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EXTRAHEPATIC MANIFESTATIONS IN PATIENTS WITH ACUTE HEPATITIS E – PAZARDZHIK, BULGARIA 2014 – 2022

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ABSTRACT

BACKGROUND: Hepatitis E is a global health issue, only partially understood. Bulgarian record started in 2019 and data is not sufficent. AIM: This research aims to analyse extrahepatic manifestation of acute hepatitis E in patients with hepatitis E from Pazardzhik region, between 2014 – 2022. MATERIALS AND METHODS: The analysis includes 247 patients with acute hepatitis E, treated at the Department of Infectious Diseases of Pazardzhik Multiprofile Hospital for Active Treatment, Bulgaria between 2014 – 2022. The methodology includes clinical observation, laboratory tests and medical imaging. The diagnosis was established by serological /ELISA for anti-*HEV* IgM, IgG detection/ and molecular-biological tests /RT-PCR for *HEV* RNA detection/. RESULTS: We observed extrahepatic manifestations in 19% (47/247) of the cases. In 60% (28/47) comorbidities were present, and 9% (4/47) were with underlying acute/chronic coinfection with another hepatotropic virus. Thrombocytopenia was found in 83% (39/47) of the patients; asymptomatic creatine kinase elevation – in 13% (6/47), acute pancreatitis – in 9% (4/47), transitory renal impairent – in 6% (3/47); 2% (1/47) had Guillain-Barré syndrome (GBS), 2% (1/47) – arrhythmia and 13% (6/47) – multiorgan involvement. While 91% (43/47) of the patients recovered, in 9% (4/47) the outcome was fatal. CONCLUSION: Extrahepatic manifestations might prevail, potentially delaying diagnosis *of HEV*-infection. Symptoms associated with comorbidities might also impede the final diagnosis. A diagnostic algorithm is needed to enhance the accurate diagnosis of HEV in patients with dubious symptoms.

Key words: *HEV*; extrahepatic manifestations; thrombocytopenia; CK elevation; acute pancreatitis;

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BACKGROUND

Hepatitis E is a global health issue. In spite of an increasing number of publications from various countries worldwide, there are questions that still remain unanswered.

Hepatitis E is caused by Hepatitis E virus (*HEV*). Mainly 4 *HEV* genotypes (*HEV* 1 – 4) cause disease in humans (1–3). HEV has no envelope, and such "bare" particles are found in gallbladder and faeces. In the bloodstream, *HEV* particles obtain host-derived lipid envelope protecting the virus from the neutralising antibodies. Presumably, the lipid envelope is significant for viral entry in otherwise inaccessible sites as cerebrospinal fluid (CSF). This gives a plausible explanation for the *HEV*-related extrahepatic manifestations (4). The latter might be the only manifestations of hepatitis E, thus complicating and delaying diagnosis. A relation between extrahepatic manifestations and co-infection with other hepatotropic viruses, mainly HBV and HCV was established (5,6). Recently, such have been observed in hepatitis E as well (7).

The first case of a *HEV* infected individual in Bulgaria was reported in 1995 by prof. Pavel Teoharov (8). By 2008 there had been only single case reports from the country (9–11). However, an increasing number of locally acquired hepatitis Ecases have been diagnosed later (12–17).

In 1965, *Austin Bradford Hill* – a British physician and statistician published "*Hill's criteria*" – pivots of the

causation between two events (18). *Pischke et al*. (19) suggested an adapted version of *Hill's criteria* for identifying the extrahepatic manifestations in *HEV* infection, as follows:

1. Strength: the stronger the association, the more likely the causality is.

2. Consistency: an association, repeatedly observed by different observers.

3. Temporality: manifestations observed shortly after or during *HEV* infection.

4. Plausibility and analogy: comparable or similar extrahepatic manifestations and underlying pathophysiological mechanisms have been already established for other viral infections, e.g., HCV infection.

5. Experimental data support a causality.

Considering the above criteria, the association between *HEV* infection and the extrahepatic manifestations is defined as possible, very probable, probable, doubtful and under debate (19). To date, *HEV* infection has been associated with neurological, renal, and haematological disorders, as well as with development of acute pancreatitis and other conditions (4,6,7,18,20–24). Only single publications from Bulgarian authors about the extrahepatic manifestations in *HEV exist*, while their incidence might be higher.

The aim of the present research **was** to analyse the extrahepatic manifestations observed during the acute period of HEV infection in patients from Pazardzhik region diagnosed between January 2014 – December 2022.

MATERIALS AND METHODS

Between January 2014 – December 2022, 247 patients with acute hepatitis E were treated at the Department of Infectious Diseases of Pazardzhik Multiprofile Hospital for Active Treatment. The methodology includes clinical observation, laboratory tests and medical imaging. The diagnosis of all patients was serologically confirmed with specific *anti-HEV IgM* and *IgG* detection (DIA.PRO ELISA Kit, Italy, diagnostic specificity >95% and sensitivity 100%). In 20% of the patients RT-PCR of serum samples for *HEV* RNA detection was also applied (RealStar – *HEV* RT-PCR, Altona Diagnostics, 22767 Hamburg, Germany, Ref.272013). The diagnostic tests were run at the National Reference Laboratory "Hepatitis Viruses" of the National Centre for Infectious and Parasitic Diseases, Sofia, Bulgaria, and the Laboratory of Virology, Acibadem City Clinic – Tokuda Hospital, Sofia, Bulgaria. The results are descriptively analysed, reported as mean (±SD) and numbers /proportions.

RESULTS

Among the 247 patients with acute hepatitis E during the studied period, 69% were males and 31% - females. The mean age *(±SD)* was 57.5 ±11.7 years with no significant difference between the two sexes (57 years for men vs. 57.8 years for women). Extrahepatic manifestations were observed in 19% (47 /247) of the patients (Table 1.). Further on, 59.6% (28/47) of patients with extrahepatic manifestations had comorbidities (Table 2.)

Thrombocytopenia defined as platelets count <150G/l was the most common extrahepatic manifestation observed (83% or 16% of all patients). The lowest platelets count was 4G/l, registered in a female patient with no comorbidities. Clinically manifested bleeding was registered in 13% of thrombocytopenic patients (5 /39) : 2 had haematemesis; 4 - puncture site hematomas; 3 developed skin haemorrhages – petechia and ecchymoses, and 3 patients had both cavitary and skin bleeding. The woman with the lowest Plt count had only petechia on the lower limbs, and was initially admitted to a haematology ward. However, the laboratory tests revealed significant transaminases elevation (ALT>1000IU/l). *HEV* infection was confirmed both serologically (*anti-HEV IgM* and *IgG* in high values) and with *HEV RNA* detection. A month after the acute illness the platelets count was normal and remained within reference range during the follow-up.The patients with haematemesis had alcohol-induced liver cirrhosis.

Creatine kinase (CK) elevation comprised 13% of extrahepatic manifestations (or 2.4 % of all patients). CK elevation was at the expense of the muscle fraction (CK-MM) with no clinical signs. The values normalised within the acute period.

Acute pancreatitis was observed in 9% of patients with extrahepatic manifestations (1.6 % of all). Its

Table 1. Patients with acute *HEV* infection: distribution according to the extrahepatic manifestations.

Extrahepatic manifestation	Number (% related to patients with extrahepatic manifestations, n=47)	Number (% related to the total number of patients, n=247)
Thrombocytopenia	39 (83%)	39 (15.7%)
Creatin kinase /CK/ elevation	6 (13%)	6 (2%)
Acute pancreatitis	4 (9%)	4 (2%)
Transient renal impairment	3 (6%)	3 (1%)
Guillain-Barré syndrome	1 (2%)	1 (0.4%)
Hemophagocytic syndrome	1 (2%)	1 (0.4%)
Cardiovascular disorders: ArrythmiasPulmonary oedema	1 (2%) 1 (2%)	1 (0.4%) 1 (0.4%)
More than one extrahepatic manifestation	6 (13%)	6 (2%)

Table 2. Patients with extrahepatic manifestations – distribution according to the accompanying diseases.

Accompanying diseases	Number (% related to patients with extrahepatic manifestations, n=47)	Number (% related to the total number of patients, n=247)
Alcoholic cirrhosis	9 (19%)	21 (9%)
Diabetes mellitus	7 (15%)	35 (14%)
Cardiovascular diseases	11 (24%)	62 (25%)
Coinfection with another hepatotropic virus	4 (9%)	30 (12%)
Other diseases	10 (21%)	34 (14%)
More than one comorbidity	15 (32%)	78 (32%)

clinical course did not differ pancreatitis with other aetiology. Two patients had liver cirrhosis, one had HAV/HEV coinfection. The disease severity depended on the clinical course of the hepatitis. The outcome of the pancreatitis was favourable in all 4 cases.

Transitory renal impairment – urea and creatinine elevation, were observed in 2% (5 /247) of all patients with hepatitis E. A patient with cirrhosis and concomitant chronic renal failure on haemodialysis, and another one who developed hepato-renal syndrome were excluded from the group. The other three (3/247, 1%) experienced transitory urea and creatinine elevation with no signs of renal injury and values returned to normal within the acute illness. None of them had medical history or sonographic data for underlying renal disorder.

Guillain-Barre Syndrome (GBS) was observed in one patient with alcohol-associated liver cirrhosis. He was initially admitted to a neurology ward due to lower limbs weakness. Electromyographic results showed demyelination and the cerebrospinal fluid analysis revealed proteinorachia 1(.2 g/l). Along with that, elevation of serum bilirubin and transaminases were registered. The diagnosis of acute *HEV* infection was established by specific antibodies detection during the acute illness – *anti-HEV IgM* and *anti-HEV IgG*. The disease ended with recovery 6 – 7 months later

Secondary hemophagocytic syndrome was observed in a female patient with severe acute hepatitis E and liver failure that developed on a damaged terrain: medical history of alcohol abuse, liver steatosis and thyroiditis. In the course of the disease, we registered fever (39ºC), low fibrinogen (1.3g/l) and cholesterol (1.14 mmol/l); elevated ferritin (1482µg/l) and triglycerides (4.2mmol/l); splenomegaly and pancytopenia with low leukocyte and thrombocyte counts and severely affected erythrocytes (Leu 1.9G/l, Plt 54G/l, Hb109g/l). The patient lost over 10

kg. The treatment was complex and continuous: 36 days inpatient period followed by a few months of convalescence. An upper endoscopy, three months after recovery, revealed oesophageal varices grade I.

Other extrahepatic manifestations – **arrhythmia* was observed in a patient who was initially admitted to a cardiology ward with paroxysmal atrial fibrillation. Due to significant transaminase elevation, additional diagnostic tests were performed including serology test for hepatitis E. ** Cardiac asthma/ pulmonary oedema* was observed in a patient with accompanying heart disease. In both patients *HEV* infection was confirmed by the detection of *anti-HEV IgM* and *IgG* antibodies, and ended with recovery.

DISCUSSION

Thrombocytopenia is a common, possibly immune-mediated haematological disorder in hepatitis E (7,19,22,24,28). Low platelets count was the most common extrahepatic manifestation among our patients. Notefully, most of them (87%) had no haemorrhage. Bleeding and its severity might not depend directly on platelets count, since the patient with the lowest (4G/l) presented only with petechia on the lower limbs. Probably, age and comorbidities, the accompanying chronic liver diseases in particular, might be of greater significance. Severe stomach bleeding was observed in two patients, both with underlying liver cirrhosis.

Other HEV-related haematological disorders include hemolytic anemia (29) and aplastic anemia (6,7,24,28,30). The established association between hemophagocytic syndrome and various viruses and bacteria has led to the definition of the so called *reactive secondary hemophagocytic syndrome* (28,31). We did not observe hemolytic or aplastic anemia in any of our patients. However, a woman with comorbidities developed a hemophagocytic syndrome in the course of the acute *HEV*-infection. The diagnosis of secondary hemophagocytic syndrome is clinically-based, the causes are yet to be recognized as well as the risk groups to be identified. Further studies are needed to elucidate the secondary hemophagocytic syndrome. Its spontaneous and malignant evolution requires timely diagnosis for successful management. To date, only sporadic cases of *HEV*-induced hemophagocytic syndrome have been published (31).

Muscle and skeletal disorders are rarely documented and are usually related to acute *HEV* infection (6,28,32,33). We registered CK elevation in few patients without symptoms and clinical signs. Changes were transient and reversible similarly to the observations documented in literature (28,32).

Acute pancreatitis is mainly associated with HEV1; however, other genotypes might cause it as well (6,7,21,27,28). HEV-related pancreatitis is clinically indistinguishable from those with different aetiology. Disease severity depends rather on the course of HEV infection. In some cases, the HEV aetiology of pancreatitis may remain unknown, especially when the patients present without jaundice. Pancreatitis in hepatitis E is more common among males. It is usually benign and treatment results in successful management (21,27). The same was observed among our patients.

** Renal manifestations* – glomerulonephritis in acute hepatitis E has been observed in immunocompetent and immunocompromised patients (7,23,24). Nevertheless, it is mainly related to chronic *HEV* infection. *HEV*-induced glomerulonephritis usually has benign course and favourable outcome (6). Perhaps, some cases remain aetiologically undiagnosed. None of the reported patients developed glomerulonephritis. In those with elevated urea and creatinine the values returned to normal within the acute period without specific treatment. Further studies of renal disorders in acute and chronic *HEV* infection are needed. Such patients should be consulted with nephrologist during the acute illness and the follow-up.

** Neurological manifestations:* The most common peripheral nervous system manifestation of HEV is development of Guillain-Barre Syndrome (GBS) (6,7,24). It is usually observed during or soon after an acute infection , affects predominantly men, and has a benign course that ends with definitive recovery. Neurological amyotrophy (Parsonage-Aldren-Turner syndrome) or brachial neuritis is the other most common neurological complication that affects mainly males as well. Nonetheless, even adequate treatment rarely leads to complete recovery (7).

Central nervous system is less commonly affected with manifestations of meningitis, encephalitis, pseudotumor cerebri, etc. (4,6,7,20,25,26). HEV RNA was isolated from CSF and serum but since the isolates showed differences, the existence of neurotropic HEV strains is still under debate (25,26). Neurologic manifestations are mainly related to HEV3 and HEV4 (4,24–26).

* There are some reports of myocarditis, thyroiditis, and other extrahepatic manifestations in the course of acute *HEV* infection (6,28,34). Cardiac asthma and arrhythmia were reported as initial manifestations of hepatitis E in two patients. Although these were the only cases and their association with *HEV* was not confirmed, they should be kept in mind and thoroughly analysed.

CONCLUSION

According to the adapted criteria of *Pischke et al*. extrahepatic manifestations in *HEV* infection are systematized as follows: *very probable* causality: *Guillain-Barre syndrome*, neurological amyotrophy, acute pancreatitis. *Probable* causality: cryoglobulinemia, haematological disorders. Further data are required to confirm the causal relationship between *HEV* and other extrahepatic manifestations. Therefore, it is important to report every extrahepatic manifestation observed in the course of or soon after an acute hepatitis E as well as in chronic *HEV* infection. Accumulation of more cases would contribute to conclude whether those events are closely related or coincidental, and what is the relative impact of comorbidities, hepatitis severity, patients' age or *HEV* genotype on the development of extrahepatic manifestations.

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AN ACUTE GASTROENTERITIS OUTBREAK IN A KINDERGARTEN

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ABSTRACT

Objective. To detect the etiological cause of an acute gastroenteritis outbreak at St. Anna kindergarten in the village of Resilovo, region Kyustendil.

Materials and Methods. A total of 22 faecal specimens from children (n = 18) and staff (n = 4) were tested. Multiplex RT-PCR with specific primer pairs detecting the most common viral causes of gastroenteritis (noroviruses, rotaviruses, sapoviruses, intestinal adenoviruses and intestinal astroviruses) was applied to detect the viral causative agent. Noroviruses were detected and sequenced and subsequent phylogenetic analysis was carried out.

Results. Genogroup II noroviruses were detected in five samples from children and one sample from staff (6/22) or in 27.3% of the specimens. According to WHO criteria, this proves that noroviruses have caused the epidemic outbreak. Detected noroviruses were subjected to sequencing and subsequent phylogenetic analysis, with data identifying genotype 17 (GII.17) as the causative agent.

Conclusion. Norovirus genotype 17 (GII.17) was first detected in Bulgaria in 2015 as the causative agent of an outbreak in a secondary school in the town of Pravets. In 2016, the circulation of this genotype was again established in sporadic cases, and in 2022 it was found to be the cause of the epidemic gastroenteritis outbreak in a kindergarten in the village of Resilovo. To protect public health, continuous monitoring and targeted search for viral intestinal agents is essential, regardless of the transient and usually mild course of the disease.

Keywords: multiplex RT-PCR, sequencing, epidemic outbreak

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INTRODUCTION

Viral enteric infections (VEI) are a serious problem worldwide that is sometimes neglected, though diarrheal diseases are at the forefront as causes of mortality (1). Symptoms of acute gastroenteritis (AGE) include general malaise, abdominal pain and cramps, nausea, vomiting, and diarrhea, usually lasting 1 to 5 days, sometimes up to 14 days. The most common viral cause of AGE are rotaviruses (RVs), but with the introduction of vaccines against them, they are increasingly being replaced by noroviruses (NoVs). Other enteric viruses such as sapoviruses (SaVs), astroviruses (AstVs), as well as a few DNA viruses such as the intestinal adenoviruses (AdVs) may also be found in cases of gastroenteritis.

Globally, 1 in 5 cases of gastroenteritis leading to vomiting and diarrhea is caused by noroviruses (2).

Norovirus is a genus from the family *Caliciviridae* and its representatives contain a positive-sense, single-stranded RNA genome. They are naked viruses with icosahedral symmetry of the capsid. The genome contains 3 open reading frames (ORFs) - ORF 1 (which encodes six non-structural proteins, including RNA-dependent RNA polymerase [RdRp]), ORF2 (encodes for the major capsid protein VP1) and ORF3 (encodes for minor capsid protein VP2) (3,4).

The genus is currently subdivided into 10 genogroups and 49 genotypes based on the complete nucleotide sequence encoding the major capsid protein VP1 (5). Genogroups are denoted by the capital Latin letter G and the corresponding Roman numeral, and genotypes are denoted by Arabic numerals.

NoV genotype 17 (GII.17) was established as the dominant one in 2014 in Italy and several other countries around the world, and its circulation in

Bulgaria was detected in 2015 as the causative agent of an outbreak in a secondary school in the town of Pravets.

In May 2022, at St. Anna kindergarten in the village of Resilovo, region Kyustendil, some of the children fell ill.

✓ On May 10th, 2022, a child was brought to the kindergarten after having vomited the previous night. Due to the subsidence of the complaints, he was not left at home (May 9th is the supposed start of the outbreak);

✓ On that day a total of 17 children were in the kindergarten; around noon (11:30 a.m.) one of the children developed symptoms of illness, 30 minutes later another child was sick, and within the next 3-4 hours 7 more children fell ill; in the remaining 5 children symptoms appeared by 9 p.m.;

✓ The symptoms were repeated fountain vomiting and abdominal pain. Fever and diarrhea were not observed;

✓ Two of the children were hospitalized with an initial diagnosis of VEI;

Based on the clinical course that led to vomiting and rapid resolution of symptoms, the infectious disease specialist at the hospital suspected a norovirus infection. To detect the etiological cause of the AGE outbreak in St. Anna kindergarten in the village of Resilovo, region Kyustendil, clinical samples were sent for investigation to the National Reference Laboratory for Enteroviruses at the National Center for Infectious and Parasitic Diseases.

MATERIALS AND METHODS

Sample collection

A total of 22 fecal specimens from children and staff (18 children and 4 staff) were collected and sent for testing in the National Reference Laboratory "Enteroviruses".

Viral RNA extraction and reverse transcription

Viral genomes were extracted from 10% (w/v in phosphate buffer saline) supernatant of stool samples using NucleoSpin® Dx Virus kit based on the spin-column procedure and following the manufacturer's instructions (Macherey-Nagel GmbH & Co KG, Düren, Germany). The reverse transcription (RT) to a complementary DNA was performed with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random primers (hexa-deoxyribonucleotide mixture) (Invitrogen, Carlsbad, CA, USA). Before reverse transcription RNAs were denatured by heating at 95°C for 5 min and cooled on ice. Reverse transcription thermal cycling conditions were as follows: 37°C for 1 h and 95°C for 5 min. Copy-DNAs were stored at -20°C until required for further analysis.

Viral detection by PCR

Monoplex and multiplex PCR assays with three primer sets comprising eight specific pairs of primers that detected RVs groups A, B and C, NoVs genogroups I and II, sapoviruses, enteric AdVs and AsVs, were applied (6). Sequences of primer pairs are presented in Table 1 (7,8,9).

The DNA amplification was conducted with 2x HotBegan™ Red-Taq Master Mix (Canvax Biotech, S.L., Cordoba, Spain). The master mix in 20 µl total volume was prepared as shown in Table 2 and the PCR was performed at 94°C for 10 min, followed by 30 cycles of 94°C for 35 sec, 50°C for 35 sec, 72°C for 1 min, and a final extension at 72°C for 7 min, and then held at 4°C. Positive controls (known cDNA from patient samples for RVs and NoVII) and negative controls were included in each run.

Electrophoresis

Visualization of the PCR products was performed by 2% gel electrophoresis. The presence of the respective viruses was determined based on the size of the expected PCR product corresponding to each virus (Table 1).

Nucleotide Sequencing and Phylogenetic Analysis

A Sanger dideoxy sequencing of the detected NoVs was performed with an automatic sequencer model GenomeLab GeXP (Beckman Coulter, USA). The primers used for sequencing were the same as the ones used for detection. They target the portion of the gene encoding VP 1 (near the ORF1-ORF2 junction, a hot point for mutation). The resulting raw sequences were processed manually using BioEdit v.7.2 computer software (10). The Norovirus genotyping tool (11) was used to determine the genotype/variant of the NoV strain, and the programs BLAST (12) and BioEdit v.7.2 were implemented to compare

Table 1. Specific primers' sequences and size of the expected PCR products for the detection of enteric viruses by multiplex RT-PCR assay.

Set	Target virus	Primer sequences 5'-3'	PCR product size (bp)	Reference
A	RVs group A	F- GGCTTTAAAAGAGAGAATTTC R - ACTGATCCTGTTGGCCATCCTTT	395	[7]
B	RVs group B	F - GGCAATAAAATGGCTTCATTGC R - GGGTTTTTACAGCTTCGGCT	814	[7]
	RVs group C	F - ATTATGCTCAGACTATCGCCAC R - GTTTCTGTACTAGCTGGTGAAC	351	[7]
	AdVs	F - TTCCCCATGGCICAYAACAC R - CCCTGGTAKCCRATRTTGTA	482	[7]
	AsVs	F - GATTGGACTCGATTTGATGG R - CTGGCTTAACCCACATTCC	409	[8]
C	NoVs geno-group I	F - CTGCCCGAATTYGTAAATGA R - CCAACCCARCCATTRTACA	330	[9]
	NoVs geno-group II	F - CARGARBCNATGTTYAGRTGGATGAG R - CCRCCNGCATRHCCRTTRTACAT	387	[9]
	SaVs	F - CTCGCCACCTACRAWGCBTGGTT R - CGGRCYTCAAAVSTACCBCCCCA	434	[9]

Table 2. Ingredients for the performance of the PCR.

Buffer and reagents	Volume		
	Set A	Set B	Set C
2 x Master Mix	10.0 µl	10.0 µl	10.0 µl
F-primer (20 pmoles/µl)	0.5 µl	x 0.5 µl each	x 0.5 µl each
R-primer (20 pmoles/µl)	0.5 µl	x 0.5 µl each	x 0.5 µl each
Sterilized D.W.	6.5 µl	3.5 µl	4.5 µl
Template	2.5 µl	2.5 µl	2.5 µl

the obtained sequence with those published in the GeneBank genome bank (http://blast.ncbi.nlm.nih.gov). The phylogenetic analysis was done by the Neighbor-Joining Method, Kimura 2-parameter, 1,000 bootstrap replications of the MEGA11 program (13).

RESULTS

Genogroup II noroviruses were detected in five samples from children and in one sample from staff (6/22) or in 27.3% of the specimens. According to WHO criteria (14), this proves that NoVs have caused the epidemic outbreak. Detected NoVs were subjected to sequencing with the same pair of primers in Table 1 that detect a part of the VP1 gene encoding the major capsid protein (near the ORF1-ORF2 junction, a hot point for mutation). Four of the samples were successfully sequenced and the genotyping tool identified genotype 17 (GII.17). When comparing the Bulgarian strains with the Genome bank, the greatest similarity was found with those established in Japan, China and Italy, and for this reason these sequences were included in the phylogenetic analysis. Subsequent phylogenetic analysis revealed that the Bulgarian strains from Resilovo were genetically related to GII.17, variant

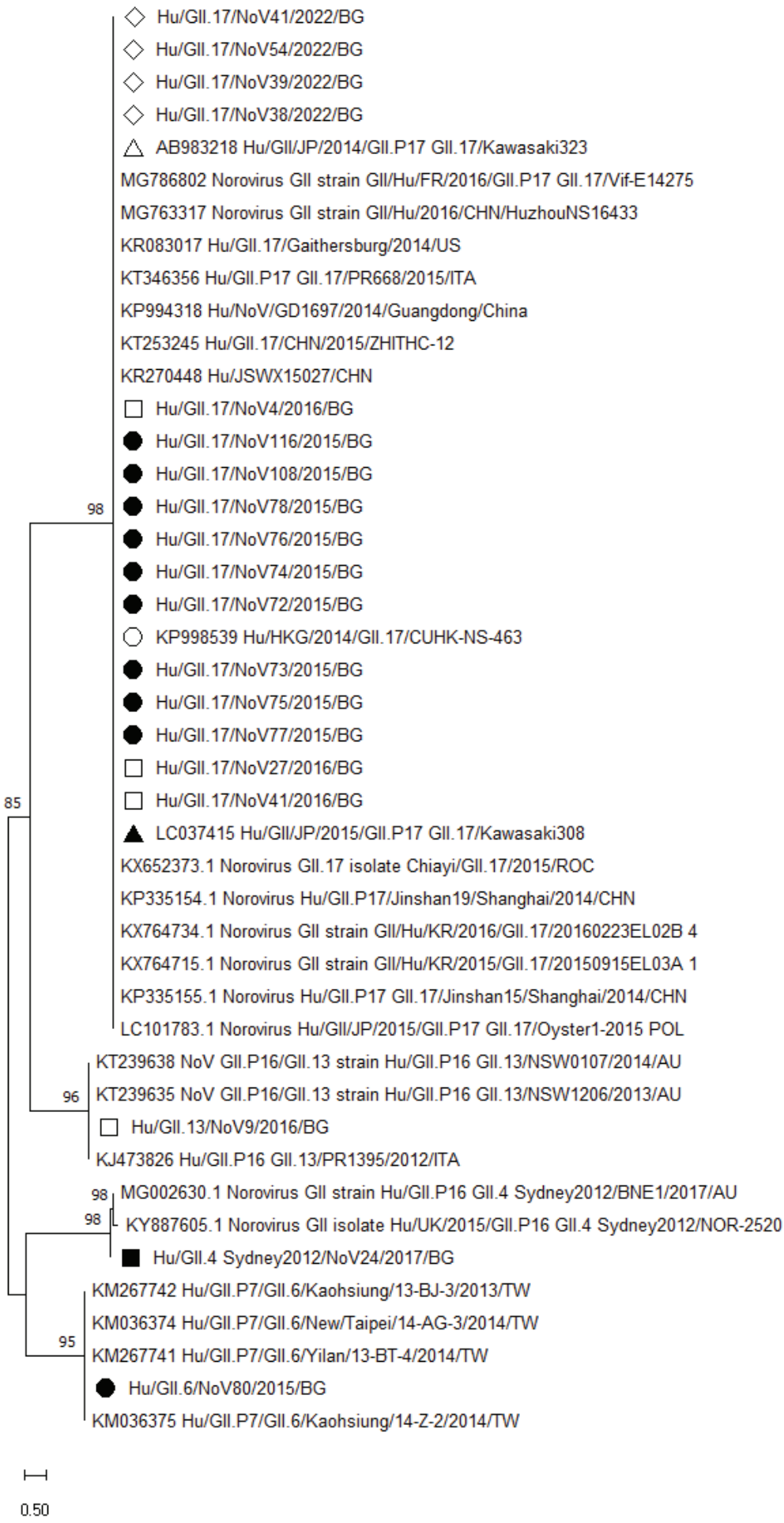

Figure 1. A phylogenetic tree showing the genetic relationships between the Bulgarian norovirus genotypes of genogroup GII and those distributed in Europe and the world regarding part of the VP1 gene encoding the capsid protein. Open circle marks the reference strain for genotype 17; open triangle - variant Kawasaki323, filled triangle - variant Kawasaki308, filled circles - the Bulgarian strains isolated in 2015, open squares – those from 2016, filled squares – from 2017 and 2022, and open diamond - from 2022.

Kawasaki323 proven in 2014 in Japan, as well as to those proven in the period 2014-2016 in the USA, China, Italy and France. Norovirus strains isolated in Pravets (2015) and from sporadic cases in our country (2016) were similar to the Kawasaki308 variant, proven in Japan in 2015, as well as in China, Poland and Korea (Figure 1).

These results conclusively demonstrated that noroviruses of genotype 17 were the cause of the acute gastroenteritis outbreak that occurred at the kindergarten.

DISCUSSION

Cases of VEI caused by rotaviruses are subject to monitoring and reporting in Bulgaria. Very rarely, other causes of acute gastroenteritis are sought. However, in case an epidemic outbreak of gastroenteritis involving the members of a closed collective (kindergarten, school, mass events with a common source of food and/or water) is suspected, samples for diagnosis of other enteric viruses are also sent to the reference laboratory.

During the hospitalization of two children attending the kindergarten in the village of Resilovo with characteristic symptoms, the infectious disease specialist of the hospital suspected a norovirus as the causative agent . Consequently, samples from children, as well as from some of the staff members were sent to NRL "Enteroviruses" for investigation.

Genogroup II noroviruses were detected in six samples (five samples from children and in one sample from staff) or in 27.3% (6/22) of the specimens. Four of the samples were successfully sequenced and the genotyping tool identified genotype 17 (GII.17).

Until recently, the dominant genotype causing epidemic outbreaks of AGE worldwide was GII.4, characterized by rapid evolution rates through mutations and recombinations. For this reason, a new variant capable of causing outbreaks appeared every 2-3 years.

For nearly 10 years, in several European and Asian countries, another genotype has been dominant in sporadic cases or epidemic outbreaks, i.e. GII.17.

NoV genotype 17 (GII.17) was first described in the late 1970s in French Guiana (15) and has periodically been associated with sporadic cases or outbreaks of AGE in Africa, Asia and Europe. During the 2014/2015 season, it replaced the dominant at that time GII.4 Sydney in several countries worldwide - China, Japan, Italy, Romania, and the Netherlands (16-21).

GII.17 was first detected in Bulgaria in 2015 as the causative agent of an AGE outbreak in a secondary school in the town of Pravets.

Subsequent phylogenetic analyses revealed that the Bulgarian strains from Resilovo were genetically related to GII.17, variant Kawasaki323 proven in 2014 in Japan, as well as to those proven in the period 2014-2016 in the USA, China, Italy and France. Norovirus strains from Pravets (2015) and sporadic cases (2016) in our country, showed similarity with the Kawasaki308 variant, proven in Japan in 2015, as well as in China, Poland and Korea.

In-depth studies, based on sequencing and phylogenetic analysis, show the specific genotype of the causative agent. The sufficiently large genetic similarity between the proven noroviruses, as well as the available epidemiological data, conclusively pointed out the cause of the epidemic outbreak.

To protect public health, continuous monitoring and targeted search for viral intestinal agents are essential, regardless of the transient and usually mild course of the disease. Also, continuous monitoring ensures the timely detection of new genotypes and/or variants with serious epidemic potential .

ACKNOWLEDGMENTS

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LABORATORY DETECTION OF COLISTIN-RESISTANT *ENTEROBACTERALES* IN TANDEM WITH ROUTINE ANTIBIOGRAM

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ABSTRACT

Since the emergence and spread of carbapenemase-producing *Enterobacterales*, particularly those carrying metallo-β-lactamases with 16S rRNA methyltransferases for which newly introduced antibiotics are inactive, colistin is a last resort therapy for life-threatening extensively drug-resistant infections. This requires that laboratories use an accurate and reliable method for routine colistin susceptibility testing. The aim of this study was to evaluate the performance and applicability of a colistin screening medium for detection of colistin-resistant isolates simultaneously with routine susceptibility testing. It was evaluated with fifty colistin-resistant and the same number of colistin-susceptible comparator isolates. Our results showed that all colistin-resistant isolates grew on DiaPlate™ EMB Agar + Colistin medium within the standard antibiogram period. In contrast, no growth was observed among the colistin susceptible comparators. DiaPlate™ EMB Agar + Colistin is a screening medium that can detect colistin- resistant *Enterobacterales* isolates when pure bacterial cultures are tested. *As this method for detecting colistin-resistant isolates uses the same inoculum as the Kirby-Bauer method, the screening test for colistin resistance can be conveniently performed on clinical isolates along with routine antimicrobial susceptibility testing.*

Keywords: *Enterobacterales*, colistin resistance, phenotypic detection

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INTRODUCTION

Polymyxins have a narrow antibacterial spectrum including some Gram-negative bacteria such as *Citrobacter* spp., *Enterobacter* spp., *Escherichia coli*, *Klebsiella* spp., *Salmonella* spp., *Shigella* spp., *Acinetobacter* spp., *Pseudomonas aeruginosa* and most strains of *Stenotrophomonas maltophilia.* They are not active against *Proteus* spp., *Providencia* spp., *Serratia* spp., *Edwardsiella* spp., *Morganella* spp., *Hafnia* spp., Gram-negative cocci, Gram-positive organisms and most anaerobic bacteria (1). In Europe, colistin was first used in 1950, but was gradually abandoned in clinical practice due to its nephrotoxicity and neurotoxicity, as well as to the availability of less toxic and clinically more effective drugs. The renaissance of colistin is linked to the emergence and rapid spread of carbapenemase-producing *Enterobacterales*, particularly those carrying metallo-β-lactamases with 16S rRNA methyltransferases, for which the newly introduced drugs are not active. For many years, colistin-based combinations have been successfully used to treat extensively drug-resistant infections. However, after the overlaying of plasmid-mediated colistin resistance to the mechanisms driven by chromosomal mutations, colistin resistance has steadily increased and it has become imperative to perform accurate colistin susceptibility testing in routine microbiological practice (2).

The recommended method for colistin susceptibility testing, by both EUCAST and CLSI, is broth microdilution (BMD), which should be performed in polystyrene microtitre plates using sulphate salt of colistin without addition of surfactants, according to the ISO standard 20776-1 (3). There are several reasons for this. On the one hand, due to colistin's large molecular weight, diffusion in agar is ineffective, making agar-based methods such as gradient strips or disk diffusion unsuitable (4). On the other hand, colistin binds to polystyrene microtiter plates, reducing the concentration of the active compound in the media. At the same time, the addition of surfactants, such as polysorbate-80, is

not an option due to the synergic effect with colistin (5). Finally, several reports have been published on the unreliability of semi-automatic systems for colistin resistance determination (4, 5). As the use of the reference method is labor-intensive, many routine laboratories still use gradient strips or semi-automated systems, despite EUCAST's warning to use only BMD (6).

Recently, a universal culture medium for screening of colistin-resistant bacteria in stool samples has been developed (7). The SuperPolymyxin™ is based on eosin-methylene blue agar (EMB) and contains colistin at an optimal concentration of 3.5 µg/ml, amphotericin B to suppress fungal growth, and daptomycin to suppress the growth of Gram-positive cocci that may still be growing on EBM medium. This medium was chosen because of its selective-differentiating characteristics, which enable additional distinction between enterobacterial species. In search of an easy and inexpensive method to detect colistin-resistant clinical isolates simultaneously with routine antibiogram, we adopted Proevska's simplified SuperPolymyxin medium. It contains only colistin and its use is convenient when pure bacterial cultures are tested (8).

The aim of this study was to evaluate the performance and applicability of a commercial DiaPlate™ EMB Agar + Colistin medium for detection of colistin-resistant isolates in tandem with routine susceptibility testing.

MATERIAL AND METHODS

The study involved one hundred clinical Enterobacterales isolates divided into two groups. The first group included a total of 50 colistin-resistant strains. Of these, 40 strains of Klebsiella pneumoniae were already confirmed with acquired colistin resistance (9); 8 isolates (Serratia marcescens, Morganella morganii, Proteus mirabilis, P. vulgaris, P. penneri, Hafnia alvei, Providencia stuartii and P. rettgeri, one isolate each) had intrinsic colistin resistance; and 2 reference strains of Escherichia coli (E. coli NCTC 13846 and EQA 4320) *possessed the mcr-1 gene. The second group comprised the same number of colistin-susceptible isolates, including 20 K.pneumoniae, 10 E.coli, 5 Citrobacter freundii and 5 Enterobacter cloacae complex.*

DiaPlate™ EMB Agar + Colistin medium (Diachim, Bulgaria), evaluated in this study, was developed to detect colistin resistance in pure culture of clinical isolates intended for routine susceptibility testing. It was therefore made from EMB agar to which only colistin sulphate was added at a final concentration of 3.5 µg/ml (7). The colistin agar plate was divided into eight parts with a marker, allowing up to 6 clinical isolates per plate to be tested (Figure 1). Each plate was always inoculated with both quality control strains: *E. coli* NCTC 13846, colistin-resistant and *E. coli* ATCC 25922, colistin-susceptible (10). The test was performed using the standard disk diffusion procedure (11). The bacterial inoculum was prepared in sterile saline to obtain a turbidity of 0.5 McFarland. Using a swab dipped in the suspension, after the excess liquid has been removed, streaks were applied to the appropriate part of the colistin agar plate. The plates were incubated at 35±1°C, 18±2h as for antibiograms. A result was considered valid if the quality control strains demonstrated the expected lack of growth for *E. coli* ATCC 25922 and the presence of confluent growth for *E. coli* NCTC 13846. The presence of growth, confluent or as isolated colonies, in the inoculated area was considered a positive result. The colistin agar plate was inoculated in parallel with the routine antibiogram using the same 0.5 McFarland bacterial suspension. In addition, the minimum inhibitory concentration (MIC) of colistin was determined by broth microdilution using the MIKROLATEST MIC Colistin strip (ErbaLachema, Czech Republic) following the manufacturer's protocol.

RESULTS AND DISCUSSION

All of the 50 colistin-resistant isolates grew on the DiaPlate™ EMB Agar + Colistin medium in 20h. In contrast, no growth was observed among the colistin-susceptible comparator isolates. The sensitivity and specificity of the DiaPlate™ EMB Agar + Colistin for detecting colistin-resistant isolates were 100%. Figure 1 demonstrates a typical growth image on the DiaPlate™ EMB Agar + Colistin medium for colistin-resistant *E. coli*, *K. pneumoniae*, *P. stuartii* and *S. marcescens*.

Our results confirmed the excellent screening capabilities of EMB agar containing colistin at an optimal concentration of 3.5 µg/ml, regardless of

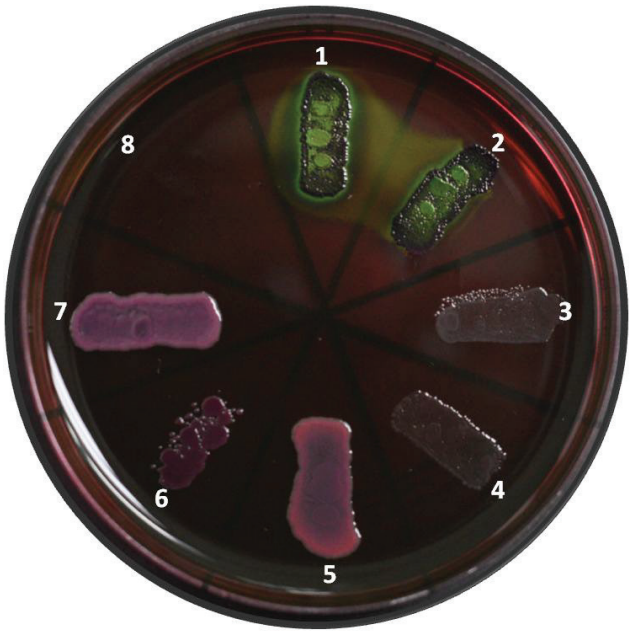

Figure 1. Detection of colistin-resistant *Enterobacterales* using the DiaPlate™ EMB Agar + Colistin medium. Clockwise: *E. coli* NCTC 13846 *mcr-1* positive, colistin MIC = 4 mg/L (1); *E. coli* EQA 4320 *mcr-1* positive, colistin MIC = 4 mg/L (2); *Providencia stuartii,* colistin MIC >16 mg/L *(3); Serratia marcescens* colistin MIC >16 mg/L *(4); Klebsiella pneumoniae* PR3759, colistin MIC >16 mg/L *(5); K. pneumoniae* PR3760, colistin MIC = 8 mg/L *(6); K. pneumoniae* PR3761, colistin MIC >16 mg/L *(7); E. coli* ATCC 25922, colistin MIC = 0.5 mg/L *(8).*

intrinsic, acquired chromosomal or plasmid-mediated resistance (7). Indeed, SuperPolymyxin™ medium is a screening medium for polymyxin-resistant bacteria with a wide range of applications, including detection of colistin-resistant carriers in human medicine and surveillance studies in veterinary medicine. Whereas, the DiaPlate™ EMB Agar + Colistin medium, evaluated here, appears to be a simple and cost-effective method for accurate and reliable detection of colistin-resistant *Enterobacterales* isolates in tandem with routine antibiogram.

CONCLISION

DiaPlate™ EMB Agar + Colistin is a screening medium that can detect all colistin-resistant Enterobacterial isolates, irrespective of resistance mechanism and level of colistin resistance. As this method for detection of colistin-resistant isolates uses the same inoculum as the Kirby-Bauer method, colistin resistance screening test can be conveniently performed on clinical isolates simultaneously with routine antimicrobial susceptibility testing.

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MOLECULAR SURVEILLANCE OF GONOCOCCAL CIPROFLOXACIN SUSCEPTIBILITY/ RESISTANCE IN BULGARIA, 2022-2023

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ABSTRACT

Background: The emergence and spread of antimicrobial resistance in *Neisseria gonorrhoeae* is a significant public health issue, with Euro-GASP conducting surveillance across the EU-EEA. The advent of molecular diagnostics for *N. gonorrhoeae* may possibly limit the surveillance data because it is dependent on gonococcal culture for phenotypic susceptibility testing. Ideally, molecular diagnostics should combine identification and resistance detection but the complexity of gonococcal molecular genetics of resistance is a major barrier for test development. Currently, resistance prediction in *N. gonorrhoeae* is accurate only for fluoroquinolones and there are commercial kits available on the market that allow antimicrobial resistance surveillance and individualized treatment.

The study examined Bulgaria's gonococcal fluoroquinolone resistance rate for 2022-2023 by molecular methods and compared it with previous years and EU-EEA trends.

Methods: The commercial ResistancePlus® GC assay was used to predict ciprofloxacin susceptibility/ resistance in 66 *Neisseria gonorrhoeae*-positive DNA samples from patients, diagnosed in 2022-2023.

Results: The identified fluoroquinolone resistance rate in 2022-2023 was 68.2%. The majority of the cases were males in the age group 20-29 (50.8%), the most common mode of transmission was MSM (77%) and 17% of the cases with known HIV status were positive.

Conclusion: This study found a higher than before fluoroquinolone resistance rate (68.2%) in Bulgaria, following the trend in Europe. In the EU-EEA, ciprofloxacin resistance increased to 65.9% in 2022. Molecular testing for predicting susceptibility/ resistance is suitable for effective antimicrobial resistance surveillance and individualized treatment decisions.

Keywords:

Bulgaria, gonorrhea, *Neisseria gonorrhoeae,* ciprofloxacin, antimicrobial resistance

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INTRODUCTION

Gonorrhoea is a sexually transmitted infection (STI) caused by *Neisseria gonorrhoeae*. Typical genital infections present as urethritis among men and cervicitis among women, but a broad spectrum of clinical presentations and complications can occur[1]. The oropharynx, anorectum and conjunctiva are sites of extragenital infection. The most common complications include epididymitis and pelvic inflammatory disease (PID), and although the frequency of disseminated gonococcal infection (DGI) decreased over the last decades[1], discrete outbreaks of DGI have recently been documented[2]. Many infected women remain asymptomatic or have only minor symptoms, resulting in delayed diagnosis, complications and uninterrupted transmission[1].

The incidence of gonorrhoea has increased in many countries around the globe during the last decade. For example, in the European Union–European Economic Area (EU–EEA) notification rates per 100 000 population increased continuously between 2014 and 2019 (from 5.9 cases in 2014 to 10.4 cases in 2019). After a decrease in 2020 to 9.5 cases, notification rates increased again in 2021 to 11.7 cases and in 2022 to 17.9 cases per 100 000 population[3]. The year 2022 marks the highest number of gonorrhoea cases in the EU-EEA over the last decade and the majority

of countries (25/28) observed substantial increases in 2022 in comparison to the previous year[4].

In Bulgaria, in contrast to the general trend in the EU-EAA, the notification rates of gonorrhoea per 100 000 population over the last ten years have been steadily decreasing (from 2.3 cases in 2014 to 0.04 in 2021[4,5]). Although our country has still one of the lowest notification rates (less than one case per 100 000 population), in 2022, a significant increase of more than 50 % was observed in comparison to the previous year[4]. In 2023, the notification rates doubled once again[6].

Current recommendations for the treatment of gonorrhea are published in the "*2020 European guideline for the diagnosis and treatment of gonorrhoea in adults*" from the IUSTI, and include parenteral therapy with a third-generation cephalosporin and accompanying treatment with azithromycin for additional coverage[7].

Alternative treatment options are limited, given the widespread gonococcal resistance to numerous antibiotic classes. For example, owing to the worldwide increase of *N. gonorrhoeae* strains resistant to fluoroquinolones, these agents now have limited clinical usefulness. Nonetheless, fluoroquinolones are highly effective against infection caused by susceptible gonococci, with cure rates of at least 98% for all anatomic sites[8]. Although their empirical use should not be recommended, these drugs could have an important role in patients with contraindications to cephalosporins, in whom no other option is feasible. In such cases, a culture isolate should be obtained so that susceptibility testing can be performed. Indeed, the above strategy for permissive fluoroquinolone use has been adopted by the British guidelines[9].

Gonococcal antimicrobial resistance remains a major problem and surveillance is necessary to address this threat. Across the EU-EEA surveillance is conducted by the European Gonococcal Antimicrobial Surveillance Program (Euro-GASP). Euro-GASP provides important data at the European level but is dependent on gonococcal culture for susceptibility testing. The use of nucleic acid amplification tests (NAATs) as routine diagnostics is increasing in many countries as well as in Bulgaria. When compared to cultures, NAATs offer several advantages including greater sensitivity, higher throughput, self-collected samples, and potential identification of more than one pathogen in one sample[10,11]. Ideally, NAATs would combine identification and resistance detection.

However, the complexity of *N. gonorrhoeae* resistance determination presents a major barrier for test development. For example, resistance to third-generation cephalosporins is predicted by four genes (*penA, penB, mtrR* and *ponA*), with the main determinant being the mosaic *penA* alleles[12]. Predicting azithromycin resistance necessitates the detection of *mtrD/mtrR* promoter mosaic 2 or semi-mosaic *mtrD* and not only 23S rRNA target mutations, which were previously the main cause of azithromycin resistance[13].

Currently, the prediction of resistance based on genetic characterization in *N. gonorrhoeae* is accurate only for fluoroquinolones, as the absence of mutations in serine codon 91 of the *gyrA* gene predicts susceptibility. A commercial test that combines pathogen identification with quinolone resistance determination is now available in Australia and Europe (SpeeDx ResistancePlus GC)[14]. The utilization of those tests in clinical settings allows the implementation of the resistance-guided therapy for gonococcal treatment with fluoroquinolones, which has already demonstrated success in some studies[15,16]. The results suggest that in the right patient population, fluoroquinolones could be very useful for treatment and could slow down antimicrobial resistance generation by reducing exposure to cephalosporins[15,16].

In our previous study from 2018-2021[17], a high prevalence of fluoroquinolone resistance in Bulgaria (59%) was detected by validated assays targeting resistance mutations in the *gyrA* and *parC* genes. This study aimed to make a comprehensive analysis of the rates of gonococcal fluoroquinolone resistance and epidemiological data of the cases in Bulgaria for 2022-2023 and to compare it with the results of previous years and the general trends in EU-EEA.

METHODS AND MATERIALS

Study population

From January 2022 to December 2023, a total of 1179 individuals (median age 30; 73.6% males and 26.4% females) attending the Center for Sexual Health

"CheckPointSofia" for voluntary and confidential HIV testing were referred to the National Center of Infectious and Parasitic Diseases (NCIPD, Sofia, Bulgaria) for gonorrhea testing based on symptoms and high-risk sexual behavior.

The routine testing was performed with Real-Time PCR (Neisseria gonorrhoeae Real-TM assay, Sacace Biotechnologies srl, Como, Italy). Following the diagnostic testing, DNA samples were stored frozen at −80 °C at NCIPD for further analysis.

During the study period, 66 *N. gonorrhoeae*-positive DNA samples (one sample per gonorrhea patient/episode) were obtained and confirmed with culture (n = 34) or with the *porA/opa* assay[18] (n = 32). When no isolate was available, confirmation by repeat testing with a PCR targeting another genetic sequence was imperative due to the suboptimal specificity of commercial assays, with the *porA/opa* assay being highly suitable for that purpose (clinical sensitivity and specificity of 100% and 99.3%, respectively). Phenotypic susceptibility testing of the corresponding *N. gonorrhoeae* isolates was performed by determining the minimal inhibitory concentrations (MIC), using gradient strips (Liofilchem srl, Italy) and interpreting the results according to EUCAST. The *N. gonorrhoeae*-positive DNA samples included genital (n = 33), pharyngeal (n = 10), ano-rectal (n = 21), and eye swabs (n = 2).

Detection of ciprofloxacin susceptibility/resistance markers

All stored DNA extracts from *N. gonorrhoeae*-positive samples were retrospectively analyzed by the commercial CE-IVD/IVDR certified ResistancePlus® GC assay (SpeeDx Pty Ltd, Sydney, Australia). The kit is intended to simultaneously detect the bacterium *N. gonorrhoeae* and the *gyrA* S91 (wild type) or *gyrA* S91F (mutant) markers that are associated with susceptibility or resistance to the fluoroquinolone antibiotic, ciprofloxacin. The assay was performed, according to the manufacturer's instructions on LightCycler® 480 Instrument II (LC480 II, Roche). All data were analyzed and reported using the ResistancePlus® GC (LC480) v1.0 analysis software.

Ethics and informed consent:

Written informed consent was obtained from all patients for epidemiological data collection and microbiological sample testing as required by the National Law and Ethics Committee at the National Center of Infectious and Parasitic Diseases.

RESULTS

***Neisseria gonorrhoeae*-positive cases**

The prevalence of *N. gonorrhoeae* among the referred individuals was 5.6% (95% CI 4.2% to 6.9%). The median age of the patients with confirmed gonorrhoea was 29 (age range 19–49). The male-to-female ratio was 21:1. The largest proportion of

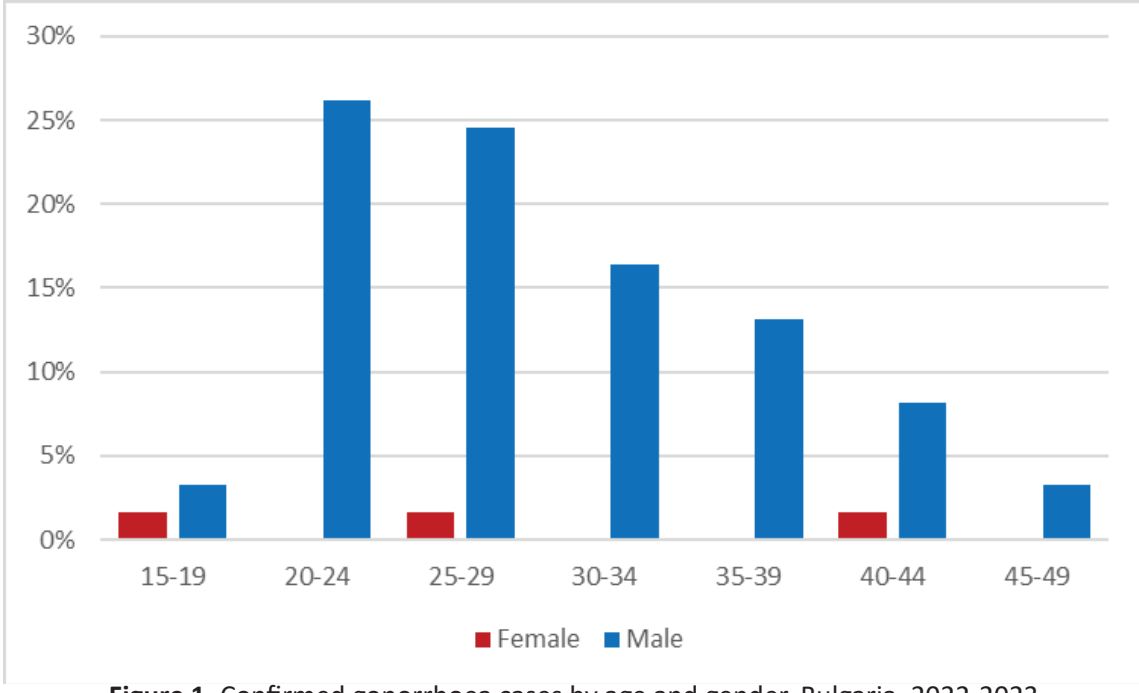

Figure 1. Confirmed gonorrhoea cases by age and gender, Bulgaria, 2022-2023

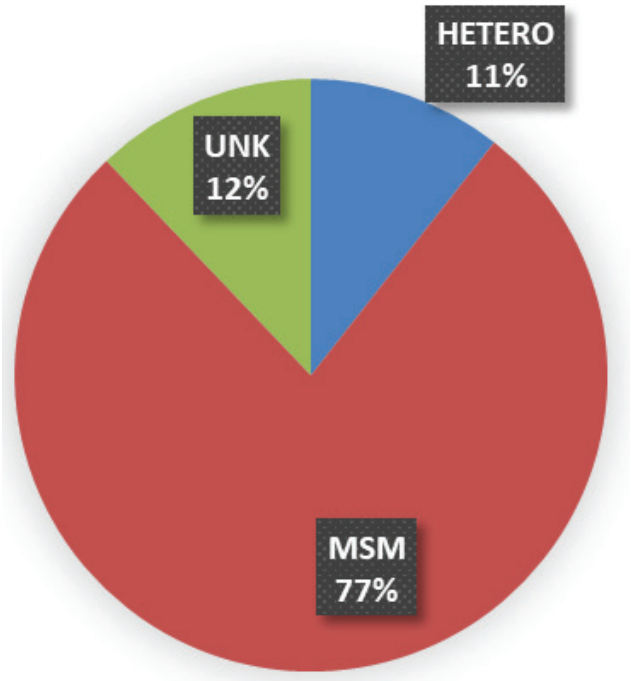

Figure 2. Confirmed gonorrhoea cases by transmission category, Bulgaria, 2022-2023

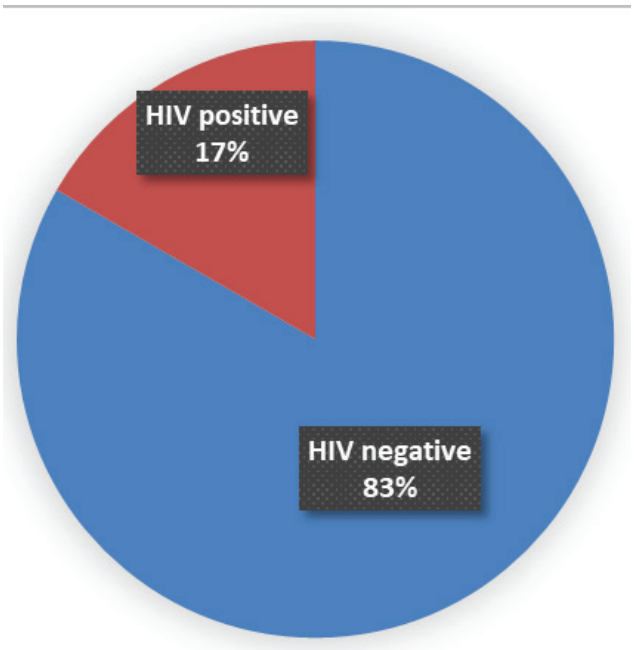

Figure 3. Confirmed gonorrhoea cases with known HIV status (n=60), Bulgaria, 2022-2023

cases was among males in the age group 20–24 years (26.2% of cases), followed by males in the age group 25–29 years (24.6%), (Figure 1).

Among the confirmed gonorrhoea cases, 77% were reported as men who have sex with men (MSM), 11% were reported as heterosexuals (57% males and 43% females), and 12% had no information on the mode of transmission (Figure 2).

Data on the HIV status of gonorrhoea cases reported in 2022 and 2023 were provided for 60 cases (91%). Among cases with known HIV status, 17% were HIV-positive (Figure 3).

Phenotypic susceptibility testing

According to the performed phenotypic susceptibility testing, of the available 34 *N. gonorrhoeae* isolates, 14 were susceptible and 20 were resistant to ciprofloxacin.

ResistancePlus® GC assay for ciprofloxacin resistance/susceptibility prediction

Detection of *N. gonorrhoeae*. The ResistancePlus® GC assay correctly identified all 66 DNA samples as *Neisseria gonorrhoeae*-positive.

Detection of *gyrA* S91 (wild type) and *gyrA* S91F (mutant). The *gyrA* S91 wild type was identified in 20 (30.3%) of the *N. gonorrhoeae*-positive samples, *gyrA* S91F was detected in 45 (68.2%) of the samples, and indeterminate *gyrA* results were obtained for one (1.5%) sample.

For the 34 *N. gonorrhoeae*-positive samples paired with *N. gonorrhoeae* isolates, ResistancePlus® GC assay detected GyrA S91F in all 20 samples resistant to ciprofloxacin according to the phenotypic susceptibility testing; and GyrA wild type was established in the other 14 samples. Accordingly, both the sensitivity and specificity of the assay for phenotypic prediction of the antimicrobial resistance was 100%.

The detected *gyrA* S91F (ciprofloxacin resistance) rate was 58.8% in the DNA samples paired with culture and 78.1% in the samples without confirmed culture (Table 1).

DISCUSSION

In this study, the prevalence of *N. gonorrhoeae* infections among individuals attending the Center for Sexual Health "CheckPointSofia" for voluntary and confidential HIV testing was estimated and gonococcal fluoroquinolone resistance rates were

Table 1. Investigation of *N. gonorrhoeae*-positive DNA samples (n=66) by ResistancePlus® GC assay for ciprofloxacin resistance/susceptibility prediction, Bulgaria, 2022-2023

	ResistancePlus® GC assay			
Samples (n)	**N. gonorrhoeae (*opa* + *porA*)**	***gyrA* S91 (wild type)**	***gyrA* S91F (mutant)**	**Intermediate**
N. gonorrhoeae-positive samples (66)	66	20 (30.0%)	45 (68.2%)	1 (1.5%)
• *Samples with paired culture (34)*	34	14 (41.2%)	20 (58.8%)	-
• *Samples without confirmed culture (32)*	32	6 (18.8%)	25 (78.1%)	1 (3.1%)

investigated by detection of *gyrA* S91 wild type or *gyrA* S91F mutant (ciprofloxacin susceptible or ciprofloxacin resistant, respectively). The prevalence of *N. gonorrhoeae* infection was 5.6% (95% CI 4.2% to 6.9%) and the detected fluoroquinolone resistance rate was 68.2%. Ciprofloxacin used for the treatment of gonococcal infections has the advantages of providing effective oral treatment of both urogenital and extragenital infections, limited side effects, and reduced selective pressure for emergence and spread of resistance to dual therapy with ceftriaxone and azithromycin[19]. Nevertheless, its current use in Bulgaria should be considered only after confirmed susceptibility.

Data on gonococcal antimicrobial resistance from the Balkan Peninsula countries is available only from Bulgaria and Greece. The detected fluoroquinolone resistance rate in Bulgaria was higher compared to neighboring Greece (66%)[3] and it had substantially increased in comparison to the previous Bulgarian study in 2021 (59%)[17]. The increase in Bulgaria is following the overall trend in Europe, where the proportion of isolates showing resistance to fluoroquinolones noticeably increased during the last several years: 65.9% in 2022 compared to that observed in 2020 (57.7%)[20].

Regarding the obtained epidemiological data, it was found that the average age of patients with gonococcal infection is decreasing in comparison to previous observations (from an average of 32 years in 2019[21] to 29 years in the current study), with almost one-third of patients identified to be less than 25 years old (31.2%). It is well known that young adults bear the highest burden of STIs . This fact may be attributed to several factors, i.e. riskier sexual behaviors, lack of awareness, social stigma, and/or limited access to sexual health services[22]. Promoting sexual education, regular testing, and safe practices are crucial in addressing this trend. In the present study, the male-to-female ratio was very high (21:1), and only several cases of gonorrhea in women were identified. The comprehensive data regarding the male-to-female ratio for Bulgaria for the last ten years are close to the EU-EEA averages (ranging from 3.5 in 2014 to 4.7 in 2020). From 2021 on, a predominance of male gonococcal patients started, exceeding a ratio of 10:1, and even - lack of confirmed cases in women[3]. Although our neighboring countries (such as Greece, Serbia and Romania) also report high male-to-female ratios (26.7:1, 12.2:1 and 10.5:1, respectively[3]) the fact is concerning, since women with asymptomatic gonorrhea may not seek healthcare services. While gonorrhea often may be asymptomatic in women, it can still lead to serious complications if left untreated[1]. Encouraging regular testing and awareness about the risks of untreated infections is crucial and should be considered in local guidelines and programs. Obtaining epidemiological data regarding transmission and HIV status for gonorrhoea patients can be challenging due to stigma and privacy concerns by patients and limited resources for surveillance and data collection by health professionals. Efforts to improve data collection should involve collaboration between healthcare

providers, laboratories, and public health agencies. In the present study, data on the mode of transmission were collected in 88% of cases, and HIV status was established in 91%. Transmission data show that in Bulgaria, the largest burden of gonococcal infection goes to MSM (77% of all cases), and epidemiological interventions to reduce incidence in this group would have an impact on enhancing the control and prevention of gonococcal infection. Of all cases with known HIV status, 17% were positive, which is above the average proportion in EU-EEA (12%[4]). The prevalence of HIV co-infection among people with gonorrhoea is an important concern. Studies have shown that the discharge of HIV in body secretions of people co-infected with gonorrhoea is significantly higher , increasing the risk of HIV transmission by 3 to 5 times[23,24]. While specific percentages may differ across populations, understanding this co-infection is crucial for effective prevention and treatment strategies.

The significant increase in gonococcal notification rates in the last two years in Bulgaria, although on a smaller scale in terms of absolute number of cases, gives grounds for public health concern. Gonorrhoea has been recognized as a significant public health problem internationally and prevention, diagnosis, and therapy strategies are used to reduce the burden of the disease[25]. Ongoing also is the improvement of antimicrobial stewardship through the implementation and use of better surveillance systems to detect antimicrobial resistance in *N. gonorrhoeae* and to inform locally appropriate treatment[17]. Sensitive and specific molecular assays for the prediction of *N. gonorrhoeae* antimicrobial resistance are needed, both to inform personalized treatment and for antimicrobial resistance surveillance, which is further highlighted by the WHO global action plan[26].

Compared to cultural diagnostics and phenotypic susceptibility testing, molecular assays such as the ResistancePlus® GC assay have many advantages, including superior sensitivity and high specificity, shorter turnaround time, automation, high throughput and potential for use as rapid tests[27]. However, a limitation of the molecular assays for the detection of resistance determinants is that they cannot detect new determinants of antimicrobial resistance. They cannot provide a complete profile of antimicrobial resistance with minimum inhibitory concentrations of the antimicrobials, either[11]. In addition, it can be very labor-intensive to detect more than one resistance determinant.

In the current study, the commercially available ResistancePlus® GC assay showed high sensitivity and specificity for the detection of *N. gonorrhoeae*, as compared with the confirmatory *porA/opa* assay[18]. The assay showed also an excellent ability to detect and distinguish *gyrA* S91 wild type and *gyrA* S91F and to predict ciprofloxacin resistance/ susceptibility compared with phenotypic testing. The results support the intended use of the assay for both detecting the ciprofloxacin resistance/susceptibility and confirming positive results of commercial PCR assays for gonorrhoea diagnostics, when no isolate is available. Furthermore, it can be effectively used for antimicrobial resistance surveillance and individualized treatment with ciprofloxacin, which is easily accessible and administered as an oral regimen[28]. Unlike our previous study where both *gyrA* and *parC* genes were investigated, in the present study only *gyrA* was targeted because it has proven to be the most effective target for the detection of ciprofloxacin resistance, i.e. mutations in *parC* are never found without concurrent mutations in *gyrA*[17,27].

However, one sample (1.5%) remained indeterminate for *gyrA*, which is likely due to low *N. gonorrhoeae* load in the clinical DNA sample and/or inhibition and cross-reactions with other Neisseria species[29] In particular, extragenital sites (such as the case) are challenging for molecular resistance prediction, because these sites frequently harbor non-gonococcal Neisseria species as commensals and many identical or very similar DNA sequences from other commensal species, including resistance determinants[30].

Finally, a statistically significant difference (p=0.049) was found in the resistance rates in DNA samples paired with culture (58.8%) and DNA samples without confirmed culture (78.1%), making the comprehensive fluoroquinolone resistance rate higher than conventionally reported (68.2%). This is of utmost importance because conventional gonococcal antimicrobial resistance surveillance is done only by phenotypic testing. Given the fact

that in EU/EEA (Bulgaria included) about half of the confirmed gonorrhoea cases are not cultured and no phenotypic testing is performed, it should be acknowledged that conventional surveillance is non-comprehensive and antimicrobial resistance could be much more prevalent than reported. This underlines the importance of a national laboratory network able perform culture studies, alongside with molecular studies in order to obtain comprehensive data about the spread of gonococcal antimicrobial resistance in our country.

CONCLUSION

The fluoroquinolone resistance rate found in this study (68.2%) was significantly higher than that observed a few years ago (59%). The increased prevalence of fluoroquinolone resistance in Bulgaria follows the general trend in Europe during the recent years. In EU-EEA the proportion of isolates showing resistance to ciprofloxacin substantially increased: 65.9% in 2022 as compared to 62.8% and 57.7% in 2021 and 2020 respectively. The molecular testing for predicting ciprofloxacin susceptibility/ resistance in gonococcal infections is very suitable for supporting effective gonococcal antimicrobial resistance surveillance, and simultaneously with diagnostics - individualized treatment decisions, thus reducing exposure to unnecessary empiric therapy and slowing the spread of resistance.

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KLEBSIELLA PNEUMONIAE – CAUSATIVE AGENT OF ENTEROCOLITIS. A BRIEF LITERATURE REVIEW.

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ABSTRACT

Gastrointestinal diseases have one of the highest incidence rates worldwide. *Klebsiella spp*., which is a part of the large family *Enterobacterales*, isolated from fecal samples is considered as a part of the normal intestinal flora, even when it presents as a monoculture. The transmission of virulent plasmids from *E. coli* strains to *Klebsiella spp*, raises the question whether those bacteria can be an etiological factor for severe diarrhoea. By using PCR methods, the *lth* gene which is coding heat-labile enterotoxin (LT) was presented in the plasmids of *Klebsiella spp* strains, and its expression was assessed.by measuring the cytopathic effect induced by the LT toxin,

For the first time in 1882, Carl Friedlander isolated *K. pneumoniae* from lung samples from patients dead of pneumonia [1]. *K. pneumoniae* species includes closely related to *K. pneumoniae Kp1-Kp7*. These phylogroups include the following subspecies of *K. pneumoniae subsp. ozaenae, K. pneumoniae subsp. pneumoniae, K. pneumoniae subsp. rhinoscleromatis, K. quasipneumoniae subsp. quasipneumoniae, K. quasipneumoniae subsp. similipneumoniae, K. variicola subsp. variicola, K. variicola subsp. tropica, K. africana and K. quasivariicola* [2,3]. Based on its capsular antigens, *Klebsiella pneumoniae* can be distinguished and classified by serotyping. Currently, 77 serotypes belong to the K-antigen and another 12 belong to the O-antigen serotype. *K. pneumoniae* can be found in nature, as well as in animals, plants, and humans. [4].

K. pneumoniae colonizes human nasopharynx and gastrointestinal tract. The bacterium is a frequent causative agent of urological tract infections (UTIs), meningitis, sepsis, and wound infections. It is for this reason that for many years the isolation of *Klebsiella spp*. from faecal samples has not been a concern to specialists and is regarded as a part of the normal intestinal flora. Even when isolated from diarrhoeal stools in monoculture, these microorganisms have not been treated as an etiological agent of gastrointestinal infections. In 1975, were obtained the first data on the ability of *Klebsiella pneumoniae* strains isolated from tropical sprue patients to secrete enterotoxin [5]. Enterotoxin activity has been shown to induce secretion of water and electrolytes and to cause structural changes in the intestinal mucosa in various animals [6]. In 1976, thermostable (ST) toxin and thermolabile (LT) toxin were discovered [7]. Later in 1983, Klipstein and Engert demonstrated that partially purified filtrates of LT toxin-producing strains were able to stimulate water and electrolyte secretion in the rat intestine. Also, oral administration of LT toxin-producing strains to piglets resulted in severe diarrhoea [8].

Colonization of gastroinsestinal tract of healthy people with unspecified pathotypes ranges from 5 to 35% in Western countries [9, 10], whereas in Asian countries the percentage is higher: from 19 to 88% [11]. During warm months, the incidence of infections with *K. pneumoniae* via the blood route is 1.5-fold higher, reflecting the increased incidence of faecal carriage in humans during summer [12]. Screening of healthy individuals is recommended for the detection of new resistant and virulent strains as well as to gain insight into strain diversity. [13].

The pathogenetic changes caused by *K. pneumoniae* in the gastrointestinal tract are no less complicated than that in urinary tract infections, meningitis, etc. To promote its growth and survival in the host, the bacterium excretes and chelates iron from the host's haemoglobin and transferrin by secretion of siderophores [14]. The host fights bacterial infection

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by secreting free iron found in plasma and binding it to an innate protective protein called lactoferrin [15]. The reduction of free iron in the host environment causes bacteria to secrete siderophores that bind competitively with iron due to its high affinity, chelating it from host proteins such as haemoglobin, ferritin and myoglobin [16]. The ability of a bacterium to secrete more than one type of siderophore increases its ability to colonize the gastrointestinal tract and prevent the neutralization of a single siderophore by the host. The most abundant siderophore secreted by *K. pneumoniae* is enterobactin due to its high affinity for iron [17]. Another factor of *K. pneumoniae* virulence is lipopolysaccharide (LPS), an endotoxin that protects bacteria from host humoral defences [18]. This is due to the ability of *K. pneumoniae* to modify the structure of its LPS, in particular the lipid A moiety, in a way that it would not activate host inflammatory responses, thus increasing bacterial virulence.

During the colonization of the gastrointestinal tract, *K. pneumoniae* causes damage to the intestinal mucosa by secreting a potent toxin called colibactin, which is also produced by some other members of the *Enterobacterales* order, including *E. coli*. The colibactin toxin induces genomic instability by disrupting the cell cycle and DNA repair machinery [19]. Colibactin also activates the senescence-associated secreted protein (SASP) phenotype that promotes tumour development [20]. Once the cell ages, it is inert to mitogenic and oncogenic stimuli, which would be beneficial. In the last decade, however, new evidence has revealed that SASP can be a double-edged sword. During cell senescence, there is increased secretion of inflammatory cytokines, chemokines, growth factors and matrix metalloproteinases (MMPs) into the surrounding tissue microenvironment. This can lead to the development of diseases associated with gastrointestinal inflammation.

K. pneumoniae is classified as an extracellular bacterium, however, in vivo and in vitro studies have also demonstrated the capacity of *K. pneumoniae* to invade and persist in intestinal epithelial cells [21]. Intracellularly, the bacterium can grow, replicate and exit the basement membrane. The potential of *K. pneumoniae* to invade and develop intracellularly, coupled with the release of colibactin toxin, can exacerbate inflammation and disease development. Prolonged exposure to virulent strains of *K. pneumoniae* and their colonization in the gastrointestinal tract can lead to the development of inflammation-induced diseases such as Crohn's disease, ulcerative colitis, and irritable bowel disease. Studies have documented an increased prevalence of *K. pneumoniae* in patients suffering from ulcerative colitis compared to healthy controls. One study revealed that increased colonization of *K. pneumoniae* in the colon would lead to colitis. *K. pneumoniae* can increase the expression of cyclooxygenase (COX)-2, IL-6, IL-1β, and TNF-α, which are potent proinflammatory stimulators [22]. They also increase the levels of lipid peroxidation in the colon [23, 24]. *K. pneumoniae* also increases the production of β-glucuronidase and LPS, which induce NO and COX-2 production in murine peritoneal macrophages and as a result suppresses host immune responses. The protective role of lactic acid bacteria such as Lactobacillus sp. and others was suggested to reduce inflammation caused by pathogenic intestinal bacteria. Thus, dysbiosis of gut microbiota, with increased population of *K. pneumonia, and* reduced population of protective lactic acid bacteria, is associated with inflammatory diseases such as Crohn's disease and ulcerative colitis [25]. Crohn's disease is an idiopathic form of inflammatory bowel disease affecting the terminal ileum that develops following chronic generalized enteritis. Affecting mainly individuals from 20 to 30 years, it has a higher prevalence in developed countries [26,27]. The pathogenesis mechanism of Crohn's disease is charcterized with recurrent subclinical infections leading to increased *K. Pneumoniae* specific antibody titers cross-reacting and binding to intestinal collagen fibres of the terminal ileum, and activating the complement pathway and proinflammatory cascades. The influx of proinflammatory cytokines into the terminal ileum induces inflammation. Recurrent infections with *K. pneumoniae* lead to a continuous cycle of damage to the colonic mucosa by various cytokines, ultimately leading to the development of Crohn's disease.

In the last decade, the role of *Klebsiella spp*. produced enterotoxins (LT) as virulence factors in the pathogenesis of diarrhoea has been revealed. These toxins are thermolabile and cytotoxic to the intestinal

epithelium, similar to the interpolable toxins produced by enterotoxigenic *E. coli (ETE*C) strains, which are an etiological factor for severe dehydrating diarrhoea in humans and animals [28,29]. The genes encoding enterotoxins are predominantly located on plasmids [30] and can therefore be transferred between gram-negative microorganisms such as *Klebsiella pneumoniae, Citrobacter freundii, Yersinia enterocolitica, Enterobacter cloacae and Aeromonas hydrophila* [31,32,33]. The production of LT toxins depends on external factors such as temperature, pH, osmotic stress and concentration of nutrients in the medium. The cytopathic effect induced by *K. pneumoniae* is weaker compared to the effect induced by enterotoxigenic strains of E. coli. Studies have shown that the gene for enteric labile toxin lth is present in *Klebsiella pneumoniae and Klebsiella oxytoca* strains isolated from patients with diarrhoea [34]. Persistent toxin infectious- and haemo-colitis syndromes are demonstrated in patients with concomitant diseases and infancy. Gastrointestinal infection by *Klebsiella pneumoniae and Klebsiella oxytoca* may proceed with the clinic of viral enteric infection. Infections caused by *K. ohutosa* present with severe toxaemia and haemocolitis in patients with concomitant diseases and infancy. However, Klebsiella bacterial enteric infection does not always proceed with hemorrhagic diarrhoea [35]. *Klebsiella spp*. enterotoxins can be important pathogenic factors causing diarrhoea in humans, which turns them from opportunistic to pathogenic bacteria, especially in the immunocompromised, and in patients with gut dysbiosis following antibiotic therapy.

In conclusion, the prophylaxis and early diagnosis of Klebsiella spp. with evidence of toxin production, especially among hospitalized patients, is of utmost importance to prevent the dissemination of antimicrobial resistance, which has been intensively increasing among the genus.

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HUMAN PATHOGENS AMONG BATS

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ABSTRACT

Bats are known to inhabit both caves and open space areas. Bulgaria is among the European countries with the highest number of bat species. The species found in the country are distributed over a wide area. They range from the Pyrenees and the British Isles to the Pacific region and the Far East. Many bat species are carriers of potential human pathogens. Bats play an important role in agriculture and act as a biological pest crop control agent. Bulgarian bat ecosystem comprises temperate climate and a wide range of abiotic factors, including humidity, darkness, sunlight, and temperature variations. The metabolism of bats and their body temperature vary significantly between the period of activity and the hibernation. Fluctuations in body temperature can potentially impact host microbiome biodiversity. Temperature variations may induce a high level of microbial mutagenesis. Additionally,the existence in large, mixed-species colonies, togeteher with a relatively long individual lifespan (4 – 16 years) and extensive travel distances, enhances the likelihood of encountering multiple pathogens in a single host organism. This, in turn, facilitates genetic variations and re-combinations among those microbial pathogens, thereby raising their potential to breach species barriers. In this review, we summarized and analyzed the available scientific information concerning the potential microbial human pathogens associated with bats, alongside with our preliminary data on the biodiversity of bats blood microbiome. Future research should focus on bats as both pathogens carriers and dynamic models for predicting emerging and re-emerging zoonotic diseases.

Key words: bats, viriome, bacteriome, metagenomics, zoonoses, vector, ectoparasites

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INTRODUCTION

Bats are the second biggest mammalian order after rodents comprising more than 1400 species or about 20% of the recent mammalian species. They are the only mammals capable of sustained flight. Taxonomically, bats are grouped in order Chiroptera. About 43 bat species are encountered in Bulgaria. Bulgaria stands out as one of the European countries with the highest diversity of bat species. The majority of European species are found in the country, with only a few exceptions including *Myotis escalerai* Cabrera, 1904, *Myotis punicus* Felten, 1977, *Eptesicus isabellinus* Temminck, 1840 and *Rousettus aegyptiacus* (Geoffroy, 1840). In Bulgaria, three bat families are present: Rhinolophidae, Vespertilionidae, and Molossidae. (1).

Bats, known for their social and communicative nature, often live in large colonies (2). This social structure, along with their ability to fly over large distances potentiates a quicker spread of potential infectious diseases compared to many other mammal groups. Typically, bat colonies consist of various species, further facilitating the development of mechanisms by which microbes can overcome species barriers.

The body temperature of bats at rest is 38 ^{0}C, while in active flight it reaches 41 ^{0}C which resembles a state of infectious process in man. On the opposite, during the hibernation period, the body temperature of the animal drops to match that of the environment, typically around 6-12°C (3). The specific bat physiology and their environmental preferences modulate a microbial flora with unpredictable potential.

In Bulgaria, as in most European countries bats are

predominantly insectivorous. This diet exposes them to pathogens carried by insects, which can be potentially transmitted further up the food chain. Insect vectors can transmit infectious pathogens between humans and animals, or vice versa. Additionally, many biting and blood-sucking ectoparasites are spread by bats, such as ticks, lice, fleas, and flies. These parasites have the potential to act as reservoirs or secondary vectors for various pathogens.

Bats have a relatively long lifespan for their size, 4 years on the average, with exceptions up to 16 years (4,5), which provides a long timeframe for carrying and potentially spreading pathogens. This extended lifespan increases the period during which they can act as reservoirs and vectors of diseases and a site of potential genetic recombination (horizontal genetic transfer) between transmitted pathogens (6).

Bats are significant reservoir hosts for a wide array of pathogens. It should be underlined that they are main or occasional reservoirs of many viral (rabies, Marburg, Ebola, Hendra, Nipah, SARS-CoV, etc.), bacterial (*Bartonella, Leptospira, Mycoplasma, Brucella, Borrelia, Coxiella, Ehrlichia, Francisella, Mycobacteria* and *Rickettsia*), fungal (*Pseudogymnoascus destructans* - white-nose syndrome, *Histoplasma capsulatum* and others) and protozoan (malaria, toxoplasmosis, leishmaniasis, trypanosomiasis) infections. Direct transmission of pathogens from bats to humans is uncommon due to limited contact. However, pathogen spread often occurs through intermediaries like ectoparasites, bat guano, and other excretions. Humans can be affected either by the pathogens or the byproducts of their metabolism, such as toxins.

Climate changes directly or indirectly affect the distribution of pathogens in bat populations, increasing the risk of contact with humans and the likelihood of zoonotic disease transmission. The present review aims to describe the pathogenic viruses and bacteria identified in bats and their parasites, such as ticks, fleas, flies, and lice from the Northern temperate zone.

Human viral pathogens associated with bats

Bats are a suitable reservoir for viral zoonoses and more than 200 viruses have been isolated from or detected in bats (7,8). Bats are vectors of about twenty viral families (9). Table 1 shows key viral families with zoonotic potential, including *Adenoviridae, Astroviridae*, *Caliciviridae*, *Coronaviridae, Filoviridae, Flaviviridae, Hantaviridae, Herpesviridae, Paramyxoviridae, Parvoviridae, Poxviridae, Rhabdoviridae* and *Retroviridae*. These families comprise viruses that can be transmitted from animals to humans.

Different species of Chiroptera could be a reservoir of mutated viruses that extend the viral host range by overcoming the species barrier between bats and humans. This was suspected for SARS-CoV-2 that caused COVID-19 pandemics in 2020 (10). COVID-19 is a disease of presumed bat origin that emerged as a result of a mutation and was transmitted to humans from bats after overcoming the species barrier. The infection is caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), a member of the viral family *Coronaviridae*. Bats are reservoirs of this family worldwide and they are vectors of more members of *Coronaviridae* e than any other taxa. It is suggested that the world pandemic started as a result of a mutation related to SARS-CoV-2 species in bats at the end of 2019 in the region of Wuhan, China. From January 2020 to April 28, 2024 the pandemic caused 7 047 396 registered deaths (https://data.who.int/dashboards/covid19/deaths?n=c) in the world, more than any infectious disease for the same period. A reservoir for SARS-CoV-2 and other similar viruses are *Rhinolophus* bats in Asia, which is one of the most common genera in Bulgaria, represented by 5 species. Possibly some mutations in bats or pangolins were the reason for overcoming the species barrier between bats and humans (11,12). Such evolution of viral fitness taking place in bats is possible and therefore investigation of bat-transmitted pathogens is very important.

Another widely spread viral family within the order Chiroptera is *Adenoviridae*. Members of this family are the cause of various diseases like conjunctivitis, gastroenteritis, and obesity (13). One of the bat vectors for *Adenoviridae* is *Pipistrellus kuhlii* spread in the South-Western and North-Eastern parts of Bulgaria. This bat species is synanthropic and its habitats are often buildings. *Pipistrellus kuhlii* and its excrements may pose an infection risk when in close contact with humans.

Table 1. Diseases and vectors of viral species identified in bats.

Viral family (representatives)	**Bat host**	**Diseases**	**Secondary/ Intermediate vectors**
Coronaviridae	*Rhinolophus*	COVID-19, MERS	pangolins, camels
Adenoviridae	*Pipistrellus kuhlii*	Respiratory Infections	n.a.
Caliciviridae	*Myotis daubentonii* *Eptesicus serotinus*	Gastroenteritis	vertebrates
Flaviviridae	Flying foxes	Ebola, Marburg	non-human primates
Flaviviridae	*Miniopterus schreibersii*	Japanese encephalitis virus (JEV)	ticks, mosquitos
Herpesviridae (*Epstein-Barr virus*)	*Myotis lucifugus*	Mononucleosis, Pneumonia. Kaposi's sarcoma, etc.	n.a.
Poxviridae	*Miniopterus schreibersii*	Smallpox	n.a.
Retroviridae	*Miniopterus, Rhinolophus*	AIDS	non-human primates
Orthomyxoviridae (H17N10 and H18N11)	New world bats	Influenza	birds
H9N2-like influenza A virus	Old world bats		

Bats together with cattle, pigs, cats, chickens, reptiles, dolphins, and amphibians are vectors of the viral family *Caliciviridae*. Members of caliciviruses are cause respiratory diseases, rabbit hemorrhagic disease (often with fatal hepatitis), and gastroenteritis (Norwalk group of viruses). *Myotis daubentonii* and *Eptesicus serotinus* are common carriers of caliciviruses and they are widely spread in Bulgaria.

Filoviridae is a viral family transmitting infections in humans and some animals. Two members of the family that are commonly known are the *Ebola virus* and the *Marburg virus*. Both viruses cause severe disease in humans and nonhuman primates in the form of viral hemorrhagic fevers (14). These two zoonoses have chiropteran vectors which are spread in Africa and are not present in our country.

Flaviviruses are a viral family whose transmission from animals to humans is mediated by arthropod vectors such as mosquitoes and ticks. They comprise the species *Zika virus*, and several other encephalitis viruses. One of the members of this family is a *Japanese encephalitis virus* (JEV) which is spread in Southeast Asia. One of the vectors of JEV - *Miniopterus schreibersii* - is a common and abundant species in Bulgaria, and in case of mutation, this disease might be transmitted to areas with a temperate climate (15).

Lyssaviruses are a genus of negative-sense single-strand RNA viruses in the family *Rhabdoviridae*. The genus *Lyssavirus* includes the rabies virus traditionally associated with the rabies disease. In Europe, bat *lyssaviruses* were detected in the United Kingdom, the Netherlands, Finland, Denmark, Poland, Czech Republic, Germany, Switzerland, France, Spain, Hungary, Italy, Slovenia, Croatia, Bulgaria, Ukraine, and Russia (7). Table 1 represents the distribution by viral family and bat species identified specifically in Northern temperate zone including Bulgaria for the last 16 years (9,16,17).

Betaherpesvirinae and *Gammaherpesvirinae* are subfamilies of *Herpesviridae* causing infections and certain diseases in animals and humans (18). Bats present in Bulgaria are common vectors of these two viral subfamilies (19). The widespread human cytomegalovirus (HCMV or HHV-5) is a Betaherpesvirus causing mucoepidermoid carcinoma. One of the gammaherpesviruses, *Epstein–Barr virus* (EBV or HHV-4), is implicated in several diseases, including mononucleosis and some cancers. Their vectors are European bat species present in Bulgaria (20).

Poxviridae is a viral family of double-stranded DNA viruses. The eradicated *Smallpox virus* belongs to this family. *Miniopterus schreibersii* is one of the vectors

of poxviruses (21).

The *Retroviridae* family of viruses includes the *Human immunodeficiency virus* (HIV), which is the etiological agent of acquired immunodeficiency syndrome (AIDS) and other zoonotic diseases. Miniopterus and Rhinolophus bats are common vectors of deltaretroviruses (22).

Human bacterial pathogens associated with bats

Most of the human bacterial pathogens associated with bats are food-borne related bacterial pathogens, zoonoses like brucellosis, tularemia (23), and atypical mycobacterial infections. Bats can spread the bacteria during their migration directly or mediated by their ectoparasites like mosquitos, ticks, fleas, and lice. Many of these pathogenic bacteria belong to Risk Group 2 or higher according to the guidelines of the National Institutes of Health (NIH) (https://absa.org/riskgroups/).

The most common bacterial pathogenic family observed in the gut of bats is Enterobacteriaceae. This family includes mainly food-borne pathogens, 1–5 µm long, Gram-negative rods (24). Many representatives of Enterobacteriaceae are part of the normal intestinal microflora and can be found in the intestines of humans and other animals, while the rest live in soil, water, or are parasitize various plants and animals. Seventeen human pathogenic genera of Enterobacteria (*Enterobacter, Citrobacter, Hafnia, Serratia, Providencia, Klebsiella, Morganella, Escherichia, Proteus, Cronobacter, Kluyveraare, Moellerella, Leclercia, Pantoea, Rahnella, Salmonella,* and *Shigella*) were identified in bats across Europe. *Myotis myotis*, *Miniopterus schreibersii*, *Rhinolophus hipposideros*, and *Myotis capaccinii* are common vectors of Enterobacteria species (25). *Yersinia pseudotuberculosis*, an enteric human pathogen, has been observed to cause outbreaks in European bat populations. Another enteric human pathogen – *Yersinia pseudotuberculosis* is not typically a pathogen of bats, but it can cause outbreaks in European bat populations (24,26). Table 2 displays the range of microbial pathogens that are present in bats.

Leptospira species like *L. interrogans* and *L. kirschneri* are pathogens that affect both humans and bats. These bacteria infect the renal tubules of animals and people and are excreted via urine into the environment. Most bat vectors of *Leptospira* are spread in tropical regions but recent studies showed the presence of this bacterium in *Barbastella barbastellus*, *Myotis bechsteinii, Myotis myotis*, and *Myotis nattereri* (27). Dogs and rodents are also carriers of this pathogen. (28,29).

Tularemia is a disease caused by *Francisella tularensis* considered a high-risk agent with the potential to be used as a biological weapon. Recently this bacterium has been identified in the bats *Pipistrellus pipistrellus, Nyctalus noctule, Pipistrellus kuhli*, and their ectoparasites (22). Other bacterial agents belonging to special pathogens are the species of the genus *Brucella*, the causative agent of brucellosis. *Brucella* species were identified in *Myotis blythii* (30). *Bartonella* is a genus of Gram-negative facultative intracellular parasitic bacteria. *Bartonella* species are transmitted by ticks, fleas, and mosquitoes. These bacteria are responsible for bartonellosis (Carrión's disease, trench fever, cat-scratch disease, bacillary angiomatosis, peliosis hepatis, chronic bacteremia, endocarditis, chronic lymphadenopathy, and neurological disorders). Bat species *M. schreibersii* and *M. blythii* are vectors of *Bartonella* species (26).

Pasteurella is a genus of Gram-negative, facultative anaerobic bacteria causing pasteurellosis, a skin or subcutaneous tissue disease like septic phlegmon which develops most often on hand or forearm after a cat bite. After infection, edema, severe pain, and serosanguineous exudate appear very rapidly. Transmission of *Pasteurella* from bats to humans is not proven, but the presence of these bacteria is common in chiropteran species like *Pipistrellus pipistrellus, Pipistrellus pygmaeus, Pipistrellus kuhlii, Plecotus auratus, Vespertilio murinus, Pipistrellus nathusii, Myotis mystacinus, Vespertilio murinus, Plecotus auritus*, and *Eptesicus serotinus* (27).

Nontuberculous Mycobacteria (NTM) are emerging pathogens causing opportunistic infections in humans and animals. Their distribution in caves and bats in Bulgaria is poorly studied. A recent study performed by Atanasova et al. (31) demonstrated that most of the NTMs were identified in bat guano (67%, n=16). Other materials positive for NTMs were water and biofilm (13%, n=3 each), sediment and clay (3%, n=1 each). The mycobacterial species diversity identified in the

Table 2. Human bacterial pathogens found in European bats.

Microbial taxa	Carriers	Diseases	Alternative vectors
Brucella spp.	*Miniopterus schreibersii, Myotis blythii*	Brucellosis	Domestic animals
Francisella tularensis	*P. pipistrellus, Nictalus noctula, P. kuhlii*	Tularemia	Arthropods, mammals
Enterobacteriacea	Chiroptera order	Gut infections	
Yersinia pseudotuberculosis	*Myotis myotis*	Far East scarlet-like fever	Animals and birds
Leptospira spp.	*Barbastella barbastellus, Myotis bechsteinii, Myotis myotis, Myotis nattereri*	Renal infections	Dogs
Pasteurella (*P. multocida Pasteurella canis, Pasteurella dagmatis, Pasteurella stomatis*)	*Pipistrellus pipistrellus, Pipistrellus pygmaeus, Pipistrellus kuhlii, Plecotus auratus, Vespertilio murinus, Pipistrellus nathusii, Myotis mystacinus, Vespertilio murinus, Plecotus auritus, and Eptesicus serotinus*	Pasteurellosis	Cats, dogs
Bartonella (*B. bacilliformis, B. quintana*, *B. henselae*)	*M. schreibersii and M. blythii*	Bartonellosis Carrión's disease, trench fever, cat-scratch disease, bacillary angiomatosis, peliosis hepatis, chronic bacteremia, endocarditis, chronic lymphadenopathy, and neurological disorders	Cats, dogs rodents

samples included: *M. chelonae* (n=3), *M. gordonae* (n=2), *M. intermedium* (n=3), *M. scrofulaceum* (n=1), *M. szulgai* (n=4), *M. fortuitum* group (n=4), mixed culture (n=4), *M. terrae* complex (n=1). Similar results were obtained by Pavlik et al. (32) by studying a total of 281 guano samples collected from caves (N = 181) in eight European countries (Bulgaria, Czech Republic, France, Hungary, Italy, Romania, Slovakia and Slovenia). The authors reported that 73 mycobacterial isolates were identified as members of three groups (*M. fortuitum*, *M. chelonae*, and *M. mucogenicum*) and four complexes (*M. avium*, *M. terrae*, *M. vaccae*, and *M. smegmatis*). A total of 20 isolates (22.5%) belonged to risk group 1 (environmental saprophytes), 48 isolates (53.9%) belonged to risk group 2 (potential pathogens), and none of the isolates belonged to risk group 3 (obligatory pathogens). The presence of NTM in cave ecosystems represents a potential source for human infection.

Recently our group has studied the blood microbiome of bats. Our results showed that a significant number of non-pathogenic and pathogenic bacterial species could be detected in bats blood. The study included shotgun metagenomics sequencing combined with a bioinformatics approach for microbial identification. Sequencing reads exceeding 10K were identified for *Mycobacteria, Bartonella, Plasmodium* and *Leishmania* species. Soil, water, and guano samples demonstrated high abundancy of mycobacterial presence. Other pathogenic species such as T*oxoplasma* spp. have also been identified but less evidently according to the number of reads. Our results demonstrated that bats in Bulgaria are a hidden or potential reservoir for malaria and atypical mycobacterial infections. Mycobacterial presence was confirmed by targeted conventional PCR.

Fungal infections in bats

Bats can potentially be carriers and spreaders of fungal infections (33). Fungal infections have been identified as either contracted by bats themselves or as bat-borne mycoses in other host species, including

humans. Research data on the fungal microflora present in the gut of different bat species and their dietary habits is limited. Reports on the occurrence of fungal species in the bats' closer environment, such as ectoparasites or bat guano, are rare. The most well-known association between bats and mycotic disease in human patients is *Histoplasma capsulatum*. Infections caused by this fungus are contracted while visiting caves inhabited by bats in tropical countries.

The distribution and impact of white-nose syndrome, caused by the psychrophilic fungus *Pseudogymnoascus destructans* (formerly *Geomyces destructans*), on European bats has yet to be fully determined (34). Dermal fungal infiltrations were observed in biopsies of bats belonging to *Myotis blythii*, *M. daubentonii*, *M. emarginatus*, and *M. myotis*. of A higher susceptibility to infection of the *Myotis* genus, especially *M. emarginatus* was suggestied, possibly due to the longer hibernation period of these bats. Genomic studies indicate that P. destructans is native to Eurasia (35). No human infections attributable to *Pseudogymnoascus destructans* have been reported. Studies have shown that the fungus grows at temperatures much lower than that of the human body. Temperature fluctuations during bat's flight and rest may pose a risk for breaching the species barrier between bats and humans.

Some causative agents of parasitoses are distributed by European bats, especially in pair with their arthropod ectoparasites like ticks and flies (36). Intracellular parasitic eukaryotic Apicomplexa with the main human pathogenetic species *Toxoplasma gondii* is spread by *M. schreibersii*. Most bat species in Bulgaria carry ectoparasitic insects that can transmit parasites from the genus *Polychromophilus*, phylogenetically closely related to the parasite that causes malaria in humans – *Plasmodium falciparum* and other plasmodia.

CONCLUSIONS

Bats are the second largest order of mammals and a significant reservoir and carrier of viral, bacterial, and fungal pathogens, some of which are particularly dangerous. The role of bats in the incidence and mutagenesis of potential pathogens and potential overcoming of the species barrier is related to the ability to fly over long distances, their longevity, colony structure, coexistence with different species including humans, and the fluctuations of their metabolism and body temperature. Investigation of the Chiropteran microbiome is very important for human health and the potential prevention of future epidemics. Currently, there are no comprehensive studies characterizing the metagenomic diversity of bats, the related blood-sucking ectoparasites, and the interaction with their surrounding environments, including caves, water, and soil. Understanding the underlying mechanisms of those process is essential for the development of strategies for mitigating the risks associated with zoonotic diseases. Combating new and emerging infections represents a significant global issue and societal challenge.

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