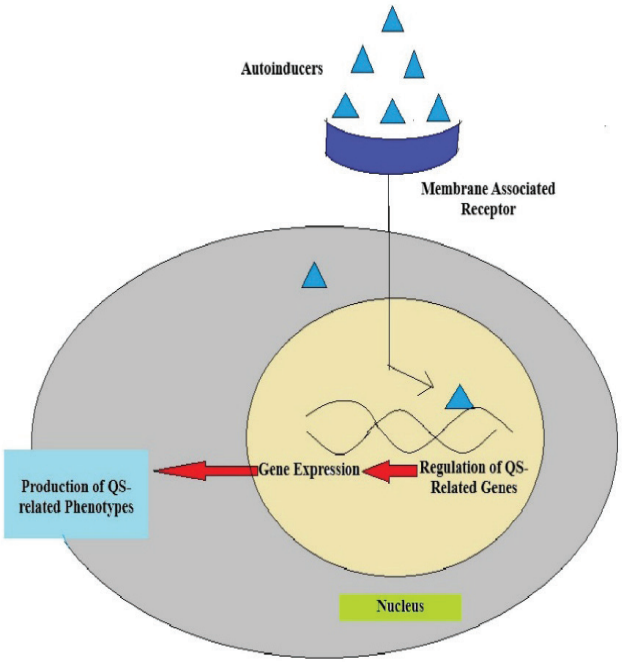

Figure 1 QS General mechanism in bacteria [5]

gens in order to coordinate the expression of a range of common traits including the production of multiple virulence factors, biofilm, and swarming motility, when the population densities of individuals attain a population level [3].

Bacteria are one of the simplest types of unicellular organisms, each being able to grow, divide, and react with its environment on its own. However, even being unicellular, bacteria can organize themselves and even share work with other cells. A complex process called quorum sensing (QS) is responsible in the attainment of such intercellular coordination. The result of this collaboration in organization can be seen when bacteria achieve a high density and create a thin gel-like material known as a biofilm.

In QS, intracellular signal transducers react to external stimuli; the extracellular signal may be diffusible components, which act directly on transcriptional regulators or via sensor kinases. The signaling molecules are produced by bacterial cells into the surrounding, and once their concentration reaches a certain level they bind to a specific receptor in the membrane, which causes a change in gene expression. The traditional activation of activity of genes in quorum-sensing (QS) leads to the increased syntheses of proteins partially contributing in the production of signalling-molecules (see Fig. 1). This enhanced expression of protein sets up a positive feedback loop forming the basis to the ubiquitous description of QS components as being autoinducers [4].

The QS process was initially explained in the bioluminescent marine bacterium; Vibrio fischeri. Here, a luxI / luxR type QS system operates: luxI encodes for an enzyme, the autoinducer synthase, to synthesize autoinducer, whereas luxR encodes for a receptor protein LuxR. During low density of microbial cells, n-acyl homoserine lactone (AHL) is produced in response to luxI gene expression. This AI (autoinducer) spreads in the medium and thus its concentration gets raised. When the threshold concentrations of AI are achieved, they interact to bind LuxR to produce a cytoplasmic AI-R complex which is a transcriptional activator which can bind DNA. This complex induces transcription of lux operon (luxCDABEG) expression and leads to an increase of the level of transcription of messenger-RNA encoding bioluminescence in the cell. At the threshold level, AI molecules regulate their own virulence factors and other virulence factors resulting in QS phenomenon [6].

It is also estimated that a large proportion (70 to 80 %) of all microbial infections are biofilm-based, and these complexes remain central to pathogenesis. The exogenous stressors are withstood through the biofilms, which serve as a barrier to receiving antibiotics and antiseptics [7].

Gram positive bacteria utilize the oligopeptides to express genes through the auto-inducer mechanisms. After release by the cell, these molecules are received by the membrane receptors of the same bacteria, a cascade of signal transduction then occurs and leads specifically to the activation of transcription of a certain gene. On the other hand, the Gram-negative bacteria regulate gene expressions density-dependently. They also release other self-activating molecule by a stimulation of Lux operon that also controls production of the major enzymes found in quorum sensing signals. These difference in controlling the gene action occur owing to both the number density and the physiological condition of the bacteria [8].

2. Mechanisms of QS

2.1 Autoinducers-Signal Molecules

QS allows the communication of bacterial cells by identifying autoinducers and secreting them. Autoinducers (AIs) are small signaling molecules which are generated at basal levels in the stationary phase of bacterial growth. These molecules serve as a population density marker and after a specific growth level

is attained, they control the expression of the corresponding genes. The signal molecules employed by a Gram-positive bacterium will be peptide derivatives in contrast to fatty acid derivatives by a Gram-negative bacterium. Most of the bacteria are capable of using both the types of AIs to control the expression of target gene [5].

Bacterial sporulation, biofilm formation, pathogenicity production, and interaction connections that involve interspecific competition, cooperation, and paternity recognition are all regulated by quorum sensing (QS) [9].

Acyl Homoserine Lactone-AHL
(Gram negative bacteria)

Quorum sensing (QS) signaling molecules, which are referred to as autoinducers vary across different bacteria species. Acyl-homoserine lactones (AHLs) are the most prevalent autoinducer secreted by gram-negative bacteria, while oligopeptides referred to as autoinducing peptides (AIPs) are most commonly secreted by gram-positive bacteria [10].

Acyl Homoserine Lactones (AHL) molecule is composed of a homoserine-lactone strand and fatty acid acyl chain (C4 to C18) (Fig.2). The AHL molecules may differ in 3- hydroxy, 3-oxo, methyl or varying levels of unsaturation based on the organisms. The LuxI-type AHL synthases are the first component in AHL signal which synthesizes the AHL molecules. In the event of AHL molecules synthesis, the molecules can be passively and actively transported in and out of the cells. The second mode of action of AHL signalling is through LuxR-type receptor proteins, which recognize AHL signal molecules and in turn, trigger the expression or repression of target genes in a QS-dependent manner. The expression of genes mediated by QS is therefore controlled by LuxR-like DNA-binding transcription factors [11].

A group of autoinducers known as acyl homoserine lactones (AHLs) is formed by around fifty Gram-negative bacteria, many of which are pathogens of clinical interest. Their synthesis combines three structural motifs: a homoserine lactone ring formed by S-adenosylmethionine, a central amide group and a variation of the chain with different length and level of oxygenation dependent on the bacteria species. Within some strains, the chain ends with a 3-oxo group; in other strains, such as V. fischeri and P. aeruginosa, it ends in a 3R-hydroxyl functional group. Aliphatic chains with 4-18 carbon atoms have essential hydrophobic characteristics that allow penetration of the various cell membranes and subsequent binding with the hydrophobic pocket of proteins as receptors.

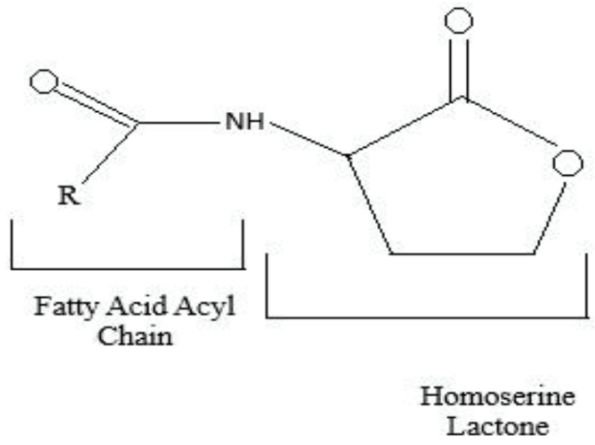

Figure 2 AHL structure [12]

Lactone, amide, and 3-oxygens functionalities, in particular, promote formation of robust hydrogen bonding networks of the receptor active site. In spite of the richness of AHL structure which differs between different species of bacteria, some analogues are shared between many organisms, such as between Vibrio fischeri and Erwinia carotovora, serving as a means of communication between bacteria which cross species boundaries, i.e. overcoming the classical species barrier [13].

Autoinducing peptides-AIP (Gram positive bacteria)

The Autoinducer Peptides (AIPs) are the primary communication mechanism of Gram-positive bacteria, which are small peptides that are frequently exposed to chemical changes. Histidine kinases are two-component membrane-bound receptors that detect these peptides [10].

Autoinducing Peptides, or AIPs, are referred to as AIP-I-II-III-IV (Fig.3). They are all more hydrophobic in their N-terminal-to-C-terminal order in their peptide architecture, but peptides might differ in their amino acid sequence. The hydrophobic side chains of the amino acids at the C-terminal locations of the sequence are present in the AIPs. AIP linear peptide analogs or the hydrolysis of the thioester moiety deactivate its activities. It was discovered that an ester moiety in the form of a thioester macrocyclic ring might prevent the deactivation of signaling. AIP loses its signaling when N-terminal exocyclic structures are eliminated [14,15].

There are two main QS systems in Gram-positive bacteria (Fig. 4). After being ribosomally generated

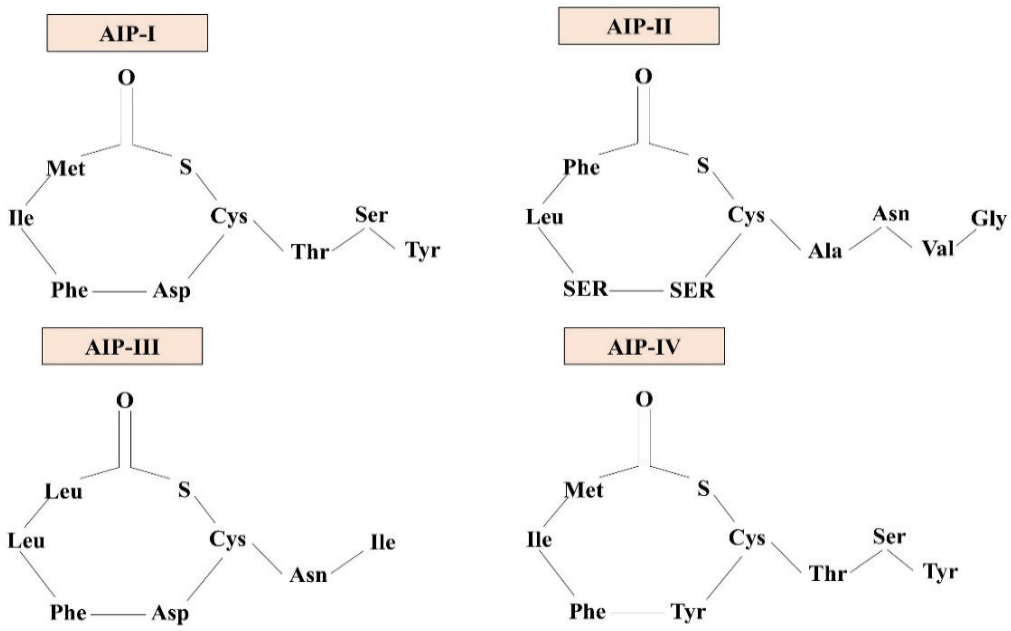

Figure 3 AIP-I, II, III, IV Structure found in Staphylococcus aureus [5].

as pro-peptides of the first pathway, AIPs undergo post-translational modification. In order to evolve into AIPs, they undergo multiple cleavages by secreted proteases after being released by specific ABC transporters. Certain cell surface receptor kinases phosphorylate a conserved His residue when they detect AIPs at a threshold concentration. By transferring the phosphoryl group to an Asp residue, the active kinase then activates an intracellular regulator receptor downstream. Lastly, the secretion mechanism of AIP and the elections of certain target genes are controlled by the activated intracellular regulatory receptor. Because it contains two essential components—the intracellular regulating receptor and the His kinase at the membrane—this system is frequently referred to as a two-component pathway [16].

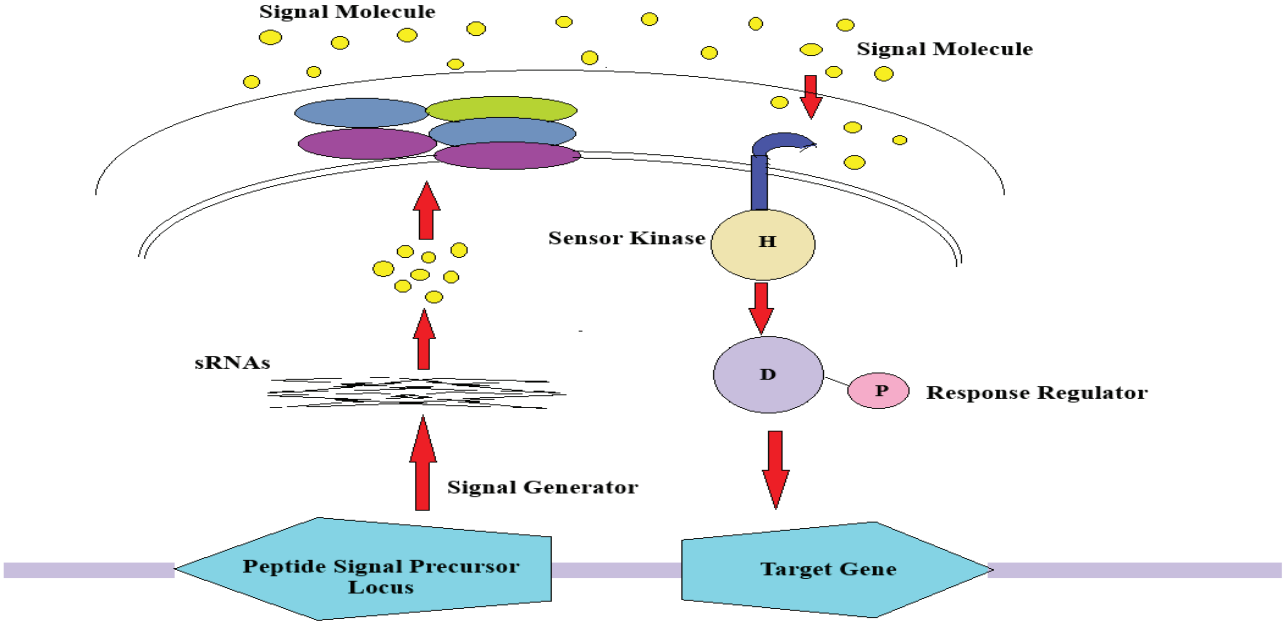

Figure 4 Two-component signal transduction and Gram-positive QS peptide signals, a general model. A response regulator protein regulates the transcription of downstream target genes, while the membrane-bound sensor kinase protein auto-phosphorylates to start the signal transduction [17].

Universal Signal AI-2

It has been reported that QS mechanisms are similar in Gram-positive and Gram-negative bacteria, but the specific autoinducers involved in these mechanisms can differ among different organisms. Saying so, there exists a group of QS systems, most noticeably DPD (dihydroxy pentanedione)/AI-2 which occurs in both Gram-positives and Gram negatives. The most versatile signaling mechanism employed by both Gram-positive and Gram-negative bacteria, DPD/AI-2, has been observed in over 50 percent of the QS-competent bacteria whose genome has been sequenced [18]. In contrast to AI (autoinducer)-1, AI-2, (quorum sensing) system occurs in both Gram-positive and Gram-negative bacteria and is believed to mediate cross-species communication [19]. In addition, autoinducer-2 (AI-2), also called furanosyl borate diester or tetrahydroxy-furan, is the common language system of both Gram-positive and Gram-negative species [9].

2.2 Receptors of QS

Signal transduction in the quorum sensing (QS) system relies heavily on receptors. Allosteric regulation takes place to control gene transcription when these receptors pick up an autoinducer. Therefore, one of the primary strategies for re-engineering bacterial behaviour is to block the activity of a receptor [20].

Bacterial group activities and population-level functions are systematized by QS (quorum-sensing) receptors and the signaling molecules that accompany them, which either directly or indirectly regulate gene expression [21].

LuxR:

With an N-terminal ligand binding domain and a C-terminal helix turn helix domain, LuxR type proteins are two domain proteins that bind DNA, typically as a homodimer via a recognition motif. It has been shown that repression is brought on by steric hindrance but that class I or II pathway proteins can induce transcriptional activation depending on protein.

The folding and ligand-binding characteristics of LuxR-type proteins are used to categorize them. AHL is necessary for Class I proteins to fold and bind them permanently. AHL can bind to the class II proteins, such as V. fischeri LuxR to organize, but the binding is reversible. Class III proteins that are independent of AHL to assemble into functional homodimers, such as Erwinia ExpR (the ortholog of SdiA) bind AHL reversibly. Escherichia coli's SdiA proteins may need an endogenous ligand, 1-octanoyl-rac-glycerol, in order to be purified without the use of AHL, suggesting that they are class III [22].

1. LuxR-type (typical)

Gram-negative bacteria use the LuxI/R type QS protein receptor system to mediate the QS process. Auto-inducers N-acyl-l-homoserine lactones (AHLs), especially those induced with LuxI type proteins, are the key auto-inducers by Gram-negative bacteria. LuxR, the AHL response regulator, and LuxI, the AHL synthase of N-acyl-l-homoserine lactone transcriptionally activate target QS genes [23].

In response to the presence of particular chemical secretions, most notably AHL generated by the LuxI

Table 1 Quorum Sensing Receptor Types [20].

Receptor Type	Signal Molecule	Communication Type	Example Receptors	Representative Bacteria
LuxR-type (typical)	Acyl-homoserine lactones (AHLs)	Intraspecies	LuxR	*Vibrio fischeri*
LuxR-solo type	AHLs or alternative signals	Intraspecies/Interspecies	SdiA	*Escherichia coli*
Two-component (Gram-negative)	HAI-1	Intraspecies	LuxN	*Vibrio harveyi*
Two-component (Gram-positive)	Autoinducing peptides (AIPs)	Intraspecies	ArgC	*Staphylococcus aureus*
RRNPP family (Gram-positive)	AIPs	Intraspecies	Rap, Rgg, NprR, PrgX, PlcR	*Bacillus subtilis*, *Streptococcus thermophilus*, etc.
AI-2 receptor	Autoinducer-2 (AI-2)	Interspecies	LuxP, LsrB	*Vibrio harveyi*, *Salmonella typhimurium*
AI-3 receptor	AI-3, epinephrine, norepinephrine	Interspecies	QseC	*Enterohemorrhagic Escherichia coli*

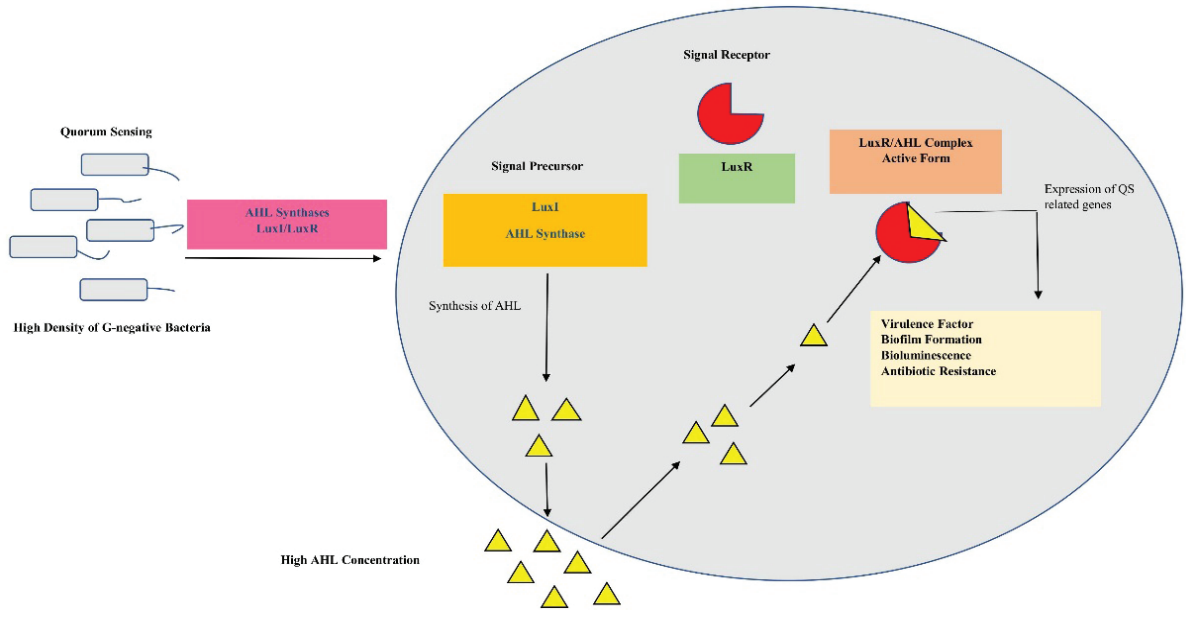

Figure 5 Mechanism of AHL through LuxR receptor [23].

(AHL Synthase) type protein, a class of common Gram-negative bacteria use LuxR-type quorum-sensing (QS) receptors to coordinate gene transcription (Fig.5). The associative mechanism of the most well-studied LuxR-type receptors allows dimerization and DNA binding to occur only when the ligand is bound.

On the other hand, a second, less studied family of proteins known as dissociatives bind to DNA when no ligand is present and unbind when a ligand is present. When compared to associative LuxR-type controllers, dissociative LuxR-type proteins are typically more operationally stable in vitro [24].

2. LuxR-solo type

The incomplete LuxR system of proteins without a corresponding synthase, commonly known as orphans or LuxR solos, is becoming more and more well-known in the field of QS. These single regulators may be distinguished by the type of origin of the ligand-endogenous or exogenous, AHL or non-AHL or neither. SdiA is part of the LuxR-type protein family which has its origin in the response regulator that controls bioluminescence in Vibrio fischeri [22]. There are two types of quorum sensing signals produced by other bacterial species and which are detected by SdiA: Exogenous Acyl-homoserine lactones (AHLs) and autoinducer-2 (AI-2) [25].

SdiA was initially identified as a transcriptional regulator of the ftsQAZ operon, which codes for proteins essential for cellular division. This operon's inducer-dependent activation speeds up cell septation while also inhibiting the actions of numerous endogenous cell-division blocking factors. It was also demonstrated that AHL exposure appears to increase the regulatory action of SdiA on the ftsQAZ operon and that the AHL mediated quorum sensing may play a role in regulation of this particular operon [26].

3. Two-component (Gram-negative)

The primary autoinducer in V. harveyi is the HAI-1 molecule, which is an AHL type [27]. The three membrane-bound two-component QS receptors in V. harveyi, include LuxN, LuxPQ, and CqsS. These receptors recognize and bind related signaling molecules HAI-1, autoinducer-2 (AI-2) and CAI-1. These molecules are the by-products of the LuxM, LuxS, and CqsA synthases, respectively [20].

4. Two-component (Gram-positive)

The formation of S. aureus biofilms has been linked to a number of regulators. These are led by the accessory gene regulator (agr), a hybrid staphylococcal quorum-sensing system that trans-up-regulates the extracellular cysteine proteases SspB and ScpA to regulate filamentous growth. The impact of agr-mediated biofilm formation on the expression of several virulence factors suggests that it plays a crucial role in staphylococcal pathogenesis. By attaching itself to

the AgrC transmembrane protein and phosphorylating it, the acrocyclic peptide AIP, which contains seven to nine amino acids, controls the Agra [28].

5. RRNPP family (Gram-positive)

The RRNPP protein family (Rgg, Rap, NprR, PlcR and PrgX), which are covalently bound to intracellular signaling peptides associated with QS in gram-positive bacteria, includes intracellular receptors associated with intracellular signaling peptides [21]. The perfect examples of RRNPP the signal transducers are PrgX regulator in Enterococcus faecalis, the NprR regulators of Bacillus cereus group, the PlcR proteins of B. cereus group, the Rap phosphatases of Bacillus subtilis and the Rgg proteins of the Streptococcus species. All the most critical cellular functions are regulated in the family of RRNPP which are sporulation, competence, virulence, biofilm development, necrotrophic life style, conjugative plasmid transmissions, and antibiotics indifferences [29].

6. AI-2 receptor

The receptors LuxP AI-2 were first found in Vibrio species and LsrB are widespread throughout enteric bacteria and microorganisms of the Rhizobiaceae, Bacillaceae, and Clostridiaceae families [30].

The LuxS enzyme is widely expressed in several bacteria and participates in the production of the AI-2 signal. As a result, AI-2 is produced as an interspecies communication signal rather than being specific to a single bacterial strain. Three validated receptors of AI-2 are LuxP protein in the Vibrio species, LsrB protein of Salmonella Typhimurium and Escherichia coli and the RbsB protein of Aggregatibacter actinomycetemcomitans. As a receptor, LsrB positively promotes internalization of AI-2 but LuxP participates in cascades of signal transduction and thus determines downstream gene expression. Following internalization, AI-2 becomes phosphorylated. This phosphorylated AI-2 then binds to the LsrR protein, which in turn causes the Lsr system to be expressed and accelerates the conversion of AI-2 to its final form [31].

7. AI-3 receptor

Like hormones, AI-3 can be considered as an extracellular signal transduced via the binary system QseBC, a histidine kinasephosphatase (QseC) and response regulator pair (QseB). In a small number of Gram-negative species, including the enteropathogenic E. coli, the periplasmic QseC domain is retained, and AI-3 resembles the eukaryotic hormones in its effects since QseC is a bacterial adrenergic receptor to the eukaryotic host hormones noradrenaline and epinephrine. The other consequence of this structural resemblance is that adrenergic receptor antagonists block AI-3. Furthermore, epinephrine/norepinephrine possesses the ability to activate the QseC/QseB cascade and become a QS signal that is then transferred to the quorum of the gut microbiota.

The human hormones noradrenaline and epinephrine are used by enterohemorrhagic E. coli O157:H7 (EHEC) to activate virulence genes that may be linked to the stress hormone cascade and irritable bowel syndrome brought on by extended stress [32].

Using histidine sensor kinase QseC, the bacteria can detect and respond to hormone-like host-produced factors like autoinducer-3 (AI-3), epinephrine (Epi), and norepinephrine (NE). The QseBC two-component system (TCS) is formed when the transmembrane C-terminus of QseC binds to the cytoplasmic membrane. However, it then undergoes autophosphorylation and the phosphate is transferred to an intracellular component known as QseB. After activation, QseB normally binds the specific sequence of DNA on the bacterial cells regulating the growth and motility, biofilm formation, and expression of virulence genes in bacteria. It acts as a virulence global regulator in enterohemorrhagic Escherichia coli, and the quorum sensing (QS) system QseBC was demonstrated to promote intracellular colonization and systemic infection [33].

3. Quorum sensing role in pathogenicity

3.1 Expression of Virulence Factor

Pathogenesis is a multilayered, interdependent process that involves multiple components interacting with one another as a pathogenic disease infects a host. Most strains follow a common path, even though the specific biology of infection can vary from organism to organism. The initial step that traps the virus by attaching itself to the host's microbiota and causing dysbiosis is adhesion. In order to alter the native cells' metabolism and evade immune responses, the invader releases a pattern of molecules, proteins, enzymes, and siderophores into the host cells after anchoring [34].

3.2 Formation of Biofilm

Biofilms are populations of microbial cells that are primarily adhered to a substrate and physically adherent to one another by a matrix of polymers, many of which are released by the microorganisms. In ad-

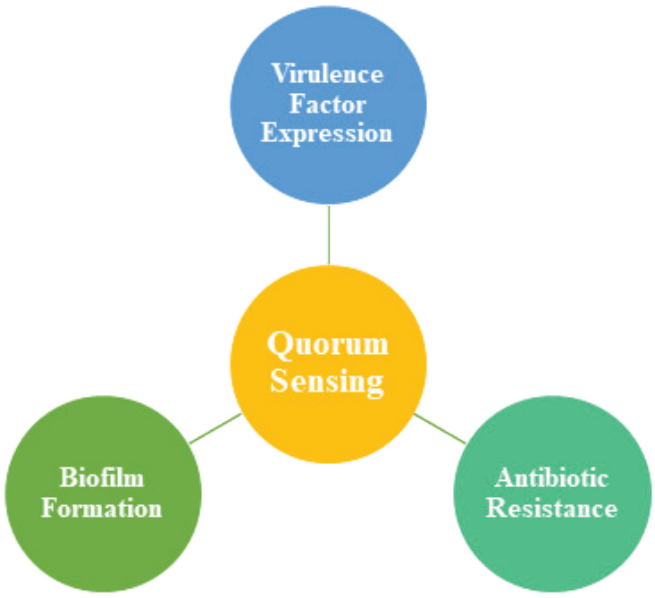

Figure 6 Quorum Sensing role in pathogenicity

dition to creating additional channels and microcolonies, the matrix provides the cells immersed inside it with physical protection as well as a microenvironmental gradient of oxygen and nourishment. These gradients are thought to produce phenotypic and genotypic plasticity and heterogeneity in populations linked to biofilms. Therefore, the created microenvironments encourage interactions between microorganisms since they are being driven inwards in the matrix and diffusion is being reduced [35].

The most advantageous of the many survival benefits that microorganisms receive from their biofilm style of life is the spread of antibiotic resistance. It may surprise you to learn that bacteria in biofilms are far more resistant to drugs than their free-living planktonic counterparts. The microorganisms in biofilms prefer to live a sessile lifestyle and constantly adapt to environmental changes. When conditions that support rapid development become available, these cells can alter their composition and return to a planktonic lifestyle [36].

The infamous biofilm-forming bacteria Pseudomonas aeruginosa successfully adheres to a variety of surfaces, resulting in persistent infections that are challenging to treat. The disease's opportunistic human pathogen, P. aeruginosa, causes severe and potentially fatal symptoms and quickly infects people with cystic fibrosis, resulting in significant morbidity and fatality rates. Because of its capacity to build biofilms, P. aeruginosa possesses the trait of being resistant to several medications.

Elements of P. aeruginosa that controls the biofilm

The extracellular polymeric substance (EPS), which contains proteins, polysaccharides, eDNA, and lipids, makes up the biofilm matrix. In addition to acting as a selective sieve that allows a small number of nutrients to enter and prevents antimicrobial probes from penetrating the matrix, the EPS helps the bacteria adhere to the surfaces. When the concentration of autoinducers in the host system exceeds a threshold, P. aeruginosa produces extracellular polymeric substances (EPS) [37].

3.3 Resistance to Antibiotics

Even though Antonie Van Leuwenhoek examined biofilms with a crude microscope as early as 1674, Bill Costerton didn't come up with the word "biofilm" until 1978. The relationship between antimicrobial resistance and biofilm production varies depending on the kind of bacteria. The growth of biofilm and strains that produce beta-lactamases was responsible for the expansion and dissemination of biofilm and multidrug-resistant Gram-negative bacilli [38].

4. Therapeutic Approach of Quorum Quenching

Alternative methods of combating such an infection are required due to the misuse and overuse of antibiotics in medicine, the development of antimicrobial resistance, the failure of antibiotics to control microbial infections, and the detrimental effects on the environment's sustainability requirements. One such option is quorum quenching (QQ), which is the mechanistic suppression, retardation, or interruption

of bacterial social behavior or communication [39].

The term quorum quenching (QQ) was coined in 2000 when the pathogenicity of the plant pathogen Erwinia carotovora was demonstrated to be highly attenuated by an enzyme AiiA lactonase in the strain Bacillus sp. 240B1. Quorum quenching disrupts bacterial communication by stopping the process of synthesizing signaling molecules, by blocking or crafting signaling molecules or by blocking receptors, one of the three critical elements that make up the QS system [40].

4.1 Strategies of Quorum Quenching

The three types of AHL (G-Negative Bacteria)-inactivating enzymes—known as lactonases, amidases, and oxidoreductases—can be categorized according to their mechanistic characteristics. In a reversible mechanism, lactonases in the AHL family hydrolyse the homoserine lactone ring to produce acyl homoserine. AHL acylases hydrolyse the AHL on the amide bonds, resulting in an irreversible reaction that produces fatty acid chains and homoserine lactone. AHL oxidoreductases, which can either oxidize or decrease the AHLs, have hardly ever been examined (refer to Fig. 7) [41].

Degradation of Signal Molecule

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Biosynthesis of Signal Inhibitor

In addition, the acyl carrier protein (ACP), the acyl component of the AHL signal, and S-adenosylmethionine (SAM), the amino donor of the synthesis of the homoserine lactone ring moiety, might be used to prevent the formation of AHL. It was discovered that the other SAM analogues, such as sinefungin, S-adenosylhomocysteine, and S-adenosylcysteine, were also efficient AHL synthesis inhibitors [43].

Other inhibitors that are utilized to prevent the synthesis of AHL have been found. For example, triclosan prevents the synthesis of AHL by preventing the enoyl-ACP reductase from producing the precursor [44].

Signal Receptor Antagonist

The formed receptor-AI complex controls the transcription of genes that control virulence, biofilm formation, conjugation, and sporulation in addition to bioluminescence and competence once the autoinducers have ultimately attached to their respective receptors. Furthermore, QS facilitates communica-

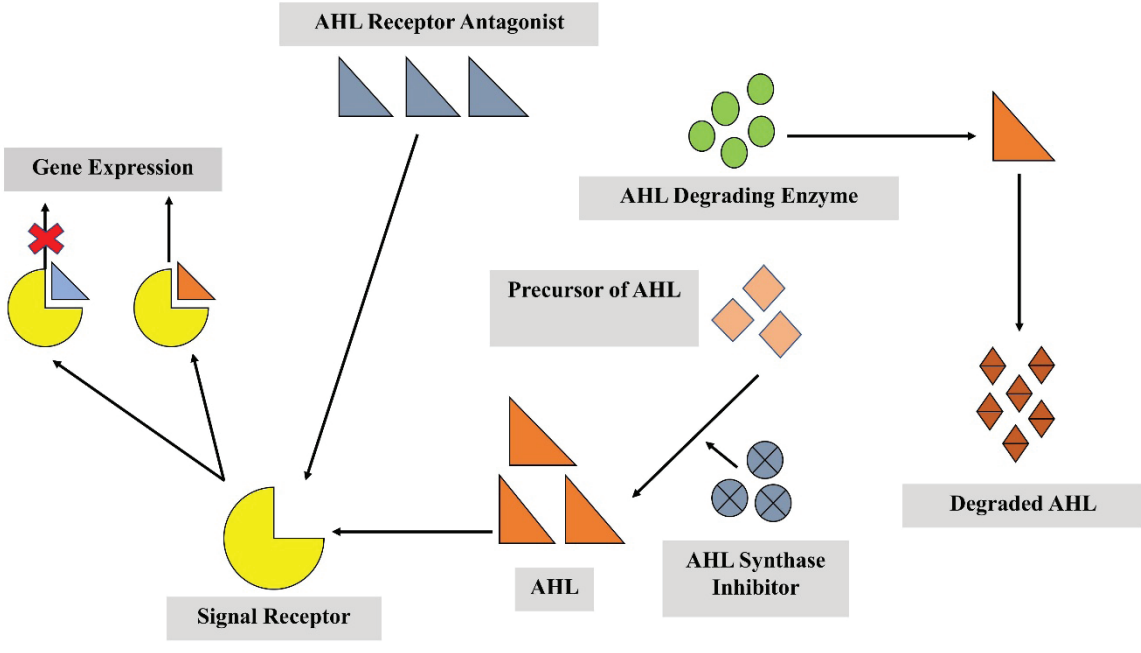

Figure 7 AHL inhibition strategies [5].

tion between bacteria of various species, and while some species lack the ability to synthesize their own AIs, they do have receptors for other species' AIs. It's interesting to note that bacterial infections' pathogenicity is much decreased by disrupting their QS systems and blocking the appropriate QS receptors [45]. By either competing with the bacteria at the receptor level or deactivating the receptor in the QS signaling, one can decrease the virulence and infection caused by the bacteria. Flavonoids and furanones have been identified as the two classes of QS inhibitors that bind to the receptors of different pathogenic bacteria. Furanones, whose halogenated derivatives are generated by the marine alga Delisea pulchra, were the first identified QS inhibitors [46].

The most prevalent AI receptor protein found in Gram-negative bacteria is LuxR-AHL. The addition of an active methylene group to the AHL also reduces the receptor's protein-signal binding by 50%, making the AHL alterations a very powerful tool for controlling processes with QS signals. Gram-positive bacteria control two systems of the QS: an active transcriptional regulator and a membrane-bound histidine kinase receptor. Currently, it is possible to block these receptors in Gram-positive bacteria against their pathogenicity by utilizing certain AIP antagonists to inhibit the receptors. Specifically, the four variant thiolactone peptides (AIP I-IV) of S. aureus, which employs the AIP-mediated QS system agr, have an impact on its bacterial activity [5].

Plant derived or Natural inhibitors of QS

Some of the secondary metabolites formed by medicinal plants target the QS system: phenols and phenolic acids, polyacetylenes, flavonoids, terpenoids, tannins, saponins, quinones, coumarins, alkaloids [47].

5. Applications and Advances in Anti-QS Therapy

The goal of anti-QS tactics is to interfere with the QS signaling pathways that allow bacteria to interact and coordinate their actions [48].

Anti-virulence drugs development

On the one hand, a new method of therapy known as precision antimicrobials was developed in order to overcome the increasing antibiotic resistance rate amongst microorganisms. Because precision antimicrobial drugs tend to target the pathogen-specific virulence determinants without affecting the resident microbiome, they are expected to establish restricted selective forces that significantly lower the risk of acquired resistance. More significantly, these antivirulence medicines target pathogen-specific virulence factors, ensuring that their activity is selective against only the disease-causing strain, rather than merely killing pathogens on a broad spectrum [49].

Nanoparticles targeting QS

In terms of therapeutic approaches, nanotechnology has been receiving a lot of interest. It was also demonstrated that nanomolecules, nanocomposites, and generally nano-and microcomposites, including composites based on Ag or ZnO, could successfully quench the quorum because they inhibited the microcolony, which decreased the creation of biofilms and changed their structure [47].

When it comes to bacterial biofilm development, metal and metallic nanoparticles like silver, selenium (SeNPs), tellurium (TeNPs), and gold (GNPs) have shown remarkable promise in the counteracting of bacterial resistance through nanotechnology. Projections reveal that nanotechnology could play a significant role in combating bacterial infections and managing them, specifically in fighting multidrug- resistant bacteria and bacterial biofilms [50].

Synergistic use with antibiotics

The QSIs do not kill or eliminate the bacteria; instead, they stop a biofilm from growing. Therefore, it is necessary to look for a synergistic interaction between QSIs and antibiotics [51]. Antibiotic resistance may be decreased by a combination therapy that uses synergistic medicines with distinct targets and mechanisms of action. Drugs like trimethoprim/sulfamethoxazole (Bactrim), tazobactam/piperacillin (Zosyn), and amoxicillin/clavulanate (Augmentin) are examples of combinations that have already received approval [52].

6. Challenges and Limitations in QS inhibitors

The organisms need to have a built-in defense system against an attack that could endanger their existence. A continuous state of AHL signals, albeit at a slower rate, can even be seen at low cell densities because quorum-sensing inhibitors (QSIs) are designed to essentially only engage QS without stimulating bacterial growth. Therefore, bacteria may be released to express their virulence once the concentration of QSI falls below the threshold. For bacteria to continue to be resistant to QSIs, they do not even need to undergo any genetic change. By keeping its QS under control until the concentrations of QSI exceed those of signal molecules, bacteria can evade QSI. It is stat-

ed that the goal going forward should be to build QSI with a lower chance of resistance. Every living thing will eventually develop defense systems because survival is their primary goal [53].

In the past few years there are a number of factors that have contributed towards the complex issue of multiple drug resistance (MDR). With periodic exposure to a particular antibiotic, multidrug and widespread drug resistance have occurred [5].

Bacteria have also evolved various forms of antibiotic resistance. First, chemical modification inhibits antibiotic effect by the secretion of enzymes which alter the chemical structure of the antibiotic either by disabling the drug molecule or by derivatizing its chemical functional groups, thus preventing the antibiotic to react with its target. Second, drug efflux pumps are an important mechanism for developing microbial resistance against antibiotics, wherein bacteria develop efflux pump proteins in the cell membrane, thus actively pumping out the antibiotic from the cell before it can reach the concentration needed for its effect. Third, bacteria can develop resistance by altering drug-target genes, either by shielding the target site against the binding action of an antibiotic, or by changing the target site itself to allow it to be less specific to the antibiotic molecule [54].

7. Future Prospects

Approaches based on CRISPER

The bacteria acquired the CRISPR-Cas system (Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated proteins) as a natural evolutionary immunity to bacteria viruses (bacteriophages). It was firstfound in E. coli in the 1980s, but its ability to actually form an adaptive immune system in bacteria, which is thought to be an analog of the mammalian system, was not realized until the 2000s. The invention of the CRISPR-Cas9 technique of gene editing in 2012 has turned the world of biotechnology upside down, enabling the editing of the genome. Scientists have recently concentrated on employing CRISPR-Cas, a novel antimicrobial intervention, to fight harmful bacteria and infections, especially those that are resistant to many drugs [55].

The CRISPR system is the most cutting-edge technology that promises to address the present issues with genome editing. It so happens that the CRISPR system is a natural immune response that protects against bacterial phage invasion. These main Cas protein and other nomenclature attributes have provided the classification of CRISPRCas systems into two classes, six types, and 19 subtypes [56]. Since its discovery, the “CRISPR-Cas9” system—a form of acquired immune system that shields several bacteria and archaea—has attracted a lot of attention [57].

By interfering with or removing the genes involved in autoinducer synthesis, detection, and downstream signaling, CCS has shown useful in controlling quorum sensing circuits [58]. Bacterial defense against viral infections depends on three key elements of the CRSIPR-Cas system: “adaptation (spacer acquisition), crRNA synthesis (expression), and target interference”. The Cas protein, sometimes referred to as the nuclease protein, is produced by the Cas gene and cleaves and destroys the foreign viral DNA [59].

Discovery of QS pathway by OMICS technologies

Through omics science, the host-pathogen relationship is consistently thoroughly and precisely studied, and the pathogen's unique proteins can be identified. Researchers are currently leveraging multi-omics data to update and reshape the microbial risk assessment role model.

Transcriptomics methodology employed in recent biofilm studies also enables the full detection of a certain set of genes that participate in antimicrobial resistance and biofilm development.

Although alterations in the whole protein profile can be detected using proteomics, nothing is known about the biological changes at the transcriptome level. The term "proteomics" describes the full expression of an organism's proteins under specific conditions.

Metabolomics is the detection, identification, and evaluation of a biological system's metabolome. Metabolite profiling can provide insights into the biochemistry, pathophysiology, and physiology of cells. Metabolomics is a useful technique for tracking the end points since, in contrast to genes and proteins, metabolite profiling is directly linked to the defined phenotype [60].

Through the deciphering of the molecular complexity of microbial life and the application of cutting-edge future technologies to these advancements, multi-omics technologies will address significant issues like food security and antibiotic resistance (ABR) [61]. Bacteria can coordinate gene expression and related metabolism through intercellular signaling, which can result in metabolic diversity in the products of bacterial metabolism. Metabolomics is the qualita-

tive and quantitative analysis of all of the low-weight molecular mass metabolites of a given organism or a given cell in a state of normal physiology [62].

By comparing samples taken under settings with and without QS induction, the transcriptome sequencing technique is fairly effective at identifying the genes linked to QS. Finding the QS-mediated expression profiles of various plant and human pathogens has been the most popular use of the technology [63].

Use of quorum sensing in the industry and agriculture beyond medicine

Quorum-sensing signaling systems are intimately linked to the network between the host plant and the associated microbial population, which is crucial for the holobiont's establishment. The balance of healthy or disease-causing bacteria and their host plants, which impart immunity and growth characteristics, is disrupted by interkingdom signaling. Although further research is need to completely understand the precise chemical process by which bacterial AHL signals impact plant performance, it is said that they do. Depending on the chemical structure of the QS signal, there is evidence that AHLs change the balance of phytohormones, mediate morphological changes of roots (elongation of the primary root, stimulation of root growth), increase salt tolerance, etc. [50].

CONCLUSION

Inhibition of quorum sensing (QS) is also a potent non-lethal approach to microbial antimicrobials because it interferes with communication networks regulating virulence and biofilm formation in microorganisms without killing microbes. Using QS-based medicines as a sustainable replacement of traditional antibiotics is an attractive prospect and emphasizes pathogenicity, biofilm formation and resistance mechanisms. The cutting-edge area in the potential application of this strategy pertains to the recent advances in the translational research efforts, including quorum quenching enzymes, natural, and synthetic enzyme inhibitors, nanotechnology-based drug and enzyme delivery vehicles, and combinatorial therapy using medications.

But multidisciplinary analysis connecting the domains of microbiology, chemistry, bioinformatics, nanotechnology and clinical sciences is important to achieve the full potent of attainment of its benefits. Future directions such as new pathway discovery via omics and fine-tuning of QS genes via CRISPR editing can accelerate clinical use. The introduction of QS-specific strategies into the process of antimicrobial therapy may completely change the landscape of the control of infections, allowing to limit the development of resistance and protecting the microbiome balance.

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CONTEMPORARY APPROACHES IN THE DIAGNOSIS AND STUDY OF *MYCOBACTERIA*: THE ROLE OF MICROSCOPY AND ARTIFICIAL INTELLIGENCE

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ABSTRACT

The genus *Mycobacterium* comprises structurally a complex of highly adaptive bacteria of major clinical importance. While tuberculosis remains a leading cause of mortality from infectious diseases worldwide, nontuberculous mycobacteria (NTM) are gaining increasing clinical relevance, particularly among immunocompromised patients. The unique structure of mycobacterial cell wall and the slow growth of these organisms necessitate the use of specialized microscopic approaches for their investigation and diagnosis.

Despite the advances in molecular diagnostics, microscopy remains a rapid and cost-effective key diagnostic tool, however limited by subjectivity and reduced sensitivity at low bacterial loads. The recent integration of artificial intelligence has significantly enhanced light and fluorescence microscopy by enabling an automated and standardized detection of the acid-fast bacilli.

The advanced high-resolution techniques, including electron and cryo-electron microscopy, provide detailed insights into mycobacterial ultrastructure and intracellular behaviour, contributing to a deeper understanding of their pathogenicity and drug resistance. This review summarizes the classical and the modern microscopic approaches and highlights their complementary roles in diagnosistic and fundamental studies of mycobacteria.

Keywords: Nontuberculous Mycobacteria, microscopy, diagnostics

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INTRODUCTION

The genus *Mycobacteria* includes structurally complex and biologically adaptive bacteria. They have a unique evolutionary strategy for survival in diverse ecological niches as well as within the human host [1-3]. The *Mycobacterium tuberculosis* complex (MTBC) remains a leading cause of mortality from infectious diseases worldwide, while nontuberculous mycobacteria (NTM) are increasingly recognized as important opportunistic pathogens, particularly in immunocompromised patients [4–6].

The unique structure of the mycobacterial cell wall, composed of peptidoglycan, arabinogalactan, and a dense layer of mycolic acids, underlies acid-fastness and limited permeability to dyes and drugs [1,2,7–9]. These characteristics necessitate the use of specific microscopic approaches for investigation. An additional challenge is the slow growth rate and prolonged generation time of mycobacteria [10–12].

Smear microscopy (SM) remains a key component of the diagnostic algorithm for tuberculosis and NTM infections, although it is typically used alongside other laboratory methods [6].

Despite the advances in molecular diagnostics, microscopy continues to play a major role in the early diagnosis of mycobacterial infections. Ziehl–Neelsen staining and its variants remain the standard technique for detecting acid-fast bacilli (AFB) using light or fluorescence microscopy [5]. Despite its high specificity (>98%), the method is semiquantitative and has limited sensitivity in paucibacillary samples [5]. Recent developments in artificial intelligence (AI) have introduced novel opportunities for automated analysis of microscopic images. AI-based algorithms enable standardized and high-throughput detection of AFB, improving the sensitivity, reproducibility, and workflow efficiency. Such technologies are particularly promising in resource-limited regions with a high tuberculosis burden. For more in-depth studies, of mycobacterial morphology, cellular organization, and cell dynamics, high resolution microscopic tech-

niques such as confocal laser scanning microscopy and electron microscopy are used [10,12].

Transmission electron microscopy enables a detailed visualization of mycobacterial ultrastructure, including the multilayered cell envelope. It provides evidence for the intracellular interactions between mycobacteria and host macrophages, allowing assessment of phagosomal membranes and defects in phagosome maturation [13].

Recent advances in cryo-electron microscopy have further expanded these capabilities by enabling the structural analysis of mycobacterial components in a near-native state without extensive chemical fixation. This approach facilitates high-resolution investigation of molecular complexes involved in cell wall organization, metabolic pathways, and mechanisms of drug resistance [13].

Traditional Diagnostic Methods:

Light and fluorescence microscopy: classical approaches and their role in mycobacterial diagnostics

Light microscopy remains a cornerstone in the diagnosis and investigation of mycobacteria [5,14]. It allows the detection of AFB using the classical Ziehl-Neelsen staining method or its variants, such as the cold Kinyoun method. These techniques are based on the irreversible binding of carbol fuchsin to mycobacterial mycolic acids, which confers acid-fastness [6]. Historically, using specialized staining techniques Robert Koch identified *M. tuberculosis* and laid the foundation for modern mycobacterial diagnostics. The Ziehl-Neelsen staining, subsequently refined through the cumulative discoveries of Paul Ehrlich, Franz Ziehl, and Friedrich Neelsen, has become the most widely accepted standard for detection of AFB [5,14]. The main advantages of light microscopy include rapid turnaround time, low cost, and ease of application even in resource-limited settings, while providing direct visual evidence of AFB in clinical specimens. It uses heat to facilitate the penetration of the primary stain into the lipid-rich, waxy cell wall of mycobacteria, therefore designated as a „hot staining" method [1,2,7]. Although this barrier limits dye penetration, once the carbol fuchsine enters the cell it is retained during acid–alcohol decolorization, allowing the clear visualization of mycobacteria as red rods against a contrasting background. Beyond detection, Ziehl–Neelsen staining provides valuable morphological information, including the characteristic cord formation by virulent *M. tuberculosis* strains, which is associated with pathogenicity [3,4]. In addition, microscopic examination allows the assessment of cellular integrity and approximate bacterial burden, contributing to treatment monitoring [5].

Despite its established role, light microscopy has important limitations. Its sensitivity is relatively low, requiring bacterial concentrations of at least 10^4 - 10^5 cells/ml for reliable detection [14]. As a result, early-stage and extrapulmonary tuberculosis may be missed. Furthermore, the method cannot distinguish between members of the *M. tuberculosis* complex and NTM, viable and nonviable organisms, or drug-susceptible and drug-resistant strains, necessitating complementary culture-based or molecular approaches [12].

Fluorescence microscopy constitutes an important extension of conventional light microscopy in mycobacterial diagnostics and research. The method relies on fluorochrome stains, including Auramine O and Auramine–Rhodamine, which bind to mycolic acid–rich components of the mycobacterial cell wall and emit bright yellow–green fluorescence when excited by UV or LED light [15,16]. This substantially enhances contrast and enables the rapid screening of large smear areas, resulting in a significantly higher sensitivity as compared to Ziehl–Neelsen staining [15,16].

In addition to improved detection, fluorescence microscopy provides valuable morphological information, allowing the visualization of subtle cellular alterations, granular forms, and aggregated structures that are not readily detected by standard light microscopy [9]. Such features are particularly relevant for nontuberculous mycobacteria, which exhibit greater morphological heterogeneity [4]. However, similar to acid-fast staining, this method does not permit species-level identification.

Recent advances in LED-based fluorescence microscopy have improved safety, reduced operational costs, and expanded applicability, particularly in laboratories with limited infrastructure [16]. A comparison of techniques for microscopy detection of genus *Mycobacterium* is presented in **Table 1**.

Ultrastructural look at the mycobacterial cell

Electron microscopy

Electron microscopy (EM) provides a resolution far beyond light-based methods, revealing structural features essential for the understanding of mycobac-

Table 1. Comparison of techniques for microscopy detection of genus *Mycobacterium*.

Method	Principle	Stain	Sensitivity*	Advantages	Limitations	Main application	Selected references
ZN microscopy	Acid-fast staining with heat	Carbol fuchsin	Moderate (10^4–10^5 bacilli/ml)	Low cost, easy, fast, standardized	Low sensitivity in paucibacillary samples; creating phenol aerosols	Routine diagnosis	5, 14, 18, 19
Kinyoun microscopy	Acid-fast staining without heat	Carbol fuchsin (high concentration)	Moderate (10^4–10^5 bacilli/ml)	Simple and safer than ZN	Slightly reduced sensitivity vs. ZN	Routine diagnosis	14, 19, 24
Auramine–rhodamine microscopy	Fluorochrome binding to mycolic acids	Auramine O / Rhodamine	High (10^3 bacilli/ml) 10% more sensitive that ZN	Increased sensitivity; rapid screening	Requires fluorescence microscope, potential toxicity of reagents	High volume laboratories	14, 16, 19
LED fluorescence microscopy	Fluorescence with LED illumination	Auramine O	High (with 5% more than Auramine–rhodamine microscopy)	Energy-efficient; durable	Higher initial cost	High volume laboratories	14, 16
AI-assisted microscopy	Automated image analysis	ZN / Fluorochrome	High	Speed, accuracy, is high efficiency	Requires validated algorithms; technical support	Screening	15,16, 23,24
Cryo-EM	Rapid freezing	Does not use conventional, heavy-metal stains	Very high	High resolution; 3D imaging	Expensive, researcher team	Advanced research	17
Electron microscopy (TEM/ SEM)	Electron beam imaging	Heavy metals	Very high	Ultrastructural detail	High cost, researcher team	Advanced research; structural studies	1, 2, 3, 10, 17, 20

***Abbreviations**: ZN - Ziehl–Neelsen; LED - light-emitting diode; AI - artificial intelligence; TEM - transmission electron microscopy; SEM - scanning electron microscopy.*
**Sensitivity is reported qualitatively and may vary depending on specimen quality, bacterial load, and operator expertise*

terial pathogenicity, physiology, and drug resistance [10,17]. Transmission EM (TEM) allows a detailed visualization of mycobacterial cell wall, [1,2,7,8]. TEM distinguishes the individual layers and demonstrates the thick, compact envelope that underlies acid-fastness. TEM has also elucidated polar growth in mycobacteria, showing that cell wall elongation occurs predominantly at one or both poles, with „new" poles exhibiting thinner, less organized layers. This structural heterogeneity contributes to differential antibiotic susceptibility and bacterial population variability, relevant for persistence and tolerance [10].

Beyond the cell wall, TEM reveals cytoplasmic inclusions, primarily lipid droplets, which serve as energy reserves and markers of persistent states. Antibiotic or nutrient stress increases the number and size of these inclusions, resulting in characteristic "foamy" morphologies associated with drug tolerance [13]. TEM is invaluable for studying drug effects on the cell wall; β-lactams induce peptidoglycan defects, while isoniazid disrupts mycolic acid layers, leading to vesicle formation and partial detachment of the lipid envelope [20]. Scanning EM (SEM) complements TEM by providing high-resolution three-dimensional surface imaging, showing topography, roughness, mucoid forms, and extracellular matrix, particularly in nontuberculous mycobacteria and biofilms - structures critical for antibiotic resistance and chronic infection [4,17].

The recent advances in cryo-electron microscopy (cryo-EM) enable the visualization of cells in a near-native state without chemical fixation, preserving ultrastructural integrity and allowing a molecular-level study of transport proteins, cell wall enzymes, and secretion systems such as ESX-1 [17]. Cryo-EM is particularly powerful for investigating resistance mechanisms, comparing structures of target proteins in sensitive and resistant strains (e.g., InhA, KatG, MmpL3, DprE1), and guiding novel drugs' development. Together, TEM, SEM, and cryo-EM provide a comprehensive understanding of mycobacterial architecture, complementing light microscopy and molecular methods by revealing the ultrastructural foundation of their biology and pathogenesis [1–3,17].

Modern optical approaches overcome the limitations

of classical structural microscopy by bridging morphological observations with functional cell biology. Confocal laser scanning microscopy, atomic force microscopy, and live-cell microscopy enable three-dimensional and dynamic investigation of mycobacterial cells, as well as the analysis of their biophysical properties [21,22]. These techniques provide critical insights into the organization of biofilms, cellular heterogeneity within populations, interactions with the immune system, and real-time adaptive responses to stress and antimicrobial agents [21,22].

In the last decade AI-based algorithms are widely applied in light, fluorescence, electron, cryo-electron, and atomic force microscopy, enabling the automated detection of acid-fast bacteria, analysis of the ultrastructure and the live-cell dynamics, and quantitative morphometric assessments.

The application of deep learning and artificial neural networks in microscopy is substantially transforming the analysis of biological images by shifting the focus from manual to automated and quantitative approaches. These technologies enable efficient image processing and enhancement, including noise reduction and high-resolution reconstruction (super-resolution), while preserving key structural features. Significant progress has also been achieved in image segmentation, where algorithms automatically recognize and distinguish cells, organelles, and microorganisms, as well as in real-time tracking of cellular behaviour [15,16,23,24].

In this context, the application of AI in TB microscopy is focused on the automated detection of AFB in sputum smears. This process is traditionally characterized by a high labour intensity and subjectivity. Contemporary approaches based on computer vision and deep learning demonstrate high diagnostic performance, particularly in resource-limited settings. Various technological solutions have been developed, including automated microscopy systems and mobile platforms integrating smartphones with conventional microscopes for real-time analysis. Object detection architectures (e.g., YOLOv7/YOLOv8) and convolutional neural networks (CNNs), such as ResNet and EfficientNet, are widely used for detection and classification [23,24].

The integration of AI into diagnostic workflows results in high accuracy (up to ~96–97%), improved detection of positive samples, and a substantial reduction in laboratory workload through automated screening, while also enhancing result standardization. Pilot studies conducted in diverse settings—including high-burden regions in sub-Saharan Africa and Asia, as well as well-equipped laboratories in Europe and North America report a sensitivity of approximately 92% and specificity of ~98% [26].

Despite these advances, important limitations remain, including the need for high-quality annotated datasets, robust quality assurance systems, algorithmic transparency, and interoperability with laboratory information systems, as well as dependence on sample quality and limited clinical validation of some solutions [13,21,22,25].

Notably, while the WHO has endorsed AI-based computer-aided detection for chest radiography (2021), no equivalent recommendation currently exists for AI-assisted smear microscopy [27]. Therefore, AI should be regarded as a complementary tool to established diagnostic methods, including Xpert MTB/RIF, rather than a replacement for expert microbiological interpretation.

CONCLUSION

Light microscopy continues to play a key role in tuberculosis diagnosis due to its accessibility, rapid turnaround time, and applicability in resource-limited settings, while fluorescence microscopy improves the sensitivity of detection. Electron microscopy provides unique high-resolution visualization of mycobacterial ultrastructure, particularly the complex architecture of the cell wall and intracellular localization.

However, each technique has inherent limitations. Conventional microscopy shows limited sensitivity in paucibacillary samples, and electron microscopy remains technically demanding and unsuitable for routine diagnostic use.

Future efforts are focused on integrating advanced imaging techniques with artificial intelligence–based tools to improve detection accuracy, standardization, and quantitative analysis in mycobacterial research.

Compliance with ethical standards

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Disclosure of conflict of interest

There is no conflict of interest to declare.

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TRICHINELLOSIS IN HUMANS IN PAZARDZHIK PROVINCE (1990-2024)

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ABSTRACT

Background. Trichinellosis is a parasitic disease acquired by ingestion of raw or undercooked meat infected with *Trichinella* larvae. The incidence of trichinellosis has decreased in the country for the last fifteen years. The aim of the study was to analyze the dynamics of trichinellosis in humans in Pazardzhik Province (in the south-central Bulgaria) for the period 1990-2024.

Material and methods. Data for this retrospective cross-sectional study were obtained from epidemiological studies and annual reports of the Laboratory of Medical Parasitology at Regional Health Inspectorate – Pazardzhik, as well as personal observations of patients with trichinellosis.

Results. A total of 472 infected persons and 16 outbreaks were registered in the province for the studied 34-year period. Most of the outbreaks were registered between 1990 and 2000, the last one occured in 2024. The source of infection was pork, wild boar meat or it was unknown but trichinoscopy of the meat was not done before consumption. Most affected were adults – 391 cases (82,8%) and city residents – 307 cases (65%). One hundred twenty-five (38%) patients had anti-*Trichinella* IgG when they were diagnosed with trichinellosis.

Conclusions. Trichinellosis remains a parasitic disease of medical importance for Pazardzhik Province. Awareness about the disease should be raised among doctors, population, especially in potentially endemic villages with registered outbreaks, pig farmers and hunters. When a case of trichinellosis is diagnosed, a detailed history can detect the source of infection. Meat inspection plays a significant role in preventing trichinellosis.

Key words: trichinellosis, incidence, epidemiology, prevention

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BACKGROUND

Trichinellosis is a zoonosis with worldwide distribution, more common in temperate climate regions. It is caused by nematodes of the genus *Trichinella*. There are nine species and four genotypes in this genus but the most common causative agent in humans is *Trichinella spiralis*. A lot of carnivores and omnivores are hosts of *Trichinella* spp. Humans acquire it by ingestion of raw or undercooked meat from pigs or other animals infected with *Trichinella* larvae (1-5).

Mandatory inspection of pork has been introduced in the country since 1910 (6). In the past, trichinellosis was a rarely diagnosed parasitic disease in Bulgaria but nowadays Bulgaria is among the most affected European countries . For the period 2019-2023, the EU notification rate was 0.01-0.03 per 100 000, in comparison to 0.79-0.02 per 100 000 in our country (7). Since 2010, a decreasing trend in morbidity has been observed, but it varies across different regions (8-11). In the 90s, Pazardzhik Province (located in the south-central Bulgaria) was one of the most affected by trichinellosis. Cases of the disease in the province were also observed in the past. In 1934, Vasil Mollov reported two trichinellosis outbreaks - in the cities of Panagyurishte and Strelcha. In 1973, 2 cases were registered in one family (12,13).

Aim. The aim of the study was to analyze the dynamics of trichinellosis in humans in Pazardzhik Province for the period 1990-2024.

Material and methods. Data used for this retrospective cross-sectional study of the prevalence of trichinellosis among the population of Pazardzhik Province, covering a 34-year period, were obtained from epidemiological studies and annual reports of the Laboratory of Medical Parasitology at the Regional Health Inspectorate – Pazardzhik as well as from personal observations of patients diagnosed and treated for trichinellosis from January 1990 to December 2024. The criteria used to classify the forms of trich-

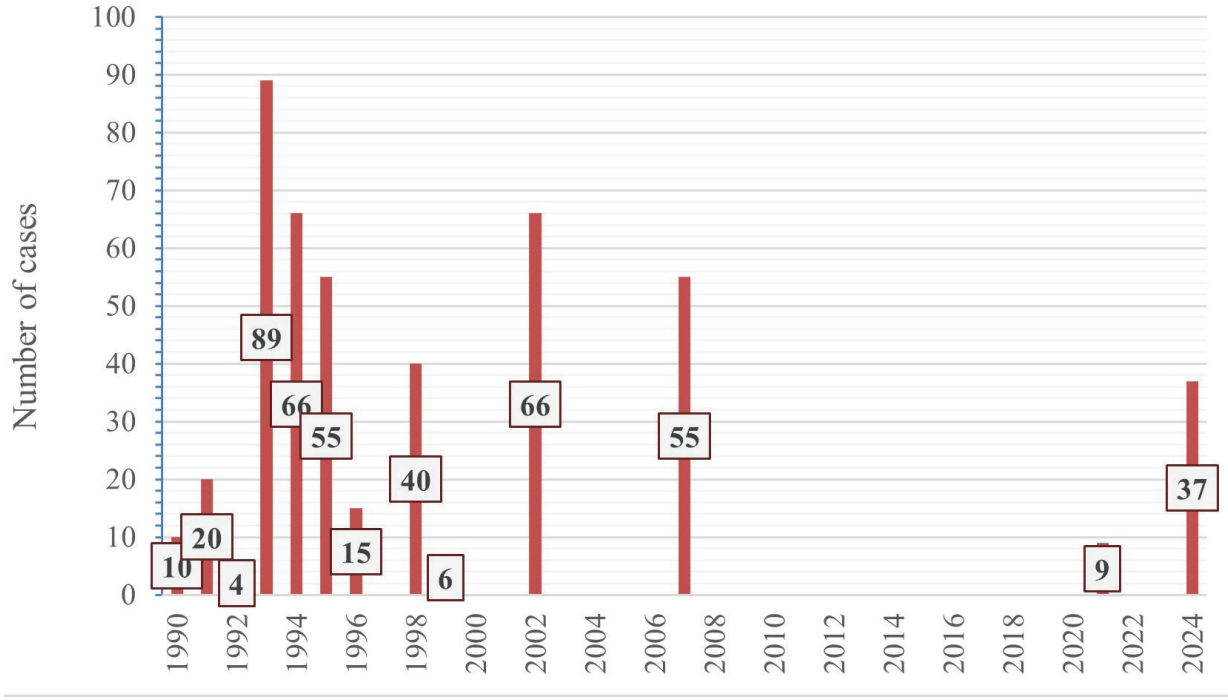

Fig. 1. Cases of trichinellosis in Pazardzhik Province (1990-2024)

inellosis were as follows: asymptomatic form – with a history of exposure to contaminated meat but without clinical signs and symptoms; abortive form – transient signs and symptoms, lasting for a few days; mild – mild signs and symptoms, without complications; moderate – presence of full blown syndrome, with rare benign and transient complications ; severe –pronounced signs and symptoms, with circulatory and/or neurological complications (14,15). Statistical analysis (t-test, mean, SD) was done using SPSS v.19.

Results. A total of 472 cases of trichinellosis and 16 outbreaks were registered in Pazardzhik Province for the period 1990-2024. All of them were observed from November to April. From 1990 to 2000, outbreaks were registered annually. The highest number of cases was in 1993: a total of 89 people were infected in two outbreaks. Two outbreaks were also registered in 1994 (a total of 66 patients) and 1995 (55 patients). Several cases in one family were registered twice - in 1992 (4 patients) and 1999 (6 patients). From 2000 to 2024, a decreasing trend in the number of trichinellosis outbreaks was observed, with only 4 outbreaks registered. The last one with 37 infected persons occurred in 2024 (Fig. 1):

The infected people were 4-75 years old (mean±SD 44,3±15,2). The age distribution showed that adults (above 19 years of age) were mainly affected (82,8% or 391 persons), while children and adolescents (under the age of 19) comprised only 17,2% (81 persons), ($p<0.01$). The highest number of patients were in the age group 40 - 49 (54 or13,8%). Females comprised were 52,3% of the patients (247 persons) vs. 47,3% males (225 persons) ($p>0.05$). During the study period, trichinellosis was diagnosed more frequently in city residents (307 cases , 65%) while village residents were 169 (35%) ($p<0.01$).

A total of 333 patients (70,5%) were tested for anti-*Trichinella* IgG , based on signs or symptoms of the disease or a history of consumption of contaminated meat. Reverse passive haemagglutination was used in 245 of the cases. It showed that 85 (35%) of the tested persons were positive. ELISA test was used in 88 of the cases, 40 (45%) of them were positive for antibodies. Regardless of the method, 125 (38%) of the tested had positive serology for trichinellosis.

Different forms of the disease were observed during the studied period. Abortive forms occurred in 146 (31%) of the patients, mild forms – in 151 patients (32%), moderate – in 175 (37%) of the patients. Few complications (in 1,5% of the infected) were registered, including single cases of myocarditis, pneumonia, and anemia and 4 cases of radiculoneuritis, but no fatal cases.

Patients diagnosed with trichinellosis between 1990 and 2000 were treated with mebendazole 20 mg/kg for 10-14 days. After this period, patients received albendazole 10-15 mg/kg for 7-14 days. Another 14 persons who consumed infected pork in 2011, took

chemoprophylaxis with albendazole for 3 days from the day following the consumption. As a result, none of these persons developed clinical features of trichinellosis later.

We found that half of the outbreaks were caused by wild boar meat (Tabl. 1):

Tabl. 1. Source of infection of trichinellosis outbreaks in Pazardzhik Province (1990-2024)

Source of infection	Number of outbreaks/%	Infected persons/%
wild boar	8 (50%)	312 (66,1%)
domestic pork	7 (44%)	150 (31,8%)
unknown	1 (6%)	10 (2,1%)
Total	**16** (100%)	**472** (100%)

The infected meat was consumed as raw fillets and sausages made of domestic pork or wild boar meat. In none of the cases trichinoscopy of the meat had been done before consumption but trichinoscopy and artificial digestion of the infected meat, which caused the last two outbreaks were conducted in the Regional Health Inspectorate - Pazardzhik. They revealed that the consumed sausages contained larvae of *Trichinella* spp. *Trichinella* larvae collected from the contaminated meat (meat product) were then subjected to species identification by polymerase chain reaction, conducted at the National Centre of Infectious and Parasitic Diseases. *Trichinella britovi* was the species identified in both cases and the source of infection was wild boar meat. In one of these cases, the wild boar was shot illegally, and in the other case, the meat was purchased from uncertain source.

Outbreaks were observed in several settlements, as shown in Fig.2 (Fig. 2):

Outbreaks occurred mainly in the northern part of the province (the southern foothills of Sredna Gora mountain) as follows: in Panagyurishte, Levski, Banya, Strelcha, Popintsi and Borimechkovo. In the middle part of the province (the Upper Thracian lowland) trichinellosis cases were registered in Pazardzhik, Septemvri, Aleko Konstantinovo, Belovo, Sestrimo. Cases were also registered in two villages in Rhodopa mountain - Byaga and Ravnogor.

DISCUSSION

Trichinellosis is a food-borne parasitic disease. It occurs as sporadic cases or outbreaks, affecting family members, relatives, and friends, mainly in autumn and winter during the hunting season or when the domestic pigs are slaughtered (6).

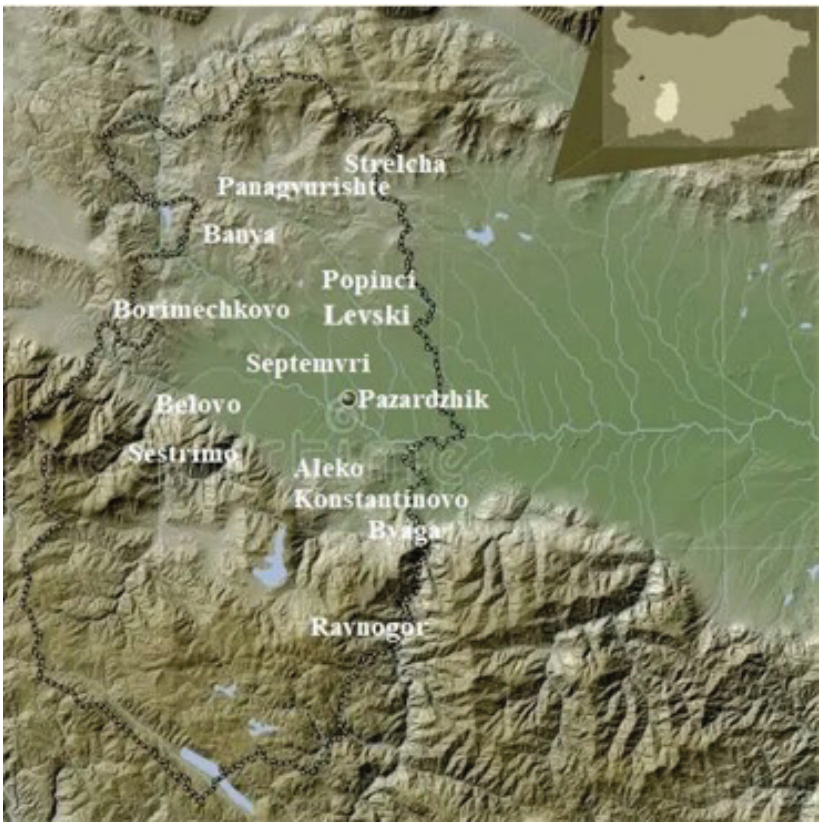

Fig. 2. Settlements with cases of trichinellosis in Pazardzhik Province (1990-2024)

Pazardzhik Province has some geographical, social, and economic characteristics which favor the spread of trichinellosis. Out of 4,458 km^2, most of the half (58%) of the province is occupied by mountains and forests. In its southern and southwestern part are the Western Rhodopes and the Rila mountain. Agricultural areas occupy 36% of the territory and have favorable soil and climatic conditions. These factors are suitable for the survival of hosts of *Trichinella* spp.: boar, fox, jackal, wolf, bear, wild cat, squirrel, goldfinch, badger. In addition, pig farming is well developed: about 50,000 pigs are raised in private farms (12,13). Outbreaks were observed among the population of mountainous areas and lowland villages of the province. In some villages more than one outbreak have been registered. The number of outbreaks has decreased in Pazardzhik province since 2000. A similar situation has been observed throughout the country for the last 15 years. It can be explained with stricter veterinary control of pig farming and meat obtained through hunting, as well as the population awareness of the disease (9).

Identifying the source of infection is of essential epidemiological and clinical importance. In our study wild boar meat was the main source of infection. According to data from Raynova (2020), in Bulgaria from 2000 to 2017, the main source of infection was also wild boar meat (in 45% of the outbreaks) (9). In Europe, wild boar meat has been a major source of trichinellosis in humans for the last ten years whereas earlier, pig meat was the main source (7, 16,17).

The most common *Trichinella* species in Bulgaria as well as in the Balkan region are *T. spiralis* and *T. britovi* (9,18,20). Species of *Trichinella* was determined only in the last two outbreaks, so we could not discuss the severity of the disease caused by different species. There are data that *T. spiralis* is responsible for more severe clinical pictures compared to *T. britovi* (9,21).

Trichinellosis affects primarily adults (3). This is explained by the fact that children are not given undercooked pork, home-made sausages or game meat (17,22). In the present study, slightly more cases were registered in women, without statistical significance. Our data differ from the data for the country, which show that men are more commonly affected (9).

Trichinellosis affected predominantly city residents. The reason is probably the intensive migration of the population from villages to cities over the last three decades. Moreover, home-made sausages are given to relatives and friends living in urban regions (17,22). Medical doctors faced some difficulties in the diagnosis of trichinellosis as signs and symptoms are not specific (6,23). History of consumption of raw or underdone meat is essential for the accurate diagnosis. Serology is widely used for diagnosis, and ELISA is the most common serological test. Initially, patients may have negative serological results because production of specific antibodies takes 3-4 weeks (14,24). In our study around 40% of the tested had anti-*Trichinella* antibodies at the time of diagnosis. A higher percentage of patients diagnosed with trichinellosis with anti-*Trichinella* antibodies was reported by Vutova et al. (79.4% of those tested with reverse passive haemaglutination and 75.9% with ELISA) (2020) (23). This difference in the results can be explained by the different sensitivity of serological tests, the time of infestation and the immune status of the infected (14,24).

When treatment with anthelmintics (albendazole or mebendazole) is initiated early during the enteral phase or acute stage, its effectiveness is greater (14,15). Probably, because of accurate etiological therapy we observed a low percentage of complications, and no fatal cases. Chemoprophylaxis with albendazole is most effective when it starts early, if possible, within 6 days following consumption of contaminated meat (25). We observed this effect in people taking albendazole the day after the consumption.

Conclusions. Although cases of trichinellosis in humans have decreased in Pazardzhik Province, it remains a parasitic disease of medical importance. Awareness about the disease should be raised among doctors, population, especially in potentially endemic villages with registered outbreaks, among pig farmers and hunters. Meat inspection plays a significant role in preventiing trichinellosis. When a case of trichinellosis is diagnosed, a detailed history can detect the source of infection. Screening of other people who have consumed the same meat products contributes to the timely diagnosis and treatment of all infected persons.

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DIAGNOSTIC CHALLENGES AND OUTCOMES OF EMPIRICAL THERAPY FOR NEUROCYSTICERCOSIS IN AN UNTREATED HIV PATIENT

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ABSTRACT

This case report describes a 35-year-old woman, Jane Doe (JD), who recently immigrated to Miami, Florida from Latin America and presented to the emergency department after a witnessed seizure. She reported one prior seizure seven years earlier in her home country, for which she did not receive anti-seizure therapy. JD also disclosed a 16-year history of human immunodeficiency virus (HIV) infection and had been off treatment for the past five years.

An extensive workup for potential infectious etiologies—including tuberculosis, syphilis, toxoplasmosis, cytomegalovirus, herpes simplex virus, strongyloidiasis, and hepatitis—was negative. Magnetic resonance imaging revealed multiple calcified supra- and infratentorial lesions with vasogenic edema and internal septations. Given these findings and JD's history of HIV infection and residence in an endemic region prior to immigration, the differential diagnosis included central nervous system (CNS) lymphoma, toxoplasmosis, and neurocysticercosis (NCC). Diagnosis was complicated by her advanced HIV disease, negative serologic testing, and the broad differential diagnosis for multiple ring-enhancing lesions in immunocompromised patients.

JD's presentation met Infectious Diseases Society of America criteria for NCC. She was started on a 14-day course of albendazole and praziquantel, as well as levetiracetam for seizure prevention and Biktarvy and Bactrim for HIV management. She was discharged after 12 days with plans for outpatient follow-up. This case underscores the importance of considering NCC in patients with untreated HIV infection and relevant epidemiologic exposures, even when serologic testing is negative.

Keywords: neurocysticercosis, HIV, case report

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INTRODUCTION

Neurocysticercosis (NCC) is a parasitic infection of the central nervous system caused by the larval stage of *Taenia solium*. The parasite is endemic in many parts of the world, including Latin America, Southeast Asia, and sub-Saharan Africa. In these regions, NCC accounts for approximately 30% of epilepsy cases and is a leading cause of new-onset seizures in adults [1]. Although not endemic in the United States (US), NCC has become increasingly prevalent with rising immigration from affected areas.

Transmission occurs through ingestion of T. solium eggs shed in human feces. Clinical manifestations vary widely depending on the number, stage, and location of cysts, as well as the host immune response. Patients most commonly present with seizures, headaches, or signs of elevated intracranial pressure. NCC can easily be mistaken for other infectious or structural brain lesions. Diagnosis typically relies on neuroimaging, supported by epidemiologic factors and serologic testing. The enzyme-linked immunoelectrotransfer blot (EITB) is the preferred serologic assay due to its high sensitivity and specificity in patients with multiple cysts, while ELISA may yield false-negative results and complicate diagnosis [2]. Diagnostic uncertainty is further amplified in individuals with untreated HIV, whose differential diagnosis or new neurologic symptoms includes a wide range of opportunistic infections and neoplasms [3].

Here, we describe the case of a 35-year-old woman with untreated HIV infection who presented with new-onset

seizures and multifocal parenchymal lesions consistent with NCC. Her case underscores the challenges of distinguishing NCC from other HIV-associated CNS diseases and highlights the importance of considering NCC in patients with relevant epidemiologic exposures.

CASE PRESENTATION

The patient is a 35-year-old woman with a history of HIV diagnosed in Cuba in 2006 who has not taken antiretroviral therapy for the past five years due to religious beliefs. She was brought to the emergency department by EMS on 12/9/22 after a witnessed seizure. According to a family member, she had been lying on a sofa when she suddenly became unresponsive and developed generalized shaking movements lasting "about 20 minutes." This was followed by a brief postictal period during which she "fell asleep for a few minutes" and then awoke drowsy and disoriented. Neither the patient nor the family reported urinary or fecal incontinence, head trauma, or tongue biting. On evaluation, the patient denied headache, visual changes, limb weakness or numbness, shortness of breath, or diarrhea.

She reported a remote history of a single seizure many years earlier in Cuba, which she attributed to stress. She denied alcohol or illicit drug use. She endorsed symptoms of anxiety and depression related to family stress but denied suicidal ideation or plan. She reported maintaining adequate oral intake but noted an unintentional weight loss of approximately 50 pounds over the past year. From an epidemiological perspective, the patient's prolonged residence in a region where Taenia solium is endemic supported consideration of neurocysticercosis, as infection can occur through fecal–oral ingestion of parasite eggs independently of pork consumption.

Admission laboratory studies (**Table 1**) were notable for normocytic anemia, leukopenia, an ESR of 71, and a lactate level of 3.4. A non-contrast head CT (**Figure 1**) demonstrated multiple calcified parenchymal lesions with areas of vasogenic edema. Her EKG showed normal sinus rhythm. The CT findings raised concern for an HIV/AIDS-related central nervous system infection or lymphoma, prompting admission to the inpatient service with early neurology and infectious disease consultation.

The patient was started on levetiracetam 500 mg twice daily. Although she tested positive for parainfluenza on admission, she remained asymptomatic without respiratory complaints. Brain MRI (**Figure 2**) revealed numerous—at least 20—supra and infratentorial lesions, several of which demonstrated calcification, peripheral enhancement, and internal septations. Vasogenic edema was also present. Given the multiplicity and characteristics of these lesions, neurocysticercosis was strongly suspected.

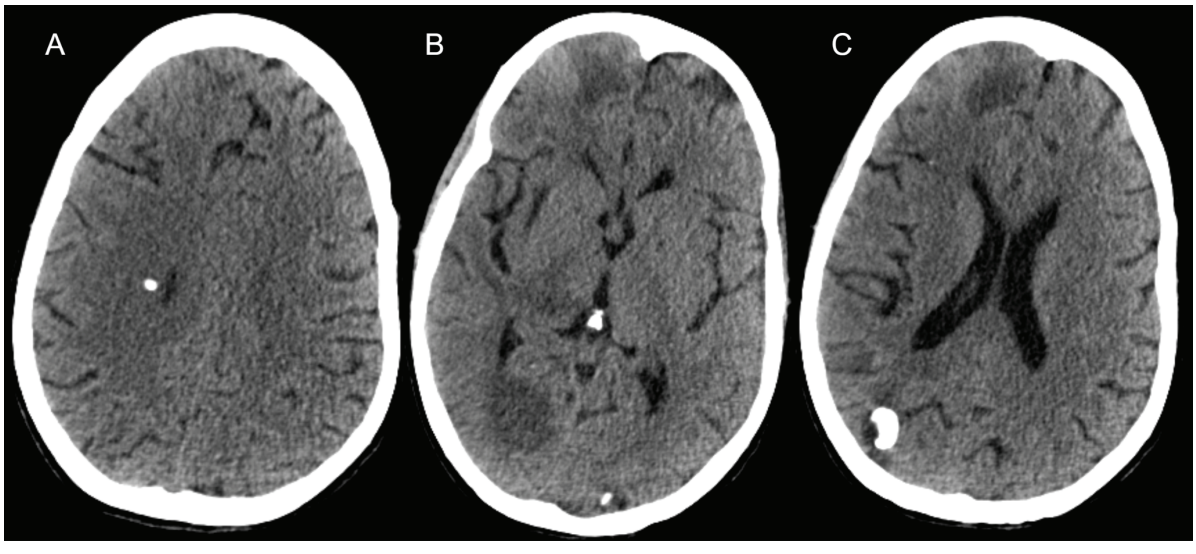

Figure 1. Brain CT without Contrast

CT Brain findings of intracranial lesions at admission. (A, B) Axial Brain CT without contrast images demonstrate numerous calcified parenchymal lesions and regions of vasogenic edema. (D) Largest lesion measuring to 18 mm on long axis.

Table 1. Admission Biochemistry Findings

Biochemistry	Reference range	Day 1
CBC with differential		
WBC (×10³/μL)	3.40–11.0	4.01
RBC (×10⁶/μL)	3.80–5.20	3.21 (L)
Hemoglobin (g/dL)	12.0–15.0	8.0 (L)
MCV (×10^{-15}L)	80-100	80.4
Hematocrit (%)	35.0–45.0	25.8 (L)
Platelets (×10³/μL)	130–360	191
Metabolic panel		
Sodium (mEq/L)	136–145	137
Potassium (mEq/L)	3.5–5.1	4.2
Chloride (mEq/L)	98–107	104
Bicarbonate (mEq/L)	21–32	26
Calcium (mg/dL)	8.5–10.1	8.6
Glucose (mg/dL)	70–126	101
Liver function		
ALT (U/L)	16–65	18
AST (U/L)	8–37	22
Total bilirubin (mg/dL)	0.2–1.0	0.1 (L)
Kidney function		
Creatinine (mg/dL)	0.55–1.02	0.68
BUN (mg/dL)	7–18	19 (H)
BUN/Cr ratio	12.0–20.0	27.9 (H)
Lactic acid (mmol/L)	0.5–2.2	3.4 (H)
Other		
ESR (mm/hr)	0-20	71

CBC = complete blood count; WBC = white blood cell; RBC = red blood cell; MCV = mean corpuscular volume; ALT = alanine aminotransferase; AST = aspartate aminotransferase; BUN = blood urea nitrogen; Cr = creatinine; ESR = erythrocyte sedimentation rate

The EEG performed on 12/12 was unremarkable. Given the patient's low CD4 count, she was started on trimethoprim-sulfamethoxazole SS daily for Pneumocystis jirovecii pneumonia (PJP) prophylaxis per Infectious Disease (ID). Due to significant cerebral edema, corticosteroid therapy was initiated on 12/15. As recommended by ID, steroids were to be administered for at least 24–48 hours before starting antiparasitic treatment for neurocysticercosis. Because several lesions were not fully calcified and were suspected to represent viable cysts, treatment for active neurocysticercosis was planned. The recommended regimen included albendazole (15 mg/kg/day, max 800 mg/day) and praziquantel (50 mg/kg/day) for 14 days, in conjunction with steroids. Ophthalmology was consulted prior to therapy initiation; the baseline exam was normal, and no papilledema was observed. Neurosurgery was consulted regarding a potential transfer to Baptist Main. However, the patient's neurological examinations remained stable throughout hospitalization, and repeat imaging showed no interval changes. After detailed discussion, neurosurgical evaluation was deemed unnecessary. The patient was transferred to the stepdown unit at WKBH for initiation of albendazole and praziquantel, with neurochecks ordered every two hours.

Initial serologic testing for neurocysticercosis was performed using an ELISA and returned negative. Infectious Disease consultants documented that ELISA testing has limited diagnostic utility for neurocysticercosis and therefore ordered confirmatory EITB testing. The EITB subsequently resulted as negative for cysticercus IgG antibodies. As noted by the performing laboratory, a negative EITB result does not exclude the diagnosis of neurocysticercosis. In the setting of advanced HIV infection with severe immunosuppres-

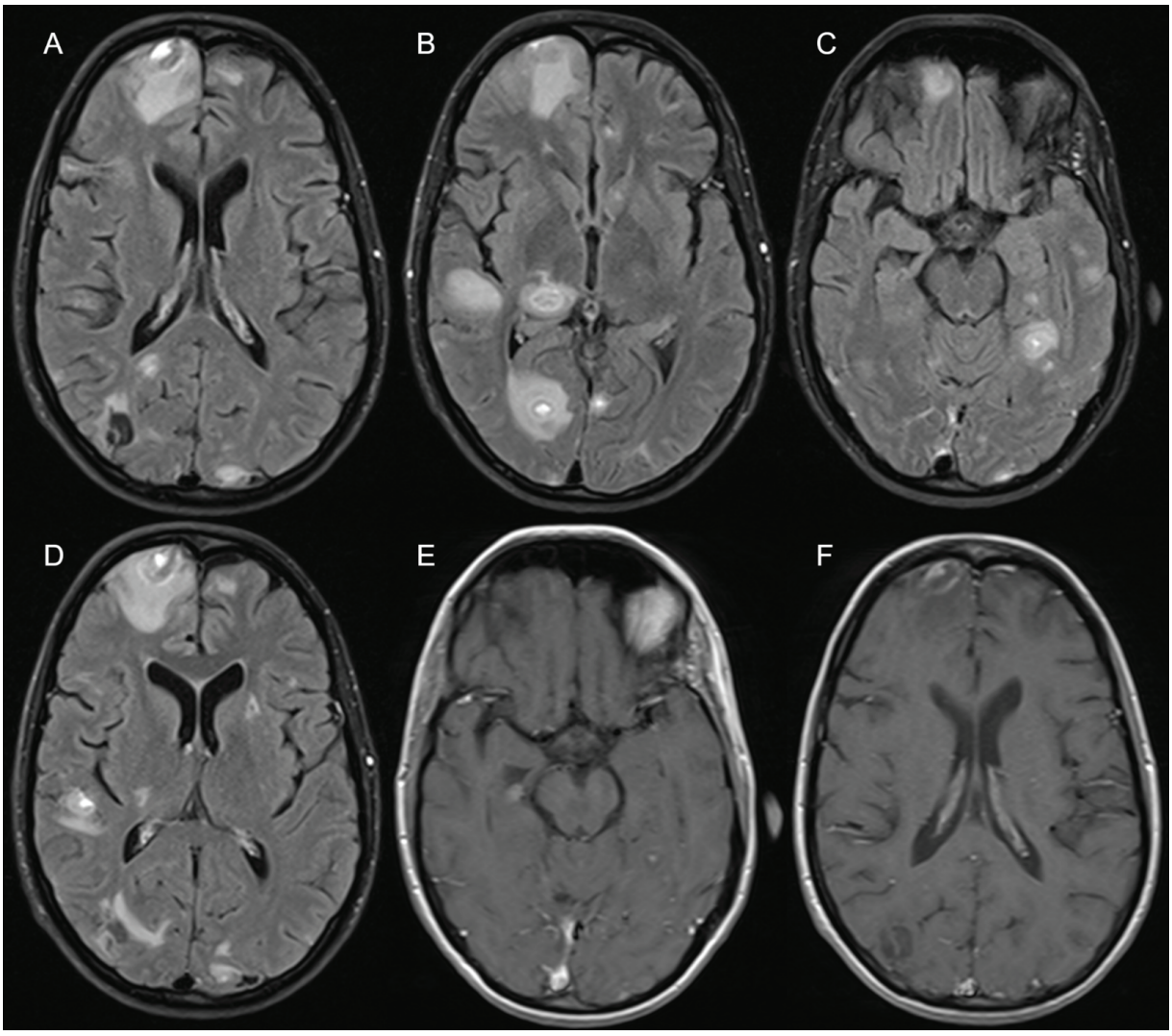

Figure 2. Brain MRI with and without Contrast

MRI findings of intracranial lesions at admission, Images demonstrate multiple supra- and infra-tentortal lesions. (A, B, C, D) Axial T2/FLAIR images show numerous lesions with classic "hole-with-dot" appearance consisting of peripheral enhancement, and eccentric scolex enhancement. Some lesions demonstrate associated calcification and internal septation as well. Vasogenic edema is associated with most of the lesions. (E, F) Axial T1 Post redomonstrates the "hole-with-dot" lesions.

sion (CD4 count 16 cells/μL), false-negative serologic results have been well described. The patient will require close outpatient follow-up with Infectious Diseases specialist. She has an appointment scheduled for next week, at which time ID will repeat a CBC and comprehensive metabolic panel. She will also need outpatient neurology follow-up; due to insurance requirements, a referral from her primary care provider will be necessary. These details, along with the importance of medication adherence, were reviewed with the patient, who verbalized understanding.

At discharge, the patient was prescribed albendazole and praziquantel to complete a 14-day treatment course, along with a Medrol Dosepak and famotidine for gastrointestinal prophylaxis during steroid therapy. She was also continued on levetiracetam 500 mg twice daily pending neurology evaluation and maintained on trimethoprim-sulfamethoxazole for PJP prophylaxis. A discharge CT scan of the brain (**Figure 3**) showed findings similar to admission, with multiple calcified lesions and modest improvement in vasogenic edema, though some edema persisted. Imaging did not show evidence of an immediate change in the viable cysts, as none of the lesions had resolved or decreased in size or number, despite initiation of antiparasitic and steroid therapy multiple days prior to her discharge.

Table 2. Infectious Panel

Infectious Panel	Day 1
Hepatitis B Surface Antigen	Non Reactive
Hepatitis B Core IgM Antibody	Non Reactive
Hepatitis A IgM Antibody	Non Reactive
Hepatitis C IgM Antibody	Non Reactive
QuantiFERON	Negative
Strongyloides IgG Antibody	Negative
Toxoplasma gondii IgG	Positive
Toxoplasma gondii IgM	Equivocal
Cytomegalovirus IgG	Positive
Cytomegalovirus IgM	Negative
Herpes Simplex Virus 1 IgG	Positive
Herpes Simplex Virus 2 IgG	Negative
Herpes Simplex Virus 1 and 2 IgM	Negative
EBV Capsid IgM	Negative
Histoplasma Antibody H Band	Negative
HIV Viral Load	992,000
CD 4 Count (/uL)	16

EBV = Epstein-Barr Virus;
HIV = Human Immunodeficiency Virus

Images

Brain CT without Contrast – 12/10

Impression: There are multiple calcified parenchymal lesions and regions of vasogenic edema. There is no midline shift or acute intracranial hemorrhage.

Brain MRI With and Without Contrast – 12/10 Impression: There are multifocal supra- and infratentorial lesions (at least 20). Several of these lesions show calcification, peripheral enhancement, and/or internal septations. Most are associated with vasogenic edema. The leading consideration is neurocysticercosis. A superimposed or previously treated toxoplasmosis infection could present with a similar appearance. Correlation with CD4 count is recommended. Short-term follow-up and comparison with any prior outside imaging, if available, are suggested.

Brain CT Without Contrast – 12/21 Impression: In this patient with HIV, multiple areas of vasogenic edema are again observed, some of which remain associated with calcified lesions. Superimposed areas of encephalomalacia are also again noted. No new findings.

DISCUSSION

Neurocysticercosis (NCC) is a leading cause of acquired epilepsy and the most common parasitic infection of the CNS worldwide [1]. However, its presentation in patients with untreated HIV remains poorly understood, complicating diagnosis and management. Clinical manifestations arise from degeneration of Taenia solium cysts within the CNS and depend on cyst number, size, and location, as well as the host immune response [1]. Reported presentations of NCC in individuals with HIV include epileptic seizures, such as in our patient, as well as headaches, focal neurological deficits, hemiparesis, and signs of increased intracranial pressure [4]. A case report of a patient with NCC presenting with symptoms of cauda equina compression, including acute urinary retention and lower extremity pain and weakness, has also been described [5]. Seizure presentation of NCC appears more likely in patients with lower CD4 counts compared with those who are HIV-negative or have higher CD4 levels [4], which may explain our patient's atypical clinical picture.

Patients with NCC and HIV may also have concomitant CNS infections at presentation, including toxoplasmosis and tuberculosis [6]. The most common HIV-associated CNS opportunistic infections globally include cerebral toxoplasmosis (including toxoplasmic encephalitis), progressive multifocal leukoencephalopathy, primary CNS lymphoma, cytomegalovirus encephalitis, cryptococcal meningitis, tuberculous meningitis, and herpes simplex virus encephalitis [7]. In patients from non–high-income settings, cerebral malaria, Chagas disease, and NCC should also be considered [7]. This wide differential, combined with HIV-related neurological complications, makes timely diagnosis of NCC challenging, especially in the context of concurrent HIV opportunistic CNS infections.

Diagnostic confirmation can be difficult. Serologic tests, including ELISA, lack sufficient reliability, and negative results are particularly common in patients with low CD4 counts [4]. Although EITB is considered the preferred serologic assay for neurocysticercosis, negative results do not exclude the diagnosis, particularly in patients with advanced immunosuppression. Clinical practice guidelines and prior studies have demonstrated reduced sensitivity of serologic testing in individuals with severe HIV-associated CD4 lymphopenia and emphasize that diagnosis may rely primarily on characteristic neuroimaging findings and epidemiologic exposure in such cases [3,4].

Imaging may show vesicular, colloidal, calcified, or racemose cysts. Most cysts are intraparenchymal, although extra-parenchymal, spinal, and subretinal lesions have been reported [4]. Multiple parenchymal lesions are the most frequent finding [8]. These imaging patterns are thought to reflect uncontrolled parasitic proliferation and blunted inflammatory re-

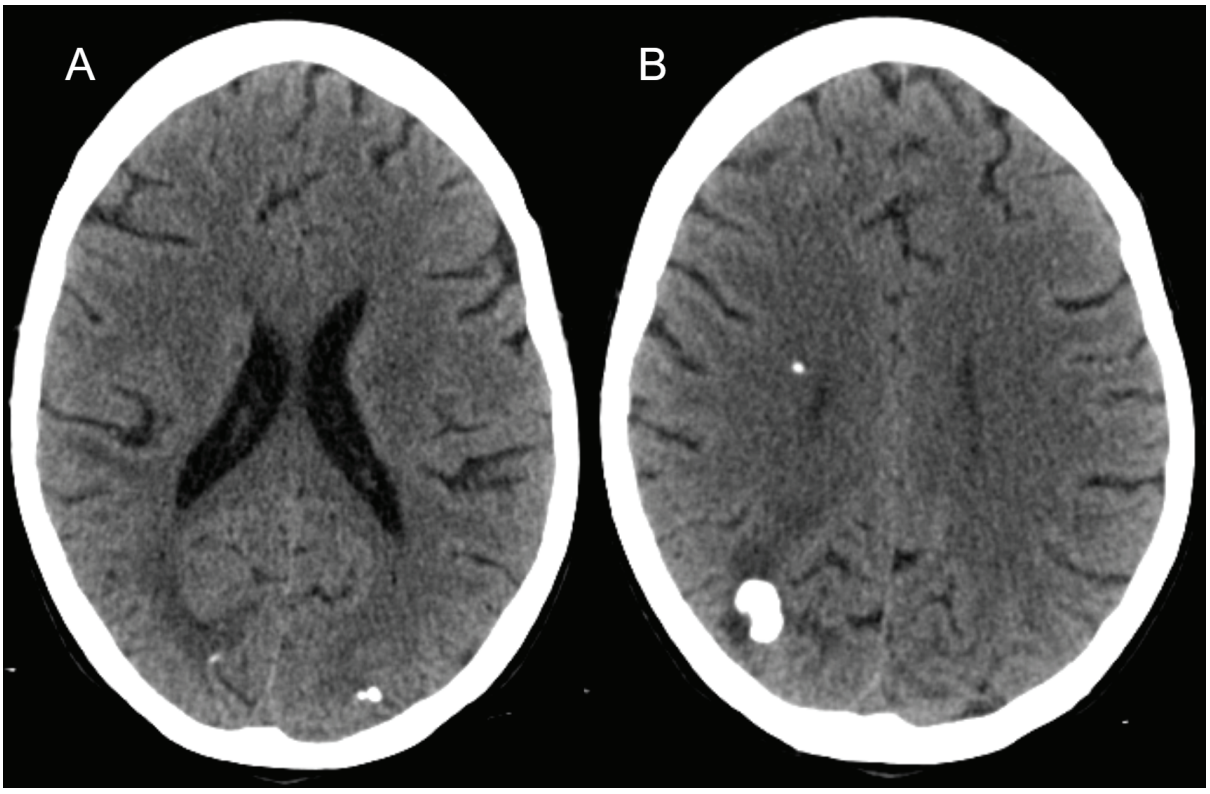

Figure 3: Brain CT without Contrast

CT Brain findings of intracranial lesions at discharge, eleven days after admission. (A, B) Axial Brain CT without contrast images demonstrate numerous calcified lesions with modest improvement of associated vasogenic edema.

sponses due to HIV-related immunosuppression [8]. Atypical lesions, such as racemose cysts, occur more frequently in individuals with CD4 counts below 500 cells/mm³ [8], which may further delay diagnosis.

Management of NCC often involves a combination of medical and surgical therapy. Reported treatments include surgery plus albendazole, surgery alone, albendazole alone, praziquantel alone, or no anthelmintic therapy [4]. Surgical interventions are typically performed for cyst removal (via craniotomy or spinal laminectomy) or for managing elevated intracranial pressure (e.g., ventriculoperitoneal shunt placement) [4]. Some patients receive adjunctive corticosteroids—most commonly dexamethasone or prednisolone [4]. The most frequently used and effective regimen is albendazole 15 mg/kg/day for 14–28 days combined with corticosteroids [4]. Combination therapy with albendazole plus praziquantel has been shown to improve cyst resolution and to increase destruction of viable parenchymal cysticerci for patients with three or more cysts [3,4]. For this reason, our patient was treated with both agents. A methylprednisolone dose pack was added to the regimen to mitigate exacerbation of symptoms secondary to inflammation from parasite death as a result of the antiparasitic medications [3]. Though our patient did not have complete cyst resolution at the time of discharge, there was some improvement noted in the vasogenic edema surrounding the lesions. The viable cysts did not seem to decrease in number or size at the time of discharge, which is logical considering the patient had just been initiated on antiparasitic treatment and had yet to complete the recommended course, and additionally cyst resolution has been shown to take months to occur. Studies have found that antiparasitic treatment destroys 60% to 80% of viable intraparenchymal cysts and complete cyst resolution occurs in less than 40% of patients [11]. Therefore, an absence of an immediate change in the viable cysts at discharge does not necessarily indicate a lack of efficacy of the treatment regimen.

Among patients presenting with seizures, only a subset received antiepileptic drugs (AEDs) [4]. Some individuals were already on antiretroviral therapy

(ART) at the time of NCC diagnosis, while others began ART concurrently with anthelmintic treatment [4]. This was true for our patient, who experienced a witnessed seizure and was initiated on Biktarvy in addition to albendazole and praziquantel. A known concern when initiating ART is the risk of immune reconstitution inflammatory syndrome (IRIS), which can convert subclinical NCC into symptomatic disease [4]. Nonetheless, anthelmintic therapy is recommended because it reduces recurrent seizure frequency and the risk of chronic epilepsy [9,10].

A further consideration is potential drug–drug interactions among AEDs, ART, and anthelmintics; an area that remains under-studied [4]. Newer AEDs, which have simpler side-effect profiles, are preferred when ART regimens include protease inhibitors [9], although older AEDs may also be effective in controlling seizures in NCC [9]. Although Biktarvy does not contain a protease inhibitor, our patient was started on levetiracetam due to its favorable safety profile and better tolerability.

CONCLUSION

Neurocysticercosis (NCC) is a rare but increasingly recognized cause of adult-onset seizures in the United States, particularly challenging to diagnose in patients with advanced, untreated HIV. Our patient's negative serology, likely due to a CD4 count of 16 cells/mm³, did not exclude NCC. Diagnosis was supported by epidemiologic history, imaging findings, and clinical course, and confirmed by improvement with antiparasitic therapy and corticosteroids. Coordinated treatment alongside re-initiation of antiretroviral therapy minimized the risk of immune reconstitution inflammatory syndrome. This case highlights the importance of detailed travel and exposure histories, timely recognition of NCC, and early intervention to prevent seizure recurrence and long-term neurological complications in immunocompromised patients.

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