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DETERMINATION OF INTRACELLULAR REACTIVE OXYGEN SPECIES IN T-CELL SUBSETS OF HIV+ PATIENTS ON CONTINUOUS cART

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ABSTRACT

Background: Reactive oxygen species (ROS) are generated at physiological levels as a result of cellular metabolism and contribute to cellular interaction and immune response. Elevated ROS may cause cell stress, damage, and apoptosis, and have been detected in different pathological states of infectious and non-infectious etiology.

Aim: To evaluate the association between intracellular ROS in T-cell subsets and HIV VL in chronic HIV infection.

Material and methods: Whole blood samples (Li-heparin, n=33) were analyzed during routine immune monitoring in two groups of HIV+ patients: A (n=21), on continuous cART for at least 2y, with sustained viral suppression (HIV VL<40 copies/ml) and group B (n=12) on cART for less than 2y, average HIV VL 92330 c/ml. Percentage and absolute counts (AC) of CD4+ and CD8+T cells were determined by flow cytometry (Multitest, BD Trucount™ tubes, FACS Canto II). Fluorometric ROS assay kit (Sigma-Aldrich) was adapted for flow cytometry analysis to detect intracellular ROS in CD4+ and CD8+ T-cells (FACSDiva 6.1.2).

Results: The average CD4AC did not differ significantly between group A and B (714 vs. 568, $p>0.05$), unlike the CD4/CD8 ratio (1.2 vs. 0.6, $p<0.01$). The mean fluorescence intensity (MFI) of CD4+T intracellular ROS was significantly lower in group A (mean MFI 1744 vs. 2492, $p<0.05$), unlike the CD8+T cell ROS content (1753 vs. 2129, $p>0.05$). Noteworthy, CD4+T intracellular ROS correlated positively with HIV VL ($R=0.5$, $p<0.05$), unlike CD8+T ROS. On the other hand, positive correlations between CD8+T ROS and cART duration, as well as age ($R=0.5$, $p<0.05$ for both) were observed in group A.

Conclusions: CD4+T ROS production may be an indicator of residual HIV activity in the settings of undetectable HIV VL. The combined effects of ageing and long-term cART affect mostly the CD8+T cell compartment.

Key words: *HIV, ROS, immune recovery, antiretroviral therapy*

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INTRODUCTION:

The existence of free radicals and their essential role in biological systems was suggested for the first time in 1954 by Commoner et al. (1). The free radicals are small molecules, which exist independently and at the same time contain one or more unpaired electrons (2, 3), which is the reason for their strong reactivity and a wide range of intra- and intercellular interactions.

Reactive oxygen species (ROS) include free oxygen radical species such as the hydroxyl radical (·OH), and superoxide anion (O_2·−) as well as non-radical forms and hydrogen peroxide (H_2O_2), which are less reactive (4, 5). The largest source of ROS is oxidative phosphorylation in mitochondria (6, 7). ROS have pleiotropic effects, modifying a wide range of cellular functions and signal-transduction pathways (6, 8) and contributing in particular to the innate and adaptive immune response: initiation of macrophages activation, antigen cross-presentation, regulation of T-cell activation and functions (5, 9, 10). On the other hand, the increased formation of various ROS or the reduction of cellular antioxidant capacity leads to „oxidative stress“, which ultimately causes apoptosis. This phenomenon is observed in various pathologies with infectious and non-infectious etiology, such as carcinogenesis (11,

12), neurodegeneration (13, 14), atherosclerosis, diabetes (15, 16) and aging (4, 17) or due to exogenous factors including ultraviolet and gamma radiation, smoke and other air pollutants, as well as several drugs and chemicals (5).

The role of oxidative stress in HIV pathogenesis has been addressed by numerous studies with somewhat inconsistent results. Reduced antioxidant capacity due to depletion of glutathione (GSH) in plasma, lymphocytes, monocytes and lung epithelial cells was described in HIV-infection. Thioredoxin (TRX) was found depleted in lymph node dendritic cells but elevated in the plasma of HIV-infected patients; reduced superoxide dismutase (SOD) levels in plasma and monocytes and H_2O_2 production in monocytes were associated with high HIV viral load (7). Indicators of elevated oxidative stress were detected in neutrophils, monocytes and astrocytes (18) in relation to TGF-β activation and induction of regulatory T cells (Foxp3+CD4+CD25+) (19).

It is well known that ROS production is induced by HIV proteins located on the viral envelope, such as transactivator of transcription (Tat), viral protein r (Vpr), negative regulatory factor (Nef) and glycoprotein 120 (gp120) (3, 20, 21, 22, 23). Nevertheless, ROS accumulation at the level of CD4+ and CD8+T cell subsets at different stages of HIV infection and during long-term combination antiretroviral therapy (cART) is poorly investigated. Contemporary cART is able to induce sustained viral suppression and immune restoration, though without complete viral elimination, bringing forward the problems of latent HIV reservoirs, low-level immune activation and accelerated ageing (24, 25, 26). ROS may increase HIV replication by reactivating LTR (long terminal repeats) in the latently infected cells through NF-κB –dependent mechanism (7). Clarifying the possible role of ROS for the activation of latent HIV in cART+ patients and understanding the mechanisms and the pathways that HIV uses to generate oxidative stress are of great importance for the successful monitoring and personalization of cART in long-term treated patients (10, 18).

Our current study aims to evaluate intracellular ROS in CD4+ and CD8+ T-cell subsets in relation to HIV VL and the residual immune activation in HIV+ patients on successful cART.

MATERIAL AND METHODS

Study design and participants

Peripheral blood samples (Lithium heparin, n=33) were obtained during routine immune monitoring of HIV+ patients registered at the Specialized Hospital for Active Treatment of Infectious and Parasitic Diseases, Sofia. Two groups were defined to evaluate the early and long-term effects of cART: Group A, patients on continuous cART for a minimum of two years, with undetectable viral load, and group B, treated less than two years, respectively **(Table 1)**.

Viral load determination

HIV viral load was determined in plasma by reverse transcription polymerase chain reaction (Abbott Real-Time HIV-1), over the range of 40 to 10,000,000 copies/ml.

Cell isolation and flow cytometric analysis:

The percentage and absolute counts (AC) of CD4+ and CD8+T lymphocytes were determined by direct multiparameter flow cytometry (Multitest CD3/CD8/CD45/CD4/TRUCount, FACS Canto II, BD Biosciences), **(Fig.1A)**.

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Paque Plus (Sigma-Aldrich). For determination of intracellular ROS in living T-cells, a cell-permeable green fluorescent sensor (λex = 490/λem = 520 nm, Sigma-Aldrich) was used, forming with intracellular ROS a fluorometric complex proportional to their quantity. Briefly, freshly isolated $5x10^4$ PBMCs were incubated for 60 min at 37°C with ROS sensor, stained with CD3APC/CD4-CF-Blue/CD8-PerCP and analysed by flow cytometry (FACSDiva 6.1.2). The same cells, not incubated with the sensor were used as negative control. ROS levels were characterized by the mean fluorescence intensity (MFI_{ROS}) of the stained sample in the FITC-channel after subtracting the background fluorescence of the negative control **(Fig. 1B)**.

Statistical analysis: Parametric data are presented as mean ± SD. Comparisons between groups were performed with one-tail unpaired T-test, and relationships between two variables were analyzed by Spearman's rank correlation coefficient. P values less than 0.05, at CI 0.95 were considered significant (SPSS Statistics v.21.0). Graphpad Prism v.9.0 was used for the graphical representation of the results.

RESULTS AND DISCUSSION:

The demographic and basic laboratory characteristics of the study groups are given in

Table 1 Demographic, clinical and laboratory characteristics of study participants. All data is represented as mean±SD.

HIV+ participants	Group A	Group B	Unpaired T-test
Number (n)	21	13	N/A
Age (years)	43 ±8	33 ±6	p<0.001***
Time after diagnosis of HIV+ (years)	10.7 ±7.6	5.8 ±5.1	p<0.05*
Time between diagnosis and start of cART (months)	15.9±39.5	12.6±25.9	p>0.05
cART duration (months)	103 ±73	13 ±9	p<0.001***
Viral load (copies/ml)	35.8 ±9.0	92330 ±2390	p>0.05
Baseline* CD4 AC (cells/µl)	358 ±243	394 ±360	p>0.05
Baseline CD4/CD8 (ratio)	0.54 ±0.59	0.53 ±0.54	p>0.05
Last CD4 AC (cells/µl)	714 ±313	568 ±383	p>0.05
Last CD4/CD8 (ratio)	1.16 ±0.80	0.61 ±0.42	p<0.01**

*At start of cART

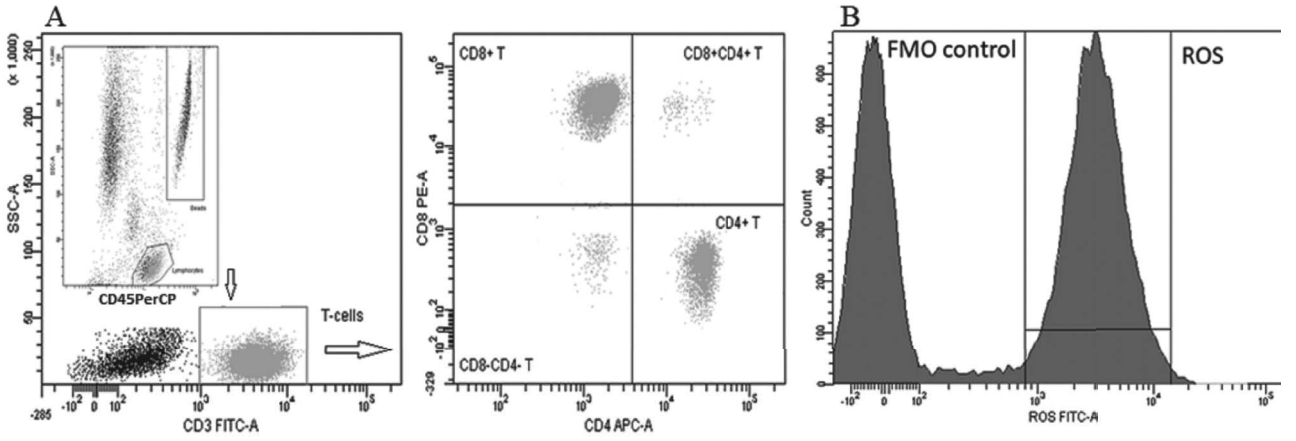

Fig.1 Flow cytometry analysis of ROS in T cells. (A) Gating strategy for CD4+ and CD8+ T-cell subsets. Lymphocytes were initially gated on side scatter properties and CD45 expression (upper left panel), T cells were then gated on the expression of CD3 (left panel), and further subdivided into CD4+ and CD8+ T cells (middle panel). **(B) Determination of intracellular ROS in CD8+ and CD4+ T cells using a ROS sensor.** MFI was measured in the FITC-channel. A Fluorescence Minus One (FMO) control was used to set markers for the ROS-positive population.

Table 1. As expected, long term-treated patients (group A) differed significantly in age, presumed disease duration (time since HIV diagnosis), and time on cART as compared to group B. Importantly, baseline CD4 AC and CD4/CD8 in group A and B (358±242 vs. 394±360 and 0.54±0.59 vs. 0.53±0.54), as well as the time interval between diagnosis and the start of cART (15.9±39.5 vs. 12.6± 25.9) were similar (p>0.05 for all comparisons) giving the grounds to compare the effect of cART with different duration. There were no significant differences between the groups regarding HIV VL, though in four patients from group B the virus was still detectable. Moreover, the last measured CD4 AC were similar (714±313 vs. 568±384, p>0.05). The only parameter which differentiated between the groups was a higher mean CD4/CD8 ratio in long-term treated patients (A) (1.16±0.8 vs. 0.61±0.42, p<0.05). In fact, 67% of patients who had recently started ART (B), and 41% of those on long-term treatment (group A) had suboptimal CD4/CD8 ratio (<0.9).

The mean fluorescence intensity of CD4+T ROS (MFI_{ROS}) in group A was significantly lower as compared to group B: (mean ±SD) 1744± 396 vs. 2492±1239, $p<0.05$. At the same time, CD8+ T MFI_{ROS} in group A was also lower than in group B (1753±542 vs. 2129±989, respectively), but the difference did not reach statistical significance ($p>0.05$) (**Fig. 2**).

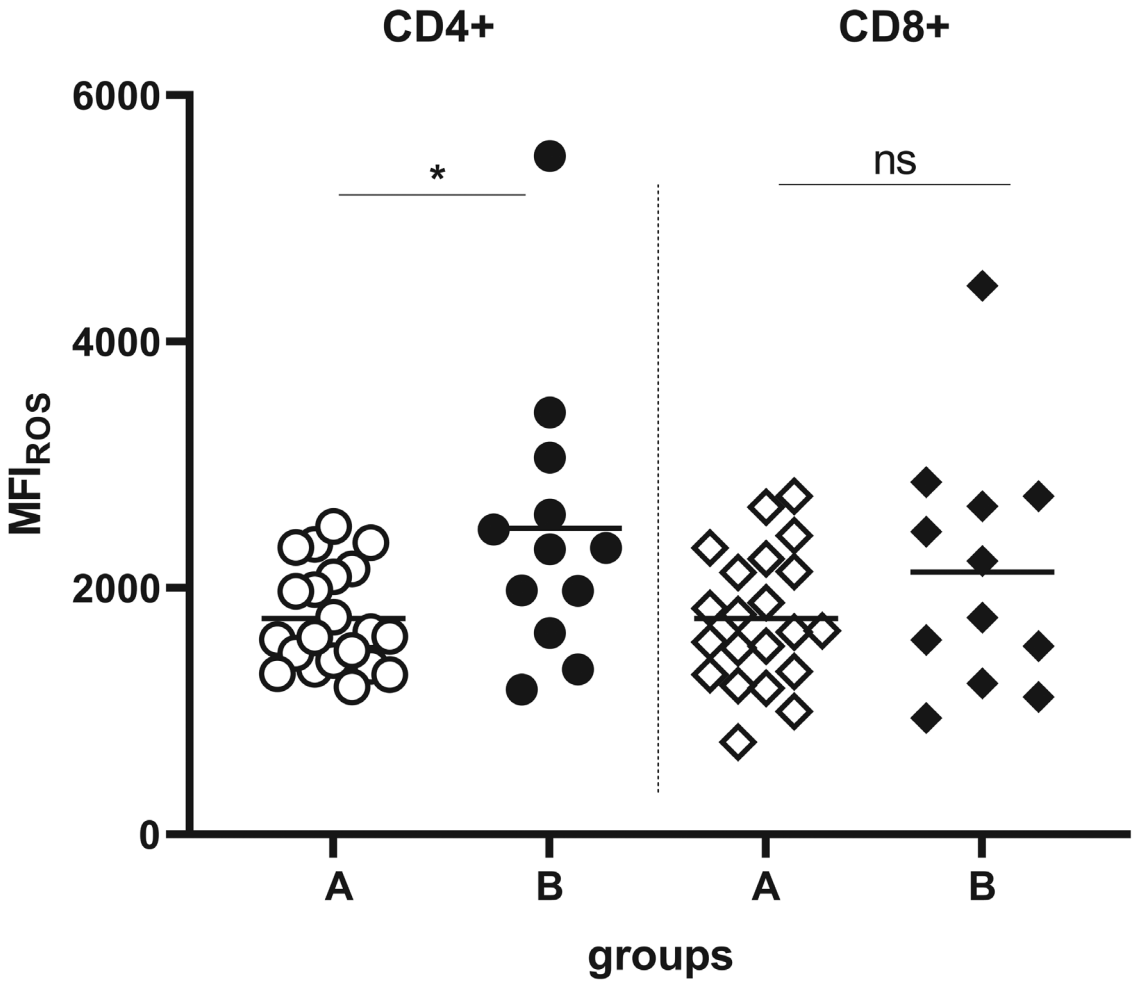

Fig. 2 Comparison of MFI_{ROS} in CD4+ and CD8+ T cells between groups A and B. The difference was statistically significant for CD4+T cells $p<0.05$ (*), unlike CD8+ T cells, ($p>0.05$).

Noteworthy, CD4+T MFI_{ROS} correlated with HIV VL ($R=0.5$, $p<0.05$) unlike CD8+T MFI_{ROS} (**Fig. 3A**). In group A, we found a positive correlation between CD8+T MFI_{ROS} and age, as well as cART duration ($R=0.5$, $p<0.05$ for both) **(Fig. 3C, D)**. Finally, CD4/CD8 ratio in group A correlated negatively with CD4+T MFI_{ROS} ($p<0.05$, $R=-0.47$) **(Fig. 3B)**. Contemporary cART suppresses plasma HIV RNA below detection limit of laboratory assays and restores CD4 T cell pool of infected patients by preventing new rounds of productive infection, depending on its baseline state. Usually, most HIV+ individuals achieve viral suppression (less than 50 copies/ml) in a couple of months after initiation of cART (27). According to accepted criteria, both studied groups demonstrated a virological and immunological response to cART. The quick restoration of CD4 AC was probably associated with the comparatively high baseline CD4AC in both groups, as observed by others as well (28). However, a number of studies have shown that neither HIV VL, nor CD4 AC are exhaustive correlates of immune restoration. The subset ratio CD4/CD8 is a more sensitive marker of ongoing low-level immune activation of variable origin in the settings of cART, involving mostly the CD8 T cell compartment and ultimately leading to its exhaustion (29). In our hands, 67% of patients who had recently started cART, and – remarkably, 41% of those on long-term treatment had a pathologically decreased CD4/CD8 ratio (<0.9) that

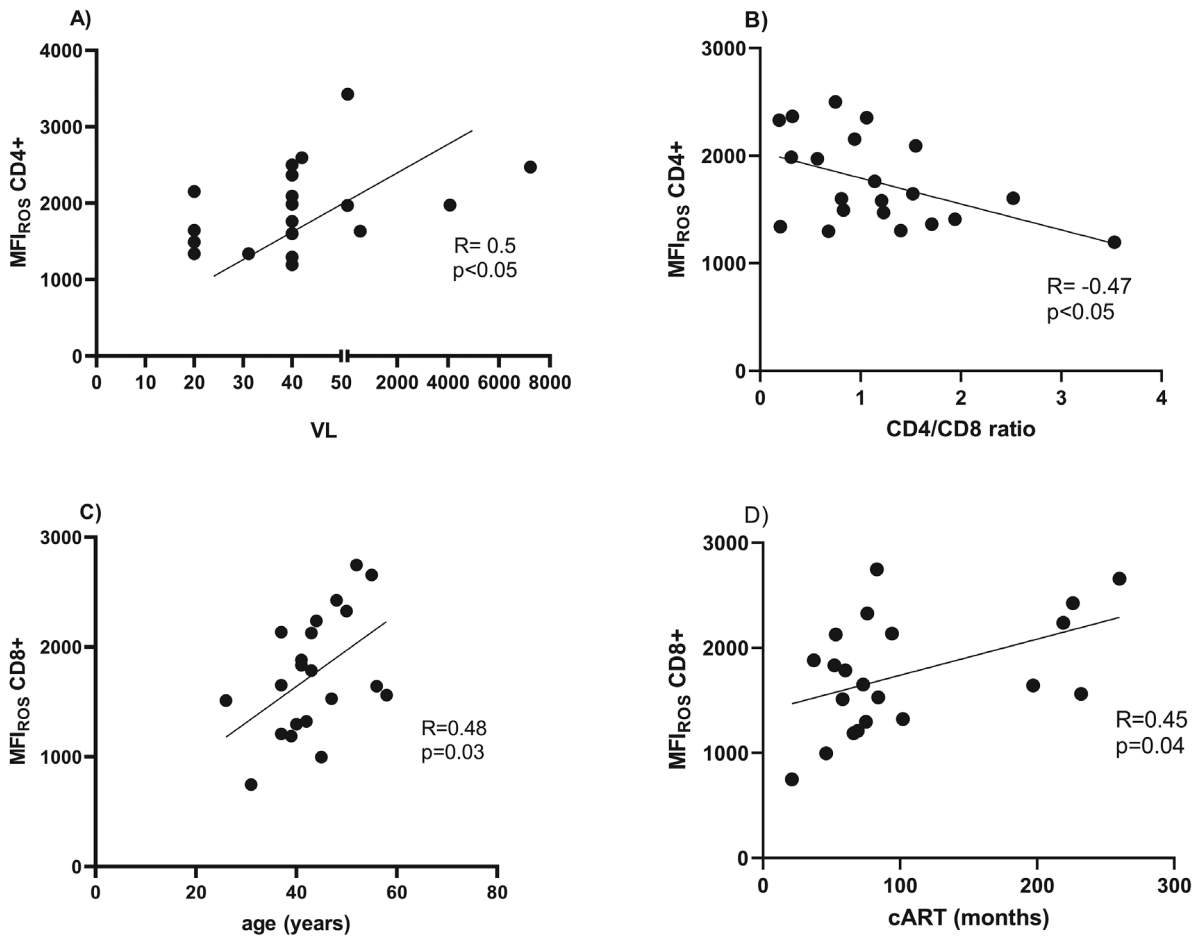

Fig. 3 Correlations of intracellular ROS: CD4+MFI$_{ROS}$ correlated positively with viral load **(A)**, and inversely - with CD4/CD8 ratio **(B)**; Intracellular CD8+T MFI$_{ROS}$ correlated positively with age **(C),** as well as with cART duration **(D).**

could reflect any immune activation, including one caused by the slowly replicating virus and affecting CD4 T cells, or one due to opportunistic infections, involving mostly the CD8 T cell compartment. In this aspect, it was interesting whether ROS content of CD4 and CD8 T cells could clarify this issue.

According to our results, it was CD4+T ROS content that differentiated between group A and B, and correlated with HIV VL. Moreover, within the group with undetectable HIV VL (A), CD4 T ROS correlated nicely and inversely with CD4/CD8 ratio, suggesting that the low level immune activation in patients with suboptimal ratio might actually reflect reactivation of latent HIV reservoirs. On the other hand, CD8 T cell ROS while not significantly differentiating between groups A and B, correlated directly with age and cART duration.

Oxidative stress has been implicated in many aspects of HIV pathogenesis, such as increased viral replication, CD4+ T-cell damage, altered immune response, and antiretroviral drug toxicity (3, 23). Most of the conflicting evidences on oxidative stress in HIV infection were reported in conditions without cART (20). A few studies reported on ROS levels in CD4 and CD8 T cell subsets of HIV+cART-treated patients. Yu et al. found that ROS accumulation in CD4+T cells was increased in HIV+ patients as compared to HIV- control subjects, while HIV infection seemed to have no effect on ROS accumulation in CD8+T cells. Further on, they did not observe any difference between CD4+ T ROS levels after 0.5-1- 2 and 3 years of cART. Interestingly though, the introduction of cART led to the abnormal accumulation of ROS in CD8+T cells as compared to healthy controls. In fact, these results are in line with ours, strengthening the idea that HIV infection mainly influences ROS accumulation in CD4+T cells, while long-term ART affects mostly the CD8 compartment (30). Unlike Yu et al. we demonstrate that long term cART significantly reduces CD4 ROS levels. Upon this background a slight increase of CD4+MFI ROS may be a sensitive early sign of HIV reactivation.

HIV replicates in highly oxidized environment. CD4+ cells shift from their resting state into an active state of immune response via a cascade of internal oxidative reactions, which stimulate HIV genes to reproduce in infected cells, while the increased metabolism of these cells provides the cellular factors that are useful for production of new viral particles (31).

The association between CD8+ ROS content and cART duration, as well as the age of patients from group A is also in line with the observations of others. Functionally exhausted CD8+ T cells in the settings of some cancers and chronic infections were shown to have signs of a broadly dysregulated metabolic state and evidence of oxidative stress as measured by the accumulation of ROS. Also, cART suppressed HIV+ patients tend to have a higher ROS content across most CD8+ T cell subsets, either related to the effects of residual infection and/or the impact of antiretroviral therapy on T cell metabolism (32). Unlike CD4+ ROS, and in line with our results, ROS content of HIV-specific CD8+ T cells in the study of Deguit et al. did not vary according to the extent or mechanism of viral control (32).

Our study has some limitations. First of all, participants in the two groups were not age-matched. This might have influenced the difference between the amount of oxygen radicals in group A (older) and group B, having in mind that ROS significantly increase with age. Second, the small number of participants and the absence of control HIV- volunteers as well as ART-naïve HIV+ patients have also limited the interpretation of the severity of oxidative stress.

CONCLUSIONS:

Our data suggest that elevated CD4+ ROS level in virally suppressed long-term treated HIV+ patients can be an indicator of residual viral activity. On the other hand, the combined effects of aging and long-term therapy predominantly affect CD8+ T cells and their ROS content might serve as an indicator of advancing functional exhaustion.

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REFERENCES:

1. Commoner B, Townsend, J, Pake G. Free Radicals in Biological Materials. Nature. 1954; 174: 689–691. doi: 10.1038/174689a0
2. Halliwell B, Gutteridge JMC. Oxigen - boon yet bane introducing oxygen toxicity and reactive species. In Free radicals in biology and medicine. Oxford University Press, 2015, 1-29. doi:10.1093/acprof: oso/9780198717478.001.0001
3. Ivanov AV, Valuev-Elliston VT, Ivanova ON, Kochetkov SN, Starodubova ES, Bartosch B, Isaguliants MG. Oxidative Stress during HIV Infection: Mechanisms and Consequences. Oxid Med Cell Longev. 2016; 8910396. doi: 10.1155/2016/8910396.
4. Ray PD, Huang BW, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell Signal. 2012; 24(5):981-90. doi: 10.1016/j.cellsig.2012.01.008.
5. Belikov AV, Schraven B, Simeoni L. T cells and reactive oxygen species. J Biomed Sci. 2015; 15-22:85. doi: 10.1186/s12929-015-0194-3.
6. Di Meo S, Reed TT, Venditti P, Victor VM. Role of ROS and RNS Sources in Physiological and Pathological Conditions. Oxid Med Cell Longev. 2016; 2016:1245049. doi: 10.1155/2016/1245049.
7. Couret J, Chang TL. Reactive Oxygen Species in HIV Infection. EC Microbiol. 2016; 3(6):597-604.
8. Anelli, T., Sannino, S. & Sitia, R. Proteostasis and "redoxtasis" in the secretory pathway: tales of tails from ERp44 and immunoglobulins. Free Radic. Biol. Med. 2015; 83, 323–330.
9. Garaude, J. Reprogramming of mitochondrial metabolism by innate immunity. Curr. Opin. Immunol. 2018; 56, 17–23.
10. Sies H, Jones DP, Reactive oxygen species (ROS) as pleiotropic physiological signalling agents. Nat Rev Mol Cell Biol. 2020; 21, 363–383 https://doi.org/10.1038/s41580-020-0230-3
11. Trachootham D, Alexandre J, Huang P. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat Rev Drug Discov. 2009; 8(7):579-91. doi: 10.1038/nrd2803.
12. Shukla Y, George J. Combinatorial strategies employing nutraceuticals for cancer development. Nutrition and Physical Activity in Aging, Obesity, and Cancer. 2011; 1229, 162-175. https://doi.org/10.1111/j.1749-6632.2011.06104.x
13. Andersen JK. Oxidative stress in neurodegeneration: cause or consequence? Nat Med. 2004; 10 Suppl: S18-25. doi: 10.1038/nrn1434.
14. Szeto H.H. Mitochondria-targeted peptide antioxidants: novel neuroprotective agents. AAPS J. 2006; 8(3): E521–E531. http://dx.doi.org/10.1208/aapsj080362.
15. Paravicini TM, Touyz RM. Redox signaling in hypertension. Cardiovasc Res. 2006; 71(2):247-58. doi: 10.1016/j.cardiores.2006.05.001.
16. Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circ Res. 2010; 107(9):1058-70. doi: 10.1161/CIRCRESAHA.110.223545.
17. Haigis MC, Yankner BA. The aging stress response. Mol Cell. 2010; 40(2):333-44. doi: 10.1016/j.molcel.2010.10.002.
18. Salmen S, Berrueta L: Immune modulators of HIV infection: The role of reactive oxygen species. J Clin Cell Immunol. 2012; 3:121. doi: 10.4172/2155-9899.1000121
19. Amarnath S, Dong L, Li J, Wu Y, Chen W. Endogenous TGF-beta activation by reactive oxygen species is key to Foxp3 induction in TCR-stimulated and HIV-1-infected human CD4+CD25- T cells. Retrovirology. 2007; 9; 4:57. doi: 10.1186/1742-4690-4-57.
20. Alimonti JB, Blake BT, Fowke KR. Mechanism of CD4+ T lymphocyte cell death in human immunodeficiency virus infection and AIDS. J Gen Virol. 2003; 7: 1649-1661.

21. Cummins NW, Badley AD. Mechanisms of HIV-associated lymphocyte apoptosis: 2010. Cell Death Dis. 2010; 1(11): e99. doi: 10.1038/cddis.2010.77.
22. Ibeh BO, Eze SE, Habu JB. Discordant Levels of Superoxide Dismutase and Catalase Observed in ART Naïve and Experienced HIV Patients in South Eastern Nigeria. J Infect Dis Ther. 2013; 1: 8-16.
23. Masiá M, Padilla S, Fernández M, Barber X, Moreno S, Iribarren JA, et al. Contribution of Oxidative Stress to Non-AIDS Events in HIV-Infected Patients. J Acquir Immune Defic Syndr. 2017; 75(2): e36–44
24. Vance DE. Aging with HIV: clinical considerations for an emerging population. The American Journal of Nursing. 2010; 110(3):42-7.
25. Eisele E, Siliciano RF. Redefining the viral reservoirs that prevent HIV-1 eradication. Immunity. 2012; 37(3): 377–388. doi: 10.1016/j.immuni.2012.08.010.
26. Vieillard V, Gharakhanian S, Lucar O, Katlama C, Launay O, Autran B, Ho Tsong Fang R, Crouzet J, Murphy RL, Debré P. Perspectives for immunotherapy: which applications might achieve an HIV functional cure? Oncotarget. 2016; 21;7(25):38946-38958. doi: 10.18632/oncotarget.7793.
27. Antela A, Rivero A, Llibre JM, Moreno S. Redefining therapeutic success in HIV patients: an expert view. Journal of Antimicrobial Chemotherapy. 2021; 76:10, 2501–2518, https://doi.org/10.1093/jac/dkab168
28. Sun Y, Fu Y, Zhang Z, Tang T, Liu J, Ding H, Han X, Xu J, Chu Z, Shang H, Jiang Y. The investigation of CD4+T-cell functions in primary HIV infection with antiretroviral therapy. Medicine (Baltimore). 2017; 96(28): e7430. doi: 10.1097/MD.0000000000007430.
29. Serrano-Villar S, Sainz T, Lee SA, Hunt PW, Sinclair E, Shacklett BL, et al. HIV-infected individuals with low CD4/CD8 ratio despite effective antiretroviral therapy exhibit altered T cell subsets, heightened CD8+ T cell activation, and increased risk of non-AIDS morbidity and mortality. PLoS Pathog. 2014; 10(5): e1004078.
30. Yu F, Hao Y, Zhao H, Xiao J, Han N, Zhang Y, Dai G, Chong X, Zeng H, Zhang F. Distinct Mitochondrial Disturbance in CD4+T and CD8+T Cells from HIV-Infected Patients. J Acquir Immune Defic Syndr. 2017; 74(2):206-212. doi: 10.1097/QAI.0000000000001175.
31. Aquaro S, Scopelliti F, Pollicita M, Perno CF. Oxidative stress and HIV infection: Target pathways for novel therapies? Future HIV Therapy. 2008; 2(4):327-338. doi. org/10.2217/17469600.2.4.327
32. Deguit CDT, Hough M, Hoh R, Krone M, Pilcher CD, Martin JN, Deeks SG, McCune JM, Hunt PW, Rutishauser RL. Some Aspects of CD8+ T-Cell Exhaustion Are Associated with Altered T-Cell Mitochondrial Features and ROS Content in HIV Infection. J Acquir Immune Defic Syndr. 2019; 82(2):211-219. doi: 10.1097/QAI.0000000000002121.

COMPARATIVE ASSESSMENT OF THE NUMBER AND IMMUNOLOGICAL STATUS OF NEWLY DIAGNOSED AND RE-ENROLLED IN CARE HIV+ IN THE PERIOD 2018–2019 AND 2020-2021

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ABSTRACT

BACKGROUND: HIV infection has not been shown to be an individual risk factor, but people living with HIV and co-morbidities are at increased risk of severe COVID-19. For HIV+ individuals with severe immunosuppression and/or uncontrolled viremia, the prognosis for the course and outcome of COVID-19 remains serious. HIV infected with low CD4+ T-cell counts before antiretroviral therapy (ART) had a higher risk of complications from COVID-19 than those with higher CD4+ T-cell counts.

The aim of the study is to make a comparative assessment of the number and immunological status of newly registered and re-enrolled in care people living with HIV in the period 2018-2019 and 2020-2021 and to look for a correlation with COVID-19 pandemic.

MATERIALS AND METHODS: The study included all people living with HIV, monitored at the Clinic of Infectious Diseases and Parasitology, "St. George" University Hospital - Plovdiv both newly diagnosed and re-enrolled in care. Patients were divided into two groups: (1) Evaluated between 01.01.2018 and 31.12.2019 and (2) between 01.01.2020 and 15.10.2021. For the purposes of the study, methods of epidemiological and clinical analysis, ELISA, Western Blot for the diagnosis of HIV/AIDS and flow cytometry for the determination of T-cell populations were used.

RESULTS: For the period 2018-2019, there were 82 newly diagnosed and 29 re-enrolled in care HIV + patients. In 42,5% (35/82) of the newly diagnosed the number of CD4 cells was over 350 cells/ µl, in 19.5% (16/82) - between 200 and 350 cells/ µl, in 7% (6/82) - between 100 and 200 cells/ µl l and in 31% (25/82) below 100 cells/ µl. In 24% (7/29) of the re-enrolled the number of CD4 cells was above 350 cells/ µl, in 7% (2/29) - between 200 and 350 cells/ µl, in 21% (6/29) - between 100-200 cells/ µl and in 48% (14/29) - below 100 cells/ µl. For the period 2020-2021, there were 63 newly diagnosed and 34 re-enrolled in care HIV + patients. In 46% (29/63) of the newly diagnosed the number of CD4 cells was over 350 cells/ µl, in 21% (13/63) - between 200 and 350 cells/ µl, in 14% (9/63) -between 100 and 200 cells/ µl and in 19% (12/63) - below 100 cells/ µl. In 30 % (10/34) of the restarted, the number of CD4 cellswas over 350 cells/ µl, in 11% (4/34) - between 200 and 350 cells/ µl, in 15% (5/34) between 100 and 200 cells/ µl and in 44% (15/34) of patients - less than 100 cells/ µl

IN CONCLUSION: During the period 2020-2021, 10% fewer new HIV + patients were registered as compared to the period 2018-2019. The late presenters, with CD4 <350 cells/ µl were 58% in the period 2018-2019 vs. 54% in the period 2020 -2021. The late presenters with advanced immune deficiency (CD4 <100cells / µl) were respectively 31% and 19%. The proportion of those re-enrolled in care with advanced immune deficiency (CD4 <100/ml) was 48% in the first group and 44% in the second group, respectively. The lower number of newly diagnosed HIV + patients could be explained with the fear of visiting hospitals, testing getting infected with SARS CoV-2. According to our data, Covid-19 pandemic did not significantly affect the immune status of people living with HIV .

KEYWORDS: *HIV/AIDS, COVID-19, Late presenters.*

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INTRODUCTION

HIV remains a major global health problem, with 79.3 million people HIV infected since 1981, of whom 39 million have died (1). In 2020, there were 37.7 million

people living with HIV (PLWH), 1.5 million new HIV infections and 680,000 deaths from AIDS. A total of 27.5 million PLWHwere on ART (73%) (1). Globally, the number of new HIV infections has been decreasing by 23% each year since 2010, but this decline is not the same for the different geographic regions (2). According to the World Health Organization (WHO), in 2020 the number of PLWH in the European region was 2.6 million (3). The number of newly diagnosed HIV+ in the WHO European Region has increased by 19% over the last decade, while the number of newly diagnosed HIV+ among the European Union /European Economic Area (EU/EEA) countries has decreased by 9% over the same period (4). In 2019, over 53% of the newly diagnosed were late-presenters, with CD4 absolute count (AC) below 350 cells / µl, the highest share of late presenters being registered in Central and Eastern European region (4). As a late presenters, late presenters with advanced or with very advanced immune deficiency are defined PLWH who have been diagnosed ,respectively, with CD4 AC <350 cells / µl , CD4 <200 cells / µland CD4 <50 cells / µl or with AIDS-defining disease, independent of CD4 AC (5, 6, 7). In clinical practice, there are two groups of late presenting PLWH : (1) Those tested, and diagnosed for the first time late and (2) Those who have discontinued treatment and follow-up for months / years and have reappeared to restart ART (5, 6, 7). The COVID-19 pandemic has caused serious disruptions in the functioning of health systems in many countries, restricting population movements and making health care very difficult (8). In some countries, up to 75% of care activities for PLWH have been reported to drop out (8).

The aim of the work was to make a comparative assessment of the number and immunological status of newly registered and re-enrolled in care PLWH in the Plovdiv region, in the periods 2018-2019 and 2020-2021, in order to assess the impact of COVID-19 pandemic on early diagnosis and adherence to ART.

MATERIALS AND METHODS

The study included PLWH monitoredat the Clinic of Infectious Diseases of the University Hospital "St. Georgi"-Plovdiv, for a period of 4 years (01.01.2018–15.10. 2021) both newly diagnosed and re-enrolled in care. The patients were divided into two groups: pre-pandemic , p from 01.01.2018 to 31.12.2019 and, pandemic, from 01.01.2020 to 15.10.2021. For the purposes of the study were used: methods of epidemiological and clinical analysis, ELISA, and Western Blot for HIV diagnosis and flow cytometry for determination of CD4 AC..

RESULTS AND DISCUSSION

The dynamics of PLWH monitored at the Clinic of Infectious Diseases for the last 4 years was as follows: during 2018-2019 there were 82 newly diagnosed and 29 re-enrolled in care.; during 2020-2021, there were 63 newly diagnosed and 34 re-enrolled in care. (Figure 1).

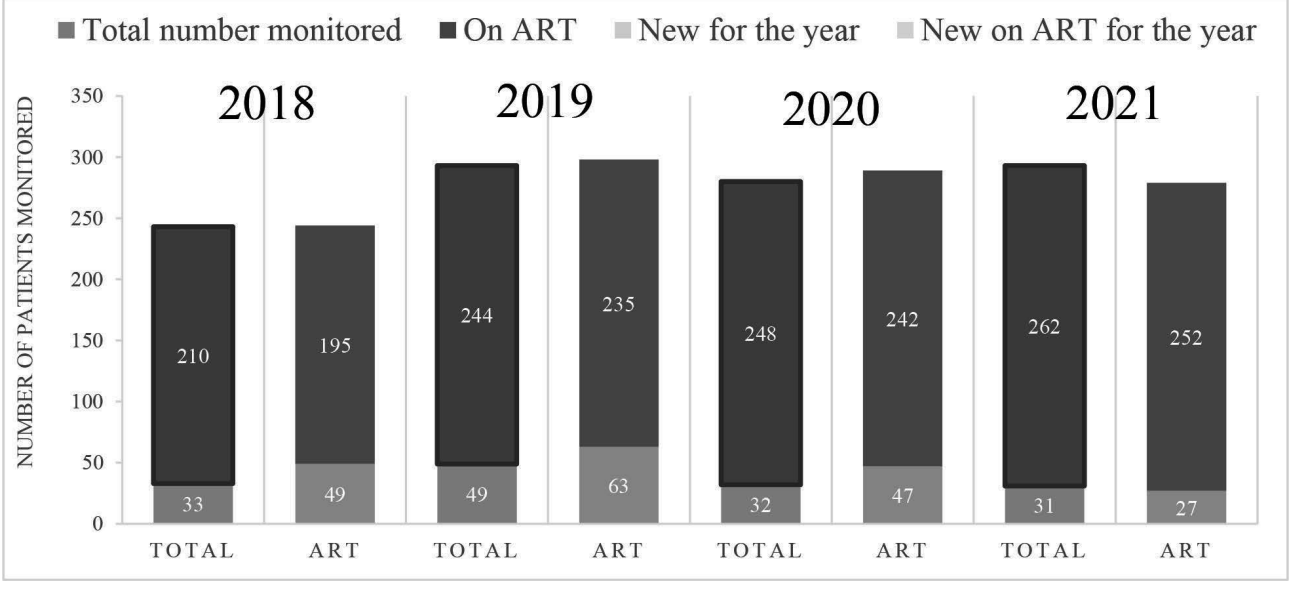


Figure 1. Dynamics of HIV+ patients monitored at the Clinic of Infectious Diseases for a period of 4 years.

For the period 2018-2019 in 42.5% (35/82) of the newly diagnosed the number of CD4 cells was over 350 cells/ µl, in 19.5% (16/82) from 200-350 cells / µl, in 7 % (6 /82) - from 100 to 200 cells / µl and at 31 % (25/82) below 100 cells / ml. In the period 2020-2021 in 29% (29/63) of the newly diagnosed the number of CD4 cells is over 350 / µl, in 21% (13/63) - from 200-350 cells / µl, in 14% (9/63) from 100 to 200 cells / µl and in 19% (12/63) below 100 cells / µl (Figure 2).

For the period 2018-2019 in 24% (7/29) of the re-enrolled in care the number of CD4 cells was over 350 cells / µl, in 7% (2/29) from 200-350 cells / µl, in 21% (6/29) - from 100-200 / µl and at 48% (14/29) below 100 cells / µl. In the period 2020-2021 in 30% (10/34) of the re-enrolled the number of CD4 cells was over 350 / µl, in 11% (4/34) - from 200-350 / µl, in 15% (5/34) of 100-200 cells / µl and in 44% (15/34) of patients - less than 100 cells / µl (Figure 3).

The number of hospitalizations, deaths and weight loss syndrome associated with HIV were more common among the newly diagnosed and restarted ART patients with immune deficiency, (CD4 <350 cells / µl, as compared to patients with CD4 above 350 c / µl, with no significant differences between the two observation periods. Cases of COVID-19 were observed equally in all patients, regardless of CD4 AC (Figures 4 and 5). The same trend was observed for coinfections with viral hepatitis B and C, and for opportunistic infections - tuberculosis and candidiasis (Figures 6, 7, 8 and 9).

IN CONCLUSION:

The number of newly diagnosed PLWH in the period 2020-2021 was 10% less as compared to the period 2018-2019. In the period 2018-2019, the late presenters (with CD4 <350 cells/ µl) were 58%, and 31% of them were with advanced immune deficiency and CD4 <100 cells/ µl. In the pandemic period 2020-2021, the late presenters were 54%, and 19% of them were with advanced immune deficiency (CD4 <100 cells/ µl). The relative share

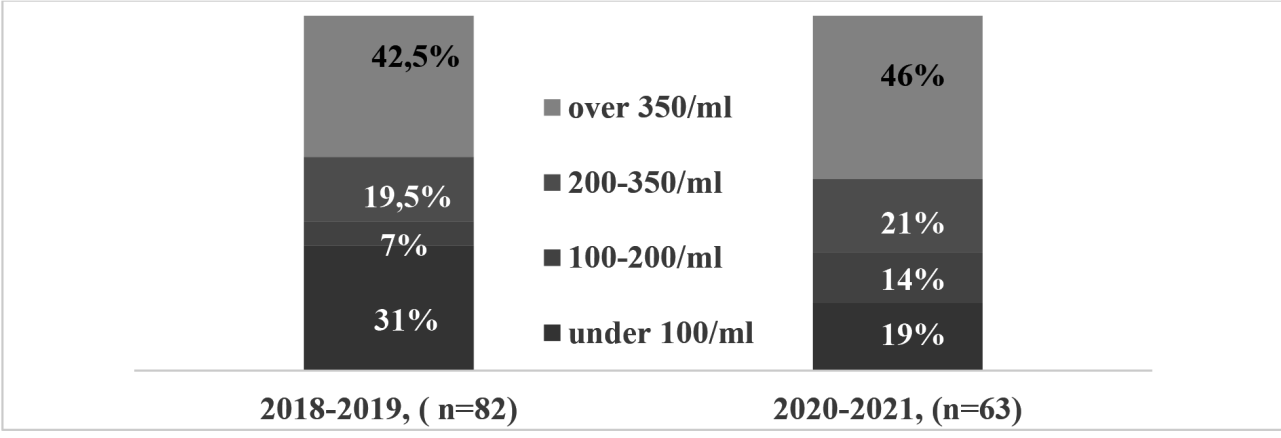


Figure 2. Immune status (CD4 cells /µl) of newly diagnosed HIV+ patients for the period 2018 - 2019 and 2020 - 2021**.**

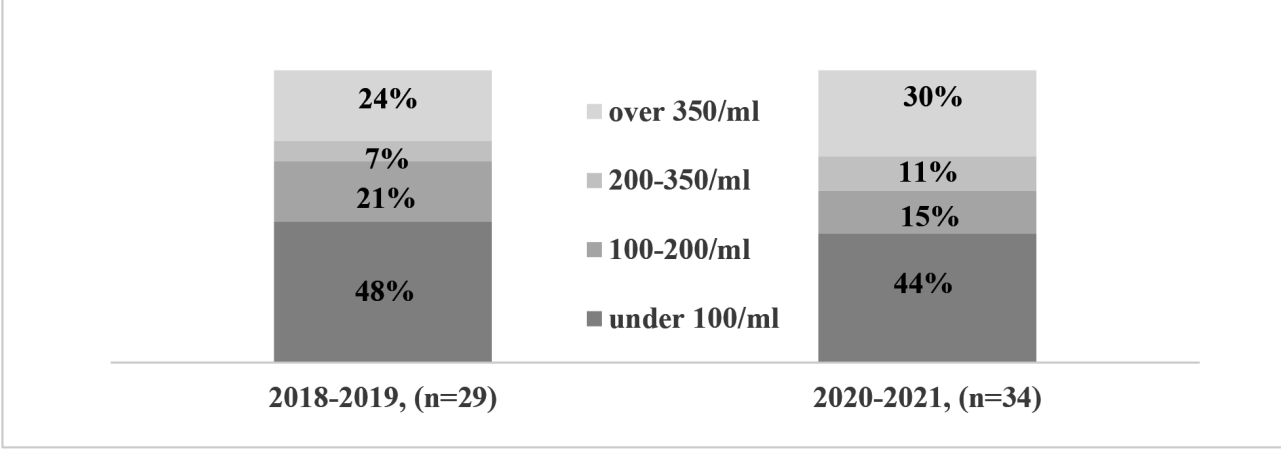
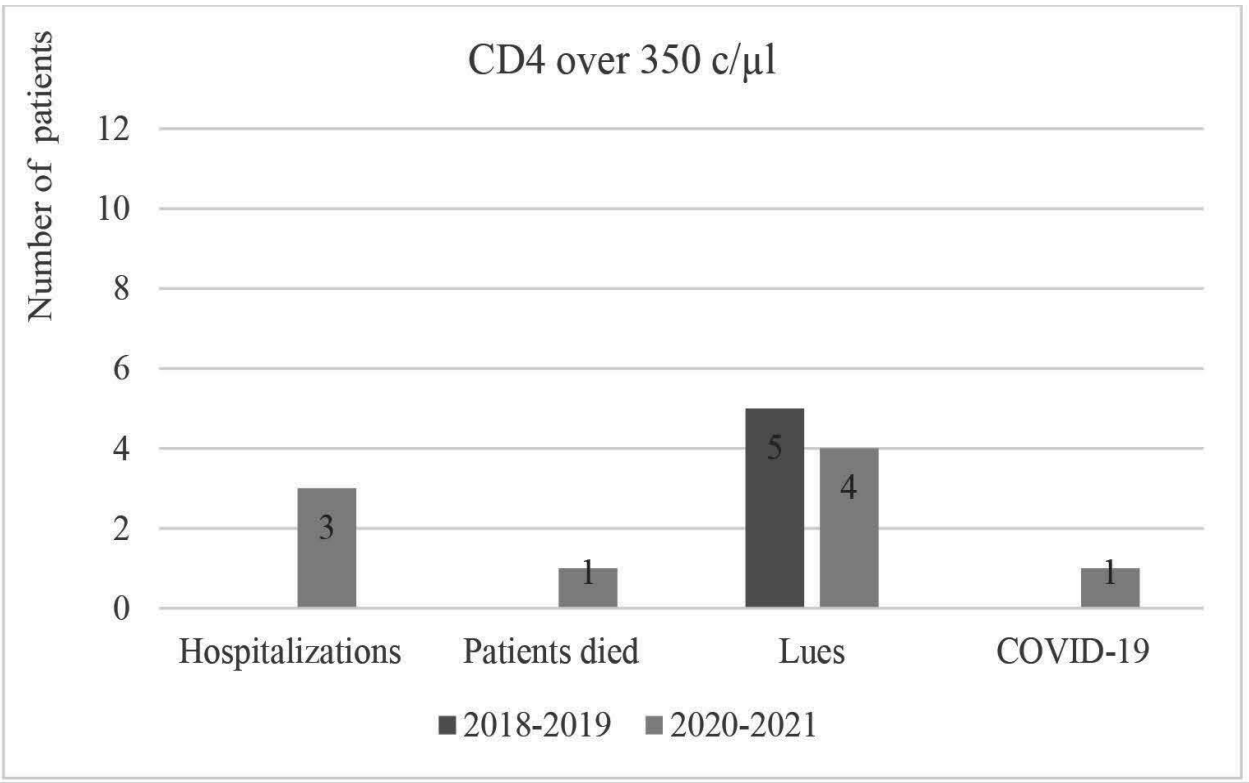


Figure 3. Immune status (CD4 cells / mm3) of restarted ART patients for the period 2018 - 2019 and 2020 - 2021.



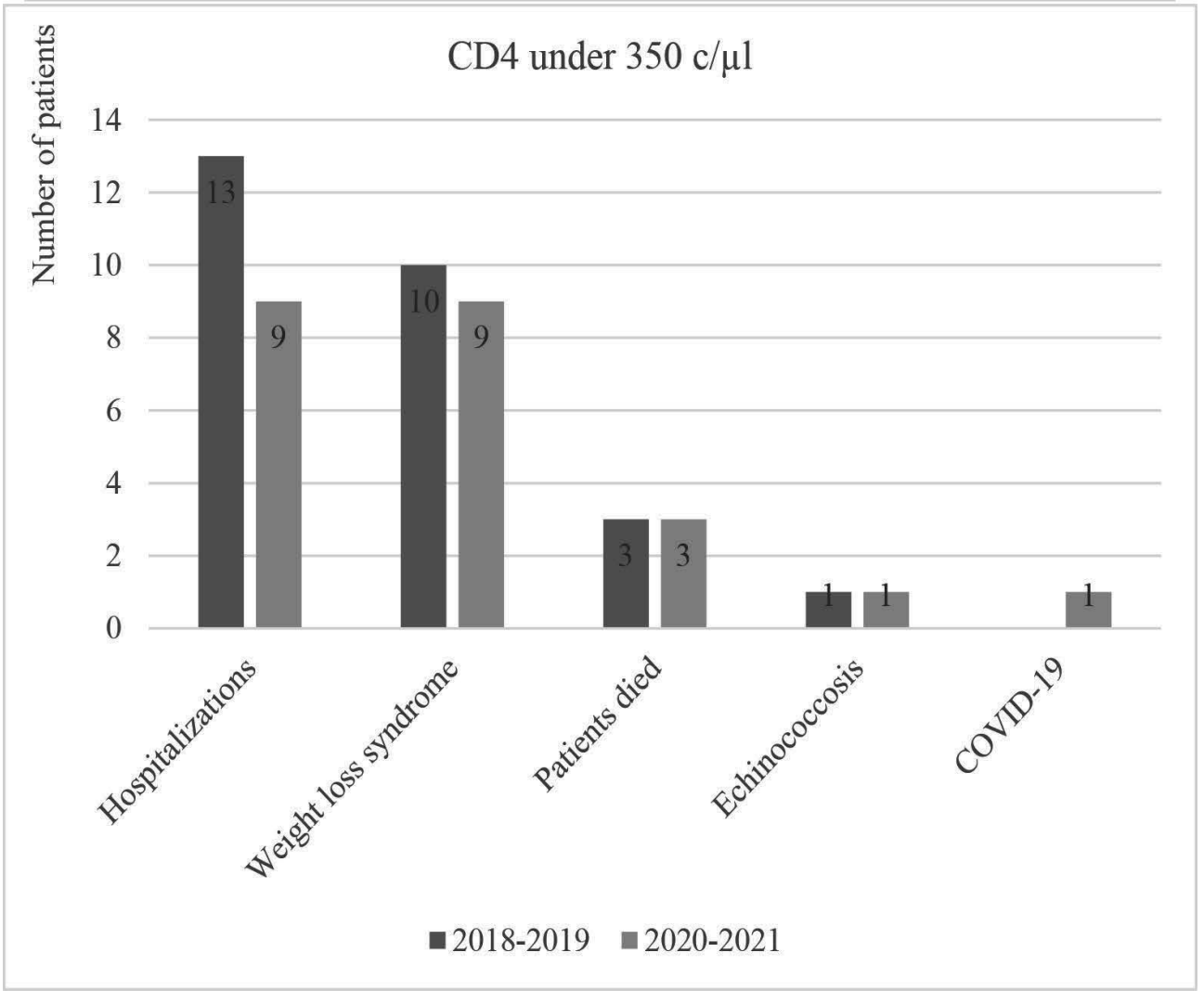


Figure 4. Comorbidity, hospitalization and fatal outcome in newly diagnosed HIV patients for the period 2018 - 2019 (n = 56) and 2020 - 2021 (n = 28).

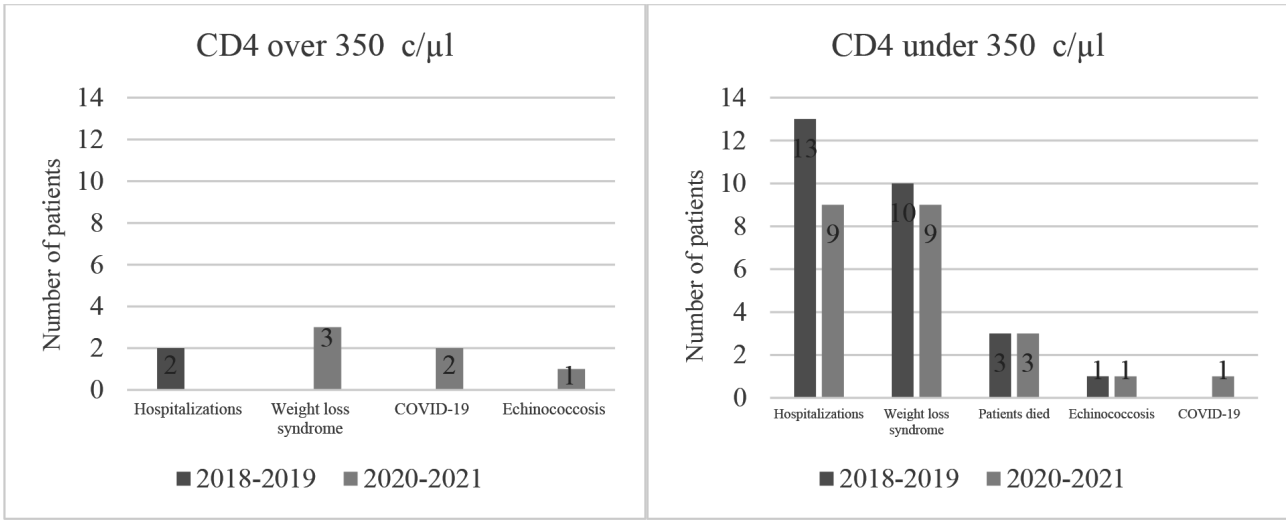


Figure 5. Comorbidity, hospitalization and fatal outcome in patients restarted on ART for the period 2018 - 2019 (n = 29) and 2020 - 2021 (n = 29).

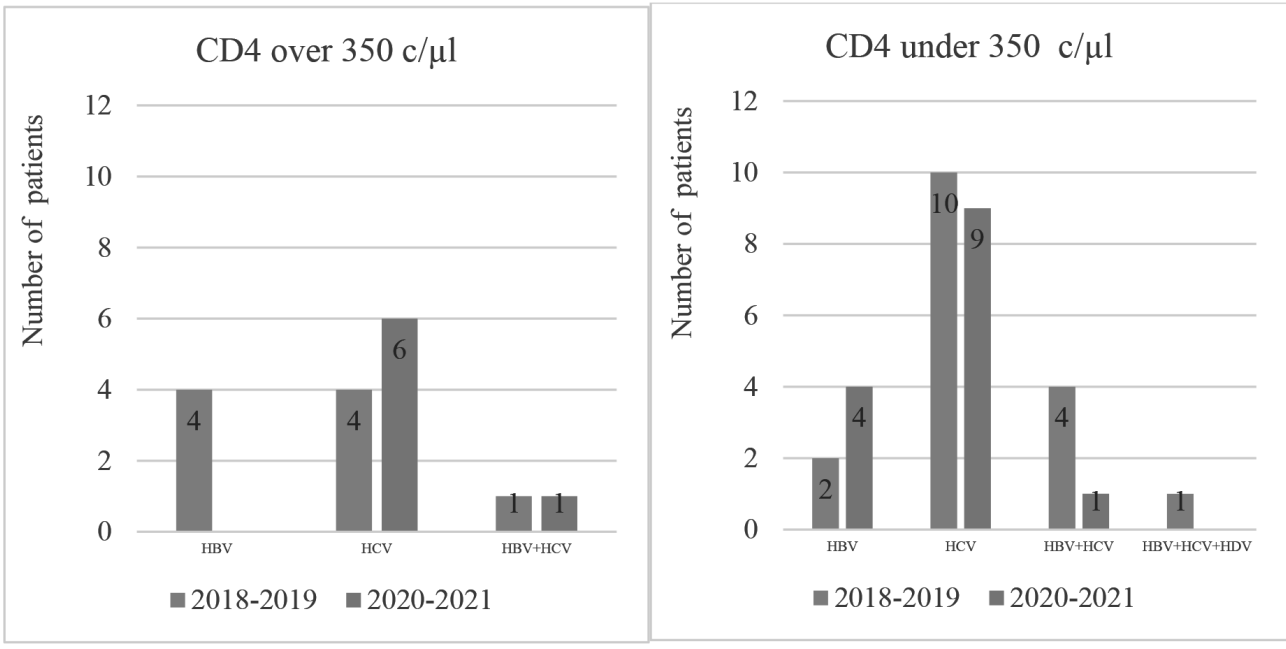


Figure 6. HIV / viral hepatitis co-infections in newly diagnosed patients for the period 2018 - 2019 (n = 26) and 2020 - 2021 (n = 21).

of PLWH re-enrolled in care with advanced immune deficiency (CD4 <100 cells/ µl) is alarmingly high, 48% in the first group and 44% in the second group. It can be assumed that the lower number of newly diagnosed in the period 2020-2021 was due to , the fear of visiting hospitals and the fear of SARS CoV2 during the COVID-19 pandemic. The relative share of HIV + patients re-enrolled in care with advanced immune deficiencywas alarmingly high, and this finding requires additional efforts to improve the adherence to treatment. A limitation of the study is the small number of patients in the two studied time periods matched by individual indicators, which does not allow to assess the statistical significance of the differences.

COMPETING INTERESTS

All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

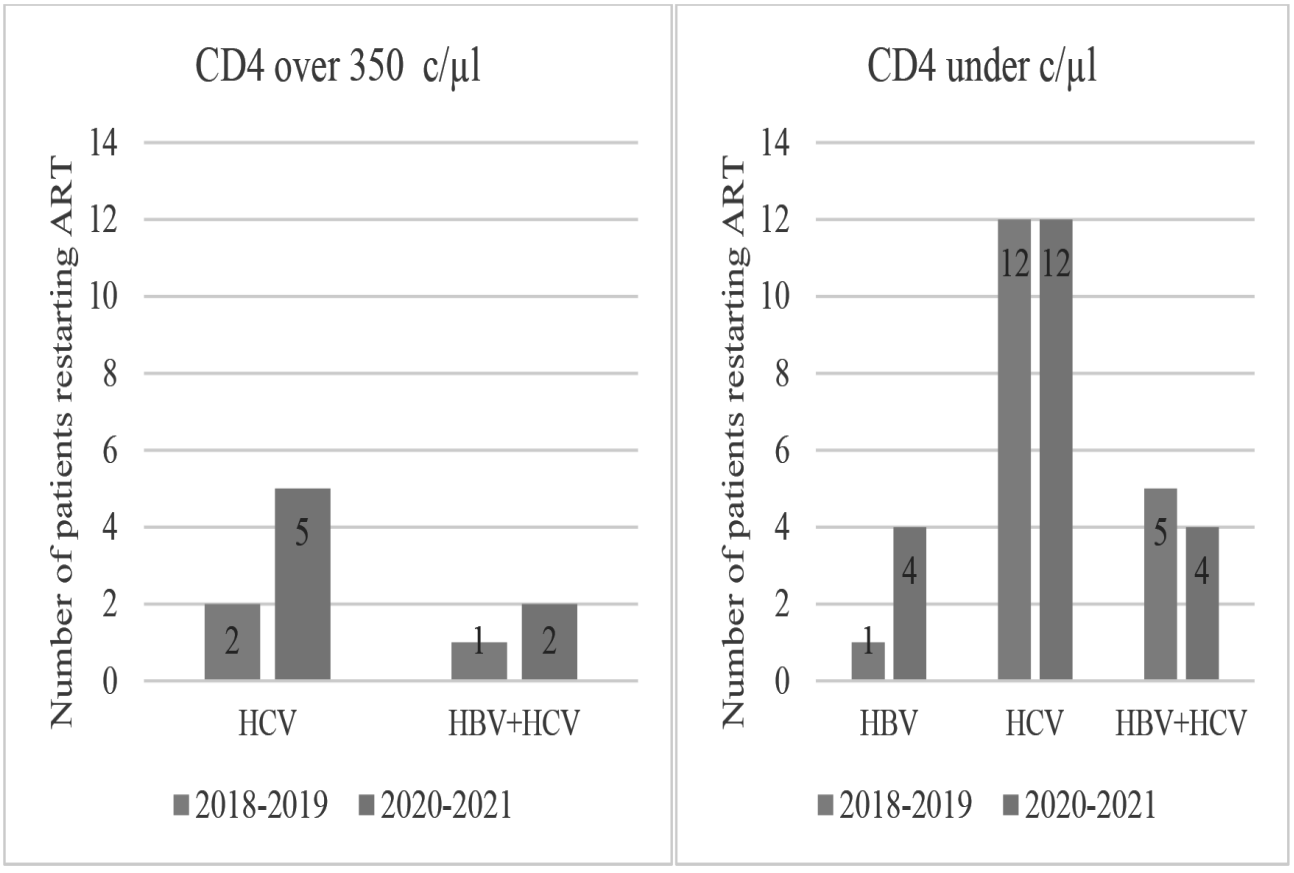


Figure 7. Co-infection of HIV with viral hepatitis in patients restarted on ART for the period 2018 - 2019 (n = 21) and 2020 - 2021 (n = 27).

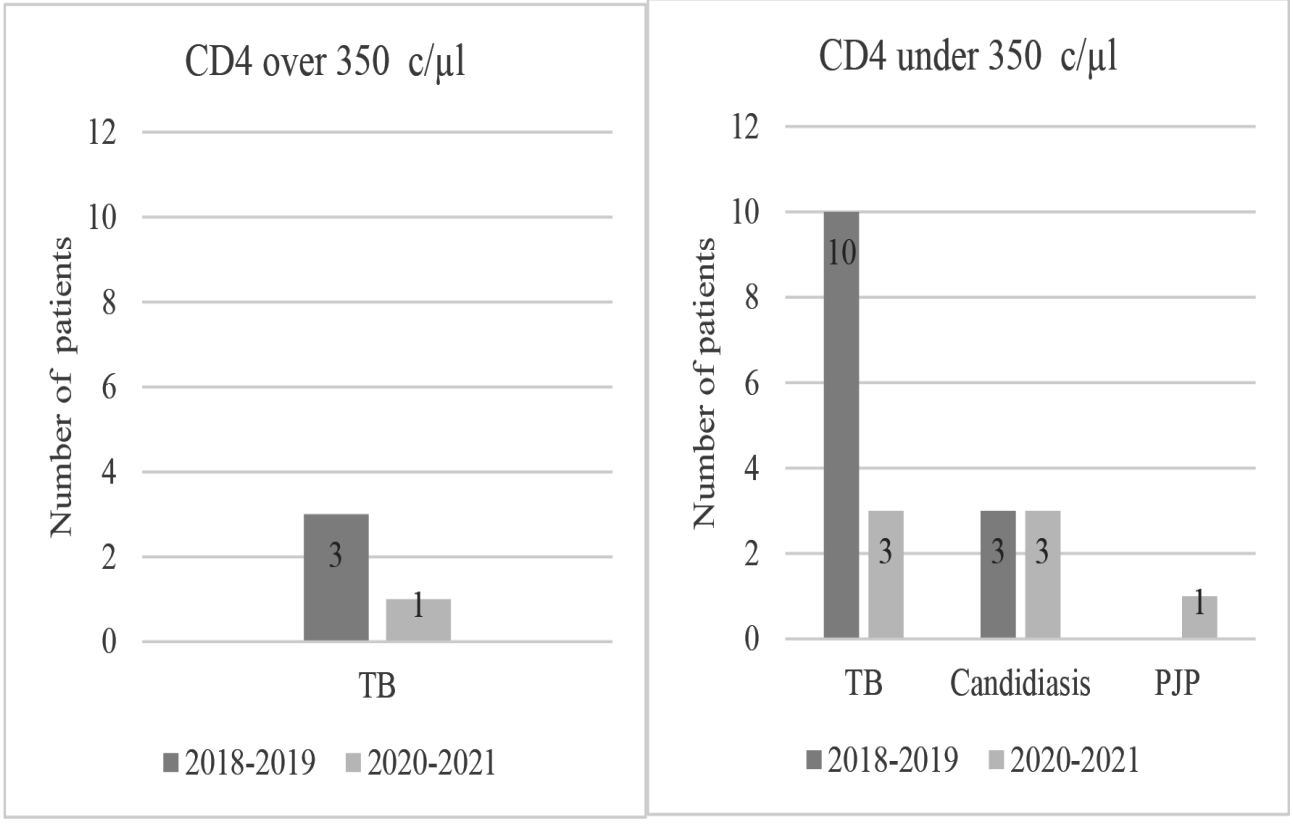


Figure 8. HIV and opportunistic infections in newly diagnosed patients for the period 2018 - 2019 (n = 16) and 2020 - 2021 (n = 8).

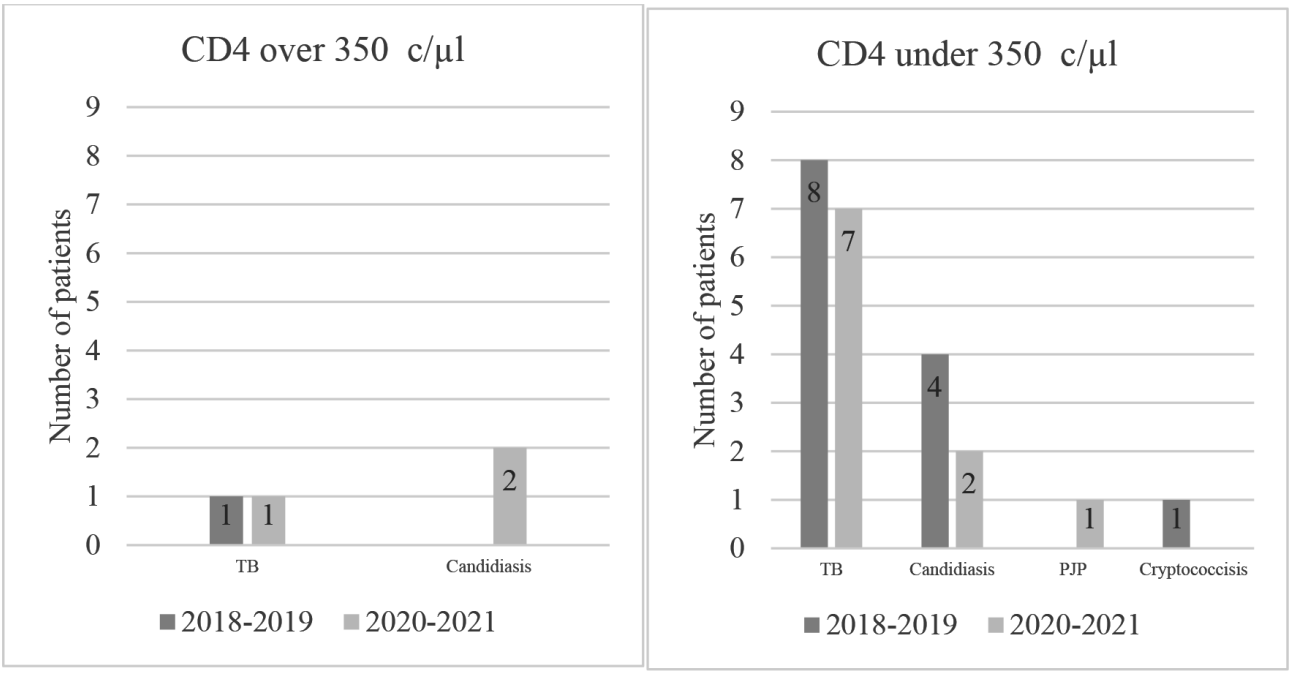


Figure 9. HIV and opportunistic infections in patients restarted on ART for the period 2018 - 2019 (n = 14) and 2020 - 2021 (n = 13).

DECLARATION OF AUTHORSHIP

Authors (PV, TV, IB, MS) participated in the design, organization, and implementation of the study. MS gave the idea of presenting the clinical case and guided the project. All authors (PV, TV, IB, MS) contributed writing the second and final draft. All authors approved the final draft.

References

1. United Nations [Official website]. UN, 24.11.2021,
2. Avert [Official website]. 24.11.2021, https://www.avert.org/.
3. World Health Organization [Official website]. WHO, 24.11.2021, https://www.who.int.
4. European Centre for Disease Prevention and Control, WHO Regional Office for Europe. HIV/AIDS surveillance in Europe 2020 – 2019 data. Copenhagen: WHO Regional Office for Europe; 2020.
5. Anitori, A. et al. Late presentation of HIV: a consensus definition. Short Communication. HIV Medicine Vol 12. 2011; (1): 61-64.
6. Mocroft, A et al. Risk Factors and Outcomes for Late Presentation for HIV-Positive Persons in Europe: Results from the Collaboration of Observational HIV Epidemiological Research Europe Study (COHERE). *PLoS Med*. 2013; 10(9):e1001510.
7. Health Protection Agency. Time to test for HIV: expanding HIV testing in healthcare and community services in England. Colindale: HPA. 2011.
8. U.S. Department of Health & Human Services and supported by the Minority HIV/AIDS Fund. [An official U.S. Government website]. MHAF Minority HIV/AIDS Fund, 19.11.2021, https://www.hiv.gov/.

PREVALENCE OF PHARYNGEAL AND RECTAL *CHLAMYDIA TRACHOMATIS* AND *NEISSERIA GONORRHOEAE* INFECTIONS AMONG MSM IN SOFIA, BULGARIA

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ABSTRACT

Sexually transmitted infections (STIs) caused by *Chlamydia trachomatis* and *Neisseria gonorrhoeae* continue to be a major public health problem. Although they mainly affect the urogenital tract, *N. gonorrhoeae* and *C. trachomatis* can also be found in the pharynx and rectum. As data on extragenital chlamydia and gonorrhea in Bulgaria are still scarce, this study aimed to (1) determine the prevalence of pharyngeal and rectal infections with *C. trachomatis* and *N. gonorrhoeae* among men who have sex with men (MSMs) from Sofia and (2) to identify risk factors related to these infections to support screening recommendations based on scientific evidence. One hundred and fifteen MSM aged 16-50 were tested by systematic sampling during a visit to a sexual health center for voluntary and confidential HIV testing in Sofia. A questionnaire was used to collect demographics and risk factors, and clinical material from three anatomical sites: pharynx, rectum, and urogenital tract (first void urine or urethral swab) was examined to detect *C. trachomatis* and *N. gonorrhoeae* infections. The prevalence of *C. trachomatis* was 8.7% in the rectal samples tested, and the prevalence of *N. gonorrhoeae* was 0.9% and 5.2% in the pharyngeal and rectal samples, respectively. Local symptoms were reported in only 16.6% of rectal gonococcal infections and in 20% of *C. trachomatis* rectal infections. Patients reporting multiple partners had a significantly higher risk of being positive (OR = 3.8, 95% CI 1.03-14). The risk of HIV-positive MSM and those having unsafe sex was also higher (OR = 1.9 95% CI 0.19-20 and OR = 4.6 95% CI 0.98-21, respectively), but the findings were not statistically significant. Overall, more than 80% of extragenital infections would remain undetected and therefore transmissible if only symptomatic cases were investigated. These results suggest that in Bulgaria HIV-positive MSM and those having multiple sexual partners and unprotected sex would benefit from screening for extragenital STIs. Larger sample surveys could provide a better characterization of risk factors to guide screening choices.

Keywords: *extragenital infections, Chlamydia trachomatis, Neisseria gonorrhoeae, MSM*

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INTRODUCTION

Bacterial sexually transmitted infections (STIs) remain a global public health concern. According to World Health Organization (WHO), the global prevalence of urogenital chlamydial infection, gonorrhoea, trichomoniasis and syphilis in adults of reproductive age (15-49 years) is high, with nearly one million newly diagnosed cases daily. In 2016, chlamydia and gonorrhoea were among the most common infections with 127 and 87 million m registered cases , respectively [1]. In addition to urogenital tract infections *N. gonorrhoeae* and *C. trachomatis* can also be detected in the pharynx and rectum [2]. In rectum gonorrhoea and chlamydia can cause proctitis and proctocolitis with symptoms such as rectal pain, bleeding, and discharge, etc. In pharynx, these infections can cause pharyngitis and lymphadenitis, but most often remain asymptomatic. An overview of existing literature shows over 80 studies published between 2000 and 2016 on the prevalence of pharyngeal and rectal infection with *N. gonorrhoeae* and *C. trachomatis* [3]. Most studies present evidence on the high prevalence of extragenital *N. gonorrhoeae* and *C. trachomatis* infections among MSM, the asymptomatic nature of these infections, as well as prevalence of extragenital infection without simultaneous urogenital

infection, supporting the need for further testing at extragenital sites. For that reason, European STIs treatment guidelines (The International Union Against Sexually Transmitted Infections (IUSTI) and the British Association for Sexual Health and HIV (BASHH)) provide recommendations for extragenital testing depending on history of exposure [4-7]. Further international guidelines (The Centers for Disease Control and Prevention (CDC) and Australian Sexual Health Alliance) additionally recommend prophylactic extragenital testing on yearly basis among specific high risk groups, such as MSM and commercial sex workers [8-10]. Given the fact that extragenital testing is not always a part of routine STIs screening, particularly in the absence of symptoms, many extragenital infections remain undiagnosed and untreated. Untreated extragenital infections could lead to more severe sequelae, such as increased HIV acquisition risk; autoinoculation from rectum to urogenital tract in women with subsequent pelvic inflammatory disease, ectopic pregnancy, infertility, disseminated gonococcal infection and sexually acquired reactive arthritis [11, 12]. As data on extragenital chlamydia and gonorrhea in Bulgaria are still limited, this study aimed to (1) determine the prevalence of pharyngeal and rectal infections with *C. trachomatis* and *N. gonorrhoeae* among men who have sex with men (MSMs) from Sofia and (2) to identify risk factors related to these infections to support screening recommendations based on scientific evidence.

MATERIALS AND METHODS

Study population

This study was performed on clinical specimens from three anatomical sites (pharynx, rectum, and urogenital tract (first void urine or urethral swabs)) and questionnaires obtained from a population of MSM visiting CheckPointSofia Sexual Health Center in Sofia for voluntary and confidential HIV testing. The CheckPointSofia Sexual Health Center in Sofia was established by the Health Without Borders Association, the successor to the Doctors Without Borders mission, and has been involved in voluntary and confidential HIV testing and counseling for more than 20 years. As this is a place for open dialogue, counseling and support for each individual regardless of sexual identity and orientation, more than 75% of annual male consultations comprise MSM.

Detection of *N. gonorrhoeae* and *C. trachomatis*

The gold standard for diagnosis of urogenital infection due to *N. gonorrhoeae* and *C. trachomatis* are the nucleic acid amplification tests (NAATs). The National Center of Infectious and Parasitic Diseases (NCIPD) has performed NAAT validation for detecting extragenital infections and offers this testing option since NAATs are the most sensitive tests for detecting *C. trachomatis* and *N. gonorrhoeae* and are recommended by CDC [10]. NAATs have demonstrated higher sensitivity but lower specificity as compared to cultivation for detecting extragenital infections [13-15]. The lower specificity of extragenital NAATs was overcome by confirmation of all positive samples with duplex PCR targeting the gonococcal *porA* pseudogene and multicopy *opa* genes [16] and PCR detecting the cryptic plasmid [17] for gonorrhea and chlamydia, respectively.

Demographic and behavioral characteristics

The demographic and behavioral characteristics were collected as follows: assigned sex at birth (male/female), sexual contacts in the last 3 months (male/female), age (in full years and grouped as 16-20, 21-30, 31-40 and >40 years), extragenital symptoms (yes/no), number of partners during the last 3 months (grouped as 0-1, 2-3 and >3 partners), having intercourse without condom in the last 3 months (yes/no) and HIV status (positive/negative).

Statistical analyses

The prevalence of extragenital *N. gonorrhoeae* and *C. trachomatis* infections with 95% confidence intervals (CI) was calculated, then a bivariate logistic regression analysis with demographic and behavioral characteristics as independent variables and extragenital *N. gonorrhoeae* and *C. trachomatis* diagnosis as the dependent variable was performed [13]. Bivariate odds rations (ORs) and 95% CIs were reported. In the statistical analysis, $p < 0.05$ was considered significant [19].

Ethics and informed consent

Written informed consents were obtained from all participants for demographic data collection and

sample testing as required by the National law and the Ethics Committee at the National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria.

RESULTS

In 2021, more than 1,120 MSM attended CheckPointSofia for voluntary and confidential HIV testing, and 115 MSM provided clinical materials from three anatomical locations and completed questionnaires. Demographic and behavioral characteristics of participants are summarized in Table 1. The mean age was 30 ± 7.8 years (age range 16-50). The majority of participants had not any extragenital symptoms (97.48%) and the median number of partners was 2 (IQ25-75: 2-3) during the last 3 months. More than two-thirds of the participants (66.1%) reported having sexual intercourse without condom in the last 3 months and 3.5% reported being HIV positive.

Table 1. *Distribution of demographic and behavioral characteristics in MSM (n=115) visiting CheckPointSofia for HIV testing in 2021*

	N	%
Age		
16-20	12	10.4
21-30	48	41.7
31-40	38	33
>40	17	14.9
Extragenital symptoms		
Yes	3	2.6
No	112	97.4
Number of partners during the last 3 months		
0-1	47	40.9
2-3	42	36.5
>3	26	22.6
Intercourse without condom in the last 3 months		
Yes	76	66.1
No	39	33.9
HIV status		
Positive	4	3.5
Negative	111	96.5

The prevalence of *N. gonorrhoeae* was 0.9% and 5.2% in the pharyngeal and rectal samples, respectively, and the prevalence of *C. trachomatis* was 8.7% in the rectal samples tested. No *C. trachomatis* was detected in any pharyngeal sample (Table 2.). The results comprised 14 (82.4%) cases of exclusively rectal infections and 1 (5.8%) exclusively pharyngeal infection, whereas 2 (11.8%) cases represented simultaneous extragenital and urogenital infection. Therefore, exclusively extragenital infections accounted for the majority of cases (n=15; 88.2%), whereas the remainder 2 (11.8%) corresponded to mixed extragenital and urogenital infections. Local symptoms were reported in 16.6% of rectal gonococcal infections and in 20% of *C. trachomatis* rectal infections which leaves the majority of extragenital gonorrhoea and chlamydia infections asymptomatic (Fig. 1.).

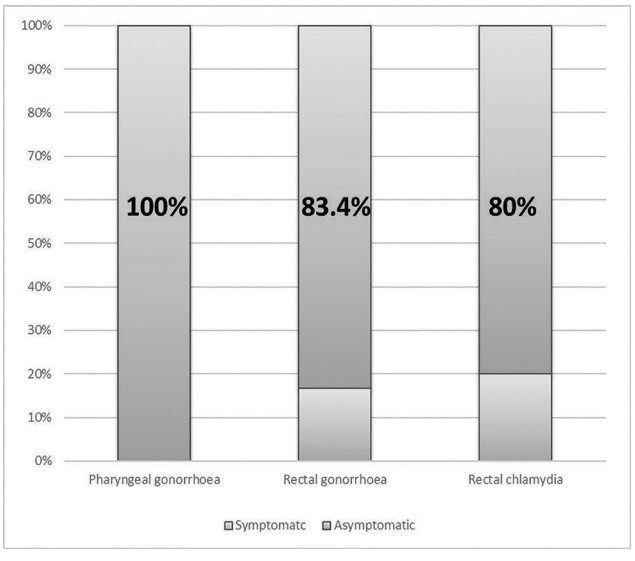

Figure 1. Distribution of symptomatic and asymptomatic extragenital infections in MSM (pharyngeal gonorrhoea n=1; rectal gonorrhoea n=6 and rectal chlamydia n=10) visiting CheckPointSofia for HIV testing in 2021

Table 2. *Prevalence of extragenital gonorrhoea and chlamydia in MSM (n=115) visiting CheckPointSofia for HIV testing in 2021*

	N	%
Pharyngeal gonorrhoea	1	0.9
Rectal gonorrhoea	6	5.2
Rectal chlamydia	10	8.7
Total extragenital infections	17	14.8 (95% CI 8.31-21)

Patients reporting multiple partners had a significantly higher risk of being positive (OR = 3.8, 95% CI 1.03-14). The risk of HIV-positive MSM and those having unsafe sex was also higher (OR = 1.9 95% CI 0.19-20 and OR = 4.6 95% CI 0.98-21, respectively), but the findings were not statistically significant (Table 3.).

Table 3. Statistical analysis of risk factors for extragenital *N. gonorrhoeae/C. trachomatis* infections

	Extragenital gonorrhoea and chlamydia (%)	OR 95% CI	P-value
Number of partners during the last 3 months			
0-1	3 (2.6%)	1	
≥2	14 (12.17%)	3.8 (1.03-14)	0.0455*
Intercourse without condom in the last 3 months			
Yes	15 (13.04%)	1	
No	2 (1.74%)	4.6 (0.98-21)	0.0524
HIV status			
Positive	1 (0.87%)	1	
Negative	16 (13.91%)	1.9 (0.19- 20)	0.5648

* Significant P-value

DISSCUSSION

To the best of authors' knowledge, this is the first Bulgarian study to investigate extragenital infections by *N. gonorrhoeae* and *C. trachomatis* among MSM. In this study, the prevalence of extragenital *N. gonorrhoeae* and *C. trachomatis* infections in MSM visiting CheckPointSofia for HIV testing in 2021 was estimated and possible risk factors for these infections were identified. The prevalence of extragenital gonorrhoea and chlamydia was 14.8% (95% CI 8.31% to 21%) and 82.3% of these infections were asymptomatic. The associated risk factors were an increasing number of partners during the last 3 months, having intercourse without condom and being HIV positive.

Exclusive extragenital gonorrhoea and chlamydia without concurrent urogenital infection accounted for the majority of cases. This means that a considerable number of extragenital infections were identified that would have been undiagnosed if urethral screening alone had been performed. Furthermore, in most cases (97.4%) the patients did not present with any extragenital symptoms. Considering that local complaints were found at extragenital sites only in 16.6% of the rectal gonorrhoea and in 20% of the rectal chlamydia infections, 83.4% of the gonococcal and 80% of the chlamydial infections would have been missed if only the patients reporting local symptoms had been tested for *N. gonorrhoeae* and *C. trachomatis* at these sites. In fact, it is known that extragenital gonococcal and chlamydial infections are usually asymptomatic [4,5,20]. According to other authors, anorectal symptoms like itching, pain, discharge and hemorrhage, are less common but more specific for infection, whereas oropharyngeal complaints, like sore throat, are more commonly reported but nonspecific [21]. Therefore, extragenital gonorrhoea and chlamydia may be missed unless they are actively investigated [22]. Undiagnosed and untreated extragenital infections can lead to

complications and constitute potential disease reservoirs [23], which may contribute to increasing transmission, incidence and antimicrobial resistance [24]. Of particular concern is the endemic spread among MSM of invasive serotypes *C. trachomatis* L1-L3 and the possible role of oropharyngeal infections in promoting resistance among Neisseria species [25,26]. The presented results confirm the relevance of testing for gonorrhoea and chlamydia at extragenital sites in MSM, regardless of local complaints.

Reducing the burden of extragenital infections by timely diagnosis and treatment is an important strategy to reduce gonorrhoea and chlamydia prevalence overall. To address this public health concern, several current international guidelines recommend testing for *N. gonorrhoeae* and *C. trachomatis* infection at extragenital sites, although different strategies have been proposed worldwide: IUSTI and BASHH recommend extragenital testing depending on the history of exposure and CDC recommends annual screening for MSM (every 3 – 6 months for high-risk MSM) at all sites, regardless of reported location of sexual contact [4-7, 10]. However, in our country, as in others [27], *N. gonorrhoeae* and *C. trachomatis* testing at extragenital sites is uncommonly performed, and thus many diagnoses and treatment opportunities may be missed. Also, unfortunately, there are currently no national guidelines specifically recommending extragenital gonorrhoea and chlamydia testing, which compromises routine and standardized screening in clinical settings. Implementation of appropriate screening programs may have substantial benefits for the public health but may find several limitations in Bulgaria, including limited institutional funding, insufficient training of physicians in addressing extragenital gonococcal and chlamydial infections and patients' lack of risk awareness and fear of judgement. Finally, larger sample surveys on this subject would be of value.

In conclusion, this study found a considerable burden of extragenital gonorrhoea and chlamydia among MSM visiting Sexual Health Center in Sofia in 2021. Exclusively extragenital infections without concurrent urogenital gonorrhoea and chlamydia accounted for the majority of cases, and most extragenital infections were asymptomatic. The presented findings reinforce the relevance of screening MSM for *N. gonorrhoeae* and *C. trachomatis* at extragenital sites, regardless of the existence of local complaints, in order to prevent these potential infection reservoirs from being underdiagnosed and untreated. Screening is of particular importance in HIV-positive MSM, and in those having multiple sexual partners and unprotected sex. This study might inform future guidelines and standardized practices concerning the screening of extragenital gonorrhoea and chlamydia among MSM in Bulgaria.

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REFERENCES

1. Rowley J, Vander Hoorn S, Korenromp E, Low N, Unemo M, Abu-Raddad LJ, et al. *Chlamydia, gonorrhoea, trichomoniasis and syphilis: global prevalence and incidence estimates, 2016*. Bull World Health Organ. 2019;97(8):548.
2. Workowski KA. *Centers for Disease Control and Prevention sexually transmitted diseases treatment guidelines*. Clin Infect Dis. 2015;61(suppl_8):S759–62.
3. Chan PA, Robinette A, Montgomery M, Almonte A, Cu-Uvin S, Lonks JR, et al. *Extragenital infections caused by Chlamydia trachomatis and Neisseria gonorrhoeae: a review of the literature*. Infect Dis Obstet Gynecol. 2016;(2016): 129-145.
4. Lanjouw E, Ouburg S, De Vries HJ, Stary A, Radcliffe K, Unemo M. *2015 European guideline on the management of Chlamydia trachomatis infections*. Int J STD AIDS. 2016;27(5):333–48.
5. Unemo M, Ross JDC, Serwin AB, Gomberg M, Cusini M, Jensen JS. *Background review for the '2020 European guideline for the diagnosis and treatment of gonorrhoea in adults.'* Int J STD AIDS. 2021;32(2):108–26.
6. Fifer H, Saunders J, Soni S, Sadiq ST, FitzGerald M. *British Association for Sexual Health and HIV national guideline for the management of infection with Neisseria gonorrhoeae (2019)*. London: British Association for Sexual Health and HIV; 2019.
7. Fitzgerald N, Anderson ER, Apea V, Curtis H, Goodall L. *2019 British Association of Sexual Health and HIV National Clinical Audit of timelines to be seen, test results and treatment for Chlamydia trachomatis*. Int J STD AIDS. 2022;33(6):604–7.
8. Dukers-Muijrers NHTM, Schachter J, van Liere GAFS, Wolffs PFG, Hoebe CJPA. *What is needed to guide testing for anorectal and pharyngeal Chlamydia trachomatis and Neisseria gonorrhoeae in women and men? Evidence and opinion*. BMC Infect Dis. 2015;15(1):1–13.
9. Australian Sexual Health Alliance, *Australian STI Management Guidelines for Use in Primary Care.* Australian Government Department of Health, Darlinghurst, Australia, 2016.
10. Workowski KA, Bolan GA. *Sexually transmitted diseases treatment guidelines, 2015*. MMWR Recomm reports Morb Mortal Wkly report Recomm reports. 2015;64(RR-03):1.
11. Marcus JL, Bernstein KT, Kohn RP, Liska S, Philip SS. *Infections missed by urethral-only screening for chlamydia or gonorrhea detection among men who have sex with men*. Sex Transm Dis. 2011;38(10):922–4.
12. De Vries HJC, de Barbeyrac B, De Vrieze NHN, Viset JD, White JA, Vall-Mayans M, et al. *2019 European guideline on the management of lymphogranuloma venereum*. J Eur Acad Dermatol Venereol. 2019;33(10):1821–8.
13. Bachmann LH, Johnson RE, Cheng H, Markowitz L, Papp JR, Palella Jr FJ, et al. *Nucleic acid amplification tests for diagnosis*

of Neisseria gonorrhoeae and Chlamydia trachomatis rectal infections. J Clin Microbiol. 2010;48(5):1827–32.
14. Schachter J, Moncada J, Liska S, Shayevich C, Klausner JD. *Nucleic acid amplification tests in the diagnosis of chlamydial and gonococcal infections of the oropharynx and rectum in men who have sex with men*. Sex Transm Dis. 2008;637–42.
15. Bachmann LH, Johnson RE, Cheng H, Markowitz LE, Papp JR, Hook III EW. *Nucleic acid amplification tests for diagnosis of Neisseria gonorrhoeae oropharyngeal infections*. J Clin Microbiol. 2009;47(4):902–7.
16. Goire N, Nissen MD, LeCornec GM, Sloots TP, Whiley DM. *A duplex Neisseria gonorrhoeae real-time polymerase chain reaction assay targeting the gonococcal porA pseudogene and multicopy opa genes*. Diagn Microbiol Infect Dis. 2008;61(1):6–12.
17. Hokynar K, Rantakokko-Jalava K, Hakanen A, Havana M, Mannonen L, Jokela P, et al. *The Finnish new variant of Chlamydia trachomatis with a single nucleotide polymorphism in the 23S rRNA target escapes detection by the Aptima Combo 2 test*. Microorganisms. 2019;7(8):227.
18. Altman DG. *Practical statistics for medical research*. CRC press; 1990.
19. Sheskin DJ. *Handbook of parametric and nonparametric statistical procedures*. crc Press; 2020.
20. Tongtoyai J, Todd CS, Chonwattana W, Pattanasin S, Chaikummao S, Varangrat A, et al. *Prevalence and correlates of Chlamydia trachomatis and Neisseria gonorrhoeae by anatomic site among urban Thai men who have sex with men*. Sex Transm Dis. 2015;42(8):440–9.
21. Dudareva-Vizule S, Haar K, Sailer A, Wisplinghoff H, Wisplinghoff F, Marcus U, et al. *Prevalence of pharyngeal and rectal Chlamydia trachomatis and Neisseria gonorrhoeae infections among men who have sex with men in Germany*. Sex Transm Infect. 2014;90(1):46–51.
22. Lutz AR. *Screening for asymptomatic extragenital gonorrhea and chlamydia in men who have sex with men: significance, recommendations, and options for overcoming barriers to testing*. LGBT Heal. 2015;2(1):27–34.
23. . Hui B, Fairley CK, Chen M, Grulich A, Hocking J, Prestage G, et al. *Oral and anal sex are key to sustaining gonorrhoea at endemic levels in MSM populations: a mathematical model*. Sex Transm Infect. 2015;91(5):365–9.
24. Annan NT, Sullivan AK, Nori A, Naydenova P, Alexander S, McKenna A, et al. *Rectal chlamydia—a reservoir of undiagnosed infection in men who have sex with men*. Sex Transm Infect. 2009;85(3):176–9.
25. Barbee LA. *Preparing for an era of untreatable gonorrhea*. Curr Opin Infect Dis. 2014;27(3):282.
26. Cole MJ, Field N, Pitt R, Amato-Gauci AJ, Begovac J, French PD, et al. *Substantial underdiagnosis of lymphogranuloma venereum in men who have sex with men in Europe: preliminary findings from a multicentre surveillance pilot*. Sex Transm Infect. 2020;96(2):137–42.
27. den Heijer CDJ, Hoebe CJPA, van Liere GAFS, van Bergen JEAM, Cals JWL, Stals FS, et al. *A comprehensive overview of urogenital, anorectal and oropharyngeal Neisseria gonorrhoeae testing and diagnoses among different STI care providers: a cross-sectional study*. BMC Infect Dis. 2017;17(1):1–10.

CURRENT APPROACHES FOR CONTROL OF ISONIAZID-RESISTANT TUBERCULOSIS

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ABSTRACT

Isoniazid (H; INH) is an important first-line drug for the treatment of active tuberculosis (TB) and latent TB infection because of its potent early bactericidal activity against *Mycobacterium tuberculosis*. Currently, TB resistant to INH, alone or in combination with other drugs, is the most common type of drug-resistant TB.

Epidemiology of INH-resistant TB, the molecular mechanisms of drug resistance, current methods for diagnosis and therapeutic regimens of this TB form are presented.

Studies in the last years have shown that resistance to INH reduces the probability of treatment success and increases the risk of acquiring resistance to other important first-line drugs.

Based on the most recent meta-analyses, the last WHO recommendations for treatment of INH-resistant TB are to include rifampicin (RIF), ethambutol, pyrazinamide and levofloxacin for 6 months, and not to add streptomycin or other injectable agents to the drug regimen. The guideline emphasizes the importance of excluding resistance to RIF before starting the regimen for INH-resistant TB because of the risk for development of multidrug-resistant TB during the treatment course.

The WHO recommendations are based on observational studies, not randomized controlled trials, and are thus conditional and based on low certainty in the estimates of effect. Therefore, further work is needed to optimize the treatment and control of INH-resistant TB.

Keywords: *Tuberculosis; Isoniazid; Drug resistance; Diagnosis; Treatment*

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INTRODUCTION

Tuberculosis (TB), an ancient communicable disease caused by *Mycobacterium tuberculosis* (MTB), is one of the top 10 causes of death worldwide. Until the Coronavirus disease 2019 (COVID-19) pandemic, TB was the leading cause of death from a single infectious agent. Based on World Health Organization (WHO) data, in 2020, an estimated 9.9 million people fell ill with TB worldwide, equivalent to 127 cases per 100,000.. Among all incident cases of TB, 8% were people living with Human immunodeficiency virus (HIV). Globally in 2020, there were an estimated 1.3 million deaths among HIV-negative individuals and an additional 214,000 deaths among HIV-positive individuals (1).

Drug-resistant TB (DR-TB) continues to be a public health threat. In MTB, drug resistance develops through spontaneous genetic mutations. The development of acquired drug resistance usually occurs when there is a large bacterial population, such as in pulmonary cavities (2) or when an inadequate drug combination or dosages of the first-line drugs (FLDs) has been prescribed (3,4). Rarely, malabsorption of anti-TB drugs may account for acquired resistance (5).

WHO currently uses five categories to classify cases with DR-TB:

- Multidrug-resistant TB (MDR-TB) – with resistance to both isoniazid (H; INH) and rifampicin (R; RIF) – the two most effective FLDs;
- Rifampicin-resistant TB (RR-TB);
- Resistant to INH and sensitive to RIF TB (Hr-TB);
- Pre-extensively drug-resistant TB (pre-XDR-TB) – MDR/RR-TB plus resistance to any fluoroquinolone (FQ) – a class of second-line anti-TB drugs (SLDs);
- Extensively drug-resistant TB (XDR-TB) – MDR/RR-TB plus resistance to any FQ, plus to at least one of the drugs bedaquiline (Bdq) and linezolid (Lzd) (1).

EPIDEMIOLOGY OF ISONIAZID-RESISTANT TUBERCULOSIS

INH (isonicotinylhydrazide) is one of the most powerful FLDs for treatment of active and latent TB infection because of its potent early bactericidal activity against MTB. INH has been in clinical use since the 1950s, and drug resistance was expected because its use became widespread, but drug-susceptibility testing (DST) for Hr-TB or special drug regimens for Hr-TB were not widely used. Indeed, for decades, no DST for any drug has been done unless patients failed treatment with FLDs or had risk factors for DR-TB.

Currently, Hr-TB alone or in combination with other drugs, is the most common type of resistance to FLDs worldwide. Based on WHO estimates, globally in 2019 an estimated 1.4 million cases were with INH-resistant TB (any type of DR-TB, including resistance to INH) – 13.1% (95% CI: 9.9–16.9%) of new TB cases and 17.4% (95% CI: 0.5–54%) of previously treated cases. The cases with Hr-TB (only resistant to INH and sensitive to RIF) were 1.1 million or 11% of all incident TB cases (6). The global burden of INH-resistant TB is keeping higher in different regions worldwide. In a representative study published in 2008, Hoopes et al. reported that the prevalence of INH-resistant TB in the United States for the period 1993-2003 had not declined, despite the downward trend in the prevalence of overall TB (7). Data from the WHO showed that resistance to INH was detected in 30% of TB cases in Eastern Europe and 14% of TB cases in West/Central Europe and Africa for the period 1994-2009 (8). Hr-TB is much more common than RR-TB and could seriously jeopardize progress in the fight against TB. This is confirmed by an analysis of aggregated DR-TB data for the period 2002-2018 across 156 countries presented in the research study by Dean et al., showing that – on average – 7.4% (95% CI 6.5–8.4) of new TB cases and 11.4% (9.4–13.4) of previously treated patients have Hr-TB. The overall prevalence of INH resistance (with or without concomitant RR) ranged between 10.7% (9.6–11.9) and 27.2% (24.6–29.9) depending on the treatment history and reached even more alarming levels in certain countries, particularly in the European and Western Pacific WHO regions.

According to the data presented in the study of Dean et al., the prevalence of Hr-TB among new TB cases in Bulgaria for the above mentioned period was between 3 and 5.9%, lower than the global prevalence (7.4%) (9).

Bulgaria is one of the 18 TB high priority countries in the WHO European Region. In 2020, 930 TB cases were notified in the country, i.e. 13.4 per 100,000 – almost double than the average for the European Union/ European Economic Area (EU/EEA) TB notification rate (7.3 per 100,000). Since 2006, the number of TB cases and the TB notification rate have been declining. A national drug resistance survey conducted in 2010 showed that MDR-TB was detected in 2.1% of new TB cases and 11.1% of previously treated TB cases. In 2020, 12 pulmonary RR/MDR-TB cases (5% of all tested with DSTs) were identified through the national TB register, which is less than expected. Out of them, 2 pre-XDR-TB cases (22.2%) were notified (10). Data from the joint review of the Bulgarian National TB Control Programme conducted in 2014 by the European Centre for Disease Prevention and Control (ECDC) and the WHO Regional Office for Europe shows that out of 1,932 TB cases reported in 2013, 951 were culture positive, of which 734 (77%) had a DST result for FLDs. The proportion of INH-resistant TB was 6.1% among new cases and 23% among previously treated cases (calculated among cases with known DST results) (11), i.e. the prevalence of INH-resistant TB in the country seems to be higher than MDR-TB rates.

MOLECULAR-GENETIC MECHANISMS OF DEVELOPMENT OF ISONIAZID-RESISTANT TUBERCULOSIS

MTB has the ability for spontaneous, slow but steady mutations, leading to the development of drug resistance. This natural phenomenon is genetically determined and varies for the different drugs. Drug intake increases the likelihood for its appearance.

Isoniazid exerts its effects only in metabolically active mycobacterial cells. The probability for development of spontaneous resistance to INH is 1 in every 10^6 cell divisions (12). Drug resistance is the result of the selection of mycobacteria with mutations among the bacterial population due to destruction of the susceptible bacteria by anti-TB drugs. The problem is exacerbated if the patient is treated with monotherapy or with combination of FLDs in suboptimal concentrations – most bacteria die, but those with mutations survive, multiply and dominate the bacterial population (13).

Table 1 presents the main MTB genes, affected by specific molecular mechanisms (mutations) leading to development of resistance to the anti-TB drugs (14).

Table 1. Anti-tuberculosis drugs and *Mycobacterium tuberculosis* genes associated with development of drug resistance.

Anti-tuberculosis drug	Gene	Responsible for encoding of:
Isoniazid (INH; H)	***inhA***	NADH-dependent enoyl-ACP reductase (mutation is related with low level of resistance to H)
	katG	Catalase-peroxidase (mutation is related with high level of resistance to H)
	ahpC32	Alkyl hydroperoxide reductase
	oxyR	Oxidative stress regulator
	kasA	β-ketoacyl-ACP M synthase
Rifampicin (RIF; R)	***rpoB***	β-subunit of RNA polymerase
Pyrazinamide (PZA; Z)	***pncA***	Pyrazinamidase
Streptomycin (STR; S)	***rpsL***	30S ribosomal protein S12
	rrs	16S ribosomal RNA
	strA	Aminoglycoside phosphoryltransferase
Capreomycin (Cm)	***tlyA***	2'-O-methyltransferase
Ethambutol (ETH; E)	***emb A*****,** ***emb B*** and ***emb C***	Arabinosyl transferases
Fluoroquinolone (FQ)	***gyr A*** and ***gyr B***	DNA gyrase

NADH: Nicotinamide adenine dinucleotide hydrogen; ACP: Acyl carrier protein; RNA: Ribonucleic acid; DNA: Deoxyribonucleic acid

Resistance to INH is usually due to a mutation in *katG* or *inhA*, and more seldom - in other genes, such as *ahpC32* (15,16).

In order to be effective against TB, INH must be activated by catalase-peroxidase, an enzyme regulated by *katG* gene,. Mutations in *katG*, most commonly at Ser315Thr (Ser → Thr), result in insufficient activation of the drug and are associated with a high level of resistance – Minimum inhibitory concentration (MIC) > 1 μg/ml (17,18).

The *inhA* gene encodes an enoyl ACP reductase involved in fatty acid synthesis in MTB. As these fatty acids are targeted by the active derivative of INH, mutations in *inhA* or its promoter region block INH binding and result in low-level INH resistance (MIC <1 μg/ml). The most frequent mutation in the promoter region is at position 15C/T (Cys → Thr) (19,20). Isolates with an *inhA* mutation are also typically resistant to ethionamide (Eto) and prothionamide (Pto) – SLDs for treatment of DR-TB (21).

According to most of the studies, risk factors for developing INH resistance include a history of TB, and origin from regions with a higher TB prevalence (Asia, Pacific Islans, etc.) (22).

DIAGNOSIS OF ISONIAZID-RESISTANT TUBERCULOSIS

Drug susceptibility tests (DSTs), which determine the sensitivity of MTB to FLDs and SLDs, and can detect the presence of DR-TB, including Hr-TB, are phenotypic and genotypic (PCR; Polymerase Chain Reaction based). DSTs are very important for developing the drug regimen, treatment outcome monitoring, disease prognosis and Drug Resistance Surveillance (DRS).

1. Phenotypic DSTs are performed with MTB strain isolated from cultures and determine if a strain is resistant to an anti-TB drug by evaluating the growth (or metabolic activity) in the presence of the drug. They are used for TB diagnosis and treatment follow-up. The reliability of DSTs varies from one drug to another. For the FLDs, DSTs are very reliable for RIF and INH, but less so for PZA and much less for ETH. DSTs for aminoglycosides, polypeptides and FQs have relatively good reliability and reproducibility. DSTs to other SLDs – para-aminosalicylic acid (PAS), Eto and cycloserine (Cs) are much less reliable and reproducible.

1.1. Automated methods – **BACTEC MGIT 960®**, using liquid culture media MGIT (Mycobacterium

Growth Indicator Tube), are currently the most widely-used option, as they are quick and reliable. It is recommended that they be performed on pure MTB cultures. The following concentrations of the FLDs are used: STR – 1.0 μg/ml; INH – 0.1 μg/ml; RIF – 1.0 μg/ml; ETH – 5 μg/ml; and of the SLDs: amikacin (Am) – 1.0 μg/ml; kanamycin (Km) – 5.0 μg/ml, capreomcin (Cm) – 2.5 μg/ml, and ofloxacin (Ofx) – 2.0 μg/ml (23).

1.2. Nitrate reductase assay (NRA) – a rapid method for detection of resistance to RIF and INH. It is a simple technique based on the capacity of MTB to reduce nitrate to nitrite, which is detected by adding the Griess reagent to solid Löwenstein–Jensen media, used for cultures of *Mycobacterium* species, and in the presence of the corresponding concentration of FLDs (RIF, INH, ETH and STR). The technique is in use since the 1980s and was developed by assoc. prof. Emil Kalfin from the Scientific Institute of Pneumology and Phthisiatry at Medical Academy, Sofia, Bulgaria. The NRA was shown to be highly sensitive and specific for detection of RIF and INH resistance when used on clinical isolates (24).

2. Genotypic DSTs – modern automated molecular technologies for rapid diagnosis of MTB using genetic material from biological samples, including determination of drug resistance. These molecular tests can only be performed by specialized laboratories with strict quality assurance in place. It is possible to test DNA of an isolated MTB strain, and also extracted DNA from the investigated clinical sample. The genotypic DSTs are used only for initial TB diagnosis but are not recommended for treatment follow-up.

2.1. Line Probe Assays (LPAs) based on DNA-STRIP® technology allow DNA detection and testing from isolated MTB strain or directly from the clinical sample, but only from the pulmonary system. This method has the advantage of giving fast results, within few hours, for smear-positive patients (referred to as direct testing, because the sputum can be directly tested). For smear-negative patients, a primary culture is needed prior to testing (referred to as indirect testing, because a culture has to be grown first from the patient's sputum).

- **GenoType® MTBDR*plus*** (Hain Lifescience GmbH, Nehren, Germany) – LPA for DSTs to **FLDs;** rapid MTB detection for 2 days; can identify mutations on ***katG*** and ***inhA*** genes associated with resistance to INH, and mutation on ***rpoB*** gene, encoding **β-subunit of RNA polymerase**, associated with resistance to RIF. Using this method, it is possible to determine the presence of **Hr-TB, RR-TB and MDR-TB**;
- **GenoType® MTBDR*sl*** (Hain Lifescience GmbH, Nehren, Germany) – LPA for DSTs to **SLDs;** can identify mutation on ***gyrA*** gene, encoding the enzyme DNA-gyrase, corresponding with resistance to FQs; mutation on ***rrs*** gene, encoding **16S rRNA**, corresponding with resistance to aminoglycosides and polypeptides. The second version of the test allows identification of mutation on ***gyrB*** gene, encoding the enzyme DNA-gyrase, associated with resistance to FQs; and mutation in the promoter of ***eis*** gene, encoding the enzyme **N-acetyltransferase**, associated with resistance to Km. Using this method, it is possible to determine the presence of **pre-XDR-TB and XDR-TB**. LPA to SLDs can be used as a triage test on smear-positive patients to guide the initial treatment of XDR-TB suspects while awaiting confirmatory results from conventional phenotypic testing. However, LPAs cannot be used as replacement tests for conventional phenotypic SLD DSTs.

2.2. Xpert MTB/RIF® (GeneXpert; Real-Time PCR, Cepheid®, Sunnyvale, USA) – automated nucleic acid amplification technology for rapid detection of MTB within 2 hours, as well as for detection of possible mutations in ***rpoB*** gene, causing RR-TB of the isolated MTB strain. The method was approved by the WHO in 2010 (25).

2.3. Whole Genome Sequencing (WGS) – where the entire genetic code of MTB strains isolated from patients is described and compared to a reference set of genomes. The WGS has been introduced routinely in England, Germany and many EU/EEA countries to guide clinical decision-making, earlier detection of resistance, and support of outbreak and epidemiological investigation. However, WGS requires sophisticated laboratory and bioinformatics infrastructure, and currently requires MTB isolation and DNA extraction before sequencing. Therefore the routine applicability of

the method beyond research especially in low resource settings is uncertain. Nevertheless, the advances in the sequencing field may rapidly turn WGS to a TB diagnostic tool (26). Between January 2017 and December 2019 Bulgaria participated in the pilot study of the European Center for Disease Prevention and Control (ECDC), evaluating the systematic use of a WGS-based approach for MTB surveillance involving all European Union/European Economic Area (EU/EEA) countries and highlighting the challenges to be considered for the future development of a WGS-based surveillance system (27).

Yordanova et al. investigated MTB isolates from 36 TB cases from all over Bulgaria in a retrospective study for the period 2015-2016. All the cases were confirmed by BACTEC MGIT 960 with mono-resistance to INH and additionally tested with GenoType MTBDR*plus* in the National Reference Laboratory of Tuberculosis (NRL TB) at the NCIPD. The authors found that only 25% of the tested MTB isolates with phenotypic INH mono-resistance had the S315T1 mutation in *katG*; all isolates were with MIC over 0.4 µg/ml. Resistance type C15T in the promoter region of *inhA* was detected in 22.22% of cases and only 1 of them showed MIC below 0.4 µg/ml. No mutations were detected in nearly half of the cases (n=19, 52.78%) and most of these isolates were with lower MIC values (n=12). The authors supposed that the resistance among the cases without mutations in *katG* or *inhA* can be explained with mutations in many other loci or genes (*furA-katG*, *fabG1-inhA*, *ahpCoxyR* intergenic region, *efpA*, *fadE24*, *iniA*, *iniB*, *iniC*, *kasA*, *nat*, *ndh*), which cannot be found using only GenoType MTBDR*plus* (28).

At the end of 2021, WGS of all isolated DR-TB strains in Bulgaria started in the NRL TB at the NCIPD, which will expand our knowledge about the mutations of MTB in the country, including those corresponding with resistance to INH.

TREATMENT OF ISONIAZID-RESISTANT TUBERCULOSIS

The management of INH-resistant TB is important because the last systematic meta-analyses have shown that resistance to INH reduces the probability of treatment success and increases the risk of acquiring resistance to other important FLDs such as RIF, thereby increasing the risk of MDR-TB. Moreover, INH-resistant TB generally requires longer treatment than drug-susceptible TB, increasing the burden of the disease (29,30).

The recommended regimens for INH-resistant TB differ among countries and have differed over time. Table 2 summarizes the previous and current international guidelines for the treatment of INH-resistant TB (22).

There is a standard code for writing out anti-TB regimens. Each drug has an abbreviation (shown in the table). A DR-TB regimen consists of two phases, separated by a slash: the first is intensive phase and the second is prolonged phase. The number shown before each phase stands for the minimal required phase duration in months . The number in subscript (e.g., 3) after a letter is the number of drug doses per week. If there is no number in subscript, treatment is daily. Alternative drug(s) appears as a letter(s) in parentheses. The drugs in the higher groups are written first followed by the others in descending order of potency.

Based on the most recent meta-analyses on the management of INH-resistant TB, in 2019 the WHO published the last key recommendations for Hr-TB treatment – with RIF-EMB-PZA-Lfx for 6 months and no addition of SM or other injectable agents to the drug regimen.

The WHO guidance emphasizes the importance of excluding resistance to RIF before starting the regimen for INH-resistant TB because of the risk for development of MDR-TB during the treatment course.

The guidelines are based primarily on individual patient data or observational studies conducted in various settings. They indicate that addition of an FQ to RIF-EMB-PZA regimens compared to ≥6 months of RIF-EMB-PZA is leading to higher treatment success rate (aOR, 2.8; 95% CI, 1.1–7.3). The addition of an FQ to a 6-month RIF-EMB-PZA regimen tended to reduce the number of deaths (aOR, 0.4; 95% CI, 0.2–1.1) and the acquisition of RIF resistance (aOR, 0.10; 95% CI, 0.01–1.2).

The main recommendations are to include Lfx rather than Mfx as a first choice because Lfx has a better safety profile than other FQs, and fewer drug interactions than Mfx; the plasma peak concentration of Lfx is not affected by the addition of RIF. Additionally, there are no contraindications for the use of Lfx with antiretroviral agents for the treatment of patients co-infected with HIV (31,32).

Table 2. International guidelines for treatment of isoniazid-resistant tuberculosis

Guideline	Recommended regimen	Duration (months)
ATS/CDC (1994)	REZ	6
	RE	12
ATS/CDC (2003)	REZ (FQ for extensive disease)	6
BTS (1998)	2 SREZ / 7 RE	9
	2 REZ / 10 RE	12
NICE (2011)	2 SREZ / 7 RE	9
	2 REZ / 10 RE	12
NICE (2016)	2 (H)REZ / 7 RE (10 months for extensive disease)	9-12
Canadian Tuberculosis Standards (2014)	2 (H)RZE / 4-7 REZ	6-9
	2 (H)RZE / 10 RE	12
	2 (H)RZE FQ / 4-7 RE FQ	6-9
WHO (2006)	REZ (FQ)*	6-9
WHO (2008)	REZ (FQ)*	6-9
WHO (2014)	REZ (FQ)* †	6-9
WHO (2018)	REZ FQ (Lfx > Mfx) ‡	6

*A fluoroquinolone may strengthen the regimen for patients with extensive disease.

†Use Xpert MTB/RIF at month 0, 2, and 3, and if RR-TB is switched to full MDR-TB treatment.

‡The new 2018 WHO guidelines recommend Lfx as the first choice, rather than Mfx.

ATS: American Thoracic Society; CDC: Centers for Disease Control and Prevention; BTS: British Thoracic Society; NICE: National Institute for Health and Care Excellence; WHO: World Health Organization; H: isoniazid; R: rifampicin; E: ethambutol; Z: pyrazinamide; FQ: fluoroquinolone; Lfx: levofloxacin; Mfx: moxifloxacin.

CONCLUSION

INH-resistant TB poses a significant challenge before public health systems. Many patients with Hr-TB would be missed by current diagnostic algorithms driven by RIF testing, thus receiving incomplete drug regimen. The development of new rapid molecular technologies is needed in order to ensure access to appropriate treatment and care.

The WHO recommendations for Hr-TB treatment are based on observational studies, but not on randomized controlled trials, and are thus conditional and based on low certainty in the estimates of the effect. Therefore, further work is needed to optimize the treatment and control of INH-resistant TB.

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REFERENCES

1. Global tuberculosis report 2021. Geneva: World Health Organization; 2021. Licence: CC BY-NC-SA 3.0 IGO.
2. Xi Y, Zhang W, Qiao R-J, Tang J (2022) Risk factors for multidrug-resistant tuberculosis: A worldwide systematic review and meta-analysis. PLoS ONE 17(6): e0270003. https://doi.org/10.1371/journal.pone.0270003..
3. Mahmoudi A, Iseman MD. Pitfalls in the care of patients with tuberculosis: common errors and their association with the acquisition of drug resistance. *JAMA* 1993;270:65-8.
4. Mitchison DA. How drug resistance emerges as a result of poor compliance during short course chemotherapy for tuberculosis. *Int J Tuberc Lung Dis* 1998;2:10-5.
5. Patel KB, Belmonte R, Crowe HM. Drug malabsorption and resistant tuberculosis in HIV-infected patients. *N Engl J Med* 1995;332:336-7.
6. Global tuberculosis report 2020. Geneva: World Health Organization; 2020. License: CC BY-NC-SA 3.0 IGO.
7. Hoopes AJ, Kammerer JS, Harrington TA, Ijaz K, Armstrong LR. Isoniazid-monoresistant tuberculosis in the United States, 1993 to 2003. *Arch Intern Med* 2008;168:1984-92.
8. Jenkins HE, Zignol M, Cohen T. Quantifying the burden and trends of isoniazid resistant tuberculosis, 1994-2009. *PLoS One* 2011;6:e22927.
9. Dean AS, Zignol M, Cabibbe AM, Falzon D, Glaziou P, Cirillo DM, et al. Prevalence and genetic profiles of isoniazid resistance in tuberculosis patients: A multicountry analysis of cross-sectional data. *PLoS Med.* 2020; 17(1): e1003008. https://doi.org/10.1371/journal.pmed.1003008.
10. European Centre for Disease Prevention and Control, WHO Regional Office for Europe. Tuberculosis surveillance and monitoring in Europe 2022 – 2020 data. Copenhagen: WHO Regional Office for Europe and Stockholm: European Centre for Disease Prevention and Control; 2022. Licence: CC BY 3.0 IGO.
11. European Centre for Disease Prevention and Control. Report of the joint ECDC and WHO review of the national tuberculosis programme in Bulgaria. Stockholm: ECDC; 2014. ISBN 978-92-9193-598-7. doi 10.2900/37158.
12. Goering R., R.I.M., Mims C. Medical Microbiology, Chapter 5.

Diagnosis and control; Resistance to Antibacterial Agents. 4th ed 2007: Mosby.
13. Pfyffer, G.E. Drug-resistant tuberculosis: resistance mechanisms and rapid susceptibility testing. *Schweizerische medizinische Wochenschrift*, 2000. 130(49):1909-13.
14. Miotto P, Tessema B, Tagliani E, et al. A standardised method for interpreting the association between mutations and phenotypic drug resistance in Mycobacterium tuberculosis. *Eur Respir J* 2017; 50: 1701354 [https://doi.org/10.1183/13993003.01354-2017].
15. Zhang Y, Heym B, Allen B, Young D, Cole S. The catalase-peroxidase gene and isoniazid resistance of Mycobacterium tuberculosis. *Nature* 1992;358:591-3.
16. Piatek AS, Telenti A, Murray MR, El-Hajj H, Jacobs WR Jr, Kramer FR, et al. Genotypic analysis of Mycobacterium tuberculosis in two distinct populations using molecular beacons: implications for rapid susceptibility testing. *Antimicrob Agents Chemother* 2000;44:103-10.
17. Ramaswamy SV, Reich R, Dou SJ, Jasperse L, Pan X, Wanger A, et al. Single nucleotide polymorphisms in genes associated with isoniazid resistance in Mycobacterium tuberculosis. *Antimicrob Agents Chemother* 2003;47:1241-50.
18. Ramaswamy S, Musser JM. Molecular genetic basis of antimicrobial agent resistance in Mycobacterium tuberculosis: 1998 update. *Tuber Lung Dis* 1998;79:3-29.
19. Dalla Costa ER, Ribeiro MO, Silva MS, Arnold LS, Rostirolla DC, Cafrune PI, et al. Correlations of mutations in katG, oxyR-ahpC and inhA genes and in vitro susceptibility in Mycobacterium tuberculosis clinical strains segregated by spoligotype families from tuberculosis prevalent countries in South America. *BMC Microbiol* 2009;9:39.
20. Palomino JC, Martin A. Drug Resistance Mechanisms in Mycobacterium tuberculosis. *Antibiotics.* 2014; 3(3):317-340.
21. Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um KS, Wilson T, et al. inhA, a gene encoding a target for isoniazid and ethionamide in Mycobacterium tuberculosis. *Science* 1994;263:227-30.
22. Jhun BW, Koh WJ. Treatment of isoniazid-resistant pulmonary tuberculosis. *Tuberc Respir Dis (Seoul)*. 2020;83:20–30. doi:10.4046/trd.2019.0065.
23. Ministry of Health of the Republic of Bulgaria. Methodological guideline on microbiological diagnosis of tuberculoosis, 2016. Approved by Order No. RD-01-72 / 10.03.2016. Available on: http://www.mh.government.bg/. In Bulgarian.
24. Martin A, Panaiotov S, Portaels F, Hoffner S, Palomino JC, Angeby K. The nitrate reductase assay for the rapid detection of isoniazid and rifampicin resistance in Mycobacterium tuberculosis: a systematic review and meta-analysis. *J Antimicrob Chemother.* 2008 Jul;62(1):56-64. doi: 10.1093/jac/dkn139. Epub 2008 Apr 10. PMID: 18407918.
25. Ministry of Health of the Republic of Bulgaria. Methodological guideline on organization of the performance of fast methods for diagnosis of tuberculosis on the territory of the Republic of Bulgaria, 2015. Approved by Order No. RD -01-237/16.10.2015. Available on: http://www.mh.government.bg/. In Bulgarian.
26. MacPherson, P. (2018). Emerging Issues in tuberculosis. K4D Reading Pack. Brighton, UK: Institute of Development Studies; p. 56.
27. Tagliani E, Anthony R, Kohl TA, et al. Use of a whole genome sequencing-based approach for Mycobacterium tuberculosis surveillance in Europe in 2017–2019: an ECDC pilot study. *Eur Respir J* 2021; 57: 2002272 [https://doi.org/10.1183/13993003.02272-2020].
28. Yordanova, S., Baykova, A., Atanasova, Y., Todorova, Y., Bachiyska, E. Isoniazid-monoresistant tuberculosis in Bulgaria. *Probl. Inf. Paras. Dis*., Vol. 48, 2020, 1, 21-24.
29. Menzies D, Benedetti A, Paydar A, Martin I, Royce S, Pai M, et al. Effect of duration and intermittency of rifampin on tuberculosis treatment outcomes: a systematic review and meta-analysis. *PLoS Med* 2009;6:e1000146.
30. Menzies D, Benedetti A, Paydar A, Royce S, Madhukar P, Burman W, et al. Standardized treatment of active tuberculosis in patients with previous treatment and/or with mono-resistance to isoniazid: a systematic review and meta-analysis. *PLoS Med* 2009;6:e1000150.
31. WHO consolidated guidelines on tuberculosis. Module 4: treatment - drug-resistant tuberculosis treatment. Geneva: World Health Organization; 2020. Licence: CC BY-NC-SA 3.0 IGO.
32. WHO treatment guidelines for isoniazid-resistant tuberculosis: Supplement to the WHO treatment guidelines for drug-resistant tuberculosis. Geneva: World Health Organization; 2018. Licence: CC BY-NC-SA 3.0 IGO.

STUDY ON TICKS REMOVED FROM PATIENTS FOR INFECTION WITH *BORRELIA BURGDORFERI* AND THEIR NUMBER DEPENDING ON TEMPERATURE AND PRECIPITATION IN 2016-2021

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ABSTRACT

Background: Deacarization, applied epidemiological measures, and climatic factors affect the abundance of ticks. On the other hand, ticks themselves are a factor of great epidemiological and epizootic importance. Studies concerning the influence of climatic factors on ticks and their infection with *B. burgdorferi* are still limited in Bulgaria. The aim was to investigate the abundance of ticks in relation to temperature and precipitation, as well as the infection with *B. burgdorferi* of ticks removed from patients during the period 2016-2021.

Materials/methods: A total of 10,907 ticks were collected from patients and classified according to species and stage of development. Nested PCR was performed targeting two sites of the spacer region between 5S and 23S of *B. burgdorferi* sensu lato rRNA. Weather data were collected from free Internet meteorological sites.

Results: It was found that 92-96% of the ticks belonged to the species *Ixodes ricinus.* PCR data were obtained for infestation of ticks in 2016-2021. Only in 2018, the highest number of ticks was observed in May, while in the other five years - in June, which was analyzed in relation to the average temperature and precipitation.

Conclusion: An increase in tick abundance was observed at average temperatures around 20°C, with rainfall on the days before the peak. During the 6-year period, the highest number of ticks was collected in June 2021, which could be explained with the high average temperatures and abundant precipitations during the preceeding spring months (April – June).

Key words: *B. burgdorferi, ticks, temperature, precipitation*

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INTRODUCTION

Lyme borreliosis is the most common tick-borne infection in North America and Europe (1). The tick *Ixodes ricinus* is the main vector. As a result of climate change, associated with global warming (2), this species is constantly expanding its distribution area. Tick-borne diseases require an established vector population, a pathogen and suitable environmental and climatic conditions across the cycle of transmission in humans (3). Climate influences the life cycle of ticks, as well as the reproduction rate of bacteria inside the vector and human hosts (4). This means that uprising temperature can reduce the incubation period of pathogens and the life cycle of vectors, thus boosting the transmission risk through elevated vector populations. Long-term seasonal changes can affect the prevalence of vector-borne diseases in Europe (5). *I. ricinus* is present throughout Europe with an expansion to higher latitudes in Sweden (6) and, to higher altitudes elevations in the Czech Republic (7), Austria (8), Norway and Germany (4). On the average, more than 1500 ticks removed from patients per year were tested for *Borrelia burgdorferi* infection during the study period. It is known that ticks are susceptible to climatic determinants as humidity and temperature. The number of ticks removed from our patients varied significantly through the years, from over 2000 in 2018 and 2020 to less than 1500 in 2016 and 2021 . In 2018 , the highest number of ticks was reached one month earlier as compared to the other years of the study. What are the reasons for the observed annual differences ? The aim of the study

was to investigate *B. burgdorferi* infection in ticks removed from patients and their numbers in relation to the average temperature and precipitation over the 6-year period.

MATERIAL AND METHODS

A total of 10,907 ticks were collected from patients as follows: 1,158 in 2016, 1,895 in 2017, 2,347 in 2018, 1,985 in 2019, 2,052 in 2020 and 1,470 in 2021. They were identified morphologically with a microscope "Leika" and determined to the species and stage of development (9). Nested PCR were performed targeting two sites from the spacer region between 5S and 23S of *B. burgdorferi* sensu lato rRNA (10). Weather data were collected from freemeteo.bg (11) and meteoblue.com (12). Diagrams were made for the four months of interest: April, May, June and July, when most of the ticks were brought to the laboratory for examination. Data on Lyme disease morbidity in humans during 2016-2020 were obtained from NCIPD (13).

RESULTS AND DISCUSSION

It was found that between 92-96% of ticks belonged to the species *I. ricinus.* There was an increase in the number of nymphs studied over the 6-year period.

PCR data for infestation of ticks from Sofia city were found in 21.03% (184/875) of the ticks investigated in 2016, 20.7% (249/1,203) of the ticks in 2017, 9.47% (155/1,636) of the ticks in 2018, 22.47% (387/1,722) of the ticks in 2019, 15.02% (273/1,817) of the ticks in 2020 and 20.41% (258/1,264) of the ticks in 2021.

A comparative study for *B. burgdorferi* infestation of ticks from 2018 to 2021 was conducted in the five locations in Sofia with the highest number of ticks removed from patients.

Table 1. *Infection of ticks with* **B. burgdorferi** *in five places in Sofia in 2018-2021*

Region in Sofia	Infection in 2018	Infection in 2019	Infection in 2020	Infection in 2021
Cemetery parks	5/50 (10%)	17/76 (22%)	13/62 (21%)	19/84 (23%)
South park	9/72 (12.5%)	12/33 (36%)	8/42 (19%)	14/56 (25%)
Borisova garden park	4/50 (8%)	9/52 (17%)	14/ 66 (21%)	9/49 (18%)
Neighbour-hood "Iztok"	4/13 (31%)	7/22 (32%)	5/33 (15%)	11/43 (26%)
Neighbour-hood Bankya	1/35 (3%)	6/23 (26%)	5/38 (13%)	7/31 (23%)

In addition, a tick infestation was compared to morbidity in humans during the same period 2016-2020.

Table 2*. Comparative study on tick infestation and morbidity in humans in 2016-2020*

Year	Infestation of ticks with *B. burgdorferi*	Number of cases / morbidity in humans in Bulgaria
2016	184/875 (21.03%)	290/ 4.05%000
2017	249/1,203 (20.70%)	402/ 5.66%000
2018	155/1,636 (9.47%)	599 / 8.50%000
2019	387/1,722 (22.47%)	375 / 5.36%000
2020	273/1,817 (15.02%)	160 / 2.3%000

The highest number of ticks in 2018 was observed during the month of May, and the peak in tick abundance in 2016, 2017, 2019, 2020 and 2021was found in June. Data on the monthly number of ticks (for April, May, June and July) were analyzed against the average temperature and precipitation during the 6-year study period (Fig. 1, 2, 3, 4, 5, 6, 7, 8).

According to our data, the species *I. ricinus* is associated with over 92% of tick bites of humans in the country. An increase in the number of nymphs studied over the years was also associated with an increase in the number of bites, due to their pronounced anthropophilia and difficult detection. Nymphs were less infected with the causative agents of Lyme disease as compared to imagos (15.93% vs. 20.29%) only in 2016. Tick infestations varied between 10.68% in 2018 and 27.58% in 2019 during the 6-year study period, showing the important role of ticks in *B. burgdorferi* transmission. A meta-analysis of the distribution and prevalence of bacteria from the group *B. burgdorferi* s.l. in European ticks (14) showed that the most commonly reported *B. afzelii, B. garinii, B. valaisiana*, and *B. burgdorferi* sensu stricto largely overlap across Europe.

The highest prevalence occurred in areas with small amplitude of the mean annual temperatures (7°C–17°C), together with a moderate spring rise of the vegetation. The study established an average of 2% infection with *B. burgdorferi* s.l. of 82,000 questing nymphs with prevalence from <1% to >46%.

The highest rate of tick infection with *B. burgdorferi* in Sofia was detected in 2019 (22.47%), and the lowest - in 2018 (9.47%). A significant decrease in tick infection was found in 2018 (9.47%), compared to 2017 (20.70%). The data were analogous to those established by us in 2003/2004, when there was a decrease from 25.42% to 7.21%, (P<0.05) (15). These data showed that statistically significant fluctuations in tick infection are observed periodically over the years.

Unfortunately, some of our most representative parks (South park, Borisova garden) were among the localities with the highest number of ticks removed from people and brought for testing (Table 1). The risk of human infection was also high (between 10 and 23%) in the spring and autumn stew, when many people visit the Cemetery parks (Table 1).

Lyme disease comprises the most important vector-borne disease burden in the European Union with an estimated of 65,000 cases per year (2). As stated in the Annual Analyses of Acute Infectious Diseases in Bulgaria (13) during the 2016-2020 period the number of Lyme borreliosis cases ranged from 160 (2020) to 599 (2018). While morbidity varied between 4.05%000 and 5.66%000 (in 2016, 2017, 2019), tick infection rates ranged between 20.70% and 22.47% (Table 2). The comparison between the rates of tick infestation and the number of Lyme disease cases in humans showed that, the lowest tick infestation rate for the 6-year period (in 2018) corresponded to the highest morbidity rate in patients. Interestingly, these facts coincided with the highest number of ticks brought to the laboratory for analysis (2,347) and with a premature rise in the number of ticks (Fig 4) in that same year (2018). It should be noted that the high incidence of human cases in 2018 was based on data for several cities for which data on tick infestation was not available.

An increase in the number of ticks was observed at an average temperature around 20°C, with rainfall of 5-10 mm on the days in May and June in 2016 and 2017 (Fig. 1, 2, 4). The highest number of ticks was detected in the month of May only in 2018, in the presence of high temperatures, without sharp fluctuations, preceded by 30-40 days of precipitation (Fig. 3, 4). The increased number of ticks in June was in associated with an increase in temperatures and precipitation from mid-May to mid-June in 2019 (Fig. 5, 8). In 2020, most of the precipitations were observed in May accompanied by a smooth rise in temperatures, and leading to a peak number of ticks in June (Fig. 6, 8). In 2021, the average spring temperature was around 20°C, with a lot of rain in April, May and June, leading to the highest number of ticks during the 6-year period in June (Fig. 7, 8). According to a climate projection by 2040–2060, a 3.8% overall habitat enlargement for *I. ricinus* is anticipated in Europe. This correlates with the increased number of ticks and their extension into higher altitudes and latitudes in many areas like the Scandinavian and Baltic countries (2). Therefore, the increased number of ticks removed from people in Bulgaria during the years of the study was not surprising.

CONCLUSIONS

Data related to number and infestation of ticks can be applied in practice in connection with the

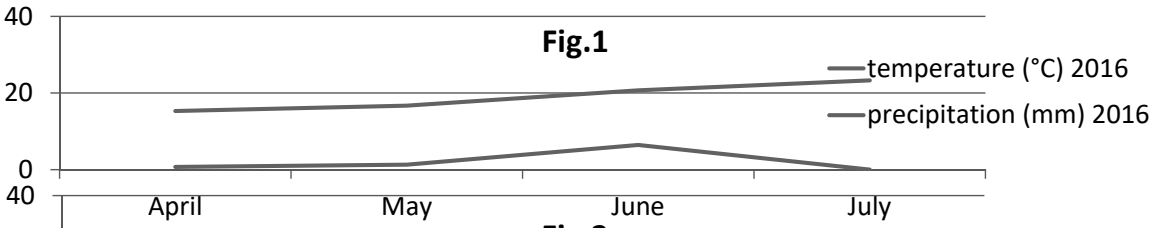

Fig.1

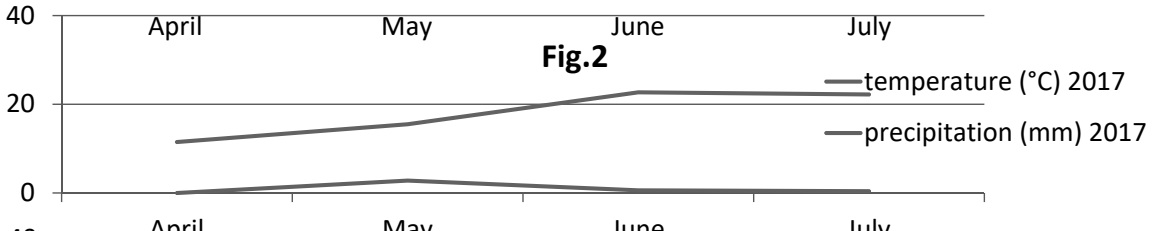

Fig.2

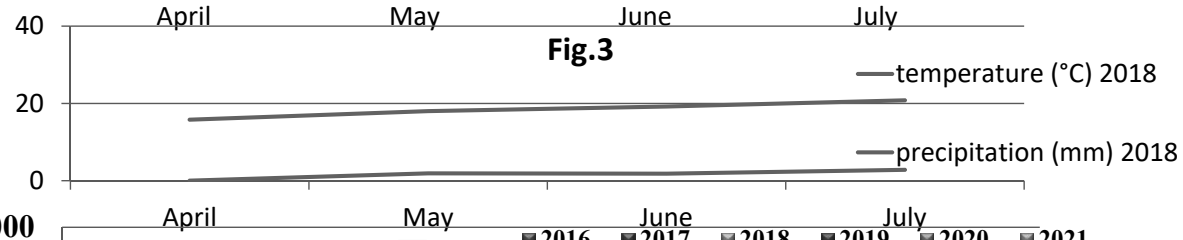

Fig.3

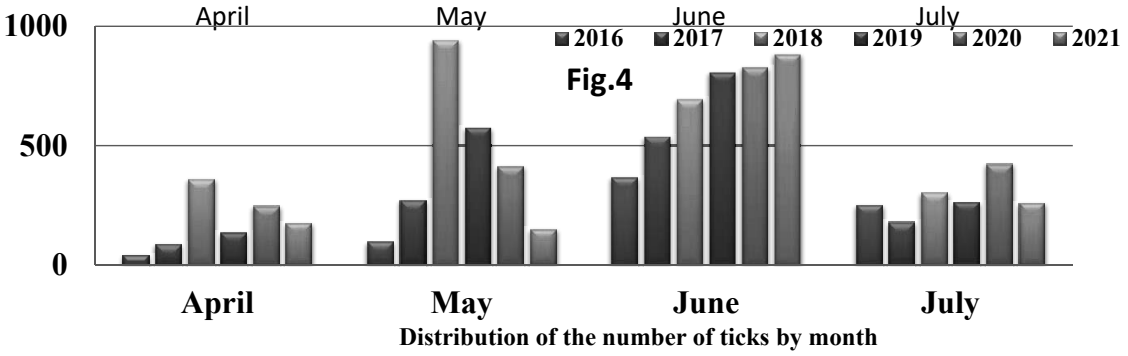

Fig.4

Distribution of the number of ticks by month

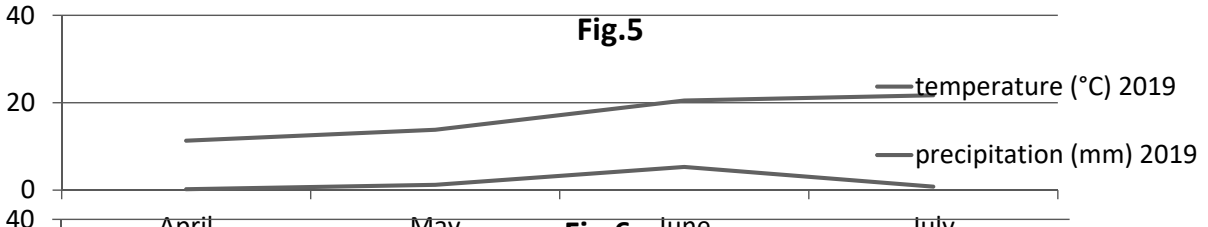

Fig.5

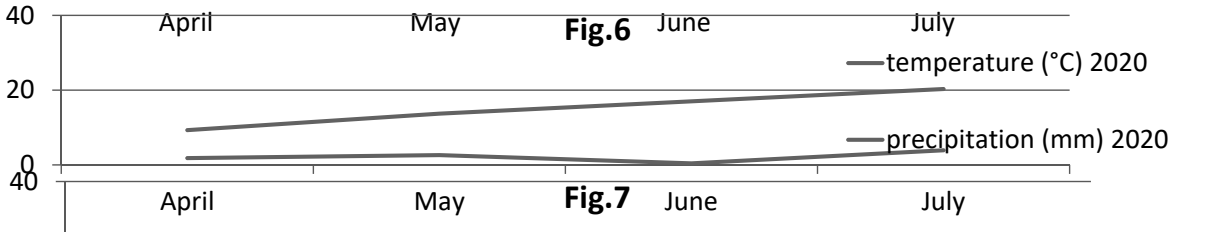

Fig.6

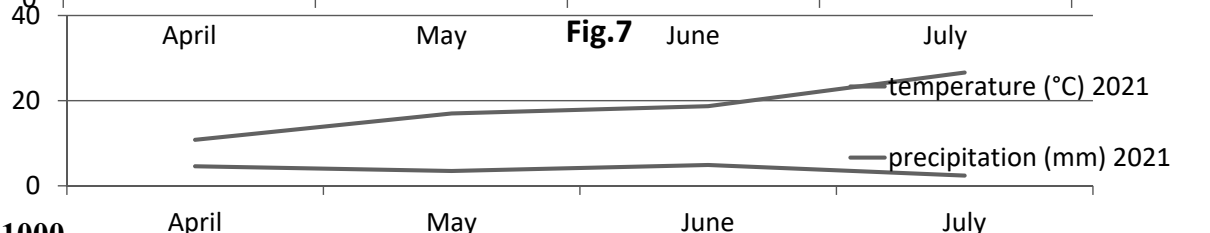

Fig.7

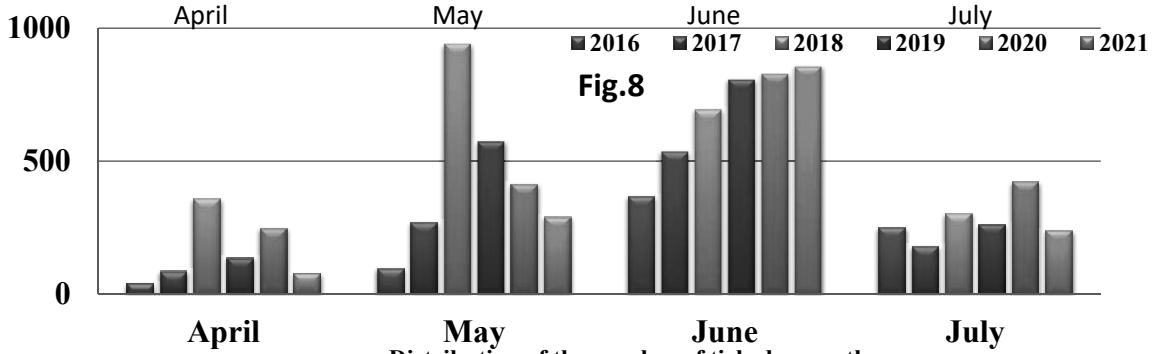

Fig.8

Distribution of the number of ticks by month

deacarization of lawns and the use of personal protective equipment. Temperature and precipitation data should also be taken into account when lawns are processed against ticks. Timely examination of the body after staying in lawns and rapid removal of ticks, especially the highly anthropophilic nymphes, reduces the risk of infection with Lyme borreliosis. This study indicates the importance of monitoring temperature and precipitation, for predicting the number and infestation levels of *I. ricinus* ticks associated with the incidence of Lyme disease

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REFERENCES

1. Mead PS, Epidemiology of Lyme disease, Infect Dis Clin North Am, 2015 Jun; 29(2):187-210.
2. Semenza JC and Suk JE, Vector-borne diseases and climate change: a European perspective. FEMS Microbiol Lett. 2018 Jan; 365(2): fnx244.
3. Randolph SE, Rogers DJ. The arrival, establishment and spread of exotic diseases: patterns and predictions. Nat Rev Microbiol 2010; 8:361–71.
4. Semenza JC, Menne B. Climate change and infectious diseases in Europe. Lancet Infect Dis 2009; 9:365–75.
5. Lindgren E, Andersson Y, Suk JE et al. Monitoring EU emerging infectious disease risk due to climate change. Science 2012; 336:418–9.
6. Jaenson TGT, Jaenson DGE, Eisen L et al. Changes in the geographical distribution and abundance of the tick *Ixodes ricinus* during the past 30 years in Sweden. Parasit Vectors 2012; 5:8.
7. Daniel M, Danielova V, Kriz B et al. Shift of the tick *Ixodes ricinus* and tick-borne encephalitis to higher altitudes in central Europe. Eur J Clin Microbiol Infect Dis 2003; 22:327–8.
8. Heinz FX, Stiasny K, Holzmann H et al. Emergence of tick-borne encephalitis in new endemic areas in Austria: 42 years of surveillance. Euro Surveill 2015; 20:9–16.
9. Померанцев БИ, Паукообразные, Иксодовые клещи (Ixodidae), Издателство Академии Наук СССР, Москва, 1950, Ленинград, том IV, вып. 2.
10. Rijpkema SG, Molkenboer MJ, Schouls LM, Jongejan F, Schellekens JF. Simultaneous detection and genotyping of three genomic groups of *Borrelia burgdorfer*i sensu lato in Dutch *Ixodes ricinus* ticks by characterization of the amplified intergenic spacer region between 5S and 23S rRNA genes. J Clin Microbiol. 1995 Dec;33(12):3091-5.
11. freemeteo.bg, Времето. Прогнози за времето. Времето сега. Предупреждения за сурово време. |
12. meteoblue.com, Времето София.
13. Analyses of acute infectious diseases in Bulgaria 2016-2020 (In Bulgarian), NCIPD, Department of Epidemiology, https://www.ncipd.org
14. Estrada-Peña A, Cutler S, Potkonjak A, Vassier-Tussaut M, Van Bortel W, Zeller H, Fernández-Ruiz N, Mihalca AD. An updated meta-analysis of the distribution and prevalence of Borrelia burgdorferi s.l. in ticks in Europe. Int J Health Geogr (2018); 17:41.
15. Гладнишка ТК, Микробиологични проучвания върху вектори и резервоари на някои предавани с кърлежи бактериални инфекции при хората, Дисертационен труд за ОНС „Доктор", научни ръководители И. Христова, Т. Кантарджиев, София, 2008.

A RETROSPECTIVE STUDY ON *E. COLI* ENTERITIS INCIDENCE IN BULGARIA FOR A PERIOD OF TEN YEARS (2011-2020)

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ABSTRACT

Intestinal diseases caused by diarrheagenic *E. coli* account for a significant proportion of infections, especially in children. *E. coli* enteritis can occur as sporadic cases but often assumes an epidemic nature. This study aims to determine the significance and prevalence of infections caused by diarrheagenic *E. coli* in Bulgaria for a period of ten years 2011-2020. **MATERIALS AND METHODS.** The etiological role and distribution of diarrheagenic *E. coli* among Bulgarian population for the last decade were determined by a retrospective analysis of microbiological and epidemiological data. **RESULTS.** Data from the studied period reveal the role of enterotoxigenic *E. coli* (ETEC) O6 as a leading etiological cause of epidemic and sporadic enteritis in the country, followed by enteropathogenic *E. coli* (EPEC) O126 and O127. There are only two reported cases of lethal hemorrhagic uremic syndrome (HUS) caused by enterohemorrhagic *E. coli* (EHEC) that happened in 2011. Most affected by diarrheagenic *E. coli* (DEC) infections are infants and young children. Neonatal meningitis in Bulgaria is rare, the etiological agents are *E. coli* O18 and O25. The most common registered DEC infections are during the summer months of May - September. Several epidemics have been registered in young children, and the etiological cause is ETEC O6. **CONCLUSION.** The main etiological agent of *E. coli* infections in Bulgaria is ETEC O6, and infants and young children are at risk. Recent DEC epidemics have not been reported. The ethiologic diagnosis of infections is underestimated, which is a risk factor for increasing the number of healthy carriers, outbreaks and unlimited agent spreading through the food chain.

Keywords: *diarrheagenic E. coli, intestinal diseases, pediatric diarrhoea*

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INTRODUCTION

Escherichia coli (E. coli) is the predominant facultative anaerobe of human colonic flora, which has gained importance due to its association with diarrheal diseases. Diarrheagenic *Escherichia coli* (DEC) strains are the main etiologic agent of moderate to severe diarrhoea in humans that is transmitted through the consumption of contaminated foods. They are classified into six pathogenic types based on specific virulence traits- Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), which constitute the subgroup of Shiga-like toxin-producing *E. coli* (STEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), and Diffusely adherent *E. coli* (DAEC) (1,2).

ETEC are the most important cause of dehydration infant diarrhoea, mainly in developing countries. In addition, ETEC are a frequent cause of travelers' diarrhoea in developed countries (3).

EPEC are an important category of DEC, which adhere to the mucosal cells of the small intestine producing pedestal-like structures (4). This results in watery diarrhoea, which is usually self-limited but can become chronic.

EAEC are an important cause of diarrhoea worldwide and are frequently observed as the aetiological agent of persistent paediatric diarrhoea in sporadic and outbreak situations (5).

EHEC cause intestinal and renal diseases. These strains express one or two Shiga toxins Stx1 and Stx2. Shiga toxin-producing *E. coli* (STEC) are also known as Vero toxin-producing *E. coli* (VTEC). The disease starts with severe crampy abdominal pain, watery diarrhoea followed by grossly bloody diarrhoea, and little or no fever. It may present in the form of sporadic cases or outbreaks. The illness is often designated as hemorrhagic colitis (1, 6).

EAEC are defined by their bricklike aggregative adherence patterns to cultured HEp-2 cells. At first, EAEC colonize the intestinal mucosa. Then, EAEC produce a mucus-mediated biofilm on the enterocyte surface. Finally, the toxins are released by EAEC, causing the inflammatory response, intestinal secretion, and mucosal toxicity (1, 7).

DAEC strains are defined based on the presence of a diffuse adherence pattern on HeLa and HEp-2 epithelial cells, where bacteria uniformly cover the cell monolayer. These strains are age-dependently involved in children's diarrhea, and can also be asymptomatic intestinal microbiota strains both in children and adults. The pathogenicity and clinical pertinence of DAEC in urinary tract infections and pregnancy complications are well established (8, 9).

The present retrospective epidemiological study aimed to evaluate the contribution of the different DEC categories to infectious diarrhoea in Bulgaria for a ten year period 2011 – 2020, to update the epidemiological data for the country and to justify the implementation of future measures for prevention and control, and for rapid and accurate diagnosis of infections caused by diarrhoeagenic *E. coli.*

MATERIALS AND METHODS

The etiological role and distribution of DEC among Bulgarian population for the last decade were determined by a retrospective analysis of microbiological and epidemiological data. Official statistics data from the National Center for Public Health and Analysis (NCPHA), data from the annual analyses of communicable diseases made by the Regional Health Inspectorates (RHI), and data from own epidemiological studies at the National Reference Laboratory, NCIPD were used.

According to Ordinance № 21/2005 on the Procedure for Registration, Communication and Reporting of Infectious Diseases of Bulgarian Ministry of Health , all DEC isolates must be sent together with clinical and epidemiological data to the NRL for Enteric Infections, Pathogenic Cocci and Diphteria at NCIPD for identification and confirmation of the bacterial agents. All data on *E. coli* isolates throughout the country are stored for a minimum of 10 years. Data on all DEC isolates reported to NRL during the studied period, were aggregated.

RESULTS

For the studied ten-year period 2011-2020, laboratory-confirmed intestinal infections by DEC, accounted for ***3 633*** *(3 633/ 530 870),* ***7,26%*** of the reported cases of acute infectious diseases (AID), influenza and acute respiratory diseases, tuberculosis, AIDS, sexually transmitted infections, and COVID-19 excluded. The greatest number of *E. coli* infections was reported in 2011 with ***514*** cases, and the lowest in 2017 with only ***240*** cases (Table 1). The seasonal distribution of DEC infections is characterized with an increased frequency during the summer months of May - September. The territorial analysis reveals the highest morbidity in the North-Eastern administrative-territorial units of Bulgaria: Varna *(48, 93%000)*, Silistra *(24, 47%000)*, Dobrich *(23, 37%000)*, Shumen *(19, 33%000)* and in the South-Eastern administrative-territorial units: Yambol *(33, 63%000)*, Sliven *(21, 35%000)* and Burgas *(8, 49%000)*. The summarized data determine the role of ETEC O6 as a leading etiological cause of epidemic and sporadic enteritis in the country, followed by ETEC O78; EPEC O126; O127, O128; O 44 (Fig.1). Except for one case in 2011, all isolates belonging to the EHEC O157 were negative for H:7 phase according to the data from primary bacteriological tests performed in the microbiological laboratories throughout the country. The characteristic complications of HUS have not been registered. In 2011, a case of enterohemorrhagic infection caused by ***E. coli*** **O157 H:7** was reported (morbidity *0.01% 000*). It refers to a 55-year-old woman from Yambol, without data of contact with a sick person or animal, or association with consumed food. No epidemiological association with the outbreak of O157 H:7 in Germany during the same year was established, either.

During the studied ten-year period, several neonatal meningitides were reported from different areas in Bulgaria. Five cases caused by *E. coli* O6 were reported in 2013 and two more caused by *E. coli* O18, in 2012. The last cases caused by *E. coli* O25 were reported in June 2020. These were three 0-20 days old infants from the same neonatal ward in the University hospital in North Bulgaria. A nosocomial infection could be suspected based on the BOX and ERIC1/2 PCR profile results obtained in the NRL. However, an epidemiological investigation report was missing, due to Covid-19 pandemics.

The affected by DEC infections were mostly infants and young children with morbidity by age groups, as follows: 0-11 months- ***141, 86%000***; 1-4 years- ***57, 59%000***; 5-9 years- ***8, 50%000*** (Fig. 2 and 3.) Out of all ***3 633*** DEC cases, ***1 805*** *(49, 68%)* were reported in females, and ***1 828*** *(50, 32%)* in males.

In the studied period 2011-2020, a total of seven outbreaks were registered. For all of them the infection source was contaminated food. The etiological agents of the outbreaks were *E. coli* O6 (more than once), O18, O168, O59 and O44 (Figure 5).

In the studied decade, **2** deaths were registered (***0, 26%*** of all AID), which determines a total lethality for the DEC intestinal infections of ***0, 46%*** **(**Table 2**).** One of the lethal cases was a 66 years old man diagnosed

with hemorrhagic *E. coli* enteritis caused by serotypes O27 and O139 in 2011, and the other - a one-year-old child from Plovdiv district, hospitalized in infectious ward in 2014. The child suffered from hydrocephalus and spina bifida. The premorbid condition has most probably contributed for the exitus Until now, there has been no reported death case of HUS caused by diarrheagenic *E. coli* in Bulgaria.

Table 1. *DEC infections morbidity in relation to reported cases of acute infectious diseases for the period 2011-2020 in Bulgaria.*

YEAR	NUMBER OF ACUTE INFECTIOUS DISEASES (without Influenza and Acute Respiratory Diseases, Tuberculosis, AIDS and Sexually Transmitted Infections, and COVID-19)	NUMBER OF DEC	DEC MORBIDITY (per 100,000)	% relative share of all ACUTE INFECTIOUS DISEASES
2011	58 259	514	6,98%000	0,88
2012	60 998	446	6,09%000	0,73
2013	67 916	333	4,57%000	0,49
2014	50 800	368	5,08%000	0,72
2015	54 471	382	5,30%000	0,7
2016	61 283	360	5,03%000	0,59
2017	52 393	240	3,38%000	0,46
2018	48 092	307	4,35%000	0,64
2019	54 397	385	5,50%000	0,71
2020	22 261	298	4,29%000	1,34
SUM	530 870	3 633	5,1%000	7,26

Table 2. *DEC infections mortality in relation to reported cases of acute infectious diseases for the period 2011-2020 in Bulgaria.*

YEAR	NUMBER of death cases of acute infectious diseases without Influenza and Acute Respiratory Diseases, Tuberculosis, AIDS and Sexually Transmitted Infections, and COVID-19	AID mortality (per 100,000)	AID total lethality (%)	NUMBER of death cases of DEC	DEC mortality (per 100,00)	DEC total letality (%)
2011	64	0,87% 000	0,11	1	0,01	0,19
2012	84	1,15% 000	0,14	0	0,00	0,00
2013	79	1,08% 000	0,12	0	0,00	0,00
2014	77	1,06% 000	0,15	1	0,01	0,27
2015	71	0,99% 000	0,13	0	0,00	0,00
2016	82	1,15% 000	0,13	0	0,00	0,00
2017	93	1,31% 000	0,18	0	0,00	0,00
2018	90	1,28% 000	0,19	0	0,00	0,00
2019	87	1,24% 000	0,16	0	0,00	0,00
2020	52	0,75% 000	0,23	0	0,00	0,00
SUM	779	1,19%000	1,54	2	0,002	0,46

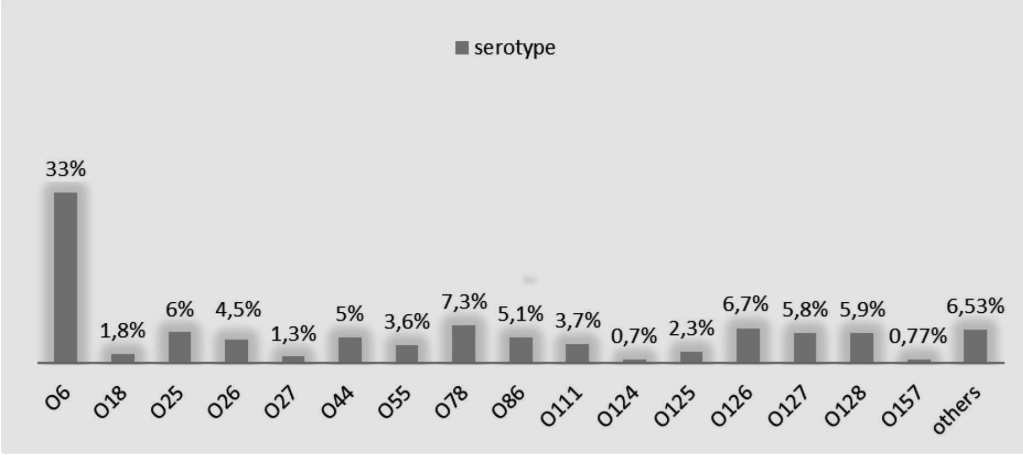

Fig. 1. Distribution of diarogenic E. coli according to the O-group for the period 2011-2020 in Bulgaria.

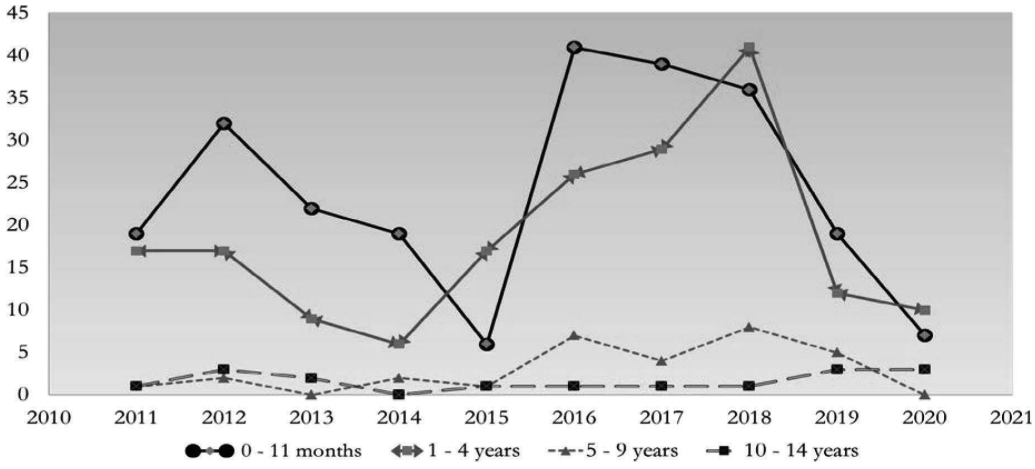

Fig 2. Distribution of DEC infections among children (0–14 years) according to age structure for the period 2011-2020 in Bulgaria.

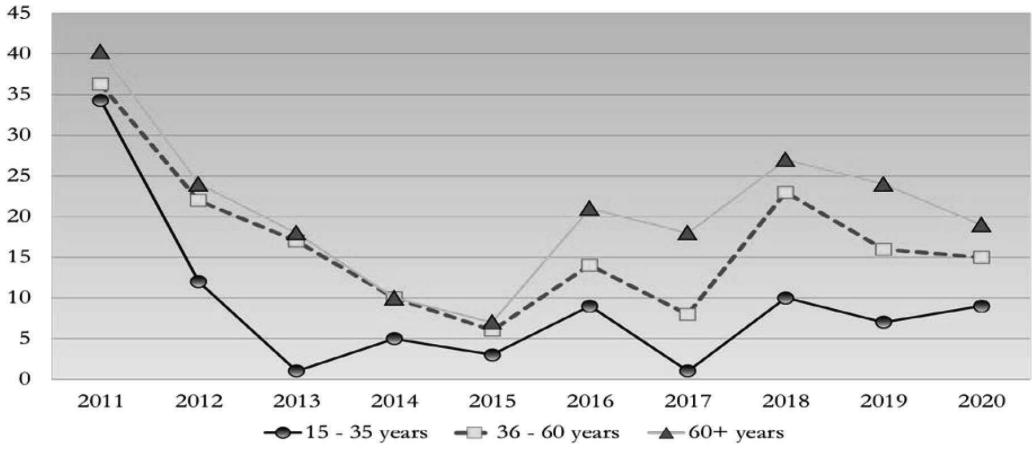

Figure 3. Distribution of DEC infections according to age structure 15–60 + years for the period 2011-2020 in Bulgaria

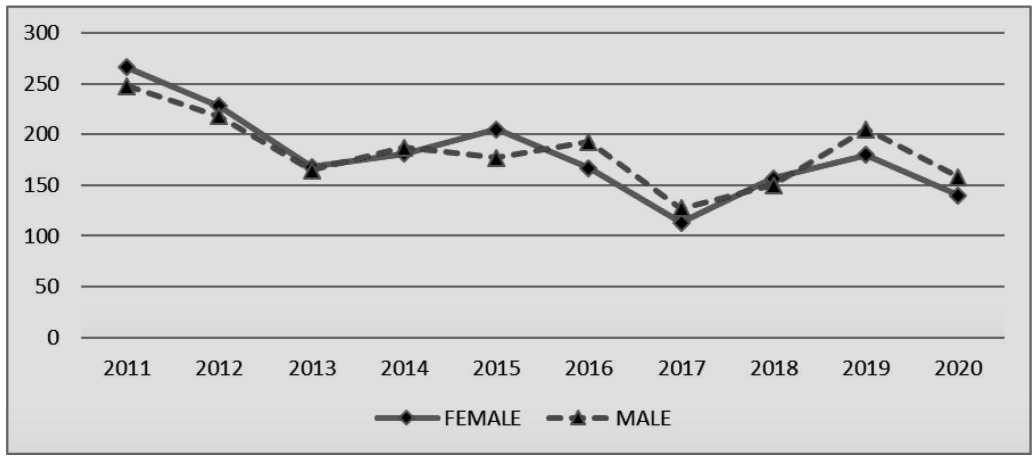

Fig. 4. Distribution of DEC cases (3 633) according to sex for the period 2011-2020 in Bulgaria.

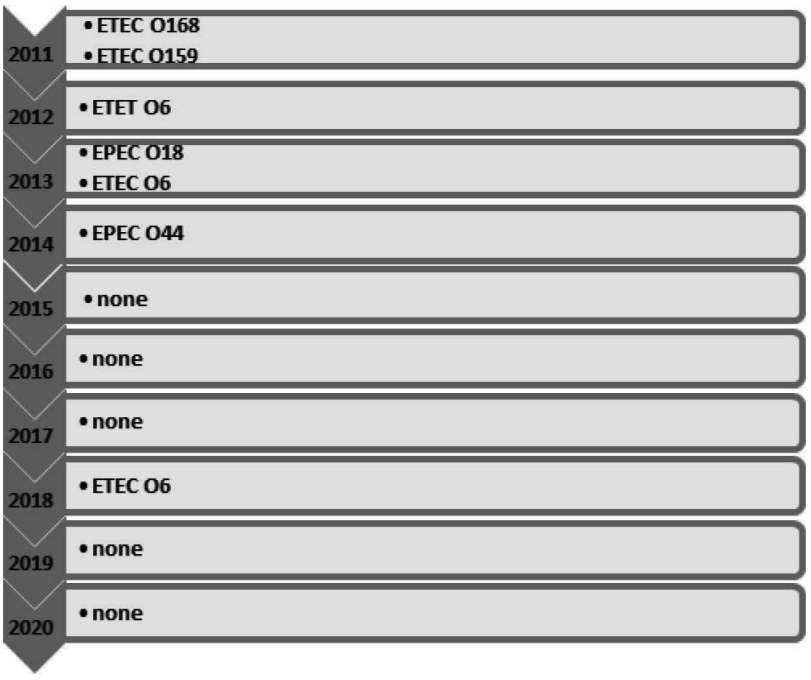

Figure 5. Annual distribution of reported DEC outbreaks for the period 2011-2020 in Bulgaria.

DISCUSSION

Escherichia coli are bacteria found in the environment, foods, and intestines of people and animals. DEC are the most common cause of a number of foodborne outbreaks, travellers' diarrhoea, chronic diarrhoea in HIV-infected patients and the main reason, neonatal meningitis and, last but not least, pediatric diarrhoea (10, 11). ETEC contribute substantially to overall morbidity, may contribute to delayed growth of infected children, and above all account for hundreds of thousands of children' deaths each year. ETEC is the predominant pathogen in DEC. Because many travellers arrive from regions with poor sanitation had been at high risk for acquisition of ubiquitously distributed ETEC infection (12 -15). The prevalence of ETEC in Bulgaria is not different from that in Europe, Asia or America. ETEC (*E.coli* O6, followed by O78) were the leading cause of pediatric diarrhoea and DEC outbreaks during the period 2011-2020. The second most frequently isolated were EPEC (*E. coli* O126, O127, O128), typical for adult diarrhoea (25-45 years) and asymptomatic carriers. EHEC and EIEC infections have a small share among Bulgarian population. Laboratory-proven cases of O157 are H: (-). Fortunately, the clinical manifestations were not related to the characteristic complications of HUS with the exception of one case of enterohemorrhagic infection in 55-years old woman without epidemiological data. At the same time, dozens of O157 H:7 cases, an epidemic that took lives, have been registered in Germany, but we do not have data that could relate the Bulgarian case with the German epidemic (16).

Neonates are at high risk of meningitis, which might lead to neurologic complications. *Escherichia coli* is the second most frequent cause of neonatal meningitis and a major cause of neonatal mortality (rates vary between 10% and 15%). Among *E. coli* strains causing extraintestinal infections in adults and children worldwide, the serotypes O1, O2, O4, O6, O7, O8, O16, O18 are predominant (17, 18, 19). Similarly, in recent years, serotypes O6 and O18 have been the most frequent cause of neonatal meningitis in Bulgaria . However, in 2020, four cases of meningitis caused by E. coli O25 were registered in one neonatal ward for a month. A molecular study using BOX and ERIC1/2 PCR was performed to prove a possibly nosocomial infection though the epidemic situation caused by Covid-19 hindered the study.

The seasonal distribution of STEC infection (May-September), as well as the most frequently affected age groups, do not stand out from the general data for Europe and the world (10, 20). The most affected age groups are infants followed by young children. Both sexes were almost equally affected at any age. The underdeveloped immune system in children and poor mastered hygiene habits are major epidemic factors in children's groups - kindergartens, training centers and others. Outbreaks reported among children's groups accounted for 90% of all cases.

Another important factor is the asymptomatic carrier state in adults involved in food preparation.

In general, the share of DEC infections in the country (*7, 26%* of the total number of AID without Influenza and Acute Respiratory Diseases, Tuberculosis, AIDS and Sexually Transmitted Infections, and COVID-19) are few as compared with reported cases from other European countries (20). Most likely this is due to reduced diagnostics in Bulgarian microbiology laboratories. Hospital microbiology laboratories perform serotyping of diarrheagenic *E. coli* mainly for pediatric and immunocompromised patients. This practice of neglecting other patients' groups leads to inaccurate data on diarrhea infections in the country, as well as increased risk of spreading the pathogens especially among asymptomatic carriers, who are a reservoir for future infections and/or epidemic outbreaks.

CONCLUSION

The diarrheal disease continues to be a health problem worldwide with a wide range of etiological agents. Among the bacterial pathogens, E. coli plays an important role (5). During the period 2011-2020, the registered cases of E. coli enteritis in Bulgaria decreased, alongside with increased number of etiologically undeciphered enterocolitis cases. Moreover, there was a significant decrease in the number of registered cases in 2020 as compared to previous years (2019 - 54,397 years, morbidity 777.10% 000; 2018 - 48,092SL., morbidity 682.15% 000; 2017 - 52 393 ff., morbidity 737.74% 000), as a result of the complex impact of factors associated with COVID-19 epidemic.

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Conflicts of interest: The corresponding author states that there is no conflict of interest.

REFERENCES.

1. James P. Nataro and James B. Kaper. Diarrheagenic Escherichia coli. Clin. Microbiol. Rev. 1998. Vol.11, №1, p 142-201.
2. Fallah N., Ghaemi M., Ghazvini K., Rad M. & Jamshidi A., Occurrence, pathotypes, and antimicrobial resistance profiles of diarrheagenic Escherichia coli strains in animal source food products from public markets in Mashhad, Iran, Food Control (2020), doi.org/10.1016/j.foodcont.2020.107640.
3. Pushker Raj. Pathogenesis and Laboratory Diagnosis of Escherichia coli-associated Enteritis. CMNEE. 1993. Vol15(12), p 89-96.
4. Samer A Al-Hilali, Ali M Almohana. Occurrence and molecular characterization of enteropathogenic Escherichia coli serotypes isolated from children with diarrhoea in Najaf, Iraq. Indian Journal of Medical Microbiology, (2011) 29(4): 383-8. DOI:10.4103/0255-0857.90171
5. Magdalena T. Nuesch-Inderbinen, Eveline Hofer, Herbert Hachler, Lothar Beutin and Roger Stephan. Characteristics of enteroaggregative *Escherichia coli* isolated from healthy carriers and patients with diarrhoea. Journal of Medical Microbiology (2013), 62, 1828–1834 DOI 10.1099/jmm.0.065177-0.
6. Julio A. Guerra, Chengxian Zhang, Jonathan E. Bard, Donald Yergeau, Natasha Halasa and Oscar G. Gómez-Duarte. Comparative genomic analysis of a Shiga toxin-producing Escherichia coli (STEC) O145:H25 associated with a severe pediatric case of hemolytic uremic syndrome in Davidson County, Tennessee, US. Guerra et al. BMC Genomics (2020) 21:564
7. Shih-Chun Yang, Chih-Hung Lin, Ibrahim A. Aljuffali, Jia-You Fang. Current pathogenic Escherichia coli foodborne outbreak cases and therapy development. Arch Microbiol (2017) 199:811–825. DOI 10.1007/s00203-017-1393-y.
8. Servin AL. Pathogenesis of human diffusely adhering Escherichia coli expressing Afa/Dr adhesins (Afa/Dr DAEC): current insights and future challenges. Clin Microbiol Rev. 2014 Oct;27(4):823-69. DOI: 10.1128/CMR.00036-14. PMID: 25278576; PMCID: PMC4187631.
9. Javadi K, Mohebi S, Motamedifar M, Hadi N. Characterization and antibiotic resistance pattern of diffusely adherent Escherichia coli (DAEC), isolated from paediatric diarrhoea in Shiraz, southern Iran. New Microbes New Infect. 2020 Oct 10;38:100780. DOI: 10.1016/j.nmni.2020.100780. PMID: 33163200; PMCID: PMC7607503.
10. Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), Division of Foodborne, Waterborne, and Environmental Diseases (DFWED). Page last reviewed: May 3, 2019.
11. David M. Gordon. The ecology of Escherichia coli. Chapter 1 in Escherichia coli Pathotypes and Principles of Pathogenesis. Second Edition. 2013. Elsevier Inc. ISBN: 978-0-12-397048-0
12. Black, R.E., 1990. Epidemiology of travelers' diarrhea and relative importance of various pathogens. Rev. Infect. Dis. 12 (Suppl. 1), S73–79.
13. Robert Steffen, MD, Epidemiology of travellers' diarrhea, Journal of Travel Medicine, Volume 24, Issue suppl_1, April 2017, Pages S2–S5,
14. Subekti, D.S., Lesmana, M., Tjaniadi, P., et al., 2003. Prevalence of enterotoxigenic Escherichia coli (ETEC) in hospitalized acute diarrhea patients in Denpasar, Bali, Indonesia. Diagn. Microbiol. Infect. Dis. 47 (2), 399–405.
15. von Mentzer, A., Connor, T., Wieler, L. et al. Identification of enterotoxigenic Escherichia coli (ETEC) clades with long-term global distribution. Nat Genet 46, 1321–1326 (2014).
16. Outbreak of Shiga toxin-producing E. coli (STEC) in Germany 27 May 2011. European Centre for Disease Prevention and Control, 2011. https://www.ecdc.europa.eu/sites/default/files/media/en/publications/Publications/1105_Risk_Assessment_Ecoli.pdf. Last accessed Feb. 21.2022.
17. Xu A, Johnson JR, Sheen S, SommersC. 2018. Draft genome sequences of fiveneonatal meningitis-causing Escherichia coliisolates (SP-4, SP-5, SP-13, SP-46, and SP-65).Genome Announc 6:e00091-18.
18. Ste´phane Bonacorsi, Edouard Bingen. Molecular epidemiology of Escherichia coli causing neonatal meningitis. IJMM. 2005. Vol. 295, 373–381. doi:10.1016/j.ijmm.2005.07.011.
19. Yanli Liu Minli Zhu Xiaoqin Fu Jiaojiao Cai Shangqin Chen Yuanyuan Lin Na Jiang Si Chen Zhenlang Lin. Escherichia coli Causing Neonatal Meningitis During 2001–2020: A Study in Eastern China. International Journal of General Medicine 2021:14 3007–3016.
20. ECDC- https://www.ecdc.europa.eu/en/escherichia-coli-ecoli/surveillance/atlas. Last accessed Feb. 21.2022.

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