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Cryptosporidiosis – diagnostic, experimental and epidemiological studies

ABSTRACT

from a dissertation for granting of the educational and scientific degree

DOCTOR

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The dissertation comprises 199 standard pages and is divided in the following sections: Introduction, Review of Scientific Materials, Purpose and Tasks, Materials and Methods, Results and Discussion, Conclusion, Deductions, Contributions. It is illustrated by means of 26 tables and 59 figures. References comprise 397 bibliographic sources, of which 55 in Bulgarian and 342 in English language.

The laboratory analyses included in the dissertation have been performed at the following locations:

- Department of Anatomy, Histology, Cytology and Biology, sector Biology, faculty of Medicine, Medical University of Pleven
- National Reference Laboratory, Diagnostics of Local and Tropical Parasites, Department of Parasitology and Tropical Medicine of the National Centre for Infectious and Parasitic Diseases, Sofia.

In connection with the dissertation, three full-text publications and four scientific announcements have been made at national fora.

The dissertation has been approved and referred for public defence by the Department Council at the Anatomy, Histology, Cytology and Biology Department of the Faculty of Medicine of the Medical University of Pleven held on the 29 November 2024.

The public defence of the dissertation will take place on the 6 February 2025 at 13.00 in Ambroise Paré hall of the Medical University of Pleven according to the rules and regulations on obtaining scientific degrees and occupation of academic positions of the Medical University of Pleven and on the grounds of Order No 3762/17.12.2024 of the Rector of the University in front of the following scientific jury:

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The materials related to the defence have been published on the website of the Medical University of Pleven: http://www.mu-pleven.bg/index.php/bg/

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List of abbreviations

CDC – Center for Disease Control and Prevention
ECDC – European Centre for Disease Prevention and Control
EFSA – European Food Safety Authority
ELISA – Enzyme-linked immunosorbent assay
GIDEON – Global infectious Diseases and Epidemiology Network
HIV – Human Immunodeficiency Virus
ICZN – International Code for Zoological Nomenclature
INFγ - interferon gamma
IgG – Immunoglobulin G
IgM – Immunoglobulin M
IgA – Immunoglobulin A
n = absolute number of studied cases
NK – natural killer cells
nPCR – nested Polymerase Chain Reaction
NTZ – Nitazoxanide
NTD – Neglected Tropical Diseases
P – value of the level of significance
PCR – Polymerase Chain Reaction
RFLP – Restriction Fragment Length Polymorphism
TD – Travel Disease
WHO – World Health Organization

I. INTRODUCTION

Cryptosporidiosis is a parasitic disease (reverse zoonotic protozoosis), which manifests itself mainly through tightening of the digestive system and diarrhoea of various degrees – from mild to severe watery diarrhoea, sometimes accompanied by life-threatening gastroenteritis. According to the data presented by the World Health Organisation, over 58 million of the cases of diarrhoea established in children on an annual basis, are connected with infections with intestinal protozoa with high levels of morbidity and mortality. In the developed countries, diarrhoea is the most frequent reason for absence from work, whereas in the developing countries it is the leading cause of death. Against this background, *Cryptosporidium* spp. is the main cause of diseases accompanied by the symptom of diarrhoea worldwide.

The disease is caused by intestinal *Coccidia*, apicomplexa protozoa of the *Cryptosporidium* genus. Sources of infection can be both animals (over 170 species of mammals, birds, reptiles, fish) and humans (infected or carrier). With their faeces they discharge oocysts containing sporozoites capable of invasion.

In many of the developed countries there are no systems in place to control cryptosporidiosis. Little research has been carried out to allow for an evaluation of its distribution, which can vary in time. In tropical countries and in the economically underdeveloped countries with low standards for hygiene, where the distribution of parasites among people is at the highest, cryptosporidiosis is a neglected parasitosis. This is due to the lack of resources to carry out testing and examination of the population.

Various ecological, climate, health, socio-economical, labour, household and other factors can influence the distribution of intestinal parasite invasions. The distribution of cryptosporidiosis is connected with the increase in migrant flows, the development of tourism, natural disasters (earthquakes, floods, draught and lack of drinking water), military actions, the presence of natural reservoirs of the infection, the low invasion dose and the high resistance of the parasite to disinfectants and antiparasitic substances. The contamination of the urban waste waters and sediments is a factor for the development of oocysts of *Cryptosporidium* spp.

The World Health Organisation has classified the four main opportunistic parasitoses — cryptosporidiosis, cyclosporidiosis, cycstosporidiosis and toxoplasmosis, which are associated with acquired immune insufficiency, as significant in terms of health. With the exception of toxoplasmosis, the remaining diseases are diagnosed more rarely, which is associated with imperfections of the diagnostics equipment, the necessity of special colouring of substances which is

not performed in all laboratories, and sometimes with the lack of professional knowledge.

The results of seroepidemiological research show that cryptosporidiosis is likely to be much more frequently occurring than diagnosed. Parasitic diseases, in particular cryptosporidiosis and giardiasis, are the cause of many cases of gastroenterological infections in Europe, including in Bulgaria, but it is suspected that such cases are not diagnosed, reported and registered. The severity of the clinical picture of such diseases remains not clear enough. There are reported data for Sofia, Varna, Plovdiv and Veliko Tarnovo, but currently there are no registered or published cases of diseases caused by cryptosporidiosis among the population of the district of Pleven.

As long as specific prophylactic activities are not at hand, diagnostics is the basis of the successful supervision and control of cryptosporidiosis. Every single case of cryptosporidiosis confirmed by a parasitological analysis is subject to mandatory reporting and registration at the Regional Health Inspectorates with a copy to the District Food Safety Inspectorates.

Cryptosporidiosis is an intestinal protozoan infection that is less known to the health network (general practitioners and specialists such as gastroenterologists, paediatricians, infectionists, etc.). Therefore, the disease is not a subject of particular attention. Patients with diarrhoea are not referred to parasitology analysis. The disease is also seriously underestimated by the directors of the Regional Health Inspectorates, the centres for diagnostics and consultation and the independent medical diagnostics laboratories. Furthermore, there is insufficient knowledge about this parasitosis among medical practitioners (nurses, midwives, medical assistants). Therefore, patients with gastroenteritis returning from seaside resorts or having visited public swimming pools are usually admitted to the infectious diseases wards of hospitals after having been diagnosed with “enteroviral infection”.

Against this background, enhanced observation and systematic analyses in connection with the epidemiology, distribution and diagnostics of cryptosporidiosis in Bulgaria in general and in the district of Pleven in particular are required, which is also one of the main purposes of the present dissertation. A significant correlation between the goals set by the European healthcare institutions in the 21 century and the present research paper is to study some diagnostic and epidemiological characteristics of cryptosporidiosis among the most vulnerable risk groups of the population of the region of Pleven.

II. PURPOSE AND TASKS OF THE DISSERTATION

1. Purpose

The purpose of the present dissertation is to study the biology, distribution, diagnostics and epidemiology of cryptosporidiosis among target groups at risk from the population of the district of Pleven and the nearby regions, as well as among animals, through the application of contemporary methods for diagnostics.

2. Tasks

The scientific purpose of the dissertation is achieved through the following tasks:

2.1 To establish the distribution of cryptosporidiosis among groups at risk from the population of the city and the region of Pleven according to the main demographic factors age, gender, place of residence and ethnical origin through the application of parasitological, immunological and molecular biology methods for diagnostics of *Cryptosporidium* spp.

2.2 To characterise the distribution of cryptosporidiosis among animals according to their age and species in the district of Pleven.

2.3 To establish the presence of *Cryptosporidia* through the application of PCR and some of its variations.

2.4 To identify the isolates of human and animal origin to the level of species/genotype through analysis of Restriction Fragment Length Polymorphism (RFLP).

2.5 To study the level of awareness of the personnel of nurseries, kindergartens and clinics at University Multi-profile Hospitals for Active Treatment from the district by means of a survey with a view to improve the level of prophylaxis of intestinal parasitic invasions (including cryptosporidiosis).

2.6 To develop a map of the areas of distribution of cryptosporidiosis in the district of Pleven.

2.7 To draft a health-awareness brochure summarising the basic information on cryptosporidiosis and containing useful recommendations for the prevention of the disease.

III. MATERIALS AND METHODS

1. Materials

1.1 Design of the study

The study is prospective and non-interventional with respect to the target groups of persons and animals under examination.

1.2 Target groups of persons under examination

The total of 1258 persons examined according to clinical and/or epidemiological data are of the age between 4 months and 70 years for a seven-year period between the years 2016 and 2023. They are divided in two groups: 1078 immunocompetent and 55 immunocompromised persons with primary or secondary immunodeficiency. The control group includes 125 clinically healthy children and adults without symptoms specific for intestinal parasitoses (Figure 1).

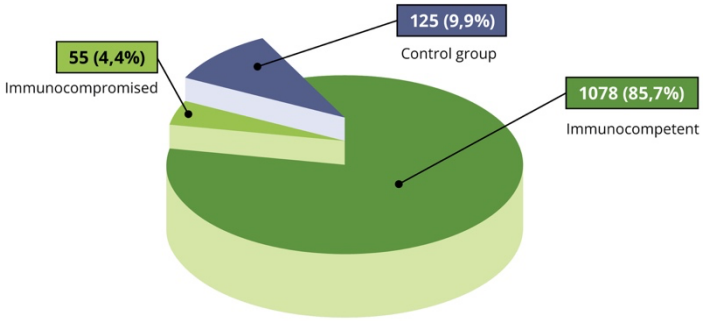


Figure 1. *Distribution of the examined persons for the period 2016-2023 in the district of Pleven*

1.2.1 Age structure

Table 1. *Distribution of target groups of examined persons according to gender, age and ethnic origin*

Target group, n=1133	Number (%)
Children - from 4 months to 18 years of age	**949 (75,43%)**
boys	486 (51,2%)
girls	463 (48,8%)
from 0 to 2 years	**134 (10.65%)**
boys	74 (55,2%)

girls	74 (55,2%)
Bulgarian	103 (76,9%)
Turkish	3 (2,2%)
Roma	28 (20,9%)
from 3 to 7 years	**751 (59,7%)**
boys	375 (49,9%)
girls	376 (50,1%)
Bulgarian	525 (69,9%)
Turkish	45 (6,0%)
Roma	181 (24,1%)
from 8 to 18 years	**64 (5,1%)**
boys	37 (57,81%)
girls	27 (42,19%)
Bulgarian	56 (87,5%)
Turkish	1 (1,6%)
Roma	7 (10,9%)
Adults from 19 to 70 years	**309 (24,56%)**
men	164 (53,1%)
women	145 (46,9%)
Bulgarian	297 (96,1%)
Turkish	9 (2,9%)
Roma	3 (1%)

1.2.2 Control group

Table 2. *Distribution of the control group according to gender, age and ethnic origin*

Control group, n=125	Number (%)
Children - from 4 months to 18 years of age	**91 (72,80%)**
boys	51 (56%)
girls	40 (44%)
Bulgarian	61 (67,0%)
Turkish	10 (11,0%)
Roma	20 (22,0%)
Adults from 19 to 70 years	**34 (27,2%)**
men	13 (38,2%)
women	21 (61,8%)
Bulgarian	27 (79,4%)
Turkish	3 (8,8%)
Roma	4 (11,8%)

The recommendations and instructions laid down in the Ethical Issues in Patient Safety Research:Interpreting Existing Guidance of the World Health Organisation have been observed in the process of working with the participants in the study. All examined persons or their parents have stated their informed consent in writing.

The study has been approved by the Committee on Research Ethics at the Medical University of Pleven (КЕНИД – ref. No 456/21.06.2017; КЕНИД – Ref. No 560/07.05.2019).

The taking of samples from organised children's institutions has been performed according to the provisions of Order No5 /06.04.2006 of the Ministry of Health regarding the diagnostics, prophylaxis and control of local parasitologists. A permission has been obtained from the Chief State Sanitary Inspector about the use of faeces samples from children in kindergartens and nurseries subject to prophylactic examinations by the Regional Health Inspectorate of the city of Pleven, Infectious Diseases Supervision Directorate, Medical Examinations section (permission by the Chief State Sanitary Inspector Ref. No 21.00.177/ 03.04.2018).

1.2 Target groups of examined animals

The animal population has been studied according to species and age in the period between 2016 and 2023. A total of 107 samples from different species of domestic animals (cows, goats, sheep) has been studied. Ninety-six of the samples were taken from animals with diarrhoea symptom. The control group includes 11 samples of animals without symptoms typical for intestinal invasions (Figure 2 and Figure 3).

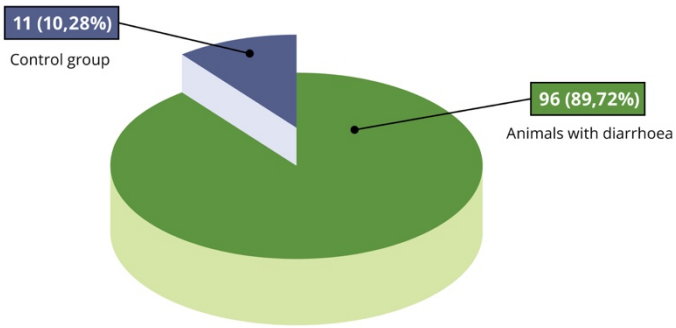


Figure 2. *Distribution of the examined animals in the period 2016-2023 in the district of Pleven*

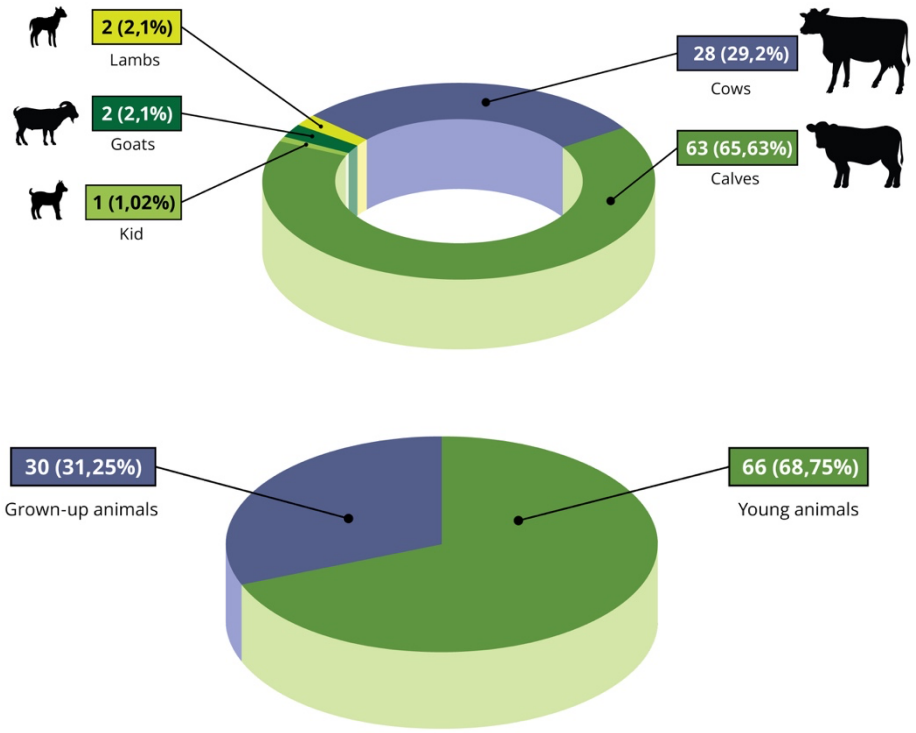


Figure 3. *Distribution of the examined samples according to the species and the age of the animal*

1.3. Territorial scope and geographical criterion for inclusion

The present study was conducted in towns and villages in the district of Pleven and in some nearby settlements. Most of the examined subjects were located in Pleven – 458 (36,4% of the total number), 270 were from smaller town in the district of Pleven (21,5%), and 459 (36,5%) were from the villages in the district. Pleven is an administrative centre of the whole district and there are many hospitals and clinics in the city, among which are two university hospitals (University Multi-profile Hospital for Active Treatment "Georgi Stranski" and University Multi-profile Hospital for Active Treatment "St. Marina Pleven"). Patients from nearby settlements are treated in the clinics of the university hospitals. There are such persons in our study, but they represent a small percentage of the total number of examined persons – 71 (5,6%) (Figure 4).

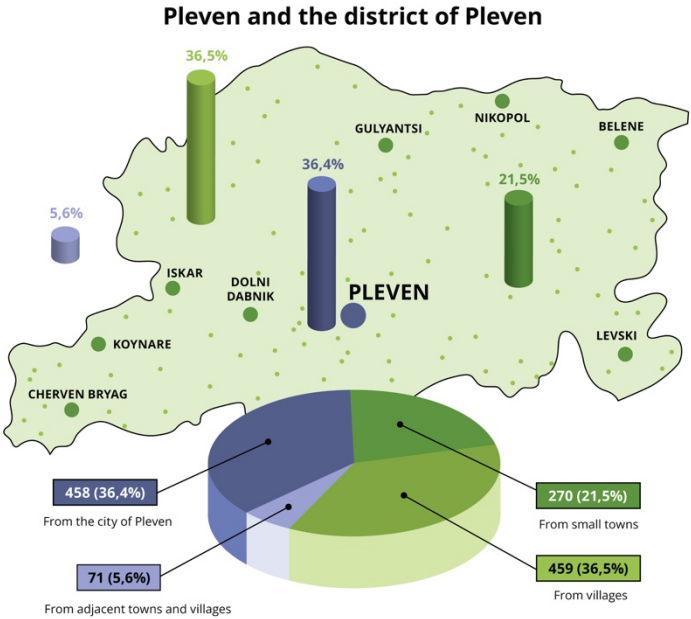


Figure 4. *Distribution of samples as per settlements within the territory of the district of Pleven*

1.4. Examined material

In order to achieve the scientific and research goals of the present dissertation, we have examined biological material in the form of faeces samples. Faeces samples from all persons and animals with or without clinical symptoms of intestinal parasitic invasions have been examined in differential and diagnostic terms through morphodiagnostic, immunological and molecular biology methods for intestinal protozoa. The faeces samples have been stored at – 80 °C in a freezer (PHCbi Ultra-Low Temperature Freezer MDF-U55V-PE) until the performance of the analyses .

1.5 Place of the scientific study

The laboratory analyses have been carried out in the Biology sector, Department of Anatomy, Histology, Cytology and Biology, Faculty of Medicine at the Medical University of Pleven and in the National Reference Laboratory “Diagnostics of Local and Tropical Parasites”, Department of Parasitology and Tropical Medicine of the National Centre for Infectious and Parasitic Diseases, Sofia.

2. Methods

In order to complete the tasks of the present study, a total of 1258 examinations for *Cryptosporidium* spp. have been carried out through the application of the following methods:

2.1 Morphological studies

2.2 Immunological methods for finding coproantigens in human and animal samples 3

2.2.1 Immunochromatographic methods

2.2.2 Enzyme linked immunosorbent assay (ELISA)

2.3 Molecular biology methods

2.3.1 Isolation of DNA of *Cryptosporidium* spp. from faeces samples of humans and animals

2.3.1.1 Isolation of DNA with a commercial kit *GeneAll® Exgene ™ Stool DNA kit* (GeneAll Biotechnology, Seoul, South Korea)

2.3.1.2 Isolation of DNA with a commercial kit *PureLink® Genomic DNA Kit*, (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA)

2.3.1.3 Isolation of DNA with a chelate agent *Chelex® 100* (Bio-Rad Laboratories, California, USA)

2.3.2 Assessment of the quality of the isolated DNA

2.3.3 DNA storage

2.3.4 Polymerase Chain Reaction (PCR), used primers and conditions for amplification

2.3.4.1 Nested PCR (nPCR) of a section of the gene for 18S ribosomal RNA of *Cryptosporidium* spp.

2.3.4.2 Nested PCR of a section of the gene for COWP (nCOWP-PCR) of *Cryptosporidium* spp.

2.3.4.3 Real time polymerase chain reaction (real time PCR) to prove *Cryptosporidium* spp.

2.3.5 Detection of amplification products – agarose gel electrophoresis

2.3.6 Purification of PCR products

2.3.7 Species identification of *Cryptosporidium* spp. through restriction fragment length polymorphism (RFLP)

2.4 Epidemiological methods

2.5 Sociological methods

2.5.1 Documentation methods

2.5.2 Survey

2.6 Statistical methods

2.7 Graphic analysis

IV. RESULTS AND DISCUSSION

1. Short description of some demographic and socio-economical indicators of the district of Pleven

1.1 Demographic indicators

The district of Pleven is the city with the largest population (226 120 people) in the Northwestern region and the ninth district in terms of territory – 3,9% of the territory of the country (Figure 5 and Figure 5a). The total number of residents in the district represents 3,5% of the total population of Bulgaria. The district comprises 11 municipalities, 14 towns and 109 villages. The district centre is the city of Pleven with 92 101 residents. The men-women ratio in the district of Pleven is 48,28% to 51,72% in favour of the women.

Figure 5. *Location of the district of Pleven within the Northwestern region (Source National Centre for Territorial Development, 2013)*

Figure 5a. *Map of the territorial distribution of the district of Pleven*

1.2 Ethnic composition

The ethnic composition of the district of Pleven is 89,7% Bulgarians, 2,3% Roma, 1,6% Turkish and 0,5% residents of other ethnic origins.

The Bulgarian ethnic origin prevails in the district of Pleven, represented by 200 197 residents or 88,5% of the persons who declared their ethnic origin. The Roma ethnic group comes second with 6999 (3,1%) residents, and the Turkish ethnic group is third — 5367 residents (2,4%). The remaining ethnic groups are insignificantly represented.

1.3 Socio-economical characteristics and infrastructure

The district of Pleven is the centre of the Northwestern economic region and has important functions for the overall economical and social development of the region. The gross domestic product (GDP) of the district of Pleven represents 30,8% of the GDP of the Northwestern region and 2,06% of the national GDP. The differentiation in the socio-economical development of the city of Pleven compared to the remaining municipalities in the region is preserved. The leading municipalities in terms of economic results in the district of Pleven are Pleven, Belene, Cherven Bryag and Levski.

The total number of childcare facilities in the territory of the district of Pleven is 115, of which 76 are kindergartens and 41 are nurseries. The social establishments for adults and children in the region are 8, and there are also 3 functioning nursing homes.

2. Results of the morphological studies for intestinal protozoa of the *Cryptosporidium* genus

The morphological diagnostics for *Cryptosporidium* spp. carried out according to the modified method of Ziehl–Neelsen (Kinyoun method) showed that 75 of the examined faeces samples from humans and 41 of the samples from animals contain oocysts (Figure 6).

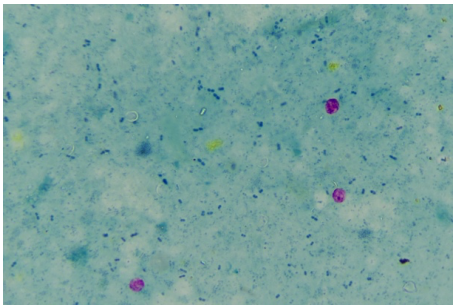


***Figure 6**. Microphotography of oocysts of Cryptosporidium spp., 1000X (by P. Dragomirova)*

Red or pink oocysts, round or oval bodies with dimensions 5-6 μm, often with granulated cytoplasm containing intracyst bodies coloured in dark grey or dark brown to black colour are visible on a cyan background.

In symptomless carriers, non-vital oocysts which do not absorb the carbolfuchsin ("ghost cells") are observed under the microscope. In our study, the lack of positive results in some of the human samples in the following PCR is most probably due to the availability of such "empty" oocysts (Figure 7).

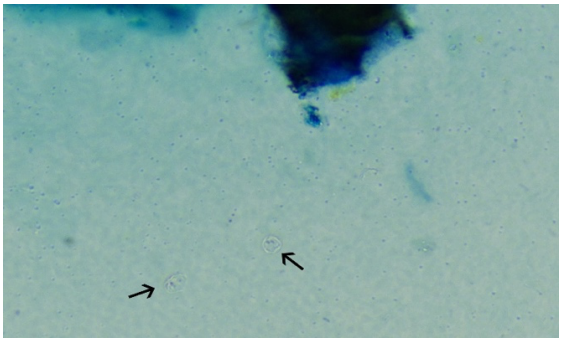


Figure 7. *Microphotography of "empty" oocysts of Cryptosporidium spp., 1000X (by P. Dragomirova)*

Polyparasitaemia was also established in the group of immunocompromised persons who tested positive for cryptosporidiosis. In 4 (4,65%) samples, the result was positive for both *Cryptosporidium* spp. and *Blastocystis* spp. (Figure 8).

- in 2 children who are symptomless carriers of *Blastocystis* spp. with less than 5 parasitic forms per observation area.
- in one child who is carrier of *Blastocystis* spp. with more than 5 parasitic forms per observation area.
- in one adult, who discharged cysts of *Blastocystis* spp. with more than 5 parasitic forms per observation area.

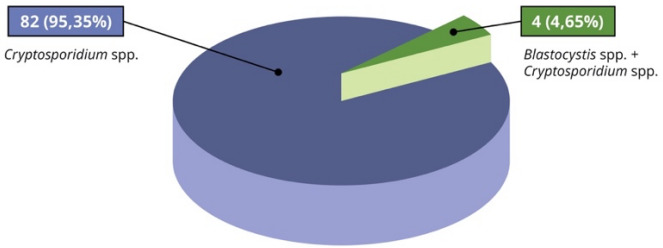


Figure 8. *Presence of polyinvasion in humans who tested positive for Cryptosporidiosis*

3. Results of immunological methods for intestinal protozoa from the *Cryptosporidium* genus

3.1 Quality coproantigen in vitro identification of *Cryptosporidium* spp. (RIDA Quick *Cryptosporidium* N1203) in human faeces samples

From the immunochromatographic tests conducted with a view to identify *Cryptosporidium* spp., 79 of the samples of the examined persons were antigen positive, and 1054 were negative (Figure 9).

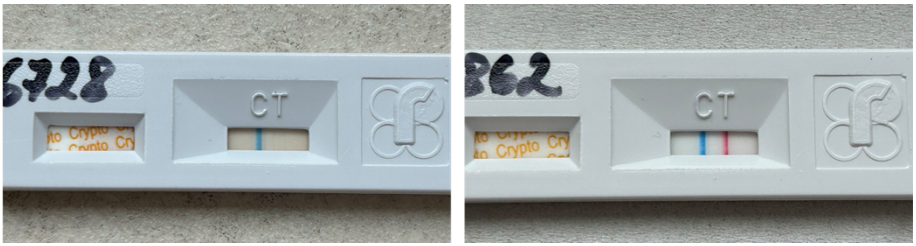


Figure 9. *Photograph of immunochromatographic tests of human faeces samples from people with negative (one blue line) and positive (one blue and one red line) result (by P. Dragomirova)*

Immunochromatographic tests are easy to perform, the result is obtained within ten minutes and they do not require any specific equipment and trained personnel. Therefore, they allow for quick on-the-spot diagnostics (in hospitals, childcare facilities, animal farms, etc.). However, immunochromatographic tests turn out to be less affordable than ELISA.

3.2 Quality coproantigen identification of *Cryptosporidium* spp. (BIO K 387 BioX Diagnostics) in animal faeces samples

Of the performed immunochromatographic tests for identification of *Cryptosporidium* spp. in animals, 37 were positive, and 59 were negative (Figure 10).

Figure 10*. Photograph of immunochromatographic tests of animal faeces samples with negative (one blue line) and positive (one blue and one red line) results (by P. Dragomirova)*

3.3 Results of enzyme-linked immunosorbent assay (ELISA) - *Ridascreen Cryptosporidium* (C1201)

Compared to immunochromatographic tests which are rapid and frequently used for diagnosing Cryptosporidiosis, but have low sensitivity and high percentage of false results, ELISA is a quick and cheap method facilitating diagnostics. As a disadvantage of this method, some authors point out variations in sensitivity and specificity, as well as the lack of particular knowledge and skills.

In the present study, ELISA turned out to be one of the main immunodiagnostic methods to detect the oocysts of *Cryptosporidium* spp. due to the simultaneous examination of a large number of samples and its higher sensitivity and affordability. The samples are not subject to additional concentration, the reading of the results with a spectrophotometer eliminates the subjectivity of the microscope examination, and the obtained results are reliable. It is possible to find *Cryptosporidium* spp. coproantigen within the framework of 10^3-10^4 oocysts per ml.

From the human samples examined through ELISA, 84 are positive, and 1049 are negative. From the examined animal samples, 40 are positive, and 56 are negative (Figure 11).

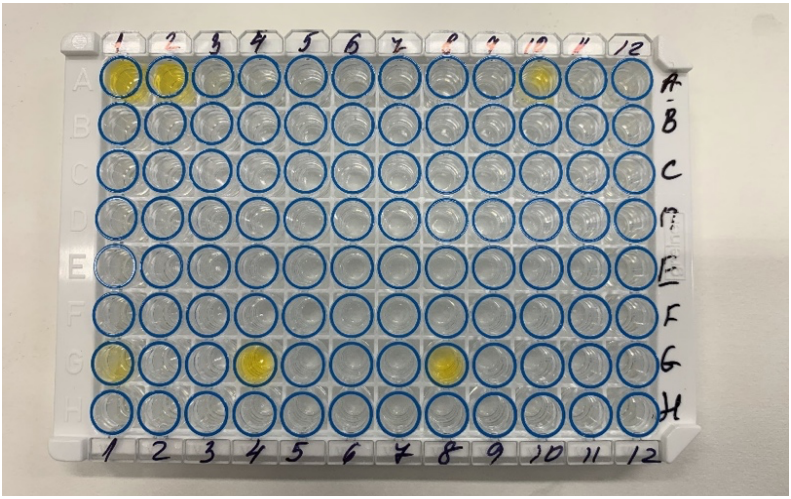


Figure 11. *Photograph of a microplate of coproantigen ELISA (by P. Dragomirova)*

After the conducted laboratory analyses it was established that the immunological methods (ELISA and immunochromatographic tests) show similar results as regards the detection of oocysts of *Cryptosporidium* spp.,unlike the morphological analyses which depend on the number of oocysts in the examined sample and from the subjective factor.

4. Results of molecular biology methods

4.1 Isolation of DNA from *Cryptosporidium* spp.

To isolate the DNA of *Cryptosporidium* spp., two methods of extraction have been applied: purification with a hard phase – silicium dioxide matrix in the form of columns (with a ready commercial kit – 2 sets: for the isolation of DNA from faeces and for the isolation of DNA from clinical materials) and with thermal processing of the sample in the presence of a chelate agent – Chelex® 100. In order to assess their effectiveness, the quantity and purity of the isolated DNA from 10 faeces samples has been determined through spectrophotometric measurement. Thus, aliquots for triple isolation of DNA have been prepared from each faeces – with two commercial sets and with the resin Chelex® 100. The obtained DNA samples were measured with a spectrophotometer ([spectrophotometer for microvolumes Biochrom, BioDrop Lite+, serial No 6491, Harvard Bioscience Co. Ltd. (Shanghai, China)]).

The proving of *Cryptosporidium* spp. in the examined faeces samples and the subsequent identification of the species has been performed in the sequence presented in Figure 12.

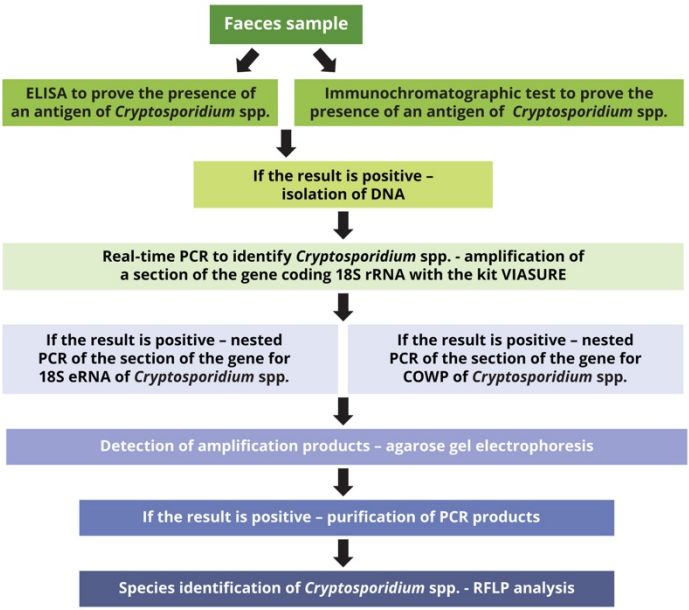


Figure 12. *Algorithm for conducting a molecular biological analysis for proving and species identification of isolates of Cryptosporidium spp.*

Legend: *ELISA – enzyme-linked immunosorbent assay; IChT – immunochromatographic test; RFLP – restriction fragment length polymorphism.*

Molecular techniques are widely used in the field of diagnostics of parasitic invasions, including for proving the presence of protozoa of the *Cryptosporidium* genus as invasive agents both in humans and animals. They significantly increase the capacity to detect cryptosporidia in the different types of examined materials (for example, clinical and water samples).

4.2 Real-time PCR on the basis of the *TaqMan* technology

The commercial kit *VIASURE Cryptosporidium*, *Giardia* & *E. histolytica Real Time PCR Detection Kit (Certest Biotec S.L., Zaragoza, Spain)* has been used to amplify the gene coding 18S rRNA, for specific and high-quality detection of the parasites in real time according to the instructions of the manufacturer. The amplifications have been carried out with the device Gentier Real-Time PCR System 96 (Xian Tianlong Science and Technology Co., Ltd.). The included internal control (Internal Control DNA (ICD)) allows for the detection of PCR inhibitors, for the monitoring of the integrity of the reagents, as well as to confirm that the nucleic acid has been successfully extracted, thus guaranteeing the reliability of the results.

4.2.1 Quality detection of DNA of *Cryptosporidium* spp.

In the present dissertation, human and animal faeces samples have been tested for the presence of the DNA of the pathogen.

Group 1 (humans): Altogether 86 persons with suspected invasion of *Cryptosporidium* spp. have been examined. An amplification of a fragment of the gene coding 18S rRNA, has been detected in the samples of 32 examined persons.

Group 2 (animals): A total of 40 animals, of which 36 calves, 3 cows and 1 kid with symptoms of the digestive tract, have been examined. A positive result of the amplification of the gene coding 18S rRNA, was obtained from 37 samples (36 calves of age between 6 days and 2 months and 1 kid of the age of 1 month).

Figure 13 presents an analysis of the result of the amplification of the examined DNA sample (patient No444), which has been proved as positive with a value of Ct below 40 (Ct=38,035) and internal control (IC) showing amplification signal (Figure 13).

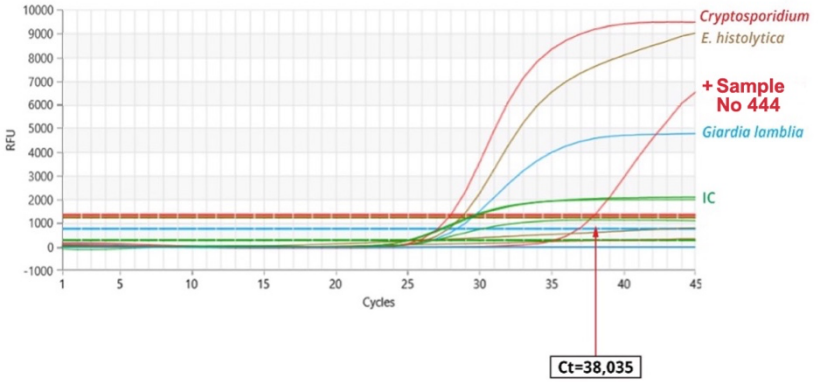
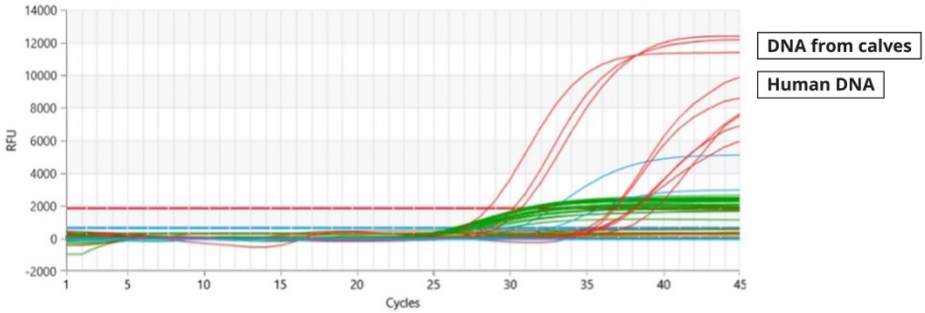


Figure 13. *Result of real-time PCR for detecting Cryptosporidium spp. in faeces sample from patient with ID No444. The positive control (K+) for the three pathogens is included in the commercial kit.*

By means of the commercial kit VIASURE, through the amplification of a section of the gene for 18S rRNA of *Cryptosporidium* spp., altogether 32 faeces samples of persons with suspected cryptosporidiosis, 36 samples of calves and one sample of a kid were identified as positive. Figure 14 shows the analysis of the results of the amplification reaction of none DNA samples isolated from faeces materials of humans (n=6) and calves (n=3).



Legend of colours	Pathogen
	Positive control for *Cryptosporidium* spp.
	Positive control for *E. histolytica*
	Positive control for *Giardia lamblia*
	Internal control

Figure 14. Result of real-time *PCR for detecting DNA of Cryptosporidium spp. in faeces samples from human (n=6) and animal (calves, n=3) origin. DNA from calves isolated from faeces samples of calves; human DNA isolated from human faeces samples.*

4.3 Species identification of *Cryptosporidium* spp. through RFLP analysis

The restriction fragment length polymorphism analysis conducted after nested PCR (nPCR-RFLP) is a specific and sensitive technique for detection of oocysts of *Cryptosporidium* in faeces sample materials. According to the data in scientific sources, the number of valid species of *Cryptosporidium* has increased significantly with the application of molecular techniques – there are 49 acknowledged species and more than 120 genotypes which are yet to be officially described (due to the lack of biological and/or genetic data). *Cryptosporidium hominis* and *C. parvum* are the species most frequently reported as causing the majority of infections in people worldwide, for example for all known typified water centres of infection with cryptosporidiosis, with the exception of one centre caused by *C. cuniculus* in the United Kingdom. Apart from *C. hominis* and *C. parvum*, other species and genotypes of *Cryptosporidium* have been proven in people through genetic methods, among which are *C. andersoni*, *C. bovis, C. canis, C. cuniculus, C. ditrichi, C. erinacei, C. fayeri, C. felis, C. meleagridis, C. muris, C. scrofarum, C. suis, C. tyzzeri, C. ubiquitum, C. viatorum, C. xiaoi, Cryptosporidium chipmunk* genotype I, *Cryptosporidium* skunk genotype, *Cryptosporidium* horse genotype and *Cryptosporidium* mink genotype.

In the present study, nPCR targeted at sections of two genes with a high extent of polymorphism have been applied, such as the hypervariable region of the gene for 18S rRNA (size of the amplicon ~434 bp), represented by many copies (20 copies of the oocyst) in the genome of the parasite, and the gene coding protein from the oocyst wall of cryptosporidia (COWP, size of the amplicon ~341 bp), represented by one copy in the genome of the parasite, which makes them a suitable marker for species identification of cryptosporidia. The high extent of polymorphism in the segments of these genes allows for the identification through RFLP analysis of the species *C. hominis* and *C. parvum*, which are found to cause the majority of human infections.

During the first stage of nPCR, a section of the gene for *18S rRNA* of *C. parvum* with size 1056 bp was amplified with the external primer pair KLJ1- F/KLJ2-R (Figure 15).

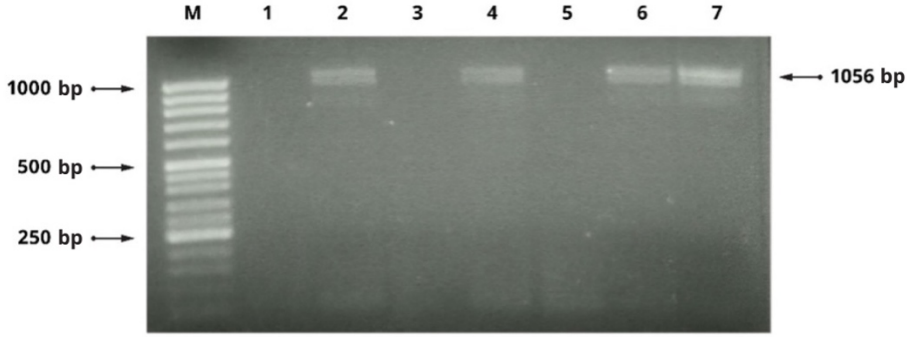


***Figure 15**. Electrophoresis analysis through 1,5% agarose gel of the amplicons from stage 1 of the nPCR with the external primer pair KLJ1- F/KLJ2-R. Line M, 50-1000-bp marker; Line 1, reference control sample of DNA of C. parvum (ATCC PRA-67DQ); lines 2-4, DNA samples isolated from faeces of patients with diarrhoea; lines 5-7, DNA samples isolated from animals faeces. At this first stage, amplification products are missing in three of the samples (lines 1, 3 and 5). The visualisation of the separated products has been performed with the fluorescent paint GelRed® Nucleic Acid Stain.*

During the second stage of nPCR, the matrix for the internal primer pair CPB-DIAGF/CPB-DIAGR is the product multiplied at the first stage. The size of the amplicons is 434 bp (Figure 16).

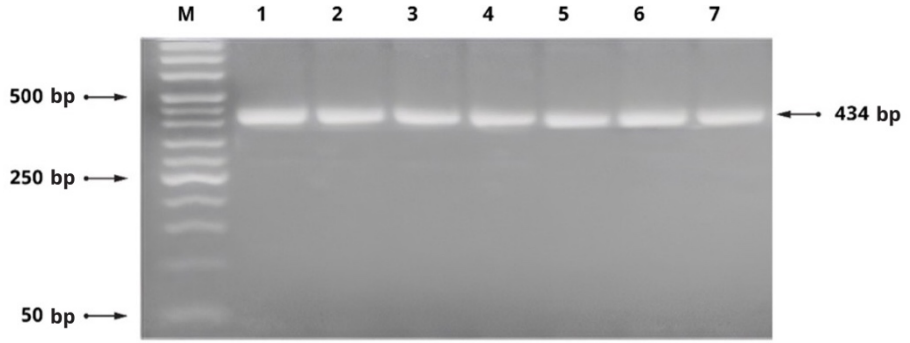


***Figure 16**. Electrophoresis analysis through 1,5% agarose gel of the amplicons (434 bp) of the second stage of nPCR with internal primer pair CPB-DIAGF/CPB-DIAGR. Line M, 50-bp marker; line 1, reference control sample of DNA of C. parvum (ATCC PRA-67DQ); lines 2-4, DNA samples isolated from faeces of patients with diarrhoea; lines 5-7, DNA samples isolated from animals faeces. The visualisation of the separated products has been performed with the fluorescent paint GelRed® Nucleic Acid Stain.*

In the present study, all isolates have been identified according to species through restrictive analysis of the amplified sections of the gene coding 18S rRNA

(with the enzymes *Dra*I, *Ase*I (*Vsp*I), *Dde*I, *Nde*I) and of the gene coding protein from the oocyst wall of *Cryptosporidium* (COWP), the enzymes *Rsa*I и *Alu*I respectively.

During the restriction analysis, the amplicons from human and animal isolates of *Cryptosporidium* obtained from the second PCR (with internal primer pair CPB-DIAGF / CPB-DIAGR), were subjected to simultaneous digestion with the enzymes *Dra*I and *Ase*I (*Vsp*I). The expected bands are of approximate size 219 (222), 112 and 104 bp (Figure 17).

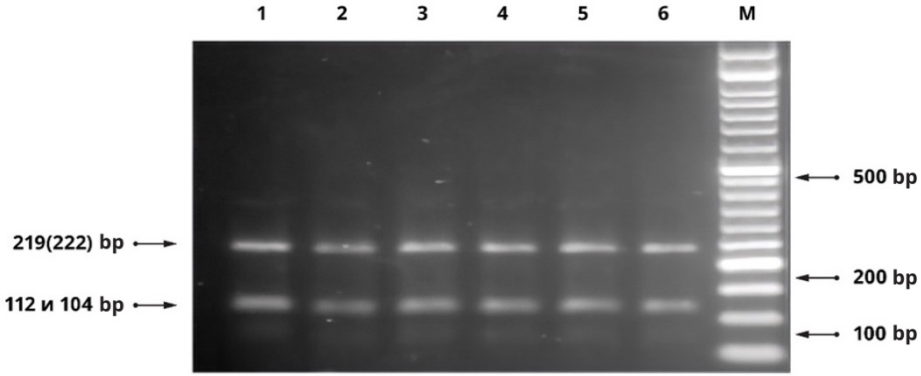


Figure 17. *Electrophoresis analysis through 2% agarose gel of the fragmented amplicons (434 bp) of stage 2 of nPCR (internal primer pair CPB-DIAGF / CPB-DIAGR), obtained after simultaneous digestion with the restriction enzymes DraI and AseI (VspI). Line M, 50-bp marker; line 1, fragmented PCR product of C. parvum (reference control sample of DNA, ATCC PRA-67DQ); lines 2-4, fragmented PCR products of human isolates of Cryptosporidium with a RFLP profile, identical with that of the reference strain of C. parvum (ATCC PRA-67DQ); lines 5 and 6, fragmented PCR products of animal isolates (calves) of Cryptosporidium with a RFLP profile, typical for that of the reference strain of C. parvum. The visualisation of the separated fragments was performed with the fluorescent paint GelRed® Nucleic Acid Stain.*

In our experimental analysis, the fragmented PCR products are of the same size as those of the products of the species *C. parvum* (219 (222), 112 and 104 bp). The difference between the sizes of the first fragment for genotype 1 and genotype 2 of *C. parvum* is 3 bp (222 bp for *C. parvum* genotype 1 and 219 bp for *C. parvum* genotype 2). The bands with size 112 and 104 bp migrate together and show as one band, which does not impact the interpretation of the RFLP analysis or the possibility to differentiate the (five) species (*C. parvum* genotype 2, *C. muris*, *C. baileyi*, *C. felis* and *C. meleagridis*).

The enzyme *Dra*I recognises the sequence 5'-TTT↓AAA-3' found in the amplified section of the gene for 18S rRNA only in the species *C. baileyi* and *C. felis.* As a result of the action of the enzyme, fragments with sizes 84 and 344 bp are obtained (if the species is *C. baileyi*) and 50 and 405 bp (if the species is *C. felis)*. The amplification products of the local isolates participating in the study and of the reference control sample of DNA of *C. parvum* (ATCC PRA-67DQ) do not contain a spot for cutting, which is determined by the sequence specific for the enzyme. Therefore, they remain undigested. This result allowed for the isolates under review to be referred to the species of *C. parvum* (Figure 18).

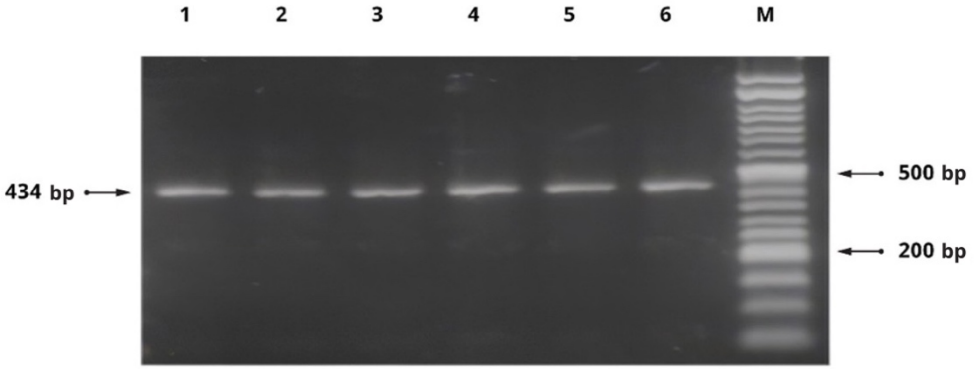


Figure 18. *Electrophoresis analysis through 2% agarose gel of the non-fragmented amplicons (434 bp) of stage 2 of nPCR (internal primer pair CPB-DIAGF / CPB-DIAGR) after digestion with the restriction enzyme DraI. Line M, 50-bp marker; line 1, fragmented PCR product of C. parvum (reference control sample of DNA, ATCC PRA-67DQ); lines 2-4, non-fragmented PCR products of human isolates of Cryptosporidium; lines 5 and 6, non-fragmented PCR products of animal isolates (calves) of Cryptosporidium with a RFLP profile, typical for that of the reference strain of C. parvum (ATCC PRA-67DQ). The visualisation of the separated fragments was performed with the fluorescent paint GelRed® Nucleic Acid Stain.*

The distinction between *C. andersoni* and *C. muris* is possible through additional digestion of the PCR products (434 bp) of the second stage of the reaction (with internal primer pair CPB-DIAGF/CPB-DIAGR) with the restriction enzyme *Dde*I. The enzyme recognises the sequence 5'- C↓TNAG-3'. Figure 19 presents an excerpt from the results of the nPCR-RFLP analysis of amplicons from 5 human and 3 animal isolates of *Cryptosporidium,* broken down in 3 fragments with sizes ~201(204), 166 and 68 bp. The slight difference (3 nucleotides) between the restriction profiles of the cryptosporidia of genotype1 (*C. parvum* human genotype (genotype H)) and genotype 2 (*C. parvum* bovine genotype (genotype B)) makes it

difficult to determine the genotype of the local isolates on the grounds of the obtained fragments. The reference DNA sample used in the present study (Quantitative Genomic DNA from *Cryptosporidium parvum* (ATCC PRA-67DQ)) was purchased from the American Type Culture Collection, ATCC for the purposes of the molecular experiments and represents quantitative genomic DNA from *Cryptosporidium parvum* Tyzzer (strain *Cryptosporidium parvum* Iowa II). The Iowa strain of *C. parvum* (genotype 2) was first isolated by H. Moon from a calf with symptoms in Ames, Iowa, and is used for different immunological, biochemical and infection studies of humans and animals and has thus become a "gold standard" isolate to which other isolates are compared. Information about the product has been published on the ATCC website (https://www.atcc.org/products/pra-67dq) and cross references have been made to the gene bank (GenBank AAEE01000000 *Cryptosporidium parvum* Iowa II, whole genome shotgun sequencing project), which contains data on the whole genome sequencing of the strain. As a result of and on the grounds of the above, as well as considering the fact that the restriction profiles of the local human and animal isolates of *Cryptosporidium* are identical with the profile of the *Cryptosporidium parvum* Iowa II strain, used as a reference strain in the present study (Figure 19), and the local isolates are referred to genotype 2 of *Cryptosporidium parvum* (zoonotic genotype), which infects humans, ruminants and some other animals.

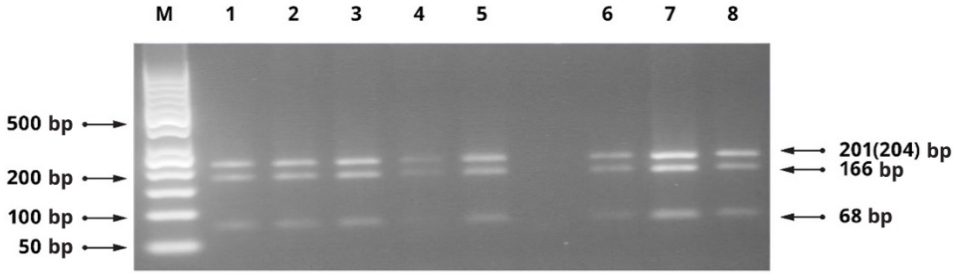


Figure 19. *Electrophoresis analysis through 2% agarose gel of amplicons (434 bp) from stage 2 of nPCR (with internal primer pair CPB-DIAGF/CPB-DIAGR) after digestion with the restriction enzyme DdeI. Line M, 50-bp marker; line 1, fragmented PCR product of C. parvum (reference control sample of DNA, ATCC PRA-67DQ); lines 2-5, fragmented PCR products of human isolates of Cryptosporidium; lines 6-8, fragmented PCR products of animal isolates (calves) of Cryptosporidium with a RFLP profile typical for that of the reference strain of C. parvum (ATCC PRA-67DQ) (201(204), 166 and 68 bp). The visualisation of the separated fragments was performed with fluorescent paint GelRed® Nucleic Acid Stain.*

With the purpose of distinguishing between the isolates of *C. parvum* and the other species of *Cryptosporidium,* a restriction analysis of the products from the amplification of the section of the gene coding 18S rRNA with the enzyme *Nde*I has been performed. The amplicons with size ~434 bp obtained from the second PCR have been cut by the restriction enzyme in the isolates of *C. parvum*. As a result of the place in the genome sequence recognised by the enzyme (the sequence 5'-CA↓TATG-3'), two fragments with sizes 293 bp and 140 bp respectively, were obtained. In the sequences of the other species of *Cryptosporidium*, such as *C. muris*, *C. baileyi*, *C. serpentis* and *C. felis*, such place is lacking and the amplification products remain whole (undigested). This shows that the examined human and animal isolates belong to the species *C. parvum.* The restriction fragments of the reference strain of *C. parvum* (ATCC PRA-67DQ) used in the study are identical with those obtained through the digestion of the amplicons from the examined human and animal isolates (Figure 20).

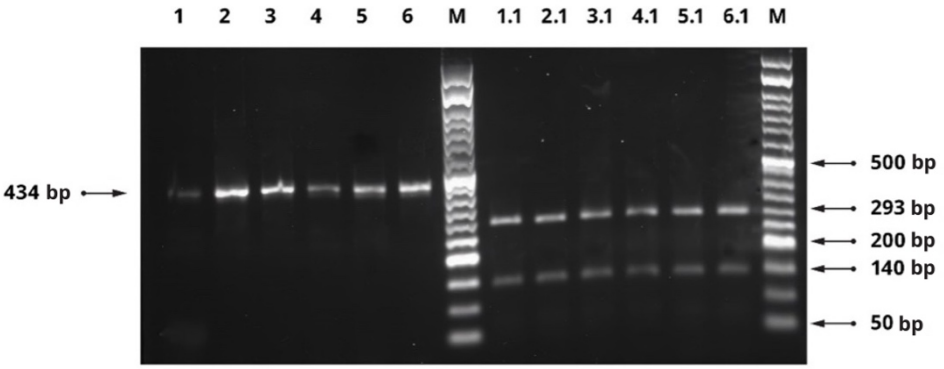


Figure 20. *Electrophoresis (2% agarose gel) of the products from nPCR (with internal primer pair CPB-DIAGF/CPB-DIAGR), subjected to the action of the restriction enzyme NdeI. The gel is coloured with fluorescent paint GelRed® Nucleic Acid Stain. Line M, 50-1500-bp DNA marker; lines 1-6, amplicons obtained from the second stage of nPCR (434 bp); line 1, reference strain of C. parvum genotype 2 (ATCC PRA-67DQ); lines 2-4, PCR products from human isolates of Cryptosporidium; lines 5 and 6, PCR products from animal isolates (calves) of Cryptosporidium; lines 1.1-6.1, fragments obtained after digestion of the amplicons (1-6) of human and animal isolates with the enzyme NdeI (~293 and 140 bp).*

Figure 21 presents a general overview of the profiles obtained through digestion of the products from the nPCR with the internal primer pair CPB-DIAGF/CPB-DIAGR, which multiplies a section of the gene coding 18S rRNA with size ~434 bp, of a calf and human isolate. The results of the performed PCR-RFLP reactions are presented in Table 3.

Table 3. *Restriction fragments obtained as a result of the digestion with enzymes DraI, VspI, DraI+VspI, DdeI and NdeI of a section of the gene coding 18S rRNA (~434 bp) of silates of animal and human origin.*

Position of the gel	Enzyme	Size of the fragments from PCR-RFLP	Identification at the level of species (genotype)
M (50-bp marker)	isolate from calf / human isolate positions **1-6 / 7-12**		
1/7	-	**~434 bp**	
2/8	*Dra*I	**~434 bp** do not contain a spot for cutting	*Cryptosporidium parvum*
3/9	*Vsp*I	fragmented PCR products **219 (222*)** and **112 bp** (missing **104 bp**)	*C. parvum* *-genotype 2
4/10	*Dra*I+*Vsp*I	fragmented PCR products **219 (222*)** and **112 bp** (missing **104 bp**)	*C. parvum* *- genotype 2
5/11	*Dde*I	three fragments with size **201(204*), 166** и **68 нд**	*C. parvum* *- genotype 2
6/12	*Nde*I	два фрагмента с големина **293 нд** и **140 нд**	*C. parvum*

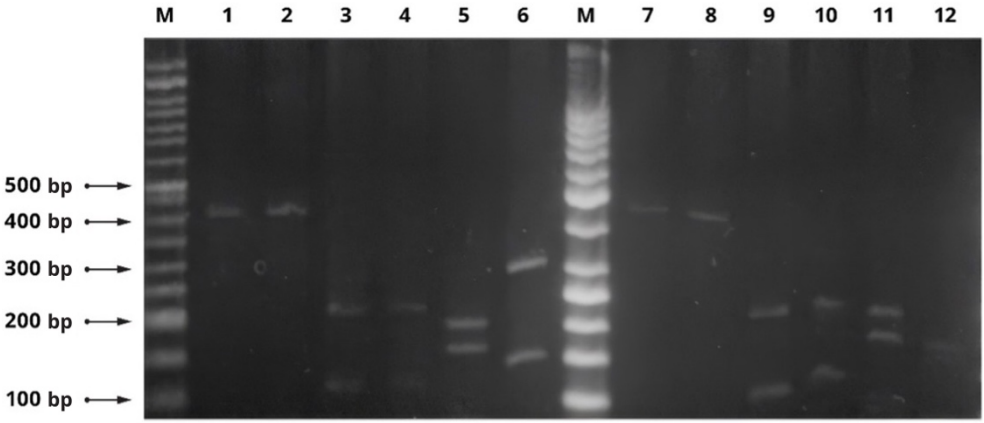


Figure 21. *Electrophoresis analysis through 2% agarose gel of amplicons before and after digestion with the restriction enzymes DraI (lines 2 and 8), VspI (lines 3 and 9), DraI+VspI (lines 4 and 10), DdeI (lines 5 and 11) and NdeI (lines 6 and 12). Lines M, 50-bp marker; lines 1-6, animal isolate (calf); lines 7-12, human isolate; lines 1 and 7,*

non-digested products from nPCR with the internal pair CPB-DIAGF/CPB-DIAGR (with size ~434 bp) of human and calf isolates.

For the amplification of the gene coding protein from the oocyst wall of *Cryptosporidium* (COWP), an nPCR *(COWP-nPCR)* has been applied. This gene is presented with one copy in the genome of the parasite. The amplification was conducted in two stages. During the first stage, the forward (PCOWPF) and reverse (PCOWPR) primers amplify a fragment of the gene for COWP with size 430 bp. A section of the COWP gene was successfully amplified in all positive samples of real-time PCR with the commercial kit *VIASURE*, 32 from humans and 37 from animals (Figure 22).

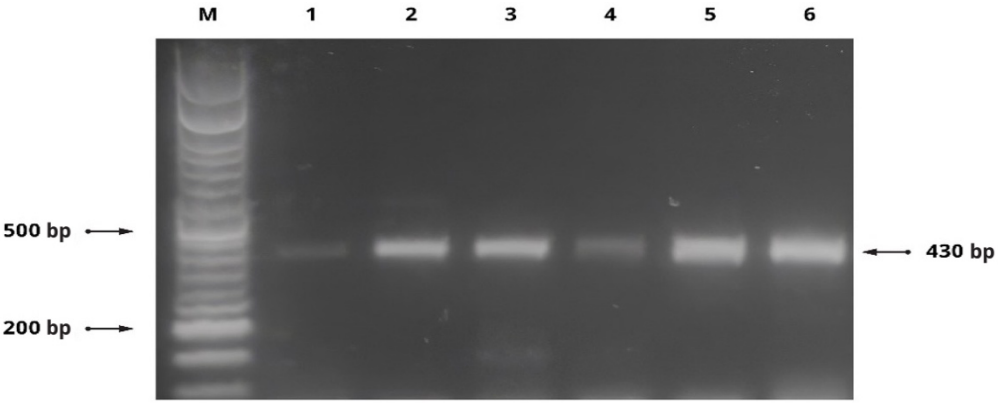


***Figure 22**. Electrophoresis analysis through 1,5% agarose gel of the products from stage 1 of nPCR (430 bp) with the external primer pair PCOWPF/PCOWPR. Line M, 50 bp marker; line 1, reference control sample of DNA of C. parvum (ATCC PRA-67DQ); lines 2-4, DNA samples isolated from faeces of patients with diarrhoea; lines 5 and 6, DNA samples isolated from animal faeces. The visualisation of the separated products was performed with fluorescent paint GelRed® Nucleic Acid Stain.*

At the second stage of the reaction, the products from the first stage serve as a matrix for the internal primers (PCOWPIF/IR) which amplify a fragment of the gene for COWP with size 341 bp. The amplicons multiplied at this second stage are then subjected to restriction analysis to determine the genotype of the local isolates of *Cryptosporidium* through RFLP analysis on the basis of digestion with the restriction enzymes *Alu*I and *Rsa*I (Figure 23).

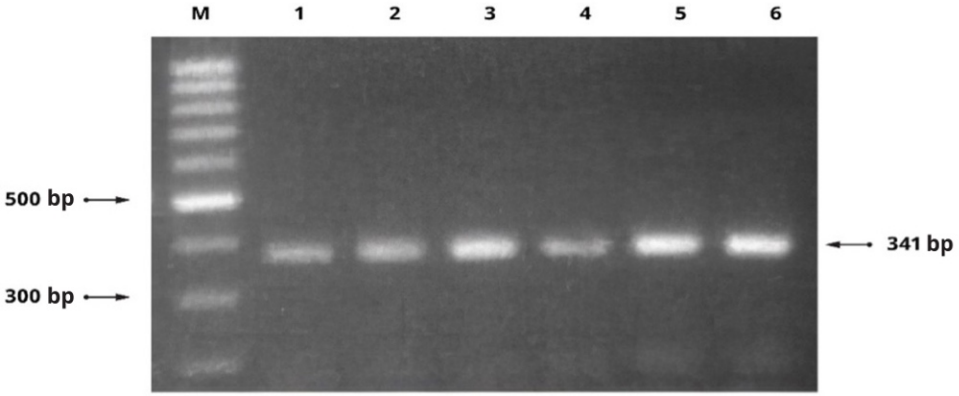


Figure 23. *Electrophoresis analysis through 1,5% agarose gel of amplicons (341 bp) of stage 2 of nPCR with the internal primer pair PCOWPIF/PCOWPIR. Line M, 100-1000 bp marker; line 1, reference control sample of DNA of C. parvum (ATCC PRA-67DQ); lines 2-4, DNA samples isolated from faeces of patients with diarrhoea; lines 5 and 6, DNA samples isolated from animal faeces. The visualisation of the separated products was performed with fluorescent paint GelRed® Nucleic Acid Stain.*

Due to the high extent of polymorphism in the COWP gene, the restriction places for the enzymes *Rsa*I and *Alu*I are used for the identification of *C. hominis* and *C. parvum* and their distinguishing from the remaining species of cryptosporidia. The results of the digestion of the PCR products (341 bp) with enzymes *Alu*I and *Rsa*I are with RFLP profiles typical for *C. parvum* genotype 2.

A number of studies of the restriction profiles obtained by digestion with *Rsa*I and with *Alu*I of the 341-bp fragment of the gene coding protein from the oocyst wall of *Cryptosporidium*, amplified by the primer pair PCOWPIF/PCOWPIR show that *Cryptosporidium parvum* is the main cause of cryptosporidiosis in humans (it has also been proven among samples of calves) and includes at least two different genotypes which represent different species of parasites in terms of taxonomy (*C. hominis* and *C. parvum*). The first genotype is encountered exclusively in naturally infected persons and in one non-anthropoid primate (genotype 1 or human genotype), whereas the second is observed in domestic animals and in humans (genotype 2 or bovine genotype).

The results of the RFLP analysis to determine the species (genotype) of *Cryptosporidium* through restriction digestion of the COWP gene fragment was performed with the enzymes *Rsa*I and *Alu*I, as described by Amar et al., 2004. The restriction fragments are separated through electrophoresis in 2% agarose gels and coloured with fluorescent paint *GelRed® Nucleic Acid Stain.* The generated fragments with *Rsa*I are of size ~311 and 30 bp (*C. parvum* genotype 2), and under

the influence of *Alu*I, the products are broken down in three fragments with sizes 193 and 129 bp. The last and smallest fragment (19 bp) does not reach the threshold for visualisation (Figure 24).

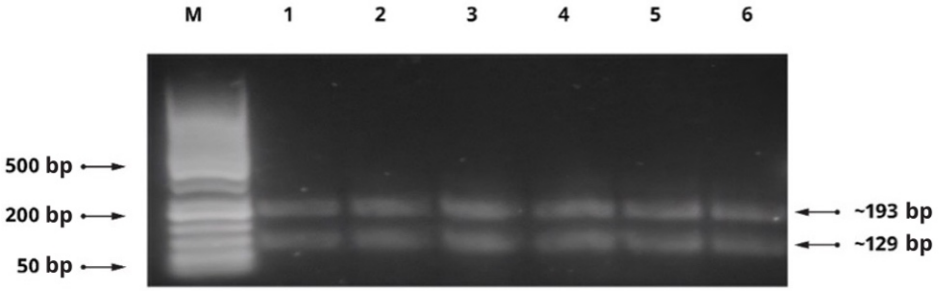


Figure 24. *Electrophoresis analysis (2% agarose gel) of amplicons (341 bp) of stage 2 of nPCR (with he internal primer pair PCOWPIF/PCOWPIR) after digestion with the restriction enzyme AluI. Line M, 50 bp marker; line 1, fragmented PCR product of C. parvum (reference control sample of DNA, ATCC PRA-67DQ); lines 2-4, fragmented PCR products from human isolates of Cryptosporidium; lines 5 and 6, fragmented PCR products from animal isolates (calves) of Cryptosporidium. The RFLP profile of all local isolates is identical with that of the reference strain of C. parvum (ATCC PRA-67DQ) (193 and 129 bp). The visualisation of the separated fragments was performed with fluorescent paint GelRed®*

The three species *C. parvum*, *C. bovis* and *C. ryanae* are the most frequently isolated species in the examination of young cattle in various studies. An age-related consistence of establishing the species *Cryptosporidium* in breast-fed calves has also been observed. The species *C. parvum* has been proven in the majority of invasions in calves weaned in advance, whereas *C. bovis* and *C. ryanae* are encountered in older or already weaned calves. The results of our study are similar to the ones published by these authors and other research teams. In the animal samples analysed within our study, the species *C. parvum* (zoonotic genotype 2) has been identified. The age of the animals at the time the samples were taken varies between 6 days and 2 months (the kid is one month old). The majority of the animals (n=29) are under one month of age, and the remaining 8 animals are older - 1 month (n=6), 38 days (n=1) and 2 months (n=1). These results correspond to the findings of other studies which describe that the calves of under 2 months of age are the prevailing population infected with *C. parvum* zoonotic species). Stockbreeders and generally the people who take care of such animals or are in contact with them, are exposed to a risk of infection with *C. parvum*. The studies of scientists from Iran who report the zoonotic transmission of *Cryptosporidium parvum* among people working close to calves, support this conclusion. In our study, we have established persons (n=3) whose samples became positive after a contact with animals infected with *C. parvum* according to three of the diagnostic methods applied.

The molecular-genetic characteristics of the isolates of the human faeces samples in the present study show that they belong to the same genotype 2 of *C. parvum*, which was established in the animal faeces samples.

The identification of *Cryptosporidium* spp. at the level of species or genotype is of crucial importance for the assessment of the potential of parasites and of the sources of invasion (humans and animals) in the light of the WHO "*One health"* initiative.

Cryptosporidium parvum is among the rare species of *Cryptosporidium* with a wide range of hosts and it is also the most significant zoonotic species. The subtyping is actively used to establish the transmission of *C. parvum* among people and animals.

5. Results of the impact of the main demographic indicators on the frequency of distribution of cryptosporidiosis among the target groups under examination

In order to establish the distribution of cryptosporidiosis among the population of the district of Pleven, a study among target groups of examined persons according to the basic demographic indicators, namely gender, age, ethnical origin and type of settlement has been conducted.

The examination of the main target group of 1133 persons of the district with the application of three main methods of diagnostics showed a total of 86 persons positive for cryptosporidiosis.

5.1 Distribution of the persons examined for cryptosporidiosis depending on the gender

A total of 1133 persons were tested for cryptosporidiosis, of which 547 women and 586 men. From the persons who tested positive for cryptosporidiosis with ELISA, 7,3% are women and 7,2% are men. The morphodiagnostics according to the modified method of Ziehl-Neelsen (Kinyoun method) showed that the positive results are equal for both genders – 7,3%. Approximate results are obtained from examination with rapid immunochromatographic tests – 6,9% women and 6,3% men tested positive.

Cramer's ratios (Cramer's V) for the three methods vary between 0,000 and 0,013 and show a weak correlation between the gender and the disease cryptosporidiosis. This is also confirmed by the p-values (0,925; 0,987; 0,668), which reveal the lack of statistically significant data in the frequency of infection of men and women.

Table 4 summarises the results from the confirmed cases of cryptosporidiosis according to the methods applied depending on the gender.

Table 4. *Distribution of the confirmed cryptosporidiosis cases depending on the gender*

		Negative n (%)	Positive n (%)	Total n (%)	Cramer's V	Sig. (p-value)
ELISA						
gender	women	2 (4,9)	39 (95,1)	41 (100,0)	0,162	0,134
	men	0 (0,0)	45 (100,0)	45 (100,0)		
Total		2 (2,3)	84 (97,7)	86 (100,0)		
Modified Ziehl-Neelsen						
gender	women	5 (12,2)	36 (87,8)	41(100,0)	0,017	0,875
	men	6 (13,3)	39 (86,7)	45 (100,0)		
Total		11 (12,8)	75 (87,2)	86 (100,0)		
Immunochromatographic rapid test - ICH						
gender	women	2 (4,9)	39 (95,1)	41 (100,0)	0,114	0,291
	men	5 (11,1)	40 (88,9)	45 (100,0)		
Total		7 (8,1)	79 (91,9)	86 (100,0)		
RT-PCR VIASURE						
gender	women	29 (70,7)	12 (29,3)	41 (100,0)	0,157	0,146
	men	25 (55,6)	20 (44,4)	45 (100,0)		
Total		54 (62,8)	32 (37,2)	86 (100,0)		
nPCR 18S rRNA						
gender	women	29 (70,7)	12 (29,3)	41 (100,0)	0,157	0,146
	men	25 (55,6)	20 (44,4)	45 (100,0)		
Total		54 (62,8)	32 (37,2)	86 (100,0)		
nCOWP-PCR						
gender	women	29 (70,7)	12 (29,3)	41 (100,0)	0,157	0,146
	men	25 (55,6)	20 (44,4)	45 (100,0)		
Total		54 (62,8)	32 (37,2)	86 (100,0)		

The correlation between the gender and the infection with *Cryptosporidium* spp. is weak, which is obvious from the low Cramer's ratios (0,017 ÷ 0,162). The p-values vary within the range 0,134 ÷ 0,875 and prove that the infection with *Cryptosporidium* spp. does not depend on the gender.

5.2 Distribution of the persons examined for cryptosporidiosis depending on their ethnical origin

The total number of persons examined for cryptosporidiosis is 1133, of which 887 are Bulgarians, 47 are Turkish and 199 are Roma.

From those tested with ELISA, 6,8% of the persons who tested positive for cryptosporidiosis are from the Bulgarian ethnic group, and 11,1% are from the Roma ethnic group. None of the three methods applied revealed positive samples among the Turkish ethnic group. Through morphodiagnostics according to the Ziehl-Neelsen method (Kinyoun method), 6,75% Bulgarians and 11,6% Roma tested positive for cryptosporidiosis. The rapid chromatographic tests show a percentage of 6,3% for the Bulgarian ethnic group and 9,5% for the Roma ethnic group.

Although Cramer's rations (0,074; 0,083; 0,086) reveal a weak correlation between the ethnical background and cryptosporidiosis morbidity, the analysis of the samples analysed by means of ELISA and the modified method of Ziehl-Neelsen reveal statistically significant differences (0,041; 0,050) in the morbidity of persons belonging to different ethnic groups. The results of the rapid immunochromatographic tests do not show significant differences between the ethnic groups (0,101).

Table 5 presents the results of the analysis of the confirmed cases of cryptosporidiosis according to the ethnic origin of the examined persons.

Table 5. *Distribution of the confirmed cases of cryptosporidiosis according to ethnic origin*

		Negative n (%)	Positive n (%)	Total n (%)	Cramer's V	Sig. (p-value)
ELISA						
ethnic group	Bulgarians	1 (1,7)	57 (98,3)	58 (100,0)	0,057	0,594
	Roma	1 (3,6)	27 (96,4)	28 (100,0)		
Total		2 (2,3)	84 (97,7)	86 (100,0)		
Modified Ziehl-Neelsen						
ethnic group	Bulgarians	8 (13,8)	50 (86,2)	58 (100,0)	0,043	0,689
	Roma	3 (10,7)	25 (89,3)	28 (100,0)		
Total		11 (12,8)	75 (87,2)	86 (100,0)		
Immunochromatographic rapid test - ICH						
ethnic group	Bulgarians	3 (5,2)	55 (94,8)	58 (100,0)	0,156	0,148
	Roma	4 (14,3)	24 (85,7)	28 (100,0)		
Total		7 (8,1)	79 (91,9)	86 (100,0)		
RT-PCR VIASURE						
ethnic group	Bulgarians	31 (53,4)	27 (46,6)	58 (100,0)	0,278	0,010
	Roma	23 (82,1)	5 (17,9)	28 (100,0)		
Total		54 (62,8)	32 (37,2)	86 (100,0)		
NPCR 18S rRNA						

ethnic group	Bulgarians	31 (53,4)	27 (46,6)	(58) 100,0	0,278	0,010
	Roma	23 (82,1)	5 (17,9)	(28) 100,0		
Total		54 (62,8)	32 (37,2)	(86) 100,0		
nCOWP-PCR						
ethnic group	Bulgarians	31 (53,4)	27 (46,6)	58 (100,0)	0,278	0,010
	Roma	23 (82,1)	5 (17,9)	28 (100,0)		
Total		54 (62,8)	32 (37,2)	86 (100,0)		

The confirmed cases of cryptosporidiosis through ELISA, the modified Ziehl-Neelsen method and the immunochromatographic methods show that Cramer's ratios are respectively 0,057, 0,043 and 0,156, i.e. only 4,3% ÷ 15,6% of the variations in the cases confirmed through these methods are attributable to ethnic origin. The p-values (0,594, 0,689 and 0,148) do not reveal significant differences between the different ethnic groups.

Significant differences between the ethnic groups (0,010) were established through the three variants of PCR – RT-PCR VIASURE, nPCR 18S rRNA and MT nPCR COWP, and, according to Cramer's ratios, 27,8% of the variations of the data are attributable to the ethnic origin of the examined persons.

5.3 Distribution of the persons examined for cryptosporidiosis depending on the settlement

From a total of 1133 examined persons, 730 live in a town, and 403 live in a village.

The results from ELISA show a higher percentage of the positive samples in persons living in a village (9,9%) compared to those who live in a town (5,8%). The morphodiagnostic modified method of Ziehl-Neelsen (Kinyoun method) show 5,5% positive samples of the residents of towns and 10,7% positive results of residents of villages. The positive results of the rapid immunochromatographic tests are 5,6% among the residents of towns and 8,4% among residents of villages.

Once again, statistically significant differences according to place of residence have been calculated (p-value = 0,009; 0,001) in the confirmed cases of cryptosporidiosis through ELISA and the modified Ziehl-Neelsen method. The use of rapid chromatographic tests do not reveal significant differences (p-value = 0,068) between the residents of towns and villages. In all three methods, between 5,4% ÷ 9,5% of the variations in the cases are due to the place of residence (Cramer's V = 0,077, 0,095 and 0,054).

Table 6 presents the results of the confirmed cases of cryptosporidiosis depending on the place of residence.

Table 6. *Distribution of the confirmed cases of cryptosporidiosis depending on the place of residence*

		Negative n (%)	Positive n (%)	Total n (%)	Cramer's V	Sig. (p-value)
ELISA						
settlement	town	1 (2,2)	44 (97,8)	45 (100,0)	0,007	0,947
	village	1 (2,4)	40 (97,6)	41 (100,0)		
Total		2 (2,3)	84 (97,7)	86 (100,0)		
Modified Ziehl-Neelsen						
settlement	town	7 (15,6)	38 (84,4)	45 (100,0)	0,087	0,421
	village	4 (9,8)	37 (90,2)	41 (100,0)		
Total		11 (12,8)	75 (87,2)	86 (100,0)		
Immunochromatographic rapid test - ICH						
settlement	town	3 (6,7)	42 (93,3)	45 (100,0)	0,056	0,601
	village	4 (9,8)	37 (90,2)	41 (100,0)		
Total		7 (8,1)	79 (91,9)	86 (100,0)		
RT-PCR VIASURE						
settlement	town	22 (48,9)	23 (51,1)	45 (100,0)	0,301	0,005
	village	32 (78,0)	9 (22,0)	41 (100,0)		
Total		54 (62,8)	32 (37,2)	86 (100,0)		
nPCR 18S rRNA						
settlement	town	22 (48,9)	23 (51,1)	45 (100,0)	0,301	0,005
	village	32 (78,0)	9 (22,0)	41 (100,0)		
Total		54 (62,8)	32 (37,2)	86 (100,0)		
nCOWP-PCR						
settlement	town	22 (48,9)	23 (51,1)	45 (100,0)	0,301	0,005
	village	32 (78,0)	9 (22,0)	41 (100,0)		
Total		54 (62,8)	32 (37,2)	86 (100,0)		

In the cases of cryptosporidiosis confirmed through the methods ELISA, Ziehl-Neelsen colouring and immunochromatographic tests of persons from different settlements, Cramer's ratios are respectively 0,007, 0,083 and 0,056, i.e. a very small percentage of the variations (0,7% ÷ 8,3%) in the cases confirmed through these diagnostic methods depend on the place of residence. The p-values (0,947,

0,421 and 0,601) indicate that there are no significant differences between the frequency of parasitic infections of the residents of towns and villages.

The use of the three versions of PCR – RT-PCR VIASURE, nPCR 18S rRNA and MT nPCR COWP showed that the place of residence influences the frequency of parasitic infection of persons (0,005). Cramer's ratios (0,301) reveal that 30,1% of the variations in the data are attributable to the place of residence of the examined persons.

5.4 Distribution of the persons examined for cryptosporidiosis according to their age

The total number of samples examined for cryptosporidiosis is 1133, of which 129 are of persons from the first age group, 665 are of persons of the second age group, 64 of the third and 275 of the fourth. From the persons tested with ELISA, 9,3% persons of the first group are positive for cryptosporidiosis, 6,9% of the second, 7,8% of the third and 6,9% of the fourth. According to the morphodiagnostics by means of the modified Ziehl-Neelsen method (Kinyoun method), 8,5% persons of the first group tested positive for cryptosporidiosis, 6,2% of the second, 7,8 % of the third and 9,5% of the fourth. The rapid chromatographic tests show the following positive results: 7,0% of the first age group, 6,2% of the second, 7,8% of the third and 7,3% of the fourth age group.

Cramer's ratios for the three methods vary between 0,023 and 0,055 and reveal a weak correlation between the age and the frequency of parasitic infection with *Cryptosporidium* spp. This is also confirmed by the p-values (0,802; 0,327; 0,900), which show that the frequency of infection does not depend on the age group.

Table 7 presents the results of the confirmed cases of cryptosporidiosis according to the age of the tested persons.

Table 7. *Distribution of the samples with confirmed cryptosporidiosis depending on the age*

		Negative n (%)	Positive n (%)	Total n (%)	Cramer's V	Sig. (p-value)
ELISA						
age	0-2	0 (0,0)	12 (100,0)	12 (100,0)	0,134	0,672
	3-7	2 (4,1)	47 (95,9)	49 (100,0)		
	8-18	0 (0,0)	5 (100,0)	5 (100,0)		
	19-70	0 (0,0)	20 (100,0)	20 (100,0)		
Total		2 (2,3)	84 (97,7)	86 (100,0)		
Modified Ziehl-Neelsen						
age	0-2	1 (8,3)	11 (91,7)	12 (100,0)	0,199	0,334
	3-7	9 (18,4)	40 (81,6)	49 (100,0)		

	8-18	0 (0,0)	5 (100,0)	5 (100,0)		
	19-70	1 (5,0)	19 (95,0)	20 (100,0)		
Total		11 (12,8)	75 (87,2)	86 (100,0)		
Immunochromatographic rapid test - ICH						
age	0-2	0 (0,0)	12 (100,0)	12 (100,0)		
	3-7	7 (14,3)	42 (85,7)	49 (100,0)		
	8-18	0 (0,0)	5 (100,0)	5 (100,0)	0,259	0,124
	19-70	0 (0,0)	20 (100,0)	20 (100,0)		
Total		7 (8,1)	79 (91,9)	86 (100,0)		
RT-PCR VIASURE						
age	0-2	6 (50,0)	6 (50,0)	12 (100,0)		
	3-7	43 (87,8)	6 (12,2)	49 (100,0)		
	8-18	0 (0,0)	5 (100,0)	5 (100,0)	0,634	0,000
	19-70	5 (25,0)	15 (75,0)	20 (100,0)		
Total		54 (62,8)	32 (37,2)	86 (100,0)		
nPCR 18S rRNA						
age	0-2	6 (50,0)	6 (50,0)	12 (100,0)		
	3-7	43 (87,8)	6 (12,2)	49 (100,0)		
	8-18	0 (0,0)	5 (100,0)	5 (100,0)	0,634	0,000
	19-70	5 (25,0)	15 (75,0)	20 (100,0)		
Total		54 (62,8)	32 (37,2)	86 (100,0)		
nCOWP-PCR						
age	0-2	6 (50,0)	6 (50,0)	12 (100, 0)		
	3-7	43 (87,8)	6 (12,2)	49 (100,0)		
	8-18	0 (0,0)	5 (100,0)	5 (100,0)	0,634	0,000
	19-70	5 (25,0)	15 (75,0)	20 (100,0)		
Total		54 (62,8)	32 (37,2)	86 (100,0)		

Among the cases of cryptosporidiosis confirmed through ELISA, the modified Ziehl-Neelsen method and the immunochromatographic methods, the p-values (0,672; 0,334 and 0,124) show that age has no significant influence on the frequency of parasitic infection with *Cryptosporidium* spp. The use of the three variations of PCR – RT-PCR VIASURE, nPCR 18S rRNA and MT nPCR COWP revealed that age has a significant impact on the frequency of parasitic infection with *Cryptosporidium* spp., which is confirmed by Cramer's ratios (0,634), according to which, 63,4% of the variations in the cases confirmed through these diagnostic methods are attributable to the age of the patients.

5.5 Distribution of the persons who tested positive for cryptosporidiosis depending on the clinical course of the disease

Table 8 shows the results of the persons who tested positive for cryptosporidiosis depending on the clinical course of the disease. The total number of positive samples is 86 (100,0%).

Table 8. *Distribution of the samples positive for cryptosporidiosis depending on the presence or absence of symptoms in the course of the disease*

		Asymptomatic n (%)	Symptomatic n (%)	Total n (%)	Cramer's V	Sig. (p-value)
ELISA						
gender	woman	31 (75,6)	10 (24,4)	41 (100,0)	0,253	0,019
	man	23 (51,1)	22 (48,9)	45 (100,0)		
Total		54 (62,8)	32 (37,2)	86 (100,0)		
ethnic group	Bulgarian	28 (48,3)	30 (51,7)	58 (100,0)	0,432	0,000
	Roma	26 (92,9)	2 (7,1)	28 (100,0)		
Total		54 (62,8)	32 (37,2)	86 (100,0)		
settlement	town	21 (46,7)	24 (53,3)	45 (100,0)	0,349	0,001
	village	33 (80,5)	8 (19,5)	41 (100,0)		
Total		54 (62,8)	32 (37,2)	86 (100,0)		
age	0-2	4 (33,3)	8 (66,7)	12 (100,0)	0,754	0,000
	3-7	46 (93,9)	3 (6,1)	49 (100,0)		
	8-18	0 (0,0)	5 (100,0)	5 (100,0)		
	19-70	4 (20,0)	16 (80,0)	20 (100,0)		
Total		54 (62,8)	32 (37,2)	86 (100,0)		

Among the examined persons who tested positive for cryptosporidiosis depending on the presence or absence of symptoms in the course of the disease, the highest Cramer's ratios were obtained for the age indicator (0,754), followed by ethnic origin (0,432). It can thus be concluded that these two characteristics have the strongest influence on the examined indicators.

6. Results of the examined animals

The total number of examined animal samples is 107, of which 11 are put in a control group in which there were no positive samples for *Cryptosporidium* spp.

Table 9 presents the results of the animals tested for cryptosporidiosis by means of the three methods applied. The total number of animals tested with the three methods is 96, of which 2 lambs, 2 goats, 1 kid, 28 cows and 63 calves.

Table 9. *Distribution of the samples examined for cryptosporidiosis depending on the species of the animal*

		Negative n (%)	Positive n (%)	Total n (%)	Cramer's V	Sig. (p-value)
ELISA						
Species	lamb	2 (100)	0 (0,0)	2 (100)	0,473	0,000
	goat	2 (100)	0 (0,0)	2 (100)		
	kid	0 (0,0)	1 (100)	1 (100)		

	cow	25 (89,3)	3 (10,7)	28 (100)		
	calf	27 (42,9)	36 (57,1)	63 (100)		
Total		56 (58,3)	40 (41,7)	96 (10 0)		
Modified Ziehl-Neelsen						
Species	lamb	2 (100)	0 (0,0)	2 (100)	0,365	0,012
	goat	2 (100)	0 (0,0)	2 (100)		
	kid	0 (0,0)	1 (100)	1 (100)		
	cow	22 (78,6)	6 (21,4)	28 (100)		
	calf	29 (46,0)	34 (54,0)	63 (100)		
Total		55 (57,3)	41 (42,7)	96 (100)		
Immunochromatographic rapid test - ICH						
Species	lamb	2 (100)	0 (0,0)	2 (100)	0,523	0,000
	goat	2 (100)	0 (0,0)	2 (100)		
	kid	0 (0,0)	1 (100)	1 (100)		
	cow	27 (96,4)	1 (3,6)	28 (100)		
	calf	28 (44,4)	35 (55,6)	63 (100)		
Total		59 (61,5)	37 (38,5)	96 (100)		

The total number of negative samples tested with ELISA is 56, of which 2 are of lambs, 2 of goats, 25 of cows and 27 of calves. Positive results were obtained in three of the groups, the largest number being that of the calves — 36. There are three positive samples among the cows, and the kid also tested positive. The total number of positive samples is 40.

Through morphodiagnostics by means of the modified Ziehl-Neelsen method (Kinyoun method), a total of 55 negative samples were found, of which 2 from lambs, 2 from goats, 22 from cows and 29 from calves. Oocysts of cryptosporidia were found in altogether 41 samples – 1 of the kid, 6 of the cows and 34 of the calves.

The rapid immunographic tests established a total number of 37 positive samples, one of which is of the kid, one of a cow and 35 of calves.

The total number of negative samples is 59, of which 2 are from lambs, 2 from goats, 27 from cows and 28 from calves.

Cramer's ratios for the three methods vary between 0,365 and 0,523 and show a moderate correlation between the animal species and the frequency of parasitic infection with *Cryptosporidium* spp. in animals. This is also confirmed by the p-values (0,000; 0,012; 0,000), which reveal a statistically proven influence of the animal species on the frequency of parasitic infection with *Cryptosporidium* spp.

Table 10 shows the results of the animal samples examined for cryptosporidiosis according to the three methods applied depending on the age of the animal. The number of tested animals is 67 young animals and 29 grown-up animals.

Table 10. *Distribution of the samples examined for cryptosporidiosis according to the age of the animal*

		Negative n (%)	Positive n (%)	Total n (%)	Cramer's V	Sig. (p-value)
ELISA						
age	grown-up	26 (89,7)	3 (10,3)	29 (100)	0,418	0,000
	young	30 (44,8)	37 (55,2)	67 (100)		
Total		56 (58,3)	40 (41,7)	96 (100)		
Modified Ziehl-Neelsen						
age	grown-up	23 (79,3)	6 (20,7)	29 (100)	0,293	0,004
	young	32 (47,8)	35 (52,2)	67 (100)		
Total		55 (57,3)	41 (42,7)	96 (100)		
Immunochromatographic rapid test - ICH						
age	grown-up	28 (96,6)	1 (3,4)	29 (100)	0,474	0,000
	young	31 (46,3)	36 (53,7)	67 (100)		
Total		59 (61,5)	37 (38,5)	96 (100)		

The total number of negative samples from those tested with ELISA is 56, of which 26 are of grown-up animals and 30 of young animals. Similar results are obtained from the modified Ziehl-Neelsen method (Kinyoun method). Of a total of 55 negative animal samples, 23 are of grown-up animals, and 32 are of young animals.

The positive samples tested with ELISA are altogether 40 – 37 of young animals and only 3 of grown-up animals. The modified Ziehl-Neelsen method shows a total of 41 positive samples – 35 from young animals and 6 from grown-up animals.

The positive samples established through rapid immunochromatographic tests are altogether 37. All samples except for one are of young animals (36). The total number of samples of examined animals which tested negative for cryptosporidiosis is 59,of which 28 are from grown-up animals and 31 are from young animals.

The cases of cryptosporidiosis confirmed through ELISA and the rapid immunochromatographic tests reveal a moderate correlation (Cramer's V=0,418; 0,474) between the age of the animal and the frequency of infection with *Cryptosporidium* spp. in animals, whereas the modified Ziehl-Neelsen method shows a weak correlation (0,293). All three applied methods show that the influence of age on the frequency of parasitic infection with *Cryptosporidium* spp. in animals is statistically significant (p-value = 0,000; 0,004; 0,000).

Figure 25 shows the results of the distribution of the positive cases in calves depending on the age. The number of positive samples in the first two groups is similar — respectively 17 in the first and 18 in the second. In the third group, there is only one registered case.

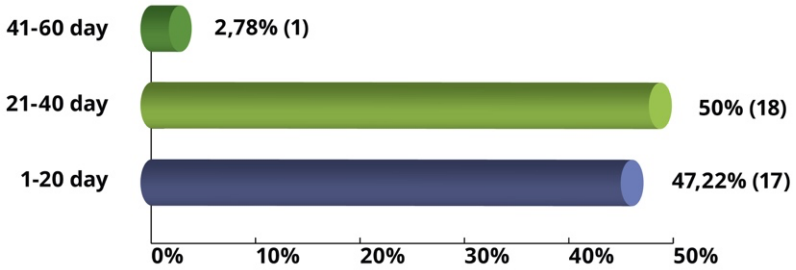


Figure 25. *Distribution of positive cases of cryptosporidiosis in calves depending on their age*

Only the animal samples which were positive for cryptosporidiosis according to the three methods were subjected to PCR tests.

In 9 of the samples of young calves, the testing with RT-PCR VIASURE revealed polyinvasion – *Cryptosporidium spp. + Giardia lamblia.* The results are presented in Figure 26.

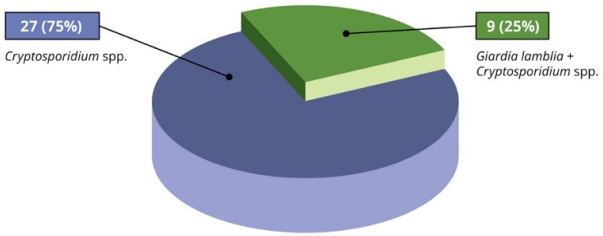


Figure 26. *Presence of polyinvasion with Cryptosporidium spp. and Giardia lamblia in calves*

7. Results of a survey conducted among the personnel of kindergartens, nurseries and clinics on their awareness regarding intestinal parasitic invasions and cryptosporidiosis

The survey has 233 participants, of which 79 work in a medical care facility, 118 in kindergartens and 36 in nurseries. The distribution of the participants by profession is, as follows: 30 doctors, 71 nurses, 56 kindergarten teachers, 42 assistant-teachers, 16 nursery teachers and 17 hospital attendants. The distribution of the participants by educational degree is, as follows: 94 have a master's degree,

58 have a bachelor's degree, 20 are specialists, 54 have high school education 54 and 7 have primary education.

As a result of the conducted survey, the following conclusions can be made:

The level of education, the profession and the type and settlement of the place of work significantly influence the knowledge of the respondents regarding intestinal parasitic invasions and cryptosporidiosis in particular.

As it can be expected, the highest level of knowledge regarding the symptoms and nature of the causes for intestinal parasitic invasions and cryptosporidiosis is observed among the respondents working in medical care facilities, the persons with higher educational degree and qualification, as well as among those who work in city and town facilities.

8. Mapping of the distribution of the *Cryptosporidium* spp. invasion among people and animals in the district of Pleven by municipality

As a result of the overall work related to the studying of *Cryptosporidium spp.* and cryptosporidiosis among people and animals, the distribution of the invasion of *Cryptosporidium* spp. among people and animals in the district of Pleven and the adjacent regions has been mapped. The map in Figures 55, 56, 57 and 58 was drafted on the basis of the results from immunological studies of faeces samples of people in the district of Pleven. Figure 27 shows the distribution of the disease in the district of Pleven among animals.

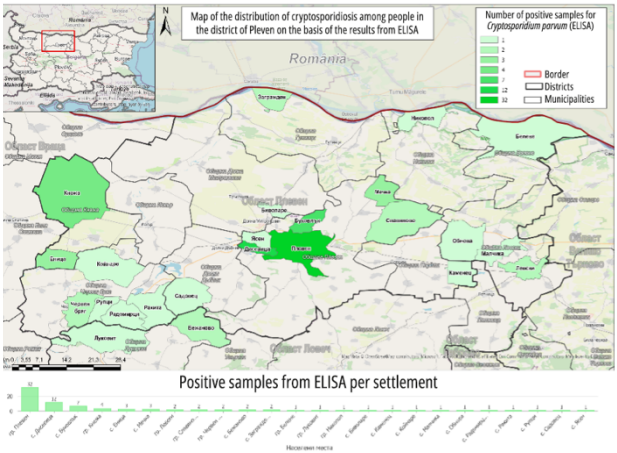


Figure 27. *Map of the distribution of cryptosporidiosis among people in the district of Pleven on the basis of the results from ELISA.*

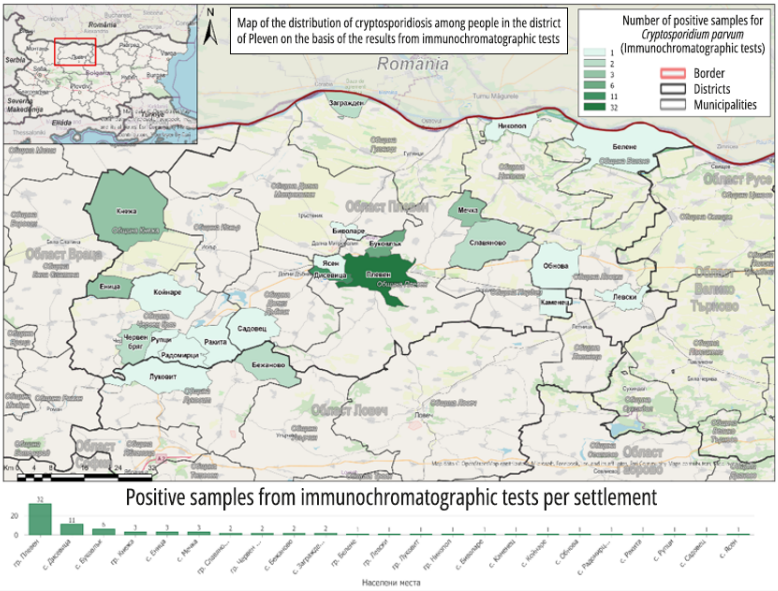


Figure 28. *Map of the distribution of cryptosporidiosis among people in the district of Pleven on the basis of the results from immunochromatographic tests.*

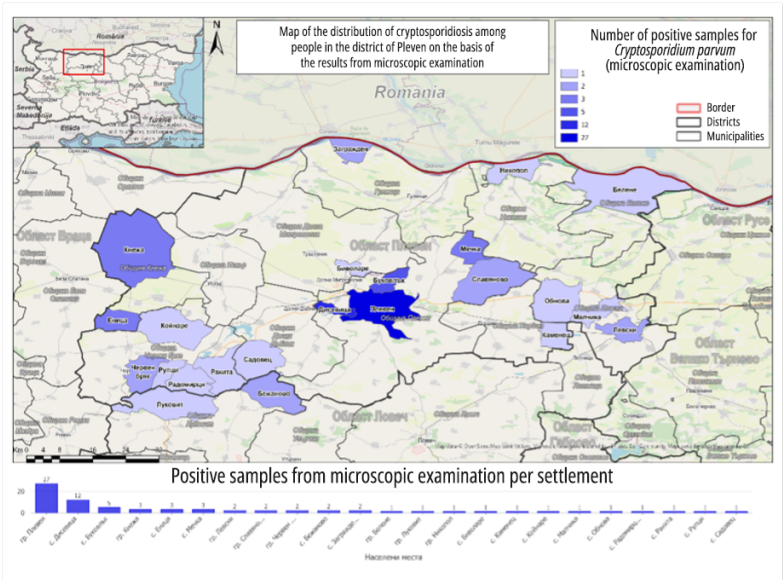


Figure 29. *Map of the distribution of cryptosporidiosis among people in the district of Pleven on the basis of the results from microscopic examination.*

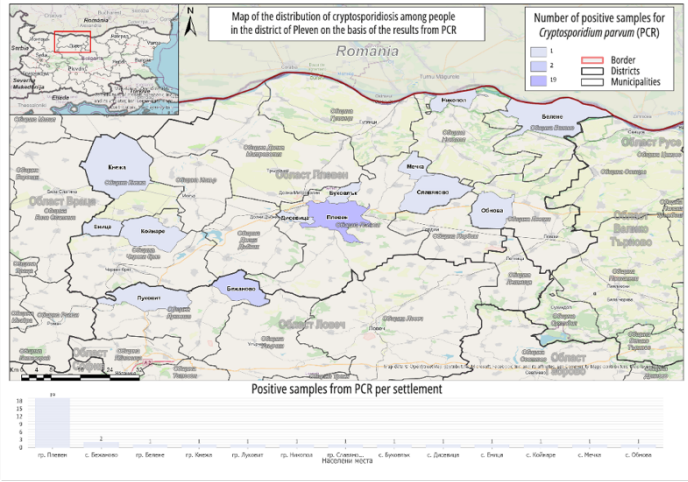


Figure 30. *Map of the distribution of cryptosporidiosis among people in the district of Pleven on the basis of the results from PCR.*

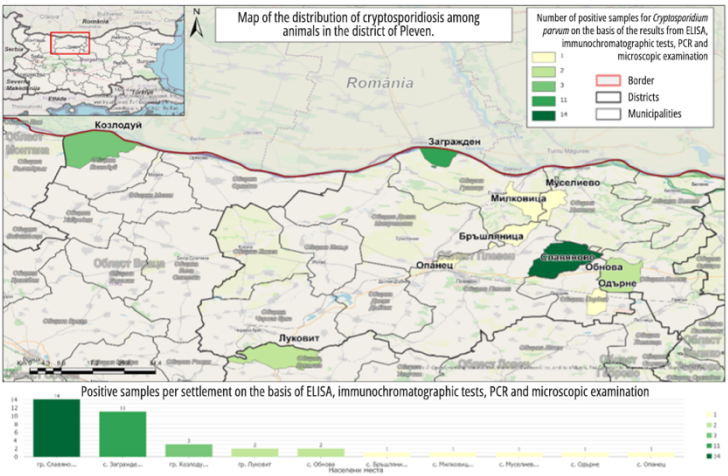


Figure 31. *Map of the distribution of cryptosporidiosis among animals in the district of Pleven.*

The mapping of the areas of distribution of cryptosporidiosis among people and animals in the district of Pleven would be a useful tool for the decision-making process of the health authorities in determining the possible risk of intestinal infections. The present study would also support the selection of adequate strategies for the purposes of public health protection in the light of the global initiative "*One health"* of the WHO.

V. Conclusion

Currently, the efforts for prevention of parasitic diseases need to be focused on improving the hygiene and sanitary conditions in order to restrict the areas of distribution of the infection with *Cryptosporidium spp.* among animal and human populations. This concerns the developing countries in particular, where the disease falls into the category of neglected zoonotic infections. The active migration processes of large populations of people accompanied by animals, the global climate changes, the tourist destinations in regions endemic to various parasitic and infectious regions all increase the risk of appearance of new diseases or the renewal of epidemic processes that have already been put under control in the past. The improved diagnostics, the development and introduction of diagnostic and therapeutic innovations, as well as the awareness of their application contribute to achieving a more realistic evaluation of the current state of a number of socially significant diseases. In the light of the *"One Health"* initiative of the WHO, all this is a new momentum in the prevention and prophylaxis of a series of infectious and parasitic diseases.

Considering that cryptosporidiosis is a zoonotic infection which falls among the top ten infections disseminated along the food chain, as well as the most frequently encountered etiopathogenic factor of epidemic outbreaks occurring in water environment, it is necessary to update the methods for epidemiological surveillance in regions with compromised sanitary and hygiene conditions. The focus should be on tracing the source of pathogenic microorganisms as an additional tool using molecular biology techniques for the precise identification of abiotic and biotic elements of the epidemic chain.

In Bulgaria, cryptosporidiosis is an underdiagnosed parasitic disease whose epidemiology, clinical course and therapy is not sufficiently known to the medical specialists. The small number of diagnosed cases diverges the attention and the possibilities for financing not only of scientific studies, but also for the administrative provisions regarding the import of etiologically active medical substances. This turns out to be a particularly serious problem in the light of the constantly increasing number of patients whose immune systems are suppressed for various reasons, including AIDS, among which cryptosporidiosis is a life-threatening parasitic disease.

VI. Deductions

In the course of the implementation of the tasks related to achieving the main purpose of the present dissertation, namely the study through contemporary diagnostic methods of the biology, distribution and epidemiology of cryptosporidiosis in an underresearched region such as the district of Pleven, the following deductions were made:

1. On the basis of the 1133 examined faeces samples from humans and animals in the period between 2016 and 2023, a distribution of the infection with *Cryptosporidium* spp. of 7,6% among particular groups of the population of the city of Pleven and the district of Pleven was observed.
2. The database accumulated on the basis of the main demographic indicators such as age, gender, settlement and ethnic origin in people is a prerequisite for an up-to-date assessment of the frequency of parasitic infection with *Cryptosporidium* spp. in the district of Pleven.
3. On the grounds of the statistical analysis and upon the application of the three main diagnostic methods, it was established that gender, ethnic origin and place of residence have little influence on the distribution of cryptosporidiosis. Long-term studies outline a weak tendency of the distribution of cryptosporidiosis, in which the persons of Bulgarian ethnic origin and the residents of towns prevail. No difference was found in terms of frequency of parasitic infection between the male and female participants in the study from the district of Pleven.
4. Cramer's ratios show that the age of the persons is in correlation withy he frequency of parasitic infection with *Cryptosporidium* spp. The largest relative share of the persons discharging oocysts is that of children between 3 and 7 years of age.
5. During the same period, a total of 96 samples of various species of domestic animals (cows, calves, goats, lambs, kids), of which 38,5% were invaded by the pathogen, was studied. It was established that the species and the age of the animal are the main factors impacting the frequency of parasitic infection with *Cryptosporidium* spp.
6. The high extent of polymorphism in the segments of the two genes used by us is an appropriate marker for the identification of the species of *Cryptosporidia*. On the grounds of genotyping on the basis of the RFLP analysis after the application of nPCR, the isolates of *Cryptosporidium* spp. of human and animal origin show restriction profiles of

Cryptosporidium parvum, identical with those of the used reference strain *Cryptosporidium parvum Iowa II* (genotype 2).

7. The molecular methods applied show that *Cryptosporidium parvum* is the main cause for cryptosporidiosis in humans and ruminants in the district of Pleven.
8. The morphological, immunological and molecular biology methods for diagnostics of *Cryptosporidium* spp. applied are the basis for discovering hidden infections among the examined target groups of persons.
9. The areas of distribution of cryptosporidiosis throughout the researched period were established my means of an analysis based on the Geographic Information System.
10. Through the survey we carried out it was found that education, profession and the place of work (town or village) influence the knowledge of the respondents about intestinal parasitic infections and, in particular, cryptosporidiosis. As it can be expected, the highest level of knowledge about the symptoms of intestinal parasitic infections and cryptosporidiosis, as well as about the nature and causes of the disease, is among those who work in hospitals, those who have a higher educational degree and qualification and those who work in towns.
11. The drafting of health education brochures and their distribution among particular risk groups and healthcare practitioners contributes to their awareness of information which is useful for both their health and their professional practice.

VII. Contributions

1. Contributions of original nature

1.1 For the first time in the district of Pleven a large-scale study for the period 2016-2023 to establish the frequency of parasitic infection with *Cryptosporidium* spp. among particular groups of people and animals was conducted through the application of routine and contemporary parasitological, immunological and molecular biology methods.

1.2 For the first time, the distribution of the infection with *Cryptosporidium* spp. was established among the target groups of the population of the city of Pleven and its district area.

1.3 For the first time in Bulgaria, genotyping on the basis of a RFLP analysis after nPCR of isolates of *Cryptosporidium* spp. from human and animal faeces samples has been conducted, which reveal the restriction profiles of *Cryptosporidium parvum*, identical with those of the used reference strain *Cryptosporidium parvum* Iowa II (genotype 2).

1.4 Three methods to isolate DNA of *Cryptosporidium* spp. from faeces samples have been applied.

1.5 An algorithm for conducting biomolecular studies of isolates of *Cryptosporidium* spp. from human and animal faeces samples in the district of Pleven was developed for the first time in Bulgaria. This algorithm can serve as a model for the study of other pathogens.

1.6 The established hidden morbidity of cryptosporidiosis in the region of Pleven among children and adults is significant in clinical and epidemiological terms and constitutes an essential contribution to extending the studies of this parasitic infection.

2. Contributions of applied and affirmative nature

2.1 Maps of the areas of distribution of *Cryptosporidium parvum* in Pleven and the district have been developed. These maps can be used within the healthcare system.

2.2 The results and analyses obtained complement and enrich the data regarding the distribution of cryptosporidiosis in Bulgaria.

2.3 As a result of the complex study, a contemporary epidemiological description for a seven-year period was created, which adds to the existing information on the species in Bulgaria.

2.4 The epidemiological significance of Cryptosporidium parvum as a cause for intestinal infections among people and animals was confirmed.

VIII. Publications, participation in scientific fora and scientific projects in connection with the dissertation

1. List of scientific publications in connection with the dissertation

1.1 Dragomirova P, Dobrev R, Boeva-Bangyozova V. Clinical Picture of Cryptosporidiosis in Humans. Childhood and Infectious Diseases 2018; X (2): 52-56. ISSN: 1313-762X

1.2 Dragomirova P, Klisarova D, Dobrev R, Dragomirov A, Nedelcheva N. distribution of *Cryptosporidium* spp. among target groups in the district of Pleven. Folia Medica 2020; 56(6): 40-46. ISSN: 1312-2193

1.3 Dragomirova P, Eneva Kr, Boeva-Bangyozova V. Cryptosporidiosis - surveillance, control and prophylaxis. Medinfo. 2018;8:50–52. ISSN: 1314-0345

2. List of participations in scientific conferences in connection with the dissertation

2.1 Dragomirova P, Petrova L, Klisarova D, Dragomirov A, Nedelcheva N, Atanasova M. A study of distribution frequency of *Cryptosporidium* among the human population and domestic animals in Pleven district. Folia Medica. 2017;59(1)19. /Abstracts/

2.2 Dragomirova P, Atanasova M. Epidemiology of *Cryptosporidium* spp. in animal population in Pleven region. XIII National Conference on Medical Biology. Varna. 2019.

2.3 Dragomirova P, Dobrev R, Boshnakova-Mihaylova J, Atanasova M. Epidemiology of *Cryptosporidium* spp. in human population in Pleven region. 45 years Medical University – Pleven". Journal of Biomedical and Clinical Research. 2019;12(1):142 /Abstracts/

2.4 Dragomirova P. Detection of Cryptosporidium oocysts by rapid immunochromatographic test. Agriculture – way of life. Trakia University - Stara Zagora, Bulgaria. 2024

3. List of participation in projects in connection with the dissertation

3.1 Project №11/2017, MU-Pleven "A study of distribution frequency of *Cryptosporidium* among the human population and domestic animals in Pleven district".

3.2 Project №3/2019, MU-Pleven "Species identification of *Cryptosporidium* spp. through nest PCR and analysis of the restriction fragments".

IX. APPENDICES

Appendix 1