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**PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
VOLUME 48, NUMBER 3/2020**

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ACKNOWLEDGEMENTS

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Identifying information regarding patients, including patients' names, initials, or hospital numbers, should be omitted unless the information is essential and the patient (or parent or guardian) gives written informed consent for publication.

PUBLIC KNOWLEDGE, ATTITUDES, AND PRACTICES TOWARDS COVID-19 PANDEMIC AMONG EGYPTIAN CITIZENS: A DESCRIPTIVE STUDY

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ABBREVIATIONS: ARDS: Acute Respiratory Distress Syndrome; CDC: Center for Disease Control and Prevention; COVID-19: Coronavirus disease 2019; KAP: Knowledge, Attitudes, Practice; MOH: Ministry Of Health; SARS-CoV-2: Severe Acute Respiratory Distress Coronavirus-2; WHO: World Health Organization

ABSTRACT

Background: Coronavirus disease 2019 (COVID-19) pandemic has been transmitted rapidly worldwide and is recognized as a main threat to human health in 2020. The best methods to prevent its spread are to know about it and act accordingly.

Aim: This study aimed to assess the knowledge, attitudes, and practices to respond to COVID-19 among a sample of Egyptian citizens.

Methods: A community-based cross-sectional online survey was conducted on 501 Egyptian citizens during the early phase of the epidemic.

Results: The study included 501 participants. Females account for 49.9% (n=250). Most of the participants (n=380, 75.8%) were between 21- 40 years old. of Transmission modes were recognized

by 96% (n=481). The signs and symptoms were identified by 90.8% (n=455). About 91% (n=453) knew that all ages were liable to get infected with COVID-19 and 96% (n=481) knew that the elderly were more prone to develop severe disease. Almost all of the participants (99%, n=494) claimed that they kept hand washing, while only 56.7% mentioned regular use of hand antiseptics. Avoiding contact with symptomatic persons was reported by 97.6% (n=490) and 91.6% (n=459) said they were putting on face masks in crowded places. The concept of remote work was accepted by 89.4% (n=448) and 97.6% (n=489) believed that preventive measures could reduce the risk of catching the infection. Almost all the participants (98.4%, n= 493) would like to know more about the disease.

Conclusion: Most of the studied sample of Egyptian citizens were knowledgeable about COVID-19, positive in their attitudes, and have appropriate practices regarding COVID-19 pandemic. However, these observations should be generalized with caution due to the limited size of the sample, and the representativeness of low socioeconomic citizens.

Recommendations: Health education programs should be implemented among Egyptian citizens to maintain appropriate and updated knowledge and practices and keep positive attitudes towards COVID-19.

Keywords: SARS-CoV-2, Egypt, Pandemic, COVID-19, Survey

INTRODUCTION

An outbreak of pneumonia of unknown etiology commenced in December, 2019 in Wuhan city of central Hubei province of China. Chinese authorities along with World Health Organization (WHO) began working collectively, the etiological agent was established to be a new virus and was named Novel Corona Virus (2019-nCoV) (1). WHO declared COVID-19 as a pandemic disease on March 11, 2020 and by the beginning of April, 2020 COVID-19 cases were reported in 203 countries around the world (2).

Fever is the most common clinical presentation of COVID-19. Other symptoms include cough, malaise, fatigue, shortness of breath, acute respiratory distress syndrome (ARDS) and cytokine storm. Worldwide concerns about the virus have risen due to its high

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transmissibility. The elderly and patients with chronic medical conditions are more prone to develop severe disease (3). To date, there is no approved specific treatment or vaccination against COVID-19. Strict infection control measures are the primary intervention to minimize the spread of the virus (4). Community awareness of how to deal with this highly infectious respiratory disease plays a critical role in controlling the pandemic. Egypt is one of the largest countries in the Arab region, Africa and the Middle East with more than 100 million citizens. Governmental efforts have been exerted to prevent the spread of the virus (5).

The Egyptian Ministry of Health (MOH) launched a COVID-19 Taskforce to steer the country's prevention, containment and mitigation measures. The Egyptian government cancelled international flights, closed schools and banned large social gatherings including certain cultural and faith practices such as mass praying, large weddings and funerals. Health authorities need timely, accurate, and actionable data to design policies and interventions in order to make evidence-based adjustments as the outbreak evolves (6).

Gathering data on public knowledge, attitudes, and practice (KAP) has long been beneficial in prevention, control, and mitigation measures during outbreaks. For example, during the 2014 Ebola outbreak, KAP surveys yielded vital information on the prevalence of misunderstanding about Ebola transmission and prevention, and the need to prevent stigmatization of Ebola survivors and foster safer case management and burial practices (7). During other recent outbreaks, such as SARS-CoV-1 or Zika virus, KAP surveys were used to evaluate how providers could better triage patient calls to fever hotlines and measure how the public responded to mitigation efforts (8&9). Hence, it is essential to study these domains among the Egyptian citizens during the current pandemic.

Taking into consideration the massive time needed to conduct a national household survey and the commonly low response rate of phone surveys, online surveys are a promising tool to analyze and track KAP among the public during fast-moving infectious disease outbreaks within a short time-frame.

SUBJECTS AND METHODS

Study design and population

This study was designed as a cross-sectional survey and was carried out between March and May 2020 in

Egypt using a snowball sampling technique. As there have been no similar published studies, the sample size calculations were based on the assumption that the likelihood of having public good knowledge and positive attitude towards preventive measures against COVID-19 was 50.0% (10). At 95% confidence interval; margin of error 5%, with a design effect of 1.0, the calculated sample size was 501 participants.

QUESTIONNAIRE DEVELOPMENT AND VALIDATION

The survey was developed by the principle author using the frequently asked questions for public posted on WHO website and translated in Arabic, the native language in Egypt. The final questionnaire was reviewed for validity with the aid of biostatistics expert and was pilot examined on 20 subjects from the target population who were not included in the study. Cronbach's alpha was calculated to be 0.7.

The survey consisted of the following parts: (1) Basic demographic data of the participants including age, gender, and education level (2) Nine multiple-choice close-ended knowledge questions tested the following aspects in relation to SARS-CoV-2 infection: methods of transmission, symptoms, vulnerable and high risk groups (3) Ten close-ended practices questions (4) Three questions to check the public attitude (5) one question about the essential source of information.

ETHICAL APPROVAL & DATA COLLECTION

Ethical approval was received from Zagazig University - Faculty of Human Medicine Institutional Review Board (IRB). The survey was carried out during March and May, 2020 when a strict lockdown was imposed by the Egyptian government to implement the social distancing policy. Thus, the online survey was selected for this study since a population-based survey was not feasible under that critical condition. Respondents were pulled across all the Egyptian governments. The questionnaire was electronically sent to the participants through e-mails, WhatsApp, and social networks. Only a single set of responses to the questionnaire was permitted for each person. Consent was implied by the completion of the questionnaire.

STATISTICAL ANALYSIS

Data management and analysis were performed using the Statistical Product and Service Solution (SPSS) version 25 (IBM SPSS Statistics, New York,

United States). Descriptive statistics were performed by reporting the number and percentages for the different categorical variables. Percentages of responses were calculated according to the number of respondents per response with respect to the number of total responses of a question. Continuous variables were expressed as mean and standard deviation (SD). Chi-square test was used for comparison of the categorical variables. All tests were two-tailed, p value ≤ 0.05 was considered statistically significant.

RESULTS

Five hundred and one participants from 23 governorates completed the survey. Demographic traits of the studied participants are shown in Table (1). Nearly half of the respondents were females ($n=250$, 49.9%). Most of the respondents ($n=380$, 75.8%) were between 21-40 years old. Approximately 69% of the participants were university graduates, 11.6% had completed postgraduate studies, and 19.6% had a high school education or less.

Results of knowledge questions are shown in Table (2). Ninety six percent of the respondents recognized the means of spread, 87% knew that the use of public toilets at work, restaurants, or malls could also transmit infection from others. About 65% were knowledgeable that avoiding uncooked animal product, unboiled or unpasteurized milk might help limiting the transmission of coronavirus. Ninety eight percent were aware that they should clean surfaces and office supplies with disinfectants and avoid public transportation or any crowded areas to limit the spread of the new coronavirus. Almost 91% could identify the signs and symptoms of the disease. About 64% percent knew that diarrhea was likely a clinical symptom of the disease. Most of the respondents ($n= 453$, 90.4%) recognized that any age may be infected and 96% ($n=481$) knew that elderly or people with chronic medical conditions ought to develop severe disease.

Results of practice and attitude questions are shown in Table (3). About 99% of the respondents said they kept hand washing. However, solely half of the respondents ($n= 284$, 56.7%) used hand antiseptics. Ninety seven percent said they used tissues when coughing or sneezing and about 98% said they practiced secure disposal of used wipes in a closed waste bin. Ninety four percent stated they used bent elbow for coughing or sneezing when tissues were

not available. Ninety four percent of the respondents claimed they avoided hand contact with mouth, nose, or eyes. About 98% stated they kept away from contact with infected people. Approximately, 92% stated they put on masks in crowded spaces. About 67% of the participants said they kept away from gathering spaces, 23% said they avoided hand shaking, 7% and 3% of the respondents stated they practice balanced food regimen and sufficient sleep as preventive measures against COVID-19 infection, respectively. About 93% said they dedicated to staying at home to stop spread of the infection.

Regarding the attitude of the participants, about 89% said they agreed to work remotely in order to preserve the workflow and employee's safety at the same time. Approximately 98% accepted the statement that using preventive measures reduces the hazard of developing the disease. Almost all the individuals ($n= 493$, 98.4%) stated they make sure to recognize more about the disease to stop it.

Based on age, the 15-20 years old respondents had the highest correct answer rate concerning the identification of the emerging coronavirus symptoms ($n=55/63$, 87.3%) compared to other groups ($p = 0.01$) as shown in Fig. (1). Regarding the practices and attitudes, all participants (100%) aged between 15 - 20 years ($n=63$), 41-50 years ($n= 42$), and over 60 years ($n=2$) claimed they were committed to staying at as home compared to 91.6% ($n=175/191$) of the participants aged 21-30 years, 89.9% ($n=170/189$) of those aged 31-40 years, and 92.9% ($n=13/14$) of those aged 51-60 years ($p = 0.05$) as shown in Fig. (2). No statistically significant differences between participants' attitude based on their age group were detected.

Based on sex, there were no statistical differences between males and females regarding knowledge questions. Yet, certain practices were statistically distinct between male and female participants as shown in Figure (3). One hundred and seventy eight females (178/250, 71.2%) claimed to avoid congested places in contrast to 156 male participants (156/251, 62%), $p = 0.03$. Twelve male individuals (12/ 251, 4.8%) stated they practiced adequate sleep as preventive measure against COVID-19 in contrast to two females (2/250, 0.8%), $p = 0.006$. Female participants were more committed to staying at home (240/250) in distinction to male participants (225/251), $p=0.005$. Regarding attitude, 234 males (234/251, 93.2%) said they agreed to work remotely

to keep workflow and employee's safety in contrast to 214 female (214/250, 85.6%), $p=0.005$.

Based on education level, both university graduates and individuals with post-graduate studies were more informed about the modes of transmission of emerging coronavirus when compared to individuals with secondary school education or less ($p = 0.02$). They were also more knowledgeable that avoiding public transportation and crowded places helped limit the spread of the new disease ($p = 0.05$). University graduates were the most expertised about COVID-19 symptoms. About 85% of the university graduates (293/345) identified correctly the symptoms of COVID-19 in comparison to 69% (40/58) and 78% (77/98) of the individuals with postgraduate or secondary school education, respectively ($p = 0.05$). Additionally, university graduates and individuals

with post-graduate studies were the best informed that all ages could be infected with the emerging coronavirus ($p = 0.0001$) and that elderly people and people with chronic medical conditions were more susceptible to severe disease when compared to individuals with secondary school education or less ($p = 0.001$). There were no statistically significant differences between participants' practices or attitude based on their level of education.

The main source of participants' information is shown in Figure (4). Most of the participants ($n=372$, 74.3%) reported social media as their major source of information, whereas 66 participants (13.2%) mentioned TV and radio, unfortunately, only 55 (11%) and 8 (1.6%) participants mentioned physicians and training courses or lectures as their source of information, respectively.

Table 1. Demographic characteristics of the study participants

Variable	No.	Percent (%)
Age (years)		
15-20 years	63	12.6%
21-30 years	191	38.1%
31-40 years	189	37.7%
41-50 years	42	8.4%
51-60 years	14	2.8%
> 60 years	2	0.4%
Sex		
Male	251	50.1%
Female	250	49.9%
Education		
Secondary or less	98	19.6%
University graduate	345	68.9%
Postgraduate	58	11.6%

Table 2. Answers to knowledge questions

Knowledge Questions	No.	%
What are the modes of infection for the emerging coronavirus?		
Droplets from the patient while coughing and sneezing	10	2%
Direct contact with the patients	4	0.8%
Touching contaminated surfaces and tools	0	0%
All of the above	481	96%
Don't know	6	1.2%
Using public toilets at work, restaurants, or malls may transmit infection from others?		
Yes	438	87.4%
No	26	5.2%
Don't know	37	7.4%
Avoiding uncooked animal product, un-boiled or unpasteurized milk helps limit the transmission of the Coronavirus?		
Yes	325	64.7%
No	53	10.6%
Don't know	123	24.5%
Should you keep surfaces clean (such as desks and tables) and office supplies (such as phones and keyboards) with a disinfectant?		
Yes	491	98%
No	8	1.6%
Don't know	2	0.4%
Avoiding public transportation and crowded places helps limit the spread of the new coronavirus?		
Yes	491	98%
No	6	1.2%
Don't know	4	0.8%
What are the symptoms of the emerging coronavirus?		
Cough	1	0.2%
Fever	24	4.8%
Dyspnea	14	2.8%
All of the above	455	90.8%
Don't know	7	1.4%
Diarrhea may be a symptom of the emerging coronavirus?		
Yes	322	64.3%
No	114	22.7%
Don't know	65	13%
Can all ages be infected with the emerging coronavirus?		
Yes	453	90.4%
No	27	5.4%
Don't know	21	4.2%
The elderly and people with previous medical conditions are more likely to develop severe disease in the case of infection with the virus?		
Yes	481	96%
No	6	1.2%
Don't know	14	2.8%

Table 3. Answers to practice and attitudes questions

Practice Questions	No.	%
Keeps hand washing?		
Yes	494	98.6%
No	7	1.4%
Do you use antiseptics for hands (Alcohol)?		
Always	284	56.7%
Sometimes	193	38.4%
No	24	4.8%
Using tissues when coughing or sneezing?		
Yes	486	97%
No	15	3%
Safe disposal immediately of used wipes and throw them in a closed waste bin?		
Yes	490	97.8%
No	11	2.2%
Using the upper arm when coughing or sneezing in the absence of a tissue?		
Yes	471	94%
No	22	4.4%
Don't know	8	1.6%
Avoid contact with eyes, nose and mouth by hand?		
Yes	472	94.2%
No	29	5.8%
Avoid direct contact with infected people or people who have symptoms?		
Yes	490	97.6%
No	12	2.4%
Wearing gags in gathering places and congestion?		
Yes	459	91.6%
No	42	8.4%
What other preventive measures did you use to prevent the emerging corona virus?		
Avoid gatherings	334	66.7%
Not shaking hands	117	23.3%
Enough sleep	14	2.8%
Balanced eating	36	7.2%
Are you committed to staying at home to prevent the spread of the Corona virus?		
Yes	465	92.8%
No	36	7.2%
Attitude Questions		
Do you agree to work remotely to maintain workflow and employee's safety at the same time?		
Agree	448	89.4%
Don't agree	40	8%
Not sure	13	2.6%
Using preventive measures reduces my risk of developing the disease?		
Agree	489	97.6%
Don't agree	4	0.8%
Not sure	8	1.6%
Make sure to know more about the disease to prevent it?		
Agree	493	98.4%
Don't agree	8	1.6%
Not sure	0	0%

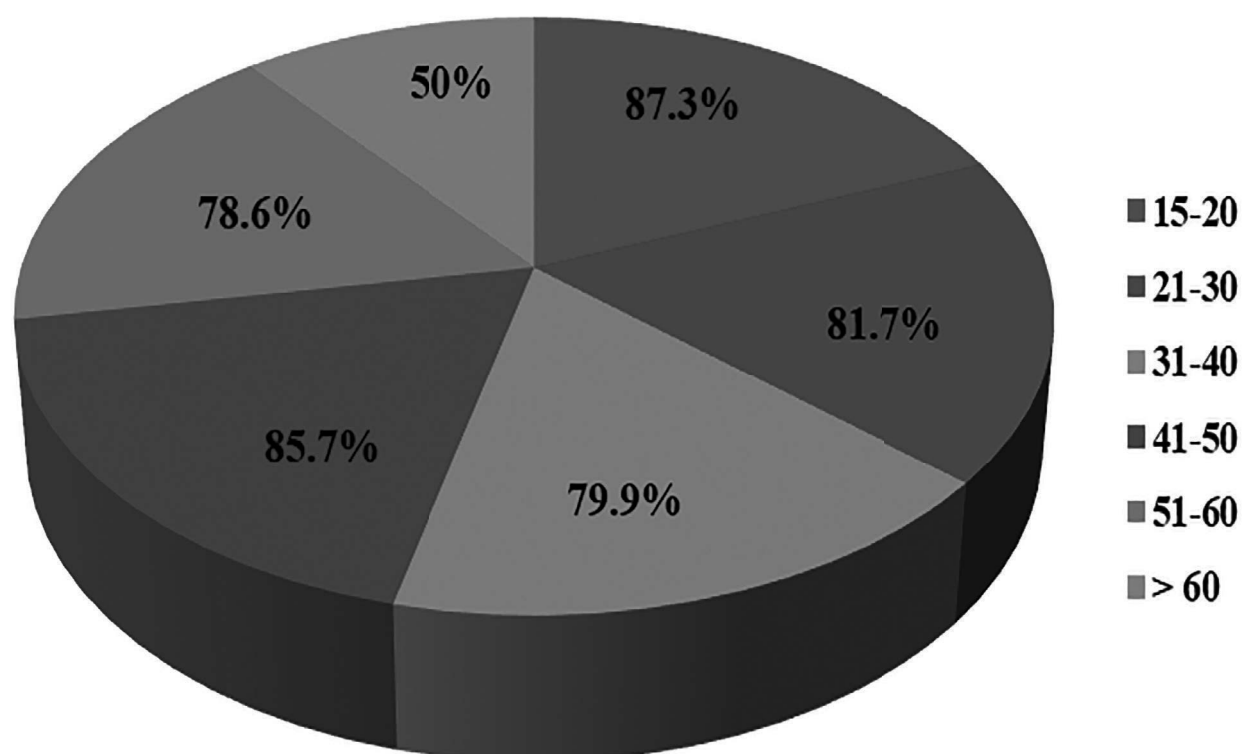


Figure 1. Correct answer rate for symptoms of the emerging coronavirus by age group.

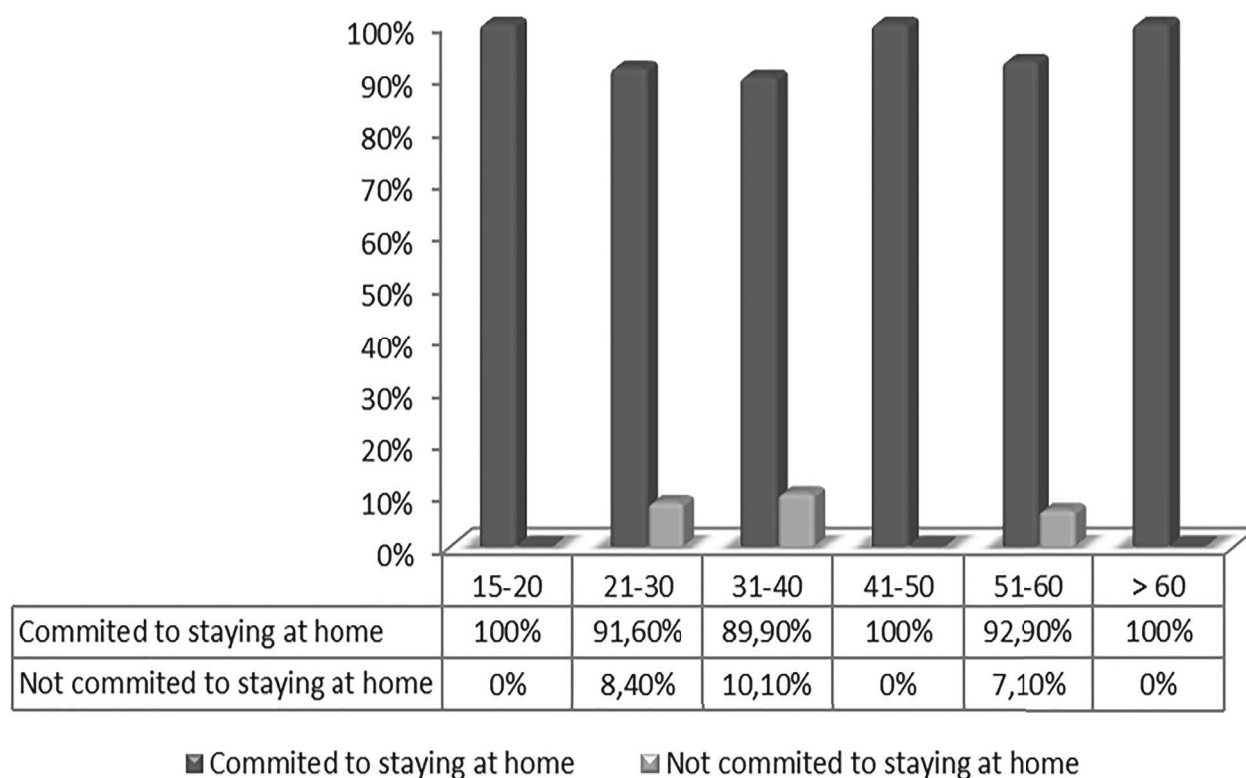


Figure2. Commitment to staying at home by age group.

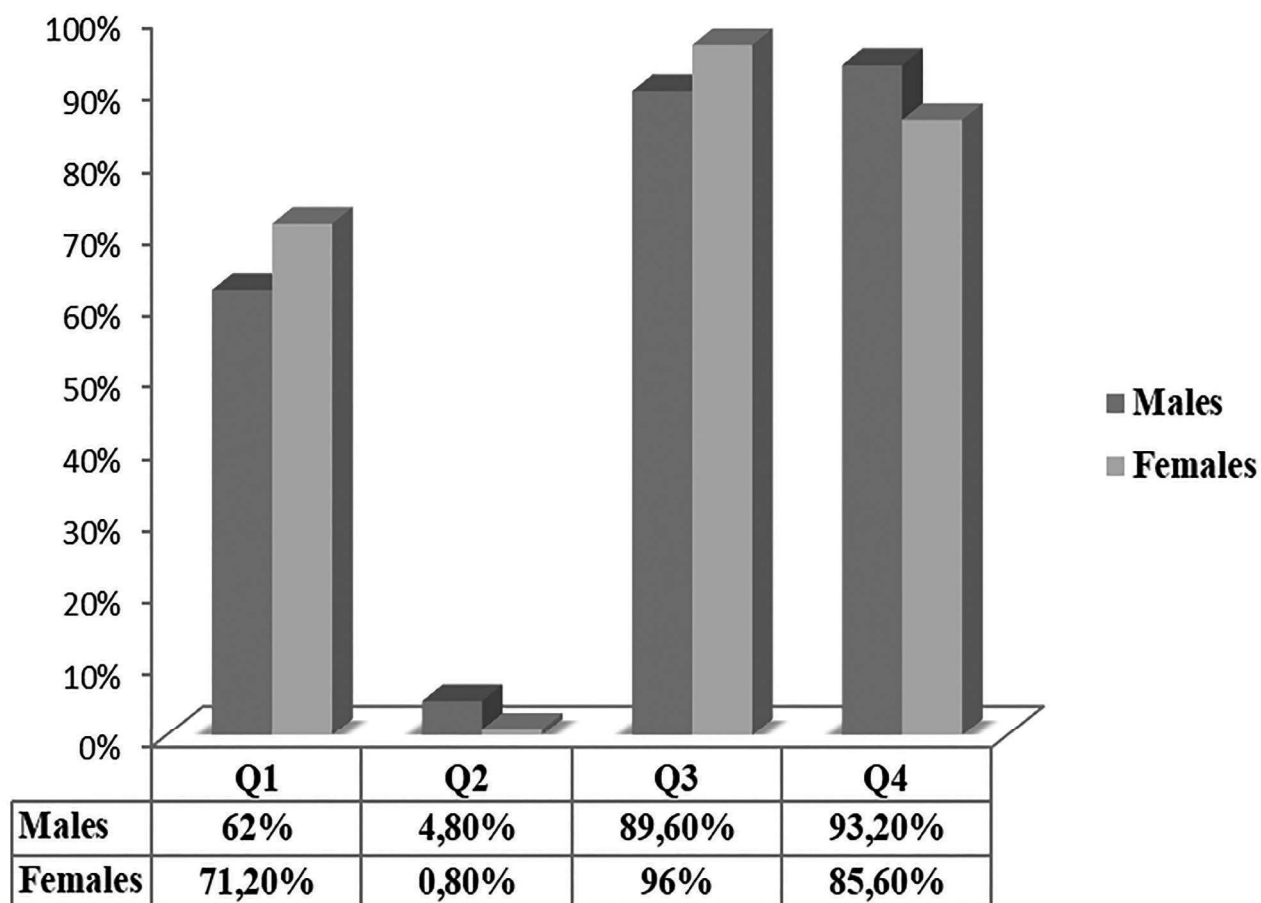


Figure3. Response differences based on gender

Q1: Avoiding congested places, Q2: Practicing adequate sleep, Q3: Are you committed to staying at home, Q4: Agreement to remote work

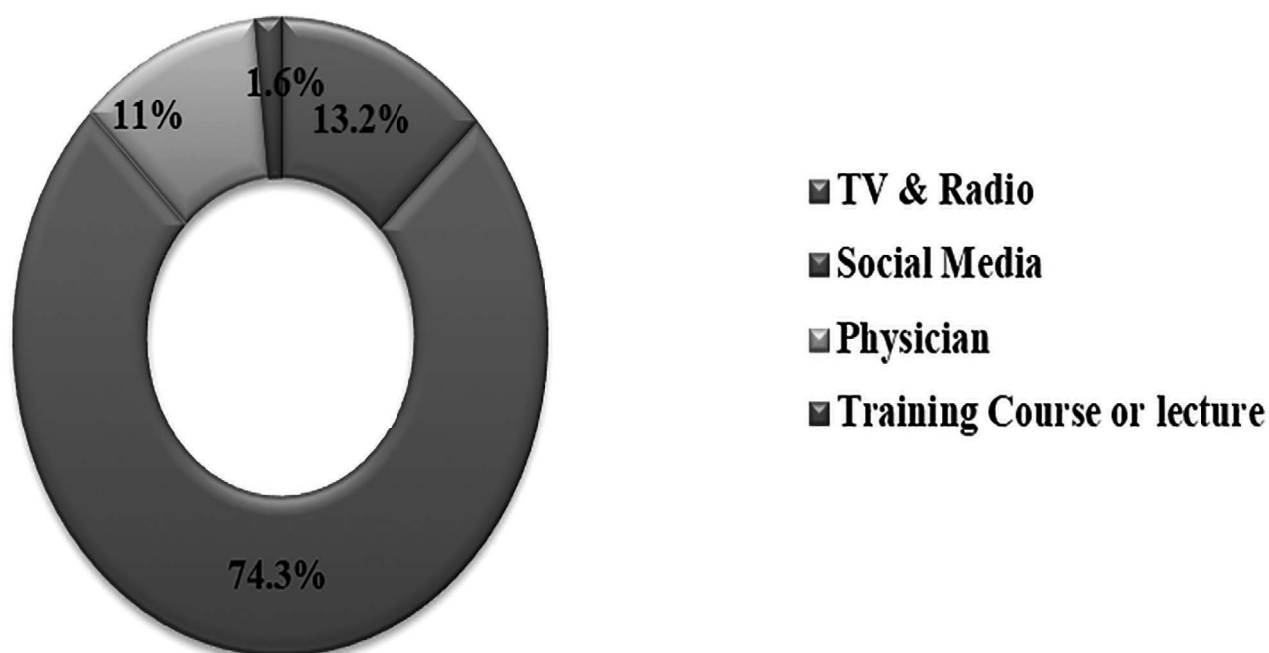


Figure 4. Major sources of participants' information

DISCUSSION

COVID-19 is an emerging disease that has had devastating consequences since it was first detected in December 2019. People in the community face multiple challenges during epidemics and pandemics. Lack of awareness regularly leads to an unconcerned attitude, which may adversely affect the preparedness to meet these challenges (11). Until now, there has been limited published data on population KAP toward COVID-19 in Egypt. Hence, this study aimed to evaluate the KAP towards COVID-19 pandemic in the Egyptian society.

We found that the vast majority of our respondents held satisfactory knowledge, ultimate practice and optimistic attitude. The high correct answer rate of COVID-19 knowledge in Egyptian citizens was unexpected. We consider that is particularly due to the sample characteristics, 82.4% of the participants holding a university degree or higher.

In agreement with our findings, Abdelhafiz et al. reported the studied sample of Egyptian participants to have a good knowledge about the disease and a positive attitude towards protective measures which was gained mainly through novel media channels (12).

Abdelhafiz et al. studied some demographic criteria and KAP aspects which were not included in our study. Regarding demographic criteria, they mentioned that individuals living in rural areas and with lower monthly income have lower knowledge about COVID-19. This may denote that extra efforts should be exerted to deliver the message to these groups, which might also have technical and/or financial difficulties getting access to the social media platforms.

Concerning knowledge aspects, Abdelhafiz et al. reported that about 34% of the studied Egyptian citizens believed that goods imported from China could be a source of virus transmission. Thirteen and 33% of the studied citizens, respectively, thought that taking antibiotics or eating garlic could prevent the spread of the disease. Additionally, about 27% (n=150/559) believed that the virus was initially designed as a biological weapon.

As regards perception and attitude, Abdelhafiz et al. reported that about 73% (n= 410/559) and about 60% (n= 335/559) of their respondents, respectively, strongly agreed to home or hospital isolation in case of contact with an infected person. This surprising

observation may point out that the Egyptian citizens do not experience a stigma of being infected with the virus, and this finding can be explained by the fact that the study participants represented educated groups using the internet. This analysis is confirmed by another question from the same study whether or not the infection with the virus was related to stigmata or feeling ashamed. Seventy one percent (n=397/559) denied feeling stigma or shame associated with COVID-19 infection. Abdelhafiz et al. also studied the economic burden of COVID-19. About 68% (n=383/559) strongly believed that they should continue receiving a salary during the period of isolation.

According to Abdelhafiz et al. about 63% (n=351/559) and 73% (408/559) of their studied sample mentioned, respectively, they were strongly willing to do the lab test or take the vaccine once available. However, we are questioning the numbers stated to accept the vaccine. We consider this observation is not identical to the current situation. Presently, as vaccination is now available for testing, and there are calls for citizens to try the vaccine, there is fear, questions about the safety of the vaccine, and lack of interest from citizens.

In agreement with our findings, Abdelhafiz et al. reported the social media channels as the major source of participants' knowledge. Most of our study participants were aged between twenty one and forty years (n=380/501, 75.8%). Interestingly, Abdelhafiz et al. also reported about 75% of their participants in the age group between eighteen and forty years, which represent more than 75% of Facebook users in Egypt. Abdelhafiz et al. reported the negative assumptions that media were exaggerating the risk. According to their findings, only 16.8% thought that media outlets exaggerate the danger of the disease. Our observations also coincide with that of Zhong et al. who reported high level of knowledge about COVID-19 among Chinese residents. This may be explained by their previous experience with the severe acute respiratory syndrome outbreak in the early 2000s and also the observation that their sample was relatively affluent and highly educated (13).

In the current study, most respondents reported taking preventive precautions by practicing hand hygiene, using tissues when coughing or sneezing with safe disposal immediately in a closed bin, or

using the upper arms when tissues are unavailable, avoiding hands contact with eyes, mouth or nose and avoiding direct contact with symptomatic people, wearing masks when going outside, and commitment to staying at home. This indicates a public willingness for behavioral changes in the face of the COVID-19 pandemic. Second, these precautions might be the result of the respondents' good information related to modes of infectivity with SARS-CoV-2. However, unfortunately only 66.6% (n=334/501) of the respondents reported no longer going to crowded locations.

Certain discrepancies between public responses in Egypt in contrast to China and American citizens were noted. The practice of Chinese residents was much cautious than reported in our study. Zhong et al. reported that nearly all his participants avoided crowded places (96.4%) and wore masks when leaving the home (98.0%) compared to (66.6% & 91.6%) reported in our study respectively (13).

However, American citizens showed the same behavior as we observed in our study. Clements et al. mentioned nearly 30% of their respondents attending gatherings or going to places with more than 50 people, opposite to advices from CDC. The authors attributed this behavior to the uncoordinated efforts of US authorities to lockdown the nation and the debate whether the federal government had constitutional authority, so that individual states were left to make decisions about "shelter at home" policies and similar efforts (14).

The same American study reported only 24% of the American citizens were using masks outside home, in contrast to 91% and 98% reported in ours and in the Chinese study respectively. This extremely lower rate of practice reported by Clements et al. was explained by lack of supply combined with hoarding behavior and ignoring governmental recommendations (14).

The optimistic attitude of the Egyptian citizens could be associated to the exceptional COVID-19 management measures such as traffic limits and the shutdown of cities, which enhanced people's self-assurance in the battle against the virus. Second, the concerted governmental efforts also contributed for the Egyptian people's confidence in overcoming the epidemic.

Our findings are also in line with Azlan et al., who suggested that Malaysians had an acceptable level of knowledge on COVID-19 and were generally positive

in their outlook on overcoming the pandemic (15). Another Nigerian study recorded good knowledge and attitudes among Nigerian participants (16).

A study from Saudi Arabia reported that the majority of the studied individuals were knowledgeable about COVID-19 with optimistic attitudes and good practices. However, in distinction to our observations, their results showed that men had less knowledge, less optimistic attitudes, and less good practices toward COVID-19 than women. They also found that older people were likely to have better knowledge and practices than younger people (17).

In contrast to our findings, a Sudanese study reported incomplete knowledge and poor practices towards COVID-19 among Sudanese residents (18). Haque et al. reported that Bangladeshi peoples had good knowledge, while their attitude and practices towards COVID-19 during the pandemic had not been impressive (19).

Implications of the study

Surveys of KAP can gather data on what is known, believed, and carried out by a specific population. Such data are necessary because unclear information and negative attitude toward infectious diseases may lead to public distress and panic. The findings of our study could be used by public health policy-makers and campaigns, health care workers, and media for targeting the populations in need for COVID-19 prevention and health education.

Limitations of the study

Being an online study, illiterates, farmers, rural and semi-urban residents, underprivileged and vulnerable, and in particular older adults were not included in the study. Thus, our sample population was obviously over-representative of well-educated people. Additionally, due to the very limited time for developing the survey during the early phase of pandemic, attitudes were measured with only three simple questions. Further research studies could use open-ended questions, in-depth interviews or focus group discussion for assessment of citizens' attitudes towards COVID-19. With the current authorities plan to initiate gradual release of the lockdown, public health experts could visit households to verify adequate KAP regarding COVID-19. In summary, our observations suggest that Egyptian citizens have had good knowledge, optimistic attitudes, and

appropriate practices towards COVID-19 during the early phase of COVID-19 pandemic.

CONCLUSION

Most of the studied Egyptian citizens were knowledgeable about COVID-19, positive in their attitudes, and had appropriate practices regarding COVID-19 pandemic. However, these observations must be generalized with caution due to the limited sample representativeness of low socioeconomic level citizens.

RECOMMENDATIONS: Health education programs should be implemented among Egyptian citizens to maintain appropriate and updated knowledge and practices and keep positive attitudes towards COVID-19.

AUTHOR CONTRIBUTIONS

Dina M. Ali is the principle investigator, and contributed to conception and design, acquisition, analysis and interpretation of data, drafting the manuscript and revising it critically. Mona S. Hamed & Lubna A. El-Korashi are co-authors, and contributed to critical revision of the data analysis and reviewing the final manuscript.

CONFLICT OF INTEREST

The authors have declared no competing interests.

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MORTALITY RATE AND PROGNOSTIC FACTORS FOR POOR OUTCOME IN HIV-INFECTED BULGARIAN PATIENTS WITH PNEUMOCYSTIS PNEUMONIA OVER A 3-YEAR PERIOD

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ABSTRACT:

Background: In spite of the use of combination antiretroviral therapy and specific prophylaxis, *Pneumocystis pneumonia* remains one of the most common AIDS-defining diseases with high mortality rate. The aim of this study was to analyze the cases of *pneumocystis pneumonia* over a three-year period by means of assessing the mortality rate and the prognostic factors for the outcome of the disease.

Material and methods: Epidemiological, clinical, laboratory and demographic data were analyzed for 13 HIV-infected patients with proved *pneumocystis pneumonia*, hospitalized at the AIDS Department over the period January 2017 - December 2019. For data processing different methods of descriptive statistics were used.

Results: All presented patients had extremely severe immune deficiency and other opportunistic infections or AIDS-related diseases. The diagnosis of *pneumocystis pneumonia* was confirmed with PCR in

76.9% of the patients and with Giemsa staining for cysts and trophozoites in 23.1% of the patients. Most of our patients were male (84.6%) and the leading mechanism of HIV infection was unprotected homosexual contact. The mean age of the patients presented was 37.4 ± 10.27 . Our results showed high PJP mortality (46.3 %), despite the applied etiological therapy.

Conclusions: *Pneumocystis jirovecii* pneumonia (PJP) continues to be a life-threatening infection in HIV-infected patients with severe immune suppression. PJP mortality remains high, and is significantly dependent on age, male sex, low CD4 T cell count, high HIV viral load, low blood albumin, and the number and severity of comorbidities.

Keywords: *Pneumocystis jirovecii pneumonia, prognostic factors, mortality*

INTRODUCTION:

Pneumocystis pneumonia remains the most common AIDS-defining disease in the United States, although its mortality rate has dropped from over 50 to 10% (1, 2, 3). The causative agent is *Pneumocystis jirovecii*, which was initially classified as a type of trypanosome, and subsequently, as a protozoa. In 1988, its DNA was found to be that of an atypical fungal species (4).

The use of combination antiretroviral therapy and prophylaxis for pneumocystosis has resulted in a dramatic reduction in the number of cases in the US and in Western Europe. However, a significant increase in the cases of *pneumocystis pneumonia* is observed in certain regions such as sub-Saharan Africa, where *Pneumocystis jirovecii* was previously a rare pathogen (5).

Until 1980, it was a rare disease, affecting mainly malnourished children with severe immunodeficiency and adults with severe immunosuppression, mainly related to chemotherapy for neoplastic diseases. With the onset of the HIV pandemic, the spread of *pneumocystis pneumonia* increased dramatically, and it is becoming the most common AIDS-defining disease in the developed countries (5, 6).

Following the introduction of co-trimoxazole chemoprophylaxis in HIV-infected patients with CD4+ T lymphocyte counts below 200 cells/mm³ since 1989, and especially after the introduction of highly-active antiretroviral therapy (HAART) in the mid-1990s, there has been a significant decline in the disease in developed countries in Europe its prevalence has

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been reduced from 4.9 cases per 100000(1994) to 0.3 cases per 100 000 (1999), (7, 8).

This is a retrospective study of patients with pneumocystis pneumonia (PJP), hospitalized and treated at the AIDS Department over a three-year period. We analyzed the patients' demographic, epidemiological, and clinical characteristics. We also analyzed the impact of some factors, such as gender, age, comorbidities, and some laboratory test values on the disease course and outcome.

MATERIAL AND METHODS:

We present data of 13 HIV-infected patients with proven pneumocystis pneumonia. They were hospitalized at the AIDS Department of the Specialized Hospital for Active Treatment of Infectious and Parasitic Diseases in Sofia over the period 2017–2019. Routine clinical, laboratory and microbiological tests were carried out in the respective laboratories of the Specialized Hospital for Active Treatment

of Infectious and Parasitic Diseases in Sofia. The immunological and virological studies regarding the HIV infection were performed at the National Center of Infectious and Parasitic Diseases.

The diagnosis of pneumocystis pneumonia in ten of the patients was confirmed by means of PCR methods: Real-time PCR with TaqMan probes for detection of *Pneumocystis jirovecii*. In the other three patients, diagnosis was confirmed by means of morphological methods: Giemsa staining for cysts and trophozoites.

Statistical analysis was performed using descriptive statistics methods (descriptives, cross tabulations, frequencies); analysis of statistical significance was carried out by means of parametric (one sample T – test) and non-parametric methods (one sample Kolmogorov – Smirnov test). The level of rejection of null hypothesis was set at $p < 0.05$.

RESULTS:

Table 1. Patients' demographic and epidemiological characteristics

Patient №	Sex	Age	Year of HIV diagnosis	Year of PJP diagnosis	Route of HIV transmission
1	Female	35	2017	2017	HETERO
2	Male	32	2014	2018	HETERO
3	Male	45	2019	2019	MSM
4	Male	44	2014	2019	MSM
5	Male	32	2019	2019	MSM
6	Male	39	2019	2019	HETERO
7	Female	27	2019	2019	HETERO
8	Male	42	2018	2019	MSM
9	Male	53	2019	2019	HETERO
10	Male	14	2009	2019	MCT
11	Male	45	2019	2019	MSM
12	Male	32	2016	2019	MSM
13	Male	47	2019	2019	MSM

Thirteen patients were monitored: 11 male (84.6%) and 2 female (15.4%), aged 37.4 ± 10.27 over the interval 14–53 years (Table 1).

The mechanism of HIV infection was as follows: heterosexual in 5 patients (38.5%), homosexual in 7 patients (53.8%), and mother-to-child transmission in 1 patient (7.7%).

The diagnosis of PJP related to the time of the diagnosis of the HIV infection was done as follows:

- In the same year: in 8 patients (61.5%);
- 2 years later: in 1 patient (7.7%);
- 3 years later: in 1 patient (7.7%);
- 4 years later: in 1 patient (7.7%);
- 5 years later: in 1 patient (7.7%);

- 10 years later: in 1 patient (7.7%).

The patients diagnosed with pneumocystis pneumonia in the year of detection of their HIV infection (8 of the patients) were late presenters, and pulmonary symptoms were the reason to be examined and detect their HIV infection. The other five patients were aware of their HIV infection, but had not been registered nor followed up at the Department of AIDS until the manifestation of their pulmonary symptoms. The patients' comorbidities are presented in Table 2.

Table 2. Patients' comorbidities

Patients' comorbidities	Number of patients	Percentage
Cryptosporidiosis	1	7.7
Candidiasis, disseminated	3	23
Orofaryngeal candidiasis	5	38.5
H. influenzae tonsillitis	1	7.7
Cyrrhosis; Aethilismus	1	7.7
Pulmonary tuberculosis	1	7.7
Kaposi sarcoma	1	7.7

Blood count, biochemical parameters and acid-base balance were examined in all patients. The variations in the values of these laboratory parameters are presented in Table 3.

Table 3. Variations in the values of some laboratory parameters

Basic lab tests	Number of patients	Minimum	Maximum	Mean	Std. Deviation
Hemoglobin	13	98.00	147.00	122.8462	15.76307
Leucocytes	13	2.40	11.10	7.0000	2.58618
Platelets	13	128.00	625.00	275.5385	130.26743
Albumin	13	15.00	35.00	22.7077	15.26458
ALAT	13	15.00	71.00	28.8462	20.15708
pO ₂	13	24.20	80.00	42.6923	15.60627

The main indicators of HIV infection were as follows: CD4+ T cells: 20 ± 26 cells/mm³ in the range 2–85 cells/mm³; mean HIV viral load (VL): $496,673 \pm 452,056$ copies/μl in the range 29,821–1,455,994 copies/μl.

The diagnosis of pneumocystis pneumonia was confirmed as follows:

- PCR: in 10 patients (76.9%);
- Giemsa staining for cysts and trophozoites: in 3 patients (23.1%).

All patients were treated with TMP/SMX for PJP at a dose of 120 mg/kg intravenously. For two of them, Clindamycin was added at a dose of 3 x 600 mg intravenously.

In eleven patients antiretroviral therapy was initiated, the other two died before initiating it.

The administered antiretroviral regimens are presented in Table 4.

Table 4. Antiretroviral regimens

ART regimen	Number of patients	Percentage
ABC/3TC + DRV/r	1	7.7
ABC/3TC/DTG	6	46.2
FTC/TDF+ DTG	1	7.7
FTC/TDF + RAL	2	15.4
3TC+TDF+LPV/r	1	7.7

In 6 patients lethal outcome was observed, with two of them having the most severe concomitant diseases: Kaposi sarcoma with visceral localization, and cirrhosis. In five of the deceased patients, the immediate cause of death was acute respiratory failure. The cirrhotic patient died of cirrhosis complications: a hemorrhagic syndrome.

The lethal outcome was significantly associated with the following indicators at a significance level for rejection of the null hypothesis $p < 0.05$:

- gender ($p < 0.0011$);
- CD4 T < 20 cells /mm³ ($p < 0.05$);
- HIV VL $> 300,000$ copies/ml ($p < 0.01$);
- number and severity of comorbidities ($p < 0.0011$);
- $pO_2 < 40$ mmHg ($p < 0.0011$);
- albumin < 30 g/l ($p < 0.011$)

DISCUSSION

Our AIDS center monitors about 1,200 people living with HIV (PLHIV), which accounts for 2/3 of all PLHIV, followed up in Bulgaria. 97% of the patients who are followed-up in our ward receive antiretroviral therapy. This retrospective study covered a small number of HIV-positive patients with pneumocystis pneumonia. All of them had severe immune deficiency and had taken neither antiretroviral therapy, nor PJP prophylaxis before their pneumocystis pneumonia

was detected. This small number of patients indicates that pneumocystis pneumonia is indeed rare among PLHIV in the era of combination ART and PJP prophylaxis (5, 7, 8).

Patients were diagnosed with the help of either PCR, or staining methods, which are the established diagnostic methods for this condition (9, 10, 11, 12, 13, 14).

We treated patients with TMP/SMX intravenously at the dose 120 mg/kg/d, and continued in accordance with the recommendations for the treatment of pneumocystis pneumonia (15). In two of the patients with rapid progression of the disease, we added Clindamycin. As Pentamidine and Caspofungin are not available in Bulgaria, This decision was based on literature data, showing a good response to Clindamycin in patients with pneumocystis pneumonia (16). Unfortunately, in both patients the disease ended with a lethal outcome.

The PJP mortality rate is high among patients not previously treated with antiretroviral therapy and with delayed diagnosis and treatment (17, 18, 19). In our study the overall mortality was high: 46.15%. All of our patients were late presenters that had not previously received ART or prophylaxis.

We observed a significant association of the poor prognosis of the disease with male sex, age over 40 years, albumin below 30 g/l, the number and severity of comorbidities, $pO_2 < 40$ mmHg, CD4 T cell count below 20 cells/mm³, and HIV VL over 500,000 copies/ μ l.

In line with our results other studies have also indicated that age > 35 years, $PaO_2 < 8$ kPa and serum albumin level < 30 g/l at admission were poor prognostic factors (20, 21, 22). Another study found that the presence of Kaposi's sarcoma as a concomitant disease was a poor prognostic factor (23), which was also confirmed in our study.

CONCLUSIONS

Pneumocystis pneumonia (PCP) is a potentially life-threatening infection that occurs in HIV-infected patients with severe immune suppression. Despite the general belief that PJP is uncommon in the era of the universal guidelines for immediate initiation of combination ART, this disease is still a problem in our country due to the still late diagnosis of the HIV infection in some patients. PJP mortality continues to be high, and significantly depends on the age, male sex, low CD 4 T+ cell count, high HIV VL, low

blood albumin, and the number and severity of comorbidities.

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FIRST CASES OF CULTURE PROVEN LEGIONNAIRES' DISEASE IN BULGARIA

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ABSTRACT

Background. *Legionella pneumophila* is the most common cause of the potentially fatal Legionnaires' disease with worldwide increasing incidence reports. The bacterium is fastidious and slow growing and most laboratories do not perform cultivation.

Aim. To present results from the first culture proven cases of Legionnaires' disease in Bulgaria.

Materials and methods. Ten lower respiratory tract materials from 10 patients were cultured for legionellae with GVPC supplement. Identification was based on growth characteristics, latex agglutination and monoclonal typing.

Results. Seven *L.pneumophila* strains (serogroup1 and serogroup6) were isolated. Five belong to monoclonal sub-types Allentown/France, Knoxville and Philadelphia. One culture-positive sputum was received in the lab 5 days after sampling. The other materials were plated on the day of sampling, but three of them were obtained long after specific therapy was started and remained negative on culture. Five from the ten patients were with fatal outcome. Three were reported to ELDSNet in real-time as travel-associated.

Conclusions. Isolation of legionellae from patients remains the diagnostic 'gold standard'. The use of selective supplement designed for water samples had no obvious impact on our results in contrast with late sampling. Isolates were from the most frequently reported *L. pneumophila* serogroups

and five of them – from the virulence-associated Pontiac sub-groups. Legionnaires' disease is still underdiagnosed in Bulgaria. Clinicians must be encouraged to send appropriate and timely obtained respiratory materials. This should happen even in cases with other positive microbiological results, since co-infection with viral, fungal or other bacterial pulmonary pathogens might be of importance for patients' treatment and health.

Keywords: *Legionella pneumophila*, Legionnaires' disease; culture from clinical samples; typing.

INTRODUCTION

Legionnaires' disease is atypical bacterial pneumonia, occurring predominantly as a result of inhalation or sometimes microaspiration of legionella-contaminated water. For decades Legionnaires' disease has been considered as underdiagnosed, despite of its individual and public health importance. The overall incidence rate in many states is increasing, but at the same time there is also high heterogeneity in reporting across EU/EEA and other countries (1,2). Members of genus *Legionella* are ubiquitous in water/moist environments, especially man-made water systems. At the same time they are nutritionally fastidious and slow growing on artificial media. Isolation of *Legionella* spp. is the 'gold standard' for diagnosis. The yield from patient cultures depends on the severity of the illness but there are also other considerations. Cultivation requires: proper and timely obtained material from the lower respiratory tract; buffered charcoal yeast extract agar with addition of growth supplements (BCYEa) and antibacterial mixture to reduce background microbiota in sputum; 3-5-7-10 days stereo-microscopic plate observation; various tests for further identification and considerable technical laboratory expertise. Another issue is that patients with Legionnaires' disease are often non-productive of sputum and in such cases invasive procedures are required to obtain lower respiratory tract sample. All of the above explains why hospital-based laboratories in our country do not perform *Legionella* spp. cultivation. Nevertheless, isolates are critically important for epidemiological investigations, where comparison between clinical and environmental strains allows identification of the infection source and thus contributes to public health prevention strategies. *Legionella pneumophila* Sg 1 is considered

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as the most pathogenic species and serogroup (Sg). It accounts for 85% of culture-confirmed Legionnaires' disease cases in Europe (1). In Bulgaria *L. pneumophila* Sg1 was first isolated from a thermal mineral lake in 1981(3) and subsequently from numerous man-made water systems (4-6). Conversely, there were no isolates from patients with Legionnaires' disease. In this paper we present results from the first culture proven cases of Legionnaires' disease in Bulgaria.

MATERIALS AND METHODS

We investigated ten patients with severe pneumonia, admitted at intensive care units and having at least one positive result for legionella infection: Legionella antigen in urine (ICT or ELISA), direct immunofluorescence staining (MonoFluo *Legionella pneumophila* IFA, BIO-RAD), antibody index > 11 in single serum sample (ELISA *L. pneumophila* Sg 1-6 Ig M+IgG, Vircell). We obtain one sample per person, as follows: sputa (n=5), bronchoalveolar lavages (n=3), pleural punctates (n=2). Sputa were digested (Digest-EUR, Eurobio Sci.) for 15 minutes at 22°C and centrifuged in 10 ml dH₂O for 5 min at 2000 rpm. Liquid materials were concentrated for 15 min at 2000rpm. The obtained native deposits were used for direct seeding without further pre-treatments (e.g. acid-treatment with HCL/KCL solution at pH 2.2). The materials were plated on charcoal yeast extract agar with growth supplements - BYCE α (Oxoid). For inhibition of other bacteria we used GVPC (Oxoid), containing glycine, vancomycin, polymyxin B and cycloheximide – a selective supplement routinely used in our laboratory for investigation of water samples for legionellae. Incubation was performed at 35°C with 2.5%CO₂ and increased humidity. Plates were observed daily up to 10 days and suspected ground glass colonies were further sub-cultured and confirmed by: l-cystein dependence, +/- autofluorescence under long-wave (365-nm) UV light, latex agglutination (Oxoid; Prolab) and for *L. pneumophila* Sg1 when appropriate for further investigation purposes - monoclonal sub-typing with the Dresden Panel (7).

RESULTS

Samples for cultivation were actively searched by us in our attempt to confirm Legionnaires' disease by the "gold standard" method – isolation of *Legionella* spp. We were able to obtain lower respiratory tract

materials from 9 males and 1 female with median age 54 yrs (range 29-72 yrs). Because of severe pneumonia, all patients were admitted at intensive care units in seven hospitals (**Fig .1**). All cases had negative results in hospital laboratories when cultured for respiratory pathogens by routine methods, but had at least one positive for legionella infection test in our laboratory. Case-based characteristics are shown in **Table 1**. They were with the following primary categorization as: confirmed cases with positive Legionella antigen in urine (n=8) and probable cases (n=2) - one with positive direct immunofluorescence staining without other tests for *Legionella* and one with a single positive serum sample but with negative urine test for Legionella antigen. Sputum samples were five, followed by three bronchoalveolar lavages and two pleural punctates. Five cases were with fatal outcome as a result of acute respiratory or multiorgan failure. One culture positive sputum sample, stored at 6°C, was received in the lab 5 days after the expectoration. All the other materials were processed on the day of sampling, but three of them were taken late (\geq 7-10 days) after specific therapy with Levofloxacin or Azithromycin was started and remained negative for legionellae up to 10 days of incubation. In the remaining 7 samples suspicious ground glass colonies, which do not grow on sheep blood agar, appeared predominantly after 3-5 days of incubation and in one case – on day 7. *L. pneumophila* was isolated from seven samples, including those from the two initially probable cases. Six isolates were identified as *L. pneumophila* Sg1 and one - as *L. pneumophila* Sg6 (**Fig .2**). Five *L. pneumophila* Sg1 strains belonging to the Pontiac group were found to be Allentown/France, Knoxville and Philadelphia monoclonal sub-types.

Our supplementary investigation revealed that none of the patients was epidemiologically related to the others, but three cases were associated with travelling during the incubation period and were notified to the European Surveillance Scheme for Travel-associated Legionnaires' disease (ELDSNet). For one of them, who traveled in Bulgaria, an environmental isolate was also available, showing complete monoclonal and sequence type match with the clinical one (data not shown). The source of infection was established for 2 other cases and instructions for appropriate behavior at the corresponding locations were given.

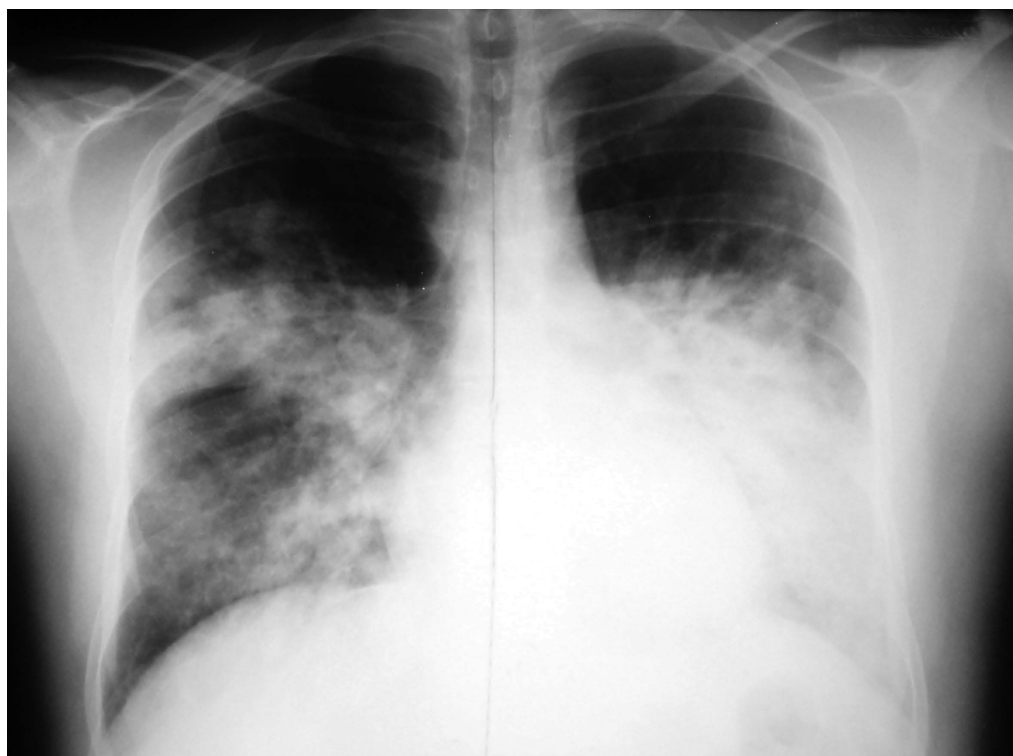


Figure 1. Radiologic manifestation of severe Legionnaires' disease in a 29 year-old patient with no co-morbidities and no risk activities during the incubation period.

Table 1. Main characteristics of the patients with lower respiratory tract samples used for *Legionella* spp. culture.

No	Gender ¹	Age	Initial diagnostic test ²	Initial category zation	Sample ³	Legionella ⁴	Final categorization	Outcome
1.	F	40	LUA	Confirmed	SP	<i>L.pn.</i> Sg 1 ⁶	Confirmed	Recovered
2.	M	60	LUA	Confirmed	BAL	<i>L.pn.</i> Sg 1	Confirmed	Dead
3.	M	58	LUA	Confirmed	BAL	<i>L.pn.</i> Sg 1	Confirmed	Dead
4.	M	72	LUA	Confirmed	SP	<i>L.pn.</i> Sg 1 ⁵	Confirmed	Dead
5.	M	50	LUA	Confirmed	BAL	<i>L.pn.</i> Sg 1 ⁵	Confirmed	Recovered
6.	M	70	DIF	Probable	SP	<i>L.pn.</i> Sg 1 ^{5,6}	Confirmed	Dead
7.	M	67	LUA	Confirmed	PP	None	Confirmed	Dead
8.	M	53	LUA	Confirmed	PP	None	Confirmed	Recovered
9.	M	41	SHT	Probable	SP	<i>L.pn.</i> Sg 6 ⁶	Confirmed	Recovered
10.	M	29	LUA	Confirmed	SP	None	Confirmed	Recovered

¹ F- female; M – male

² LUA – Legionella urinary antigen; DIF – direct immunfluorescence; SHT- single high titer

³ SP- sputum; BAL - bronchoalveolar lavage; PP - pleural punctuate

⁴ *L.pn.* Sg – *Legionella pneumophila* serogroup

⁵ clinical strains for comparison with corresponding environmental ones

⁶ strains from a travel associated cases

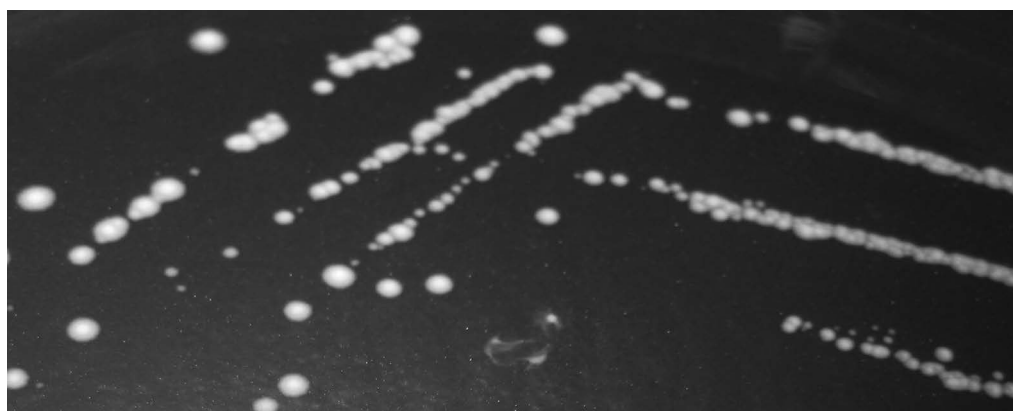


Figure 2. Pure culture of Legionella pneumophila Sg1 from BAL sample

DISCUSSION

Legionnaires' disease accounts for 2% to 15% of all community-acquired pneumonias requiring hospitalization in Europe and North America (8). *L. pneumophila* has been found to be a common cause of bacterial pneumonia, just after *S. pneumoniae* in patients who required intensive care unit admission (9). The case-fatality rate in Legionnaires' disease varies between 10–40% and may approach 50% in nosocomial outbreaks (10). Five of our patients were with fatal outcome, most probably as a result of late diagnosis and/or co-morbidities.

Because of the fastidious legionella nature (lack of laboratory capacity) and their slow growth (3–10 days), cultivation of patient materials is rarely requested and requires active lab approach. Clinicians prefer the easier and quicker test for detection of legionella antigen in urine where depending on the format used, results are obtained within minutes or several hours. However, these tests are mainly designed for detection of *L. pneumophila* Sg1 and thus Legionnaires' disease caused by other *Legionella* spp. or non- Sg 1 *L. pneumophila* could be overlooked. Our results show one such case with *L. pneumophila* Sg 6 infection.

Selective combinations recommended for patient specimens differ from the GVPC designed for water investigation and routinely used by us for environmental testing and in the present study. This had no obvious impact on our results. Delayed obtaining of the material, long after initiation of appropriate anti- *Legionella* therapy, appears to have a more significant negative impact as was the case with our three culture- negative samples. Based on positive isolation results, the two initially probable cases were re-categorized as confirmed ones. Timely obtained and/or appropriately stored and sent materials are crucial for legionella isolation. The disease is not only a personal health issue, but can cause epidemic outbreaks (11–16) with great economic and public impact (17,18). Comparison between clinical and environmental isolates is essential for infection source location, implementation of appropriate control measures, and thus - solving a multiauthority problem.

We have succeeded in culturing *L. pneumophila* from seven out of ten materials. These numbers may look insignificant but are important for such a neglected in our country infection, as well as for

further epidemiological investigations. Five from our *L. pneumophila* Sg1 isolates expressed the virulence-associated epitope recognized by the MAb 3/1 (Dresden Panel). Such strains present a problem in the Mediterranean countries and the UK for both community-acquired and nosocomial cases (7). We were able to locate the infection source in three of our cases, one of which was part of an international cluster so that the result contributed to its limitation.

Legionnaires' disease still remains an important cause of potentially preventable morbidity and mortality in EU/EEA with 23% notification increase in 2018 vs. 2017 and no indication of decreasing burden (1). In cases of atypical pneumonia, clinicians must be aware of the importance to send a set of materials for legionella testing. These must include at least urine, combined if possible with a lower respiratory tract sample, both obtained before/early after starting anti-*Legionella* therapy.

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APPLICATION OF PCR METHOD FOR DETECTION AND SPECIES IDENTIFICATION OF TOXOCARA SPP.

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ABSTRACT

The ascaridoid nematodes of dogs and cats *T. canis* and *T. cati* have wide distribution and are causative agents of Toxocariasis - a disease in human and animals. Human disease has several clinical forms with different clinical manifestations such as visceral, ocular, neuro and covert toxocariasis. The morphological methods used to differentiate the two species, especially to identify their eggs or larvae, may lead to inaccurate diagnosis. This requires the use of more reliable methods, such as PCR, for identification of *Toxocara* species.

The aim of our research was to develop an in-house PCR method for species identification of *Toxocara* and to determine its applicability on different parasites stages.

We applied the method used by Khademvatan et al. (2013), with some modifications for detection of different forms of *Toxocara* - eggs, larvae and adult parasites. We used species-specific oligonucleotide primers from the ITS2 gene sequence of the ribosomal DNA - Tcan1/NC2 for *T. canis* and Tcat1/NC2 for *T. cati*.

The presence of a band with the size of 380 bp, specific for *T. canis*, was found for all stages of the studied parasite.

The described method will allow species differentiation of Toxocariasis causative agents and improve the diagnosis of the disease, as well as

determine the actual spread and reservoirs of these parasites.

Keywords: *Toxocara* spp., PCR, ITS-2, molecular identification.

INTRODUCTION

Toxocara are ascaridoid roundworms that are important for both animal and human health. The main representatives of these parasitic helminths are *T. canis* and *T. cati*, and the adult worms of the two species inhabit the lumen of the small intestine of the definitive host, dog and cat, respectively. Humans can be infected via accidental ingestion of embryonated eggs present in the soil or contaminated food, as well as by ingestion of encapsulated larvae present in improperly cooked tissues of paratenic hosts (1, 2). In the human body larvae can not develop to mature adult worms, they migrate via the bloodstream and are localized in different organs (muscle, liver, brain and eyes), which causes the development of various clinical symptoms. The following clinical forms have been described: visceral toxocariasis (3), ocular toxocariasis (OLM), (4), neurotoxocariasis (5), covert or common toxocariasis (CT), (6), and asymptomatic form (7).

The determination of the species specificity of *Toxocara* larvae from animal and human tissues is very difficult because of their small size and few morphological features that can be used for identification (8). Ascaridoid nematodes are usually identified on the basis of their morphological characteristics and predilection sites within hosts, but there are limitations in traditional methods, especially with regard to their stages - eggs and larvae. To overcome the limitations of traditional (morphology-based) determination, various molecular methods based on ribosomal and mitochondrial markers - both for identification of *Toxocara* spp., have been developed (9). Various studies have shown that the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA) is useful genetic marker for accurate identification of different genetic groups (9, 10). To distinguish *T. canis* and *T. cati* from closely related species the high genetic variability inside the molecular markers, such as the internal transcribed spacer 2 (ITS-2), is used (10, 11).

The aim of the present study was to develop an in-house polymerase chain reaction (PCR) method for the identification of *Toxocara* species and to deter-

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mine its applicability to various parasite stages – mature forms, larvae and eggs.

MATERIAL AND METHODS

Parasites

Adult parasites of *Toxocara* spp. were submitted for determination to the Department of Parasitology and Tropical Medicine, National Center of Infectious and Parasitic Diseases (NCIPD) by a veterinary clinic in Sofia. One part of the adult *Toxocara* spp. worms were homogenized in phosphate-buffered saline (PBS, pH 7.2) and kept frozen at -20° C until the PCR reaction was performed.

From another part of the adult parasites (female), *Toxocara* spp. eggs were collected using a scalpel, and washed by centrifugation three times with Tyrode's solution (NaCl - 8 g, KCl - 0.2 g, CaCl₂ anhydrous - 0.2 g, MgCl₂ · 6 H₂O - 0.05 g, glucose - 2 g, NaHCO₃ - 1 g, distilled water up to 1 l). Portions of the washed parasitic eggs were frozen at -20° C until PCR was performed and the rest were incubated for 1 month to obtain *Toxocara* larvae.

Cultivation

Toxocara spp. eggs were incubated in petri dishes for 1 month in 1% formalin solution at room temperature. Their status was monitored every 72 hours under a microscope to check the state of the eggs and the development of the larvae within them. At the end of the period, the eggs were washed three times with Tyrode's solution and centrifuged at 1000 rpm for three minutes. The protein shell of the *Toxocara* eggs was partially lysed with NaOCL solution and water (in a ratio of 1:1 for 12 h) at room temperature (12). The resulting suspension was washed again at the same speed with Tyrode's solution to obtain a neutral pH. The larvae collected in this way were distributed in mattresses for cultivation in DMEM (Gibco) media (prepared according to the manufacturer's instructions) under anaerobic conditions / 37° C (method described by de Savigny et al. (1975),(13). The vitality of the larvae was monitored daily, and at the end of the first week the nutrient medium was replaced with a new one. After a fortnight incubation, the medium was removed and the sediment containing *Toxocara* larvae was collected and frozen at -20° C until use.

Isolation of DNA and PCR

DNA from mature forms, parasitic eggs and larvae of *Toxocara* spp. was extracted using a commercial QIAamp DNA Kit (Qiagen), following the manufactur-

er's instructions. Samples were initially subjected to 3 freeze-thaw cycles for 20 min. Proteinase K digestion was performed overnight at 55°C under continuous shaking conditions (14) on Thermomixer compact (Eppendorf) at 450 rpm.

PCR conditions and primer sets were as previously described (14, 15). Briefly, primers hybridize to the internal transcribed spacer 2 (ITS2) gene and were designated as Tcan1 (5'-AGTATGATGGGCGCGCCAAT-3') and NC2 (5'-TAGTTTCTTTTCTCCGCT-3') for *Toxocara canis* and Tcat1 (5'GGAGAAGTAAACTC-3') and NC2 for *Toxocara cati* (14, 15, 16).

PCR was performed in a final volume of 25 µL of the reaction mixture containing 2.5 µL of 10x PCR buffer, 0.2 mM of each dATP, dTTP, dCTP and dGTP, 25 pmol of each primer, 3 mM MgCl₂, 1.25 U of Taq DNA polymerase (Fermentas) and 20 ng of template DNA. PCR amplification was done on the GeneAmp PCR System 2700 Amplification Apparatus (Applied Biosystems) under the following conditions: an initial cycle at 94°C for 30 s; followed by 35 cycles including denaturation at 94°C for 60 s; hybridization at 58°C for 30 s and extension at 72°C for 30 s; as well as 1 final extension cycle at 72°C for 10 min. Upon completion of amplification, 20 µl of the PCR products were added dropwise to a 1.8% agarose gel containing ethidium bromide in 1x Tris-acetic acid-EDTA buffer (Fermentas),(17).

RESULTS

PCR amplification follows the one described by Khademvatan et al. (2013), which we performed with some modifications, presented in detail in Materials and Methods section. The method was developed for the study of parasitic eggs in the environmental fecal samples of stray cats (17), which we applied to the three stages of the parasite - adult forms, eggs and larvae (Fig. 1).

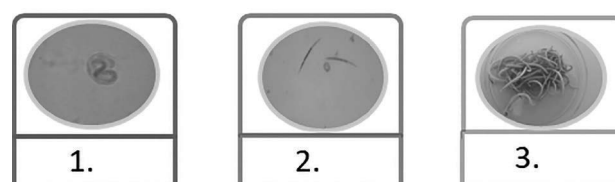


Figure 1. *Toxocara* - eggs (1), larvae (2) and adult worms (3), tested with the developed PCR method.

PCR of *Toxocara* - eggs

The applied PCR assay using primers Tcan1/NC2 and Tcat1/NC2 in the study of parasitic eggs obtained

from adult female forms by the method described by Khademvatan et al. (2013), revealed the presence of bands of about 380 bp in length, specific for *T. canis* (lines 3, 4, 5 in Figure 2, DNA isolated from *Toxocara* eggs).

To determine the sensitivity of the reaction, DNA was extracted from aliquots of different amounts of parasitic eggs - a sample containing 8 eggs (line 3), a sample with 4 parasitic eggs (line 4) and with only 1 *Toxocara* egg (line 5). Electrophoretic analysis of PCR products showed the presence of a product of approximately 380 bp specific for *T. canis* in all samples containing parasite eggs.

The results showed that PCR was able to detect the presence of *T. canis*-specific DNA in all tested dilutions of the parasitic egg suspension, including the one egg-suspension.

PCR of *Toxocara* – larvae

Examination of parasitic larvae obtained after *in vitro* cultivation of *Toxocara* eggs in the applied PCR method showed the presence of the same specific bands (380 bp) found in the test of eggs characteristic of *Toxocara canis* (lines 6, 7 and 8 in Figure 2). The presence of the characteristic band was observed in the reaction mixtures containing 8 (line 6), 4 parasitic larvae (line 7) and a single larva (line 8).

PCR of adult *Toxocara* worms

To overcome the strong cuticle sheath of the adult forms of *Toxocara*, two methods of pretreatment were used before the PCR reaction: 1) freezing and thawing at -70° C three times for 20 min and 2) sonication for 3 min.

When the first method (triple freezing and thawing) was applied to the adult forms of the parasites a sample from marital forms examination a very weak band on the agarose gel appeared following electrophoresis in (line 2). Processing the mature forms by ultrasonic disruption resulted in a lack of specific bands (line 1), indicating the need to apply additional processing methods in order to retrieve a larger amount of DNA, and to optimize amplification conditions.

The optimized by us PCR protocol showed for all studied *Toxocara* stages PCR products of approximately 380 bp in length, which identified parasites as *T. canis*.

No amplification was observed in the negative control (Fig. 2).

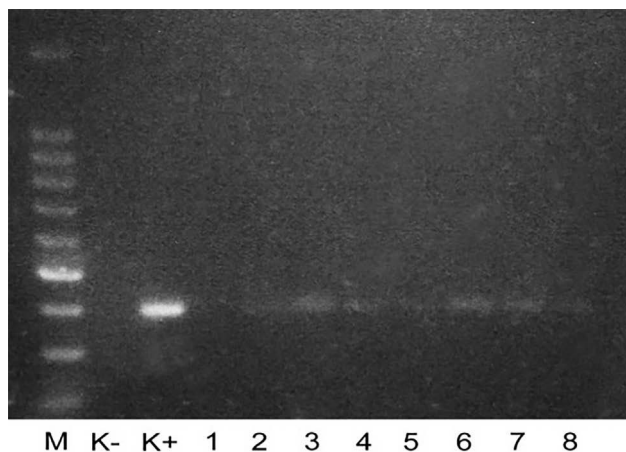


Figure 2. Agarose gel electrophoresis on 1.8% gel of ITS-2-PCR products from *Toxocara* isolates. M, marker (100 bp DNA Ladder); K(-), negative control; line 3, K(+), positive control for *T. canis*; Lines 1, 2 *Toxocara* adult forms; lines 3-5, *Toxocara* eggs; lines 6-8, *Toxocara* larvae, products ~ 380 bp in length.

DISCUSSION

T. canis and *T. cati* are species of human and animal health significance. Besides them, other Ascaridoid nematodes, such as *T. malaysiensis* and *Toxascaris leonine* cause gastrointestinal infections in mammals of the families Canidae and Felidae (18) and their zoonotic potential is still unclear. To overcome the limitations of traditional (morphology-based) taxonomy various molecular methods based on ribosomal and mitochondrial markers for identification of *Toxocara* spp. and diagnosis of toxocariasis have been developed (9), including conventional and real-time PCR techniques for amplification of different targets (11, 16, 19).

Sequences of ITS1 and ITS2 rDNA regions were demonstrated to be reliable markers for distinguishing *T. canis*, *T. cati*, *T. malaysiensis*, and *Toxascaris leonine* (16, 20). ITS-2 sequence-based analyses were found useful for differentiation between adult worms of *T. canis*, *T. cati*, and *Toxascaris leonina*, as well as other ascaridoids that can be found in human tissues (first demonstrated by Jacobs et al. (16)). Molecular techniques for diagnosis of toxocariasis in bronchoalveolar lavage have been developed in experimentally *Toxocara* spp. infected mice, in order to improve the diagnosis in patients with pulmonnary signs and symptoms (21). Data published on toxocariasis show a widespread prevalence of this helminthosis, which is still a neglected and poorly evaluated problem (22). Our studies among healthy individuals in Bulgaria show 8.0% prevalence of

Toxocariasis found in ELISA, and and 4.0% in WB (23). Studies on the prevalence of *T. canis* in animals in our country show that this is one of the most common infestations in dogs (12%) (24), especially in small puppies. The exact determination of environmental contamination with the *Toxocara* eggs is important for effective prevention and control of the disease. However, distinguishing ascarid eggs of different species based only on microscopic examination is difficult, due to their similar morphology and size. In addition, invasions in cats with adult *T. canis* have been reported (25). Khademvatan et al. (2013) found *T. canis* eggs in 4 (6.34%) of 63 *Toxocara*-positive fecal samples from stray cats (17), and Fahrion et al. (2011) showed that dogs shed significant amounts of *T. cati* eggs, probably as a result of coprophagia (26). Therefore, the need for more sensitive and specific techniques to overcome the above mentioned disadvantages is obvious.

In our study, PCR based on amplification of the ITS-2 gene was used for identification of *Toxocara* eggs, larvae, and marital forms to species level. The methodology was applied by Khademvatan et al. (2013) for the characterization of *Toxocara spp.* eggs in feces of stray cats (17). With some modifications and optimization in our conditions, it was applied for species determination of *Toxocara* at all stages - eggs, larvae, and adult forms of the parasite. In all cases, the species *T. canis* was distinguished by the characteristic band of approximately 380 bp in length. The electrophoresis following PCR reaction on the adult forms of the parasite visualized a very weak band, indicating the need for additional sample processing and optimization of the amplification conditions in order to retrieve a larger amount of DNA . Our results show that the PCR method applied and optimized by us can be used for species identification of different forms of *Toxocara* parasites found in tissues of paratenic hosts, including humans. It can be also used for confirmatory diagnosis of toxocariasis at the species level, as well as for determining the causative agents of environmental pollution (soils, sands and sludge sample).

CONCLUSION

Toxocariasis remains one of the most widespread and economically important zoonoses. The parasites of genus *Toxocara* have an exceptional capacity to survive for a long time in the environment and in the

host, and are adapted to a number of domestic and wild definitive and paratenic hosts.

The limitations of morphological methods for determination of *Toxocara* species require the use of more specific methods. The present work is a pilot first in the country examination of the applicability of PCR technique for species-specific identification of the eggs and larvae of *T. canis* and *T. cati*. This molecular assay could be a useful tool not only for diagnosis of toxocariasis in humans and animals, but also for contamination assessment of environmental samples such as sand, soil, as well as wastewater treatment plant (WWTP) sludge.

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DYNAMICS OF THE ETIOLOGICAL STRUCTURE AND SENSITIVITY TO ANTI- BACTERIAL AGENTS OF SALMONELLOSIS IN BULGARIA FOR THE PERIOD 2016 – 2019

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ABSTRACT

Background. In recent years, some features of salmonellosis have been noted: an increase in morbidity all around the world, which is accompanied by an increase in the number of isolated serotypes from humans, animals and food; an increase in sporadic diseases and multi-countries *Salmonella* outbreaks.

Material and methods. For the period 2016 - 2019 in the National Reference Laboratory for Enteric Infections, Pathogenic Cocci and Diphtheria 2 696 *Salmonella* isolates from all over the country have been confirmed. All of the strains were epidemiologically and microbiologically examined, and tested for antimicrobial resistance.

Results. Salmonellosis is caused by several dominant for Bulgaria serotypes - is for S.Enteritidis and STyphimurium take the leading place, followed by *Salmonella* 1,2,5,12: i: -, Infantis, Paratyphi B Java, Kottbus, Corvallis, Derby, Dublin.

Conclusions. Given the widespread transfer of *Salmonella* through the exchange of food and animals, and international tourism, the microbial landscape of

salmonellosis is becoming more diverse, though the leadership of S. Enteritidis and S. Typhimurium has been maintained in the recent years, both worldwide and Bulgaria.

Keywords: *salmonellosis, serotype, salmonella, antibiotic susceptibility.*

INTRODUCTION

Salmonella infections are a major cause of gastroenteritis worldwide. Approximately 2.8 billion cases of diarrhoea are reported each year. The transmission of *Salmonella* is often associated with the consumption of contaminated water and food of animal origin (eggs, meat, dairy products, etc.) and this is facilitated by poor hygiene conditions (1). S.Enteritidis is the most commonly isolated *Salmonella* serotype, followed by S. Typhimurium according to WHO, CDC, ECDC and FoodNet. Besides, they are the leading cause of food epidemics, followed by S. Agona, S. Infantis, and last but not least S.Typhi. The latter is still taking thousands of children's lives in endemic areas of Asia and Africa (2, 3, 4). The number of antibiotic-resistant *Salmonella* strains is increasing every year. Resistance to ampicillin, amoxicillin and trimethoprim/sulfamethoxazole is more common in developing countries. In all cases, a strict assessment of the actual needs for antibiotic therapy, consideration of the current aetiology and the antibiotic susceptibility of the dominant *Salmonella* serotypes are required (5, 6). The leading place of salmonellosis among intestinal bacterial infections, the growing incidence of salmonellosis all over the world, the high susceptibility of children, as well as the severity of the clinical course, determine the importance of the problem and the need for its thorough investigation.

Our study presents the etiological dynamics of salmonellosis in Bulgaria for the period 2016 - 2019 and the sensitivity of the isolated strains to the most commonly used antibacterial agents.

MATERIALS AND METHODS

For the period 2016 - 2019 in the National Reference Laboratory for Enteric Infections, Pathogenic Cocci and Diphtheria 2 696 *Salmonella* isolates from all over the country were confirmed, of which: 718 in 2016, 796 in 2017, 586 in 2018 and 596 in 2019. To trace the etiological dynamics of salmonellosis in Bulgaria, we conditionally divided the period into two smaller

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ones - first period January 2016 - December 2017, the second period January 2018 - December 2019. The identification of *Salmonella* serotypes was performed phenotypically - agglutination of a slide with antisalmonella sera (Sifin - Germany) and genetically - PCR analysis for identification of *S. Enteritidis* and *S. Typhimurium*, as well as for differentiation of *S. Paratyphi B var. Java*, as described in our previous studies (7, 8). Demonstration of epidemiological relatedness of *Salmonella* isolates and confirmation of epidemic outbreaks was performed by PFGE analysis (pulse gel-electrophoresis) according to the protocol of PulsNet, CDC, Atlanta (9).

Antimicrobial susceptibility

Due to the widespread prevalence of resistant *Salmonella* strains worldwide, treatment must be guided by the antimicrobial susceptibility of each specific isolate of *Salmonella sp.* The isolates of *Salmonella sp.* (from faeces, urine, blood and wounds) were tested for antimicrobial susceptibility to cefotaxime- CTX (30µg), cefoxitin- FOX (30µg), ceftazidime- CAZ (30µg), ampicillin- AMP (10µg), amoxicillin/ clavulanic acid- AUG (20/10µg), amikacin- AMK (30µg), gentamicin- GEN (10µg), tetracycline- TE (30µg), chloramphenicol- CL (30µg), ciprofloxacin- CIP (5µg) and trimethoprim/sulfamethoxazole- SXT (1,25/ 23,75 µg) by the disk diffusion method according to EUCAST.

RESULTS AND DISCUSSION

During the first subperiod (January 2016 - December 2017) the number of salmonellosis cases in the country was 1,514, 468 of which in children aged 0-12 years; the incidence was 10.63%, and the mortality rate was 0.14% with two deaths of salmonellosis according to the data from the Epidemiology Department of NCIPD and the National Center for Public Health and Analysis (NCPHA). During this period *S. Enteritidis* represented 55.47% of all serotypes, followed by *S. Typhimurium* - 21.62%, of which 5.72% monophasic *Typhimurium* (*Salmonella* 1,4,5,12: i :-) and *S. Infantis* - 3.81 %. The remaining 19.1% were isolated cases of *Paratyphi B var. Java*, *Kottbus*, *Corvallis*, *Derby*, *Dublin*, *Typhi* and others, presented in detail in Figure 1a. Figures 1a and 1b present the dynamics of the etiological structure during the two compared periods. For the first period, 17 epidemic outbreaks were reported in Bulgaria. Epidemiological studies have identified a

factor in the transmission of the infection to healthy carriers (service personnel in organized teams and events) and contaminated food. A total of 227 individuals were infected, with *S. Enteritidis* being the leading etiological agent in 12 of the epidemics, followed by *S. Typhimurium* in 4 and *S. Infantis* – in one epidemic outbreak.

During the second period (January 2018 - December 2019) the of 1,182 salmonellosis cases were registered, of which 627 in children aged 0-12 years; the incidence was 8.41%, and the mortality rate was 0.34% according to the Epidemiology Department of, NCIPD and NCPHA. During this period, the relative spread of *S. Typhimurium* increased (35.17%) at the expense of *S. Enteritidis* (41.01%). *Salmonella* 1,4,5,12: i - comprised 13,02% of the cases, due to several registered food epidemics among children. *Salmonella Infantis* (5.98%) maintained its dominant position with a slight increase in isolated cases. During the same period 2018 - 2019 an epidemic outbreak of a rare for Bulgaria serotype - *London* was registered (3.89%), which dramatically changing dramatically of serotype dynamics for the country. The remaining 13.95% of salmonellosis cases are detailed in Figure 1b. A total of 11 epidemic outbreaks were reported during the second period, (six fewer than the first period), as follows: 5 epidemics caused by *S. Enteritidis*, 3 epidemic outbreaks - by *S. Typhimurium*, 2 - by *Salmonella* 1,4,5,12: i - and one with a total of 198 patients - by *S. London*,. The etiological dynamics of the reported salmonellosis outbreaks in the country for the two compared periods 2016 - 2017 and 2018 – 2019 is shown in Figure 2.

Salmonellosis cases are reported throughout the year, with the highest number of affected - 916 cases (60.45%) in June - October for the first period and 734 cases (62.06%) in May - September for the second period . This is due to the seasonal occurrence of food epidemics during the warmer months. In both periods the group of children (0-12 years) was the most affected, and the incidence in men was higher than in women, as published by other authors (10, 11).

In Bulgaria, cases of Typhoid fever are mostly sporadic. The last reported epidemic of *S. Typhi* was in 2014, affecting a risk group of elderly people in a closed group. For the two compared periods three sporadic, laboratory-proven cases of *S. Typhi* were reported in Bulgaria, each of them imported from

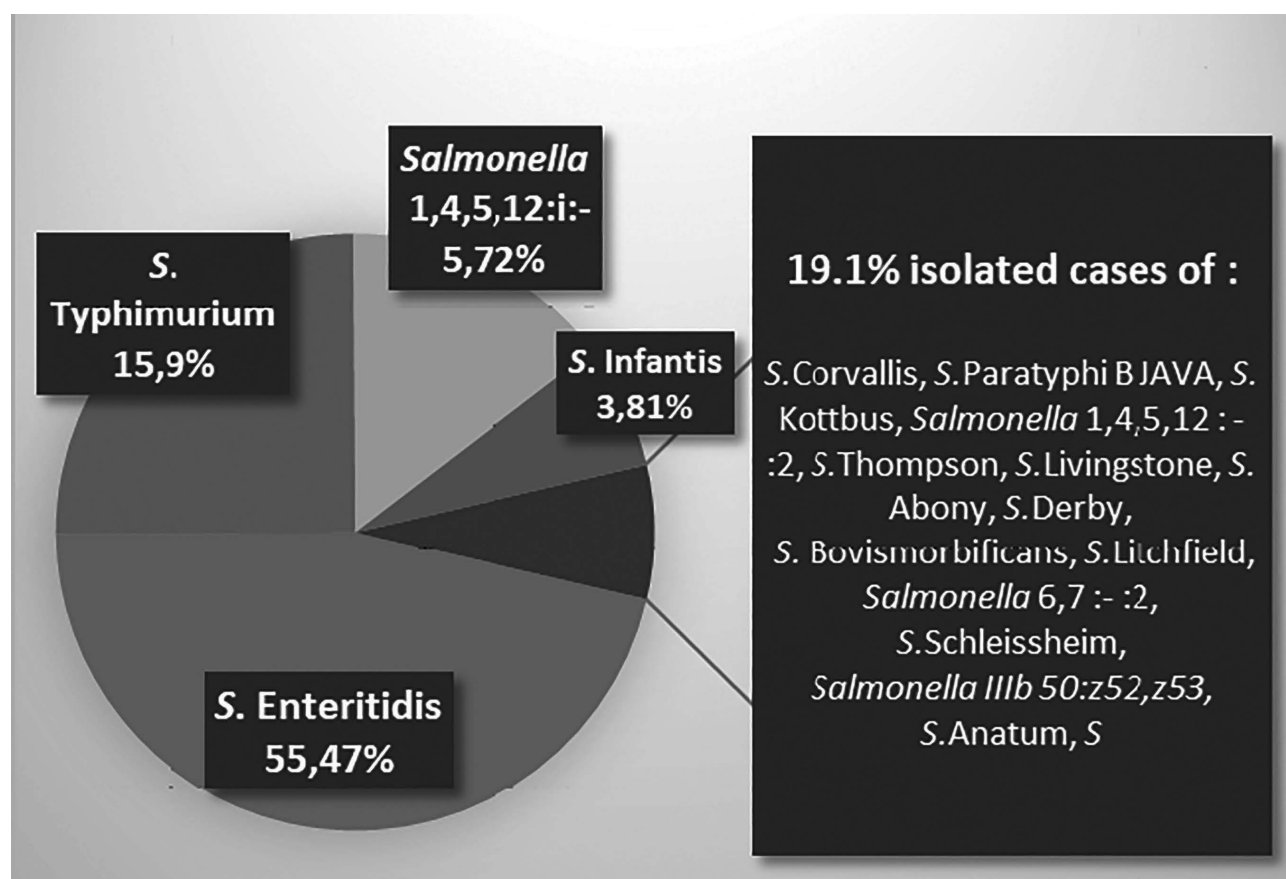


Figure 1a: Etiological structure of Salmonellosis in Bulgaria for the period 2016 - 2017.

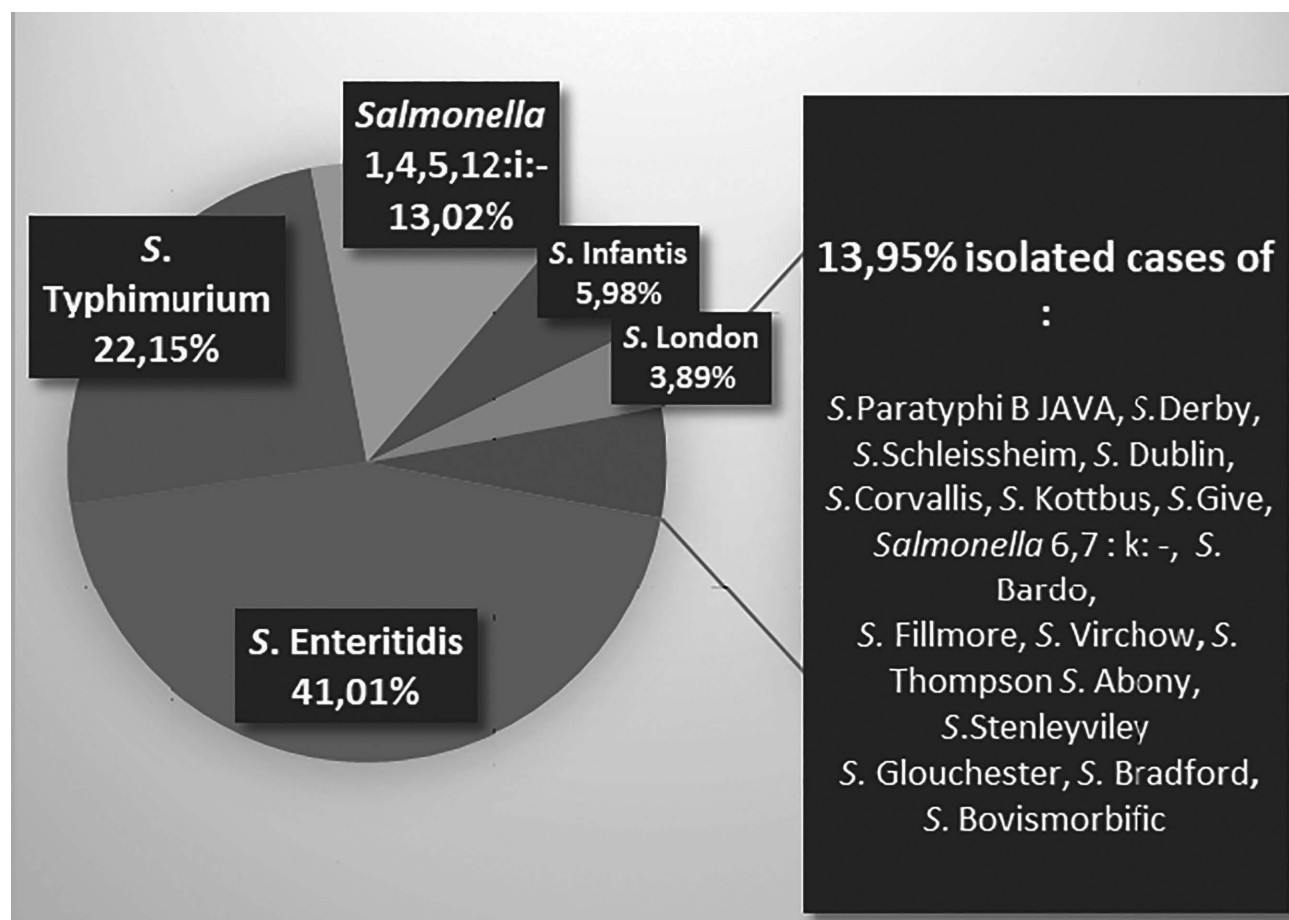


Figure 1b: Etiological structure of Salmonellosis in Bulgaria for the period 2018 - 2019.

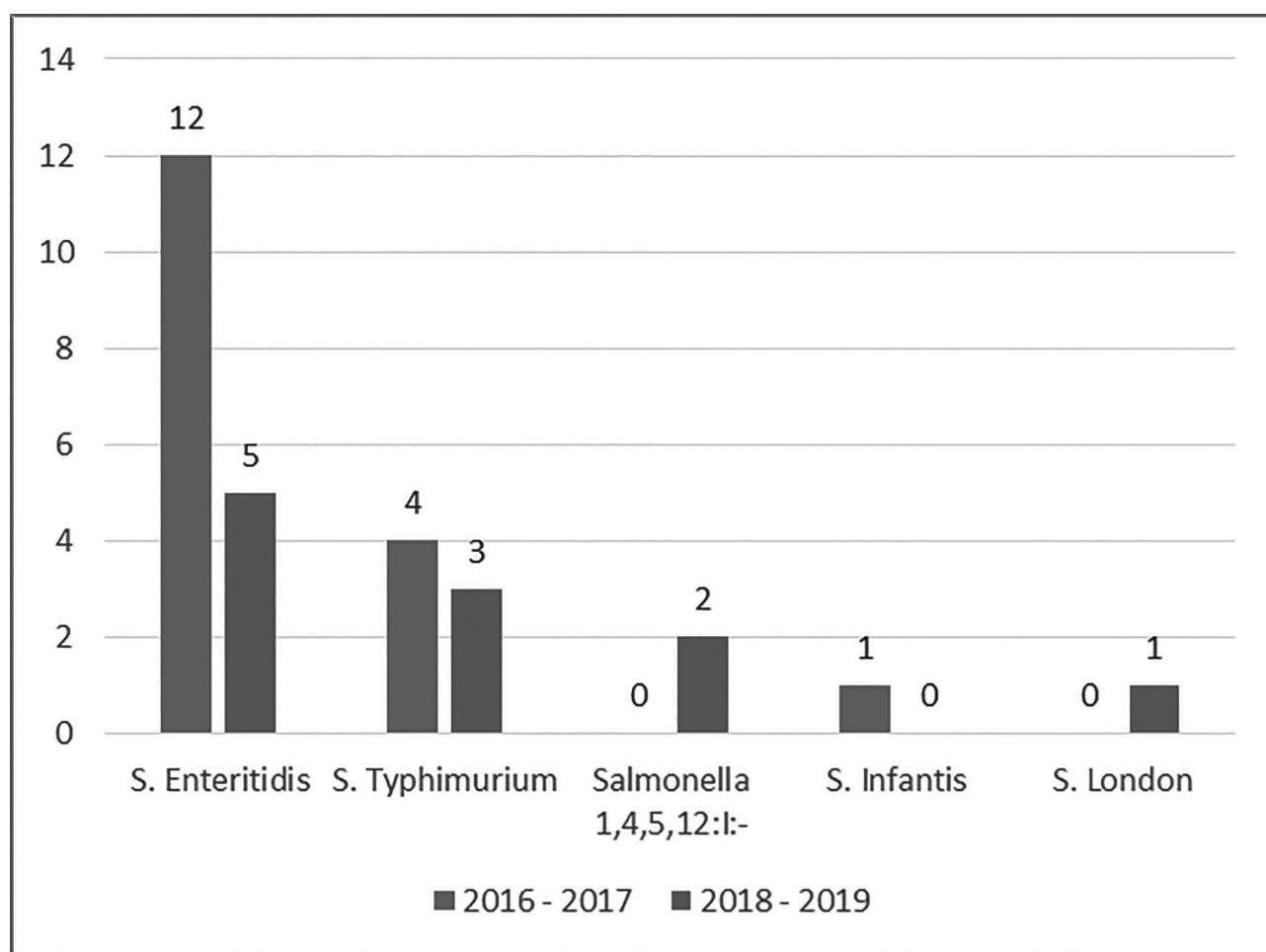


Figure 2: Dynamics of the etiological causes of the registered epidemics of salmonellosis in Bulgaria for the two compared periods 2016 - 2017 and 2018 - 2019.

an endemic region - India, Morocco, Uganda. All the three isolates of *S. Typhi* demonstrated sensitivity to the tested antibiotics, which facilitated the treatment of this dangerous systemic infection.

It is important to note that a large part of the Bulgarian population neglects the diarrhoea syndrome, and does not look for medical help, thus leaving an important part of diarrhoeas etiologically indeterminate, reflecting on the number and dynamics of salmonellosis cases in the country.

Despite the frequent reports of a worldwide increase in *Salmonella* resistance to fluoroquinolones (12, 13), the antimicrobial susceptibility demonstrated by *Salmonella* isolates studied in this investigation, was encouraging - 90% of isolates were sensitive to ciprofloxacin. According to the data of the NRL for Enteric Infections for the two compared periods, the studied representatives of *S. Enteritidis* showed a good sensitivity to the most often used antibacterial agents - CTX, FOX, CAZ, AMP, AUG, AMK, GEN, TE, CL, CIP, SXT (90%). Still, some *Enteritidis* strains were resistant to these antibiotics, most often to

GEN and AMP. *S. Typhimurium* is often resistant to AUG and SXT, and more rarely – to CIP, which could be associated with the animal origin of the serotype and the frequent use of antibiotics in agriculture. It is impressive The 100% antibiotic sensitivity of certain epidemiologically related *Salmonella* isolates from the various epidemic outbreaks in the country should be mentioned (*S. Infantis*, and *S. Enteritidis* from the first period and *S. London* and *Salmonella* 1,4,5,12:i- from the second period). The antibiotic susceptibility of isolates from an epidemic is one of the phenotypic criteria for proving the relatedness of these isolates (14). In our study, the phenotypic method was only complementary to the established gold standard for bacterial outbreaks examination - PFGE analysis, which has been successfully applied in NRL for Enteric Infections for years. The antibiotic susceptibility of the other serotypes did not change significantly. Particular attention is paid to isolates belonging to *S. Paratyphi B* var. Java due to the possible manifestation of more severe clinical symptoms resembling Typhoid fever. The most common Java strains resistance is to

AMK and GEN (30% of isolates).

It is of The need for antibiotic therapy requires careful assessment in order to avoid the development of dysbacteriosis and the appearance of resistance among the circulating *Salmonella* serotypes.

During the studied period 2016 - 2019, the largest relative share in Bulgaria had *S. Enteritidis* among the isolated strains in Bulgaria, which is also the most prevalent salmonella serotype in Europe and the world according to ECDC, CDC and WHO. *Salmonella* Enteritidis is a leading etiological cause of food and water-borne epidemics, both in Bulgaria and in other EU member states (15, 16, 17). There is an obvious tendency of increase the number of laboratory-confirmed single-phase isolates of *Salmonella* Typhimurium (1,4,5,12: i: -) but at the moment the two-phase Typhimurium (1,4,5,12: i: 1, 2) is still leading in Bulgaria. The sensitivity of both varieties *S. Typhimurium* to AUG, CIP and SXT are lower, which hardly justifies their inclusion in the therapeutic process.

In general, the susceptibility of *Salmonella* to antibacterial drugs remains high, despite the growing number of resistant strains in the world. That could be due to a more moderate consumption of antibiotics in Bulgaria compared to other European countries as France, Italy, Belgium and others. (18, 19, 20).

CONCLUSION

The etiological structure of salmonellosis in Bulgaria does not differ significantly from that in other European countries. The relatively high susceptibility of *Salmonella* strains to the most commonly used antibiotics in the country should not be reassuring. A constant control and monitoring of salmonellosis is indispensable for the good medical practice.

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THE ROLE OF BIRDS IN THE TRANSMISSION OF LYME DISEASE A LITERATURE REVIEW

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ABSTRACT

During the vast distances in migration, sometimes through thousands of kilometers, the birds carry ticks very far away from their nesting sites. Up to this moment 136 species of hard ticks Ixodidae have been frequently found on birds. Larvae, nymphs and adults of 25 species of the genus *Ixodes*, parasitize on birds. By transporting ticks infected with *B. burgdorferi* s.l., the birds introduce the infection indirectly to new geographical regions. Once imported, the spirochetes may adapt to the local ecological areas and reservoir hosts. Migratory birds which carry *Borrelia* spp. in their tissues or blood, are able to infect the ticks in the new area. This determines the reservoir competence of the birds and their participation in the ecology and the epidemiology of Lyme disease. The selective transmission of *Borrelia garinii* from birds to ticks has been detected; as well as the fact that migratory birds are able to carry Lyme disease as a latent infection for several months.

Keywords: birds, ticks, *Borrelia* spp., reservoir competence

BIRDS AS DISSEMINATORS OF THE VECTORS OF LYME DISEASE

The risk for humans in certain regions to be infected with *Borrelia* spp. and to develop Lyme borreliosis (LB) largely depends on the tick abundance. In Europe, the primary vector tick is the widely spread *Ixodes ricinus* (1), in Asia - *Ixodes persulcatus* (2), and in the USA - *Ixodes scapularis* and *Ixodes pacificus* (3).

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Mammals are the most common hosts for Ixodid ticks (4). A total of 136 hard ticks have been found on birds (Aves) so far. Larvae, nymphs and adults of 25 species of the genus *Ixodes* parasitize on birds, including the vectors of Lyme disease (5). During their long-distance migration, sometimes through thousands of kilometers, birds carry ticks far away from the nesting habitats. The slow feeding process of ticks for several days, makes their passive transportation possible.

I. ricinus is a polyphagous that feeds on a broad range of vertebrates (6). There is a large number of studies on its distribution and host-spectrum in Russia. Among birds, some species of Galliformes and Passeriformes, such as the song thrush (*Turdus philomelos*), the tree pipit (*Anthus trivialis*) and the Eurasian jay (*Garrulus glandarius*), serve as hosts for nymphs (7). Larvae prefer to suck the blood of the common chaffinch (*Fringilla coelebs*), the European crested tit (*Parus cristatus*) and the European robin (*Erithacus rubecula*).

A survey in Cyprus showed that the larvae and nymphs of *I. ricinus* parasitize on migratory passerines (8). *Haemaphysalis punctata* and *Haemaphysalis concinna* larvae were also found. These two species may play the role of vectors of Lyme disease (9, 10).

Almost 100 million birds migrate every spring to or through Sweden to their breeding sites (11). At the same time, they carry a great number of ticks. Out of 22 998 studied Aves, 2% were infested with 967 ticks (12). The most-abundant tick species was *I. ricinus* (98.3%). The authors estimated that more than 6.8 million new ticks were carried to Sweden by the migratory birds every spring. Every year, during the autumn flights 4.7 million ticks passively leave the country and move to the south. The taiga tick, *I. persulcatus*, was found for the first time in the Scandinavian region during the study period, one nymph from willow warbler (*Phylloscopus trochilus*). *I. persulcatus* is one of the most opportunistic hard ticks. It takes blood meal on more than 200 species of mammals and over 120 species of birds (13). Birds are apparently preferred by nymphs (14). An interesting fact is that the ones that mainly parasitize on humans are the *Ixodes persulcatus* adults (15), unlike *I. ricinus* and its nymphal stage which is the one that most frequently bites humans (16).

Ixodes scapularis, widespread in the USA and Canada, feeds on more than 125 vertebrates, of which 57

are birds (17). Immature stages were found on birds (18, 19, 20). A little less diversity of hosts has *Ixodes pacificus*. A review article showed a total number of 108 vertebrates, 48 of which are Aves (21).

BIRDS AS RESERVOIRS FOR *BORRELIA BURGDORFERI* SENSU LATO (S.L.)

Together with the knowledge on the feeding behavior of the vectors and the competent reservoirs among mammals (22, 23), assumptions appear that some Passeriformes may be also a source of *Borrelia* spp., causing Lyme disease in humans. In the early 1980s entomologists at the Connecticut Agricultural Experiment station isolated spirochetes from the blood of six species of birds (18). Spirochetes were also detected in the larvae and the nymphs of *I. dammini* (*scapularis*), removed from some of bird species in the investigated area. The identification of the spirochetes was still unknown. Two years later, again in Connecticut, spirochetes were isolated and identified as *Borrelia burgdorferi*. The isolates were from a liver of a veery (*Catharus fuscescens*) and *I. dammini* larvae derived from other passerines (24). The recovery of *B. burgdorferi* from the liver of a veery was the first isolation of this pathogen from a bird. To confirm the infectious nature of *B. burgdorferi* to birds, the veery liver isolate was inoculated in laboratory bred chicks. *Borreliae* were successfully isolated from the kidney and the spleen of an inoculated chick. During field investigations in Minnesota, spirochetes were observed in the blood culture from a song sparrow *Melospiza melodia* (25). The bird was naturally infected and the isolate was confirmed as *B. burgdorferi* by immunofluorescence assay. Ticks feeding on song sparrow were not tested, even though it was already noticed that *Melospiza melodia* is a species that is frequently infested (26). Another study on the reservoir role of birds was conducted on an island near Massachusetts (27). It was proven that one of the most common birds on the island (*Dumetella carolinensis*) was not a source of infection for ticks but only a blood meal source. None of the individual birds in the study were able to infect the larvae, although *Dumetella carolinensis* is frequently attacked by ticks and the rate of tick infection with *B. burgdorferi* on the island is significant (28). These results proved that the high density of a certain bird species in a certain area and the frequent tick infestation of birds do not mean that the bird should be considered as a competent reservoir. Most

probably that bird species is not susceptible to Lyme disease infection and it is not capable of transmitting the pathogens to vectors. The authors concluded that in the endemic LB areas a regular research on the infection of the common bird species with spirochetes was necessary.

An increase in LB cases in Scandinavia during the 90s lead to determination of the role of migratory birds in the maintenance of the natural infection cycle. Around 1.3 million potentially infected with *B. burgdorferi* s.l. ticks leave Sweden during the bird migration every autumn. Larvae of *I. ricinus* were examined for *Borrelia* spp. by molecular genetic methods of polymerase chain reaction (PCR) (12). *B. garinii* was the most frequently found among ticks, in 23 of 33 infected ticks (70%), followed by *B. burgdorferi* s.s. (20%) and *B. afzelii* (9%). Results showed that all three *Borrelia* species associated with LB in Europe could be detected in bird transported ticks. Moreover, seven out of eight analyzed Turdidae birds carried larvae infected with *B. burgdorferi* s.l. The authors assumed that some ground foraging species are hosts of both ticks and LB spirochetes. Another study in Sweden (2006) also identified *B. garinii* as the most dominant genospecies in ticks feeding on Passeriformes, especially among the larvae (29). Considering the rare transovarial transmission of Lyme disease agents (30), the researchers supposed that the infected larval stage most probably got spirochetes by birds and the putative reservoir role of birds depends on their feeding habits.

Spirochetes of the genus *Borrelia* were found in *Ixodes uriae* ticks collected from Alcidae seabirds, on an island in the Baltic Sea (31). DNA of the spirochete-infected *I. uriae* ticks was isolated, purified and amplified by PCR. A presence of *B. burgdorferi* was detected. *B. burgdorferi* DNA was also observed in a biopsy from the foot web of one razorbill. One of the two ticks found on the same bird was also positive for *Borrelia* spp. It contained *B. burgdorferi* DNA with the same size of the flagellin gene fragment as the *Borrellia* DNA of the biopsy. According to the authors the results supposed bird involvement in the transmission cycle of *B. burgdorferi* s.l. This is also supported by the fact that the island is free from mammals. The evidence that *I. uriae* parasitizes on razorbills and is capable of harboring *B. garinii* (32), prompted the hypothesis that the presence of seropositive Faeroe Islanders who were regularly

bitten by *I. uriae* might be due to a transfer of *B. garinii* to humans by this tick species (33). The principal vector of Lyme disease, *I. ricinus*, is not established on the Faroe Islands. The major participants in the enzootic cycle are *I. uriae*, *B. garinii* and seabirds such as puffins.

All existing knowledge on the reservoirs of *B. burgdorferi* s.l. in Europe was collated in a review article from 1998 (34). A total of 16 species of Passeriformes, Charadriiformes (Alcidae) and Galliformes (Phasianidae) were listed in the paper, which appear capable of participating in the natural transmission of *B. burgdorferi* s.l. The criteria for reservoir status were also described. To identify the species which maintain the circulation in the specific area, the researchers recommended the method of xenodiagnosis or a combination of other methods: collection of engorged larvae from wild animals and their examination after moulting under laboratory conditions; analysis of blood meal remnants in the gut of unfed nymphs for identification the host that the nymphs fed on as larvae; isolation of *B. burgdorferi* or detection of *B. burgdorferi* DNA in vertebral tissue; detection of antibodies to *B. burgdorferi* in vertebrate blood.

Richter et al. (2000), who explored the reservoir competence of the American robin (*Turdus migratorius*) in North America, stated that the competent reservoir host tolerates feeding by both subadult stages of vector ticks (35). These authors considered that the competent animal reservoir for LB agents had three major characteristics: 1) readily acquires infection from vector ticks; 2) permits spirochetes to proliferate; 3) readily infects vector ticks.

SELECTIVE TRANSMISSION OF BORRELIA BURGDORFERI S.L. BY BIRDS

Together with the studies on the reservoir role of birds, some researchers formulated the hypothesis that the transmission cycle of various *Borrelia* spp. in the nature is associated with specific vertebrate animal groups (36). This hypothesis was supported by the results for Japan, showing that more than 60% of *I. persulcatus* larvae from birds were infected with *B. garinii*. No isolates of *B. garinii* were found among the isolates from rodent-feeding *I. persulcatus* larvae. Only *B. afzelii* were detected in them. The observations suggested that two enzootic cycles in nature maintain borreliae specifically: birds-ticks and

rodents-ticks. Another Japanese study in 2000 year showed that *B. garinii* is most probably imported in the country by migratory birds from North-East China via Korea (37).

Researches on the transmission of borreliae in England showed the very high ability of *Phasianus colchicus* to infect ticks with *B. garinii* and *B. valaisiana* (38). Over 50% of *I. ricinus* nymphs detached from pheasants carried both *Borrelia* spp. A xenodiagnostic experiment was applied in order to assay the spirochetal transmission from rodents to ticks. Non-infected *I. ricinus* larvae were placed to feed on wild rodents captured from the pheasant breeding territory. The diagnostic method indicated that rodents were not capable of transmitting *B. garinii* to the larvae, although a later performed biopsy of the internal organs showed that 19% (9/47) of them harbored *B. garinii*. The rodents only transmitted *B. burgdorferi* s.s. Data for pathogen presence in questing adult ticks from the same site showed a prevalence of *B. garinii*, followed by *B. valaisiana*. That data was similar to the one obtained for the ticks from pheasants. This gave basis to researchers to think that pheasants, which are the major host for nymphs in the area, selectively infect with *B. garinii* and *B. valaisiana*. The researchers considered that different genospecies of *B. burgdorferi* s.l. could be maintained in some regions of Europe by distinct transmission cycles involving the same vector tick species but different vertebrate host species. The assumptions for an existing association between the birds and *B. garinii* were based on the optimum temperature growth of the borreliae causing LB. The optimum temperature for *in vitro* cultivation of Lyme disease spirochetes is between 34 and 37°C (39). This might mean that *in vivo* growth is not possible in some animals with relatively high body temperature. The average body temperature of some Passeriformes may reach 40,6°C (40). Most probably *B. garinii* tolerates a higher one (41).

The experimental results obtained for the common pheasants are somewhat contradictory to the results for the reservoir competence of the great tit *Parus major* (42). In south Belgium, the ticks from *Parus major* carried five genospecies of *B. burgdorferi* s.l. and some of them were associated with mammals *B. afzelii* and *B. spielmanii*. The authors explained that it could be due to the fact that the European *B. afzelii* strains have a longer co-evolutionary history with

the native birds than with the introduced pheasants. Findings of Polish, German and Spanish researchers also proved the presence of *B. afzelii* in bird-derived ticks (43, 44, 45). Swiss researchers found that migratory birds infect *I. ricinus* with one more *Borrelia* species (46). It was *B. lusitaniae* which is distributed mainly in Southwestern Europe (47) and North Africa (48). *B. lusitaniae* was the third most prevalent genospecies in Switzerland following *B. valaisiana* and *B. garinii*. Only *B. lusitaniae*-infected larvae were detected in that study. The authors concluded that migratory Aves appear to be reservoir hosts for *B. lusitaniae*. *B. lusitaniae* was also found in non-engorged, field-collected Bulgarian *I. ricinus* ticks (49).

Association of *B. garinii* and *B. valaisiana* with Aves was observed several times in Central and Western European countries (50, 51, 52). Czech researchers revealed that although only three bird species (common blackbird, song thrush and great tit) mainly infect the ticks, they have the potential to spread millions of spirochetes in urban areas (53). In a three-year period, blood samples of 1254 Passeriformes belonging to 42 species were examined in a neighboring Polish region, an endemic area of *B. burgdorferi* s.l. infection in ticks (54). *B. burgdorferi* s.l. was detected in 4.2% of all blood samples. Nine birds were positive. Although in this study ticks were not tested, the results indicated that the incidence of infected birds was higher in April-June, when *I. ricinus* abundance is increased. Later, in July-August, the incidence of infected birds was lower and it corresponded to the decreased abundance of parasites. In conclusion, the authors assumed a presence of infected ticks in the observed region of northeastern Poland, as well as bird hosts susceptible to the pathogens. *Borrelia* infection in bird-derived ticks was examined a decade later (2016) in the same region (43). *B. garinii* was the most prevalent genospecies, in 16 of all 35 ticks, or 45.7 %.

The important role of birds in the epidemiology of Lyme disease is also supported by findings for Germany (55). The prevalence of *B. burgdorferi* s.l. in Thuringia is 25% as showed the testing of 141 bird-derived *Ixodes ricinus*. The majority of the ticks carried *B. garinii* (53%), followed by *B. valaisiana* (28%). Unusually high *Borrelia* prevalence (29%) was found in larvae. Among the unfed ticks in the same area, a total of 256 *Ixodes ricinus* were tested, and

Borrelia DNA was found in 39 of them (15.2%). The bird-associated *B. valaisiana* was the predominant species.

In Western Europe, data about Spain indicated that the common blackbird and the song thrush were the most frequently infested. (45). Ticks detached from birds were not examined, but only questing nymphs collected in the same site by dragging a white flannelette over the vegetation. Vertebrate DNA in the blood remnants in the nymph gut was identified by PCR amplification. Sixty-one nymphs were processed. Unambiguous pathogen and/or host detection was only achieved for 25 specimens. Most blood remnants (17 out 25) were from Passeriformes and Galliformes. A few blood meal samples were from rodents, wild boars and *Cervus* sp. *B. afzelii*, *B. garinii* and *B. valaisiana* were detected as well.

A recent article summarized the data for tick infestation of Passeriformes in 11 European countries - Czech Republic, Estonia, Finland, Germany, Greece, Hungary, Netherlands, Portugal, Slovenia, Spain and Sweden (56). A total of 2,308 ticks were collected from 843 birds. A total of 656 *Ixodes* ticks were analyzed for *B. burgdorferi* s.l. and 244 were positive (37.2%). *B. burgdorferi* s.l. prevalence in larvae was 20% (22/110), and in nymphs 41% (214/521), almost twice as higher. *Ixodes ricinus* was the most infected tick species, 40.2% (210/522). The fieldfare *Turdus pilaris* turned to be carrying ticks with the highest *Borrelia* prevalence (92%), followed by the common blackbird (58%). Sequencing the *flaB* gene showed that the most prevalent genospecies was the *B. garinii* (60.7%, 116/191), followed by *B. valaisiana* (23.6%, 45/191), *B. afzelii* (9.4%, 18/191), *B. turdi* (5.2%, 10/191), *B. lusitaniae* (0.5%, 1/191) and one novel genospecies (0.5%, 1/191). The article also focused on the genetic characterization of *B. garinii* at the European and transcontinental scale. The diversity of the common avian-associated *B. garinii* genospecies and potential phylogeographical patterns were determined using a multilocus sequence typing scheme (a technique in molecular biology). Little overlap of the sequence types among the continents Europe, Asia and North America was found but no structuring geographical population in Europe was noticed. Furthermore, according to the authors, no pattern of geographical population structuring was observed according to the isolation source (bird-derived ticks or questing ticks/human isolates), probably due to *B. burgdorferi* s.l./

or ticks' dispersal promoted by birds. These results taken together provided evidence that birds act as important reservoirs for *B. garinii* and are the main source of infection of this genospecies to ticks and ultimately to humans.

HOW LONG DO THE BIRDS REMAIN INFECTIOUS?

Experiments of Kurtenbach et al. demonstrated that pheasants can be infected and that their infectivity for ticks may persist as long as 10 weeks. (57). In North America, four birds American robins infected with *B. burgdorferi* s.s. by lab nymphs *I. dammini* were used for determination of their subsequent infectivity for larvae (35). After the larvae molted, the resulting nymphs were examined by dark-field microscopy. The birds were able to infect 88% of laboratory-raised ticks (xenodiagnostic larvae) for at least 3 weeks. Infectivity waned by 2 months and disappeared by 6 months. The researchers also determined that American robins tolerated reinfection by tick-borne spirochetes. Six months after the birds were initially infected, each of the four American robins was exposed to the bites of eight infected nymphs. All four robins regained infectivity for ticks within the next 2 weeks. It was revealed that the birds remained tolerant to reinfection and became infectious again. The authors concluded that probably American robins tolerated reinfection in the nature as well, because they forage in ground, and are frequently infested by vector ticks. Several years later (2005), the reservoir competence of American robins in North America was proven again (58). In relation to the reactivation of *Borrelia* infection in birds an experiment was conducted under stressful conditions for birds by simulating their migration (59). It turned out that the redwing (*Turdus iliacus*) was able to carry Lyme disease as a latent infection for several months. As a result of migratory restlessness, the infection could be reactivated and passed on to tick vectors. Ticks can feed on birds with reactivated infections anywhere through the migration flight, to infect themselves and to pass the disease on to other organisms.

STUDIES IN BULGARIA

Bulgarian studies on the role of birds as disseminators of Ixodid ticks are very few and were conducted more than 40 years ago. There are no researches on the detection of *Borrelia* spp. in ticks feeding on birds

or in tissues of birds. Data is available on the coastal strip of the Bourgas region for the early seventies (60). Ixodidae specimens collected from birds showed a prevalence of *Haemaphysalis punctata* larval and nymphal ticks. The most frequently infested bird species were the pheasants, *Corvus frugilegus* (rook) and *Perdix perdix* (grey partridge). During the Crimean-Congo hemorrhagic fever outbreak investigation in Pazardzhik region, mostly immature stages of *Hyalomma plumbeum* and *Haemaphysalis punctata* were found on birds (61). The common blackbirds, the grey partridges, *Upupa epops* (Eurasian hoopoe), *Motacilla fl. feldegg* (Black-headed yellow wagtail), *Athene noctua* (Little Owl), *Pica pica* (Eurasian magpie), *Garrulus glandarius* (Eurasian jay), and *Charadrius dubius* (Little ringed plover) were indicated as hosts. *I. ricinus*, reported years later as a European vector of Lyme disease, was not established as an avian ectoparasite back then. However *Haemaphysalis punctata* was established, which may play a role of a secondary vector of Lyme disease in Bulgaria (62).

CONCLUSION

The surveillance of infected questing ticks and reservoir hosts of *B. burgdorferi* s.l is the main part of the LB surveillance. The observation of spatial and temporal dynamics of pathogens in tick and vertebrate animal populations was defined as an essential part for preliminary assessment of LB incidence in humans and disease burden (63). The most frequent disseminated manifestation of Lyme disease in Europe is the neuroborreliosis. In Norway, for example, extensive data of annual disease incidence from 1995 to 2017 showed that 69% of disseminated Lyme borreliosis cases were neuroborreliosis (64). The bird-associated species *B. garinii* are considered to be their causal agent. Studies carried out during the period 2003-2008 found that *B. garinii* is dominant among ticks from migratory birds in Norway (65, 66). These results emphasize again the key role of birds in the epidemiology of LB.

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