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# The first report of animal genotypes of *Cryptosporidium parvum* in immunosuppressed and immunocompetent humans in Slovakia

Elena Hatalová[1] | Alexandra Valenčáková[1] | Lenka Luptáková[1] | Michaela Špalková[1] | Jana Kalinová[1] | Monika Halánová[2] | Veronika Bednárová[2] | Juliana Gabzdilová[2] | Kinga Dedinská[2] | František Ondriska[3] | Vojtech Boldiš[4]

[1]The University of Veterinary Medicine and Pharmacy in Košice, Košice, Slovakia

[2]University of Pavol Jozef Šafárik, Košice, Slovakia

[3]Trnava University, Trnava, Slovakia

[4]Medirex, Bratislava, Slovakia

**Correspondence**
Alexandra Valenčáková, The University of Veterinary Medicine and Pharmacy in Košice, Košice, Slovakia.
Email: alexandra.valencakova@uvlf.sk

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## Abstract

The aim of our study was to determine species and genotypes of *Cryptosporidium* in patients suffering from immunosuppressive illnesses, but also in immunocompetent patients suffering from diarrhoea. A total of 80 samples of faeces were collected from both immunosuppressed and immunocompetent patients. The immunosuppressed patients (65 samples) — 35 adult patients (group A) and 30 children (group B) were hospitalized at the Clinic of Oncohemathology. Samples from immunocompetent humans (15 samples, group C) were taken from patients with clinical signs of acute diarrhoea. With the use of molecular methods targeting the 60 kDa glycoprotein (GP60) gene region, we have identified multiple genotypes of *Cryptosporidium. parvum* and *Cryptosporidium. hominis* in immunocompromised, but also in immunocompetent individuals (*C. hominis* IbA10G2, IeA12G3T3; *C. parvum* IIaA10G1R1, IIaA11G2R1, IIaA12G2R1, IIaA13G1R1, IIaA14G1R1, IIaA14G2R1, IIaA17G1R1 and IIaA18G1R1). This is the first report of the occurrence of genotypes IIaA10G1R1, IIa12G2R1 and IIaA18G1R1 in human hosts.

**KEYWORDS**
*C. hominis*, *Cryptosporidium parvum*, Genotypes, immunosuppression

## 1 | INTRODUCTION

*Cryptosporidium* spp. are intracellular protozoan parasites with a cosmopolitan distribution, infecting a wide range of host species — amphibians, reptiles, fish, birds and mammals, including humans (Fayer, Morgan, & Upton, 2000; Valenzuela et al., 2014). More than 90% of human *Cryptosporidium* infections are caused by species: *Cryptosporidium parvum* and *Cryptosporidium hominis*; although other species and genotypes have been also reported in humans (Petrincová et al., 2015; Ryan, Zahedi, & Paparini, 2016; Xiao, 2010). The transmission of infection occurs directly by person-to-person (anthroponotic transmission), animal-to-human (zoonotic transmission), animal-to-animal; or indirectly via contaminated water or food. (Efstratiou, Ongerth, & Karanis, 2017; Karanis, Kourenti, & Smith, 2007; Kosek, Alcantara, Lima, & Guerrant, 2001). *Cryptosporidium* infections are also classified as waterborne infections, due to the easy spreading of infectious stages — oocyst — through the water. Oocysts are extremely hardy and resistant to various forms of water disinfection, making them difficult to remove from water (Fayer, Dubey, & Lindsay, 2004). Cryptosporidiosis occurs mainly in immunocompromised patients, but *Cryptosporidium* spp. can also infect immunocompetent humans as well. In immunocompetent individuals, cryptosporidiosis manifests as a self-limiting gastroenteritis with a loss of appetite, weight loss and slight fever (Valenzuela et al., 2014). However, patients suffering from some type of immunocompromised condition have an increased probability of *Cryptosporidium*

  

infection. In these patients, cryptosporidiosis can manifest as severe protracted diarrhoea with chronic malabsorption, malnutrition and may even cause death (Assefa, Erko, Medhin, Assefa, & Shimelis, 2009; Idris, Dwipoerwantoro, Kurniawan, & Said, 2010; Valenzuela et al., 2014). While immunosuppression and diarrhoea are recognized side-effects in treating malignant diseases, relatively few studies have been conducted to estimate the prevalence of *Cryptosporidium* in patients suffering from oncologic illnesses (Al-Qobati, Al-Maktari, Al-Zoa, & Derhim, 2012). In the last decade, several molecular methods detecting *Cryptosporidium* have been developed. Polymerase chain reaction (PCR) is used as a reliable method for detecting *Cryptosporidium* in clinical samples. Several PCR protocols targeting various regions of the genome of *Cryptosporidium* are used. The most common targeted regions are, for example, the SSU rRNA region, *Cryptosporidium* oocyst wall protein (COWP), internal transcribed spacer region (ITS), thrombospondin-related adhesive protein (TRAP) and beta-tubulin. For the identification of *Cryptosporidium* genotypes, the 60 kDa glycoprotein gene (GP60) is amplified (Leetz, Sotiriadou, Ongerth, & Karanis, 2007). Also, microsatellite and minisatellite markers are used (Feng et al., 2011).

Genotyping *Cryptosporidium* based on the GP60 gene coding the 60 kDa glycoprotein (GP60, also called GP40/15) is a popular method in subtyping *Cryptosporidium* spp. GP60 subtyping is nowadays used for several species (*C. hominis*, *C. parvum*, *C. meleagridis*, *C. cuniculus*, *C. ubiquitum*, *C. erinacei* and other; Alves et al., 2003; Koehler, Whipp, Haydon, & Gasser, 2014; Kváč et al., 2014; Li et al., 2014; Stensvold, Beser, Axén, & Lebbad, 2014). GP60 primers used for genotyping have higher sensitivity and detection rate in amplifying the DNA of *Cryptosporidium* than primers targeting another region (especially when host specific *Cryptosporidium* are expected in the sample, e.g., *C. parvum* and *C. hominis* in samples from humans; Hatalová et al., 2017). Also, these primers are more specific than primers amplifying the SSU sRNA region, which can produce non-specific amplifications or false-positive results, due to similarities in SSU sequences of *Cryptosporidium* and other pathogens (infraphylum *Apicomplexa*, gregarines; unpublished results).

In this study, we have focused on the identification of *C. parvum* and *C. hominis* species and genotypes in patients hospitalized at the Clinic of Oncohemathology (University of Pavol Jozef Šafárik, Košice) and also in immunocompetent patients suffering from acute diarrhoea.

## 2 | MATERIALS AND METHODS

### 2.1 | Population study

A total of 80 samples of faeces were collected from both immunosuppressed and immunocompetent patients. The immunosuppressed patients (65 samples) — 35 adult patients (group A) and 30 children (group B) were hospitalized at the Clinic of Oncohemathology (University of Pavol Jozef Šafárik, Košice). Samples from immunocompetent humans (15 samples, group C) were taken from patients with clinical signs manifested as acute diarrhoea (approval for the study was obtained from the Ethics Committee of the Faculty of Medicine at P. J. Šafárik University in Košice (No. 104/2011). The study was performed in accordance with the ethical standards as laid down in the Declaration of Helsinki of 1975 and revised in 2008). Samples were stored at −20°C until DNA extraction.

### 2.2 | DNA isolation

Approximately 100 mg of faeces was homogenized and the oocysts were disrupted at 3,543 *g* for 90 seconds with the addition of 0.5 mm glass beads, 1.0 mm zircon beads and 300 μl of lysis solution in Precellys 24 homogenizer (Bertin Technologies). DNA was extracted using the DNA-Sorb-B nucleic acid extraction kit (AmpliSense, Russia). Isolated DNA was stored at −20°C until the use in PCR.

### 2.3 | Nested PCR using GP60 primers (amplification of the GP60 gene)

#### 2.3.1 | PCR reaction mix

In both reactions, the total volume of the PCR reaction mixtures was 50 μl, with 5 μl of the DNA sample. Primers with a concentration of 0.2 μM and 5 U Taq DNA polymerase (FIREpol) were used.

The 60 kDa glycoprotein gene (GP60 gene) was amplified and analysed for identifying *Cryptosporidium* spp. genotypes and subgenotypes.

The GP60 gene was used as a marker because it has a higher detection rate in diagnosing *C. parvum* and *C. hominis* than primers targeting the SSU rRNA region (Hatalová et al., 2017).

#### 2.3.2 | Nested PCR

GP60 primers were used for amplifying the GP60 region. For the first reaction, outer primers GP60 F1 (5′- ATG AGA TTG TCG CTC ATT ATC-3′) and GP60 R1 (5′- TTA CAA CAC GAA TAA GGC TGC-3′) were used at annealing temperature 55°C, with a product size of 980–1,000 bp. For the second reaction, inner primers GP60 F2 (5′- GCC GTT CCA CTC AGA GGA AC-3′) and GP60 R2 (5′- CCA CAT TAC AAA TGA AGT GCC GC-3′) were used at annealing temperature 58°C. PCR was run in thermocycler XP Thermal Cycler Blocks, with an incubation of 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 55/58°C for 1 min/45 s and elongation at 72°C for 1 min. A final elongation step of 72°C for 7 min was included to ensure the complete extension of the amplified products.

### 2.4 | Gel electrophoresis and DNA sequencing

Final products of a product length of 450 bp were evaluated by electrophoresis on 1.5% agarose gel dyed with GelRed™ Nucleic acid gel stain (Biotium) in TAE buffer. Samples were dyed with Red load™ PCR loading buffer (Top-Bio). Polymerase chain reaction (PCR)

products positive on the gel were sent for DNA sequencing. Final sequences were compared with homologous sequences stored in GenBank using Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

## 2.5 | Rules of genotyping *C. parvum* and *C. hominis*

Genotypes of *C. parvum* and *C. hominis* were identified using the rules of genotyping *C. parvum* and *C. hominis* by Xiao, 2010. The GP60 gene shows similarity with microsatellite markers by having tandem repeats of serine-coding trinucleotide (TCA, TCT, TCG) at the 5′ end of the gene. In addition, there are sequence differences in non-repeating regions of the gene, distinguishing *C. parvum* and *C. hominis* in subtype families. The subtypes in each family differ mostly in the number of tandem repeats of trinucleotide. Name of the subtype family starts with the subtype family designation (Ia, Ib, Ic, etc. for *C. hominis* and IIa, IIb, IIc etc. for *C. parvum*). A family designation is followed by the number and abbreviated form of the tandem repeats (TCA represented by letter A, TCG represented by letter G and TCT represented by letter T). Some subtype families also have repeats of certain sequences after tandem repeats. The *C. hominis* Ia family is further identified by the copy of a 15 bp repetitive sequence represented by the letter R (5′-AA/GGACGGTGGTAAGG-3′), with the last copy being a 13 bp AAA/GACGGTGAAGG. *C. parvum* subfamily IIa is identified by copies of a 6 bp sequence ACATCA.

Examples of GP60 subtype families and representative sequences are summarized in Table 1.

## 2.6 | Phylogenetic tree construction

The consensus sequences were edited and assembled using program BioEdit. Then sequences were aligned using Alignment ClustalW2 with all reference sequences from GenBank. Trees were constructed by method Maximum likelihood using the program MEGA 6. Bootstrap values were calculated from 1,000 replicates. We used *C. tyzzeri* and *C. meleagridis* as outgroup.

# 3 | RESULTS

From a total of 80 samples, 20 samples were identified as positive either with *C. hominis* or *C. parvum*.

**TABLE 1** Examples of *Cryptosporidium parvum* and *Cryptosporidium hominis* subtype families

| Species | Subtype family | Dominant repeat | Other repeat |
|---|---|---|---|
| *C. hominis* | Ia | TCA | AA/GGACGGTGGTAAGG |
| | Ib | TCA, TCG, TCT | |
| | Ie | TCA, TCG, TCT | |
| *C. parvum* | IIa | TCA, TCG | ACATCA |
| | IIb | TCA | |
| | IIc | TCA, TCG | |

*Note.* Source: Xiao, 2010, modified.

In group A (immunocompromised adults), *C. parvum* genotype IIaA14G1R1 was identified in one sample, *C. parvum* genotype IIaA16G1R1 also in one sample, genotype IIaA17G1R1 in three samples and genotype IIaA17G1R1 in one sample.

In group B (immunocompromised children) *C. parvum* genotype IIaA13G1R1 was identified in one sample and genotype IIaA17G1R1 was identified in three samples. Also, *C. hominis* was identified as genotype Ie12G3T3 in one sample.

In group C (immunocompetent patients with acute diarrhoea) following genotype of *C. parvum* were identified: IIaA10G1R1, IIaA11G2R1, IIaA12G2R1, IIaA13G1R1, IIaA14G1R1, IIaA14G2R1, IIaA17G1R1, meaning each positive sample was a different genotype. *C. hominis* genotype Ie12G3T3 was identified in one sample and IbA10G2 in one sample.

All results are summarized in Table 2.

## 3.1 | Phylogenetic analysis

For the phylogenetic analysis, we have used the amplified GP60 gene for distinguishing individual subtypes of *C. parvum* and *C. hominis*. The phylogenetic tree was constructed using Maximum likelihood tree method in program MEGA 6 (Molecular Evolutionary Genetics Analysis software). *C. meleagridis* and *C. tyzzeri* were selected as out-group. After local alignment in the BioEdit program, sequences containing 204 bp were used. We have identified nine subtypes of *C. parvum* from our samples. All *C. parvum* subtypes are clustered together in one tree clade, together with reference sequences of various subtypes identified as *C. parvum* subtype IIaA17G1R1, IIaA16G1R1, IIaA14G1R1, IIaA13G1R1, IIaAG2R1 and

**TABLE 2** *Cryptosporidum* species and genotypes identified in samples

| Group | No. of examined samples | No. of positive samples | Identified species and genotypes |
|---|---|---|---|
| A | 35 | 6 | *C. parvum* IIaA14G1R1 (*n* = 1) |
| | | | *C. parvum* IIaA16G1R1 (*n* = 1) |
| | | | *C. parvum* IIaA17G1R1 (*n* = 3) |
| | | | *C. parvum* IIaA18G1R1 (*n* = 1) |
| B | 30 | 5 | *C. parvum* IIaA13G1R1 (*n* = 1) |
| | | | *C. parvum* IIaA17G1R1 (*n* = 3) |
| | | | *C. hominis* IeA12G3T3 (*n* = 1) |
| C | 15 | 9 | *C. parvum* IIaA10G2R1 (*n* = 1) |
| | | | *C. parvum* IIaA11G2R1 (*n* = 1) |
| | | | *C. parvum* IIaA12G2R1 (*n* = 1) |
| | | | *C. parvum* IIaA13G1R1 (*n* = 1) |
| | | | *C. parvum* IIaA14G1R1 (*n* = 1) |
| | | | *C. parvum* IIaA14G2R1 (*n* = 1) |
| | | | *C. parvum* IIaA17G1R1 (*n* = 1) |
| | | | *C. hominis* IeA12G3T3 (*n* = 1) |
| | | | *C. hominis* IbA10G2 (*n* = 1) |

IIa11G2R1. *Cryptosporidium. parvum* subtype IIaA17G1R1, diagnosed in samples IS1, IS2, IS4, IS9 and IS15 (obtained from immunosuppressed adults) samples BL7, BL8, 2H and 3H (obtained from immunocompetent adults) and samples 28 and 29IV (obtained from immunosuppressed children). By comparing genetic variability of the sample sequences with reference sequences, substitutions on five positions were detected in samples 28 and 29IV, while in samples IS1, IS4, IS15 and BL7 only in two nucleotide positions. In sample 2H (*C. parvum* subtype IIaA12G2R1), 31 substitutions were detected. Samples IS2 and BL8 (subtype IIaA14G1R1) have a very low number of substitutions in comparison to reference samples (5–7 substitutions). By comparison of sample IS9 (subtype IIaA16G1R1) with reference sequences, substitutions were located in 18 positions. In another tree clade, reference sequences of *C. parvum* subtype IIA12G2R1, IIaA14G2R1 and IIAa11G2R1 are clustered together with sequences from samples BL1, BL5 (immunocompetent patients) and IS3 (immunosuppressed patient). These samples are characterized by low genetic variability in comparison with sample BL2 which is genetically more distant. By comparing sequence from sample BL2 (subtype IIaA11G2R1) with reference sequences of the same subtype, substitutions in 30 positions were detected, which relocated the sample to a different clade. Sample BL5 (IIaA10G1R1) has 25 substitutions in comparison with the reference sequence. On the main clade, a subclade was formed, containing two samples: 20 (*C. parvum* IIaA17G1R1) and 25 (*C. parvum* IIaA13G1R1), both obtained from immunosuppressed children. In the sequence of sample 20 (*C. parvum* IIaA17G1R1), 20 substitutions were detected, relocating it to a different clade. By analysis of the sequence of sample 25 (*C. parvum* IIaA13G1R1), as much as 81 substitutions were detected (which can be explained by poor sequencing results from this sample), relocating it to a different clade than reference sequences and one other sample of the same subtype. We can find sample 29II (*C. hominis* IeA12G2R1) on the same clade (which we also explain by poor sequencing results). Other samples diagnosed with *C. hominis* — 4H (*C. hominis* IbA10G2) and 1H (*C. hominis* Ie12G3T3) obtained from immunocompetent adults are clustered on the same clade with a bootstrap value of 86. Sample 4H shows low genetic variability in comparison with reference samples of the same subtype and sample 1H is clustered with subtypes IeA12G3T3 and show low genetic variability.

The Phylogenetic tree is represented by Figure 1.

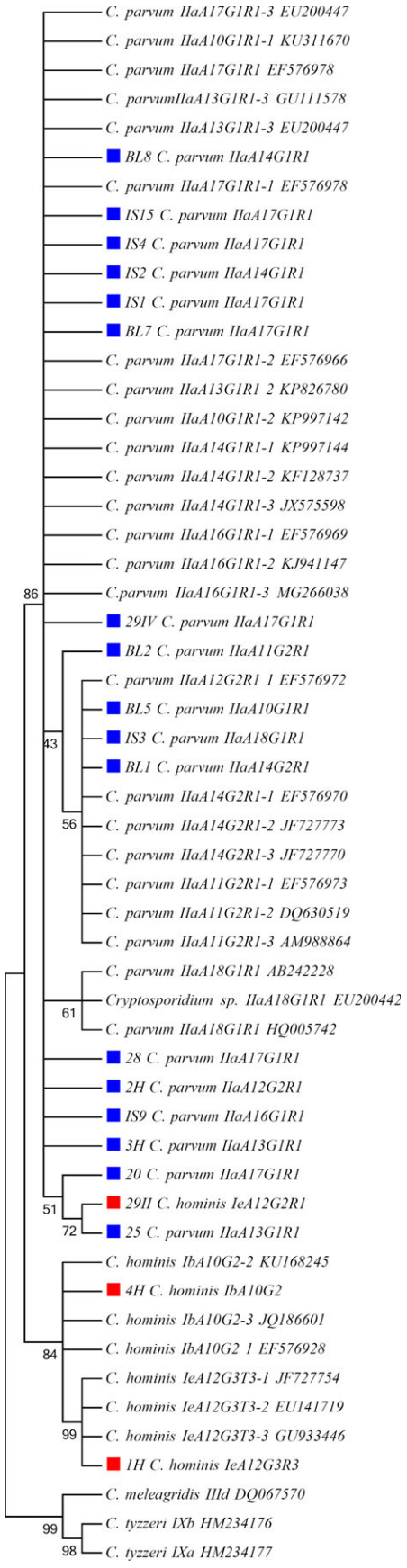


**FIGURE 1** Phylogenetic tree constructed using the Maximum likelihood method [Colour figure can be viewed at wileyonlinelibrary.com]

## 4 | DISCUSSION

Worldwide, cryptosporidiosis is one of the most important causes of diarrhoea and death in immunocompromised patients, mainly AIDS patients, organ transplant patients and patients on immunosuppressive medications. In this study, we have confirmed that cryptosporidiosis can also occur in a clinical form in immunocompetent persons. The transmission of infectious stages of the parasite in hospitalized patients remains unclear. The oocysts of *Cryptosporidium* are commonly transmitted via the faecal-oral route, where contaminated

water plays a key role in their pathogenesis (Fayer & Xiao, 2008). Although drinking water in the distribution system is processed by various treatment systems, oocysts are resistant to the most common form of water treatment — chlorination, and their small size enables them to bypass various water filtration systems (Jakubowski, 1995).

In our study, we have identified three different genotypes of *C. parvum* in the group of immunosuppressed adults, two genotypes of *C. parvum* and one genotype of *C. hominis* in the group of immunosuppressed children, and surprisingly five different genotypes of *C. parvum* and two genotypes of *C. hominis* in immunocompetent patients suffering from diarrhoea.

*Cryptosporidium hominis* genotype IbA10G2 diagnosed in one immunocompetent patient is a common genotype infecting humans and was also identified in Spain, Sweden, Scotland and the Netherlands in clinical samples, but also in water (Azcona-Gutiérrez et al., 2017; Bjelkmar et al., 2017; Deshpande et al., 2015; de Lucio et al., 2016; Roelfsema et al., 2016). This genotype was also previously identified in Slovakia (Ondriska et al., 2013).

*Cryptosporidium hominis* genotype IeA12G3T3, also found in our study in one immunocompetent patient and one immunosuppressed child was also identified in China, Tasmania and the America (Chalmers et al., 2005; Feng, Li, Duan, & Xiao, 2009; Koehler, Whipp, Hogg et al., 2014).

*Cryptosporidium parvum* genotype IIaA11G2R1 identified in this study in one immunocompetent patient was also identified in Slovenia (Soba & Logar, 2008).

*Cryptosporidium parvum* genotype IIaA16G1R1 identified in our study in one patient suffering from oncological illness was diagnosed in patients in several countries of the European Union — Slovenia, Estonia, Romania, and also in other parts of the world — in Canada, Mexico and Tasmania (Iqbal, Goldfarb, Slinger, & Dixon, 2015; Koehler, Whipp, Hogg et al., 2014; Lassen, Ståhl, & Enemark, 2014; Soba & Logar, 2008; Valenzuela et al., 2014; Vieira et al., 2015). There are also studies confirming this genotype in water basins in Slovakia and in rivers in Romania (Imre et al., 2017; Kalinová et al., 2017).

*Cryptosporidium parvum* genotype IIaA17G1R1 found in three immunosuppressed patients, three children with oncological illness and one immunocompetent patient was also identified in humans in the United Kingdom, Iran and Slovenia (Chalmers, Smith, Hadfield, Elwin, & Giles, 2011; Ranjbar, Baghaei, & Nazemalhosseini Mojarad, 2016; Sharbatkhori, Nazemalhosseini Mojarad, Taghipour, Pagheh, & Mesgarian, 2015; Soba & Logar, 2008). This is the most common genotype identified in this study.

Iqbal, Lim, Surin, and Sim (2012) identified *C. parvum* genotypes IIaA13G1R1, IIaA14G1R1 and IIaA14G2R1 in AIDS patients in Malaysia. These genotypes were also identified in our study, one in an immunosuppressed child (IIaA13G1R1). Genotype IIaA14G1R1 was identified in one immunosuppressed patient and one immunocompetent patient. Genotype IIaA14G2R1 was found in one immunocompetent patient. Genotype IIaA14G1R1 were also identified in Slovenia, genotype IIaA14G1R1 also in the United States (Herges et al., 2012; Soba & Logar, 2008). Genotypes IIaA14G1R1 and IIaA14G2R2 were also identified in Spain and Italy (Azcona-Gutiérrez et al., 2017; Del Chierico et al., 2011).

Furthermore, we have also identified *C. parvum* genotype IIaA10G1R1 ($n$ = 1) and IIaA12G2R1 ($n$ = 1) in immunocompetent patients. *C. parvum* genotype IIaA18G1R1 was found in one immunosuppressed patient. All three genotypes were previously identified only in animal hosts (cattle and mice; Wielinga et al., 2008; Danišová et al., 2017; Del Coco et al., 2014; do Couto, Lima Mde, & do Bomfim, 2014; Tomazic et al., 2013; Misic & Abe, 2007). This is the first report of the occurrence of all three genotypes in human hosts.

The diagnosis of various genotypes of *C. parvum* and *C. hominis* in this study shows high adaptation and mutation capability of *Cryptosporidium* spp., many of them different only in one tandem repeat on the GP60 locus. Another possibility exists in the transmission of the infection — the free movement of people in the European Union, especially between countries in the Schengen Area, which can explain the identification of same genotypes in Slovakia, Slovenia, Estonia, Spain, Romania and the United Kingdom. As patients suffering from immunocompromising diseases increases worldwide, cryptosporidiosis can cause a major health concern.

Animal-to-human transmission of *Cryptosporidium* is known to occur. The diagnosis of genotypes previously identified only in the animal host (IIaA10G1R1, IIaA12G2R1 and IIaA18G1R1) again confirms the zoonotic potential of these parasites. These genotypes pose a potential risk for humans coming in close contact with these animals. In case of cattle, the risk of infection is higher in farmers, veterinarians and animal technicians. In case of genotypes identified in mice, the infection may be transmitted to humans especially in areas with increased prevalence of rodents, poor hygienic standards and insufficient rodent control (Roma settlements, slums). Both animals can also come in contact with farming land and drinking water supplies, causing food-borne and water-borne infections.

The advantage of genotyping *Cryptosporidium* is in the ability to study geographic variations and the relationship between demographic and epidemiological data (Peng et al., 2001). The diagnosis of *Cryptosporidium* is essential for the surveillance of the infection, as for its prevention and control.

Cryptosporidiosis is a protozoan infection, causing mild to severe diarrhoea in various hosts. This infection is not routinely monitored in the majority of countries around the globe. Due to the free movement of humans, animals, food and animal feed between various countries, especially countries in the Schengen Area, and the absence of routine screening, these pathogens can easily pass borders. This poses a severe danger, primary for immunocompromised individuals, in whom the infection can have fatal consequences. But, as we proved in this study, *Cryptosporidium* spp. can infect also immunocompetent patients, in which it can occur as a hidden infection and is diagnosed only by targeted sample collecting and specific laboratory procedures, increasing the risk of transmission not only to a patient suffering from diseases compromising immunity but also to other susceptible individuals in the population.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ETHICAL APPROVAL

Approval for the study was obtained from the Ethics Committee of the Faculty of Medicine at P. J. Šafárik University in Košice (No. 104/2011). The study was performed in accordance with the ethical standards as laid down in the Declaration of Helsinki of 1975 and revised in 2008. Participation in the study was voluntary and anonymous and informed consent was obtained prior to the medical examination.

## ORCID

*Elena Hatalová* http://orcid.org/0000-0003-0929-4642

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