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EVALUATION OF THE APPLICABILITY OF THE O.K.N.V.I. RESIST-5 AND THE KPC&MBL&0XA-48 DISK TESTS IN A ROUTINE MICROBIOLOGY LABORATORY

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ABSTRACT

Background: The global spread of carbapenemase-producing *Enterobacterales* (CPE) and the increasing emergence of clinical *Enterobacterales* harboring multiple carbapenemases of different molecular classes have prioritized the use of rapid molecular detection methods in routine microbiology laboratories. The aim of this study was to evaluate the applicability of the immunochromatographic O.K.N.V.I. RESIST-5 and the KPC&MBL&0XA-48 disc tests in a clinical microbiology laboratory. **Material and methods:** The tests were performed with 50 CPE belonging to 8 species and producing 7 molecularly characterized carbapenemases. Six of these isolates carried two different carbapenemases. To assess the specificity of the assays, 18 non-carbapenemase-producing but carbapenem-resistant *Enterobacterales* (non-CP CRE) were also included. Both tests were performed from a common overnight culture on Mueller-Hinton agar with inoculum harvested around an ertapenem disk. **Results:** The O.K.N.V.I. RESIST-5 correctly detected all 56 carbapenemases, including KPC-2 in *Klebsiella pneumoniae*; OXA-48 in *Serratia marcescens*, *Citrobacter freundii*, *Enterobacter hormaechei* and *K. pneumoniae*; NDM-1 in *Escherichia coli*, *Morganella morganii*, *E. hormaechei*, *C. freundii*, *S. marcescens* and *K. pneumoniae*; VIM-1 in *Proteus mirabilis*; VIM-4 in *C. freundii* and *S. marcescens*; VIM-86 with and without NDM-1 in *Providencia stuartii*, and NDM-5 with and without OXA-232 in *K. pneumoniae*. The KPC&MBL&0XA-48 disc tests correctly confirmed KPC, OX-48-like and most MBL except VIM in *P. mirabilis*. Furthermore, the combination disc tests failed to detect OXA-48-like in pairs with MBL in *K. pneumoniae*. **Conclusions:** The O.K.N.V.I. RESIST-5 multiplex lateral assay is an excellent test for rapid diagnostic of CPE in routine microbiology laboratories. It is easy to handle and provides results with 100% sensitivity and specificity when an inoculum around ertapenem disc from routine antibiogram was used.

Keywords: *Enterobacterales*, carbapenemase detection, O.K.N.V.I. RESIST-5, KPC&MBL&0XA-48 disc tests

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INTRODUCTION

The dissemination of carbapenemase-producing *Enterobacterales* (CPE) and the increasing emergence of clinical *Enterobacterales* possessing multiple carbapenemases of different molecular classes have prioritized the use of rapid molecular detection methods in the routine microbiology laboratory (1). Another argument is that some of the few available therapeutic options (e.g. ceftazidime-avibactam) are active against certain classes of carbapenemases but inactive against others (2). Furthermore, as has been reported, prompt initiation of adequate therapy appears to be a determining factor in the management of CPE infections (3).

Several methods are currently used in clinical microbiology laboratories to identify and characterize carbapenemase types, such as culture-based phenotyping methods and molecular methods using gene amplification. However, phenotypic assays are time-consuming, whereas the molecular method needs expensive equipment and high expertise.

Recently, a multiplex lateral flow immunochromatographic assay, O.K.N.V.I. RESIST-5 (CORIS BioConcept, Gemblux, Belgium), was

developed to detect the five major carbapenemases (OXA-48-like, KPC, NDM, VIM, and IMP) identified in *Enterobacterales* worldwide (1). The newly introduced immunochromatographic tests detect carbapenemase-specific epitopes using monoclonal antibodies and are a rapid alternative, taking only 15 minutes without additional equipment. Previous studies of various lateral flow assays detecting different spectra of carbapenemases have shown high sensitivity and specificity for OXA-48-like, KPC, and NDM carbapenemases (4,5). Furthermore, the influence of different culture media and antibiotic discs on test sensitivity was analyzed. It was found that the best results were obtained with inoculum harvested around an ertapenem or meropenem disk or from zinc containing agars (4,5).

The aim of this study was to evaluate the applicability of the O.K.N.V.I. RESIST-5 immunochromatographic assay and the KPC&MBL&0XA-48 disc tests in a routine microbiology laboratory.

MATERIAL AND METHODS

This study collection included 50 clinical isolates of CPE with molecularly characterized carbapenemases by using whole genome sequencing or PCR and sequencing and previously reported in our studies (6–9). Of these, 36 isolates belonged to the *Enterobacter* spp. *Serratia marcescens*, *Citrobacter freundii*, *Providencia* spp., and *Morganella morganii* (ESCPM) group. They consisted of 11 VIM-4 producers (10 *S. marcescens* and 1 *C. freundii*), 10 NDM-1 producers (4 *C. freundii*, 3 *S. marcescens*, 2 *M. morganii* and 1 *Enterobacter hormaechei*), 7 OXA-48 producers (5 *E. hormaechei*, 1 *C. freundii* and 1 *S. marcescens*), 6 VIM-86-producing *Providencia stuartii*, and 2 *P. stuartii* producing VIM-86 and NDM-1 (6). Of the remaining 14 study isolates, 6 *Klebsiella pneumoniae* produced NDM-5 and OXA-232, 3 *Escherichia coli* produced NDM-1, 2 *Proteus mirabilis* produced VIM-1, 1 *K. pneumoniae* was NDM-1 producer, 1 *K. pneumoniae* was OXA-48 producer, and 1 was *K. pneumoniae* KPC-2 producer (7–9). Eighteen non-carbapenemase-producing but carbapenem-resistant *Enterobacterales* (non-CP CRE) were also included to assess the specificity of the assays. This group consisted of 8 *E. hormaechei*, 4 *K. pneumoniae*, 3 *E. coli*, 2 *S. marcescens* and 1 *Klebsiella aerogenes* and was characterized in our previous study (7).

To evaluate the applicability of O.K.N.V.I. RESIST-5 and the KPC&MBL&0XA-48 disc kit for detection and confirmation of carbapenemases in a routine microbiology laboratory, both tests were performed from a common overnight culture on Mueller-Hinton agar (MHA) for each isolate. KPC&MBL&OXA-48 disc tests (Liofilchem, Roseto degli Abruzzi, Italy) were performed as described in our previous study (10). EDTA, cloxacillin, or phenylboronic acid were used as β-lactamase inhibitors with meropenem in combination disc tests for phenotypic differentiation of metallo-β-lactamases, KPC, or AmpC. Results were interpreted according to the manufacturer's recommendations. Immunochromatographic O.K.N.V.I. RESIST-5 assay was performed according to the manufacturer's instructions. Briefly, colonies were harvested around an ertapenem disc from the MHA plate, resuspended in LY-A buffer, and then transferred to the lateral flow test. One assay contained two lateral flow cassettes, one to identify NDM, KPC and OXA-48-like, and the other for IMP and VIM. A positive result is indicated by a red band next to the letters of each carbapenemase. In addition, the minimum inhibitory concentrations (MICs) of meropenem, ertapenem and imipenem were determined by broth microdilution using the MicroScan NM-EN52 panel (Beckman Coulter, Inc., Brea, CA, USA) and the Micronaut-S MDR plate (Merlin Diagnostika GmbH, Bornheim, Germany) by following the manufacturer's protocols. Susceptibility testing results were interpreted in accordance with EUCAST clinical breakpoints v14.0 (11). *E. coli* ATCC 25922 was used for quality control.

RESULTS

All 56 carbapenemases present in the 50 CPE isolates were correctly identified with the O.K.N.V.I. RESIST-5 assay (Table 1, Figures 1 to 5). Of the 50 carbapenemase producers, six isolates expressed two different carbapenemases and all were correctly detected (Table 1, Figures 4b and 5b). All non-CP CRE were negative in the tests. Overall, the sensitivity and specificity of O.K.N.V.I. RESIST-5 for detecting carbapenemases were both 100%. Regarding the KPC&MBL&OXA-48 disc tests, the results of this study confirm our previous findings for VIM MBLs

Table 1. Results from the O.K.N.V.I. RESIST-5, KPC&MBL&OXA-48 disk tests and carbapenem susceptibility testing for 50 CPE with molecularly characterised carbapenemases

Isolate (reference no.)	Year of isolation	Species	Car-bapene-mase(s)	MIC values (mg/L)[a] for:			KPC&MBL&OXA-48 disc tests[b]						O.K.N.V.I. RESIST-5 results
				MEM	ETP	IPM	TMO	MRP	MR+CL	MR+BO	MR+ED	Interpreta-tion	
KP746-PR3337 (9)	2017	*K. pneumoniae*	KPC-2	>128	>1	>8	8	6	6	16	6	KPC	KPC
KP1335-PR2899 (8)	2014	*K. pneumoniae*	OXA-48	0.5	>1	1	6	22	22	23	22	OXA-48-like	OXA-48-like
CF1843 (6)	2014	*C. freundii*	OXA-48	2	>1	2	6	22	22	22	22	OXA-48-like	OXA-48-like
SM585 (6)	2016	*S. marcescens*	OXA-48	4	>1	8	6	21	21	23	21	OXA-48-like	OXA-48-like
EH1401 (6)	2018	*E. hormaechei*	OXA-48	4	>1	2	6	21	21	21	21	OXA-48-like	OXA-48-like
EH3113 (6)	2018	*E. hormaechei*	OXA-48	4	>1	4	6	20	20	20	20	OXA-48-like	OXA-48-like
EH3371 (6)	2018	*E. hormaechei*	OXA-48	2	>1	2	6	22	22	22	22	OXA-48-like	OXA-48-like
EH273 (6)	2020	*E. hormaechei*	OXA-48	2	>1	2	6	23	23	23	23	OXA-48-like	OXA-48-like
EH1872 (6)	2020	*E. hormaechei*	OXA-48	2	>1	2	6	23	23	23	23	OXA-48-like	OXA-48-like
EC3517 (7)	2012	*E. coli*	NDM-1	32	>1	8	6	7	7	9	23	MBL	NDM
MM4395 (6)	2018	*M. morganii*	NDM-1	2	>1	13	20	22	22	24	27	MBL	NDM
SM4015 (6)	2018	*S. marcescens*	NDM-1	32	>1	>8	11	15	15	16	23	MBL	NDM
SM4949 (6)	2018	*S. marcescens*	NDM-1	128	>1	>8	6	6	6	9	23	MBL	NDM
SM4487 (6)	2019	*S. marcescens*	NDM-1	64	>1	>8	6	6	6	8	22	MBL	NDM
EH10088 (6)	2020	*E. hormaechei*	NDM-1	32	>1	8	10	14	15	17	24	MBL	NDM
CF4015 (6)	2018	*C. freundii*	NDM-1	16	>1	8	9	15	16	17	23	MBL	NDM
EC52491 (7)	2018	*E. coli*	NDM-1	16	>1	8	14	16	16	18	27	MBL	NDM
EC52492 (7)	2018	*E. coli*	NDM-1	8	>1	4	14	17	17	19	27	MBL	NDM
CF1976 (6)	2021	*C. freundii*	NDM-1	32	>1	>8	11	14	15	16	21	MBL	NDM
CF2068 (6)	2021	*C. freundii*	NDM-1	16	>1	8	12	15	15	17	23	MBL	NDM
CF2341 (6)	2021	*C. freundii*	NDM-1	16	>1	8	12	15	15	17	22	MBL	NDM
MM231 (6)	2023	*M. morganii*	NDM-1	2	>1	8	20	22	22	24	27	MBL	NDM
KP740 (7)	2023	*K. pneumoniae*	NDM-1	64	>1	8	8	12	12	14	24	MBL	NDM
KP166 (7)	2023	*K. pneumoniae*	NDM-5	64	>1	>8	10	10	10	12	22	MBL	NDM
KP3112 (7)	2023	*K. pneumoniae*	NDM-5	64	>1	>8	8	6	6	6	20	MBL	NDM

[a] MIC, Minimum inhibitory concentration; MEM, meropenem; ETP, ertapenem; IMP, imipenem

[b] mm sone diameters KPC&MBL&OXA-48 disc kit; TMO, temocillin; MRP, meropenem; MR+CL: meropenem+cloxacillin; MR+BO, meropenem+phenylboronic acid; MR+ED, meropenem+EDTA

Isolate (reference no.)	Year of isolation	Species	Car-bapene-mase(s)	MIC values (mg/L)[a] for:			KPC&MBL&OXA-48 disc tests[b]						O.K.N.V.I. RESIST-5 results
				MEM	ETP	IPM	TMO	MRP	MR+CL	MR+BO	MR+ED	Interpreta-tion	
KP3648 (7)	2022	*K. pneumoniae*	NDM-5 + OXA-232	128	>1	>8	6	8	8	11	14	MBL	NDM + OXA-48-like
KP146 (7)	2023	*K. pneumoniae*	NDM-5 + OXA-232	64	>1	8	6	6	6	9	13	MBL	NDM + OXA-48-like
KP448 (7)	2023	*K. pneumoniae*	NDM-5 + OXA-232	128	>1	>8	6	6	6	7	12	MBL	NDM + OXA-48-like
KP3161 (7)	2023	*K. pneumoniae*	NDM-5 + OXA-232	128	>1	>8	6	6	6	8	12	MBL	NDM + OXA-48-like
PM1421 (7)	2007	*P. mirabilis*	VIM-1	1	1	8	20	26	26	28	29	Negative	VIM
PM1502 (7)	2021	*P. mirabilis*	VIM-1	1	1	8	19	25	25	27	28	Negative	VIM
CF2748 (6)	2014	*C. freundii*	VIM-4	2	>1	4	6	22	22	24	27	MBL	VIM
SM502 (6)	2014	*S. marcescens*	VIM-4	2	>1	8	6	22	22	24	28	MBL	VIM
SM1281 (6)	2015	*S. marcescens*	VIM-4	16	>1	>8	8	18	18	20	26	MBL	VIM
SM666 (6)	2017	*S. marcescens*	VIM-4	32	>1	>8	6	15	15	18	26	MBL	VIM
SM681 (6)	2018	*S. marcescens*	VIM-4	64	>1	>8	6	9	9	12	23	MBL	VIM
SM791 (6)	2018	*S. marcescens*	VIM-4	16	>1	>8	6	17	17	20	27	MBL	VIM
SM2238 (6)	2018	*S. marcescens*	VIM-4	32	>1	>8	6	6	6	8	23	MBL	VIM
SM2704 (6)	2018	*S. marcescens*	VIM-4	128	>1	>8	6	6	6	9	23	MBL	VIM
SM3131 (6)	2018	*S. marcescens*	VIM-4	64	>1	>8	6	8	8	11	24	MBL	VIM
SM1524 (6)	2020	*S. marcescens*	VIM-4	32	>1	>8	6	10	10	12	22	MBL	VIM
SM2942 (6)	2020	*S. marcescens*	VIM-4	32	>1	>8	6	10	10	12	23	MBL	VIM
PS316 (6)	2017	*P. stuartii*	VIM-86	16	>1	>8	14	18	18	20	22	MBL	VIM
PS314 (6)	2019	*P. stuartii*	VIM-86	16	>1	>8	13	18	18	20	25	MBL	VIM
PS995 (6)	2019	*P. stuartii*	VIM-86	16	>1	>8	15	18	18	20	25	MBL	VIM
PS3722 (6)	2019	*P. stuartii*	VIM-86	16	>1	>8	15	14	14	17	22	MBL	VIM
PS2654 (6)	2020	*P. stuartii*	VIM-86	16	>1	>8	11	17	17	20	24	MBL	VIM
PS3347 (6)	2020	*P. stuartii*	VIM-86	16	>1	>8	13	17	17	20	24	MBL	VIM
PS567 (6)	2019	*P. stuartii*	VIM-86 + NDM-1	32	>1	>8	10	16	16	18	24	MBL	VIM + NDM
PS1396 (6)	2019	*P. stuartii*	VIM-86 + NDM-1	64	>1	>8	10	11	11	13	24	MBL	VIM + NDM

[a] MIC, Minimum inhibitory concentration; MEM, meropenem; ETP, ertapenem; IMP, imipenem

[b] mm sone diameters KPC&MBL&OXA-48 disc kit; TMO, temocillin; MRP, meropenem; MR+CL: meropenem+cloxacillin; MR+BO, meropenem+phenylboronic acid; MR+ED, meropenem+EDTA

Figure 1. Example of a positive test for KPC carbapenemase using O.K.N.V.I. RESIST-5: left, cassette for OXA-48-like, KPC and NDM; right, cassette for VIM and IMP. C, control band; O, OXA-48-like; K, KPC; N, NDM; V, VIM; I, IMP carbapenemases. **Antibiogram:** Positive combination disc test for KPC-producing *K. pneumoniae* KP746 using KPC&MBL&OXA-48 disc kit. Clockwise: MRP, meropenem; TMO, temocillin; MR+ED, meropenem with EDTA; MR+BO, meropenem with phenylboronic acid; MR+CL, meropenem with cloxacillin.

Figure 2. Example of a positive test for OXA-48-like carbapenemase using O.K.N.V.I. RESIST-5: left, cassette for OXA-48-like, KPC and NDM; right, cassette for VIM and IMP. C, control band; O, OXA-48-like; K, KPC; N, NDM; V, VIM; I, IMP carbapenemases. **Antibiogram:** Positive combination disc test for OXA-48-like-producing *S.marcescens* SM585 using KPC&MBL&OXA-48 disc kit. Clockwise: MRP, meropenem; TMO, temocillin; MR+ED, meropenem with EDTA; MR+BO, meropenem with phenylboronic acid; MR+CL, meropenem with cloxacillin.

Figure 3. Example of a positive test for NDM carbapenemase using O.K.N.V.I. RESIST-5: left, cassette for OXA-48-like, KPC and NDM; right, cassette for VIM and IMP. C, control band; O, OXA-48-like; K, KPC; N, NDM; V, VIM; I, IMP carbapenemases. **Antibiogram:** Positive combination disc test for NDM-producing *K. pneumoniae* KP740 using KPC&MBL&OXA-48 disc kit. Clockwise: MRP, meropenem; TMO, temocillin; MR+ED, meropenem with EDTA; MR+BO, meropenem with phenylboronic acid; MR+CL, meropenem with cloxacillin.

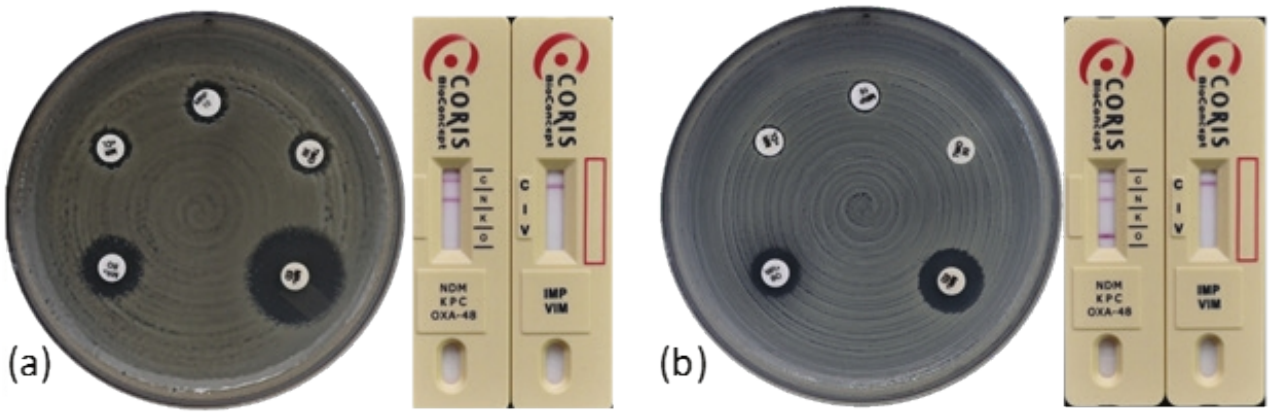

Figure 4. **(a)** Example of a positive test for NDM carbapenemase using O.K.N.V.I. RESIST-5: left, cassette for OXA-48-like, KPC and NDM; right, cassette for VIM and IMP. C, control band; O, OXA-48-like; K, KPC; N, NDM; V, VIM; I, IMP carbapenemases. **Antibiogram:** Positive combination disc test for NDM-producing *K. pneumoniae* KP166 using KPC&MBL&OXA-48 disc kit. Clockwise: MRP, meropenem; TMO, temocillin; MR+ED, meropenem with EDTA; MR+BO, meropenem with phenylboronic acid; MR+CL, meropenem with cloxacillin. **(b)** Example of a positive test for NDM and OXA-48-like carbapenemases using O.K.N.V.I. RESIST-5: left, cassette for OXA-48-like, KPC and NDM; right, cassette for VIM and IMP. C, control band; O, OXA-48-like; K, KPC; N, NDM; V, VIM; I, IMP carbapenemases. **Antibiogram:** Positive combination disc test for NDM and OXA-48-like-producing *K. pneumoniae* KP146 using KPC&MBL&OXA-48 disc kit. Clockwise: MRP, meropenem; TMO, temocillin; MR+ED, meropenem with EDTA; MR+BO, meropenem with phenylboronic acid; MR+CL, meropenem with cloxacillin.

from *P. mirabilis* strains, which were not detected. Furthermore, no co-production of OXA-48-like was detected in four isolates of *K. pneumoniae*. These isolates, which carried NDM-5 and OXA-232 carbapenemases, were interpreted as MBL producers.

The results obtained with the O.K.N.V.I. RESIST-5 and KPC&MBL&0XA-48 disc tests are illustrated in

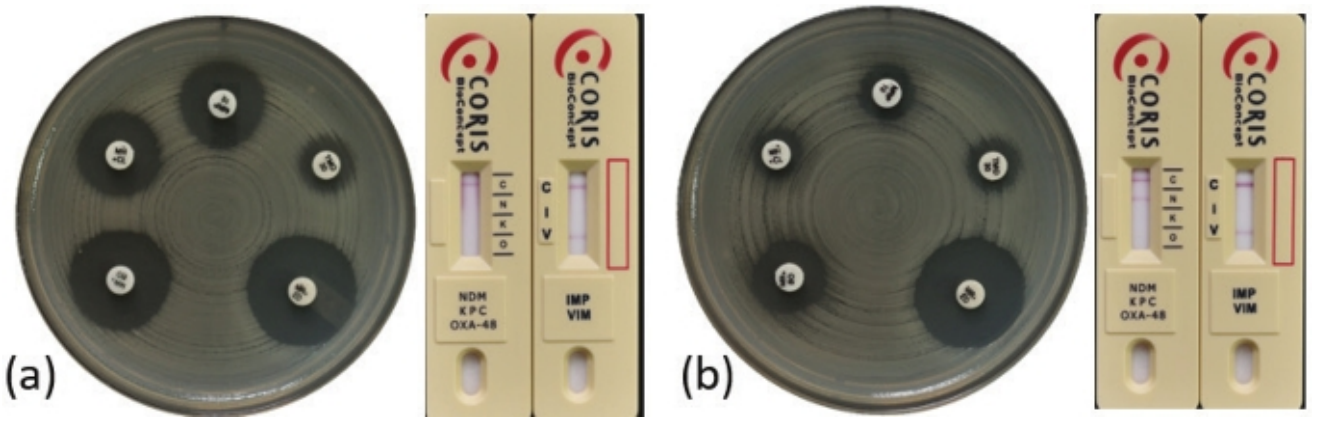

Figure 5. **(a)** Example of a positive test for VIM carbapenemase using O.K.N.V.I. RESIST-5: left, cassette for OXA-48-like, KPC and NDM; right, cassette for VIM and IMP. C, control band; O, OXA-48-like; K, KPC; N, NDM; V, VIM; I, IMP carbapenemases. **Antibiogram:** Positive combination disc test for VIM-producing *P. stuartii* PS2654 using KPC&MBL&OXA-48 disc kit. Clockwise: MRP, meropenem; TMO, temocillin; MR+ED, meropenem with EDTA; MR+BO, meropenem with phenylboronic acid; MR+CL, meropenem with cloxacillin. **(b)** Example of a positive test for NDM and VIM carbapenemases using O.K.N.V.I. RESIST-5: left, cassette for OXA-48-like, KPC and NDM; right, cassette for VIM and IMP. C, control band; O, OXA-48-like; K, KPC; N, NDM; V, VIM; I, IMP carbapenemases. **Antibiogram:** Positive combination disc test for NDM and VIM-producing *P. stuartii* PS1396 using KPC&MBL&OXA-48 disc kit. Clockwise: MRP, meropenem; TMO, temocillin; MR+ED, meropenem with EDTA; MR+BO, meropenem with phenylboronic acid; MR+CL, meropenem with cloxacillin.

comparative aspect in Figure 1 for KPC, in Figure 2 for OXA-48-like, in Figure 3 for NDM, in Figure 4 for NDM with and without OXA-48-like, and in Figure 5 for VIM with and without NDM.

DISCUSSION

The O.K.N.V.I. RESIST-5 multiplex immunochromatographic assay can detect the five most common carbapenemases. In a collection of 50 molecularly characterized clinical isolates, O.K.N.V.I. RESIST-5 performed excellently, detecting 7 carbapenemases produced by 8 species, with sensitivity and specificity of 100% for all OXA-48-like, KPC, NDM and VIM variants. Our results are consistent with data reported for previous versions of the assay when colonies were harvested around an ertapenem or meropenem disc on an MHA plate (4,5). It is notable that most false-negative results reported in the literature involved VIM or NDM producers that exhibited low carbapenem MIC levels (4,5,12). This could be related to the low expression of VIM or NDM enzymes, which can be overcome with the use of inoculum harvested around an ertapenem or meropenem disc, as antibiotic selection pressure leads to an increase in carbapenemase production. Based on these findings, Greissl et al. recommend the use of routine antibiogram to perform O.K.N.V.I. RESIST-5 if disk diffusion is the primary method of susceptibility testing, or the use of MHA or sheep blood agar with an ertapenem or meropenem disk for purity control when an automated susceptibility testing system is used. In such an approach, the results of the antibiogram and the identified carbapenemase are obtained simultaneously, which would help physicians to prescribe adequate therapy and appropriate infection control measures.

CONCLISION

Our results show that the O.K.N.V.I. RESIST-5 assay has excellent performance in detecting all carbapenemase genes present in the collection we studied. For best results, isolates should be harvested around an ertapenem or meropenem disc from a routine antibiogram. In addition, the O.K.N.V.I. RESIST-5 is a rapid, simple and efficient tool for implementation in the clinical microbiology laboratory.

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EVALUATION OF POPULATION-LEVEL IMMUNITY TO SARS-COV-2 ACROSS BULGARIA (END OF 2023)

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ABSTRACT

Background: The COVID-19 pandemic in Bulgaria was characterized by a high mortality rate and vaccination efforts yielded suboptimal results. Understanding population immunity is important as new SARS-CoV-2 variants continue to emerge. This study aimed to assess the seroprevalence of SARS-CoV-2 antibodies in the general population of Bulgaria and examine demographic variations in antibody presence.

Materials and methods: In December 2023, 1895 serum samples were randomly collected from healthy individuals across all 28 provinces. Samples were tested for SARS-CoV-2 spike protein-specific IgG antibodies using ELISA method. A subset of the positive samples was subsequently tested for neutralizing antibodies. Seroprevalence was analyzed by sex, age group, and geographic region. Statistical analyses were conducted using SPSS v.26.

Results: Overall seroprevalence was 91.9%, with similar rates between males (91.6%) and females (92.0%). Seroprevalence was highest in the 18-39 age group (95.1%) and lowest in those over 65 (89.3%). Regional seroprevalence ranged from 80.0% to 98.3%. Among samples tested for presence of neutralizing antibodies, 90.5% were positive, indicating effective immune response.

Conclusions: The high seroprevalence suggests widespread prior exposure to SARS-CoV-2 and/or vaccination among the Bulgarian population. The strong presence of neutralizing antibodies might provide potential protection against severe disease. Targeted interventions towards older age groups could be appropriate in order to sustain immunity as COVID-19 remains a public health concern.

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INTRODUCTION

The coronavirus disease 19 (COVID-19) pandemic began in early 2020 and was caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1). In Bulgaria, the government quickly implemented various public health measures in order to control the spread of the virus including lockdowns, social distancing, and mass testing (2-6). A major breakthrough in the fight against the virus was the rapid development and implementation of vaccines, with the vaccination campaign in Bulgaria beginning at the end of 2020 (7).

Despite the introduced public health measures, Bulgaria's pandemic response was hindered by a combination of public misinformation and noncompliance, healthcare system weaknesses, vaccine hesitancy, and instability (8-11). These factors resulted in Bulgaria having one of the highest COVID-19 death rates and one of the lowest vaccination rates in Europe (12-15).

However, the true extent of the virus's spread, particularly among individuals who may have had mild or no symptoms, remains unclear. Seroprevalence studies are an important epidemiological tool in the efforts to understand the spread of the virus. These studies can be helpful in evaluating the actual exposure to the virus and they are also crucial for assessing the overall immunity of the population, whether acquired through natural infection or vaccination. This is especially important in the setting of low vaccination coverage and rising portion of vulnerable population in regards to respiratory diseases in the country.

The aim of this study is to estimate the proportion of the population that has developed antibodies against

SARS-CoV-2 and to identify demographic variations in seroprevalence across Bulgaria. By testing for specific IgG antibodies against the spike protein, both naturally acquired and vaccine-induced antibodies can be detected. Further testing for SARS-CoV-2 neutralizing antibodies helps in the assessment of the functional immunity in seropositive individuals. The seroprevalence data obtained can provide insight about the current state of immunity in the general population and guide strategies to mitigate future outbreaks.

MATERIALS AND METHODS

Serum samples (n=1895) were randomly collected from healthy citizens over 18 years of age from all 28 administrative Bulgarian provinces. Samples were collected in December 2023 by polyclinic laboratories from individuals visiting for routine health screenings. Information on the sex and age of participants was recorded. The study included 778 males and 1114 females and the mean age of participants was 54.79 (SD±17.20) years. For analysis of the results, participants were divided into three age groups: 18-39 (n=407), 40-64 (n=868), and over 65 years of age (n=620).

All samples were tested for specific IgG antibodies against the Spike (S) protein of SARS-CoV-2. Positive samples from nine provinces chosen at random (Burgas, Haskovo, Kardzhali, Pernik, Silistra, Sofia-region, Targovishte, Yambol and Vidin), were additionally tested for specific SARS-CoV-2 neutralizing antibodies. Both tests were conducted utilizing ELISA techniques with commercially available kits, according to the manufacturer's instructions. Screening for IgG antibodies was performed with Anti-SARS-CoV-2 ELISA (EI 2606-9601 G), Euroimmun, Germany, and neutralizing antibodies were tested with cPass SARS-CoV-2 Neutralizing antibody detection kit (L00847), GenScript, China.

The results for specific IgG antibodies were calculated semiquantitatively as a ratio between the extinction of the tested sample and the extinction of the calibrator. The interpretation of the results was as follows: positive, if the ratio was ≥1.1, and negative, if the ratio was <1.1. A ratio ≥ 0.8 to < 1.1 was interpreted as borderline. The presence of neutralizing antibodies was evaluated qualitatively as the result of signal inhibition (in percentages, %). Calculations were performed using the following formula: % Signal inhibition = 1-(OD of sample/OD value of Negative control) x 100, OD=optical density. Following the manufacturer's instructions, a cut off value of 30% was applied, with results considered negative when signal inhibition was <30% and positive when signal inhibition was ≥ 30%.

The statistical analyses were performed with IBM SPSS Statistics v.26. The Analysis of Variants (ANOVA) method was used to compare differences between the groups. A p-value <0.05 was considered statistically significant.

Ethical approval for this study was obtained from the Institutional review board at NCIPD (approval number 5/17.10.2023).

RESULTS

Presence of specific anti-SARS-CoV-2 IgG antibodies

Table 1. SARS-CoV-2 seropositivity among the Bulgarian population by December 2023.

		Tested samples, n	Positive and borderline, n (%)	95% CI	p
Age, years					
	18-39	407	**387 (95.1%)**	92.8-97.1	-
	40-65	868	**800 (92.1%)**	90.2-93.8	ns
	over 65	620	**554 (89.3%)**	86.5-91.5	0.003
Sex					
	male	778	**713 (91.6%)**	90.1-93.9	-
	female	1117	**1028 (92.0%)**	90.4-93.6	ns
Total		**1895**	**1741 (91.9%)**	**90.7-93.2**	

CI=Confidence Interval

Table 2. COVID-19 prevalence in Bulgaria (December 2023).

Province	Tested samples, n	Positive samples, n	Seroprevalence, %	95% CI
Overall	**1895**	**1741**	**91.9%**	**90.7-93.2**
Blagoevgrad	63	61	96.8%	92.3-99.9
Burgas	96	92	95.8%	92.1-99.9
Dobrich	60	55	91.7%	85.1-98.8
Gabrovo	95	88	92.6%	87.9-98.1
Haskovo	100	91	91.0%	85.3-96.6
Kardzhali	65	61	93.8%	88.2-99.8
Kyustendil	50	42	84.0%	73.8-94.1
Lovech	60	59	98.3%	94.4-99.9
Montana	60	53	88.3%	79.8-96.2
Pazardzhik	100	93	93.0%	88.0-98.0
Pernik	70	56	80.0%	70.6-89.3
Pleven	60	58	96.7%	91.0-100
Plovdiv	70	65	92.9%	87.0-98.9
Razgrad	60	58	96.7%	91.0-100
Ruse	60	51	85.0%	76.0-94.0
Shumen	82	77	93.9%	88.7-99.1
Silistra	60	54	90.0%	82.4-97.6
Sliven	60	58	96.7%	92.3-100
Smolyan	60	59	98.3%	94.4-100
Sofia	60	55	91.7%	85.1-98.9
Sofia-region	80	69	86.3%	78.4-93.6
Stara Zagora	60	54	90.0%	82.4-97.6
Targovishte	80	76	95.0%	90.2-99.8
Varna	60	55	91.7%	85.1-98.9
Veliko Tarnovo	64	56	87.5%	78.8-95.2
Vidin	40	38	95.0%	88.2-99.9
Vratsa	60	58	96.7%	91.0-100
Yambol	60	49	81.7%	72.3-91.7

CI=Confidence Interval

was detected in 91.9% (1741/1895) of the tested population. Of those, 89.5% (1697/1741) were positive and 2.4% (44/1741) – borderline. No specific antibodies were detected in 8.1% (154/1895) of participants. Results are presented in Table 1.

The seroprevalence among male and female participants was similar, with 91.6% (713/778) of males and 92.0% (1028/1117) of females testing positive or borderline for anti-SARS-CoV-2 antibodies. A slight decline in seropositivity was observed across the three age groups: 95.1% (387/407) in the 18-39 age group; 92.1% (800/868) in the 40-64 age group and 89.3% (554/620) among participants aged 65 years and older. Statistically significant differences were found between the first and the third group (p=0.003).

The COVID-19 seroprevalence across the Bulgarian provinces ranged from 80.0% to 98.3%. The lowest seroprevalence rates (<85%) were detected in Pernik, Yambol, Kyustendil and Ruse. The highest seroprevalence rates (>95%) were found in Burgas, Pleven, Razgrad, Sliven, Vratsa, Blagoevgrad, Lovech and Smolyan. Details for each province are given in Table 2.

We further tested for presence of virus neutralizing antibodies in 571 of the positive samples from

Table 3. SARS-CoV-2 virus neutralizing antibodies in samples, positive for presence of specific anti-S IgG antibodies.

		Tested samples, n	Positive, n (%)	95% CI
Age, years				
	18-39	126	**115 (91.3%)**	87.3-96.7
	40-65	262	**241 (92.0%)**	88.7-95.3
	over 65	183	**161 (88.0%)**	83.3-92.7
Sex				
	male	231	**209 (90.4%)**	86.1-93.9
	female	340	**308 (90.5%)**	86.8-93.2
Total		**571**	**517 (90.5%)**	**87.5-92.5**

randomly selected provinces and confirmed neutralization activity in 90.5% (517/571). The demographic patterns were parallel to those for the specific anti-spike IgG antibodies: similar results were seen between males and females, while across the age groups, the same tendency for decline of the immune response with age was observed (Table 3). However, no significant differences were found between the age groups.

DISCUSSION

In this study, an overall 91.9% seroprevalence rate of SARS-CoV-2 at the end of 2023 was found across the general population of Bulgaria with high prevalence rates observed in all provinces. These results suggest that a significant proportion of the Bulgarian population has either been exposed to the virus and/or has been vaccinated. Taking into consideration the relatively low vaccination rate in the country, with only about 30-31% of the total population having completed the primary vaccination series (16), this finding is probably a reflection of a widespread natural infection. A high proportion (90.5%) of the samples tested for neutralizing antibodies against SARS-CoV-2 were positive, meaning that the antibodies present are effective in preventing cell attachment and replication of the virus. Therefore, despite the challenges faced during the vaccination campaign, a high level of antibody presence in the population has been achieved, which might provide some protection against severe outcomes in cases of reinfection (17-20).

No significant differences were found between male and female participants which echoes findings from previous studies (21-23). In general, the differences in antibody prevalence by sex observed in the literature have not been consistent (24, 25) and systematic and meta-analysis studies have established that sex is not significantly associated with presence of anti-SARS-CoV-2 antibodies (26, 27).

The relatively lower seroprevalence rate observed in the older population (>65 years) might be due to different factors, including a weaker immune system and fewer social interactions in high-risk settings. This result suggests that additional immunization efforts in this demographic group could be beneficial for maintaining effective protection against the virus. On the other hand, the relatively higher prevalence in the 18-39 and 40-65 age groups may be attributed to the fact that these individuals are more likely to be socioeconomically and socially active. While similar findings have been observed in previous studies (27, 28) other reports have found that higher seroprevalence rates were associated with older age (24,29,30). However, most of those studies were conducted in 2020 and 2021 when older individuals were more likely to have been recently exposed to the virus or vaccinated and their antibody levels had not started to wane yet. It should also be taken into consideration that older individuals were often prioritised during the early stages of COVID-19 vaccination campaigns in many countries.

Many studies have been conducted on the durability of antibody response against SARS-CoV-2, mostly concluding that antibodies remain detectable at least 3-6 months after infection (20,31,32). Some studies

have found that anti-SARS-CoV-2 IgG antibodies could remain positive over 1 year in convalescent individuals (33-35). In Bulgaria, the last significant COVID-19 peak was seen at the end of 2022 and the beginning of 2023 (36). Afterwards, with the declining number of cases, COVID-19 started fading from media and social consciousness, and thus, testing and vaccination rates significantly decreased. Despite that, new SARS-CoV-2 variants continued to emerge and circulate among the population, including the EG.5 descendant lineage of XBB.1.9.2 and BA.2.86 (37, 38). In this study, samples were collected at the end of 2023, but it is difficult to say whether the observed results could be attributed to a robust long-lasting immunity or were due to new cases passing undetected throughout the year. One of the limitations of our study is the lack of epidemiologic data regarding past infections and immunizations of the participants. Another limitation of the study is the lack of longitudinal follow-up which hinders the deeper understanding of the duration and stability of SARS-CoV-2 specific immunity.

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CASE REPORT ON TWO CONSECUTIVE PATIENTS WITH NEUROINVASIVE WEST NILE VIRUS INFECTION IN AN INTENSIVE CARE UNIT

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ABSTRACT

We present the clinical course, and treatment of two consecutive cases of neuroinvasive West Nile virus (WNV) infection in the Clinic of Intensive Care, an Intensive Care Unit (ICU) at the Military Medical Academy - Sofia. Clinical and epidemiological data, microbiological, laboratory, molecular methods, and imaging techniques were used. Both patients resided in Sofia, Bulgaria, and had not travelled in recent months. The first case was a 60-year-old man who have had mental status changes, fever, and progression of existing Parkinson's disease. Antibodies to WNV were present in cerebrospinal fluid (CSF). His condition worsened with the development of sepsis and respiratory failure and he ended up lethally. The second case was a 72-year-old man who had fever and lower dyspeptic syndrome for one week, mental status changes, with adynamia to inability to walk independently, and head, hand and tongue tremors. CSF analysis showed mild pleocytosis with proteinorachy. Antibodies to WNV were present in serum, and PCR for WNV in urine was positive. The patient was admitted to ICU due to worsened mental and neurological status, coma and development of acute respiratory failure, necessitating intubation and assisted pulmonary ventilation. The patient ended lethally 13 days later. Neuroinvasive WNV infection can cause substantial morbidity, particularly among older adults, and high mortality, in the presence of comorbidities. Physicians should include West Nile virus infection in the differential diagnosis of aseptic meningitis and encephalitis, should perform appropriate laboratory tests, and report immediately the cases to the public health authorities.

Keywords: West Nile virus, fever, neuroinvasive disease, encephalitis

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INTRODUCTION

West Nile virus (WNV) is an RNA virus classified as arbovirus. WNV belongs to *Flaviviridae* family including Dengue, Yellow fever, and Japanese encephalitis virus. WNV was named after the region where in 1937 was isolated for the first time in Uganda. Today, WNV is one of the arboviruses with the largest geographical spread and can be found in all continents [1]. The disease impacts countries in all parts of Europe, except for the Northern one [1, 2]. Several cases of West Nile encephalitis have been reported in Bulgaria in previous years [3-5].

WNV is transmitted through an enzootic cycle involving mosquitoes and birds, which act as vectors and amplifying hosts, respectively. Several bird species serve as effective reservoirs for infected mosquitoes. Among mosquitoes, Culex species play the key role for transmission [6]. The disease is most commonly transmitted to humans through bites from infected mosquito. Humans, as well as other mammals, are accidental hosts and do not cause significant additional spread of the virus as they do

not develop a sufficiently prolonged or high viremia [6, 7]. Human-to-human transmission is uncommon but can happen after hem transfusion, organ or tissue transplantation, and mother-to-child transmission during pregnancy or lactation [8].

Approximately 80% of human WNV infections are asymptomatic [9]. Some 20% of infected persons can have symptomatic viral infection and less than one percent can develop West Nile neuroinvasive disease [9]. In rear cases, WNV infection could cause nerve disorders involving demyelination such as Guillain-Barré syndrome [10].

The incubation period of WNV infection is 2 to 6 days and up to 21 days in immunocompromised hosts. West Nile fever (WNF) is characterized by a sudden presentation of symptoms, including headache, eye pain, fever, malaise, myalgia, fatigue, rash, vomiting, and diarrhea [10]. The symptoms may vary from a mild self-limiting health issue from which patients heal for a week, to a prolonged disorder that can become severely debilitating [11, 12].

West Nile neuroinvasive disease (WNND) presents with a critical clinical course, possible fatal outcome, and is commonly associated with neurological complications in survivors. Advanced age, malignancies, alcohol abuse, some comorbidities such as arterial hypertension, diabetes mellitus, hematologic diseases, renal disease, as well as genetic factors increase the risk of developing WNND [10, 13]. The mortality rate of WNND is up to 17% [14, 15].

Neuroinvasive disease develops when WNV crosses the blood-brain barrier and affects specific groups of neurons, especially in the deep nuclei, brainstem, and anterior horn of the spinal cord [16]. The clinical characteristics of WNND vary and may include encephalitis, aseptic meningitis, and polio-like syndrome [12]. More than a third of WNND patients develop meningitis, some 55-60% will have encephalitis, and nearly 5-10% will have polio-like syndrome; the presentation varies based on the region or the season. Patients could also develop overlap syndromes [16].

The symptoms of WNV encephalitis are close to other viral brain inflammations. A key finding in West Nile virus encephalitis is muscle weakness (reported for 30-50%), frequently accompanied with lower motor neuron symptoms, flaccid paralysis, and hyporeflexia without sensory abnormalities [12]. Other WNND symptoms include cranial nerves involvement (notably the seventh) and motor disorders.

The diagnosis of WNND is based on factors such as exposure to the vector, residence in an endemic area, summer season, and clinical symptoms. Laboratory methods for diagnosis of WNV infection are mostly indirect, based on serology as well as the direct detection of the virus. A cerebrospinal fluid (CSF) sample is needed in case of neurological sequelae. Indirect detection of WNV-specific IgM and/or IgG antibodies is mostly grounded on the principles of Enzyme-linked immunosorbent assay (ELISA) or indirect immunofluorescence assay (IIFA) [1]. WNV infection can be confirmed by detection of the viral genome by real-time polymerase chain reaction (RT-PCR) [17].

PCR testing of serum or cerebrospinal fluid have limitations for diagnosis of WNV in immunocompetent patients because the peak of viremia occurs three to four days before the onset of symptoms. The sensitivity of PCR for WNV is low in CSF (57%), and serum (14%) [18]. PCR sensitivity may be greater in immunocompromised patients with a weakened antibody development and prolonged viremia [16]. CSF testing results in WNND patients are nonspecific and could show pleocytosis (prevalence of lymphocytes or neutrophils) with increased protein and normal glucose levels [19].

Imaging tests, such as magnetic resonance imaging (MRI), are also nonspecific and the results from different series are inconsistent. In some series, MRI results showed no abnormalities, whereas in others, up to 70% of patients displayed abnormal findings [20-22].

At present, there is no specific treatment for WNV infection and disease management relies on supportive measures. Analysis of the literature reveals the availability of several effective antiviral drugs against the WNV pathogen [23]. Thus, experts have demonstrated the activity of ribavirin and interferon alfa-2b *in vivo* [24] and *in vitro* [25,26]. High doses of ribavirin were shown to inhibit WNV replication and cytopathogenicity in human nerve cells *in vitro* [25]. Israeli researchers have constructed a homologous immunoglobulin [27] providing effective treatment

for severe cases of WNV.

Remdesivir can effectively inhibit RNA-dependent RNA polymerases from viruses that cause infections such as Zika, West Nile fever, Yellow fever, Japanese and tick-borne encephalitis, and Dengue. Thus, Remdesivir or its derivatives have the potential of a broad-spectrum treatment effective against various RNA viruses [28, 29].

All reviewed guidelines for encephalitis treatment recommend the empiric initiation of antibiotics and acyclovir as soon as possible while awaiting diagnostic test results [30].

Case 1

A 60-year-old man was admitted to the ICU with complaints of high fever of up to 40°C lasting 3-4 days, severe weakness, low level of urine (oligouria) and shortness of breath. According to his relatives, he had been bedridden for the past few days, with restricted movements of the whole body, stiffness, confusion, and difficult speaking. He had a history of Parkinson's disease and arterial hypertension.

The initial examination found a male in severely impaired general condition, in contact, completely inadequate, and disoriented. He was febrile (39°C) and bedridden. A moderately marked tremor, perioral cyanosis, and reduced subcutaneous adipose tissue were observed. The patient had prolonged expiration, crepitations at the lungs' bases, and single dry wheezings. Oxygen saturation was 81% on ambient air. Cardiovascular system examination showed a rhythmic heart rate of 115 beats per minute. Arterial pressure was 117/84 mmHg. Neurological status showed a marked neck rigidity with rigidly increased muscle tonus in all four limbs. The patient was somnolent with absent pathological reflexes, and grossly unperturbed sensation. The lung x-ray showed pneumonia on the left; a mildly marked cerebellar atrophy was detected by MRI.

The results of patient's serum laboratory tests (Table 1) showed: leukocytosis, elevated creatinine, C-reactive protein (CRP), creatine phosphokinase (CK), ferritin, lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and Myoglobin as well as low serum potassium. The presence of protein, ketone bodies, and bilirubin were found in urine. Uroculture and throat swab showed no bacterial growth. *Staphylococcus hominis*

Table 1. Patients' laboratory results

Parameter	Reference interval	Case 1	Case 2
Leukocytes, n*10^9/L	3.5-10.5	19.2	14.45
Creatinine, μmol/L	74-130	148	217
CRP, mg/L	0-5	102.12	45.54
Serum potassium, mmol/L	3.5-5.5	2.9	3.9
CK, U/L	15-180	8675.0	1931.0
Ferritin, ng/ml	30-400	1016.0	2016.0
LDH, U/L	135-225	665.0	1055.0
Myoglobin, ng/mL	25-70	>4102.0	>4102.0
AST, U/L	5-40	233.8	210.7
ALT, U/L	5-40	75.7	66.9
Serum WNV IgM, ELISA	<1.1	4.6	(+)
Serum WNV IgM, CLIA	<1.1	10.7	(+)
Serum WNV IgG, ELISA	<1.1	(–)	(–)
CSF WNV IgM, ELISA		(+)	
CSF WNV IgG, ELISA		Borderline	
CSF WNV PCR		(–)	
Urine WNV PCR		(–)	(+)
CSF leukocytes, n*10^6/L	<5	3	12
CSF protein, g/L	0.15-0.45	0.678	0.996

was isolated from haemoculture.

Serological tests revealed WNV specific IgM antibodies: 4.6 by ELISA and 10.7 by Chemiluminescence immunoassay (CLIA) (reference value < 1.1), and negative WNV IgG. The remaining specific serology tests were negative except for a positive Lyme disease IgG.

The Arbovirus Reference Laboratory at the National Center for Infectious and Parasitic Diseases (NCIPD) detected WNV specific IgM antibodies in serum and CSF and borderline IgG by ELISA; PCR in urine and CSF were negative. Cerebrospinal fluid analysis showed $3x10^6$/L leukocytes (reference values < $5x10^6$) and slightly elevated protein: 0.678g/L (reference interval 0.15-0.45).

Six days after the admission, the patient was intubated and placed on assisted pulmonary ventilation due to a marked respiratory failure and a comatose state. Despite the resuscitative measures, he ended lethally with the presentation of acute cardiovascular and respiratory failure.

Case 2

A 72-year-old man presented with a history of fever up to 38.5°C and lower dyspeptic syndrome lasting for one week. He was treated by his general practitioner with cefuroxime as an outpatient. After COVID-19 infection in 2021, the patient had a residual tremor of the right hand, mainly on purposive movements. Arterial hypertension was reported as a concomitant disease. Since two days the tremor had increased, and also appeared in the other arm, head and tongue, the patient developed severe adynamia to inability to walk, worsening of the general condition, and confusion, due to which the patient was admitted to the hospital. On the following day the patient became inadequate, disoriented, difficult to contact, with aphasia, low oxygen saturation and was transferred to the ICU. Initial assessment revealed a progressive general cerebral manifestation to coma, acute renal failure, and development of severe respiratory failure, necessitating intubation and placing on assisted pulmonary ventilation.

Lung X-ray showed stasis and small pleural effusions, with the subsequent development of bilateral pneumonia. Contrast computer tomography scan of the brain showed evidence of cerebral atrophy and long dated focus of lacunar ischemia in the right middle cerebral artery basin. ECG evidenced absolute arrhythmia and atrial fibrillation. Neurological status showed no meningoradicular irritation syndrome; craniocerebral nerves had no abnormalities; both pupils were isocoric, equally responsive to light. Static tremor of both hands was observed and tendon-anterior reflexes were generally attenuated for all four limbs.

Serum laboratory tests showed elevated C-reactive protein (CRP), LDH, myoglobin, ferritin; creatinine, AST and ALT (Table 1). Uroculture, hemoculture, and throat swab showed no bacterial growth. CSF showed elevated protein and mild pleocytosis.

Serological examination demonstrated specific IgM antibodies to West Nile virus (CLIA), and negative IgG. Specific WNV IgM antibodies in serum were confirmed at the Reference Arbovirus Laboratory of NCIPD. The PCR for West Nile virus in urine was positive. PCR test of CSF for Meningitis and Encephalitis Panel (S. pneumoniae, H. influenzae, Mycoplasma pneumoniae, Escherichia coli, Listeria monocytogenes, Neisseria meningitides, Streptococcus agalactiae, Enterovirus, Herpes simplex 1/2, Human herpes virus 6, Human parechovirus, Varicella zoster virus, Criptococcusneoformans, Streptococcus pyogenes) was negative.

Despite the treatment, the patient ended lethally on the 13th day after the admission with symptoms of cerebral edema, acute cardiovascular and respiratory failure.

DISCUSSION

In our article, both patients had encephalitis, which was a diagnostic challenge because all tests for the usual agents responsible for encephalitis were negative. Initial CSF examinations revealed mild proteinuria in both cases and mild pleocytosis in the second case. MRI showed no specific changes in the first case and was not feasible in the other case. In both cases, the diagnosis was made based on the risk factors, the season (August), symptoms, and laboratory findings. Confirmation of WNV aetiology was performed at NCIPD by ELISA and RT-PCR in serum, urine, and CSF. Both patients had not visited endemic areas and the WNV infection occurred in Sofia, making it a consideration in the differential

diagnosis of a neuroinfection.

While waiting for the results of the serological, CSF, and imaging tests empirical therapy for potential bacterial and viral meningoencephalitis was initiated with Ceftriaxone, Vancomycin and Acyclovir as well as symptomatic and causative anti-oedema therapy (dexamethasone, hepatoprotectors, diuretics, glucose-electrolyte solutions). After the positive result for WNV, the antiviral treatment was changed and Ribavirin was added to the therapy. Unstable hemodynamics was controlled with constant infusion of catecholamines.

These two reported clinical cases indicate that in advanced age and in the presence of comorbidities, WNND can have a severe clinical course and fatal ending. The mortality rate among patients can be as high as 17% [14, 15].

CONCLUSION

WNV infection is associated with considerable morbidity, particularly among the older individuals. Suspicion should be upheld in febrile patients with encephalitis or aseptic meningitis of unknown aetiology, admitted to the ICU. This is particularly important during the summer season when the disease can be spread by mosquitoes. More research is needed to identify the factors predicting a critical course requiring intensive care.

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A RARE CASE OF FAMILIAL ECHINOCOCCOSIS AFFECTING ALL FAMILY MEMBERS: CASE REPORTS AND REVIEW OF LITERATURE

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ABSTRACT

Echinococcosis is a parasitic disease that affects humans, caused by the larval stage of the *Echinococcus* tapeworm. The disease is a major health problem in many parts of the world, including Bulgaria. It has a long incubation period and can affect various organs, but most commonly the liver and lungs. In this article, we present cases of echinococcosis diagnosed in all members of the same family, highlighting the importance of early diagnosis and the need for effective prophylactic measures. Regardless of the degree of endemicity, cases of familial echinococcosis are rare in medical practice. Therefore, a comprehensive epidemiological study is needed to establish the causes of such a phenomenon. In conclusion, seroepidemiological research on echinococcosis and imaging (ultrasound and X-ray) of seropositive individuals should be performed among risk groups to establish hidden morbidity, particularly among communities, where familial echinococcosis is more prevalent.

Key words: familial echinococcosis; serological tests; cyst; surgical procedure

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INTRODUCTION

Human echinococcosis is a parasitic zoonosis caused by helminths belonging to the genus *Echinococcus*. The two clinical forms of greatest medical and social significance worldwide are cystic echinococcosis (CE) and alveolar echinococcosis (AE). Cystic echinococcosis, also referred to as hydatid disease or hydatidosis, results from infection with a species complex centred on *Echinococcus granulosus sensu lato*. Alveolar echinococcosis is caused by infection with *E. multilocularis* (1). Most human cases of AE in Europe are recorded in France, Germany, Switzerland, Austria, Lithuania, and Poland. In 2020, 77% of the 114 cases were recorded in Germany and France (2). Cystic echinococcosis (CE) is significantly more frequent in humans, and has an endemic distribution in several countries from the Mediterranean and Eastern Europe, Central Asia, China, North and Sub-Saharan Africa, and South America (3). The endemicity of CE is attributed to biotic and abiotic factors. A range of behavioral and socioeconomic factors (e.g. agricultural activities including traditional sheep rearing and farming practices, contact with dogs, geophagy, outdoor activities or contaminated food or water) that may facilitate the ingestion of *E. granulosus* eggs influence the transmission of this infection in humans (4). Human CE is a foodborne parasitic disease that can have a severe prolonged clinical course, leading in some cases to disability. Relapses following treatment are common, with potential for permanent disability and even death (5). It is of utmost importance to implement adequate surveillance and control measures for CE, in order to limit the spread of this infection in the community.

Cystic echinococcosis (CE) is endemic in Bulgaria, affecting individuals of all sexes and age groups. Regrettably, in recent years the highest notification rates among the Member States of the European Union have been registered in Bulgaria. While Bulgaria accounts for most cystic echinococcosis cases in the EU in 2020 (39%), there has been a 65% overall decrease of registered cases from 269 in 2016 to 95 in 2020 (2).

The transmission mechanisms suggest that multiple family members may be affected, with patients often unaware of the disease for an extended period of time due to the lack of specific symptoms. This paper

presents a rare case of familial echinococcosis affecting all family members (n=4) and reviews literature concerning this issue. The cases are presented chronologically according to the time of diagnosis.

CASE REPORTS

Case 1

A 43-year-old male residing in a small town in the northeastern region of Bulgaria. He has completed only the primary education level and was employed as a livestock farmer. The patient presented with a constellation of symptoms, including fever up to 40°C, productive cough, chest pain, shortness of breath, and a 12-kg weight reduction over eight months. The results of clinical-laboratory tests revealed: erythrocyte sedimentation rate (ESR) 73 mm/h, Hb 139 g/l; Er 4.75x1012/l; Eo 0.03 x109/l. A chest X-ray was performed due to suspicion of pneumonia, revealing two oval, smooth-walled formations, within the upper lobe of the right lung, each measuring 80 mm in length. The patient was admitted to a surgical clinic for diagnostic evaluation. A CT scan of the chest and abdominal organs was performed. Imaging revealed the presence of four cystic formations in both lobes of the lung - two oval, smooth-walled formations measuring 80 mm in the upper lobe of the right lung and two with the same characteristics at the base of the left lung. A large cyst measuring 120 mm was identified in the liver. Immunodiagnosis was carried out by an enzyme-linked immunosorbent assay (ELISA), which showed the presence of anti-echinococcal IgG antibodies.

A one-stage echinococcectomy was performed. Pathohistological analysis of the extracted cystic formations described chitinous membranes, which confirmed the diagnosis. The patient underwent postoperative chemoprophylaxis with albendazole in a dose of 800 mg/24h for 6 months.

According to the epidemiological survey, the patient had a dog that was not regularly dewormed and was fed with raw animal products. The man was in the habit of consuming unwashed fruit and vegetables and was not aware of the disease and its transmission mechanisms.

As a part of the anti-epidemic measures, the three other family members were tested for echinococcosis. Immunodiagnostic tests and imaging were performed, and all three were diagnosed with hydatidosis.

Case 2

Seventeen-year-old female, daughter of Case 1. She had a history of dermal hypersensitivity of unknown etiology since 2-3 years ago treated with antihistamines and corticosteroids. The woman had clinical symptoms of pneumonia lasting about a month. The serological test performed as a apart of the epidemiological survey was positive for echinococcosis. The chest x-ray showed three round shadows in the left lobe with smooth and sharp outlines measuring 60x70 mm and two cystic formations with connection to the hilus and a dense shadow in the hilus itself, as well as atelectasis on the right side. Pulmonic echinococcosis was concluded. Abdominal ultrasound revealed the presence of multiple cysts of varying sizes and shapes, some of which - septated. Four cysts were observed between the spleen and the diaphragm. The young patient was hospitalized for further diagnostic evaluation and treatment in the Pediatric Surgery Department of the regional hospital. On admission to the medical facility, the general condition of the patient was satisfactory, with low fever and persistent, dry, nonproductive cough. Physical examination revealed pure vesicular breathing with single dry wheezes. The liver was painless, with a soft-elastic consistency, enlarged (3-4 cm below the costal arc). The clinical-laboratory analyzes showed: ESR 110 mm/h; Hb 143 g/l, Er 4.85 x 1012/l, Leu 16.9 x 109/l. A differential leukocyte count on a peripheral blood smear showed: St 3%, Sg 45%, Ly 32%, Mo 3%, Eo 17%. Liver biochemistry: aspartate aminotransferase (ASAT) 27 U/L (10-40); alanine aminotransferase (ALAT) 63 U/L (10-35); gamma-glutamyl transpeptidase (GGTP) 203 U/L (6-54). The diagnosis of multiple echinococcosis was based on imaging data, positive serological tests for echinococcosis, clinical-laboratory analyses, and the history of a family member with proven echinococcosis. A CT scan of the head showed no formations suspicious for echinococcal cysts. In the right lung lobe, in the VI segment, bilaterally well-shaped elliptical formations with thin walls, water-equivalent content and an average size of 40x45 mm were visualized. One of the formations was drained

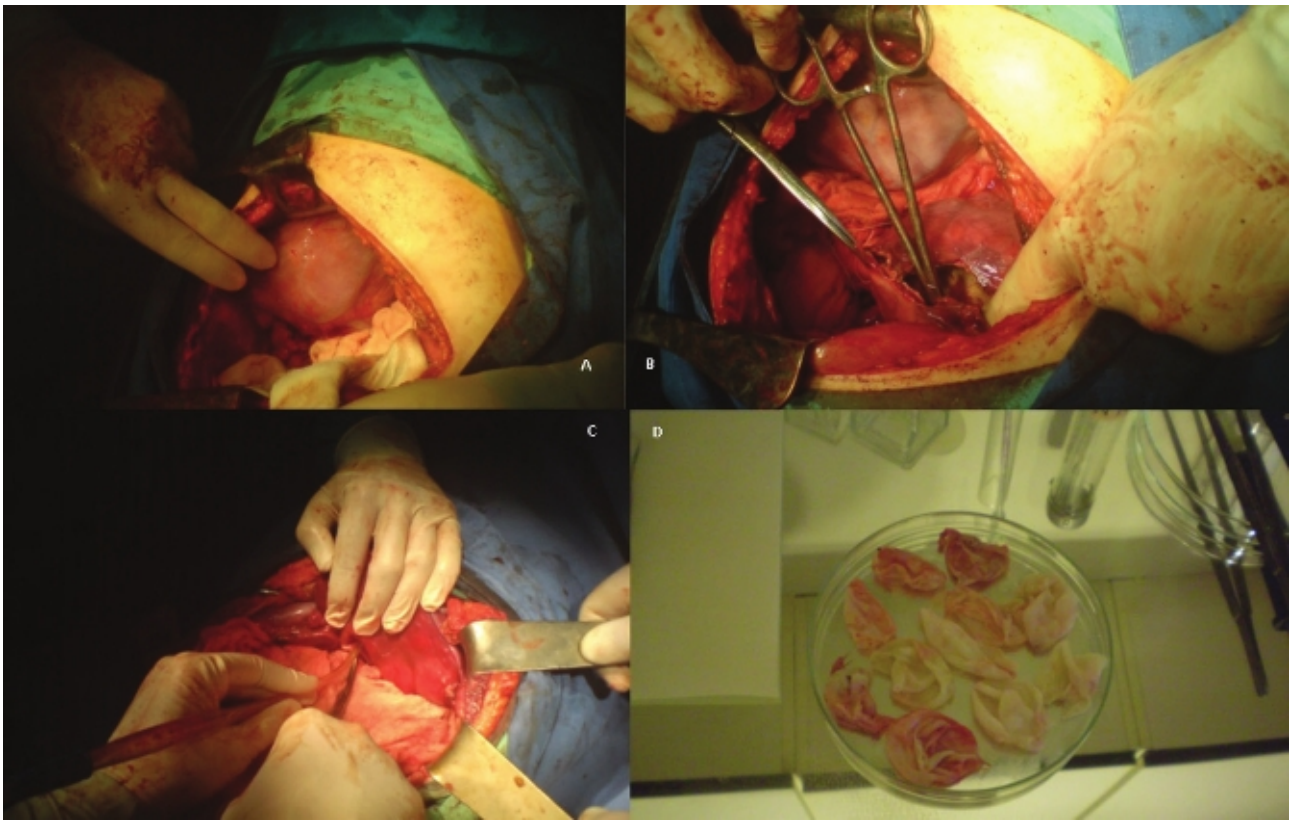

Figure 1. The stages of surgical intervention (A, B, C) and the capsules of extracted echinococcal cysts (C) are presented.

into the bronchial tree. There was evidence of inflammation around a cyst with a diameter of 9x10 mm in the right lower lobe. CT-scan of the abdominal cavity revealed multiple cystic formations in the liver, with thin walls and sizes between 7 and 10 mm. Cysts were also found in the left lateral subdiaphragmatic area. Five echinococcal cysts were surgically removed from the right lung and four from the right lobe of the liver (Fig. 1).

The surgical material was subjected to histological examination, which revealed the presence of an echinococcal cyst, a cyst capsule, and cyst fluid. The presence of viable and invasive protoscolices was confirmed (Fig. 2).

The patient underwent a second operation one month after the initial procedure, during which three

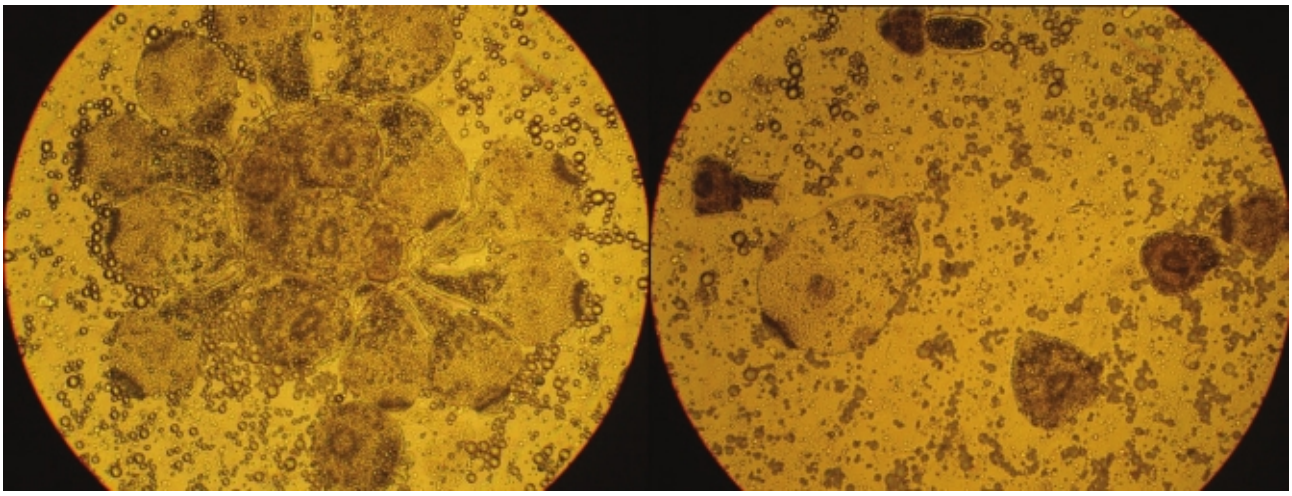

Figure 2. *Echinococcus granulosus* protoscolices in echinococcal fluid from surgically removed cysts (magnification x 400).

echinococcal cysts were removed from the left lung. A third operation was performed two months after the second operation, during which 24 echinococcal cysts were removed from the liver. Postoperatively, six one-month courses of albendazole were administered by the recommended regimen 10 mg/kg of body weight daily in 2 divided doses

Case 3

A 39-year-old woman, the wife of Case 1, with primary education level and no employment, was examined for epidemiological indications. Serological data showed the presence of specific anti-echinococcal antibodies. Ultrasonography of the abdominal organs showed the presence of an approximately 8 cm cyst with daughter cysts involving segments VII and VIII of the liver.

The peripheral blood parameters were within the reference ranges. The patient presented no subjective symptoms but was referred to the regional hospital for admission and treatment. A cyst was subsequently removed via surgical intervention.

The epidemiological study revealed that the woman had frequent contact with the family's pet yard dog, which had not been dewormed. Furthermore, the patient had no knowledge of CE disease and its transmission mechanisms.

Case 4

A 22-year-old woman, daughter of Case 1 living in the same household. She had a secondary education level and was currently unemployed. She had no complaints and was actively diagnosed as a part of the epidemiological survey conducted due to her father's illness. Echinococcosis was proven by positive serological tests for antibodies to *Echinococcus granulosus* and imaging results. Ultrasonography visualized multiple cysts of different calibres in the liver, measuring between 15 and 50 mm, with well-formed walls, some of them annular, and calcium deposits.

Given the multiple hepatic echinococcosis, the relatively small sizes of the cysts and the ultrasound data of partial devitalization in two cases, only conservative chemotherapy was applied with albendazole in a dose of 10 mg/kg body weight daily in 2 divided doses, for six month. The disease course was favorable, with involution of echinococcal cysts.

Again, the epidemiological survey established lack of knowledge about the zoonosis and the mechanisms of its transmission.

DISCUSSION

The term "echinococcosis" is used to describe a synanthropic and naturally occurring zooanthroponosis. The epidemic and epizootic processes are sustained within the synanthropic focus, facilitated by the prevailing lack of health education, the irresponsibility of domestic animals owners and the high population of stray dogs. Humans serve as accidental intermediate hosts for the echinococcal tapeworm, yet they have no epidemiological significance for its spread. However, when infected, they experience significant health damage. Disease cases are typically sporadic and disseminated in both rural and urban settings. With a notable spread of the disease nationwide, it is possible to observe parasitic outbreaks limited to individual households, with several disease cases within them (6, 7).

Nevertheless, the available literature data on such cases is relatively scarce. Two comparable studies were published for Bulgaria. One of them presents a clinical series of 15 families, with either both spouses affected (in 3 families), or - both spouses and the child, (in 9 families), or - the mother and the child (4), or - the grandmother and a grandchild (2). All cases were diagnosed at the same time and the source of infection was assumed to be the same (8). The second study covered six families (with a total of 26 individuals) with two CE patients in each of them - a total of 12 patients. Eight patients (66.7%) were 19 years old, and four (33.3%) were children and adolescents. CE had most often liver localization (7 patients, 58.3%), followed by pulmonary (2, 16.7%), combined lung and liver localization (2) and combined lung and spleen - in one of the patients (8.3%). Three of the families were from the Roma ethnic community. According to the history, five of the families were keeping a pet dog, but did not consider the risk of infection and did not deworm it regularly (9). Similar cases have been described in Romania and Turkey (10, 11).

Our case study is similar to the published ones. First, the presence of multiple cases of cystic echinococcosis in the same family may be attributed to the fact that all patients were exposed to the same risk factors, such as contact with potentially infected

dogs, ingestion of water and food that may have been contaminated with *Echinococcus granulosus* eggs, and lack of adequate hygiene habits. Additionally, the patients resided in endemic regions with developed sheep breeding (10). It is imperative to consider the educational, health-related, and cultural peculiarities of the affected individuals. In the present case the parents had only a primary level of education, one daughter was a student, and the other one had completed secondary education and was unemployed. None of the patients demonstrated any awareness of CE or the importance of deworming measures, or the risks associated with raw meat feeding of domestic dogs. Another similarity between the Bulgarian studies, is that of 22 families with more than one family member affected, 13 (59%) were from a minor ethnic community (Roma, Turkish). In our opinion, the cultural and social characteristics of these groups exert a considerable influence on the transmission of the disease among the population.

A further common conclusion of the published studies is the importance of timely family screening when diagnosing a case of CE in a family member. An effective evaluation of all family members at the time of the first case of hydatid disease diagnosis provides an opportunity for rapid diagnosis of the disease in other family members (11).

In our case, all family members were diagnosed within a month of the initial case, and immediate treatment was promptly initiated. Except for one case (the 22-year-old daughter), all other family members underwent surgical treatment, followed by anti-relapse drug prophylaxis. The younger daughter had to undergo three surgical procedures due to the extensive number of echinococcal cysts and their multiorgan localisation. Except for the mother, all other family members were afflicted with multiple echinococcosis. The father and the younger daughter also presented with multiple organ involvement. This suggests a high level of exposure and concentration of ingested parasite eggs.

CONCLUSION

The seroepidemiological research on echinococcosis and imaging (ultrasound and X-ray) studies of seropositive individuals should be performed among risk groups to establish hidden morbidity, with particular attention to ethnic communities where familial echinococcosis is more prevalent. Targeted examination of environmental samples for possible persistent contamination with echinococcal eggs and associated risk of spreading the disease among human and animal hosts is recommended during epidemiological investigations of outbreaks of familial echinococcosis.

ACKNOWLEDGMENTS

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BABESIOSIS IN HUMANS: A BRIEF LITERATURE REVIEW

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ABSTRACT

Babesiosis is a tick-borne parasitic disease caused by the intraerythrocytic protozoan *Babesia* spp. and transmitted primarily by *Ixodes* ticks. The geographical distribution of the parasites coincides with the regions where their tick vectors are prevalent. More than 50 cases of human babesiosis have been reported in Europe, mainly associated with *Babesia divergens*, which causes acute disease in cattle and is transmitted by *Ixodes ricinus*. In contrast, the incidence of the disease in the USA is approximately 2000 cases per year, with the main causative agent being *Babesia microti* and the tick vector being *Ixodes scapularis*. Although babesiosis is primarily an animal disease, humans can also become acutely ill, particularly splenectomized and immunocompromised individuals. Clinical manifestations range from asymptomatic to severe disease with symptoms including fever, chills, hemoglobinuria and anemia. There is a risk of potentially fatal complications such as acute respiratory, renal or multi-organ failure, particularly in vulnerable populations. Diagnosis is primarily based on light microscopy and PCR testing, while serological methods are more appropriate for epidemiological studies. Treatment regimens typically include a 7-10 day course of either atovaquone plus azithromycin or clindamycin plus quinine. Human cases are associated with outdoor activities or living in rural areas during the warm months when tick activity is at its peak. Because of the increasing incidence in endemic regions and the potentially serious clinical consequences, babesiosis should be considered in the differential diagnosis of febrile illnesses of unknown origin.

Keywords: babesiosis; etiology; distribution; treatment

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INTRODUCTION

Babesiosis is an infectious disease caused by the intraerythrocytic protozoa of the *Babesia* species. The principal mode of transmission is via a transmissible vector - the *Ixodes* tick. *Babesia* infects primarily animals, with humans acting as opportunistic hosts. The parasite is also called 'pyroplasma' due to its distinctive pear-shaped morphology observed in infected erythrocytes. The prevalence of *Babesia* infestation is low and confined to specific geographical regions. The majority of individuals do not present with any discernible symptoms; however, in some cases, patients may exhibit severe clinical manifestations and a high mortality rate. In general, the disease affects predominantly patients who have undergone splenectomy, those with a compromised immune system, and the elderly. The diagnosis of babesiosis is not straightforward and requires caution, particularly in regions where the disease is endemic. It is recommended that patients are treated regardless of the presence or absence of symptoms, in order to prevent disease progression and transmission. (1)

Morphology and life cycle

More than 100 different *Babesia* species have been identified (2), of which several have been confirmed to cause human infection: *B. crassa*-like agent, *B. divergens*, *B. duncani*, *B. microti*, *B. motasi*, *B. venatorum*, *Babesia divergens*-like and *Babesia microti*-like pathogens (3).

Babesia spp. can be classified in different ways. Taxonomically, they are part of the phylum *Apicomplexa*, subclass *Piroplasmea*, order *Piroplasmida* (4). Using morphological characteristics, they can be grouped into small *Babesia* (trophozoites are 1.0 to 2.5 µm) and large *Babesia* (2.5 to 5.0 µm) (5), although size has limited taxonomic value (2). Another classification is

based on the capacity for transovarial transmission. *Babesia sensu strictu (s.s.)* demonstrates such transmission, and *Babesia sensu latu (s.l.)* doen not. New molecular taxonomy has classified *Piroplasmidia spp.* into different clades using analysis of 18S rRNA gene sequences obtained from public databases (2). Currently, there are at least ten main lineages, of which four clades include *Babesia spp. – B. microti* is part of Clade I, and *B. divergens* is part of Clade X (6). Morphologically, *Apicomplexan* spp. (like *Babesia, Plasmodium, Toxoplasma, Cryptosporidium* and others) are characterized by the presence of an apical complex (7), located anteriorly in all invasive stages, such as sporozoites and merozoites (6). It consists of multiple structures, including rhoptries and micronemes - membranous organelles containing substances responsible for the attachment to and invasion of host cells. During this process, apicomplexan parasites create a parasitophorous vesicule, in which they reside, using the host cell's plasma membrane (7). Unlike others, the parasitophorous vesicule of *Babesia spp*. disintegrates soon after the parasite invades the host cell, therefore it resides directly in its cytoplasm (6).

Babesia spp. need two hosts to complete their development - vertebrates (including man) and tick vectors. The parasite's life cycle is complex, with two asexual and one sexual reproduction cycles. Stages of development include merogony, gamogony and sporogony, which are typical for the phylum *Apicomplexa* (6).

Babesia-infected ticks introduce sporozoites into vertebrae hosts by blood meal. Sporozoites enter the host's erythrocytes and develop into trophozoites (ring forms), which undergo merogony and turn into merozoites. They egress, infect other erythrocytes and either undergo a further merogony cycle or a sexual commitment to transition into intraerythrocytic gametocytes. In further blood meal, the host's erythrocytes containing gametocytes enter the tick and develop into gametes, referred to as ray-bodies, inside the vector's gut lumen (6). *Babesia spp.* exhibit two gamete types (different from other piroplasmidian micro- and macrogametes) (8), the fusion of which creates an ookinete. Ookinets invade the tick gut cells, undergo a meiotic division and develop into kinetes. Kinetes reach other organs in the tick, including ovarian cells, which mediates transovarial transmission in *Babesia sensu stricto* species (6). Primary kinetes multiply asexually to create secondary kinetes, which invade the salivary gland cells, where they turn into a multinucleated sporoblast. This syncytium is dormant during the tick's ecdysis and mediates babesial transstadial transmission. Infective sporozoites are procured by sporogony once the adult tick initiates blood-feeding on a naive host, during which multiple sporozoites get inoculated in the vertebrate bloodstream (8).

Hosts

B. divergens was thought to have a narrow host range and to be typical for cattle (9). Now it has been proven to have one of the widest host ranges for a *Babesia* species through experimental infections in different splenectomized and non-splenectomized animals, including primates, deer, sheep, gerbils and rodents. Furthermore, naturally infected reindeer, red deer and roe deer have been reported, although it has not been proven with absolute certainty that the causative agent is *B. divergens* (10).

Bovine babesiosis is the most economically significant arthropod-transmitted pathogen affecting cattle due to the mortalities, abortions, and reduced production of meat and milk caused by this disease. The most economically relevant pathogens are *B. bovis* and *B. bigemina* in tropical and subtropical areas, as well as *B. divergens*, mainly found in Europe from Scandinavia to the Mediterranean, but also in North Africa (2).

B. divergens is transmitted by *Ixodes ricinus*. The species' habitat is restricted to areas with an average annual rainfall of 100cm or more due to its requirement for high humidity. A suitable microhabitat can be found in woodland, rough hill scrub and damp low-lying land, unlike well-maintained pastures, where the conditions needed are rarely provided (9). According to different studies, this tick is found primarily in urban and peri-urban areas such as city parks, gardens and forest patches. It should be noted that climate change leads to a wider distribution of *I. ricinus* in regions with higher latitudes and altitudes (11).

There is a geographical overlap between countries reporting human babesiosis cases, regions with

infected cattle, and *I. ricinus*-infested areas. Human cases occur between May and September, which corresponds with the peak activity of *I. ricinus* ticks (11). People at the highest risk of acquiring babesiosis are those who visit rural areas where cattle are kept, such as farmers, foresters and hikers (12). Furthermore, the risk of infection through a tick bite increases because all stages (larvae, nymph and adult) can transmit *B. divergens* and *B. venatorum* (11).

Ixodes scapularis is the main tick vector in North America and is found in the north- and southeast, upper Midwest and mid-Atlantic states of the United States and in Canada, but *I. ricinus* is also proven to be a competent vector for *B. microti* and *B. venatorum* (11). The main reservoir of *B. microti* is the white-footed mouse (*Peromyscus leucopus*) (13).

Epidemiology

The first case of human babesiosis was described in 1957. In former Yugoslavia (now Croatia), a 33-year-old splenectomized farmer died after an acute illness, characterized by fever, hemoglobinuria and anemia. Parasites found in blood smear were identified as piroplasms, resembling *B. bovis* (14). The second case of human babesiosis was reported in California in 1968 when a splenectomized individual exhibited malaria-like symptoms without any evidence of malaria exposure. A diagnosis of babesiosis was later confirmed through serological evidence (17). In the U.S., the primary causative agent of human babesiosis is *Babesia microti*, which is endemic mainly in the Northeast and upper Midwest (13). The incidence is 2000 cases a year, although the number is believed to be higher (18).

Since then, more than 50 cases of human babesiosis have been reported in Europe, of which nearly 1/4th in France. The other cases were registered in the following countries: Great Britain, Ireland, Spain, Portugal, Italy, Germany, Austria, Switzerland, Russia, Poland, the Czech Republic, Sweden, Denmark, Norway, Finland, Turkey (15) and Hungary (16). The majority were caused by *B. divergens*; 5 were caused by *B. venatorum* and 24 by *B. microti* , 11 autochthonous and 13 imported cases acquired in the Americas (15).

An imported case of human babesiosis was described in Bulgaria in 1995. The patient was 34 years old and had a history of residing in Sudan for six months, as well as a tick bite two months before the onset of the illness. Symptoms included fever, chills, fatigue, loss of appetite, jaundice and hepatomegaly for more than a week before hospitalization. Laboratory tests showed hemoglobinuria and elevated CRP, AST, ALT, total and direct bilirubin levels. *Babesia* infection was diagnosed using light microscopy. The patient was treated with Chloroquine orally for five days, with doses of 1g on the first day and 500mg on the subsequent days. The treatment was successful, and the patient was discharged in improved condition. Since then, no further cases of human babesiosis have been registered in Bulgaria (19).

Clinical manifestations and complications

B. divergens infection

Splenectomized and immunocompromised patients

B. divergens affects mainly splenectomized (15,20,21) or hyposlenic individuals (22,23). The incubation period varies from 1 to 3 weeks after vector bite (20). The onset is sudden with fever (22, 24-26), chills (24,26,27), sweats, headache (28), myalgia (24), abdominal (25-27) and back pain (22). (20) Hemoglobinuria and jaundice (23, 25-27) are commonly seen due to massive intravascular hemolysis (20). Other symptoms include nausea (23-25), anorexia (25), malaise (23), vomiting (26,29), diarrhea (29), cough (24,26), hematuria (25,27), oliguria (22), proteinuria (25), fatigue (22,24,28), arthralgia (24), skin rash (24), petechiae (27).

Life-threatening complications can occur, such as acute respiratory (25-27), renal (23, 25-27) and multi-organ failure (22), coma (27), DIC (30), shock (26), hemophagocytic syndrome (31), hospital-acquired pneumonia (23), atrial fibrillation (27), QT-prolongation (26). Rapid renal failure due to babesiosis has been associated with pulmonary edema (20).

The most common laboratory findings are anemia, lymphopenia, thrombocytopenia, elevated levels of ASAT and evidence of inflammation (24). Higher levels of LDH (26,28) and signs of hemolysis (22,26,28) can also be established.

Normosplenic patients

Acute illness caused by *B. divergens* has also been reported in normosplenic individuals, some of whom with no prior remarkable medical history. In those cases, the clinical manifestation was moderate (32) to severe (33-35). One case had a lethal outcome (36).

Symptoms included fever with chills, headache, arthromyalgia (32) in milder cases, fatigue (35), nausea, abdominal pain, dark urine (33), jaundice, (35) mild hepatomegaly, acute renal failure (34), cough and dyspnea (35) in more severe cases. One of the patients experienced a relapse of the disease after 18 days of treatment (34). Laboratory findings included leukopenia, elevated liver enzymes and CRP in milder cases (32) and anemia (34), thrombocytopenia (33,34), leukocyte left shift with immature neutrophils (35), low haptoglobin, hematuria (34), elevated creatinine, total bilirubin, direct bilirubin and LDH (34) in more severe ones.

Furthermore, seropositivity for *B. divergens* in individuals without a diagnosis of human babesiosis has been shown in different serological surveys: in Belgium (33% in patients with tick-borne disease) (37), in Italy (5,1 % in individuals with professional risk and 1,4% in less exposed individuals) (38), in Midwestern Germany (3,6% in individuals with clinical or serological evidence of Lyme borreliosis) (39), in Sweden (6,9% in individuals positive for *Borrelia burgdorferi* antibodies and 1% in healthy volunteers) (40).

B. microti infection

Studies in white-footed mice and hamsters suggest that the time for transmission after tick attachment may be 36 to 54 hours, but this has not been studied in humans. The incubation period for tick-borne disease varies from 1 to 4 weeks (41).

The course of *B. microti* infections varies from asymptomatic to severe (42). This condition is observed mainly in normosplenic patients (15). Most cases are mild to moderate, with a gradual onset. Clinical manifestations typically include fever, chills and sweats, malaise, fatigue, anorexia, headache, arthromyalgia and cough. Gastrointestinal disturbances (nausea, vomiting, abdominal pain) and other symptoms (e.g. conjunctival injection, sore throat, pallor, weight loss, and depression) are less common. Physical examination shows minimal changes like mild spleno- and hepatomegaly and slight jaundice. Immunosuppressive medication and conditions (HIV-coinfection, malignancies, and splenectomy) are associated with a more severe disease. Furthermore, such patients are more likely to experience a prolonged, relapsing course of illness and have a higher mortality (42). In asplenic individuals, the clinical manifestation is similar to *B. divergens*, often fulminant and lethal (15). Complications include acute respiratory, hepatic, renal and heart failure, DIC and splenic infarction (42).

Asymptomatic parasitemia can be found in individuals who have either no symptoms or a subclinical manifestation, and it may persist for months to years. Such carriers have been identified through different serosurveys, showing a disparity between seroprevalence and the number of reported cases. Asymptomatic parasitemia in blood donors may lead to transfusion-transmitted babesiosis (42), which has a longer incubation period (1 to 6 weeks) (41) and can occur at any time of the year (29).

In Europe, seropositivity for *B. microti* has been found in different countries, for example - in Sweden (10,4% in individuals positive for *Borrelia burgdorferi* antibodies and 1,52% in healthy volunteers) (40), in Poland (23,1% in employees of National Forests) (43), in Belgium (9% in patients with tick-borne disease) (37), in Italy (4,8% in individuals with professional risk; 4,2% in less exposed individuals) (38), in Midwestern Germany (5,4% in of individuals with clinical or serological evidence of Lyme borreliosis) (39).

Diagnosis

Babesiosis should be considered in patients with fever of unknown origin or signs of hemolytic anemia (15), history of residing in *Babesia* endemic areas, tick-bite or exposure to tick-infested areas (5), absence of recent travel to malaria-endemic regions (9), splenectomy (5) and potential immunocompromising factors (15).

Laboratory findings in symptomatic patients may be non-specific, such as normochromic normocytic anemia, thrombocytopenia and occasionally leukopenia, as well as elevated liver enzymes

(aspartate aminotransferase [AST], alanine transaminase [ALT], alkaline phosphatase) (44). Evidence of intravascular hemolysis may be present - elevated LDH levels, total and indirect bilirubin levels, and reduced haptoglobin (18). Furthermore, if alongside with hemolytic anemia, the Coombs test is positive and procalcitonine levels are elevated, babesiosis is to be suspected, and further diagnostic tests should be performed (44).

Light microscopy

Light microscopy of Giemsa-stained thin blood smears is used for detecting *Babesia spp* (44). Various forms can be seen, including rings, pear-shaped parasites and Maltese cross forms (5), which are pathognomonic of babesiosis (18). Tetrads are more commonly seen in *B. microti* but are still rare (44). The size of merozoites varies from 1,5 to 2 µm in *B. microti* and from 1 to 3 µm in *B. divergens*, depending on the host (5), the mean length of pyriforms in human erythrocytes being approximately 2µm (9). Merozoites have a (sub)central position and polyparasititsm is common, with up to 8 parasites in a single erythrocyte (9).

The main differential diagnosis is malaria because *Plasmodium spp.* also shows intraerythrocytic rings. Typical for malaria is the parasitic pigment (hemozoin), although early parasitic stages may lack it (44). Furthermore, schizonts and gametocytes, seen in malaria, are absent in *Babesia* infections (45). Parasitemia in *B. divergens* ranges from 1 to 80% (20), though it should be noted that in the early stages of the disease in immunocompetent patients, it can be lower than 1% (44). Therefore, a review of at least 200–300 fields in thin blood smears is recommended, but the exact number of fields has not yet been standardized. An examination of thick blood smears may be helpful, although parasites could be missed due to their size (18).

Serological diagnosis

Serological testing is not suitable for diagnostic purposes (18). On one hand, specific antibodies become detectable 1 week after the onset of acute *B. divergens* infection, which may lead to false-negative results and delay of treatment. On the other hand, false-positive results have been observed in patients with connective tissue disorders such as systemic lupus erythematosus and rheumatoid arthritis. Cross-reactivity between different *Babesia spp.* (*B. divergens* and *B. venatorum*), as well as between *Babesia* and other *Apicomplexa* parasites (*Plasmodium* and *Toxoplasma*) were also described (21). Moreover, the differentiation between active and past infection is challenging because most patients remain seropositive for a year or more after acute illness. Therefore, serological methods are suitable only for epidemiological studies (18).

Molecular diagnosis

PCR assays targeting the 18S rRNA gene can be used to detect *Babesia* parasites. Both clotted and EDTA-treated blood samples can be used for this test (15). These methods have higher sensitivity and equal specificity compared to microscopic examination of blood smears and hamster inoculation (5). The detection limit of PCR assays is approximately 1-3 parasites per µL of blood, which is lower than the microscopic detection limit (15). The detection of *Babesia* DNA indicates the presence of parasitemia (21).

PCR assays for both *B. microti* and *B. divergens* have been developed. These assays typically amplify highly conserved sequences, which contain species-specific regions. By analyzing the sequences of the amplified fragments and comparing them to a database of known sequences, the infective agent can be conclusively identified (5).

Treatment

Currently, atovaquone, azithromycin, clindamycin and quinine are used as antibabesial drugs. The mechanism of action of atovaquone in *Apicomplexa* parasites is through targeting the cytochrome bc1 complex of the mitochondrial electron transport chain. Azithromycin is associated with protein synthesis inhibition, including the translation machinery in the apicoplast. Clindamycin is thought to have the same target of action as azithromycin. The exact mechanism of action of quinine against *Babesia* is different from its action against malaria parasites, where it interferes with hemozoin formation. *Babesia* species do not produce hemozoin, which suggests that quinine's action in babesiosis is mediated through other pathways. According to different studies, quinine may potentially inactivate critical biological

functions in various parasite organelles such as the plasma membrane, endoplasmic reticulum, and mitochondria, or it may act as a DNA intercalator, though the latter hypothesis is less supported (46).

Two reatment regimens are used: atovaquone plus azithromycin, which is preferred, and clindamycin plus quinine as an alternative. Courses should last at least 7–10 days and are longer for immunocompromised patients. Asymptomatic patients typically do not require treatment (47).

For mildly to moderately ill adult patients, treated in outpatient settings, the preferred regimen is atovaquone (750 mg p.o. twice a day) combined with azithromycin (500 mg p.o. on the first day and 250 mg p.o. daily on subsequent days). Alternative regimens are clindamycin (600 mg p.o. three times a day) or quinine (650 mg p.o. three times a day) (47).

For hospitalized adult patients with severe disease, the preferred regimen includes atovaquone (750mg p.o. twice a day) and azithromycin (500mg i.v. daily). Clindamycin (600mg i.v. four times a day) or quinine (650 mg p.o. three times daily) can be used as alternatives. The administration of either regimen should continue until symptoms subside, after which the patient should receive oral medications at outpatient treatment doses to complete the 7 to 10-day course. In severely ill patients and immunocompromised ones, higher doses of azithromycin have been administered - 1000 mg, followed by 500mg daily (47).

Supportive care including antipyretics, vasopressors, blood transfusions, exchange transfusions for high-grade parasitemia (>10%), mechanical ventilation, or dialysis may be necessary for some patients (47).

In immunocompetent patients, symptoms usually resolve during the 7–10 days of treatment, and blood smears become negative. In contrast, highly immunocompromised patients require longer courses and close monitoring, including daily blood smears until parasitemia is below 4%, followed by weekly checks. Treatment should continue until parasites are undetectable on smears for two consecutive weeks (47).

CONCLUSION

Babesiosis is a rare infection globally and in Bulgaria. Diagnosis can be challenging, as the infection can be confused with malaria or other tick-borne diseases. Therefore, it is advisable to adopt a multidisciplinary approach when diagnosing suspected infections. This entails the involvement of specialists in both infectious diseases and medical parasitology, to prevent any potential omissions. In the light of rising incidence of human cases observed in recent years and the potential of babesiosis to manifest as a severe disease with a fatal outcome, it is imperative to consider it in the differential diagnosis of febrile conditions of uncertain origin.

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SURVEILLANCE OF PARASITIC DISEASES IN BULGARIA: ANNUAL EPIDEMIOLOGICAL ANALYSIS FOR 2023

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ABSTRACT

The incidence and number of cases of several parasitic diseases subject to mandatory reporting and registration under the Ministry of Health (MoH) 2005 Regulation 21 increased after the end of the global pandemic of COVID-19 in 2023. In addition, parasitic infections imported from endemic tropical regions are recorded annually in the country. A significant number of people are screened for parasitic diseases each year. The primary indication for screening is prophylactic, followed by epidemiological and clinical indications. This report aims to analyse the dynamics of parasitic diseases in 2023. Data from the periodic and annual reports of the RHI, medical institutions and NCIPD were used as inputs. In 2023, 647 781 people were tested for parasites, of whom 2.0% were diagnosed with various parasitic infections. Cystic echinococcosis increased from 89 cases in 2022 to 117 cases in 2023. The annual incidence of the disease increased from 1.3% in 2022 to 1.81% in 2023.

Regarding soil-transmitted parasitic diseases, 447 people were diagnosed with ascariasis and 50 with trichuriasis. Among the 458 764 people tested, the prevalence of enterobiasis was 1.74%. Out of 1 305 suspected cases, 10 individuals were diagnosed with imported malaria. Unfortunately, the disease was fatal for one patient of Bulgarian nationality.

Analysis of the data showed no discernible trend in the incidence of parasitic diseases in 2023. However, cases of cystic echinococcosis show an upward trend. For the first time, no epidemic outbreaks of trichinellosis were recorded in 2023.

Keywords: parasitic diseases; incidence; zoonoses

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INTRODUCTION

Parasitic infections continue to represent a significant global health concern. These infections have a substantial impact on morbidity and mortality rates in developing countries, while also being prevalent in developed countries. Early diagnosis and treatment of parasitic diseases are of critical importance for reduction of morbidity and mortality, as well as for prevention of their transmission within the community (1). Approximately 25% of the global population is affected by one or more parasitic infections, with parasitic zoonoses transmitted via food or vectors being the primary concern. Moreover, zoonoses and communicable diseases that are common to humans and animals are receiving increasing attention on a global scale. The significant changes in climatic conditions, agricultural activities, demography, dietary habits, alongside with the intensified international travelling and trade, deforestation and urbanisation play a considerable role in the emergence and re-emergence of previously eliminated parasitic zoonoses (2).

The principal aim of this concise analysis is to present an overview of the human parasitic pathology in the country and to delineate some trends and projections about its prevalence.

MATERIALS AND METHODS.

The study encompasses the period between January and December 2023 and was conducted at the Department of Parasitology and Tropical Medicine, at the National Centre of Infectious and Parasitic Diseases (NCIPD), Sofia, Bulgaria. The data set comprised the annual reports of the Regional Health Inspectorates (RHIs), including information

about parasitological examinations conducted in the respective regions of the country, the identified cases of infection, and aggregated data on the examined population, including age, sex, and place of residence. In the case of infections subjected to mandatory registration and reporting, epidemiological survey cards were also provided.

To evaluate the infection burden and severity of involvement by nosological entity, indicators such as prevalence (%), morbidity and mortality per 100,000 population, and spatial distribution by districts were analyzed. In addition, the preparedness of the public health system for surveillance, and adequate response to parasitic disease outbreaks in the country was assessed.

RESULTS

PARASITIC INFECTIONS WITH LOCAL SPREAD

In 2023, a total of 647 781 persons were tested in the various parasitology laboratories in the country (including RHIs, SMDLs and NCIPD), and 13 535 (2%) were diagnosed with parasitoses (Table 1).

Cystic echinococcosis: In 2023, 104 (89%) primary cases of cystic echinococcosis (CE) and 13 (11%) cases of postoperative recurrence were registered. The most affected age groups were 10-14 years and 45-49 years with 13 cases each. The 30-34 years and 55-59 years age groups were also highly affected with 12 and 10 cases of CE respectively. The proportion of children and adolescents (0-19 years) affected was 25.6% (n=30). The distribution of echinococcal cysts according to their respective organ localizations was as follows: 75 individuals (64.1%) had liver cysts, 26 (22.2%) had cysts with pulmonary localization, nine patients (7.7%) were with multiple echinococcosis, and seven (6%) had extrahepatic-pulmonary localization (spleen, kidney, peritoneum). The annual incidence rates had a very uneven territorial distribution. In 2023, the highest incidence rates were registered in the districts of Sliven (8.15‰), Kardzhali (6.37‰) and Shumen (3.9‰).

Trichinellosis: During the analyzed period no inoutbreaks of trichinellosis were recorded. Sofia RHI reported a single sporadic case.

Taeniasis: During the year under review, six taeniasis cases were reported, with *Taenia saginata* identified as the causative agent. A single case of taeniasis was reported in four regions of the country (Varna,

Table 1. Diagnosed parasitic infections with local spread in 2023.

Nosological unit	Number examined	Number of positives	Incidence per 100,000 / Prevalence in %
Zooanthroponoses with epidemic risk			
Echinococcosis	1327	117	1.8 per 100,000
Trichinellosis	87	1(sporadic)	0.02 per 100,000
Taeniasis ((beef tape-worm)	7953	6	0.09 per 100,000
Soil-transmitted helminth infections			
Ascariasis	551261	447	6.9 per 100,000
Trichuriasis	551261	50	0.7 per 100,000
Community-Acquired Parasitic Diseases			
Enterobiasis	458764	7983	1.74%
Giardiasis	442205	673	10.4 per 100,000
Hymenolepiasis	406921	110	1.7 per 100,000
Urogenital trichomoniasis	4294	271	4.2 per 100,000
Opportunistic parasitic infections			
Visceral leishmaniasis	29	2	0.03 per 100,000
Toxoplasmosis	16322	1680	10.29%
Blastocystosis	339676	1800	0.53%
Cryptosporidiosis	150	0	0
Pneumocystosis	88	17	0.26 per 100,000

Plovdiv, Razgrad and Haskovo), and two cases were identified in the capital, Sofia.

Soil-transmitted helminthiases (STH): A total of 497 cases of soil-transmitted helminthiases with traditionally local distribution (ascariasis and trichuriasis) were recorded by the country's parasitology laboratories, with significantly predominating ascariasis cases (Table 1). The parasitology departments of the RHI maintained a record of 147 STH-endemic settlements in nine country districts (six have been removed from the record out of regulatory requirements). Prophylactic examinations were conducted in 36 settlements (24%), with 118 688 individuals tested. Of these, 36 cases (0.03%) were diagnosed with ascariasis. All infected individuals were treated, and subsequent control examinations demonstrated that the treatment had been 100% effective. As in previous years, cases of trichuriasis were recorded primarily in nursing homes for individuals with mental disabilities.

Enterobiasis: Enterobiasis cases were predominantly concentrated among preschool children (n = 3705, 46.5%), school-aged children and adolescents (n = 2989, 37.4%), with a significantly lower relative proportion observed among adults (n = 1286, 16.1%).

Giardiasis: Out of the 673 cases of giardiasis, 403 (60%) were in children in childcare and early school age (up to 9 years). A higher prevalence was recorded in the districts of Burgas, Sliven and Yambol.

Hymenolepiasis: Over 95% of all cases of hymenolepiasis were documented in three country regions. The highest notification rates were observed in Sliven (n = 43), Yambol (n = 35) and Sofia-city (n = 27), predominantly among social home residents and minority groups. Most infected individuals (n = 76, 69%) were of preschool and primary school age.

Visceral leishmaniasis: During 2023, two cases of visceral leishmaniasis were registered. One was local (Kolarovo village, Petrich municipality), while information was lacking for the other one, which appeared in the database of the National Center for Public Health and Analyzes (for V. Tarnovo region), (no epidemiological survey card). Both patients were male and in the age group 45-49. One of the cases was fatal.

Blastocystosis: In 2023, 339 676 individuals were screened for blastocystosis, with 1 800 cases diagnosed (0.53% prevalence).

Cryptosporidiosis: During 2023, 150 patients were tested in the laboratories of Sofia-Capital, Varna and Plovdiv, and no positive cases of infection were identified.

Pneumocystosis: In 2023, 88 suspected patients were tested for pneumocystis pneumonia. A total of 17 cases (19.32%) were diagnosed as positive and were all confirmed by real-time PCR at NCIPD.

IMPORTED PARASITIC INFECTIONS

In 2023, a total of 3 097 individuals were tested for imported parasitic infections. Of these, 110 were Bulgarian citizens, while the remaining 2 987 were of foreign nationality. Of them, 174 individuals (164 foreigners and 10 Bulgarian citizens) were diagnosed with different parasitic infections.

Malaria: A total of 1 305 individuals were tested for malaria in seven districts of the country and at NCIPD. Of them, 115 were of Bulgarian nationality and 1 190 were foreigners, mostly refugees residing in the districts of Sofia-city and Sliven. In 2023, ten cases of imported malaria caused by *P. falciparum* were recorded, nine in Bulgarian citizens and one in a foreigner. Unfortunately, one of the infected Bulgarians from Varna died, most probably due to delayed seeking of medical care.

Other imported parasitic pathogens: In 2023, 1 792 individuals were tested for imported parasitic infections different from malaria, and all of them were foreigners. A total of 164 cases (9%) were identified, with the following parasitic species diagnosed: *B. hominis* (n = 65), *G. intestinalis* (n = 38), *A. lumbricoides* (n = 5), *H. nana* (n = 3), T. trichiuris (n = 1), *Ancylostoma spp.* (n = 1), *Entamoeba coli* (n = 27) and *Iodamoeba butschlii* (n = 14). No indigenous secondary outbreaks were documented, and prompt treatment was provided to the infected.

DISCUSSION

The transmission of parasites from the environment to different hosts, including birds, mammals and humans, occurs through the food chain and different vectors. In numerous instances, birds and animals serve as reservoirs and play a pivotal role in the transmission of pathogens to humans, thereby contributing to the emergence of zoonotic pathology. Given the involvement of multiple sources, these

diseases are now regarded as One Health issues and represent a challenge for the control of zoonotic diseases. The One Health approach is an integrated and unifying methodology aiming to achieve a sustainable equilibrium between the health of humans, animals and ecosystems (3). Of the 20 Neglected Tropical Diseases (NTDs) listed by the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC), 13 are of parasitic origin, thereby indicating their global impact (4). Although most parasites are not associated with acute disease, they have a detrimental influence on animal and human health and productivity through widespread morbidity. In some cases, this can ultimately result in mortality among affected individuals. Parasitic diseases are a pervasive global health concern. However, they are particularly prevalent in tropical and subtropical regions (5). It is estimated that approximately 25% of the worldwide population is affected by one or more parasitic infections, with parasitic zoonoses transmitted by foodborne and vector-borne routes representing a significant public health concern. Noteworthy, parasitic diseases predominantly affect the world's most impoverished and disadvantaged populations, who often lack access to adequate healthcare (2).

As to the endemic parasitic zoonoses, cystic echinococcosis (CE) is of the greatest medical importance in Bulgaria. In recent years, there has been a decline in its incidence, with the most significant decline occurring between 2020 and 2022. This period coincided with the peak of the pandemic caused by SARS-CoV-2. Due to the possibility of gaps in diagnosis and registration, the data may not be entirely indicative (Fig. 1).

As reported by the European Centre for Disease Control (ECDC), 16 countries reported 299 confirmed cases of CE caused by *E. granulosus sensu lato* in 2022. As was the case in 2020 and 2021, the number of reported cases of CE in 2022 was significantly lower than the average annual number of cases reported in 2018 and 2019 (299 cases in 2022 compared to an average of 430 cases in 2018-2019). The highest number of cases were reported in Bulgaria and Germany (89 and 96 cases, respectively), accounting for 62% of all cystic echinococcosis cases reported in 2022. Among the 208 cases for which the age was known, 35% of those diagnosed with cystic echinococcosis were between 25 and 44 years, with 29% -between 45 and 64. Of the 205 cases of cystic echinococcosis with known sex, the majority (59%) were female. Among the 127 cases with known import status, 59% have originated outside the EU/ EEA in 2022 (6). Although the data are for 2022 (the annual analysis for 2023 has not been published yet), it is important to highlight some trends that are relatively constant over time. These include the fact that cystic echinococcosis predominantly affects people of working age, and according to our survey data for 2023, the age groups with the highest relative prevalence are 10-14 years and 45-49 years with 11% each, followed by the group of people between 30-34 years with 10%. The trend for a high relative proportion of affected children and adolescents is also

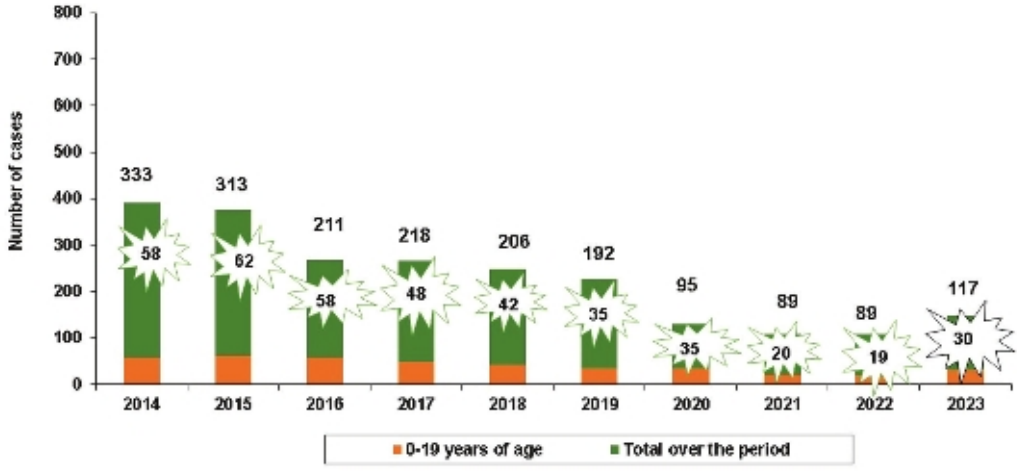

Figure 1. Registered cases of cystic echinococcosis for 2014 - 2023 (total and in persons in childhood and adolescence).

maintained, with 25.6%, 21.3% and 22.5% for 2023, 2022 and 2021, respectively (7). These data are of great concern because the infection is asymptomatic over a long period, which gives reason to believe that infection occurs at a relatively very young age.

Given that the primary means of transmission of the infection in humans is the consumption of water or food contaminated with tapeworm eggs or hands contaminated by contact with soil, it is imperative to prioritize the testing of environmental samples, particularly in regions with a high prevalence of the disease. Furthermore, greater efforts are required to increase the awareness of the disease, particularly among children. We contend that the principal reason for the persistently elevated levels of recorded incidence is the insufficient veterinary control on deworming of stray, yard and shepherd dog populations and meat production (predominantly in sheep) on private farms. Data on recorded CE cases over the next two - three years will indicate whether the decline in incidence observed in 2020-2022 was due to an improved health situation or was unfortunately related to gaps in diagnosis and treatment associated with the past Covid-19 pandemic.

Another zoonozis that has posed a significant public health concern for decades is trichinellosis. The greatest risk of trichinellosis is associated with the consumption of undercooked meat from pigs reared under uncontrolled husbandry conditions (unrestrained/free-range) or from hunted wild boar. For a considerable period, our country has been among the leading EU/EEA Member States in terms of recorded annual incidence. The data from the European Surveillance System (TESSy) for 2022 indicate that 28 European Union/European Economic Area (EU/EEA) countries have reported 39 cases of trichinellosis, representing a 49% decrease as compared to 2021. The notification rate in the EU/EEA is 0.01 cases per 100,000 population. The highest notification rates in the EU/EEA are reported by Latvia and Bulgaria, at 0.16 and 0.13 cases per 100,000 population, respectively (8). Considering those data, the information available for 2023 offers grounds for cautious optimism, given the absence of documented outbreaks and the occurrence of only one isolated case. In 2022, only one outbreak with nine cases was recorded, while in 2021, three outbreaks with 29 cases were documented, respectively (7, 9).

As part of the food- or water-borne diseases human cases of bovine tapeworm are recorded annually in Bulgaria. In 2023, six taeniasis cases were recorded, with the causative agent identified as *Taenia saginata*. In the preceding two years, there were two cases (in 2022) and nine cases (in 2021), respectively. The epidemiological studies identified data on the consumption of sausages that had not undergone thermal processing and were purchased by private individuals. However, the source of the infection remains unclear. Nevertheless, it is irrefutable that the occurrence of infection cases is attributable to the consumption of animal products that have not undergone veterinary inspection. This reinforces the question of the quality control of meat and meat products, as with trichinellosis, produced primarily in small private farms and sold to other individuals without legal control.

Soil-transmitted helminth infections represent a significant global health burden, disproportionately affecting the most vulnerable communities, particularly those with limited access to resources and services. The parasites are transmitted by eggs in human feces, which contaminate the soil in areas with poor sanitation (10). Following improvements in living conditions, the availability of treatment and the implementation of targeted control and health education programs, these infections have been almost completely eradicated in Western Europe. Currently, soil-transmitted helminths are predominantly found among marginalized populations in economically disadvantaged countries in Central Asia and Eastern Europe. Additionally, they are detected among marginalized populations in Central Europe, where environmental and socioeconomic conditions facilitate transmission (11). In Bulgaria, Ascaris lumbricoides and Trichuris trichiura are endemic to specific regions. The data for 2023 does not reveal a trend of dynamic change in the number of STH cases registered and the incidence per 100,000 population as compared to 2022. Overall, the incidence of ascariasis and trichuriasis in the last five years decreased slightly compared to the previous five years and is in the range that does not require mass deworming programmes in the population (Fig.

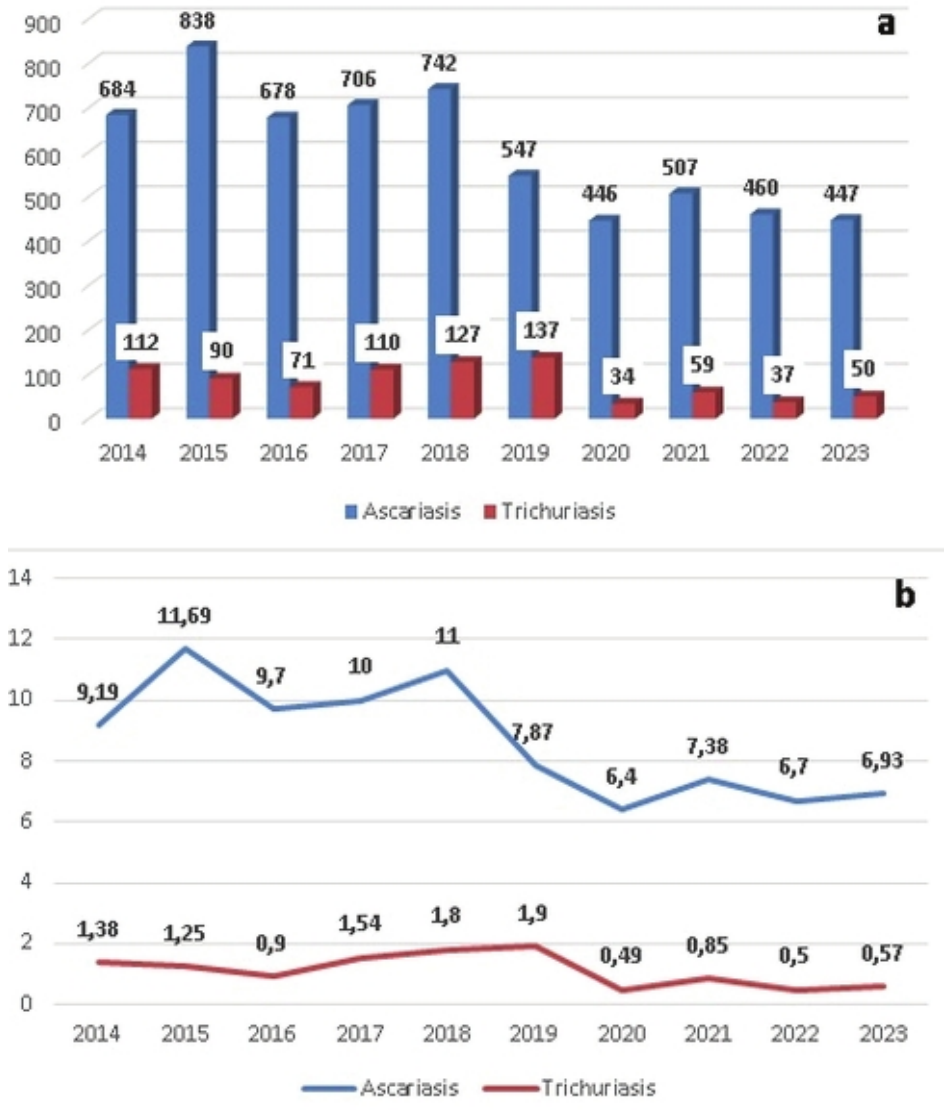

Figure 2. a: Number of cases of ascariasis and trichuriasis registered for the period 2014-2023;
b: Incidence of ascariasis and trichuriasis for the period 2014-2023 per 100 000 population.

2 a, b). Therefore, surveillance and control measures for this group of diseases in the country are adequate and no additional measures are needed.

Among the parasitic infections with a contact mechanism of transmission, enterobiasis is the most prevalent one globally, as well as in Bulgaria. The highest prevalence of the infection is observed in children, with recent studies indicating that 12.9% of children worldwide are infected with *E. vermicularis* (12). Our data for 2023 is comparable, with over 80% of those infected being preschoolers, school-aged children, and adolescents. The elevated proportion of preschool-aged children diagnosed with enterobiasis is attributed to the fact that those attending organized childcare facilities are subjected to annual parasitological examinations. No significant difference in the registered morbidity of giardiasis and hymenolepiasis was observed as compared to previous years. For both diseases, the infected were mostly children of preschool and primary school age.

Like the majority of vector-borne zoonotic diseases, the epidemiology of visceral leishmaniasis is characterised by an uneven temporal distribution of cases and a high degree of unpredictability (Fig. 3). However, given the life-threatening nature of the disease and the annual registration of sporadic local or imported cases, it is imperative that the healthcare system is prepared and has a clear understanding of the diagnostic and treatment algorithms for such cases.

Notwithstanding the alteration in the taxonomic classification of *Pneumocystis jirovecii* and the

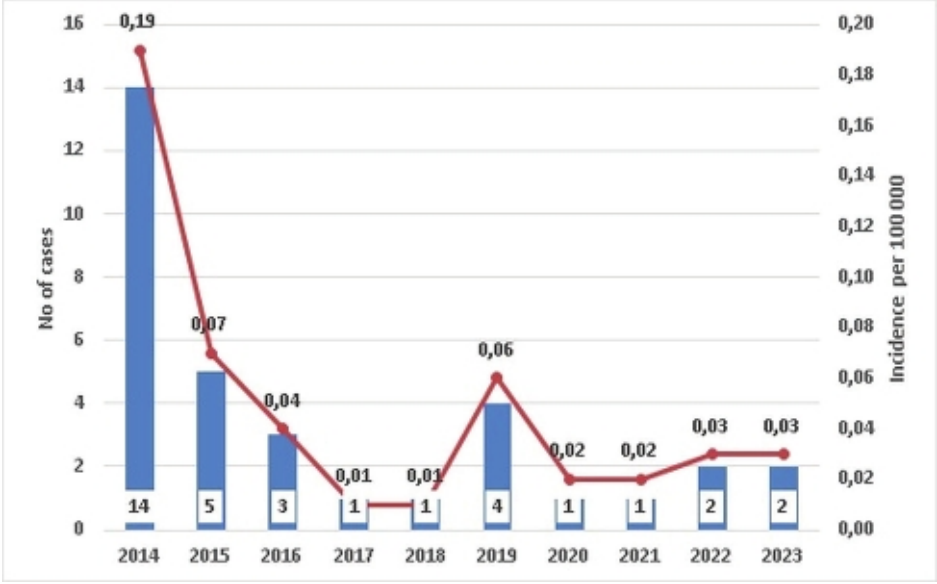

Figure 3. Number of cases and incidence per 100,000 population of visceral leishmaniasis in the period 2014–2023.

current categorization of this pathogen as a fungus causing opportunistic infections, the diagnosis is primarily conducted at the National Reference Laboratory for Diagnosis of Parasitic Diseases at NCIPD. Following the introduction of advanced diagnostic techniques, such as real-time PCR, the detectability of the infection has increased significantly. In 2023, 17 cases of pneumocystis pneumonia (PJP) were diagnosed, compared to 10 in 2022, 11 in 2021 and 18 in 2020 (9). The majority of cases were diagnosed in individuals infected with human immunodeficiency virus (HIV) and in other conditions resulting in immune deficiency. However, Pneumocystis jirovecii pneumonia (PJP) has also been identified in immunocompetent individuals (13).

Malaria is the most significant imported parasitic disease. Between 2014 and 2023, 110 cases of imported malaria were registered in the country with 10 cases occurring in 2023. A review of data from previous years suggests that the predominant cause of imported malaria cases is *P. falciparum*, all recorded imported malaria cases in the country in 2023, and 65% of the cases in the last decade were caused by this plasmodium. It is regrettable to note that a fatal outcome was also recorded in a case last year. Over the 10 years, six fatal cases were reported (Table 2).

All deaths occurring over the past decade have been attributed to complicated tropical malaria. The mean time for establishing an etiological diagnosis in fatal cases was 8 days, with the interval between the initial clinical manifestations and the lethal outcome

Table 2. Registered cases of imported malaria (2014-2023) and the disease outcome (Harizanov et al. 2024).

Year	No of cases	Recovered	Deceased	Case fatality rate
2014	10	9	1	10%
2015	19	20	0	0
2016	28	27	1	3.6%
2017	8	8	0	0
2018	8	8	0	0
2019	8	8	0	0
2020	5	5	0	0
2021	9	7	2	22%
2022	5	4	1	20%
2023	10	9	1	10%
Total	**110**	**104**	**6**	**5%**

averaging 11 days (across all affected). It was observed that none of the patients who had malaria and subsequently died had received chemoprophylaxis during their residence in an endemic area. These data demonstrate that travelers to malaria-endemic areas are not familiar with the mechanisms of infection and prevention measures remain inadequate. This deficiency has clinical and epidemiological consequences. There is a potential risk of the re-emergence of local malaria transmission in Bulgaria. The current level of vulnerability is moderate and is determined by the number of malaria cases imported from endemic countries. In our country, a high level of susceptibility is maintained, due to the presence of suitable climatic and faunal conditions that facilitate the local spread of the disease for the major part of the year. To mitigate the consequences of import and prevent the return of malaria to Bulgaria, it is necessary to maintain a high level of surveillance and control measures regarding this disease (14).

Concerning the remaining parasitic pathologies imported into the country, no notable differences were observed in comparison to the diagnosed local cases, except for one instance of hookworm infection. Importantly, due to control measures concerning the risk groups arriving from endemic countries (predominantly refugees and illegal economic migrants) as previewed in Bulgarian legal framework, no secondary outbreaks of endemic diseases among the local population were recorded as the infected individuals have been promptly treated.

In conclusion, the structure and dynamics of parasitic pathology registered in 2023 do not exhibit significant differences as compared to previous years. However, it is essential to maintain vigilance, particularly in the settings of a declining number of medical parasitologists in the country. According to us, it is necessary to secure the RHIs with personnel trained in this field, given that they are responsible for the primary control measures related to local and imported parasitic infections in the country. Furthermore, providing treatment for tropical malaria and visceral leishmaniasis, which can prove fatal in some cases is of vital importance. In our view, efforts are required to optimize the delivery system for unregistered but highly effective drugs for treatment of life-threatening parasitic diseases.

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SALIVARY SPECIFIC IgE TO D1 AND G6 IN PATIENTS WITH RHINITIS WITH OR WITHOUT *S. AUREUS* COLONIZATION

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ABSTRACT

Globally, allergic rhinitis impacts roughly 25% of children and 40% of adults. Immunoglobulin E (IgE) plays a crucial part in allergic inflammation, with two sources: spontaneously produced IgE, and IgE stemming from reactions to environmental allergens. The damaging effects of *S. aureus*, as well as Staphylococcal enterotoxins (Ses), have been proven in numerous airway illnesses. As superantigens, Ses generate intense Th2 inflammation, with 70-80% of IgE being locally synthesized. Our study aimed to determine if any correlation existed between local and systemic specific IgE responses in rhinitis patients – both treated and untreated. Furthermore, we sought to pinpoint significant disparities in serum allergy-specific IgE levels between *S. aureus* positive and negative patients. Results: From 70 patients with a relevant rhinitis history spanning at least two years, we found that in our rhinitis cohort, 36 were sIgE-negative for d1 in blood samples but positive in saliva samples ($\chi2 = 19.76$, $\alpha = 0.181$), while 25 tested negative for g6 in blood samples but positive for g6 in saliva samples ($\chi2 = 6.89$, $\alpha = 0.86$). No significant difference emerged between serum allergy-specific IgE levels in *S. aureus* positive and negative rhinitis patients ($\chi2 = 0.38$). Similar results were noted within the saliva samples. However, mucosal-specific IgE levels were lower among patients receiving active therapy ($p < 0.001$ for both d1 and g6). Conclusion: There is no correlation between mucosal-specific IgE levels and systemic-specific IgE levels or *S. aureus* carriers. We observed that salivary-specific IgE levels were lower in patients undergoing active treatment compared to untreated patients.

Keywords: mucosal specific IgE; systemic specific igE; local allergic rhinitis; *S. aureus* colonization; IgE production

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INTRODUCTION

Allergic rhinitis, an increasingly prevalent disease, affects roughly 25% of kids and 40% of adults worldwide. Most cases appear before reaching the age of 20. This illness involves a Th2-mediated immune response, where Th2 lymphocytes trigger the synthesis of cytokine subsets that stimulate IgE antibody production by activated B-cells [1].

Staphylococcus aureus (S. aureus) frequently colonizes the upper respiratory tract, and staphylococcal enterotoxins (Ses) can act as superantigens. These antigens bypass crucial immunological processes like antigen processing and presentation by antigen-presenting cells (APC), binding directly outside the peptide-binding pocket. This may leads to excessive production of T cell cytokines by T cells regardless of specificity of their T cell receptor . Staphylococcal enterotoxin B (SeB) is typical T-cell superantigen. The intensity of such a response can reach a degree known as a cytokine storm [2]. The secreted cytokines IL-4, IL-5, and IL-13 are responsible for isotype switching and the preferential synthesis of IgE. Recent research indicates that IgE to SEs is functional. Additionally, these cytokines activate type 2 innate lymphoid cells (ILC-2) which produce extra IL-5 and IL-13, resulting in eosinophilic inflammation [3].Eosinophils are actively involved in allergic inflammation. They infiltrate tissues in the late phase of allergic inflammation. Locally resident eosinophils are regulated by Il- 33 and epithelial derived innate cytokines thymic stromal lymphopoetin (TSLP). These cytokines secreted by activated epithelial cells directly activate eosinophils. Upon activation they

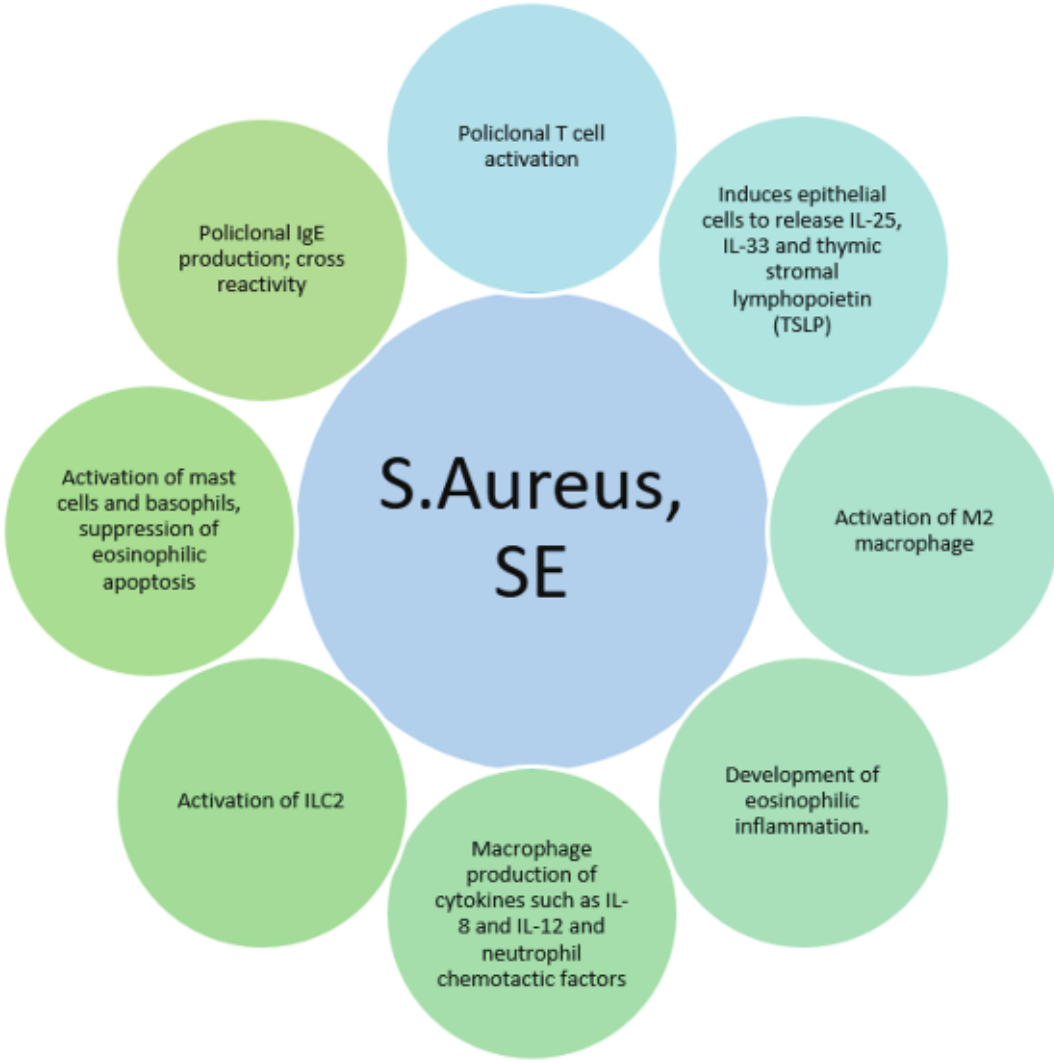

Fig. 1. Role of *S. aureus* and Se in development of inflammation: ***S.****aureus-Staphylococcus aureus* and Se-Staphylococcal enterotoxin- serve as superantigens; Il- 33 and IL25- epithelial derived innate cytokines; TSLP- thymic stromal lymphopoetin epithelial derived during initial stage of allergic inflammation ; M2 macrophage- type 2 macrophages- upon activation release Il8, Il12, neutrophil chemotactic factor and drive the inflammatory cascade; ILC2- Innate lymphoid cells type 2- express cytokine receptors and pathogen-associated receptors of the innate immune response during the early phase of inflammation. Activated ILC 2 secrete IL 4 and IL 13 which potentiate Th 2 inflammatory type.

release eosinophil peroxidase (EPO) and eosinophil derived neurotoxine (EDN). Furthermore, both EPO and EDN stimulate dendritic cells to maturation and antigen presentation [4]. Figure 1 presents the role of *S. aureus* on the development of Th 2 inflammation.

Small doses of antigen (10 micrograms is optimal) adsorbed on mucosal surfaces initiate the IgE response in genetically predisposed individuals [5]. Mature naïve B cells encounter the antigen in the lymph nodes engulfed by mature antigen-presenting cells and presented on their surface for recognition by T cells. After interaction with antigen specific T cells activated B cells migrate to the follicle and extra – follicular region, proliferate, form germinal centers, and differentiate into plasma cells. Moreover, somatic hypermutation (SHM), clonal expansion, and class switching to IgE are realized in musosa [6].

Sensi observed that the production of IgE increased at a faster rate in the nasal mucosa than in the serum of House Dust Mite (HDM) patients following allergen stimulation [7, 8]. Antigen-specific IgE antibodies have been detected in the nasal mucosa 24 hours post Nasal Allergen Provocation Test (NAPT), signifying local antibody synthesis in the nasal mucosa. Since

the 1970s, literature has indicated that 70-80% of IgE is locally produced[9, 10]. In fact, determination of local IgE in the absence of systemic IgE has diagnostic and therapeutic consequences. .

In 2000, Klein Jan and Cameron identified local IgE synthesis in the nasal B cells and plasma cells of individuals suffering from Allergic Rhinitis (AR) [7]. Subsequently, Rondon devised the current definition of Local Allergic Rhinitis (LAR) [7]. Gelardi documented the existence of allergen-specific IgE (asIgE) in the nasal mucosa of patients with allergic rhinitis, non-allergic rhinitis, and healthy controls. The authors reported local IgE detection across all three groups and inferred that local IgE generation could be a normal response to environmental allergens[11].

In a clinical context, both allergic rhinitis (AR) and local allergic rhinitis (LAR) [11] exhibit characteristics common to each type of rhinitis, such as rhinorrhea, sneezing, and nasal itching. LAR refers to patients with a history of allergic disease who have negative skin and blood tests for allergy. These individuals usually display localized signs of atopy, including the presence of sIgE in nasal secretions, positive nasal allergen provocation tests, and responsiveness to allergen-specific immunotherapy. Although multiple endotypes of rhinitis exist, distinctions between them are not consistently well-defined. The precedence of local IgE production over systemic sensitization remains uncertain. Patients with AR may exhibit nasal reactivity to various allergens despite the absence of allergen-specific IgE on the skin or in serum for both allergens, a condition known as "double allergic rhinitis" [1].

Skin prick tests and ssIgE immunoassays are the predominant laboratory tests employed for identifying potential allergens; however, no definitive "gold standard" laboratory test exists for diagnosing AR. The aim of our research was to evaluate the potential relationship between *S. aureus* nasal colonization and local as well as systemic specific IgE production in patients with rhinitis, irrespective of whether they have received treatment.

MATERIALS AND METHODS

Study design

An observational study was carried out utilizing intricate clinical and laboratory methodologies on a cohort of 70 patients possessing a clinically significant history of rhinitis for at least two years. Inclusion criteria consisted of a comprehensive evaluation of atopic status, incorporating specific IgE to Derp1 and g6 in saliva, alongside serum-specific IgE against inhalant allergens and a clinically relevant rhinitis history with or without concomitant

Table 1. Inclusion and exclusion criteria:

Criteria	Inclusion	Exclusion
Age	Over 6 years (cooperation in the collection of sputum samples)	Less than 6 years
Active rhinitis	History of previously confirmed allergic rhinitis or persistent rhinitis complaints for at least 2 months.	History of previous antibiotic therapy (local or systemic) for persistent rhinitis symptoms.
Comorbidities	Asthma and other disorders related to atopic march	Nasal polyposis
Therapy	Application of intranasal corticosteroids and/or immunotherapy is permissible Lack of active therapy (intranasal corticosteroids and/or immunotherapy) is not a contraindication for inclusion.	Biologicals
Other		Pregnancy

Children and adults with clinically significant history of rhinitis for at least two years .Blood and saliva samples were gathered during polen season when when active production of a specific IgE is expected. All patients must have active symptoms. Previous infectious disease or administration of antibiotics or biological therapy were exclusion criteria. Nasal polyposis as a different clinical entity was also an exclusion criteria. Local steroid therapy and application of antihistamines were permissible as not disease-modifying drugs. Immunotherapy was not an exclusion criteria Local biomarkers investigated in this study might serve as valuable biomarker throughout the course.

asthma. A thorough medical history was gathered for all patients, encompassing the type of therapy administered during sample acquisition and the presence of comorbidities. All participants were guided on the proper technique for procuring unstimulated saliva samples to facilitate subsequent scrutiny of local IgE production. Additionally, nasal swabs were obtained from every individual involved in the study. Full list of inclusion and exclusion criteria is presented in Table 1.

Collection of saliva and blood samples was performed during polen season. Specific IgE in saliva and serum samples is measured at only one time point. Another limitation is lack of healthy controls. Study design is presented on Fig2.

Immunological assessment

Serological evaluations were conducted utilizing the Euroline Allergy Profile Inhalation and Enzyme Allergo Sorbent Test (EAST) by Euroimmun® (Medizinische Labordiagnostica, AG, 2014, Luebeck, Germany), encompassing prevalent aero-allergens. The EUROLINE method yields semi-quantitative results demonstrated via the EAST system in seven categories, ranging from class 0 to 6 (<0.35 kU/L EAST class 0 to >100 kU/L EAST class 6). Analysis of specific IgE antibodies pertaining to d1-Dpt allergen and g6-timothy allergen in saliva specimens was performed using the ImmunoCAP system (Thermo Fisher Scientific, Uppsala, Sweden). All serological examinations were completed within an accredited Laboratory of Clinical Immunology.

These two methods are categorized in the subsequent data evaluation by EAST classes from 0 to 6 class. Such an approach would allow a comparative analysis between blood and saliva samples. In fact, the ImmunoCAP system detected

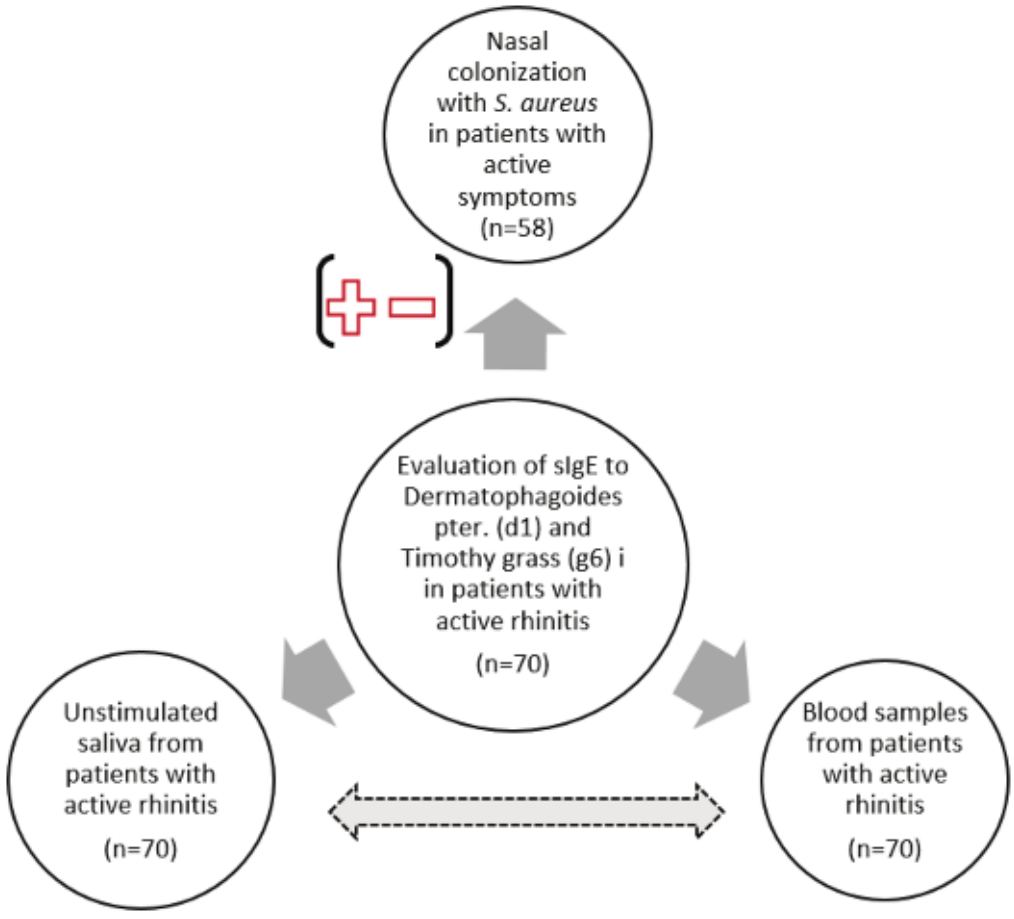

Fig. 2. Study desing: Unstimulated saliva (n=70), serum from blood samples (n=70) and nasal swabs of patients (n=58) with clinical features of active rhinitis were collected and analyzed. Saliva and serum samples (n=70) were analyzed using ImmunoCAP system (quantitative method) and Euroline Allergy Profile Inhalation (semi-quantitative method), respectively, and results compared. Nasal swabs (n=58) were cultured and subsecuently analyzed for *S. aureus* using matrix-assisted laser desorption ionization-time of flight mass spectrometry.

much lower concentrations of the specific IgE to d1 and d6 lower than 0.35 KU/L. Such quantities are a practically imperceptible for Euroline system. It is speculative to claim that such a low concentrations of specific IgE in saliva samples have prominent clinical significance considering the study included patients with active rhinitis symptoms. In fact, it is necessary to emphasize that all participants in this study have clinically confirmed symptoms . On the other hand active treatment is typical for most included patients. According to literature in majority of studies in this field local sIgE response have been measured in nasal swabs. However, we should note that the collection of saliva is less traumatic for the patients than collection of nasal swabs. Saliva could be a suitable material in evaluation of atopy most probably among children.

Sample processing and identification.

Nasal samples were streaked onto Columbia Agar containing 5% sheep blood (BD™ Becton Dickinson GmbH, Heidelberg, Germany) and incubated for 18-20 hours at 35°C. Additionally, the nasal swabs were cultivated in tryptic soy broth (Soybean-Casein Digest Medium, BD™ Becton Dickinson GmbH, Heidelberg, Germany) for enrichment purposes. Broth cultures that emerged were subsequently plated on Columbia agar plates only if the initial swab culture remained sterile. Single colonies exhibiting morphological characteristics similar to *S. aureus* were restreaked to obtain pure cultures and identified utilizing matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics, Billerica, MA, USA). In summary, a single colony from these pure cultures was placed on a polished steel MSP 96 target (Bruker Daltonics, Billerica, MA, USA) and covered with 1 µL of saturated-cyano-4-hydroxycinnamic acid (HCCA) matrix solution (Bruker Daltonics). Strains that remained unidentified

Table 2 Demographic and clinical characteristics of subjects:

Disease		Gender		Age
		Male	Female	
Allergic rhinitis	Count	16	26	
	%	66.7%	56.5%	
	Mean			26.21
	Maximum			53.00
	Minimum			6.00
	Standard Deviation			12.63
Asthma and allergic rhinitis	Count	0	3	
	%	.0%	6.5%	
	Mean			37.67
	Maximum			41.00
	Minimum			34.00
	Standard Deviation			3.51
Rhinitis	Count	8	17	
	%	33.3%	37.0%	
	Mean			34.84
	Maximum			52.00
	Minimum			19.00
	Standard Deviation			9.56
Total	Count	24	46	
	%	100.0%	100.0%	
	Mean			29.79
	Maximum			53.00
	Minimum			6.00
	Standard Deviation			12.11

From a total of 70 patients analyzed, 42 had allergic rhinitis (M/F: 16/26; mean ± SD age: 26.21 ± 12.63 yr) and 25 were with persistent rhinitis sumptoms and negative serum samples for any inhalant allergen (M/F: 8/17; mean ± SD age: 34.84 ± 9.56 yr).

underwent resubmission using the extended protocol involving 1 µL of 70% formic acid. The acquired mass spectra were analyzed employing a microflex LT mass spectrometer (Bruker Daltonics), utilizing the research-use-only (RUO) software workflow and reference library MBT v. 4.1.100. All isolates were successfully identified with scores exceeding 2.0.

Statistical methods

Statistical analyses were conducted utilizing SPSS®, IBM 2009 version 19 and Graph Pad Prism version 9.0.0 . Descriptive statistics were employed to delineate the clinical and demographic features of patients, treatment status, and immunological parameters. One-way ANOVA analysis and correlation analysis were calculated between category characteristic. A significance threshold of $\alpha = 0.05$ was established. If $p < \alpha$, the null hypothesis is rejected.

RESULTS

Demographic and clinical characteristics

Seventy patients were included in the study. Demographic and clinical characeristics of the study group are shown on Table 2

As per the research methodology, all participants had a documented history of rhinitis persisting for a minimum of two years. Among them, 42 subjects (60%) were previously diagnosed with allergic rhinitis. Concurrent diagnoses of rhinitis and asthma were reported by three patients, while 25 participants (37,5%) exhibited rhinitis symptoms of unidentified etiology. In group with LAR (n=20) were not receiving therapy at the time of inclusion in the study and n=40 were receiving intranasal corticosteroids or had started sublingual immunotherapy. In addition, we tested 12 volunteers. Specific IgE for any inhalant allergen in saliva samples was detected in all of them. Such a result guided our subsequent analysis on the effect of therapy on the amount of IgE in saliva samples.

S. aureus colonization in patients with rhinitis

Nasal samples were evaluate for *S. aureus* in order to to assess its influence on the production of a specific IgE.A total of 58 nasal swabs were obtained and analyzed following the procedure outlined by the manufacturer. *S. aureus* was identified in 23 rhinitis-afflicted participants. Meanwhile, 39 serum samples tested positive for at least one specific aeroallergen. Observing the relationship between *S. aureus* carriage and specific IgE presence revealed no significant distinction between the serum allergen-specific IgE levels in *S. aureus*-positive and *S. aureus*-negative rhinitis patients ($\chi 2 = 0.38$) A comparable analysis of the saliva samples yielded analogous outcomes, as delineated in figure 2 . We performed a one-way analysis of variance ANOVA ($F= 0.40$, $p= 0.749$) and did not found any statistically significant differences between groups with *S. aureus* colonization in the nasal cavity and without colonization. Locally produced specific IgE to Dermatophagoides pter. (d1) and Timothy grass (g6) seem to be independent processes. No significant differences were found between groups as shown in figure 3

Local and systemic specific IgE distribution

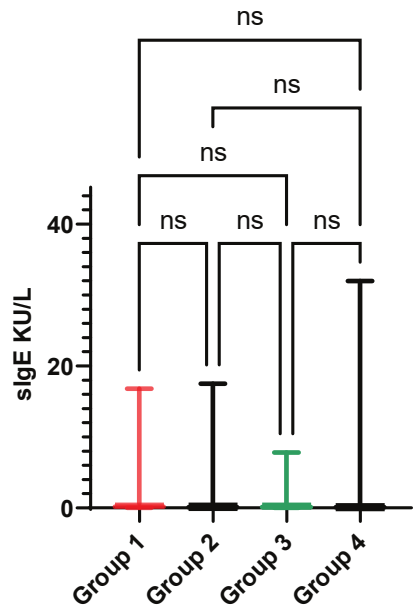

Fig. 3. *S. aureus* colonization and S IgE in saliva samples: Patients were divided into four groups according to two criteria: colonization with *S. aureus* and detection of d1 or g6 specific IgE in saliva samples**. Group 1**: ***S. aureus*** positive nasal swab/ d1 sIgE detected in saliva; **Group 2**: *S. aureus* negatve nasal swab/ d1 sIgE detected in saliva; **Group 3**: *S. aureus* positive nasal swab/ g6 sIgE detected in saliva; **Group 4**: *S. aureus* negative nasal swab/ g6 sIgE detected in saliva. No significant differences were found between groups.

In an immunological examination, the local and systemic detection of specific IgE antibodies to allergens Dermatophagoides pteronissinus (d1) and Timothy grass (g6) allowed for the categorization of patients into distinct groups. We utilized the quantification of specific IgE in saliva and serum samples as a distinguishing factor for these classifications: No specific antibodies detected (IgE < 0.35 KU/L for serum samples, or even lower for saliva samples); Weak antibody detection (0.7 KU/L < IgE < 3.5 KU/L); Defined antibody detection (3.5 KU/L < IgE < 17.5 KU/L); Strong antibody detection (17.5 KU/L < IgE < 50 KU/L); High antibody titer (17.5 KU/L < IgE < 50 KU/L); Very high antibody titer (50 KU/L < IgE < 100 KU/L). In our study, we identified d1-specific IgE in the saliva samples from 60 patients. Among these individuals, 36 demonstrated no detectable d1-specific antibodies in their serum samples. Cases of timothy grass sensitization (g6) 44 participants revealed the presence of IgE in saliva samples. However, 20 of these individuals exhibited no specific antibodies against g6 in their serum samples. Relationships between serum and salivary IgE to the two allergens are presented in figure 4.

Analysis of variance of the variance among serum and saliva specific Ig E to Dermatophagoider pter. and Timothy grass showed a statistically significant dependence between saliva d1 sIgE vs. Serum g6 sIgE and serum g6 sIgE vs. Saliva g6 sIgE (F 7.380, p<0.0001). Comparison of all other variables showed no statistical significance. Such a result may be due to the time of sample collection. During polen season active production of a specific IgE is expected in genetically susceptible individuals.It is scientifically acknowledged that IgE can constitute a normal immune response to various antigens without

Treatment and Dermatophagoides pter. sIgE in sa

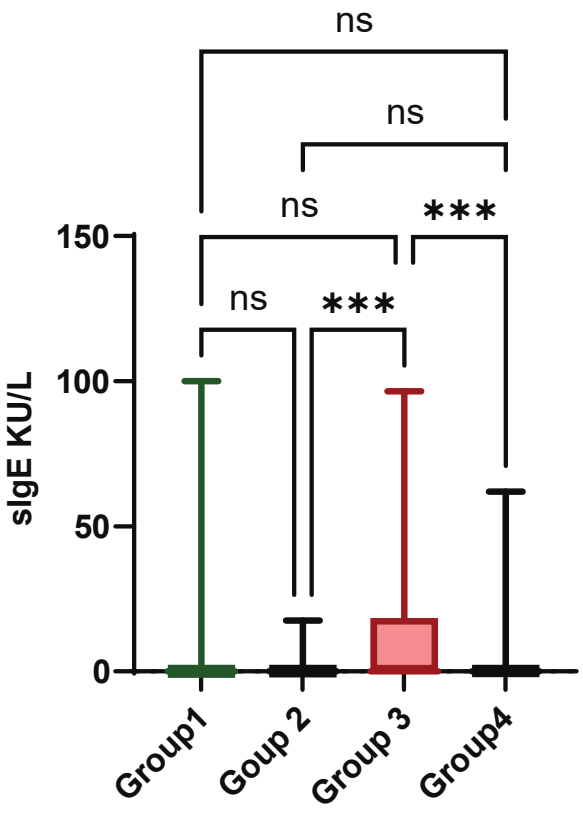

Fig. 4. Serum and saliva sIgE levels: Patients were divided into four groups according to the detection of specific IgE in saliva and blood samples. **Group 1:** Patients with positive serum samples to Dermatophagoides pter. (d1); **Group 2:** Patients with positive saliva samples to d1; **Group 3:** Patients with positive serum samples to Timothy grass (g6); **Group 4:** Patients with positive saliva samples to g6

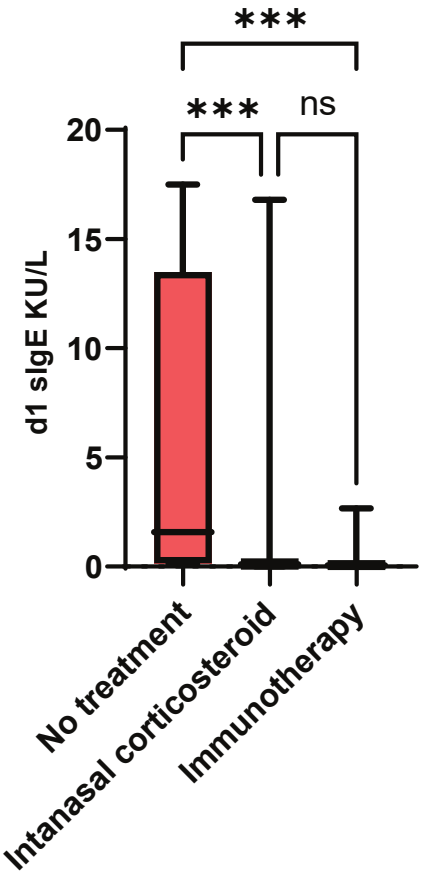

Fig. 5. Influence of therapy: Higher concentration of specific IgE in saliva samples in untreated patients was detected. No superiority of either therapy was found.

being linked to clinical manifestations. However, it is important to emphasize that our study's inclusion criterion is the presence of persistent rhinitis symptoms for at least two months. This fact directs our focus towards whether patients are undergoing treatment or not. A significant percentage of the participants in our research received nasal steroid treatment or immunotherapy, which may be attributed to the inclusion criteria for clinically relevant rhinitis. Among them, 47.5% (n = 32) reported using intranasal steroids, 25.7% (n = 18) were undergoing immunotherapy, and 28.6% (n = 20) were not receiving any treatment.

In order to evaluate the influence of therapy on the local production of specific IgE, we applied a one-factor analysis. Results are shown on fig 5.

Tukey's multiple comparisons test showed a higher concentration of specific IgE in saliva samples in untreated patients. No superiority of either therapy approaches was found ($F=11.43$, $p<0.0001$)

DISCUSSION

Immunoglobulin E (IgE) is widely recognized as a crucial mediator in allergic inflammation and helminth infections. There is a significant overlap in serum levels of total IgE between allergic and non-allergic individuals, which may be attributed to geographical variations in the prevalence of exposure to helminth infections, as the concentration of IgE in human serum rapidly escalates during such an infection. Moreover, "growth curves" of total IgE levels are established during childhood in children without atopy by determining total IgE levels at various time points during growth. Indeed, the trajectory of total IgE during different time points in childhood is strikingly similar in never-allergic and allergic children, and this process is independent of the absolute concentration of total IgE. These total IgE levels are derived from two distinct sources: spontaneously produced IgE ("normal," baseline IgE) and specific IgE generated in response to environmental allergens ("abnormal" or atopic IgE). Seasonal exposure to allergens can trigger a rapid increase in allergen-specific IgE levels, which can consequently elevate total IgE levels. As a result, higher IgE levels can be sustained through repeated contact with allergens. Repeated exposure to inhalant or food allergens serves as a prime example of maintaining this process. Furthermore, populations of IgE-secreting plasma cells are significantly higher in the nasal mucosa compared to peripheral cell populations. This fact highlights the local production of secretions as an especially intriguing focus for research on key components in the pathogenesis of diseases with primarily organ-localized manifestations, such as allergic rhinoconjunctivitis. Comparative analyses of specific IgE immunoglobulin levels in serum and saliva have been performed for various food allergens, such as shrimp, eggs, soy, wheat, chestnut, peanuts, kiwi, banana, tomato, and cocoa. In the majority of foods, no significant difference was observed between serum and saliva levels. However, the mean concentration of IgE antibodies against cow's milk and papaya in serum was markedly lower than that in saliva ($p<0.05$), whereas the mean concentration of IgE antibodies against fish and corn in serum was notably higher than that in saliva ($p<0.05$) [12-15].

Another potential mechanism for maintaining the total IgE pool is polyclonal T cell activation through microbial stimulation. Gram-positive microorganisms has been identified in the role of superantigens. As superantigens may also served: coagulase-negative staphylococci, beta-hemolytic streptococci (groups B, C, and G), *Mycoplasma arthritidis, Yersinia enterocolitica, Yersinia pseudotuberculosis, Plasmodium falciparum, Clostridium perfringens, Candida albicans, and Toxoplasma gondii. S.aureus* strains secreted up to 24 different superantigens. In case of group A streptococcal strains 11 types are reported [16].

S. aureus superantigens (SAgs) stimulate large proportion of T cells by cross-linking their T cell receptor[17].

The pathogenic role of *S. aureus* and its Ses has been recognized in various airway diseases such as chronic rhinosinusitis with nasal polyposis and severe asthma [3]. Furthermore, scientific literature indicates that toxic shock syndrome toxin (TSST-1) amplifies the allergen-specific IgE production in vitro. This effect was regulated by IFNγ and IL-4 concentrations. During the pollen season restimulation of in vivo-primed peripheral blood mononuclear cells with TSST-1 induced the allergen-specific IgE production in vitro. Outside the polen season additional exogeneous IL4

is needed to induce allergen- specific IgE production in vitro[17].

S. aureus might exacerbate local eosinophilic inflammation, thereby intensifying the overall nasal symptom score. Some studies have suggested that nasal S. aureus or specific IgE in serum against Ses are correlated with an increased risk of asthma and heightened symptom severity [18]. By functioning as superantigens, SEs can provoke intense Th2 inflammation. Nobusuke Hohchi documented the impact of *S. aureus* colonization in a mouse model of allergic rhinitis wherein the Ovalbumin-sensitized *S. aureus*-inoculated (AR-SA) group exhibited elevated eosinophil and neutrophil counts, IgE and IgG1 levels, and expressions of IL-4 mRNA and IL-5 mRNA compared to control groups. Furthermore, higher *S. aureus* counts were detected in the nasal mucosa of patients in the same group [19]. It is fascinating to explore the possibility that *S. aureus* contributes to the increased production of serum-specific IgE in response to various inhalant allergens by amplifying the Th2 immune response. Our findings suggest that there is no association between *S. aureus* colonization and serum-specific IgE levels, as well as saliva-specific IgE levels, in patients with rhinitis ($\chi2 = 0.38$ for serum samples and $\chi2 = 0.113$ for Dermatophagoides pter d1 and $\chi2 = 0.474$ for thymothy grass g6 in saliva samples, $F= 0.40$, $p= 0.749$).

The majority of serum IgE is derived from mucosal surfaces [9]. During the initial development of allergic rhinitis, allergen provocation triggers IgE production at the mucosal level, with a minor fraction eventually entering the blood stream . According to manufacturers' instructions, specific IgE concentrations of 0.35 KU/L or higher become detectable. The process of entering circulation is likely influenced by multiple factors such as allergen exposure frequency, age, and presence or absence of modifying therapy. Conversely, various studies in this field have followed patients for different periods; long-term studies remain scarce.

Ramadani et al. demonstrated that local IgE production rates are sufficient to saturate the entire IgE receptor system on mast cells present at the site before ultimately entering the bloodstream . In cases of allergic rhinitis, symptoms may manifest only when specific local IgEs are present. "Local allergic rhinitis" (LAR) is a recognized endotype of rhinitis involving nasal allergen provocation tests (NAPT) in patients exhibiting typical allergic rhinitis symptoms but testing negative for skin prick tests (SPT) or serum-specific IgE against any allergen [7, 12]. The exact prevalence of LAR remains uncertain; however, it ranges from approximately 25% to 45% among patients with chronic, non-infectious rhinitis [7, 12]. Several research studies have suggested that local allergic rhinitis (LAR) does not progress to allergic rhinitis (AR) accompanied by systemic atopy over time. Conversely, other investigations have posited that "local IgE" levels may be a risk factor for systemic IgE-dependent reactions [7, 8]. Mortada et al. in their review [8] provide data from a 10-year follow-up of LAR patients, indicating that only small percentage of them developed systemic sensitization. On the other hand LAR is considered as a significant risk factor in the emergence of asthma. Another systemic review describes a high frequency of LAR among children [15] Terada et al described that nasal allergen reactivity was present in 16.1% of children under 16 years old with non-allergic rhinitis (NAR) and frequency of LAR may increase with age [9, 15]. There is potential for the initial stage of allergic rhinitis to exhibit serum concentrations of free IgE too low for detection by current IgE tests [12]. Indeed, immune responses to allergens in healthy individuals necessitate disparate antibody production, consisting of low IgG1, IgG4, and secretory IgA (sIgA) levels, with or without a minimal amount of IgE present [20].

It is hypothesized that local IgE production, with initial negative Skin Prick Test (SPT) and IgE test, represents a stage of allergic rhinitis. This stage may persist for an extended duration, particularly among individuals with very low IgE production. Treatment using monoclonal anti-IgE antibodies, which bind to allergen-specific IgE present in the circulation, might lead to elevated serum IgE levels. Consequently, low producers may become detectable. In our study, patients receiving omalizumab were not included.

In our cohort of patients diagnosed with rhinitis, 36 individuals demonstrated specific IgE-negativity for Dermatophagoides preronissinus (d1) in blood samples and positivity in saliva samples ($\chi2 = 19.76$, $\alpha = 0.181$), whereas 25 patients were negative for g6 in blood samples and positive for g6 in

saliva samples ($\chi 2 = 6.89$, $\alpha = 0.86$). In accordance with previous research, our study verified the independent manifestation of a local IgE-specific response and systemic sensitization to the same or another allergen. In addition we performed analyses of variance and found statistically significant dependence between saliva dermatophagoides pter. (d1) sIgE vs. Serum timothy grass (g6) sIgE and serum thymothy grass sIgE vs. Saliva timothy grass sIgE (F 7.380, $p<0.0001$). Such a result may be due to the point of sample collection. All samples are gathered during polen season. Earlier investigations confirmed Local Allergic Rhinitis (LAR) among children exhibiting significantly higher nasal sIgE levels (nasal sIgE > 0.35 kU/L according to EAST classification system) compared to control subjects and positive results in the Nasal Allergen Provocation Test (NAPT). Dust mites represent the most prevalent allergens in LAR. In our study population of sixty patients, we identified IgE specific to dermatophagoides pter. (d1) in saliva samples. Among these individuals, thirty-six did not display specific antibodies to Dermatophagoides pter. (d1) in their serum samples. As for timothy grass (g6) sensitization, forty-four participants exhibited salivary IgE specificity toward timothy grass (g6). Within the same group, twenty individuals lacked specific antibodies against timothy grass in their serum samples. In contrast, Miranda reported undetectable levels of specific IgE in saliva samples among atopic and non-atopic children [21]. However, follow-up individuals at least in two time points would contribute to clarifying the dynamic of local IgE response in atopics and non-atopics.

When the constituents of saliva originate from the bloodstream, the concentrations of biochemical and immunological components present in the saliva can mirror their levels in the blood. There is a growing interest in substituting blood samples with salivary samples for analyzing various biomarkers, owing to the non-invasive nature of saliva collection and the elimination of risks linked to blood sample acquisition [13]. Saliva can be gathered and assessed as either unstimulated or stimulated from specific glandular pairs. Unstimulated whole saliva refers to the initial saliva found in the oral cavity for a majority of a 24-hour period [13]. Identifying diverse biomarkers in saliva can potentially serve as an invaluable technique in monitoring the management of chronic diseases. The ImmunoCAP system is recognized as the optimal approach for quantifying specific IgE in nasal swabs, with 0.12 kUA/L established as the threshold value [22].

In our study, we observed reduced levels below 0.12 kUA/L in the patient group (0.04 KU/L was the lowest level of detection). It is crucial to note that these findings were primarily conducted within the context of implemented therapy for most participants. Specific IgE levels were assessed at a single time point during the pollen season, underlining the importance of continuously monitoring local-specific IgE production in patients with rhinitis. The non-invasive nature of this test renders it an appropriate biomarker for therapy evaluation, disease management, and diagnostic precision.

Egger [23] stated that fluticasone propionate application did not significantly influence the increase in systemic allergen-specific IgE production following allergen exposure. Wilson [24] suggested that grass pollen immunotherapy could hinder the seasonal rise of basophils and eosinophils in the nasal epithelium among individuals with allergic rhinitis. It has also been reported that immunotherapy can increase the ratio of IFN-γ to IL-5 micro RNA cells within nasal mucosa [25].

The primary treatment for individuals with asthma and allergic rhinitis involves the local administration of corticosteroids. In a study conducted by Jerome Kerzerho, the impact of systemic and local corticosteroid administration on mucosal tolerance development in patients with bronchial asthma was examined . The results demonstrated that inhaled corticosteroids enhanced the development of Treg cells [26]. Our research further revealed that specific IgE levels in saliva were significantly associated with the course of treatment for Dermatophagoides pter. (d1) and Tymothy grass (g6) saliva samples, as indicated by single factor analysis: $F(2,57) = 21.85$, $P < .001$ for Dermatophagoides pter. (d1), and $F(2,41) = 12.42$, $P < .001$ for Timothy grass (g6), with Eta Squared values of 0.411 for d1 and 0.377 for g6. Tukey's multiple comparisons test showed a higher concentration of specific IgE in saliva samples in untreated patients ($F=11.43$, $p <0.0001$).

CONCLUSION

In the examined cohort, localized IgE production to allergens d1 and g6 plays a significant role in the progression of rhinitis. It is important to note that mucosal-specific IgE concentrations do not exhibit a correlation with systemic specific IgE levels or *S. aureus* colonization. Our findings reveal a reduced presence of salivary-specific IgE in patients undergoing active treatment compared to those without treatment. This observation highlights the potential utility of local specific IgE as a reliable biomarker for individuals suffering from rhinitis within an academic context.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by ETHICS COMMITTEE IRB 00006384 (protocol code PROTOCOL № 3/2024 and May 09, 2024)."

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CONFLICT OF INTEREST STATEMENT (AUTHORS)

I certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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I certify that have no personal or financial conflict of interest with authors of the manuscript provided me for review.

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AND ANIMALS IN RESEARCH

I certify that this study involving human subjects is in accordance with the Helsinky declaration of 1975 as revised in 2000 and that it has been approved by the relevant institutional Ethical Committee.

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I certify that this study involving animals followed the institutional and national guide for the care and use of laboratory animals.

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