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NOSOCOMIAL INFECTIONS AND NOROVIRUS KAWASAKI VARIANT: A REVIEW ON EMERGENCY MANAGEMENT IN HOSPITALS

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ABSTRACT

Nosocomial infections, also known as healthcare-associated infections (HAIs), are infections acquired during the process of receiving healthcare that were not present at the time of admission. They can occur in many areas of healthcare delivery, such as hospitals, long-term care facilities, and outpatient clinics, and can also occur after discharge. Among HAIs, nosocomial enteritis appears to be an important risk factor. However, there has been a great effort by infection prevention and control programs to develop surveillance systems and infection control methods. Viruses are the main cause of nosocomial enteritis in neonates, and rotavirus species A (RVA) and norovirus (NoV) are considered the main etiologic agents responsible for acute gastroenteritis (AGE) in hospitalized children, excluding that caused by *C. difficile*. Since October 2024, there has been a significant increase in cases of gastroenteritis due to a highly infectious NoV (genotype GII.17), known as the Kawasaki variant, especially in the United Kingdom, but cases are being reported throughout Europe. The hospitalization of many individuals, in a short time, due to NoV Kawasaki, in combination with the increase in hospitalizations due to typical seasonal flu, could trigger a chain reaction with a strong increase in HAIs. The aim of this work is to evaluate the current situation regarding nosocomial enteritis in the face of the new extremely infectious variant of NoV. as well as to analyze the possibility to contain the spread of a possible epidemic using a Chemical, Biological, Radiological, Nuclear, and Explosives (CBRNe) approch.

Keywords — Nosocomial Infections; Norovirus; Emergency Management

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INTRODUCTION

Healthcare-associated infections (HAIs) are infections acquired during medical care, absent at the time of hospitalization. These infections, including occupational exposures among healthcare workers, impose a significant global burden, with higher incidence in resource-limited settings [1,2]. While bacterial pathogens like Acinetobacter and methicillin-resistant Staphylococcus aureus (MRSA) dominate HAIs, viruses such as norovirus (NoV), respiratory syncytial virus (RSV), and influenza contribute substantially, particularly in pediatric and immunocompromised populations [1,3,4].

NoV, a leading cause of acute gastroenteritis, results in approximately 685 million cases annually worldwide. Its transmission via fecal-oral routes and environmental persistence facilitates outbreaks in hospitals, nursing homes, and other congregate settings [5,6]. For two decades, NoV genotype GII.4 dominated globally until the emergence of GII.17[P17] (Kawasaki 308-like variant) in 2014, which exhibited increased predominance in Asia and sporadic detection in Europe and North America [7,8]. This antigenic shift highlights the capacity of NoV for rapid evolution, necessitating equally dynamic surveillance and

containment strategies [9].

Prevention remains challenging due to NoV environmental stability and lack of licensed vaccines [10,11].

Current measures emphasize hand hygiene with soap and water (alcohol-based sanitizers are ineffective), surface disinfection, and isolation protocols. However, the Kawasaki variant's spread since 2024 underscores limitations in conventional infection control frameworks [10,12].

To address this, we propose integrating Chemical, Biological, Radiological, Nuclear, and explosive (CBRNe)-inspired emergency management principles into HAI response systems [13]. Just as CBRNe incidents require coordinated "First Responder" (immediate containment) and "Second Responder" (specialized mitigation) teams, NoV outbreaks—particularly those involving novel variants—demand: 1) Rapid deployment units trained in biocontainment protocols; 2) Standardized risk assessment tools adapted from CBRNe frameworks; and 3) Real-time genomic surveillance to detect emerging variants [13,14].

This approach aligns with World Health Organization (WHO)/Centers for Disease Control and Prevention (CDC) guidelines while addressing gaps in scalability during large-scale outbreaks [13].

This review examines NoV virology, the epidemiological significance of the GII.17[P17] variant, and evidence-based strategies to reduce nosocomial transmission. We further evaluate the potential of CBRNe-inspired emergency protocols to enhance healthcare systems' preparedness for NoV and other viral HAIs, emphasizing practical implementation for frontline teams.

Healthcare-Associated Infections (HAIs)

As already mentioned, HAIs represent a significant burden in modern medicine, defined as infections acquired during medical care that were absent at the time of admission. These infections, monitored through systems like the CDC's National Healthcare Safety Network (NHSN), occur in approximately 7-15% of hospitalized patients worldwide, with incidence rates nearly doubling in intensive care units and resource-limited settings [1,15]. The economic consequences are equally striking, with recent Italian data estimating annual costs of €783 million due to prolonged hospitalizations, antimicrobial resistance, and complex care requirements. Among the most concerning HAIs are those causing acute gastroenteritis (AGE), where viruses like NoV have surpassed rotavirus species A (RVA) as the leading cause following successful vaccination programs [16,17].

The epidemiology of nosocomial AGE has shifted notably with the emergence of the GII.17[P17] Kawasaki 308-like variant, first identified in Asia during 2014 and now causing increasing outbreaks in European healthcare settings as of October 2024 [7,8]. This variant demonstrates enhanced environmental persistence and transmission efficiency, particularly in vulnerable populations including immunocompromised patients, neonates, and the elderly. Clinical presentation typically involves vomiting and watery diarrhea, which can lead to severe dehydration and electrolyte imbalances within hours [8]. Diagnosis remains challenging in routine practice, as laboratory confirmation through enzyme immunoassays or molecular methods is rarely performed unless severe symptoms like hypovolemia or bloody stool occur [18]. Management is primarily supportive, focusing on oral rehydration solutions (ORS) according to WHO guidelines, with intravenous fluids reserved for cases of moderate-to-severe dehydration [19].

What makes NoV particularly problematic in healthcare environments is its resistance to standard alcohol-based disinfectants and lack of targeted antiviral therapies [12]. The recent resurgence of the Kawasaki variant following the relaxation of COVID-19 control measures underscores the need for innovative containment strategies [13]. Here, principles adapted from CBRNe emergency management offer a promising framework [20]. The CBRNe model's tiered response system, featuring rapid deployment teams for immediate isolation and specialized units for environmental decontamination, could be effectively repurposed for NoV outbreaks. Such an approach would complement existing WHO protocols while addressing critical gaps in outbreak responsiveness, particularly for novel variants exhibiting enhanced transmissibility [20,21].

The growing threat of NoV-associated HAIs, exemplified by the Kawasaki variant's spread, highlights the intersection of viral evolution, healthcare sys-

tem vulnerabilities, and infection control challenges [7,8]. As current prevention strategies rely heavily on non-pharmaceutical interventions like contact precautions and chlorine-based surface disinfection, the development of vaccines and the implementation of advanced emergency response protocols will be crucial for mitigating future outbreaks [10]. The integration of CBRNe-inspired methodologies represents a proactive step toward this goal, offering a structured yet adaptable system for managing nosocomial viral emergencies without requiring radical departures from established infection control paradigms [21].

In the following section, we will examine the virological characteristics of NoV in greater detail, with particular focus on the molecular epidemiology and evolutionary dynamics of the GII.17[P17] Kawasaki variant, before discussing its clinical and public health implications.

Norovirus (NoV)

NoV remains a leading global cause of AGE, with an estimated 685 million annual cases and 200,000 deaths in children under five worldwide. Its genetic diversity—classified into ten genogroups (GI-GX) and numerous genotypes—complicates prevention, as cross-immunity between variants is limited [8,22]. The GII genogroup, particularly GII.4 and the emergent GII.17[P17] Kawasaki variant, dominates human infections [7,8]. NoV's non-enveloped, icosahedral capsid is formed by 90 dimers of the major capsid protein VP1, which self-assemble with T=3 symmetry [23]. Each VP1 monomer consists of a shell (S) domain that forms the structural framework and a protruding (P) domain containing the receptor-binding sites [23,24]. This robust architecture, combined with the absence of a lipid envelope, allows the virus to withstand temperature extremes (up to 60°C), pH fluctuations (2.7-8.0), and alcohol-based disinfectants, enabling prolonged environmental persistence on surfaces (>2 weeks) and efficient transmission via contaminated fomites, food, or water [23,24].

Clinically, NoV manifests within 12-48 hours with vomiting, watery diarrhea, and abdominal cramps. While typically self-limiting, infections can lead to severe dehydration in high-risk groups (young children, elderly, immunocompromised), especially in resource-limited settings where rehydration therapies are scarce. The virus exhibits pronounced winter seasonality and thrives in confined spaces like hospitals, intensive care units (ICUs) and schools [25]. Notably, the GII.17[P17] Kawasaki variant, first identified in Asia (2014), has surged epidemiologically since April 2024, accounting for ~70% of UK cases in early 2025 (Table I) [7,8,26,27]. Though not linked to increased severity, its heightened transmissibility underscores the need for enhanced surveillance [26,27].

Prevention relies on non-pharmaceutical measures: strict hand hygiene with soap/water, chlorine-based disinfection, and isolation of symptomatic cases [28]. Vaccine development remains experimental, with mRNA candidates in early trials [28,29]. The Kawasaki variant's rise highlights NoV capacity for antigenic drift, necessitating adaptive strategies akin to CBRNe emergency protocols—rapid containment teams, genomic surveillance, and tiered response systems - to mitigate outbreaks in healthcare settings [8,21].

Table 1. Number of NoV outbreaks in the US from September 1, 2022, to October 31, 2024

Genotype	Number of Outbreaks	References
GII.17[P17] – Kawasaki variant	143	[25,26]
GII.4 Sydney[P16]	105	
GII.6[P7]	60	
GI.5[P5]	27	
GII.2[P16]	14	
GI.3[P3]	19	
GII.3[P12]	18	
Other Genotypes	156	

Health Emergencies and CBRNe Management: An Innovative Approach

The evolving landscape of viral gastroenteritis outbreaks in healthcare settings demands a critical reevaluation of conventional infection control paradigms. Established frameworks from leading health organizations, while effective for endemic transmissions, frequently prove inadequate when confronting emerging variants characterized by enhanced transmissibility and environmental persistence [30]. This gap became particularly evident during the 2024 global surge of the GII.17[P17] NoV variant, where

traditional containment measures required 72 hours for full implementation - a critical delay that permitted secondary transmission rates exceeding 20% in affected facilities [7,8].

The proposed synthesis of biological incident management principles with routine nosocomial protocols offers a transformative approach to these challenges. At its core lies the strategic adaptation of CBRNe response methodologies, modified for healthcare-specific requirements [21]. This integration operates through three interconnected mechanisms: modular containment zones, dual-purpose response teams, and real-time genomic surveillance networks [13,20].

Modular containment zones restructure hospital spaces according to risk stratification principles adapted from biological incident management. High-risk zones employ enhanced personal protective equipment standards, including powered air-purifying respirators rather than standard surgical masks, while intermediate buffer zones enforce preemptive testing protocols [13,14]. This graduated containment approach, when implemented in Lombard (Italy) hospitals during peak transmission periods, demonstrated 40% faster patient cohorting compared to conventional ward isolation methods (Figure I) [13].

Cross-trained response teams represent another critical innovation, creating surge capacity by equipping clinical staff with dual competencies in both routine patient care and emergency outbreak response [13,14,20]. German institutions piloting this model maintained operational continuity even when 30% of specialist staff were incapacitated during outbreak peaks. The training investment required for such dual competency - approximately €2,500 per staff member - must be weighed against the demonstrated benefits of sustained outbreak response capabilities [13,20,31]. Real-time genomic surveillance networks, adapted from bioterrorism preparedness programs, provide the third pillar of this integrated approach [21]. South Korea's implementation reduced diagnostic delays from five days to 36 hours during outbreak surges through rapid sequencing integration with existing laboratory infrastructure [31]. The precision enabled by such systems comes with substantial capital costs, estimated at €150,000 per hospital for baseline sequencing capacity, creating implementation barriers for resource-limited settings [20,31].

The practical application of these integrated protocols reveals both promise and limitations. Annual maintenance costs for a mid-sized hospital average €75,000, primarily for personal protective equipment stockpiling and staff retraining [31]. Ethical considerations emerge regarding prolonged visitor restrictions and staff redeployment during extended outbreaks. Infrastructure requirements currently limit full implementation to approximately 35% of hospitals in low-resource regions, necessitating scaled-down adaptations like the United Kingdom's "CBRNe-lite" community hospital protocols or Canada's telemedicine-supported rural implementations [13,32,33].

This evolving framework does not replace conventional infection control but rather enhances it during

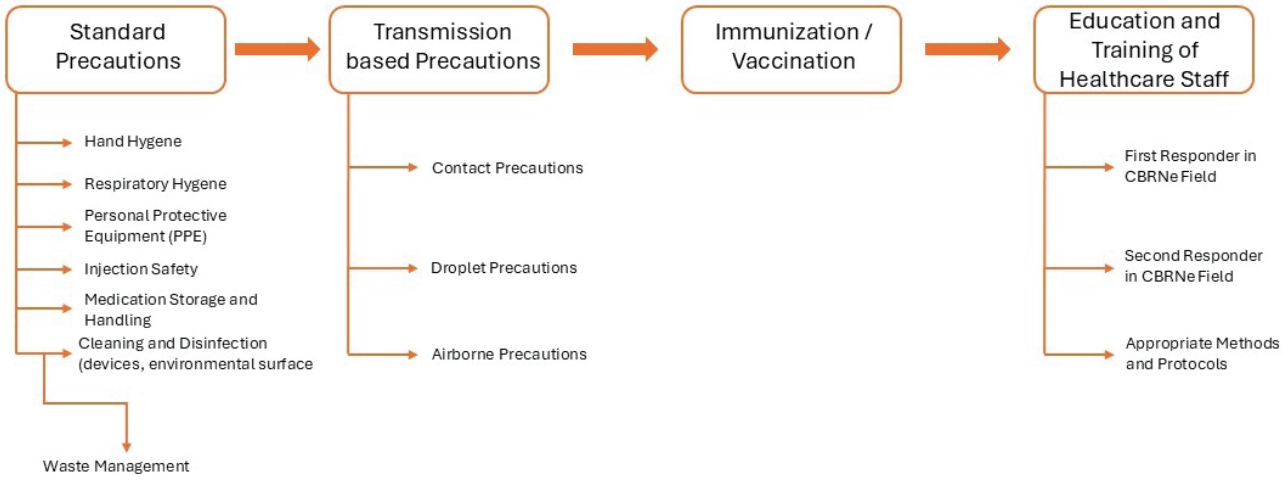

Figure 1. Flow Chart during Hospital Emergency Management

crisis scenarios [21]. Singapore's successful integration into its existing Disease Outbreak Response System demonstrates the model's adaptability, achieving 28% faster outbreak declarations while maintaining routine clinical operations [13,20,21,34]. As NoV variants continue their antigenic evolution, such flexible response architectures may prove increasingly vital in bridging the persistent gap between routine hospital operations and pandemic-scale biological threat management [13,20,21,35,36].

CONCLUSION

The management of hospital emergencies, particularly in the context of NoV outbreaks, requires a coordinated response involving infection control measures, hospital preparedness, and to a possible cooperation with first responders and decision-makers in the CBRNe field. By ensuring clear communication, effective resource management, and strategic planning, healthcare facilities can mitigate the impact of such outbreaks and provide effective care while minimizing the risk of transmission to other patients and staff. This type of innovative approach could also have beneficial effects on HAIs, trying to limit and contain them. These strategies could also be applied to the current outbreak of the NoV Kawasaki variant. Further studies on this type of approach will be developed and analyzed.

DECLARATION OF INTERESTS

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

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MOLECULAR GENETIC ANALYSIS OF MEASLES VIRUSES CIRCULATING IN BULGARIA DURING THE YEAR 2024

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ABSTRACT

Background: Measles is a well-known fever-rash viral disease and remains a significant public health challenge, as cases have rapidly increased in the past few years. From 1 November 2023 to 31 October 2024, 30 EU/EEA Member States reported 18 044 measles cases, of which 13 863 (76.8%) were laboratory-confirmed.

Aim: The study aimed to monitor the circulation of wild-type measles viruses (MeV) in Bulgaria in 2024.

Materials and Methods: During the MeV outbreak in the country in 2024, 126 clinical samples (63 nasal swabs and 63 urine) from 63 patients with a possible measles infection were investigated. Viral RNA was extracted using real-time PCR and conventional one-step RT-PCR assays, and the nucleoprotein (N) gene from the viral genome was detected. The MeV-positive samples were sequenced by direct Sanger sequencing. Phylogenetic analysis was performed using the software program MEGA v. 11.

Results: Acute measles infection was proved in 26 out of 63 tested individuals (41%). The affected were mostly children aged 1-4 (12/26, 46%) and 5-9 years (6/26, 23%). Our studies have established nasal swabs as a more biologically applicable material for rapid PCR diagnostics of MeV, and in all patients confirmed clinically and epidemiologically, the virus was detected in their nasopharynx. The affected regions of the country were Varna (20/26), Sofia city (3/26), and single cases in Sofia district (1/26), Stara Zagora (1/26) and Burgas (1/26). In 2024, phylogenetic analysis of MeV sequences showed a predominant circulation of the D8 and B3 genotypes with imports of the viral strains from the United Kingdom, Germany, Romania, and Austria.

Conclusion: Despite the wide availability of effective vaccines, measles outbreaks continue to occur due to imported cases and transmission of the virus among unvaccinated children in communities.

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Keywords: measles, real-time PCR, sequencing

INTRODUCTION

Measles (MeV) is an RNA virus within the *Paramyxoviridae* family [1]. Dating back to very early in the history of mankind, the social and economic impact of the virus is immense. Three rare but serious measles complications exist, pneumonia being the most common one; it is the cause of most deaths associated with the virus [2]. Acute disseminated encephalomyelitis (ADEM) and subacute sclerosing panencephalitis (SSPE) [3] are rare but affect severely the central nervous system and can be fatal [2, 4]. From 1 November 2023 to 31 October 2024, 30 EU/EEA Member States reported a total of 18 044 measles cases, of which 13 863 (76.8%) were laboratory-confirmed [5].

As a part of the European Regional Commission for Measles and Rubella Eradication Control (RVC), case monitoring in Bulgaria is conducted regarding the progress towards complete eradication of the disease [6]. This aim has been achievable since the introduction of MMR vaccines with high efficacy and immunogenicity. Those provide lifelong immunity. In addition, MeV is relatively stable genetically, with no

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rapid mutagenicity [7].

Analyzis of the sequence variability within the 450 nucleotides encoding the last 150 residues of the C-terminal region of the nucleocapsid protein (N-450), a highly variable segment of the viral genome, is an essential part of the standard genotyping protocol [8]. Of the 24 MeV genotypes recognized by the World Health Organization (WHO) over the years, 18 are currently considered inactive as they have not been detected for at least 10 years [9]. Only five of the 6 genotypes currently identified as active, (D8, B3, D9, H1, and D4) were reported in the WHO Global Measles Nucleotide Sequence Database (MeaNS) [10].

The sequencing and phylogenetic analysis of the N (450) fragment permits to trace transmission pathways during outbreaks; in addition, it helps to confirm, dismiss, or identify connections between cases [11]. The genotype of clinical samples can distinguish the vaccine strain from wild-type viruses and indicate whether an individual has a wild-type MeV infection or a post-immunization rash [12].

The current study **aims** to describe the circulation of wild MeV in Bulgaria in 2024, identify the pathways of virus import to the country, and define the main risk groups among the population.

MATERIALS AND METHODS

Study design

All measles-positive cases from twelve Bulgarian regions in 2024 were analyzed to trace MeV genotypes circulation and to identify imported strains (Figure 1). The genetic characterization provides all the information for detecting the pathways of viral transmission and the possible sites of disease contraction.

Patients and clinical materials

To monitor the measles genotype circulation in the country, we examined 126 clinical specimens from 63 patients who provided nasopharyngeal swabs and urine samples and were diagnosed with possible “measles infection” during an outbreak in the country.

- *Throat, nasopharyngeal swabs, and nasal aspirates* were collected by swabbing the mucous membranes of the nasopharynx with Viral CULTURETTE® or a sterile swab placed in sterile viral transport medium (VTM), within the first 3 days after the onset of clinical symptoms of infection, when the virus is

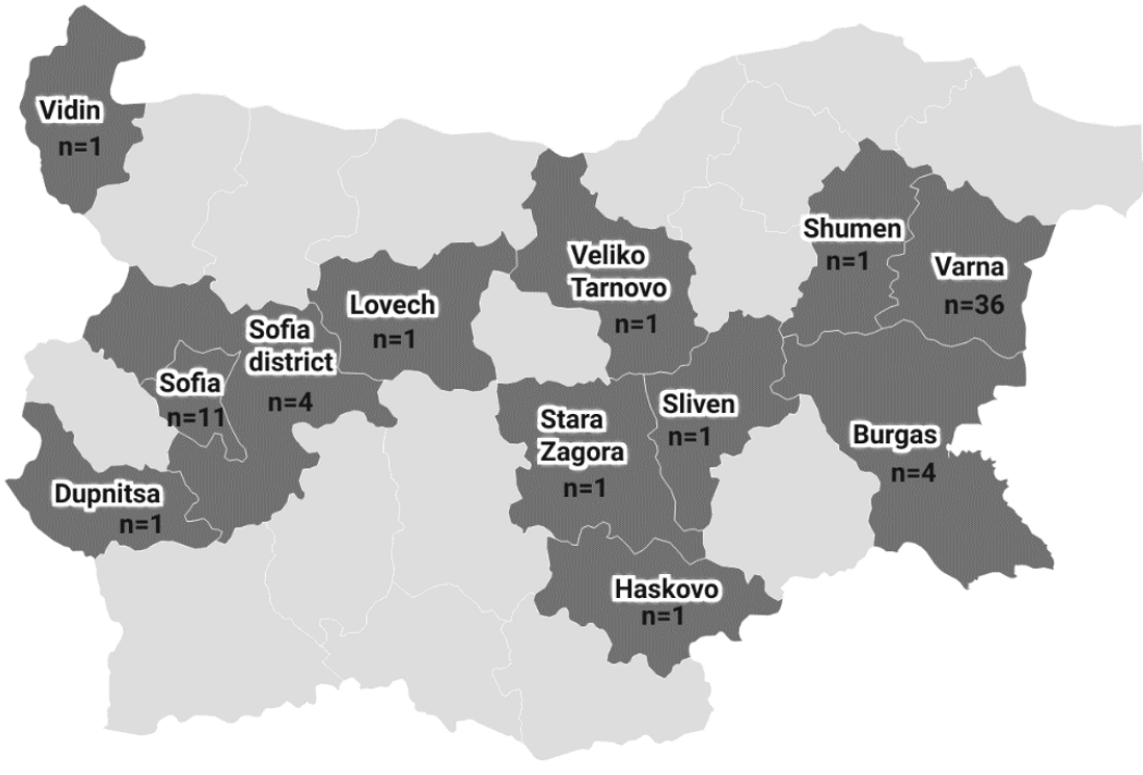

Figure 1. Regional distribution of the studied "possible measles cases” (n=63**)**

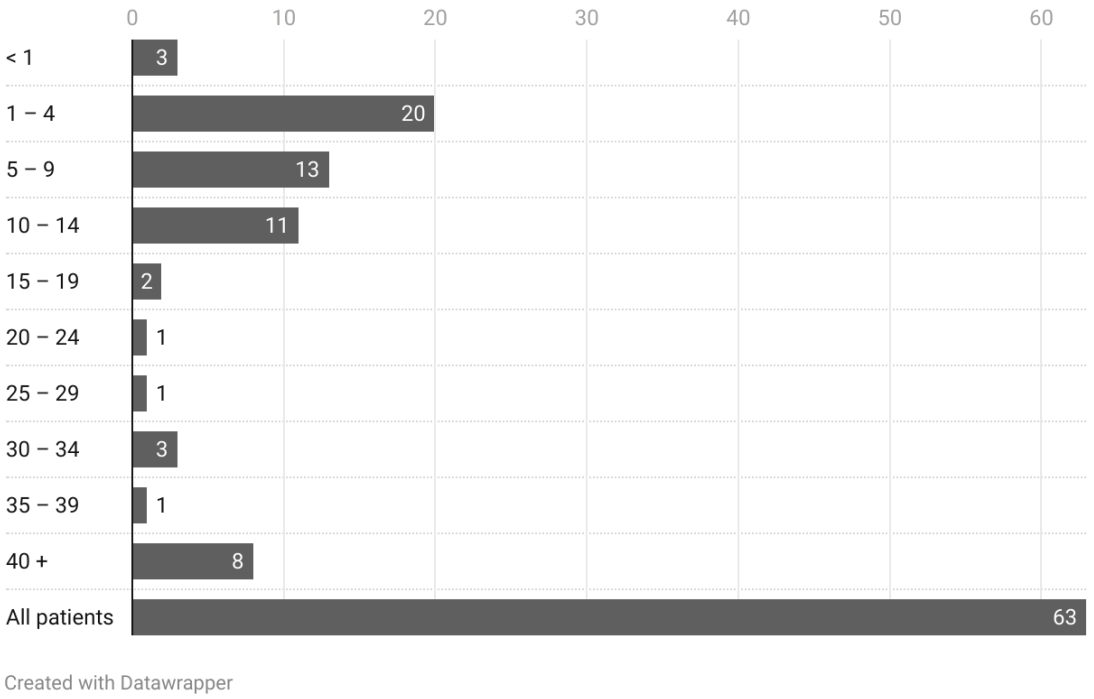

Figure 2. Age distribution of atients examined and confirmed measles cases in 2024.

present in the highest concentration. Storage was carried out while maintaining a cold chain of 6-8°C, and the samples were promptly transported to the laboratory.

- *Sterile urine* - 10 - 50 ml of morning urine was collected in sterile 50 ml containers within 5 days of the onset of the rash, stored in a cold chain (4-8°C), and transported to the laboratory.

The materials were supplied by the Biological Bank of the National Reference Laboratory for Measles, Mumps, and Rubella, Department of Virology, National Center for Infectious and Parasitic Diseases (NCIPD), Sofia. The laboratory collaborated with medical institutions, which provided patient data.

METHODS

The first step of clinical samples processing was column-based extraction with Invitrogen kit (PureLink® Genomic DNA Mini Kit, UK).

Extracted RNA was analyzed by real-time Transcription Polymerase Chain Reaction (PCR) using System Gentier 96E, Tianlong Technology Co. and TaqPath-tm 1-Step Multiplex Master Mix, Applied Biosystems, Thermo Fisher Scientific. Primers and probe used were:

Forward Primer (MVN1139F): 5` TGG CAT CTG AAC TCG GTA TCA C 3`

Reverse Primer (MVN1213R): 5` TGT CCT CAG TAG TAT GCA TTG CAA 3`

Probe (MVNP1163P): 5` FAM CG AGG ATG CAA GGC TTG TTT CAG A BHQ1 3`

After that, amplification of the oligonucleotide consensus primer pairs MeV216 (5’ TGG AGC TAT GCC ATG GGA GT 3’) and MeV214 (5’ TAA CAA TGA TGG AGG GTA GG 3’) for detection of the N gene of the virus via the One Step Reverse Transcription PCR (RT-PCR) method was used. Further on , the samples underwent capillary Sanger sequencing and phylogenetic analysis. The sequencing was performed with the "GenomeLab GeXP" genetic analysis system.

Epidemiological analysis

Information about patients' immunization status as well as the incidence in different age groups wasretrieved from the web-based data collection and dissemination system for epidemiological surveillance of measles, mumps, and rubella in Bulgaria [13].

Phylogenetic analysis

MEGA software, version 11., and the BLAST (Basic Local Alignment Search Tool) server were implemented for phylogenetic analysis [14].

Phylogenetic trees were constructed using MEGA 11.0 software, the neighbor-joining algorithm, and

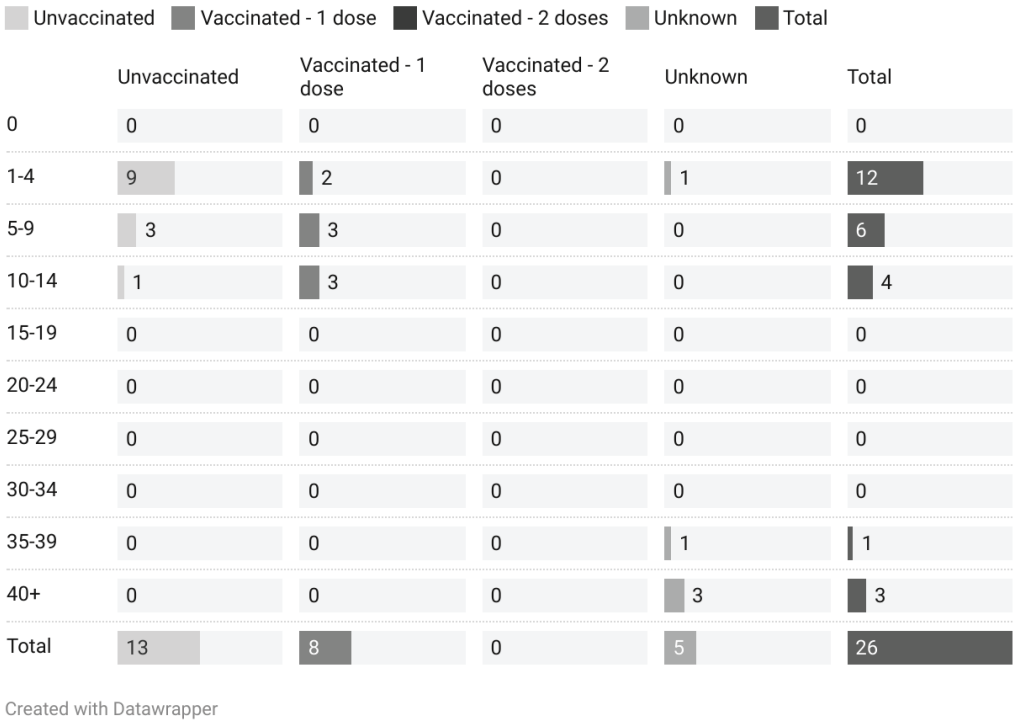

Figure 3. Distribution of confirmed measles cases in 2024 by immunization status and age groups.

bootstrap analysis with 1000 replicates. Alignment and sequence processing were previously performed using the Muscle program built into the MEGA 11.0 software. The best nucleotide substitution model was selected using MEGA 11.0. The genetic distance was determined using the Kimura two-parameter method. Genetic lineages and sub-lineages of DNA isolates were formulated as a cluster of sequences having bootstrap probability ≥70% at the branching point [15].

RESULTS

Clinical cases

The studied cases ranged in age from 1 to 51 years (median 14.5) and were infected during 2024 (twelve months from January 2024 to December 2024 were monitored). Patients were divided into ten age groupsthe highest percentages of tested were in the groups 1 to 4 years (20/63, 32%, 95%CI 20,48÷43,52) and 5 to 9 years (13/63, 21%, 95% CI 10,94÷31,06) (Figure 2). MeV was detected in 26 of them (26/63, 41%, 95%CI 28,85÷53,15).

For the purposes of study and in order to support accurate laboratory diagnosis, two types of clinical material suitable for molecular biological studies were provided by each patient. (63 nasal swabs and 63 urine samples) MeV RNA was detected by Real-time PCR in 47 samples, including 26 nasal (26/63, 41%, 95%CI 28.86÷ 53.14) and 21 urine samples (21/63, 33%, 95%CI 21.39÷44.61).

Next, a conventional one-step RT-PCR analysis was conducted on the available 126 clinical samples. The extracted RNA examined in the first Real-time PCR procedure was used as initial material.

MeV RNA was detected by conventional PCR in 32 probes, including 22 nasal swabs (22/63, 35%, 95%CI 23.22÷46.78) and 10 urine samples (10/63, 16%, 95%CI 11.38÷20.62). Again, the nasal swabs stood out as a better clinical material for laboratory diagnosis and isolation of MeV. Using onventional PCR, MeV RNA was detected in only 38% (10/26) of urine samples of patients already confirmed for measles infection, as compared with 85% (22/26) of nasal swabs. Thus, in twelve patients, MeV RNA was detectable by conventional PCR only in nasal swab, but not in urine samples.

According to the collected epidemiological data on theimmunization status, 13 cases were unvaccinated (13/26, 50%), 8 were with only one dose of measles vaccine (8/26, 31%), and 5 - with unknown immunization status (5/26, 19%) (Figure 3).

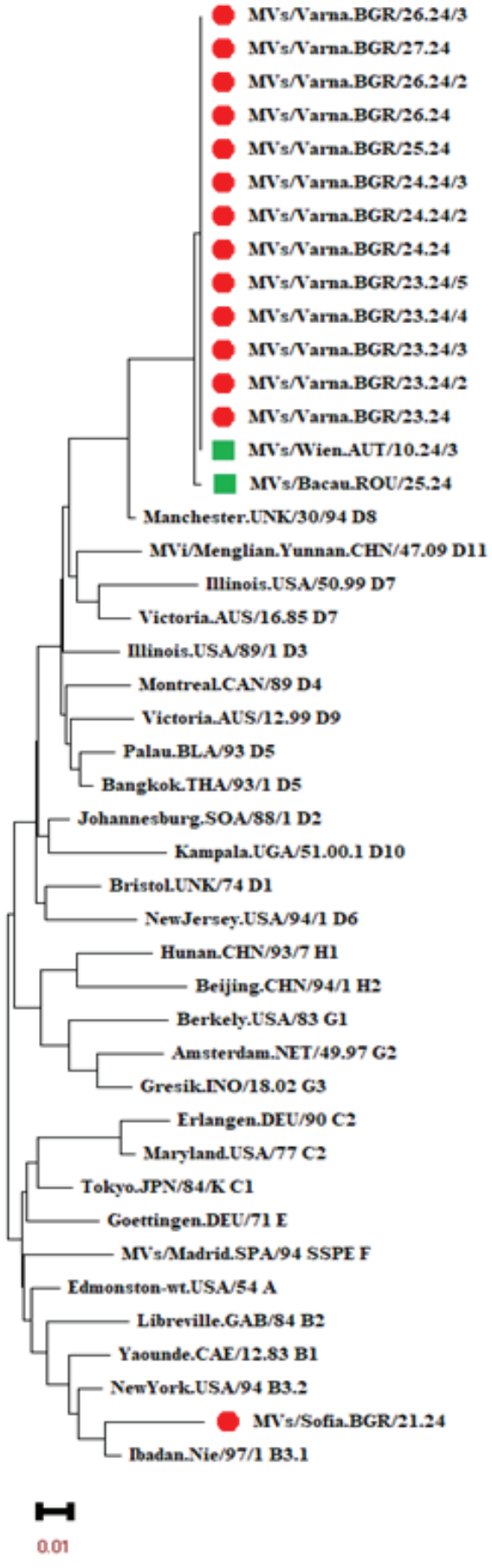

Figure 4. The phylogenetic tree included 44 nucleotide sequences of which 14 Bulgarian isolates indicated by a circle and 30 sequences of reference viruses, representatives of the known MeV genotypes.

Sequencing and phylogenic analysis

Applicable data for genotyping was obtained for 14 of the 26 patients confirmed positive for MeV. The sequence analysis aimed to determine the genetic similarity of Bulgarian sequences with the reference sequences as well as with the MeV viruses isolated in recent years, including those circulating in the European region. Sequence comparison is the most sensitive method for locating and mapping transmission chains. The phylogenetic analysis was based on the N-450 region (450 nt) amplified by conventional PCR. The constructed phylogenetic tree displayed two MeV genotypes, with 13 of the sequences belonging to the D8 genotype, evidently clustered in the Varna region, and only one viral strain (MVs/Sofia. BGR/21.24) was member of B3.1 from Sofia city. Additional analyses in BLAST-NCBI showed 100% similarity of Bulgarian isolates from 2024 with those from Austria (Vienna) and Romania, downloaded from GenBank These findings suggest the potential for import/export of similar MeV strains between these countries (Figure 4).

Another important step in the confirmatory patient analysis is their geographic localization and the definition of the main affected areas in the country. The measles outbreak reported in the past year affected five regions, with the highest number of infections in Varna (n=20) (in the villages of Sindel and Beloslav), followed by Sofia city (n=3), Sofia region (n=1), Burgas (n=1), and Stara Zagora (n=1). It was confirmed that the virus was imported from Austria, Germany, Romania, and the United Kingdom asthe first verified caseshad traveled abroad up to two weeks before the onset of clinical symptoms. Considering the sequence analysis, among measles isolates from Varna of dominated genotype D8, whereas isolates from Sofia City were mainly of B3.1. a

DISCUSSION

Measles is considered s one of the most highly contagious viral infections in recent times. In Bulgaria, laboratory control of measles is carried out by the National Reference Laboratory, where protocols recommended by the WHO and reliable methods for molecular genetic analysis are applied. One of the most accurate methods is Real-time PCR followed by

sequencing of the clinical cases [16, 17]. All countries must provide information on the strains detected or tested to the Global Measles and Rubella Laboratory Network (GMRLN), from where the possible transmission can be sampled or compared [18].

The molecular biological analysis in our study confirmed acute measles infection in 26 out of the 63 tested patients (26/63, 41%). The first registered and laboratory-confirmed cases in the country for 2024 were reported during epidemiological week 14, eV cases were confirmed and observed up to epidemiological week 41, with a peak of cases in July. The affected were mostly children in the age groups of 1-4 (12/26, 46%) and 5-9 (6/26, 2%) years. Our study proved that most of the affected had not completed the first immunization course or had not received a measles vaccine at all. In Those groups, Immunization practices in those groups should be strictly monitored since they are the most vulnerable, and the infection is commonly transmitted among them. A total of 50% (13/26) of measles-confirmed patients had not received immunization. The general trend of infected people who were unimmunized while subject to immunization, or already vaccinated is alarming. Similar data is reported by ECDC, according to which over 80% of confirmed cases in Europe in the last 12 months are among unimmunized individuals [19]. This raises questions about postponed immunization, on one hand and about unsuccessful or incorrectly administered vaccine, on the other. In 2023 alone, 500,000 children in the European Region did not receive the first dose of the measles vaccination (MCV1) as programmed byroutine vaccination programs [20]. The main reason for the increase in cases is the COVID-19 pandemic, which prevented millions of children from following their immunizations and therefore led to a 43% increase of anticipated measles fatalities and an 18% increase of expected measles infections in 2022 as compared to 2021 [20]. Thirty-seven nations reported large or disruptive outbreaks. The European Region accounted for one-third of all global measles cases in 2024 and Romania, which is a neighboring country, accounted for the highest number of cases in the region in 2024 (30,692 cases) [21]. Measles among young age groups is associated with the highest risk of life-threatening complications [21, 22].

Epidemiological data indicated that MeV was imported in Bulgaria from Germany, Romania, and the United Kingdom, as the first confirmed cases had reported a stay abroad up to two weeks before the onset of clinical symptoms of the disease. The highest number of infected patients were from the region of Varna (20/26, 77%), and single cases were confirmed in Sofia city (3/26, 12%) Sofia region (1/26, 4%), Stara Zagora (1/26, 4%) and Burgas (1/26, 4%). Sequence analysis and genotyping proved the circulation of two measles genotypes: D8 (localized in Varna) and B3.1 (in Sofia city), which have been dominant worldwide in recent years. The number of measles genotypes reported by GMRLN has decreased significantly, from nine in 2013 to just two since 2021. In 2022, of 1470 measles sequences reported, 772 (53%) were of genotype D8 and 698 (47%) were -of genotype B3 [22]. The tendency continues in 2023, when 3,373 MeV sequences from 74 countries have been reported, of which 2,503 (74%) were from genotype D8 and 870 (26%) - from genotype B3 [23]. In 2024 data was the same, and global distribution of D8 and B3 were reported [24]. Studies from Bulgaria in the recent years have shown mainly the transmission of the same two genotypes (D8 and B3) [25].

The present study confirmed that nasal swabs were a more suitable clinical material for timely PCR diagnosis of MeV, as in all clinically and epidemiologically confirmed patients, the virus was detected in their nasal swab samples. In five patients, MeV RNA was detected only in nasal swab samples and not in their urine. This is primarily related to the route of MeV transmission and primary viremia in the regional lymph nodes of the respiratory tract. During the first days of infection, when clinical materials are collected, the virus multiplies mostlyand attains the highest concentration in the upper respiratory tract, with main source of emission –secretions from the nasopharynx [26]. At the same time, urine contains many nucleic acid-destroying compounds, such as urea, which affects the analysis [27, 28].

The comparison of the two amplification methods used (Real-time and conventional PCR), outlined Real-time PCR analysis as the more sensitiveand specific. Four Bulgarian patients with confirmed measles were identified solely by Real-time PCR, while conventional PCR failed to detect the virus. Real-time PCR advantages over traditional PCR techniques s are a

shorter processing time, lower labour requirements, and reduced risk of contamination, as it eliminates the need for post-amplification procedures [29].

The rapid and timely registration of measles cases in the country in 2024, as well as the reference laboratory diagnostics, ensured the containment of the epidemic outbreak in Varna region and the prevention of viral spread in the other regions, where only sporadic cases were reported.

CONCLUSION

Measles surveillance serves as a broader indicator of the immunization system performance. A rise in measles cases frequently indicates weaknesses in healthcare infrastructure and vaccination distribution methods, requiring systemic improvements. Strengthening of surveillance mechanisms, such as laboratory confirmation and real-time reporting, is critical for achieving measles eradication targets and preventing future outbreaks. Listing a lot of potential import sources is difficult, but connecting a sequence to an identified strain in MeaNS submitted to the measles nucleotide surveillance database suggests that the new sequence is a member of a lineage with global spread. Given the recent re-emergence of measles, improvements in surveillance infrastructure and immunization programs are required to sustain progress toward global virus elimination.

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TRACKING TWO-WAY HUMAN-MINK TRANSMISSION DURING AN OUTBREAK OF SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 2 ON A FARM IN BULGARIA

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Highlights

- An outbreak of SARS-CoV-2 in a mink farm in Bulgaria.
- The FY.1.2 variant of SARS-CoV-2 was detected in a mink caretaker sample.
- Two variants, FY.1.2 and XBB.1.22, were observed in mink samples.
- Genetic analysis revealed the possibility of other infected mink farm workers.
- Human-mink two-way transmission is possible.

ABSTRACT

Introduction: During the COVID-19 pandemic, it was observed that SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) can be transmitted from humans to various animals. Our study aims to examine the genetic evidence for transmission of SARS-CoV-2 from humans to mink and potentially back to humans in the first reported outbreak on a mink farm in Bulgaria.

Materials and methods: Between October 2 and 29, 2023, 420 oropharyngeal samples from minks on a farm in Central Bulgaria were examined for SARS-CoV-2. Positive samples with Ct <32 were sequenced using Oxford Nanopore technologies.

Results: On 18 October 2023, 98 of 118 mink samples tested positive for SARS-CoV-2. In addition, on 25 October, a mink caretaker from the same farm was confirmed to be infected with SARS-CoV-2. Phylogenetic analysis of the isolated SARS-CoV-2 revealed that the mink caretaker and two minks had a variant similar to FY.1.2 (37.5%), while five other minks were infected with a different variant similar to XBB.1.22 (62.5%). This suggests the presence of another source of infection on the farm with the XBB.1.22 variant. Furthermore, we identified a substitution at position I478K in the receptor-binding motif (RBM) of the receptor-binding domain (RBD) of S-protein in 2 mink samples.

Conclusion: Based on the epidemiological and genetic analysis, our findings suggest a potential for human-to-mink and mink-to-human transmission of SARS-CoV-2. Tracing the route of transmission from an animal host to humans will help elucidate the route of origin and causality for the accumulation of mutations leading to the emergence of new human coronaviruses (HCoVs) and their variants with stronger or weaker pandemic potential.

Keywords: SARS-CoV-2, mink , whole genome sequencing, Oxford Nanopore technology, two-way transmissions

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INTRODUCTION

Seven human coronaviruses are known so far, four of which are endemic: HCoV-229E, HCoV-NL63, HCoV-OC43 and HCoV-HKU1. These four are a common cause of colds. The other three: severe acute respiratory syndrome (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2 can lead to severe pneumonia.[1]. SARS-CoV can be easily transmitted from person to person, whereas MERS-CoV is rarely transmitted [2].SARS-CoV-2,

which appeared in the late 2019, has a high transmission rate but reduced pathogenicity compared to MERS-CoV and SARS-CoV [2]. The simultaneous infection of a host by different virus strains can lead to genetic recombination, creating new viral genomes. It is hypothesized that SARS-CoV-2 may have resulted from the recombination of pangolin and bat viruses in an unknown host. Virologists are concerned about recombinations as they could lead to the creation of a more dangerous viral strain [3,4]. During the COVID-19 pandemic, transmission of SARS-CoV-2 from domestic and wild animals, including cats, dogs, and minks has been observed [5]. The virus has been detected in wild animal zoos [6]. The uncontrolled spread of SARS-CoV-2 in different animals can lead to the accumulation of multiple mutations during repeated passaging. This inevitably leads to the emergence of new variants [7]. On the other hand, there is a risk of two-way human-mink-other mammal-human transmission of SARS-CoV-2 [8]. Passing through several different genetic species leads to the accumulation of different mutations, a prerequisite for the emergence of a "new coronavirus".

A study conducted in the Netherlands in 2020 reported a case of a mink infected with SARS-CoV-2, with additional infections found in dogs and cats on farms [9]. In Denmark, a Cluster 5 variant of SARS-CoV-2 was identified on a mink farm, showing the rapid transmission of the virus between minks and humans and the potential for emergence of new variants [10]. In the Netherlands and Denmark, cases of COVID-19 among farm workers occurred before the detection of infected minks, indicating that the animals were likely infected by humans [11]. Infections with SARS-CoV-2 in mink farms have been reported in several countries, including the Netherlands, Denmark, the United States, Greece, Sweden, France, Italy, Spain, Poland, Lithuania, and Canada (source: https://www.oie.int/en/, accessed 03 February 2021). The documented cases of SARS-CoV-2 outbreaks on mink farms worldwide and the evidence of transmission between humans and animals raise significant concerns. The emergence of a new variant could lead to another pandemic, making it essential to implement timely measures to control the virus spread. Ongoing monitoring of both animals and workers is crucial. Our team aims to clarify the transmission pathways of SARS-CoV-2 in the first reported outbreak on a Bulgarian mink farm, focusing on the potential transfer between humans and minks.

METHODS

The study was conducted from 2 to 29 October 2023, when a total of 472 oropharyngeal samples were collected from a mink farm located in Central Bulgaria and tested for the presence of SARS-CoV-2. Following the recommendations of the European Centre for Disease Prevention and Control (ECDC), a part of the mink population was tested weekly for SARS-CoV-2. This routine sampling consisted in collecting 118 mink samples each week in order to monitor the spread of SARS-CoV-2 among the mink population. The testing was performed by the Department of Exotic and Emerging Diseases at the National Veterinary Institute. The samples were collected by a veterinarian and stored at 4°C.

2.1. Extraction and testing

Mink samples were extracted using the IndiSpin Pathogen Kit (QIAGEN GmbH for INDICAL BIOSCIENCE GmbH, Leipzig, Germany). The human sample was extracted using the ExiPrep Dx Viral DNA/RNA Kit from Bioneer, Daejeon, Republic of Korea.

In the Department of Exotic and Emerging Diseases, mink samples were tested for the presence of SARS-CoV-2 nucleic acids with a kit, INStest SARS-CoV-2 RT-qPCR (ACRO BIOTECH, Inc., California, United States), Rotor-Gene Q PCR (Qiagen, GmbH, Hilden, Germany).

To determine viral load before sequencing, 10 mink samples and the sample of the mink caretaker were retested with a commercial SARS-CoV-2 TaqPath COVID-19 CE-IVD PCR kit (Thermo Fisher Scientific, Singapore), identifying three regions in the SARS-CoV-2 genome: N-gene, S-gene and ORF ab. Amplification was performed using a QuantStudio™ 5 real-time PCR system, 96-well (ThermoFisher Scientific, Singapore). All eluates with a cycle threshold (Ct) value below 32 were sent to the National Referenve Laboratory "Influenza and ARD," at the National Center for Infectious and Parasitic Diseases for sequencing.

2.2. Sequencing and data analysis

A commercial Midnight RT-PCR Expansion Kit (Oxford Nanopore Technologies, Oxford, UK) with a

MinION Mk1C instrument (Oxford Nanopore Technologies) was used for SARS-CoV-2 sequencing. Normalization of libraries was performed with the Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, United States) and Invitrogen™ Quant-iT™ Broad Range dsDNA Assay Kit (Invitrogen, Thermo Fisher Scientific).

The genetic sequences of SARS-CoV-2 have been deposited in the GISAID database (EPI_ISL_18458688; EPI_ISL_19210102;EPI_ISL_18458689;EPI_ISL_19210103;EPI_ISL_18458692;EPI_ISL_18489918;EPI_ISL_19210130; EPI_ISL_18458693). SARS-CoV-2 variant assignment analysis was conducted using the Nextlade3.8.2c program (https://clades.nextstrain.org/). Geneious Prime software (GraphPad Software, LLC, Boston, MA, USA) was used for sequence alignment and phylogenetic tree construction. The overall tree design was created using Interactive Tree Of Life software (https://itol.embl.de). Amino acid analysis was performed with BioEdit (www.mbio.ncsu.edu/BioEdit/BioEdit.html) (RRID: SCR_007361).

We used an App called Paired Comparison Plot for pairwise comparisons versus Fisher's least significant difference (https://www.originlab.com/). Statistical significance was set at 0.05.

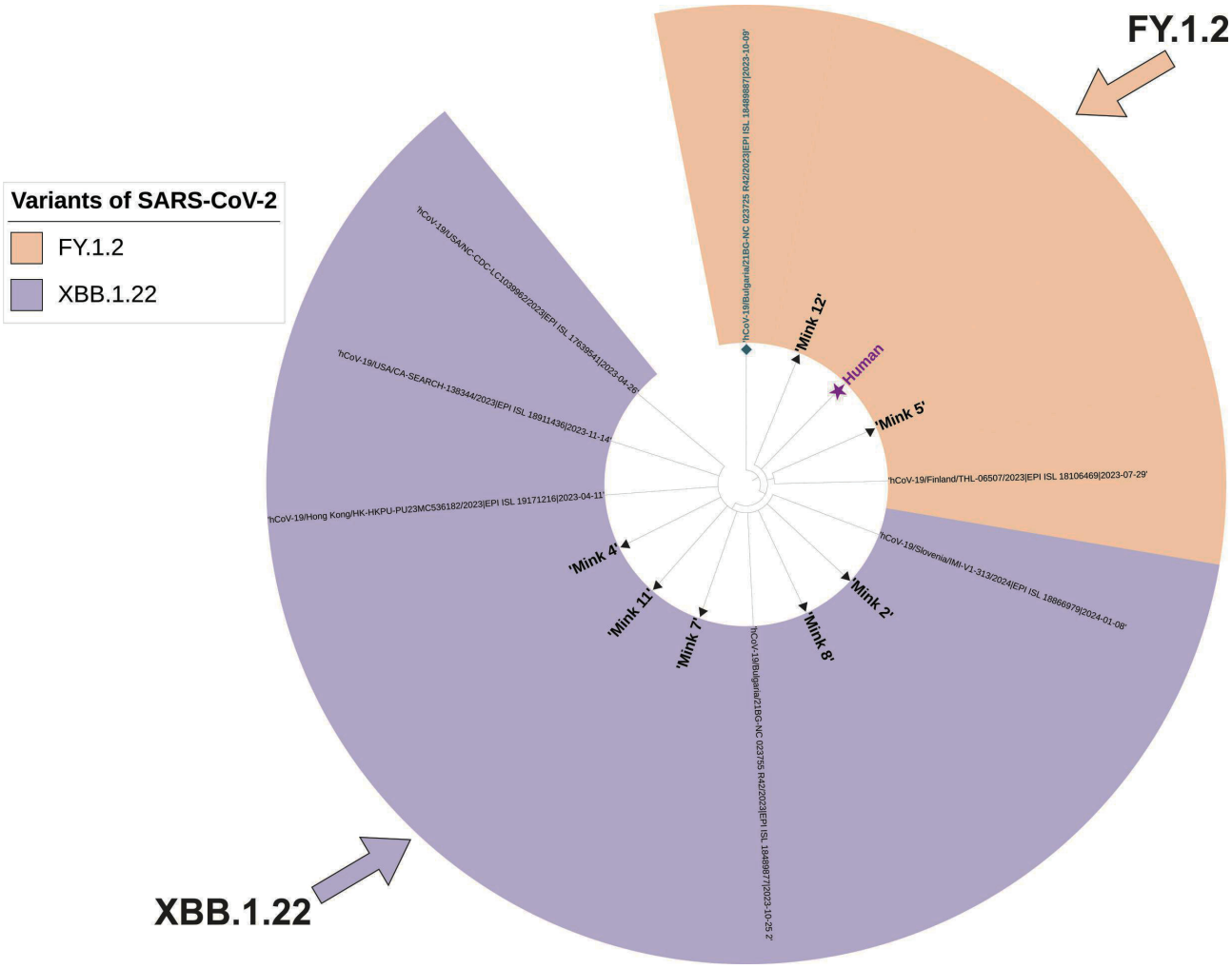

Figure 1. Phylogenetic tree based on the analysis of mink complete sequences published in GISAID. The tree was rooted against the Bulgarian sequence EPI_ISL_18489887 isolated during the same period marked with the symbol "◆". Other GISAID-derived sequences were used as reference strains. The isolated sequence from the person who cared for the minks is marked with the symbol "★" font and is called "Human" for short (EPI_ISL_18458688). The mink samples are labeled according to the names given by the veterinary laboratory marked with the symbol "▼". "Mink 2" (EPI_ISL_19210102), "Mink 4" (EPI_ISL_18458689), "Mink 5" (EPI_ISL_19210103), "Mink 7" (EPI_ISL_18458692), "Mink 8" (EPI_ISL_18489918), "Mink 11" (EPI_ISL_19210130), and "Mink 12" (EPI_ISL_18458693).

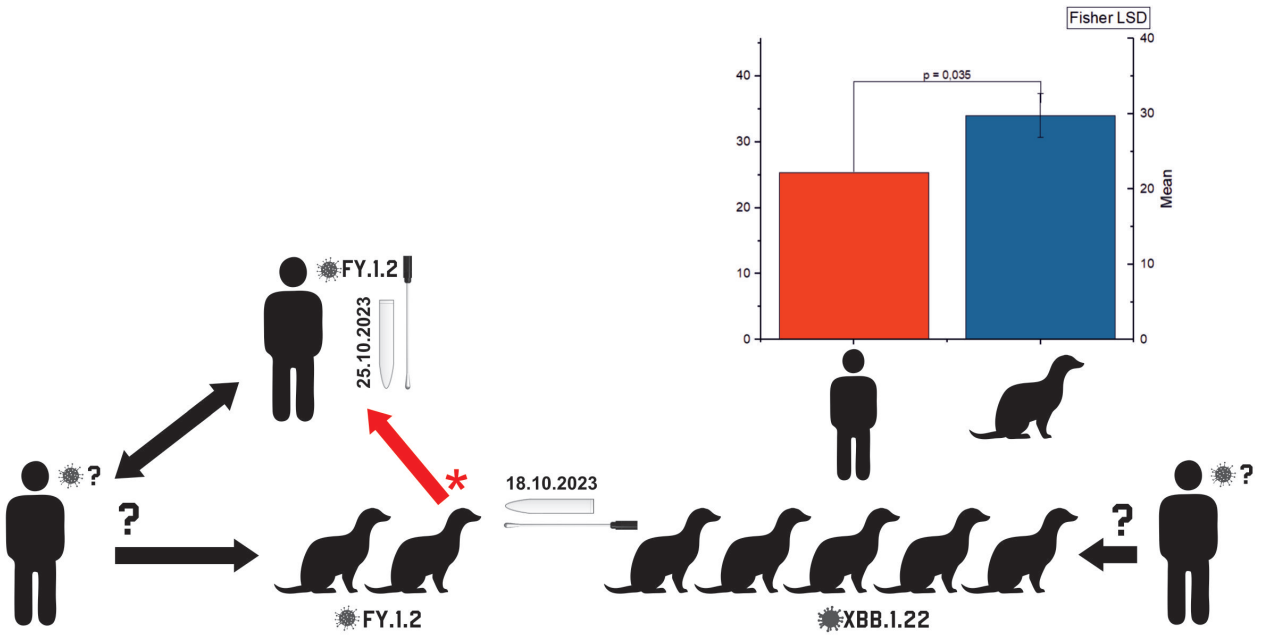

Figure 2. Transmission path of the SARS-CoV-2 infection in a mink farm on the territory of Bulgaria. The question mark indicates suspected transmission routes based on the comparison of SARS-CoV-2 Ct values from the mink caretaker and the 10 samples subjected to sequencing analysis. The arrow with a sign „*„ indicates the reverse transmission route of infection from mink to human.

RESULTS

3.1. Epidemiological data

Between 2 and 29 October 2023, 472 oropharyngeal samples from minks were tested for the presence of SARS-CoV-2. On 18 October, 98 (83%) of 118 samples were found positive. However, the examination of 118 samples from other minks during the next week returned negative results. No other minks tested positive for SARS-CoV-2 in the weeks that followed. No deaths due to the infection were registered among minks infected with SARS-CoV-2.

On 25 October, the Regional Health Inspectorate received a report of a mink caretaker from the same farm with slightly elevated temperature of 37.5°C and respiratory symptoms, without a cough. A respiratory sample was sent to the National Laboratory "Influenza and ARI", where infection with SARS-CoV-2 was confirmed. The mink caretaker was a 51-year-old man with no known comorbidities. He was quarantined for home treatment. No contact persons infected with SARS-CoV-2 were identified.

3.2. Tracing the path of infection by phylogenetic analysis

Initial PCR screening showed that only 10% of SARS-CoV-2 positive samples were suitable for sequencing analysis. Therefore, whole genome sequencing (WGS) was performed on 10 mink samples and one mink caretaker sample on 26 October. The Omicron variant of SARS-CoV-2 was identified in the human sample and in seven mink samples. The FY.1.2 subvariant was detected in the mink caretaker's sample and two mink samples (37.5%), while a different subvariant, XBB.1.22 (62.5%), was observed in five mink samples (Figure 1). The genetic integrity of three out of ten mink samples was insufficient to identify subvariants of SARS-CoV-2. The remaining positive mink samples had Ct values above 32 and were not sequenced.

Based on the temporal sequence of events and the evolutionary path of the SARS-CoV-2 variants, we hypothesize that other mink caretakers or mink contacts were infected with the two different variants FY.1.2 and XBB.1.22. They have probably transmitted the infection to the minks from which these two variants of SARS-CoV-2 were islolated.

In addition, the SARS-CoV-2 variant FY.1.2 detected in the mink caretaker was the same as the one found in two of the minks. The minks tested positive for SARS-CoV-2 on 18/10/2023, while the caretaker - on 25/10/2023. The Regional Health Inspectorate also reported that on October 25, 2023, the first signs of infection were registered in caretakers of minks.

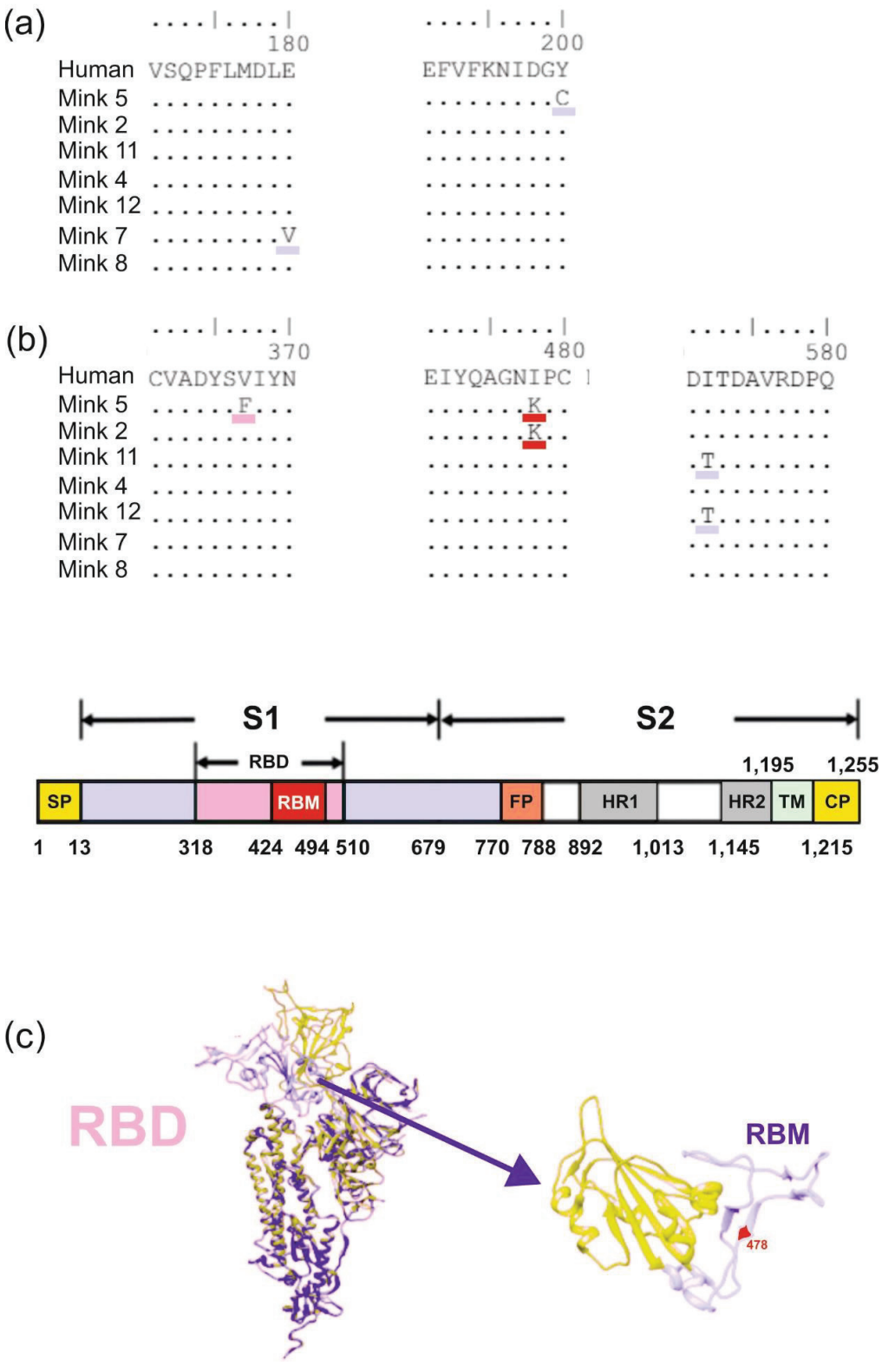

Figure 3. Amino acid analysis of the SARS-CoV-2 spike protein (S) isolated from a mink sample during an outbreak on a farm in Central Bulgaria: (a) cross-sections of the S analysis of the spike protein of SARS-CoV-2 isolated from samples of minks 5, 2, 4, 7, 8, 11, and 12 in the places of established replacement. An alignment was performed to the strain isolated from a person caring for these mink (EPI_ISL_18458688). (b) The structure of the S-protein; (c) The structure of RBD is depicted in yellow and purple, and the RBM is in purple, the site of the established mutation at position 478 is represented in red.

Based on this, we hypothesize that a transmission has occurred from a mink infected with the FY.1.2 variant to the positive mink caretaker (the path indicated by an arrow from mink to human in Figure 2). Regarding ше viral load, we observed that the mink caretaker's Ct value was lower than the mean Ct value observed in 10 mink samples that were subsequently sequenced (22.2 vs. 29.7 ± 2.9; p = 0.035). The remaining 88 minks had a Ct value in the range of 32 - 38.

3.3. Amino acid analysis

The S protein sequences isolated from seven mink samples were compared with those of the mink caretaker. The analysis identified five different substitutions in four of the mink samples. Specifically, one substitution was observed in mink 2 (I478K), three in mink 5 (Y200C; V367 F; I478K), two in mink 7 (E180V; I572T), and one in mink 11 (I572T) (Figure 3a). The substitutions at positions 367 and 478 were located in the receptor-binding domain (RBD) (Figure 3b). The sequences from minks 2 and 5 harbored a substitution at position 478 of the receptor-binding motif (RBM) (Figure 3c).

DISCUSSION

During the early stages of COVID-19 pandemic, there were reports of SARS-CoV-2 outbreaks on mink farms [12,13,14]. Minks are vulnerable to infection and can carry the virus without symptoms [13]. Direct contact with infected minks poses a risk for SARS-CoV-2 transmission to humans [11]. This interspecies transmission can result in genetic mutations and the emergence of new SARS-CoV-2 variants [15]. Similar to our study, other researchers have aimed to trace the potential transmission routes of SARS-CoV-2 between humans and minks [6,16,17]. Despite existing research on this topic, it remains relevant and important. A study reported that human angiotensin-converting enzyme 2 (ACE2) receptors have a higher binding affinity to the SARS-CoV-2 RBD as compared to mink ACE2s. Therefore, only SARS-CoV-2 variants with a higher binding affinity would contribute to viral entry and replication in minks and thus acquire higher transmissibility [18]. SARS-CoV-2 is believed to have originated from animals in 2019. By the end of 2020, it had significantly adapted to humans, leading to the emergence of the alpha, beta, gamma, delta, and Omicron variants. The Omicron variant, which appeared in November 2021, caused the largest wave of COVID-19, spawning multiple subvariants. The XBB line, comprising over 300 subvariants, was dominant in 2023 [19]. The XBB.1.22 subvariant was found in 71.4% of mink samples in our study. Other studies in the Netherlands and Denmark reported the transmission of infection from minks to humans [20]. Our study identified similar subvariants of SARS-CoV-2 in some mink samples and a mink caretaker (FY.1.2), suggesting an evolutionary mechanism of mutation accumulation. Human-to-mink transmission of the infection is assumed. Studies have shown that the period from the SARS-CoV-2 infection to the appearance of the first symptoms takes an average of 5.6 to 6.7 days [21,22]. People diagnosed with COVID-19 can transmit the virus from 1 - 2 days before, upto 8 - 10 days after the symptoms onset (https://www.ecdc.europa.eu/en/infectious-disease-topics/z-disease-list/covid-19/factsheet-covid-19). Omicron showed peak virus shedding 3–6 days after symptom onset, with viable virus cultured by day 10 [23]. Our observations suggest that another mink caretaker was the primary source of FY 1.2 infection on the farm, and third parties infected with XBB 1.22 likely transmitted the infection to minks. The higher SARS-CoV-2 viral load of the mink caretaker, along with the eight-day gap between the appearance of his symptoms and the detection of positive mink samples, suggests the possibility that the human was infected by these minks. In support of this claim, high viral load coincides with the peak development of infection [24]. The farm's minks showed attenuation of the infection, and no other humans in contact with the mink caretaker were identified as infected with SARS-CoV-2.

The S protein of SARS-CoV-2 is highly variable and plays an essential role for viral infectivity and virulence [25]. Amino acid analysis was performed to identify substitutions that may have occurred during potential transmission between humans and minks or vice versa [26]. This analysis reveals substitutions in the receptor-binding sites. RBM (438–506) directly interacts with the ACE2 receptor and plays a crucial role in determining its affinity [27]. A substitution at position 478 found in two mink samples could potentially affect the binding affinity of ACE2.

Therefore, we can conclude that the mutations were introduced during the possible transmission of infection from humans to mink, which changes the binding affinity between RBD and ACE2. Other studies have reported no such mutation occurring I478K. In 2020, mutations Y453F and F486L were reported in the sequence of SARS-CoV-2, for which the first source was most probably a mink from a farm in the Netherlands [24]. Those mutations were located in the RBM and were close to the one we found. In a subsequent study, the authors reported the prevalence of Y453F mutation in human samples in Denmark [28]. The transmition of such mutations poses a risk because they can affect the neutralizing ability of virus-specific antibodies [29].

The emergence of new mutations at receptor-binding sites is crucial for SARS-CoV-2 transmission between humans and animals. However, our study has limitations, as only 10% of positive mink samples could be sequenced, hindering our understanding of the infection pathways. Improved farm monitoring and more frequent sampling are necessary for future research. Additionally, cycle threshold (Ct) measurements may not accurately reflect primer amplification efficiency across different detection kits. Despite these challenges, the study highlights the importance of continuous monitoring and the potential of new SARS-CoV-2 variants of to pose pandemic threats.

CONCLUSIONS

The genetic analysis of the SARS-CoV-2 genome in both minks and humans suggested that there may have been other infected workers at the mink farm in addition to the caretaker who tested positive. This raises the possibility of SARS-CoV-2 being transmitted from humans to minks and then back to humans. This situation highlights the potential for mutations to occur within the spike protein, and affect the neutralizing ability of virus-specific antibodies thus increasing the public health risks.

CRediT authorship contribution statement

IT: conceptualisation, methodology, analysis, research design. **IT and NK**: writing the original manuscript**. ST and LL:** recruitment and testing of mink samples. **IT, IM, II, LG, IS DD**: Implementation of the experiments. **IT, IM, NK, IA, LG, II, IS, and DD:** Analysis of the results. **IC, II** project management and task execution. **NK, II, and IC**: review of the final manuscript.

Declaration of competing interest

The corresponding author affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned have been explained. The authors have no conflicts of interest to declare. No funding was received for this work.

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Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the Ethics Committee of 00006384, November 2022 (SPREAD AND CLINICAL IMPACT OF MONO- AND CO-INFECTIONS WITH ENDEMIC CORONAVIRUS 229E, OC43, NL63 AND HKU1 DURING THE COVID-19 PANDEMIC).

Database linking

https://gisaid.org/(EPI_ISL_18458688;EPI_ISL_19210102;EPI_ISL_18458689;EPI_ISL_19210103;EPI_ISL_18458692;EPI_ISL_18489918;EPI_ISL_19210130; EPI_ISL_18458693).

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MAST CELLS, THE EFFECTOR CELLS OF IGE-MEDIATED ALLERGY

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ABSTRACT

Background

Mast cells, are the primary responder cells of IgE-mediated allergy and while extensive research has been done to elucidate their precise structural and functional characteristics, mast cells are a type of tissue resident cell which makes their isolation and use for various research and diagnostic procedures in the field of allergy and immunology very complicated. Recently, attempts have been made to devise a method measuring mast cell degranulation through allergen stimulation for allergy diagnosis – Mast cell activation test (MAT). The aim of this article is to summarize existing knowledge on mast cells, the MAT, and the related Basophil activation test (BAT) on which the MAT is based on.

Methods

We analyzed data from 51 relevant articles.

Results

We made an extensive literature review of available data on the cytology, biochemistry and role of mast cells in the pathogenesis of allergic diseases—anaphylaxis, allergic rhinitis, and bronchial asthma, as well as available data on the MAT and how it compares to the BAT.

Conclusion

While much key information is available on mast cells, further research is required to expand on their function and role in allergy. Further research is needed to adequately compare MAT and BAT and create standardized protocols for the application of MAT in clinical practice and allergy research.

Key words: Mast cells, degranulation, IgE-mediated allergy, Basophil activation test, Mast cell activation test.

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Mast cells are a type of granulocytes that originate from the myeloid stem cell.

They are part of the immune and neuroimmune systems and play an important role in triggering the inflammatory cascade (1).

Mast cells play a key role in the development of IgE-mediated allergy and anaphylaxis, as they are the primary cell type responsible for releasing histamine in the body (2).

Additionally, they have an important protective role, participating in the development of immune tolerance, and in innate immunity against bacteria, toxins and parasites. They are also involved in angiogenesis and wound healing (3).

Phylogenetically, mast cells are found in primitive reptiles that lived 276 million years ago. Their study as cells of the human body began in 1863 with the discovery of granulated cells from Recklinghausen. Later, in 1878, in his doctoral dissertation, Paul Ehrlich described a new type of granular cells in connective tissue. He noted their "tendency to accumulate around preformed structures in connective tissue and mucous membranes," such as blood vessels, nerves, secretory ducts, sites of inflammation, and neoplastic foci. Initially, Ehrlich believed that these cells originated from connective tissue cells that had absorbed large amounts of nutrients, and therefore gave them the name "Mastzellen", meaning "well-fed cells" in German (4).

Mast cells are unique among hematopoietic cells because they mature in peripheral tissues rather than in lymphoid organs or the bloodstream. Their progenitors are released from the bone marrow and complete their development as tissue-resident cells (5).

Mast cell progenitors (MCPs) circulate from the bone marrow to the tissues, where their migration is

mediated by the interaction of integrins with vascular cell adhesion molecules (VCAM-1) and (MAdCAM-1), as well as chemokine receptors (e.g., CXCR2), which mediates their homing to the tissues where they mature (6).

Unlike basophils, mast cells exhibit different phenotypic and functional subtypes depending on their location, protease secretion, and reactivity.

Based on the types of proteases released upon degranulation, human mast cells are classified into two main categories: tryptase-positive mast cells (MCT), which secrete tryptases, and tryptase-chymase-positive mast cells (MCTC), which secrete both (7).

MCT are the primary type of mast cell found in the pulmonary alveoli, the mucosa of the small intestine, and the eyes. During mucosal inflammation, a selective increase in MCT is observed in the affected epithelial surface. MCTC are typically found in normal skin, blood vessels, submucosa, and synovium. Both types of mast cells are involved in local or systemic allergic reactions (8).

Microscopically, mature human mast cells range from 7 to 20 μm in diameter and appear as round or spindle-shaped cells with round or oval nuclei. They stain purple with aniline dyes, a property known as "metachromasia". They are most commonly identified by immunohistochemical analysis using monoclonal antibodies against mast cell-specific proteases.

The cell membrane features thin 1–2 μm microprotrusions (microplicae). Immature mast cells have a multilobed nucleus, while mature mast cells have a nucleus without nucleoli, with loosely condensed chromatin. The most remarkable characteristic of mature mast cells is their abundant cytoplasmic secretory granules, which constitute about half of their volume (9).

The granules contain numerous preformed mediators (Table 1.).

At an ultrastructural level, the secretory granules of human mast cells contain proteoglycans, of which Heparin is the most abundant. It stabilizes the β-tryptase tetramer. Neutral proteases, acid hydrolases, and histamine are bound to heparin by ionic bonds.

Depending on its size and location, each human mast cell contains 1-10 pg of histamine (10).

Histamine is synthesized in the Golgi apparatus by decarboxylation of the amino acid histidine by the enzyme histidine decarboxylase (HDC). After exocytosis at neutral pH, histamine dissociates from mast cell proteoglycans.

Once released into the extracellular environment, histamine is rapidly metabolized (half-life ≈1 min) through methylation or oxidation.

The initial product, N-methylhistamine (MHA), can be excreted through the kidneys, or deaminated by

Table 1. Types of preformed mediators and their biological properties in mast cell granules**.**

Mediator	Biological properties
Histamine	Bronchoconstriction; tissue edema; ↑ vascular permeability; ↑ mucus secretion; ↑ fibroblast proliferation; ↑ collagen synthesis; ↑ endothelial cell proliferation; differentiation and activation of dendritic cells.
Heparin	Anticoagulant; mediator binder; releases growth factors; activates fibroblasts; ↑ endothelial cell migration.
Tryptase	Breaks down inhalant allergens and cross-linked IgE; generates C3a and bradykinin; breaks down neuropeptides; activates TGF-β; increases basal heart rate and contractility of airway smooth muscle; ↑ fibroblast proliferation and collagen synthesis; ↑ ICAM-1 expression on epithelial cells and CXCL8 release; promotes histamine release from mast cells; attracts neutrophils.
Chymase	↑ mucus secretion; degradation of extracellular matrix; processing of type I procollagen; converts angiotensin I to angiotensin II; reduces T cell adhesion to airway smooth muscle; activates IL-1β; degrades IL-4; releases membrane-bound SCF.

the enzyme monoamine oxidase, to be excreted as N-methylimidazoleacetic acid (MIAA). Histamine can also be oxidized by diamine oxidase (histaminase) to imidazoleacetic acid (IAA) (11).

Histamine acts through four different G-protein coupled receptors (GPCRs) - from H1 to H4. Its binding to these receptors mediates effects that include: vasodilation and increased vascular permeability; spasm of bronchial and gastrointestinal smooth muscle; increased gastric acid secretion and itching (12).

The majority of effects associated with histamine in allergic reactions, such as pruritus, vascular effects and contraction of smooth muscle (bronchospasm), are mediated by the H1 receptor.

Histamine also exerts both stimulatory and inhibitory effects on immune cells: enhancement of antigen presentation by dendritic cells; suppression of TNF-α and IL-12 synthesis, and increased production of IL-10 by monocytes and dendritic cells; regulation of Th1, Th2 and Treg cell balance; chemoattractant activity for T cells and eosinophils (13).

Activation of H2 receptors primarily leads to increased gastric acid secretion and also plays a role in the allergic immune response – increased mucus production in the airways; vasodilation and constriction of bronchial smooth muscle (14).

H3 receptors are found mainly in the central and peripheral nervous system as presynaptic receptors controlling the release of histamine and other neurotransmitters. This is the only variety of histamine receptor mast cells don't possess. Yet, it has been implicated in neuro-inflammatory disease, with mast cells still playing a role (15,16).

H4 receptors are expressed by leukocytes, including mast cells, and mediate mast cell chemotaxis in vivo. H4 receptors influence the induction of dendritic cells in the Th2 response in a mouse model of asthma and may also play a role in the pathogenesis of itching (17).

In mast cell granules, neutral serine proteases are represented by two main families: tryptases (with trypsin-like activity) and chymases (with chymotrypsin-like activity). They contribute to the development of symptoms during the immediate-type allergic reaction (18).

Mast cell activation occurs primarily through three main receptors:

- **The high-affinity IgE receptor (FcεRI)**, which mediates the pathogenesis of clinical manifestations in IgE-mediated allergy.

IgE-dependent activation of mast cells leads to the release of preformed inflammatory mediators stored in their secretory granules (histamine, neutral proteases, preformed cytokines and proteoglycans – Table 1.). In addition, mast cells activated by FcεRI also secrete newly synthesized lipid mediators which are products of endogenous arachidonic acid metabolism, such as prostaglandin (PG) D2, leukotrienes (LT): LTB4 and LTC4, as well as the parent molecule of cysteinyl leukotrienes (cys-LTs). Furthermore, activated mast cells synthesize and secrete numerous proinflammatory cytokines.

The ultimate result of cell activation is plasma extravasation, tissue edema, bronchoconstriction, leukocyte recruitment, and persistent inflammation, with clinically recognizable symptoms of anaphylaxis, urticaria, angioedema, and dyspnea (19).

- The second receptor is the so-called **Mas-like G protein-coupled receptor X2 (MRGPRX2)**. It is primarily expressed by mast cells, which contain tryptase and chymase (MCTC), in the connective tissue of the skin,

MRGPRX2 is classified as an orphan receptor, meaning that its recognized ligands have not yet been fully identified (20). Studies conducted so far have shown that it responds to a wide range of molecules. For example, complement anaphylatoxins C3a and C5a, certain neuropeptides like substance P (SP), and antimicrobial peptides such as LL-37 can stimulate this receptor and induce mast cell activation independently of IgE receptor (FcεRI) cross-linking. In addition, it has been shown that some of the drugs that trigger anaphylaxis and pseudoallergic reactions are also agonists of the MRGPRX2 (21).

- **Toll-like receptors (TLRs)** are widely expressed on mast cells.

Mast cells respond to TLR activation by secreting cytokines, chemokines, and lipid mediators. Several studies have shown that activation via TLR2 can also trigger mast cell degranulation. Moreover, stimulation through TLR ligands can act synergistically with FcεRI signaling, enhancing the cellular response to allergens in vivo (22).

IgE-mediated activation is well-studied. It occurs

through cross-linking of two high-affinity IgE receptors (FcεRI) by allergens. This process triggers intracellular signaling pathways leading to degranulation, with the release of preformed mediators, activation of phospholipid metabolism, and transcription of specific genes, resulting in the production and secretion of cytokines, chemokines, and growth factors (23).

Mast cell degranulation occurs in several phases: Enlargement of granules; liquefaction of the crystalline structure; fusion of granules with each other and with the cell membrane and and finally, exocytosis and release of mediators into the extracellular space (24).

Recent studies on the mechanism of mast cell degranulation have revealed that FcεRI engagement is associated with activation of the AKT/PKC (protein kinase C) signaling pathway, leading to phosphorylation of the nuclear factor kappa-β kinase β-subunit (IKK-β) inhibitor. This initiates mast cell degranulation. The process is slow and occurs after fusion of the granules into large, irregularly shaped granules and the formation of membrane complexes comprised of synaptosomal-associated protein 23 and syntaxin-4 (SNAP23-STX4), which mediate exocytosis. Due to the large size of the granules, mediators are released slowly and thus can mediate immune responses at sites distant from the initial site of mast cell degranulation. Additionally, an inflammatory component contributes to the onset of allergic inflammation (25).

Interestingly, after nearly complete degranulation, human lung mast cells are able to survive and undergo regranulation for a period of 15 minutes (26).

A key aspect of IgE-mediated activation and degranulation of mast cells is the activation of phospholipid metabolism (arachidonic acid pathway) and the synthesis of newly formed mediators, such as prostaglandin (PG) D2, leukotrienes (LT): LTB4 and LTC4, whose biological effects play a central role in the initiation and maintenance of allergic inflammation (27).

Another less well-known mechanism of mast cell activation and degranulation is through IgE in its monomeric form. The binding of monomeric IgE to FcεRI in the absence of a specific antigen may lead to increased surface expression of FcεRI, which stabilizes the expression of the receptor on the cell surface and enhances the functional responses of the cells to activation through FcεRI aggregation. In this regard, it has been found that human mast cells, isolated from umbilical cord blood, activated with monomeric IgE, release chemokines (CCL1, CCL3) and growth factors (GM-CSF), but not histamine (28).

However, another study showed, that in similar condition human lung mast cells secreted histamine, leukotrienes (LTC4), and chemoattractants (CXCL8), which is a sign of complete degranulation (29).

In recent years, numerous studies have been conducted on the so-called non-immunological (direct) degranulation. It was found that this process primarily occurs upon engagement of the Mas-related G protein-coupled receptor X2 (MRGPRX2) by various ligands, including vasoactive intestinal polypeptide (VIP); endothelin 1; poisons; signaling molecule substance P (SP); anaphylatoxin complement fractions C3a and C5a; certain medications, etc.

This type of degranulation is direct, rapid with short-term release of small, spherical granules that are unstable and do not transport their cargo to the regional lymph nodes. Thus, direct degranulation is induced quickly and completes in less than five minutes. In this case, the main effector molecules are proteases in the granules. They have direct functions in the tissues - degradation of potentially harmful endogenous proteins, such as vasoactive intestinal polypeptide (VIP) and endothelin 1; and exogenous substances, such as poisons. They can also break down signaling molecules like substance P (SP) (30).

In such cases, rapid release of proteases may be sufficient to limit the effect of such substances and restore tissue homeostasis without causing unnecessary damage. There is no release of inflammatory mediators and no allergic inflammation develops (21,25).

The role of mast cells in the pathogenesis of allergic diseases is well known. They are key cells in the development of skin, food and inhalant allergies, which occur through IgE-mediated signaling. Most often, their biological effects are associated with the development of rapid allergic and anaphylactic reactions.

In anaphylaxis, systemic activation of mast cells is observed. The clinical manifestations

are mediated primarily by tryptase contained in mast cell granules. Alpha-tryptase (α-tryptase) is the most abundant enzyme in mast cells and its increased release is observed in mastocytosis. In IgE-mediated degranulation, β-tryptase is mainly released. Measuring β-tryptase levels is considered the best marker for systemic activation of mast cells in anaphylaxis (31).

Allergic rhinitis can be defined as an IgE-mediated, mast cell-dependent disease. It is characterized by continuous mast cell activation in the nasal mucosa, and the biological effects following mast cell degranulation may explain much of the symptomatology and pathology of allergic rhinitis. Evidence supporting this includes an increased number of mast cells in the epithelium; elevated expression of Th2 cytokines in mast cells; and increased expression of IL-4, which is affected by the application of topical corticosteroids (32).

The atopic phenotype of bronchial asthma results from the interaction between allergens, specific IgE and hyperreactive mast cells.

The early phase of the asthmatic reaction is due to mediators released by human pulmonary mast cells: histamine - during the first 2 minutes of the reaction onset, and later - prostaglandins and leukotrienes. These mediators lead to bronchoconstriction, mucosal edema, and mucus secretion

In the late phase of the asthmatic reaction, the following changes are observed: Increased concentrations of histamine, PGD2, LTC4, but in different proportions compared to the early asthmatic reaction; Decreased levels of tryptase and recruitment and activation of inflammatory cells (33,34).

Accumulated knowledge shows that mast cells have unique and diverse functions; they play a protective and regulatory role, and are also involved in the development of allergic inflammation.

The wide range of potent effector mechanisms of mast cells determines their broad distribution in the organism and their evolutionary conservatism.

The application of therapies targeting their key mediators will continue to be a major part of the treatment of allergic diseases, and the repertoire of such treatments is likely to expand (35).

Since mast cells play a key role in the development of IgE-mediated allergy, the most commonly used diagnostic methods are based on demonstrating their degranulation. Thus, the main in vivo tests such as skin tests and the various provocation tests for demonstrating allergen-specific sensitization are based on the functional determination of mast cell degranulation in different tissues after exposure to a specific allergen.

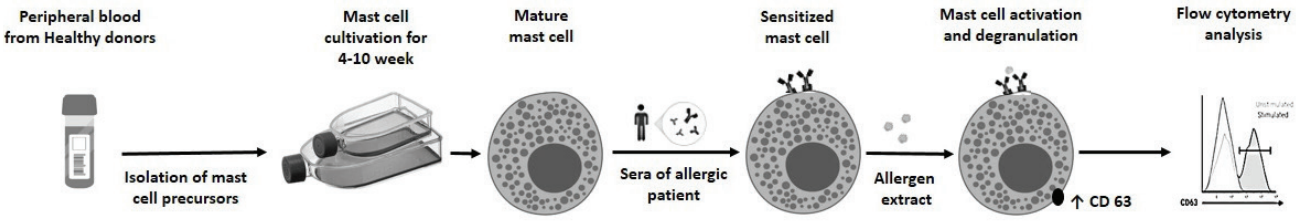
Numerous attempts have been made to develop in vitro methods for detecting mast cell degranulation in response to various allergens. Modern in vitro methods are based on measuring released mediators, such as tryptase (36) or histamine (37) or using the meso scale discovery (MSD) ELISA-based assay platform (38).

Mast cells are usually difficult to detect by flow cytometry and therefore their quantification is not of essential clinical significance. Considering the low mast cell content in peripheral blood, in order to ensure adequate analytical sensitivity, it is recommended to collect a large number of events (~500,000). The significant granularity of mast cells and their autofluorescence can further complicate assay design (39,40).

With the discovery of lysosomal-associated membrane protein (LAMP)-3, or CD63, as a marker of basophil degranulation, a flow cytometric basophil activation test (BAT) was developed in 1991. This test analyzes basophil degranulation in response to specific stimulation with a particular allergen (41).

CD63 is a glycoprotein that, in resting basophils, is located on cytoplasmic granules. During allergen-stimulated basophil degranulation, CD63 increases its expression on the cell membrane and can be determined by flow cytometry as a marker of the immediate-type allergic reaction (42). Currently, BAT is widely used for the diagnosis of IgE-mediated allergy, and various protocols have been developed to enable the identification of basophils and the quantitative assessment of their degranulation (43).

In recent years, several researchers have developed a new alternative in vitro test based on BAT designed to evaluate the direct degranulation of mast cells, known as the Mast cell Activation Test (MAT) (44,45). An important feature of the test is that, since mast cells are primarily tissue-resident cells, the test is performed on a cell culture of human mast cells



(hMCs) - Fig 1.

After passive sensitization with patient's serum, the cells are activated directly with the relevant allergen and their degranulation is measured by flow cytometry. In cases of sensitization, patients demonstrate allergen-specific and dose-dependent degranulation, which is measured by the expression of surface activation markers, such as CD63 (46).

Initial comparisons between BAT and MAT showed that despite the more complicated methodology of MATq, it is much more sensitive. MAT shows markedly higher levels of surface expression of the activation marker CD63 after allergen stimulation, even at concentrations up to 2 log lower than those used in BAT (Table 2).

Moreover, in BAT, 10-15% of non-responders are observed, whose basophils do not respond to IgE-mediated activation, making the test inapplicable for these patients.

The BAT methodology requires the use of fresh blood, ideally processed within 12 hours of its collection. In MAT, patient sera are used, which can be frozen and used repeatedly in the course of routine laboratory testing (47).

One of the greatest advantages of MAT is the possibility of studying non-immune (direct) degranulation, which will lead to a more precise diagnosis of drug allergy.

The disadvantages of MAT include its high cost and the time required to generate a cell line from mature mast cells, which ranges from 4 to 10 weeks depending on the protocol (47).

MAT, being a novel testing tool, is still not routinely used in allergy diagnostics, unlike BAT, whose application in this field spans close to two decades, during which, a lot of practical data have been obtained. For example, in the area of drug allergy testing, BAT can accurately predict negative result values, as well as obtain positive results, even after other testing has produced a negative (48,49). In food allergy diagnostics, specificity, as well as sensitivity are very high, and testing could be carried out by using either crude allergen extracts or single molecules, limiting the potential number of oral provocations these patients could undergo in the course of the diagnostic algorithm (50).

Still, BAT is a supplemental tool in allergy diagnostics, providing further information and an additional

Table 2. Comparison between BAT and MAT.

Characteristics	BAT	MAT
Non-responders (%)	10%–15%	No
Optimal time to perform the test after sample collection	12 h.	Does not depend on time
Possibility of repeatedly examination	No	Yes
Passive sensitization required	No	Yes
Stable response to FcεRI	Yes	Yes
Possibility to test MRGPRX2	No	Yes
Releasing of mediators	++	+++
Donor-dependent variability	Yes	Yes
Standardized protocols	Yes	No
Cost of the test	+	+++
Routine use in clinical practice	Yes	No

layer of security for practitioners when diagnosing allergic disease in patients at high risk for anaphylactic reactions. It is still not recommended to be used as a single diagnostic tool due to various limitations, such as differences in cut-off values and lack of standardized interpretation protocols for certain allergens (51). MAT, in a similar way, could expand the scope of allergy testing but more data is needed on its potential applications and relevant shortcomings in clinical practice.

For now, it is difficult to make a definitive analysis of the superiority or weaknesses of the two methods, since the reproducibility of the tests is still not good, mainly due to the lack of standardization of the protocols according to which BAT and MAT are performed.

One thing is clear - both methods are gaining increasing importance in the diagnosis of IgE-mediated allergy and their use is beginning to be regularly recommended in guidelines and program documents.

However, for these methods to be incorporated into clinical practice as routine diagnostic tools for allergies, further studies are needed regarding their standardization and validation.

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VIRULENCE FACTORS OF *E. COLI*, ASSOCIATED WITH URINARY TRACT INFECTIONS

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ABSTRACT

Urinary tract infections (UTIs) are the most common type of infections second only to respiratory tract infections. Millions of UTI cases are reported each year, affecting in- and outpatients. The most frequent causative agents of UTIs are the enteric Gram-negative bacteria, among which *Escherichia coli* (*E. coli*) dominates. While most strains of *E. coli* are harmless and indeed play a beneficial role in gut health, some strains (uropathogenic *Escherichia coli*, UPEC) can cause infections when they are translocated to generally sterile body areas, such as the urinary tract.

This review presents the wide range of virulence factors of UPEC, involved in the urinary tract colonization, infection development and host tissue invasion. Cell-associated and extracellular key virulence factors such as adhesins, invasins, iron acquisition factors, factors mediating serum resistance, toxins and structural components are discussed in detail. Also, the review focuses on the process of biofilm formation, another crucial virulence factor in UPEC, responsible for UTI persistence, reoccurrence and antimicrobial therapy failure. The regulatory mechanisms involved in biofilm production are also discussed.

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Escherichia coli (E. coli) is a Gram-negative bacterial species, a member of the order *Enterobacterales.* It is an opportunistic organism, part of the human microbiota, with the potential to cause intestinal and extraintestinal infections, including severe invasive complications [1]. Members of the species belong to several phylogenetic groups, A, B1, B2, C, D, E, F and clade I [2, 3]. Nonpathogenic strains of *E. coli* are most commonly from A and B1 groups, while those causing extraintestinal infections are usually assigned to B2 and D groups [4, 5]. Extraintestinal pathogenic *E. coli* (ExPEC) can cause a variety of infections usually associated with penetration in primary sterile anatomical areas or with severely immunocompromised patients. The ExPEC group is currently represented by UPEC (uropathogenic *E. coli*), NMEC (neonatal meningitis-associated *E. coli*), SEPEC (sepsis-associated *E. coli*) and AREC (avian pathogenic *E. coli*) [4, 5].

Urinary tract infections (UTIs) are among the most common bacterial infections in both community and hospital settings, with over 150 million cases diagnosed annually worldwide [6]. In over 80%, UTIs are associated with *E. coli* [7]. Most UTIs are caused by the highly heterogeneous UPEC group. UPEC strains due to their multiple virulence factors, usually encoded on pathogenicity islands, plasmids and other mobile genetic elements, migrate from the intestinal tract, colonize the urinary tract and cause different types of UTIs [4, 5]. The virulence factors related to UTIs pathogenesis are extremely diverse and serve different functions (adhesins, invasins, iron acquisition factors, factors mediating serum resistance, toxins, structural components and etc.) [8]. They are divided into two major groups - cell-associated and extracellular virulence factors.

Cell-associated virulence factors

1. Outer membrane: lipopolysaccharides and proteins

Lipopolysaccharides

Lipopolysaccharides (LPS) are a major component of the outer membrane of *E. coli*. Due to strong immunogenicity, they play a key role in the activation of host immune system. LPS are composed of lipid A,

responsible for the toxicity of the structure, R antigen and the O antigen, which is the major bacterial somatic antigen [9]. Based on the O antigen specificity, there are more than 180 different *E. coli* serotypes, with UPEC belonging mainly to O1, O2, 04, O6, O7, O8, O14, O15, O16, O18, O21, O22, O25, 075 and O83 [10-15]. Amongst them, there is an *E. coli* subtype, which belongs to O25 serogroup and a widely spread and hypervirulent sequence type 131 – *E. coli* O25b/ST131. It is often associated with UTIs and subsequent complications such as sepsis. Furthermore, the majority of the isolates are multidrug-resistant and render the therapy with beta-lactams, fluoroquinolones and aminoglycosides ineffective [3]. Typically, uropathogenic serotypes of *E. coli* have the ability to inhibit the induction of certain cytokines in the epithelial cells, specifically IL-6 and IL-8 [16, 17]. In addition, the resistance to serum bactericidal effect (serum resistance) is also associated with specific O antigens typical for UPEC (O6, O8) [18].

Outer membrane proteins

Outer membrane protein A (OmpA) is one of the major outer membrane proteins of *E. coli.* It is associated with multiple structural and physiological functions, including maintenance of the cell shape and stability, biofilm formation, adhesion, colicins, bacteriophages receptor and F-dependent conjugation [19]. OmpA has been documented in all ExPEC strains [20]. It is believed that OmpA plays a key role in the intracellular bacterial colony formation and long-term persistence of *E. coli* in the bladder [21]. Another important outer membrane protein is associated with the *traT* gene – a transfer surface exclusion lipoprotein, which confers serum resistance and prevents bacterial death [3].

2. Flagella

E. coli is a motile organism with motility, mediated by peritrichial flagella. These surface structures, composed of the protein flagellin, are of particular importance for the UPEC virulence. The flagella are directly related to the bacterial cell adhesion and colonization of the urinary tract and biofilm production during the course of the UTI [22, 23]. A direct link has been demonstrated between the flagella production and the fimbriae production in UPEC strains, and the regulation of expression of these structures are highly linked and coordinated [24]. This is mediated by the Pap X repressor localized in the P fimbrial operon of UPEC, with Pap X overexpression leading to inhibition of the *flhDC* gene and to a reduction in flagella production and respectively to decreasing the bacterial cell motility [25, 26].

3. Adhesive structures

The bacterial attachment to the uroepithelial cell is critical for the onset and subsequent development of UTI [27]. The ability of *E. coli* to adhere to the uroepithelium, to resist to urinary flashing and colonize the urinary tract is associated with the expression of specific adhesins. These thin and extracellularly located protein filamentous organelles are called fimbriae or pili. The loss of the ability of bacterial cell to attach to and, respectively to colonize the anatomic region by adhesin-mediated recognition of specific receptors is often sufficient to render it avirulent [28]. Of note, adhesive structures (type 1, P, S fimbriae) mediate both the bacterial cell attachment process and cytokine production by T-lymphocytes, play an important role in tissue invasion and inflammation [29, 30].

Type 1 fimbriae, P, S and F1C fimbriae and some afimbrial adhesins are the most common adhesion factors found in UPEC [31-33]. They occur more frequently in UPEC than in commensal strains of *E. coli* [34].

Type 1 fimbriae

Type 1 fimbriae are extremely prevalent among both UPEC (86-100%) and commensal isolates [35-37]. They are considered to be one of the most important and critical virulence factors [38]. Type 1 fimbriae are encoded by the highly conserved *fim* operon including 9 genes (*fimA, B, C, D, E, F, G, H, I*) and its expression is under phase variation control [39, 40]. Products of the *fim* operon are structural (FimA, FimI, FimF, FimG), adhesion (FimH) and regulatory proteins (FimB, FimE) [41]. The *fimH* gene mediates the binding of the fimbriae via the FimH adhesin, located at the end of the structure, to specific D-mannose-containing receptors, found mainly on epithelial cells of the lower urinary tract and less in the renal parenchyma [42, 43]. An additional function of type 1 pili, particularly in cases of pyelonephritis, is to aid bio-

film formation by binding to urothelial uroplakin [44-46]. Studies involving targeted inactivation of FimH adhesin conducted in human and mouse models have shown a significant reduction in the potential of UPEC strains to colonize the urinary tract [47-48].

Besides type 1 fimbriae (mannose-dependent), *E. coli* also possesses type 2 fimbriae (mannose-resistant), represented by P, S and Dr fimbriae.

P fimbriae

Similar to type 1, P fimbriae are also extracellularly located, however, they do not associate with D-mannose-containing receptors but exhibit specificity for glycosphingolipid-containing structures (α-D-galactopyranosyl-1,4-β-D-galactopyranoside) [49]. Such receptors are found on epithelial cells in the kidney and renal tubules [45, 50, 51]. This explains why P fimbriae are more frequently reported in cases of ascending UTIs and pyelonephritis (61 - 91%), and much less frequently in cases of UTIs (< 30%) or in *E. coli*, representatives of the normal gut microbiota [33, 50, 53-58]. Some authors also report P fimbriae particularly strongly associated with pyelonephritis in childhood [52, 53, 55, 59].

The P fimbriae are encoded by the *pap* operon (*papIBAHCDJKEFG),* which contains structural (*papA, papJ, papG*) and regulatory genes (*papI, papB*) [41, 60]. The papG gene encodes the terminal PapG protein directly responsible for the adhesion process [58]. Based on its specificity, PapG exists in four variants (PapI, PapII, PapIII, PapIV), with PapII being the most common among UPEC strains [58, 60].

It was also found that nonpathogenic *E. coli* isolates, acquiring the *pap* operon, also acquire the ability to colonize kidney tissue [58].

S fimbriae

S fimbriae are adhesins that are encoded by the *sfa* operon [61, 62]. They exhibit specificity for a**Capsule**

In *E. coli*, more than 100 capsular polysaccharide antigens have been identified. The main function of the capsules is related to inhibition of phagocytosis and complement bactericidal activity [78]. The similarity between some polysaccharide K antigens in UPEC and tissue structures in the host organism explains the antigen mimicry phenomenon and their difficult recognition by the immune system as foreign antigens [79]. On the other hand, the UPEC polysaccharide capsule, coating the bacterial cell surface, can inhibit the adhesion process to the corresponding epithelial cells. However, binding of type 1 fimbriae to mannose-containing receptors has been shown to result in down-regulation of the *kps* operon, followed by reduced production of capsular substance and thus aiding the adhesion process [80].

Extracellular virulence factors

1. Toxins

Haemolysins

Hemolysin A (HlyA) and cytotoxic necrotizing factor 1 (CNF1) of *E. coli* are the two main toxins responsible for bacterial tissue invasion and dissemination, as well as for the dysfunction of local immune responses [32, 33].

HlyA is mainly associated with the destruction of host cells, thus releasing nutrients and other factors, such as iron, which are critical for bacterial growth. HlyA is a calcium-dependent toxin that in high concentrations forms pores in the cell membranes of the host organism leading to cell lysis. In low concentrations, HlyA can induce apoptosis in the epithelial cell and thus promote the spread of infection [29, 81]. Encoded by the *hlyCABD* operon, the toxin is found in approximately 50% of UPEC [33, 82, 83] and its expression is associated with increased clinical severity of UTIs [47]. HlyA genes have been detected more frequently in patients with pyelonephritis (38%) than in those with cystitis (15%) [33].

HlyE is another toxin of the haemolysin group [84]. Its production and action are mediated by the cytoplasmic enzyme HlyF, which increases the formation of outer-membrane vesicles containing HlyE. The presence of *hlyF* in the UPEC genome is associated with more severe UTIs such as pyelonephritis accompanied by urosepsis and induction of an exacerbated inflammatory response, a specificity that distinguishes the *hlyF* positive strains from the typical UPEC [84-86].

Cytotoxic necrotizing factor 1 (CNF1)

The effects of CNF1 are closely related to its ability to bind to specific cellular receptors (BCAM), inducing RHO GTPases activation, responsible for the control of multiple eukaryotic cell functions (actin structure formation, motility, phagocytosis, etc.) [87-90]. The

CNF1 production is associated with the induction of apoptosis of bladder epithelial cells with subsequent exfoliation, with bacterial invasion and persistence, and involvement of new cells [47, 91, 92]. The genes encoding CNF1 have been documented in about 1/3 of UPECs and are more frequent in uropathogenic than in commensal strains of *E. coli* [93, 94].

Other toxins

Spa (Serine protease autotransporter), Sat (Secreted autotransporter toxin) and Vat (Vacuolating autotransporter toxin) are also toxins found in UPEC associated with kidney injury. They exhibit cytotoxic activity against uroepithelium, cause vacuolization of renal glomeruli and tubules, exhibit marked proteolytic effects against some complement factors, degrade mucin and promote the colonization stage [95-99].

2. Iron acquisition systems

Siderophores

Iron (Fe^{3+}) is essential for life and proper functioning of all living organisms [100]. This element plays a key role in all essential processes in the bacterial cell, including the "virulence" behavior [101-103].

The human body uses several mechanisms to restrict pathogenic organisms' access to free iron: in tissues and cells, the iron is stored as ferritin, and in the blood, transferrin binds to iron with high affinity [104-106]. Stored as part of the heme (a cofactor of hemoglobin, myoglobin and cytochromes), the iron ions, are in a form inaccessible to microorganisms [101].

However, bacteria develop mechanisms that allow them to acquire iron from the host organism and thus survive and cause infections. The secretion of siderophores, iron-chelating molecules is such a mechanism. The siderophore high affinity for iron ions, especially trivalent iron (Fe^{3+}), allows its capture by ferritin and transferrin [107-108]. After iron binding, the complex is recognized and transferred into the bacterial cell via specialized transport systems. This mechanism allows bacteria to obtain the necessary amount of iron even when its level in the organism is very low. Siderophores belong to 5 main classes - catechols (cateholates), phenols (phenolates), hydroxamic acids, alpha-hydroxycarboxylates and a mixed type containing different siderophores [34]. *E. coli* has several siderophore systems - enterobactin and salmochelin (catechol siderophores), yersiniabactin (phenolate siderophore) and aerobactin (mixed type). Enterobactin is found in both pathogenic and non-pathogenic *E. coli* and its role in virulence is of less importance compared to other siderophore systems. One reason for this is that enterobactin can be inactivated by certain defense mechanisms of the host organism associated with the Lipocalin-2 protein [109]. On the other hand, the modification of this siderophore by glycosylation leads to the formation of salmochelins that manage to escape the action of Lipocalin-2 [110].

In contrast to enterobactin, the production of aerobactin, salmochelin and yersiniabactin has been demonstrated predominantly in UPEC and much less frequently in commensal *E. coli* strains [8]. Aerobactin is the most frequently studied siderophore system in uropathogenic *E. coli* and dominates as a survival mechanism [31, 111, 112]. Compared to enterobactin, aerobactin is much more efficient in iron capture and even at very low concentrations can stimulate bacterial growth [108].

The siderophores salmochelin and yersiniabactin have also been attributed an important role in the pathogenesis of *E.* coli-associated UTIs. The expression of *iroN*, encoding the salmochelin-siderophore receptor IroN, is associated with a 5- to 10-fold increase in the invasiveness in the urothelial cells [113]. Yersiniabactin has been attributed also to be important for biofilm production in *E. coli* and for the increased bacterial resistance to phagocytosis [108, 114].

In addition to siderophores, UPECs use the Hma and ChuA transporters, which are involved in the iron uptake directly from extracellular heme [8, 108]. Another transporter type that also delivers iron to the interior of the bacterial cell is SitABCD [115].

Other extracellular virulence factors

Curli protein

Curli protein is an amyloid protein encoded by the *csg* gene and secreted by many bacterial species, including *E. coli*. This protein is involved in different processes, including biofilm production, in which curli is a major component, but also in the adhesion,

colonization, invasion and in the development of an inflammatory response mediated by the release of some cytokines (IL-6, IL-8, TNF-alpha) [116-117]. Curli protein is also involved in the intercellular interaction and communication under biofilm conditions [116-117].

Intracellular Bacterial Communities (IBCs)

A specific feature of UPEC is their ability to reproduce intracellularly [8, 118]. Only after adhesion to the urothelial cell, E. *coli* can enter the host cell and form mature IBCs as a result of active replication, followed by leaving the damaged cell, infecting new cells and spreading the infection [70, 119]. The process of IBCs formation is accompanied by a change in bacterial cell morphology from coccoid to rod-shaped and filamentous shape [119]. IBCs are mediated initially by the FimH adhesin, related to type 1 fimbriae, which recognizes specific receptors on bladder epithelial cells (uroplakin, integrin), followed by activation of RHO GTPases and a process of bacterial endocytosis [120]. The *E. coli* capsular polysaccharide antigen contributes significantly to intracellular survival and IBCs formation [121].

The ability of UPEC to form IBCs is a mechanism to evade the host immune response [56]. It is thought that IBCs are a key mechanism for the development of *E. coli* UTIs [8], including recurrent UTIs [119]. The last are associated with the Quiescent Intracellular Compartments (QICs), located in cells of the underlying transitional epithelium, containing a small number of viable but non-replicating bacterial cells that can be re-activated [56, 122].

Biofilm production

In unfavorable living conditions bacterial biofilm production is an important survival mechanism. It protects the microbial cells from the innate defense factors (complement, phagocytosis), specific (immune) defense mechanisms of the host organism and is among the most important antimicrobial resistance mechanisms [123]. Biofilm production mediates microbial colonization of various medical devices, including urinary catheters, contributing to increased morbidity from both acute and chronic infections [124].

Regarding UPEC, the biofilm production underlies the pathogenetic mechanism of UTIs and significantly contributes to the UTIs persistence and recurrence and catheter-associated UTIs (CAUTIs), accounting for about 40% of all nosocomial infections [125-129]. It is biofilm formation that is one of the most important mechanisms determining the high levels of antibiotic resistance, often accompanying the UTIs [125, 127, 130].

The biofilm is a 3D community of microbial cells embedded in a self-produced extracellular polymeric substance attached to biological or non-biological surfaces [129, 131]. Under biofilm conditions, the bacterial population differs significantly from the free-living (planktonic) cell [130, 132]. Biofilm associated bacteria reduce their motility and metabolic activity to conserve energy and nutrients [130, 132]. Besides protection, this viscous substance anchors the bacterial colony to the site of infection, and the increased release of extracellular bacterial toxins provides additional nutrient release at the site of infection [133].

According to the amount of biofilm secreted, strong, medium and weak biofilm producers are differentiated among UPEC. The biofilm formation in *E. coli* is a complex process consisting of several sequential stages: a stage of reversible attachment; a stage of irreversible attachment and early biofilm development; biofilm maturation and a stage of biofilm dispersal [123, 124]. During the first stage (the reversible attachment), which is typical for freely living bacterial (planktonic) cells, the flagella production is particularly important. It ensures the cell motility and overcoming the hydrodynamic and van der Waals forces and thus mediates the attachment to the surface of epithelial cells or foreign bodies [124]. Catheters, stents or the rough stone surfaces are ideal for biofilm attachment [123]. In the reversible attachment stage, two types of bacterial populations are found, those that can permanently form flagella and those in which the expression of genes encoding these structures is repressed [124].

When the bacterial cells find optimal conditions for a "sessile" lifestyle, the attachment becomes irreversible, and the process is mediated by a diversity of microbial structures with adhesive function (type 1 pili, F pili, curli, colanic acid) [134]. Besides to epithelial cells and/or artificial surfaces, bacteria attach

to each other, which further strengthens and stabilizes the structure, a process associated with the outer membrane protein Ag43 [135-136]. The type 1, P and S pili, the Dr and F1C adhesins are thought to be critical for biofilm formation, although various studies have shown different distribution of genes encoding adhesins in *E. coli* biofilm producers [29; 57; 64, 137-139]. A systematic review and meta-analysis on virulence factors among 1888 UPEC isolated from patients with UTIs over a 10-year period (2000 - 2019) showed a prevalence of CSH (80%) and *fimH* (75.3%) among the genes encoding the group of adhesive factors [140]. Tewawong's study also found the dominance of *fimH* (91.8%) but also of *pap* genes (79.3%) and demonstrated a very high relative proportion of UPEC isolates carrying a combination of adhesins (80.3%), with the *fimH* + *pap* combination identified in 69% [141].

The inhibition of the irreversible attachment by an antibody-mediated mechanism or by downregulation of pili-encoding genes, can dramatically reduce biofilm formation [45]. The cyclic-diguanylic acid (c-di-GMP), theconcentration oh which is increased at this early phase of biofilm development, is of great importance for the microbial transition from planktonic to biofilm (sessile) form [117]. In addition, the attached bacterial cells actively replicate and increase in number, which is associated with the induction of the intercellular Quorum sensing (QS) communication system [123].

During the maturation a matrix substance is produced, and the biofilm is differentiated into a growing, well-structured 3D microbial community, with a defined architecture and spatial arrangement [124]. The mature biofilm is a dense structure, very difficult to eradicate [130, 142]. The main components of the matrix are water, exopolysaccharides, proteins, DNA and lipids [143]. It is the exopolysaccharide component that is specific and distinguishes the microbial biofilm from the planktonic bacterial form [117]. The stability and the shape of the biofilm are primarily mediated by this component represented by poly-β-1,6-N-acetyl-D-glucosamine, cellulose and colanic acid [129, 144-147]. The matrix polysaccharides are also involved in other processes: they contribute significantly to the adhesion of the cells to each other and to various surfaces and host cells, provide protection against defense host factors, mediate resistance to antimicrobials and to desiccation, act as a mechanical barrier and a reservoir of nutrients and mediate the interactions between bacterial cells [117, 143, 148, 149]. The cellulose production is specifically responsible for the formation of a rigid biofilm, and the colanic acid forms a capsule around the bacterial cells, protecting them from adverse external conditions. However, colanic acid can also inhibit biofilm formation, which is associated with masking of Ag43 and AidA [150]. In addition, by coating highly immunogenic structures, exopolysaccharides (especially cellulose) significantly reduce the immune response against the pathogen [8]. Several authors have reported that increased production of this matrix component is associated with the development of more severe and persistent UTIs [151-154].

Although with indirect effects, the LPS and the capsular polysaccharides (O and K antigens) also contribute to biofilm formation. They mediate the interaction between the bacterial cells and the environment and especially mediate the adhesion process through interaction with cell surface structures [155].

The matrix DNA and proteins are also involved in binding to and colonization of biotic and abiotic surfaces [143] and perform a variety of functions in the biofilm maturation stage (desiccation resistance, protection, nutrient supply, exchange of virulence factors, etc.) [143, 149, 156].

Except the exopolysaccharides, the autotransporter proteins are critical for the biofilm maturation and intercellular interactions [155]. Antigen 43 (Ag43), a key autotransporter, mediates the adhesion of cells to one another, the processes of auto-aggregation and formation of the 3D biofilm structure. AidA and TibA proteins have a similar function [150].

Due to unfavorable living conditions in the biofilm during its final stage (scarce nutrients, low O_2 concentration, pH changes, accumulation of toxic products, and other stressful conditions), individual daughter cells detach from the biofilm, migrate, and adhere to adjacent, new surfaces [123, 157-160]. The process of active dispersion is mediated by proteins within the matrix, responsible for its enzymatic degradation [143, 161, 162]. Passive dispersion can also occur, but under external interference, including human influence [161].

The transformation of *E. coli* from planktonic to biofilm form and vice versa is a complex process, whose regulation is strongly dependent on 3′,5′-cyclic diguanylic acid, the two-component CpxA/CpxR signaling system, the three-component protein regulatory system RcsCDB and quorum sensing [124]. The high level of 3′,5′-cyclic diguanylic acid blocks the flagellar proteins, resulting in motility loss. In addition, 3′,5′-cyclic diguanylic acid is involved in the curli, cellulose and Poly-β-1,6-N-acetyl-D-glucosamine syntesis [163]. The CpxA/CpxR system also influences motility, and this effect is mediated by cell surface chemical composition changes via OmpC activation [164]. Additionally, CpxA/CpxR inhibits curli production [165]. The RcsCDB system regulates the gene expression of structures associated with different functions - adhesive (Ag43, curli), mediating motility (flagella) and protection (capsules) [166].

Furthermore, during the process of biofilm formation in *E. coli*, multiple genes encoding stress tolerance, related to survival in adverse conditions and to biofilm protection are expressed. Products of such genes are the Hfq protein (promotes biofilm production in adverse conditions), YcfR/BhsA (induces indole production and makes biofilm resistant to acids, temperature and peroxide), AriR (an acid-resistance regulator protein associated with indole production), the sigma S factor (affects the expression of regulatory and structural genes associated with biofilm production and degradation) [168, 169].

Quorum sensing (QS)

QS is a bacterial communication system, mediated by the release of chemical signaling molecules called autoinducers or quormons, which allows bacteria to communicate and function as a multicellular organism, and coordinates their behavior [117, 169]. QS has a leading role on cell division control, bacterial movement, biofilm formation, upregulation of genes encoding virulence factors, as well as on the gene transfer between bacterial cells in the biofilm, and on the pathogen and host organism interactions through the immune response modulation [129, 136, 170-172]. The QS system is dependent on the cell density in the biofilm and coordinates bacterial behavior to maximize benefits to the microbial population in the biofilm, including optimal nutrient utilization, increased pathogenicity, and survival [173]. A minimum population number threshold is required for QS activation [160]. An inducer-receptor mechanism associated with the QS system is involved in the gene control mechanisms [173-176]. The Gram-negative bacteria use N-acyl-homoserine lactones (AHLs)-associated inducers and their corresponding receptors (LuxRs) [177, 178]. At low biofilm cell density, the inducers are secreted at very low, non-detectable levels, but as the bacterial population increases, their amount is sufficient to bind to the corresponding receptors, forming complexes involved in the gene expression control [179-183]. AI-2 is one of the most important autoinducers, directly related to *E. coli* biofilm production. Upon reaching optimal bacterial density, AI-2 production is inhibited, a process, related to *luxS* gene down-regulation [123, 171]. The AI-2 effect of increasing significantly the biofilm mass has been shown to be mediated by a specific QS motility regulator (MqsR) [172].

The biofilm in *E. coli not only* successfully prevents most of the humoral and cellular defense mechanisms of the host, but also through various mechanisms can render bacterial cells in the biofilm up to 1000-fold more resistant to antimicrobials than the planktonic forms [124]. The weak penetration of antibiotics in the biofilm-related bacteria, mediated by the matrix substance as a physical barrier and by other biofilm components such as polysaccharides, enzymes and DNA, binding or degrading the antimicrobial agents, is one of the most important mechanisms [185-187]. Another factor contributing to high antimicrobial resistance in biofilm conditions are the bacterial cells of "persister" phenotype, found in the biofilm, which are characterized by a very slow growth. Once the action of the antibiotic agent is discontinued, microbial cells with this phenotype can reactivate and cause infection [186, 188]. The presence of "persister cells" is associated with the chronic course of some UTIs [187]. Furthermore, the over-expression of some efflux pumps under biofilm conditions [187, 189], as well as high levels of horizontal gene transfer due to over-population and close physical contact between bacterial cells [124, 190], also contribute significantly to *E. coli* antimicrobial resistance and to the spreading of genetic resistance and virulence determinants under biofilm conditions.

In conclusion, *E. coli* is a well-studied enteric organism with a potential to cause many different types of infectious syndromes, among which the UTIs are most common. Apart from being able to become resistant to routinely used and strategic antimicrobials, this organism is also capable of harboring a wide range of genes, associated with increased virulence. In combination with the high frequency and severity of *E. coli* infections, and the related mortality, this places *E. coli* among the bacterial pathogens of highest public health importance.

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IMMUNOBLOT REACTIVITIES OF DIFFERENT LYME DISEASE SPECIFIC ANTIGENS TO ANTI-BORRELIA BURGDORFERI IGG ANTIBODIES

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ABSTRACT

Background

Lyme disease is a vector-borne disease with increasing incidences in last years. It is the most common tick-borne infection in Bulgaria, caused by *Borrelia burgdorferi* sensu lato (*Bb*sl) complex after a bite from *Ixodes ricinus* tick. The aim of this study is to identify the most common antigens targeted by specific IgG antibodies produced in response to*Bb*sl infection in humans.

Material and methods

Serum samples (n=220), pre-tested as positive or borderline by ELISA for specific anti-*Borrelia* IgG antibodies, were tested with a commercially available confirmatory immunoblot kit.

Results

The most common specific anti-*Borrelia* IgG antibodies reacted against: p41 (97.1%), VlsE (average of 62.1% across the three variants: VlsE Bg, VlsE Bb and VlsE Ba), p83 (32.4%), p39 (30.4%) and OspC (22.6%) among the positively interpreted immunoblot tests. The weak positive bands among the positively interpreted blot tests were: OspC (17.7%), VlsE (average of 10.5% across the three variants), p21 (9.8%), p39 (8.8%), p83 (6.9%).

Conclusions

This study analysed the most common antigens targeted by specific IgG antibodies synthesised after *Bb*sl infection in humans from all 28 provinces of Bulgaria. It was probably cross-reactive antibodies that appeared in some cases with weak positive bands. The data obtained from the confirmed cases of Lyme disease, based on the different distribution of immunoblot bands allow for a better understanding the peculiarities of this disease. The use of new specific antigens for confirmatory tests based on selected conserved fragments within *Bb*sl leads to development of more sensitive and specific serological analyses.

Keywords: Lyme disease, immunoblot, antibodies

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INTRODUCTION

Lyme disease (LD) is recognized as the most common tick-borne infection in Europe, North America and Asia with increasing geographic distribution in association with global warming (1). It is a multisystem, chronic disease transmitted by infected *Ixodes* spp. ticks, and its aetiological agents are spirochetes of the *Borrelia burgdorferi* sensu lato complex (*Bb*sl). LD incidence has been ever-increasing in the last years and this can have significant consequences for public health.

The laboratory diagnosis of the disease mainly relies on serological tests for detection of specific IgM and IgG antibodies against *Bb*sl. There is a great diversity of *Bbsl* in Europe and most of the immunogenic proteins are heterogeneous, which is why the use of whole cell lysate with only one genospecies as antigen carries the risk of obtaining a false-negative result (2, 3, 4, 5, 6). On the other hand, *Bbsl* produces many proteins homologous among microorganisms, which may lead to false-positive results. The insufficient specificity of diagnostic tests for LD required the introduction of a standard two-tiered testing in 1995, and updated later, based on: an enzyme immunoassay (EIA), or an indirect immunofluorescent antibody assay (IFA) or chemiluminiscent assay; followed

by a second-tier orthogonal EIA (modified two-tiered testing, MTTT) or immunoblot assay confirmation of positive and equivocal results from first-tier level (2, 7, 8, 9, 10, 11).

The aim of the study is to identify the most common antigens targeted by specific IgG antibodies produced in response to *Bb*sl infection in humans.

MATERIAL AND METHODS

Serum samples (n=220), pre-tested as positive or borderline with ELISA for the presence of anti-*Borrelia*-specific IgG antibodies, were collected in December 2023, from all 28 provinces of Bulgaria. Adults over 18 years of age were included. No additional patient information was available.

The samples were tested using the immunoblot method with a commercially available kit: Anti-Borrelia EUROLINE-RN-AT (IgG), DN 2131-3201 G according to the manufacturer's instructions. Immunoblot strips included the following fourteen specific antigens: VlsE Ba, VlsE Bb, VlsE Bg, p18, p19, p20, p21, p41, p58, OspC (p25), p39, p83, Lipid Bb, and Lipid Ba. After completion of the test procedure, the stained test strips were evaluated with EUROLineScan Software v. 3.4.37 (YG 0006-0101, Euroimmun). The evaluation of the bands was as follows: positive if the intensity units were ≥19; ≥ 12 but < 19 were interpreted as borderline; and negative if the intensity units were <12, according to the set software of Anti-Borrelia EUROLINE-RN-AT (IgG), Euroimmun, Germany. A positive result was indicated by one of the following: one positive VlsE band; one borderline VlsE band and one positive non-VlsE band; two or more positive non-VlsE bands.

Ethical approval for this study was obtained from the institutional review board at the National Center of Infectious and Parasitic Diseases (NCIPD) (approval number 5/17.10.2023).

RESULTS

A total of 220 samples, positive/borderline in ELISA for specific anti-*Bb*sl IgG antibodies, were tested with a confirmatory immunoblot method. 102/220 (46.4%) of the samples were positive and 118/220 (53.6%) were negative for the presence of anti-*Borrelia* IgG according to the immunoblot test results. Analysis of the reactive immunoblot bands showed that the most common anti-*Borrelia* IgG antibodies targeted p41 (flagellin) in 201/220 (91.4%), followed by VlsE Bb in 72/220 (32.7%), VlsE Ba in 66/220 (30.0%) and VlsE Bg in 58/220 (26.0%) among all tested samples (Fig. 1).

A positive p83 band was observed in 35/220 samples (15.9%). The OspC and p39 bands were each

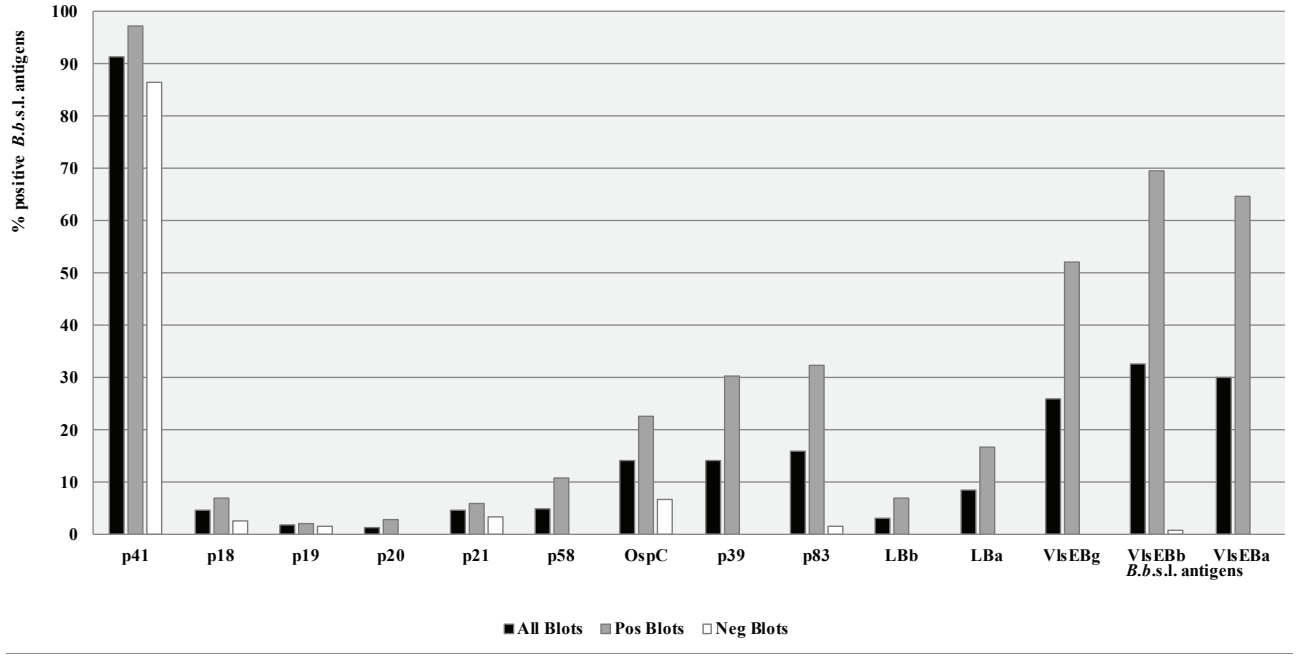

Figure 1. Antigens inducing positive IgG antibody response against of *B. burgdorferi* s.l.: frequence of detection in all blots, in blots interpreted as positive, in blots interpreted as negative

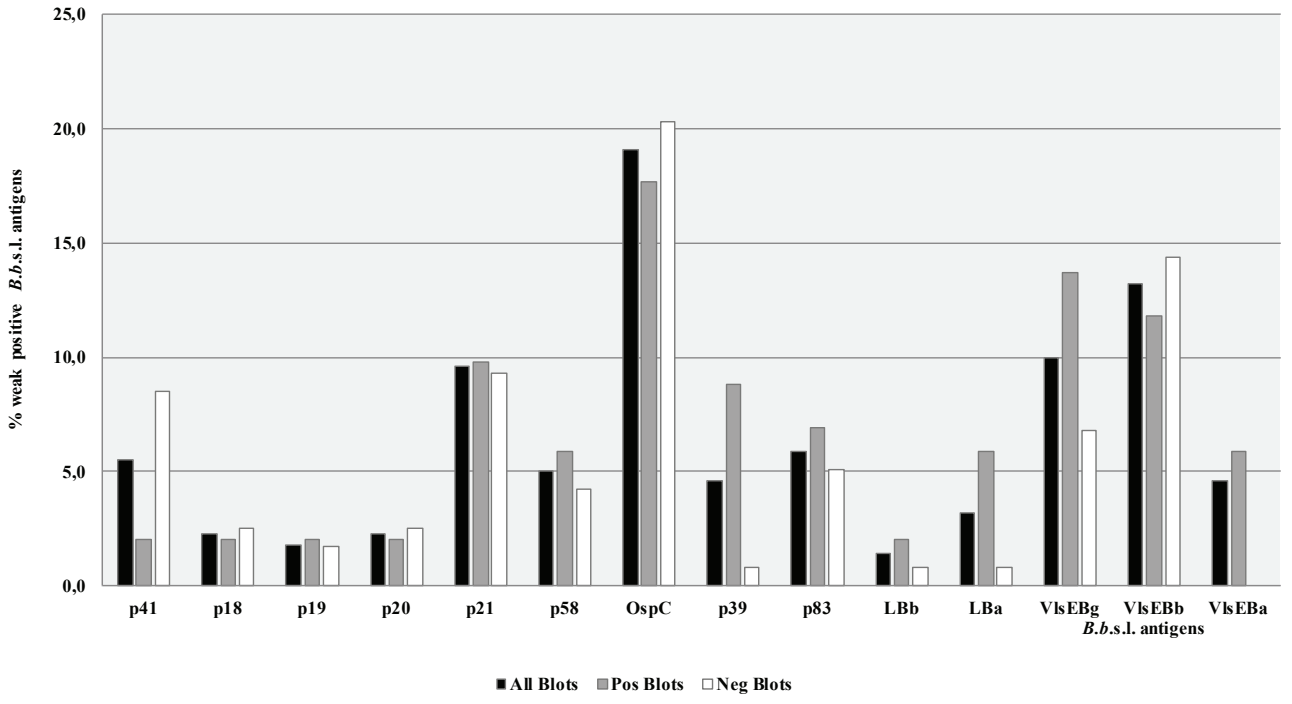

Figure 2. Antigens inducing a weak positive IgG antibody response against of *B. burgdorferi* s.l.: frequence of detection in all blots, in blots interpreted as positive, in blots interpreted as negative

detected in 31/220 samples (14.1%). A positive LBa band was identified in 19/220 samples (8.6%), while 11/220 (5.0%) had a positive p58 band. The p18 and p21 bands were each found in 10/220 samples (4.6%), and the LBb band was observed in 7/220 samples (3.2%). The least frequent were the p19 band in 4/220 samples (1.8%) and the p20 band in 3/220 samples (1.4%).

Among the positively interpreted blot tests, there were no differences with the combination of the reactive bands, compared to the set of reactive bands from all examined blot tests: 99/102 (97.1%) had a positive p41 band, followed by VlsE Bb - 71/102 (69.6%), VlsE Ba - 66/102 (64.7%), VlsE Bg - 53/102 (52.0%), p83 - 33/102 (32.4%), p39 - 31/102 (30.4%) (Fig. 1). OspC was positive in 23/102 (22.6%), LBa - in 17/102 (16.7%), and p58 - in 11/102 (10.8%). LBb and p18 were both detected in 7/102 (6.9%) and the least frequent were: p21 - in 6/102 (5.9%), p20 - in 3/102 (2.9%) and p19 - in 2/102 (2.0%).

Only seven positive bands were observed among the negatively interpreted blot tests: p41 - 102/118 (86.4%), OspC - 8/118 (6.8%), p21 - 4/118 (3.4%), p18 - 3/118 (2.5%), p19 and p83 - 2/118 (1.7%) (Fig. 1).

The most common weak positive bands among all tested samples were: OspC - 42/220 (19.1%), followed by: VlsE Bb - 29/220 (13.2%), VlsE Bg - 22/220 (10.0%), p21 - 21/220 (9.6%), p83 - 13/220 (5.9%), p41 - 12/220 (5.5%), p58 - 11/220 (5.0%), p39 and VlsE Ba - 10/220 (4.6%), LBa - 7/220 (3.2%), p18 and p20 - 5/220 (2.3%), p19 - 4/220 (1.8%) and LBb - 3/220 (1.4%) (Fig. 2).

The weak positive bands among the positively interpreted blot tests were as follows: OspC - 18/102 (17.7%), VlsE Bg - 14/102 (13.7%), VlsE Bb - 12/102 (11.8%), p21 - 10/102 (9.8%), p39 - 9/102 (8.8%), p83 - 7/102 (6.9%), p58, LBa and VlsE Ba - 6/102 (5.9%), p41, p18, p19, p20 and LBb - 2/102 (2.0%) (Fig. 2).

Finally, the weak positive bands among the negatively interpreted blot tests were: OspC - 24/118 (20.3%), VlsE Bb - 17/118 (14.4%), p21 - 11/118 (9.3%), p41 - 10/118 (8.5%), VlsE Bg - 8/118 (6.8%), p83 - 6/118 (5.1%), p58 - 5/118 (4.2%), p18 and p20 - 3/118 (2.5%), p19 - 2/118 (1.7%), p39, LBb and LBa - 1/118 (0.8%) (Fig. 2).

DISCUSSION

There is not yet much data related to the most common reactive immunoblot bands corresponding to antigens targeted by *Bb*sl - specific IgG in infected humans in Bulgaria. Immunoblots are not routine diagnostic tests as they require specialized laboratory

equipment. Immunoblot tests are currently available in two formats: western blot and line blot. Western blot involves electrophoretically separated proteins of *Bb*sl (typically whole-cell lysate). When whole cell lysate is used, similarly sized proteins move in parallel, which can reduce specificity. Therefore, purified recombinant proteins are currently used for the second tier of LD assays (9, 10). By utilising purified recombinant antigens, line blots reduce the potential for cross-reactions due to co-migrating proteins.

Despite the efforts in this direction, the serodiagnosis of LD is still challenging. Some of the antigens currently used for diagnosis of this disease are involved in cross-reactions. In general, this is due to the complex antigenic structure of the *Bb*sl. There is a significant heterogeneity among the *Bb*sl species prevalent in Europe (*B. afzelii*, *B. garinii*, and *B. burgdorferi*), particularly regarding the most relevant for serodiagnosis antigens (3, 11). Cross-reactivity can occur with a single antigen like flagellin (12) or with entire pathogens, such as *T. pallidum* (13, 14), *Yersinia* spp. (15, 16, 17), cytomegalovirus (CMV), Epstein-Barr virus (EBV) (2). Knowledge of these antigens - their potential for cross-reactions and the use of purified recombinant or chimeric proteins, is crucial for the correct diagnosis and treatment of LD.

*Bb*sl-specific IgM can be detected two to four weeks after the appearance of erythema migrans - a pathognomonic symptom observed during the early stage of disease, or a longer period after the tick bite in cases without erythema, while IgG rise four to six weeks after the onset of infection (18). In many cases, IgG levels peak in six months and may remain elevated for months to years. Eg, 62% of patients with lyme arthritis during the first diagnosic testing showed a presence of specific IgG antibodies even 10 years later (19).

The sensitivity of serological tests increases during the later stages of the disease, because the immune response matures with the continuation of the infection (3, 20). An extensive IgG response against multiple antigens is observed, with reactivity against DbpA (p18), OspC (p21 - p25), OspD (p28), OspA (p30), VlsE, BmpA (p39), FlaB (p41), p45, p58, p66 and p93 (3). IgG antibodies directed against specific OspC, VlsE and p41 antigens are commonly detected early in the course of infection (3). In the second stage, IgG antibodies are directed against highly specific p39 and p58 membrane antigens. Other antigens are added in the late stages of the disease: late-phase marker p83 (major extracellular protein) (21) and immunogenic lipids. The 39 kDa and 41 kDa proteins were found to be some of the most significant markers for LD (21). Some manufacturers offer kits containing engineered immunoreactive antigens designed to increase the test specificity. Such antigens (p18, p19, p20, p21 and p58), are included in the Euroimmun strips used in our study.

Several diseases can be misdiagnosed as LD due to cross-reactivity, and these are most often syphilis, relapsing fever, EBV and CMV infections (2). Patients with elevated rheumatoid factor are also often misdiagnosed with LD (22 - 26). Studies showed that rheumatoid factor is reactive with many *Bb*sl antigens: BmpA, VlsE, OspC, OppA2, LA7 (p22), BBA64 (p35), p37 (25 - 28).

The flagellar protein FlaB (p41, 41 kDa), localized on Bbsl chromosome, is one of the first proteins targeted by antibodies after infection with *Bb*sl (29). It builds the flagellum core and is highly conserved among a large number of microorganisms (2). P41 was the most common positive reactive band against *Borrelia*-specific IgG among all blot tests in our study: 97.1% of the positively interpreted, and 86.4% of the negatively interpreted tests. P41 was very rare among the weak positive reactive bands both in the positively and negatively interpreted blot tests. Bands with intensity under 19 units are not considered signifcant in the IgG immunoblot (29). According to some studies, p41 has about 40% sequence identity with the *Treponema* spp. flagellin, the cross-reactive epitopes being located between positions 131 - 234 (2). Low levels of IgG against flagellin are present in sera from persons with infections other than LD: syphilis, human granulocytic anaplasmosis (HGA), yersiniosis, EBV and CMV. This suggests that in some cases in our study the appearance of p41 band could be due to cross-reacting antibodies (2, 29).

Outer surface-exposed protein C (OspC, 21 - 25 kDa) is an immunodominant lipoprotein antigen for *Bbsl* (30). It is necessary for *Bbsl* transmission to mammals as it binds plasminogen, as well as tick saliva protein Salp 15 (2). However, antibodies cross-reacting with OspC can be observed in some viral infections. The

prevalences of EBV and CMV among the adult human population are very high - about 90% and 83%, respectively (22, 23, 31). Studies from Poland have established that infection with these two viruses generated antibodies that cross-reacted with p41 and OspC in immunoblots (22, 32). Generally, it was observed that serum samples from patients with EBV infection cross-reacted mainly with the OspC protein of *Bbs*l, while anti-CMV IgG cross-reacted with a wider variety of *Bbs*l antigens, such as OspC, FlaB, BmpA and VlsE (2). Antigenic similarity was found between the OspC and FlaB antigens of *Bbs*l and YopD of *Yersinia* spp. and two-way cross-reactivity was presented (15, 16). It was also shown that IgG from patients with HGA cross-reacted with *Bbs*l proteins, such as OspC, p37, FlaB, OspA and OspF (25). Cross-reactions with *Bbs*l antigens, like OspC, p37, BmpA and OspF were also observed in patients with oral infections (2). The locus of OspC is probably the most genetically diverse locus in all *Bb* genospecies (33). In our study, OspC was positive in 22.6% of the positively interpreted blot tests and in 6.8% of the negatively interpreted blot tests. It was the most common weak reactive band among both positive and negative blot tests, 17.7% and 20.3% respectively. It is considered as significant for the IgG response when the intensity units are greater than 19 (29). It is possible that in our study, the observed weak positive OspC bands could, in some cases, be due to cross-reactive antibodies.

Another immunodominant antigen for *Bbsl* is the variable major protein-like sequence expressed (VlsE, 34 - 35 kDa), which is a surface-exposed outer membrane lipoprotein encoded by a gene located on the linear plasmid lp28-1 (30). VlsE helps the spirochete evade the host immune response by undergoing antigenic variations. The expressed region of the VlsE gene contains six variable regions that recombine with multiple unexpressed cassette sequences located on the same linear plasmid. This results in a high degree of antigenic variation among *Bbs*l clones infecting a host (30). VlsE contains a highly conserved IR6 region that is used in lyme immunoblots as a target antigen (2). Strong immunoblot reactivity to a recombinant VlsE protein was obtained with LD patients' sera, but not with sera from patients with syphilis and autoimmune conditions (34). Another study provided strong evidence for the key role of the vls locus for *Bbs*l persistence in the human host despite antibiotic treatment (35). Other studies have shown the importance of VlsE antigenic variation for persistent infection of natural reservoir hosts (36) and it was observed that the VlsE recombination system operates only in mammal models of infection, but not in culture or in the tick vector (33). Currently, multiple purified recombinant proteins are used for European LD immunoblot tests, as in Europe LD can be caused by different *Bbsl* species - *Borrelia afzelii, Borrelia garinii,* and *Borrelia burgdorferi* sensu stricto (9, 37). Recombinant VlsE proteins were also used in our study - VlsE Ba (*Borrelia afzelii)*, VlsE Bb (*Borrelia burgdorferi* sensu stricto) and VlsE Bg (*Borrelia garinii*). Reactive VlsE Bb bands were present in 69.6% of positive immunoblot tests, VlsE Ba - in 64.7% and VlsE Bg - in 52.0%. This shows their importance as highly specific diagnostic antigens.

Another immunogenic antigen which may induce antibody response is p18 (Dbp A/B) surface protein. P18 is an adhesin encoded by a gene located on the linear plasmid lp54. It binds to decorin and is involved in the spread of *Bbsl* in mammals (2). P18 and VlsE are the most widely used proteins in commercial diagnostic tests, mainly for detection of IgG antibodies (2). Because of the high variability of DbpA antigens within the *Bbsl*, for diagnostic purposes they are added in different variants from different genospecies. In our study, the p18 band was positive in 4.6% of all tested samples, in 6.9% of all positive samples and in 2.5% of all negative samples.

The Basic membrane protein A (BmpA, p39) is one of the most commonly applied specific markers for LD, both on IgM and IgG immunoblot strips (29). Its gene is localised on the borrelial chromosome, it binds to mammalian lamin and is highly conserved. BmpA plays a role in the development of lyme arthritis (2). Studies have shown that BmpA cross-reacts with antibodies against syphilis, relapsing fever, CMV and parvovirus B19, and is recognized by rheumatoid factor (2). In our study, the p39 band was positive in 14.1% of all tested samples and in 30.4% of all positive samples. None of the negative samples had a reactive p39 band.

In a study on the seroprevalence of LD in Eastern Slovakia in 2018, the most commonly found antibodies were against VlsE (80.2%), p41 (66.7%), p18

(56.3%), p58 (31.3%), and p39 (30.2%) (38). According to our study, the most common specific anti-*Borrelia* IgG antibodies were established against p41 (97.1%), VlsE (average of 62.1%), p83 (32.4%), p39 (30.4%) and OspC (22.6%). In comparison, p58 and p18 were reactive in 10.8% and 6.9% of positive blots, respectively. Wide variations in different combinations of reactive proteins were observed, which could be due to multiple factors specific to host, pathogen and environment.

CONCLUSIONS

This study analysed the most common antigens targeted by specific IgG antibodies synthesized as a result of *Bb*sl infection in humans from all 28 provinces of Bulgaria. The immunoblot can provide more detailed information on antibody response patterns to different antigens of *Bb*sl and adds to the true picture of the disease in patients with Lyme borreliosis. The immunoblot might also be used to identify the immunodominant antigen, part of it and to apply chimeric molecules for the development of more sensitive and specific serological analyses. The design of new specific antigens from selected conserved fragments within *Bb*sl eliminates the problem of cross-reactivity with commonly found antigens of other pathogens. It was shown that the precise selection of protein fragments from VlsE and FlaB increased the specificity without reducing the sensitivity of the test (39, 40). The assessment of the manifested symptoms of LD and the detection of highly specific antibodies, based on purified recombinant or chimeric proteins, without cross-reactive epitopes is very important for the correct diagnosis and treatment of this disease. The study of data related to the incidence and seroprevalence of LD, together with the peculiarities of the immune response allow a better understanding of the pathogenesis of this disease and the development of key strategies for protecting human health.

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COMPARISON OF AUTOMATED AND MANUAL DNA EXTRACTION METHODS IN ISOLATING *ENTEROBIUS VERMICULARIS* DNA.

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ABSTRACT

The aim of the present study was to compare automated and manual DNA extraction method from *Enterobius vermicularis* (human pinworm) eggs.

Materials and methods. Scotch tape samples positive for *Enterobius vermicularis* eggs via microscopic examination, collected from 16 patients aged between 2 and 12 years, were included in the study. Using manual spin-column-based and automated procedures, the isolation of parasite DNA was carried out under identical conditions including: number of eggs, type and quantity of storage solution before the extraction process, and sample pretreatment (number of freeze-thaw cycles). The concentration and purity of the isolated DNA with both methods was measured with a spectrophotometer. Nested PCR was applied to amplify a segment of the mitochondrial gene encoding for the cytochrome c oxidase subunit 1 (*cox*1) of *E. vermicularis*, and the products were separated by agarose gel electrophoresis.

Results. Higher concentrations of DNA with excellent purity (A260/A280 – 1.0-3.0 were obtained with the automated extraction method. The DNA extracts obtained with the Nucleic Acid Automated Extraction System contained fewer contaminants than those isolated by the manual procedure as detected by analyzing the A260/A230 and A260/A280 ratios. As compared to the manual extraction method, a greater sensitivity in percent yield of positive samples was demonstrated by the automated method. All samples (n=16) subjected to the automated isolation were PCR positive and products with the expected size of 379 bp were detected in 100% of the samples vs. 2 false negatives (12.5%) with the manual extraction procedure.

Conclusion. Data from the comparative study of the two methods for DNA extraction from *E. vermicularis* eggs showed that the automated extraction procedure provides an excellent quality and yield of isolated DNA samples as compared to the manual processing. The extracted DNA has a lower content of organic or carbohydrate contaminants, including proteins, which is a prerequisite for successful conduct of subsequent molecular genetic analyses.

Keywords. *Enterobius vermicularis*, DNA extraction, PCR.

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INTRODUCTION

Enterobiasis is one of the globally prevalent intestinal helminth infections in humans, mainly affecting children. Its causative agent is *Enterobius vermicularis*, and humans are its only host. Poor hygiene habits including nail biting, finger sucking, as well as not washing hands after using the toilet contribute to the cosmopolitan spread of the parasite.

Infection occurs through the ingestion of mature eggs, from which a larva is released following the small intestine entry. There it reaches sexual maturity and migrates to the colon. The parasite lives in the lower half of the small intestine and the cecum. After fertilization, male worms die and females migrate to the rectum. At night, they actively come out through the anus to deposit their eggs in the perianal folds. Under suitable conditions, including moisture and temperature of 37°C, the eggs mature within 5-6 hours and are already infective. The female can lay up to 15,000 eggs per day, and the lifespan of *E.*

vermicularis is about 3-4 weeks (1). The diagnosis of *E. vermicularis* infection is made by *pinworm* eggs demonstration after microscopic examination of a scotch tape obtained from the patient's perianal skin folds or, *less commonly*, when eggs and/or female pinworms are detected in stool samples (2,3).

Molecular tools are widely used to track pathogen transmission, to assess the epidemiology in the general population and in specific subgroups, to distinguish persistent from recurrent infections. PCR-based analyses are employed to detect and identify parasitic organisms, including pinworms. They can help to determine the pathogenetic role of the parasite in some gastrointestinal disorders. The application of PCR for pinworm detection in human stool samples has been reported (5,7,8). These publications were focused on the diagnostic application of the PCR method in patients with suspected *E. vermicularis* infection.

Mitochondrial (mt) DNA is a commonly used genetic marker of molecular and phylogenetic studies (9,10,11). The complete mtDNA of *E. vermicularis* has been sequenced and published by Kang, S et al.(12) Three distinct clusters (subtypes) A, B and C in *E. vermicularis* isolates from samples of captive chimpanzees and humans from Asian and European countries, based on phylogenetic studies using the gene encoding mitochondrial cytochrome c oxidase subunit 1 (*cox*1) have been identified (5, 7, 13). A and B genotypes were isolated from humans (14) and captive chimpanzees (*Pan troglodytes*), while genotype C has only been identified in chimpanzees (15).

The process of DNA extraction, which constitutes the initial stage of the PCR procedure, is of paramount significance. The quality and integrity of the isolated materials are pivotal in determining the outcome of the subsequent PCR experiment.

The objective of the present study is to compare the efficacy of automated and manual methodologies for the extraction of deoxyribonucleic acid (DNA) from Enterobius vermicularis (human sedge) eggs.

MATERIALS AND METHODS

Patients infected with E. vermicularis

Sixteen patients with microscopically proven *E. vermicularis* infection were included in the study. All patients were children, 9 (56.3%) girls and 7 (43.7%) boys, aged 2 – 12 (age distribution: 4 in the group of 0-4; 10 children in the group of 5-9; 2 in the group of 10-14 years). In the cohort of patients participating in the study, a triple testing of samples was performed.

Specimen preparation

Samples were obtained from the patient's perianal region using transparent tape and subsequently examined under a light microscope at 100x and 400x magnification. The egg-positive samples (slides) were stored at 4°C until DNA extraction. A sample was considered positive if at least one *E. vermicularis* egg was found. For collection of pinworm eggs from the adhesive tape surface, the slide was placed in a petri dish and soaked in sterile saline solution. The eggs were then recovered from the tape surface by scraping with a disposable scalpel under a light microscope and transferred to 200 µL of sterile saline solution into a clean 1.5 mL *microcentrifuge tube*. Distilled water was used to avoid inhibiting factors in the subsequent molecular processing of the samples. Samples were subjected to three freeze-thaw cycles in order to destroy the egg shell. Five eggs recovered from each slide were aliquoted into two 1.5 mL microcentrifuge tubes for DNA isolation using the two methods (manual and automated) and stored at -20°C until use.

Manual spin-column-based extraction of DNA

Manual DNA extraction was conducted using the PureLink Genomic DNA Mini Kit (Invitrogen, Thermo Fisher Scientific, North America) according to the manufacturer's guidelines. Two hundred microliters (µl) of ATL Digestion buffer and 30 µl of proteinase K (20 mg/mL) were added to a microcentrifuge tube containing the eggs. The DNA was then eluted in 100 µl of elution buffer and stored at -20°C until needed.

Automated extraction of DNA

DNA extraction was performed using the Techstar Nucleic Acid Automated Extraction System (model YC702, China). Parasite DNA was isolated using the Nucleic Acid (DNA/RNA) Extraction Kit from Techstar, following the manufacturer's instructions. The kit includes two types of wash buffer, proteinase K (30 µl, 20 mg/mL), and elution buffer (100 µl). The volumes of the reagents used were identical to those in the manual extraction method. The extracted DNA was stored at 4°C for short-term use (2-3 days) and at -20°C for long-term storage.

DNA yield and purity.

DNA was extracted from five *E. vermicularis* eggs per sample and quantified utilizing a Biochrom microvolume spectrophotometer (BioDrop µLite+, Harvard Bioscience Co. Ltd.; Shanghai, China). Initially, the elution buffers provided with each extraction kit were employed to blank the spectrophotometer prior to the measurement process. Subsequently, the absorbance of the DNA samples was recorded. The spectrophotometer computes the concentration of DNA for each sample based on the absorbance measured at 260 nm (A260), which is then multiplied by a conversion factor of 50 µg/ml per 1 A260 unit for double-stranded DNA. To assess the purity of the DNA extracts, absorbance was measured at 280 nm (A280) and 230 nm (A230). The average ratios of A260 to A280 (A260/A280) and A260 to A230 (A260/A230) were calculated for all samples. Samples exhibiting A260/A280 ratios between 1.8 and 2.0 are regarded as free from significant contamination (6,16).

***E. vermicularis*-specific PCR**

To compare the performance of different extraction methods, the PCR amplification success with *E. vermicularis*-specific primers was used. Nested PCR targeting a 379-bp segment of mitochondrial *cox*1 gene of *E. vermicularis* was applied according to Piperaki et al. (2011) (4), using two sets of primers. Primer sequences of the outer primer pair are as follows:

EVM1 5'-TTTTTGGTCATCCTGAGGTTTATATTC-3',

EVM2 5'-CCACATTATCCAAAATAGGATTAGCC-3'.

Primers for the inner amplification reaction have the following sequences:

EVIF 5'-TTGGTCATCCTGAGGTTTATATTC-3',

EVIR 5'-TCCAAAATAGGATTAGCCAACA-3'.

The first amplification reaction was performed in a 25-µl volume, using AmpliTaq Gold 360 Master Mix (AppliedBiosystems by Thermo Fisher Scientific) containing enhancer, 0.2 mM of each primer, and 3 µl DNA. The thermal cycling protocols were as follows: an initial denaturation at 95°C/ 10 minutes, followed by 45 cycles of 95°C/1 minute, 57°C/1 minute, and 72°C/10 minute; final extension step at 72°C for 10 minutes.

Three µl of the first PCR were used as a template for the inner amplification reaction (25-µl volume). The conditions of this second reaction differed from the first one in the annealing temperature (53°C), and the number of cycles (30). One positive (*E. vermicularis* DNA from adult pinworms) and one negative (PCR water instead of template DNA) control were included in all reactions to check for amplification success and DNA carry-over contamination, respectively. PCR amplifications were performed in a GeneExplorer Thermal Cycler instrument (Hangzhou Bioer Technology Co., Ltd.). PCR products were separated by agarose gel electrophoresis on 3% gel. A 50 bp marker (GeneRuler™ 50bp DNA Ladder, Thermo Scientific, Lithuania) was used to determine the sizes of the resulting products. Ten µl of the products from the second PCR reaction were mixed with 2 µl of fluorescent dye (peqGREEN DNA/RNA dye, VWR International GmbH, Germany) and loaded on the gel, visualized on a UV transilluminator, and photographed using SYNGENE gel documentation system (GelVue Model No. GVM20, Synoptics Ltd, UK).

RESULTS

Specimens

Sixteen transparent Scotch tape slides that tested positive for eggs were included in the study. Each slide was examined under direct light microscopy, confirming the presence of at least one egg, which validated their positive status. The study aimed to compare the PCR results of DNA samples extracted using manual and automated methods. Therefore, the samples from the 16 patients were prepared in duplicates, ensuring an identical number of parasite eggs in each sample. All manipulations during the pretreatment phase, including an equal number of freeze-thaw cycles, were carried out under consistent conditions.

Comparison of DNA Extraction methods

Two methods for isolation of genomic DNA from *E. vermicularis* eggs were applied. The comparison between the procedures was performed using a PCR assay developed by Piperaki et al. (2011), which optimized for our conditions (5). *E. vermicularis* DNA was detected in all 16 samples (n=16, 100%) processed by automated extraction method and the presence of a 379-bp band was determined for all examined samples. Two specimens (12.5%) obtained by manual extraction procedure were read as false negatives by PCR. This may be due to loss of sensitivity, associated with DNA yield and purity. DNA samples isolated by

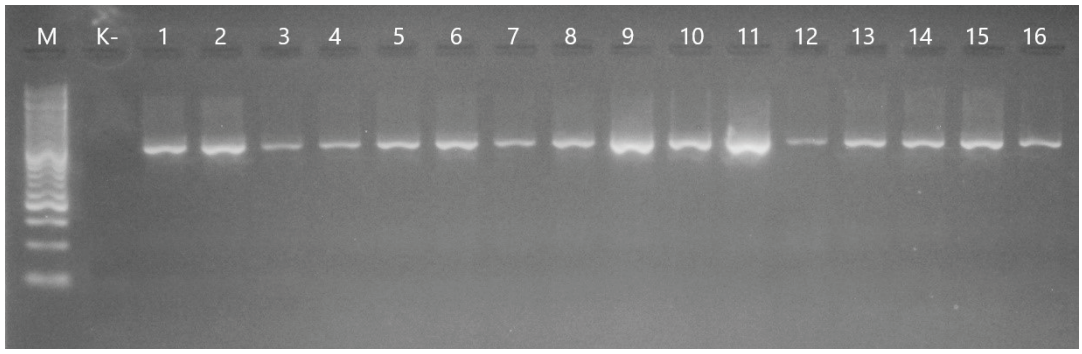

Fig. 1. Amplified PCR products analyzed by 3% agarose gel electrophoresis - automatic isolation. Starts: 1-DNA marker, bands across 50bp, 2-negative control, 1-16 *E. vermicularis* positive samples. Presence of band ~379 bp

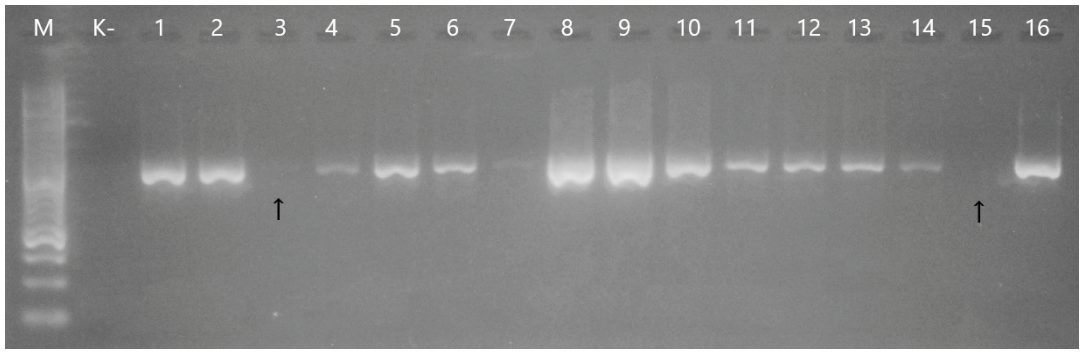

Fig. 2. Amplified PCR products analyzed by 3% agarose gel electrophoresis – manual extraction. Starts: 1-DNA marker, bands across 50bp, 2-negative control, 1-16- samples – 3 and 15 without presence of band ~379 bp

the automated procedure showed highest concentrations: 6 μg/ml and excellent purity (A260/A280 – 1.0 – 3.0). They contained less contaminants, compared to the manual extraction (fig.1 and fig.2). The manual extraction procedure using the PureLink Genomic DNA Mini Kit yielded DNA with ratios ranging from (A260/A280 – 0.6 – 2.0). The DNA concentration values were in the range of 1 – 5 μg/ml.

DISCUSSION

This study was conducted to compare the performance of DNA extraction methods (automated and manual procedures) with regard to DNA yield and purity by using *E. vermicularis*-specific PCR. The sensitivity of PCR assays can be influenced by different factors, such as DNA yield, DNA purity from PCR inhibitors, and DNA damage. The selection of the appropriate DNA extraction method is essential for the detection of the target DNA region of the studied pathogen. The adequate quality and purity of the isolated DNA are essential for its long-term storage and to guarantee good-quality results in subsequent analyses. We tested the effects of two different procedures used for isolation of genomic DNA from pinworm eggs on the sensitivity of the detection of a segment of mitochondrial *cox*1 gene of *E. vermicularis*. Using *E. vermicularis*-specific primers, the automated extraction method resulted in higher rate of amplifiable *E. vermicularis* DNA, which is expressed as 100% PCR amplification success.

A good correlation between the manual and automated extraction methods has been reported (17,18,19). Good average values of DNA concentration and purity were obtained by Utaminingsih (20) using the column-based method for DNA isolation and the extracted DNAs were successfully used as templates in their real-time PCR analysis.

Contamination of the samples is a factor responsible not only for the low quality of the isolated product, but for an insufficient yield of DNA (that is needed for the subsequent steps in genotyping) as well. The literature review showed that the automated instruments have been used for the DNA isolation for nearly two decades (21, 22, 23, 24). DNA extraction methods that utilize an automated procedure for DNA extraction were reported as effective in

Table. 1 Purity and concentracion of automatic and manual extraction

Sample / yields and purity	Automatic extraction		Manual extraction	
	A260/A280	concentration	A260/A280	concentration
1	2	6 μg/ml	0,6	4 μg/ml
2	3	6 μg/ml	0,6	3 μg/ml
3	2	6 μg/ml	0,67	2 μg/ml
4	1,25	5 μg/ml	0,67	4 μg/ml
5	1,8	5 μg/ml	0,67	4 μg/ml
6	1	4 μg/ml	0,7	3 μg/ml
7	2	5 μg/ml	2	3 μg/ml
8	2	5 μg/ml	2	5 μg/ml
9	2,5	5 μg/ml	1,5	5 μg/ml
10	1,5	4 μg/ml	0,9	3 μg/ml
11	3	6 μg/ml	0,63	5 μg/ml
12	1,5	4 μg/ml	0,83	3 μg/ml
13	1,8	5 μg/ml	0,63	4 μg/ml
14	2	5 μg/ml	0,83	3 μg/ml
15	2,23	5 μg/ml	0,6	2 μg/ml
16	2,2	5 μg/ml	1	4 μg/ml

removing PCR inhibitors (25). Compared to the manual extraction method, a greater sensitivity in terms of positive samples yield was demonstrated by the automated method (26, 27). The results of the present study support the findings of these authors. The automated DNA extraction method that we applied yielded DNA with higher purity than the manual extraction. The automated extraction system reduced contamination by using a unidirectional autosampler, UV lamps for subsequent sterilization, leading to DNA yields in required amounts for performing further analyses. Based on the observations on the sensitivity of PCR analyses in the current study we considered that the automated isolation of *E. vermicularis* DNA from the parasite eggs has some advantages over the manual method. It is faster as it provides the opportunity to process a larger number of samples in a shorter time. The products obtained contained sufficiently low amounts of impurities to be classified as pure products based on the A260/280 ratio and to be used for subsequent molecular approaches, including PCR assays and sequencing.

Considering the fact that all 16 samples were subjected to the same pretreatment conditions and procedures before applying the manual and automated DNA extraction methods from parasite eggs, the following reasons may contribute to the negative PCR results obtained for 2 of the samples: a) insufficient purification of organic contaminants such as ethanol; b) egg shell proteins that have failed to break down further; c) contamination of the columns used for isolation. We believe that the automated washing and overall processing lead to the more efficient contaminant elimination.

Genetic methods enhance diagnostic accuracy by facilitating the identification of diverse genotypes. This, in turn, enables the determination of the geographical distribution of the parasite using molecular epidemiological approaches.

CONCLUSION

The results from our comparative study of two DNA extraction methods from pinworm eggs of *E. vermicularis* showed that the automated extraction procedures provided excellent quality and yield of isolated DNA samples as compared to manual extraction. The DNA extracts had a lower content of organic carbohydrate and protein contaminants, which is a prerequisite for the successful conduct of subsequent molecular genetic analyses. The automated method was more time-efficient when processing multiple samples, and demonstrated a higher sensitivity. Therefore, the automated DNA extraction method provides a favorable option for

high-throughput clinical laboratories.

The Techstar Nucleic Acid Automated Extraction System used in our study is capable of automatically processing 1-32 samples simultaneously. It provides robust and reliable results in obtaining genomic DNA from samples containing pinworm eggs of *E. vermicularis* for downstream applications such as PCR and sequencing. The extracted DNA was free of PCR inhibitors as determined by the positive PCR results.

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A RARE CASE OF LABORATORY-CONFIRMED COCCIDIOIDOMYCOSIS IN BULGARIA, OCCURRING AFTER A 20-YEAR INTERVAL

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ABSTRACT

Coccidioidomycosis is a systemic fungal infection with no local distribution in Europe , associated with travelling to endemic regions of the world. The disease is defined as local but of global importance due to the increasing number of travellers to endemic areas.

We present a rare case of coccidioidomycosis in a 52-year-old woman living in the state of Arizona, USA. The patient had no disease symptoms, but computed axial tomography performed at the annual follow-up showed a small dense mass in the right lung. Positron emission tomography was also performed for suspicion of neoplasia, without conclusive evidence of malignancy. On her annual holiday in Bulgaria, the woman decided to consult a microbiologist at the National Centre of Infectious and Parasitic Diseases in Sofia, based on her information about the disease and the regions at risk of infection. The patient was referred to a consultation with a pulmonologist. As recommended by the pulmonologist, the lung nodule was surgically removed and subsequent histological and microbiological studies (Sabouraud agar medium culture) confirmed the diagnosis of coccidioidomycosis.

This is only the second case of this systemic mycosis registered in Bulgaria, showing that the diagnosis is difficult due to the lack of specific symptoms. A multidisciplinary approach is essential for the rapid diagnosis and timely treatment, which in turn is a prerequisite for a favorable outcome of the disease.

Keywords: coccidioidomycosis; endemic mycoses; case report

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INTRODUCTION

Coccidioidomycosis is caused by fungi of the genus *Coccidioides*. The latter contains two species, *Coccidioides immitis* and *Coccidioides posadasii*. also known as 'endemic mycoses' Together with *Histoplasma* and *Blastomyces*, *Coccidioides* only occur in certain geographical regions [1]. Coccidioides are 'dimorphic fungi', meaning they can grow in the human body like yeast, but in the environment (soil) they grow like moulds and form spores called arthroconidia. When the integrity of the soil is disturbed (agriculture, construction, wind, etc.), the spores become airborne and can be inhaled into human lungs. This can cause coccidioidomycosis, known as "valley fever". This form is often mild and asymptomatic. One to 3 weeks after the exposure, flu-like symptoms may occur, such as fever, weight loss, cough, fatigue, headache, chest pain, and myalgia [2, 3].

Coccidioidomycosis is not transmitted from person to person [4]. The asymptomatic forms can be detected during routine chest imaging. Pulmonary nodules are seen that can persist for years and be metabolically active, making them difficult to differentiate from malignant nodules [1].

The severity of the disease varies depending on various factors (immune deficiency, transplantation, comorbidities, pregnancy, diabetes, age, etc.). Infection can progress from community-acquired pneumonia to disseminated form spreading throughout the body (skin, bones, liver, brain, heart, meninges). One of the most aggressive forms is the cutaneous manifestation with ulcers, skin lesions and abscesses (erythema nodosum and erythema multiforme). Mediastinal fibrosis is rare and is associated with hemoptysis. Most people recover from pneumonia without complications. A small percentage may develop lung cavities that resolve spontaneously or are surgically removed. Prolonged antifungal therapy is

needed because of the risk of relapse [1, 3, 5].

Coccidioides inhabit soils with a limited geographical distribution. In the USA, the endemic regions are California, Arizona, New Mexico, Utah and Texas. The endemic region for coccidioidomycosis extends south into the deserts of Mexico and parts of Central and South America [1]. The disease is not endemic in Bulgaria, and isolated imported cases acquired after travelling to endemic areas have been reported [5].

By presenting a clinical case of an extremely rare infection in Bulgaria, we would like to point out that the appearance of imported cases is not excluded, and knowledge of diagnostic algorithms and treatment can be essential for a favorable outcome of the disease.

Case presentation

We describe a clinical case from 2024 of a 52-year-old woman who has lived for 10 years in the state of Arizona, North America, known for its mostly dry climate. She worked in the field of advertising, has been an active smoker for 30 years, and did not report any allergies, alcohol consumption, elevated blood glucose level, or concomitant diseases. In addition, she was living in a house with a flower garden, where she often cultivated flowers.

A prophylactic imaging study conducted in late 2023 (computed tomography scan of the lung) revealed the presence of a pulmonary nodule (8x11 mm) situated in the superior lobe of the right lung. The oval lesion had a small excentric cavitation. There were no other focal or infiltrative changes, lymphadenomegaly, or evidence of pleural effusion. Follow-up of the nodule in three to six months was recommended.

A follow-up CT scan six-months later revealed that the lesion had slightly increased in size (12x12 mm) while maintaining the same characteristics. The nodule was characterized by clearly demarcated borders, with no evidence of pleural or pericardial effusion, and without compression or dislocation of trachea and main bronchi (Fig. 1).

To elucidate the etiology of the lung formation, a positron emission tomography (PET-CT) scan was performed, which revealed no evidence of enlarged and metabolically active cervical, axillary, mediastinal and inguinal lymph nodes. No evidence of pleural or pericardial effusion was observed, and no patholog-

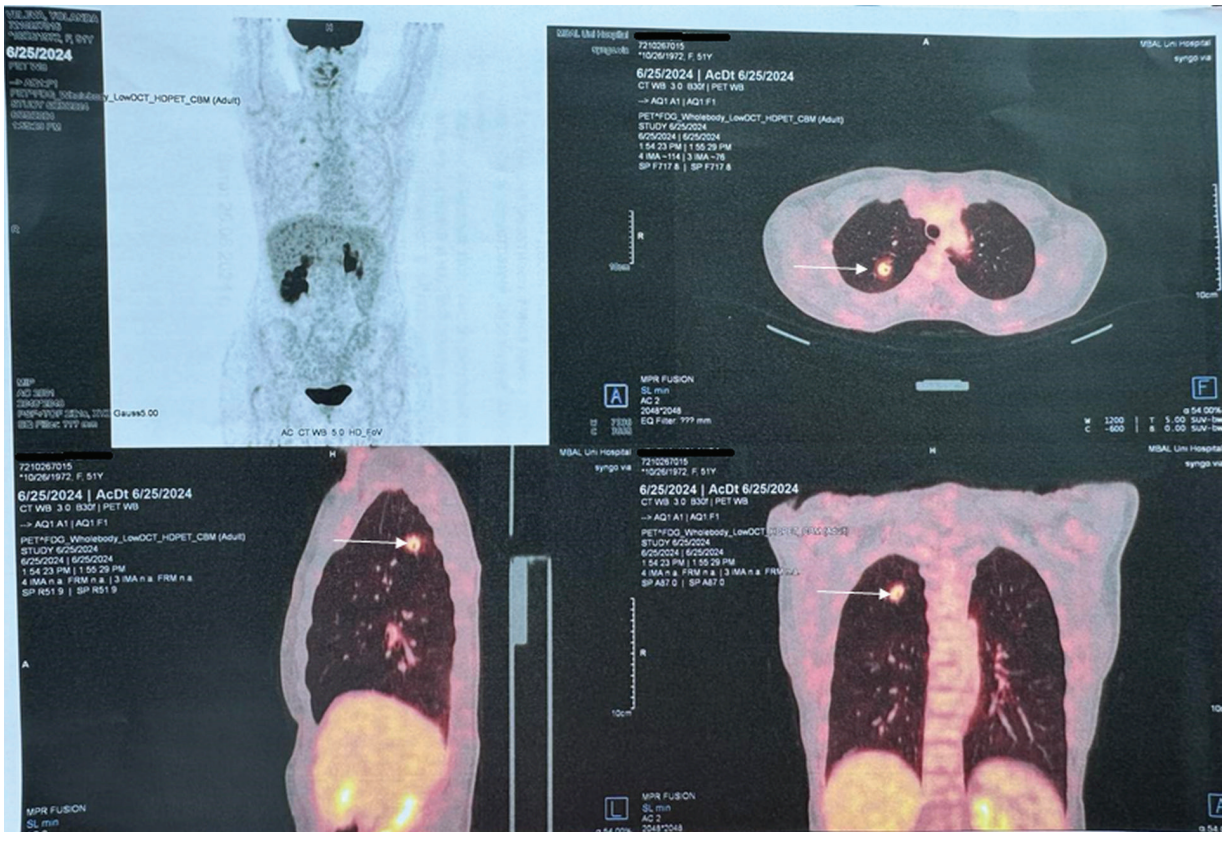

Fig. 1 Axial CT image showing an oval lesion with small eccentric cavitation in different sections (white arrow).

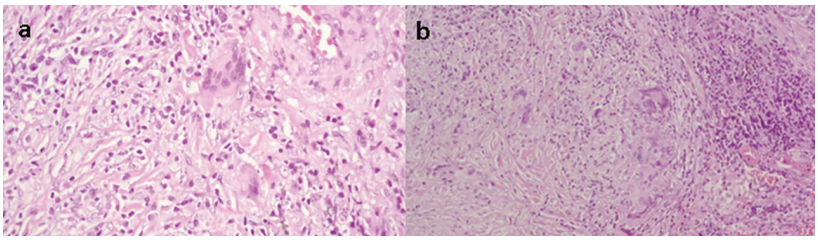

Fig. 2 (a, b) Histological slides of a surgically removed lung nodule showing a caseous granuloma with a concentration of inflammatory cells.

ical foci were identified in the abdomen and pelvis minor. In conclusion, the described lesion could not be definitively related to a primary neoplastic process, and there was no evidence of lymphogenous or haematogenous dissemination. The patient did not present any subjective complaints at the time of the imaging studies.

In the summer of 2024, the patient came to Bulgaria during her annual leave. Based on her personal information, she visited the National Reference Laboratory "Mycoses" at the National Centre of Infectious and Parasitic Diseases (NCIPD), Sofia, regarding a possible "valley fever" infection. As an initial screening serological immunodiffusion tests were conducted for detection of antibodies to the endemic fungi *Coccidioides*, *Histoplasma* and *Blastomyces* using the following procedure:

- Phenol agar is spilled in a few petri plates, and immediately refrigerated at 4°C.
- Smooth-edged wells are formed, and the following are dropped:

• Control serum (commercial kit IMMY, USA)
• Antigen (commercial kit IMMY, USA)
• Patient's serum

Precipitation lines are sought following incubation in a wet camera at 25°C for a few days.

The results were negative.

To further refine diagnosis, we recommended a microbiological and histological examination of bronchoalveolar lavage or of biopsy material from the lesion. According to the recommendation of a pulmonologist, the pulmonary nodule was excised through thoracoscopy , and the biopsy material was delivered to the Mycoses Reference Laboratory. A histological examination of the biopsy material (lung parenchyma fragment) revealed the presence of a caseous granuloma comprised of giant multinucleated cells of the Langhans type, lymphocytes, and epithelioid cells (Fig. 2).

The morphological picture was comparable with that of specific granulomatous inflammation, and a differential diagnosis for tuberculosis was conducted. The results of the tuberculosis test were negative. At the same time, the culture of the biopsy material on Sabouraud agar medium at 30°C demonstrated on the fifth day the expansion of an unpigmented fungus with substrate and aerial mycelia, which became clearly visible after another two to three days. At macroscopic observation, the fungus was found to be similar to Coccidioides (Fig. 3).

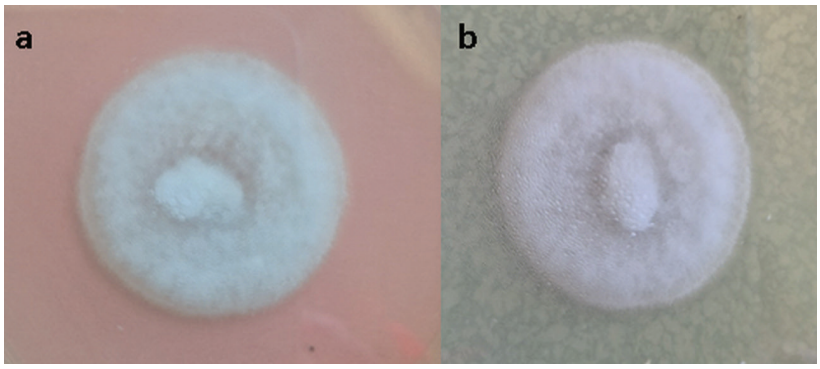

Fig. 3 (a, b) Growth of unpigmented fungus with substrate and aerial mycelium on Sabouraud's medium

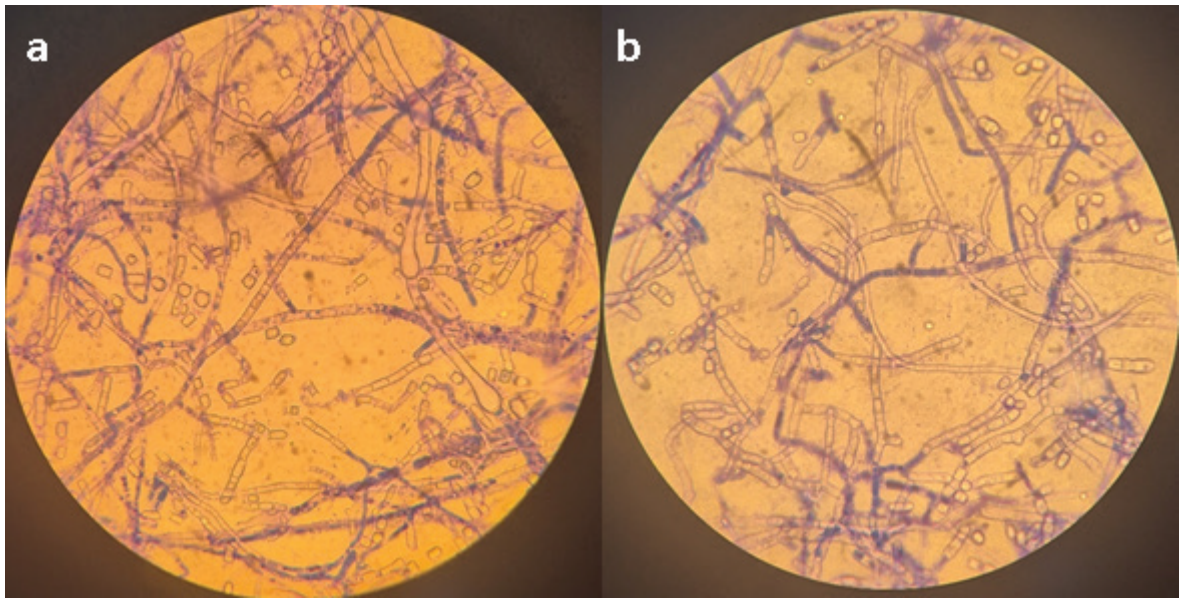

Fig. 4 (a, b) Hyphae of Coccidioides with the presence of arthroconidia

Microscopic evaluation confirmed the identification by visualising the characteristic hyphae and the presence of arthroconidia (Fig. 4).

A microscopic examination was conducted following occupational safety requirements, given the infectious nature of the fungus spores. The patient was prescribed antimycotic therapy with fluconazole for six months, given the elevated risk of recurrence (7), and follow-up has not yet been completed.

DISCUSSION

Coccidioidomycosis is a disease of limited prevalence in certain geographic regions but is of global importance given the potential exposure of increasing numbers of travellers and residents in endemic areas [1]. It is estimated that 60% of coccidioidomycoses are asymptomatic, while the remaining cases present with clinically manifested respiratory disease. In some cases, this can lead to disseminated life-threatening conditions, particularly among individuals with impaired immune systems [6].

The case presented is the second diagnosed in Bulgaria. The first was described in 2004 in a woman who underwent a surgical intervention for nodular lesions in the lung. Histological slides of the lesions showed morphological changes typical of infection. The aetiological diagnosis was based on the detection of specific antibodies against *Coccidioides immitis* by immunodiffusion with fungal antigen. The epidemiological history showed that the disease developed about one month after a stay in the state of Arizona, USA. The patient recovered after 9 months of therapy [7]. Although the travel history is important for diagnosis of endemic mycoses, especially in non-endemic countries, it is not always easy to obtain [8]. The diagnosis of coccidioidomycosis is based on a combination of epidemiological information, clinical findings, physical examination and laboratory/radiological data. All patients should have a carefully recorded history and physical examination, including assessment for possibly disseminated disease [1]. Various microbiological diagnostic methods exist. In the present case, we used a culture method and microscopic detection of the pathogen. Immune-based diagnostic method was used in the other case described in the country 20 years ago . In both cases, the epidemiological history and the histological examination of specimens obtained after surgical interventions provided the clue for a targeted microbiological study. Similar data were obtained in a study of endemic mycoses in Italy, where most cases were diagnosed by histology and/or culture. In recent years, PCR diagnostics has become essential, while serological and antigenic tests are of limited diagnostic value [9].

In Europe, the infection is generally rarely seen, mainly in people returning from areas where

coccidioidomycosis is known to be endemic. The last published case was a 26-year-old Dutch man who returned from a trip to California, North America, in 2017 [10]. In 2016, one case of pulmonary coccidioidomycosis was reported in Ireland in a patient also residing in California [11]. To date, ten cases have been described in Italy, six of which after travelling to the United States and South America and four – without a travelling history. Only one case has been reported in an immunocompromised person with AIDS [9]. All published cases from Europe, as well as the Bulgarian one, were pulmonary coccidioidomycosis and all had a favourable outcome after treatment. Most of them, including the present one, were treated with fluconazole in generally accepted doses and regimens.

Given the increasing number of international travelers and the relatively large proportion of individuals with compromised immunity due to causes other than HIV infection (organ transplantation, cancer, systemic corticosteroid use, etc.), clinicians should be particularly cautious in approaching individuals with pulmonary symptoms and evidence of residence in a region endemic for coccidioidomycosis. In our experience, though from a single case, culture diagnosis has a sufficiently high sensitivity and specificity when appropriate clinical material is available. It is important to observe strictly workplace safety rules because of the high risk for the laboratory personnel performing the diagnostic tests. The timely application of contemporary therapy minimizes the possibility of infection dissemination, complications and, consequently, poor prognosis.

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