Annals of Parasitology 2019, 65(3), 237-243
doi: 10.17420/ap6503.205

Original papers

Molecular identification of *Cryptosporidium parvum* in rabbits (*Oryctolagus cuniculus*) in Nigeria

Adekunle B. Ayinmode, Victor I. Agbajelola

Department of Veterinary Parasitology and Entomology, University of Ibadan, Ibadan, Nigeria

Corresponding Author: Adekunle B. Ayinmode; e-mail: ayins2000@yahoo.com

ABSTRACT. Rabbits are commonly reared by households and farmers in Nigeria as a source of meat, but there is no information available on *Cryptosporidium* genotypes occurring in rabbits in Nigeria. Fecal samples were collected from 107 rabbits and examined by modified Ziehl-Neelsen technique for the presence of *Cryptosporidium* oocysts. An infection rate of 3.7% (4/107) was obtained and all microscopy-positive samples were genotyped and subtyped to determine the circulating *Cryptosporidium* species using sequence analysis of the 18S rRNA gene and 60-kDa glycoprotein (gp60) gene, respectively. All the four microscopy-positive samples were identified as *C. parvum* by 18S rRNA gene. However, analysis of the gp60 gene revealed the presence of *C. parvum* subtype IIc, which is commonly found in humans in two isolates. These findings indicate natural infection of rabbits with *C. parvum* and underscore the need to investigate the probable role of animal hosts in the epidemiology of *Cryptosporidium* infection. This is the first report on genetic characterization of *Cryptosporidium* infecting rabbits in Nigeria.

Keywords: rabbits, cryptosporidiosis, *Cryptosporidium parvum*, genotyping, Nigeria

Introduction

Cryptosporidium, an intracellular apicomplexan parasite infecting mammals including humans, was first reported in rabbits in 1912 [1], but the microscopic and ultra-structural appearance was only described in 1979 [2]. The *Cryptosporidium* isolate in rabbit was first identified as the rabbit genotype in China [3] and then in Czech Republic [4] but was later renamed *Cryptosporidium cuniculus* [5].

Although *C. cuniculus* has been described as genetically related to *C. parvum* and *C. hominis*, it is most related to *C. hominis* [3,4,6] and has been implicated in human cryptosporidiosis outbreaks in the United Kingdom [5,7,8].

C. cuniculus can be identified by sequence analysis of the small subunit (SSU) of ribosomal RNA (18S rRNA) gene, actin genes, *Cryptosporidium* oocyst wall protein (COWP) gene and 60-kDa glycoprotein (gp60) gene [5]. Subtype families Va and Vb have been described in *C. cuniculus* based on the sequences outside the tandem repeat region of the gp60 gene, with Va subtypes mostly detected in human cases and Vb subtypes in rabbits, humans and recently in Australia kangaroo [7,9].

Several reports exist on the genetic characteristics and zoonotic potential of *C. cuniculus* worldwide [10–15]. However, there are knowledge gaps in understanding of the natural occurrence of other *Cryptosporidium* species in rabbits.

Rabbit farming in Nigeria is largely traditional and operated as smallholder backyard production systems in which the animals are reared basically for family consumption [16]. However, it is now growing at a fast rate being largely accepted as an alternative source of animal protein to meet the demands of the growing human population.

The aim of this study was to identify and characterize the genotype and subtype of *Cryptosporidium* species infecting rabbits in Ibadan, Nigeria.

Materials and Methods

Sampling. Fecal samples were randomly collected from 107 out of at total of 204 adult New Zealand white rabbits in a farm located in Ojoo in Ibadan, Southwestern Nigeria between February and August 2016. Fresh droppings from individual cages were obtained and kept at 4°C prior to microscopy and DNA extraction.

***Cryptosporidium* oocyst detection.** Fecal samples were examined for the presence of *Cryptosporidium* oocysts after making smears stained with modified Ziehl-Neelsen technique stain [17]. Slides were examined using ×100 objective for the presence of bright pink roundish oocysts that indicate positivity for *Cryptosporidium*. Oocysts from all positive samples were then purified using the sucrose gradient concentration method [18].

***Cryptosporidium* genotyping and subtyping.** Ultra-pure® DNA Kit (Roche, Indianapolis, USA) was used to extract DNA from purified oocysts and *Cryptosporidium* species were detected by a nested polymerase chain reaction (nested-PCR) amplification of a ~590 bp fragment of the 18S rRNA gene using already described primers for the primary and secondary amplification [4]. The 50 µl PCR reaction mixture contained 21.6 µl nuclease-free water (Roche, Indianapolis, USA), 25 µl master mix (Roche, Indianapolis, USA) containing pre-mixed Taq polymerase, $MgCl_2$ and dNTPs, 1.2 µl each of forward and reverse primers and 1 µl DNA template. *Cryptosporidium hominis* DNA (TU502) and ultra-pure PCR water were used as positive and negative controls, respectively. The cycling protocol for both primary and secondary amplification included an initial denaturation step of 94°C for 5 minutes, followed by 45 cycles of 94°C for 30 s (denaturation), 58°C for 30 seconds (annealing) and 72°C for 30 seconds (extension), with a final extension of 72°C for 10 minutes.

Subtyping was achieved through amplification of a 400-bp fragment of the 60-kDa glycoprotein (gp60) gene using already described primers for the primary and secondary amplification [19]. The constituents of the 50 µl PCR reaction mixture the cycling conditions were the same as for 18S rRNA

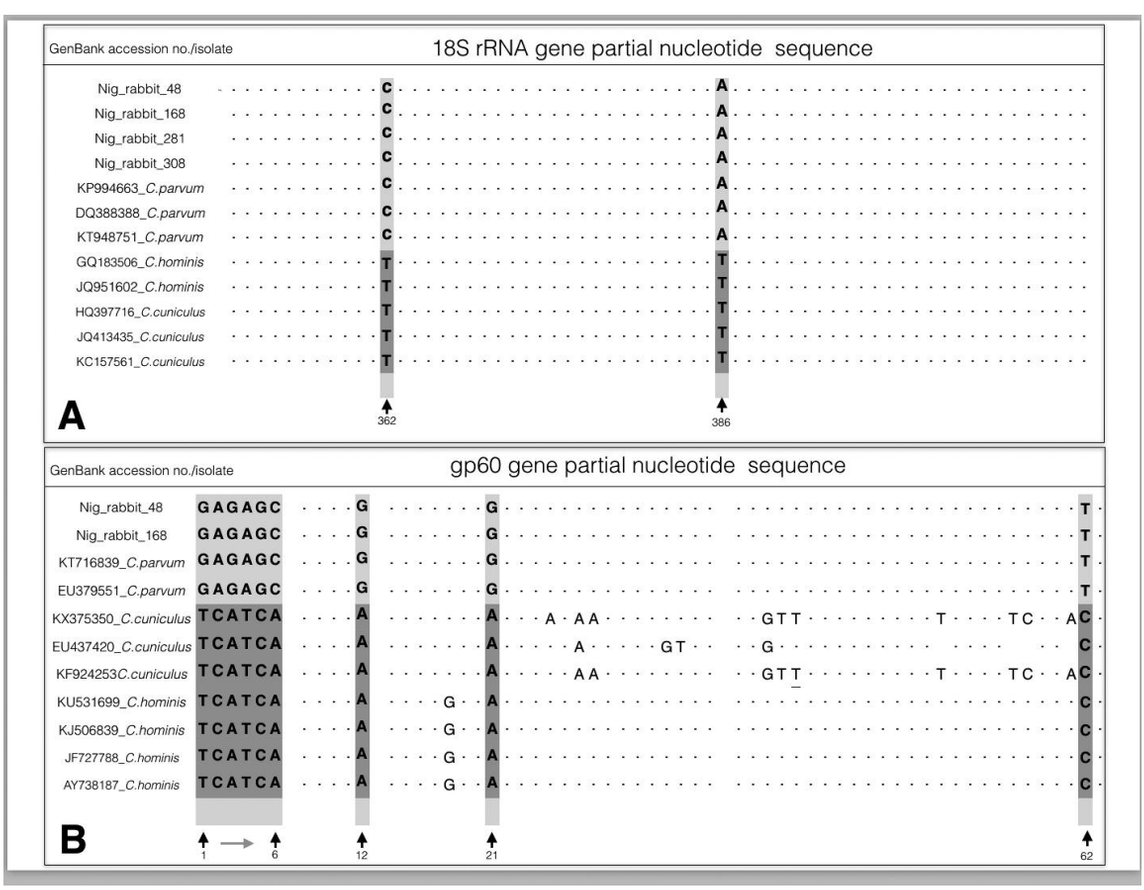

Fig. 1. Nucleotide sequence similarities and differences in partial 18S rRNA (A) and gp60 (B) genes between isolates from this study and reference sequences of *C. parvum*, *C. hominis* and *C. cuniculus* differences. Dots indicate base identical to those in obtained isolates.

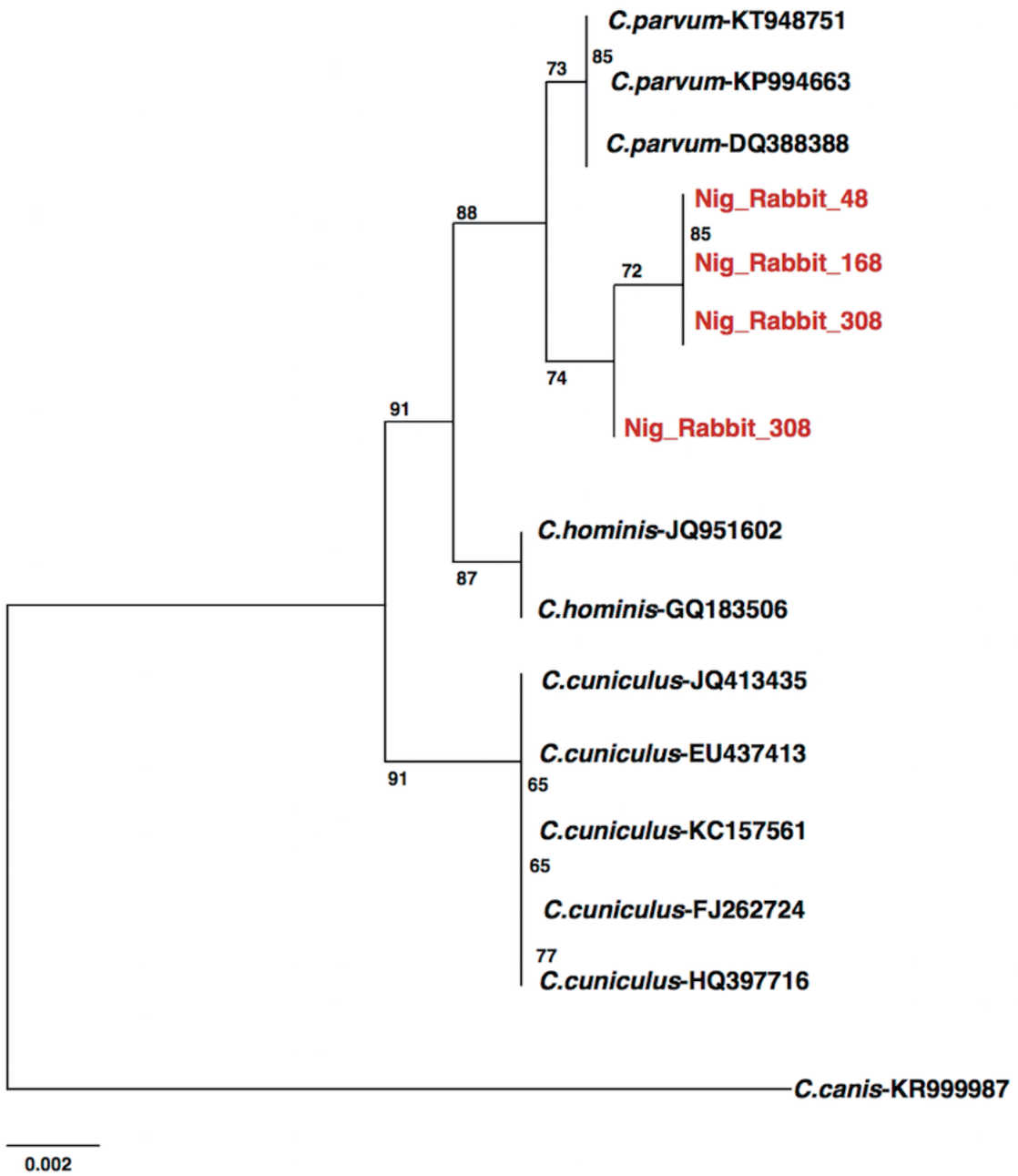

Fig. 2. showing the phylogenetic relationships obtained among *Cryptosporidium* species from the present study and those already deposited in the Genbank based on the neighbor-joining analysis of the partial sequences of the 18S rRNA locus. The numbers at the nodes represent bootstrap values of 1000 replicates (> 50%).

gene amplification. Sequencing of secondary products of both 18S rRNA and gp60 genes was performed at a commercial sequencing facility using secondary primers. The sequences were compared with *Cryptosporidium* sequences found in GenBank (http://www.ncbi.nlm.nih.gov/) using BLAST and sequence alignments were performed with Bioedit alignment editor (version 7.0.9.0). Sequences obtained from analysis of the 18S rRNA gene were assigned GenBank accession numbers KY189296, KY189298, KY189300 and KY189301, while, those from the gp60 gene were assigned accession numbers KY189297 and KY189302.

Phylogenetic analysis. Analysis of partial sequences obtained for both 18S rRNA and gp60 genes were carried out using MEGA 6 software package (www.megasoftware.net). Obtained sequences were compared with selected reference

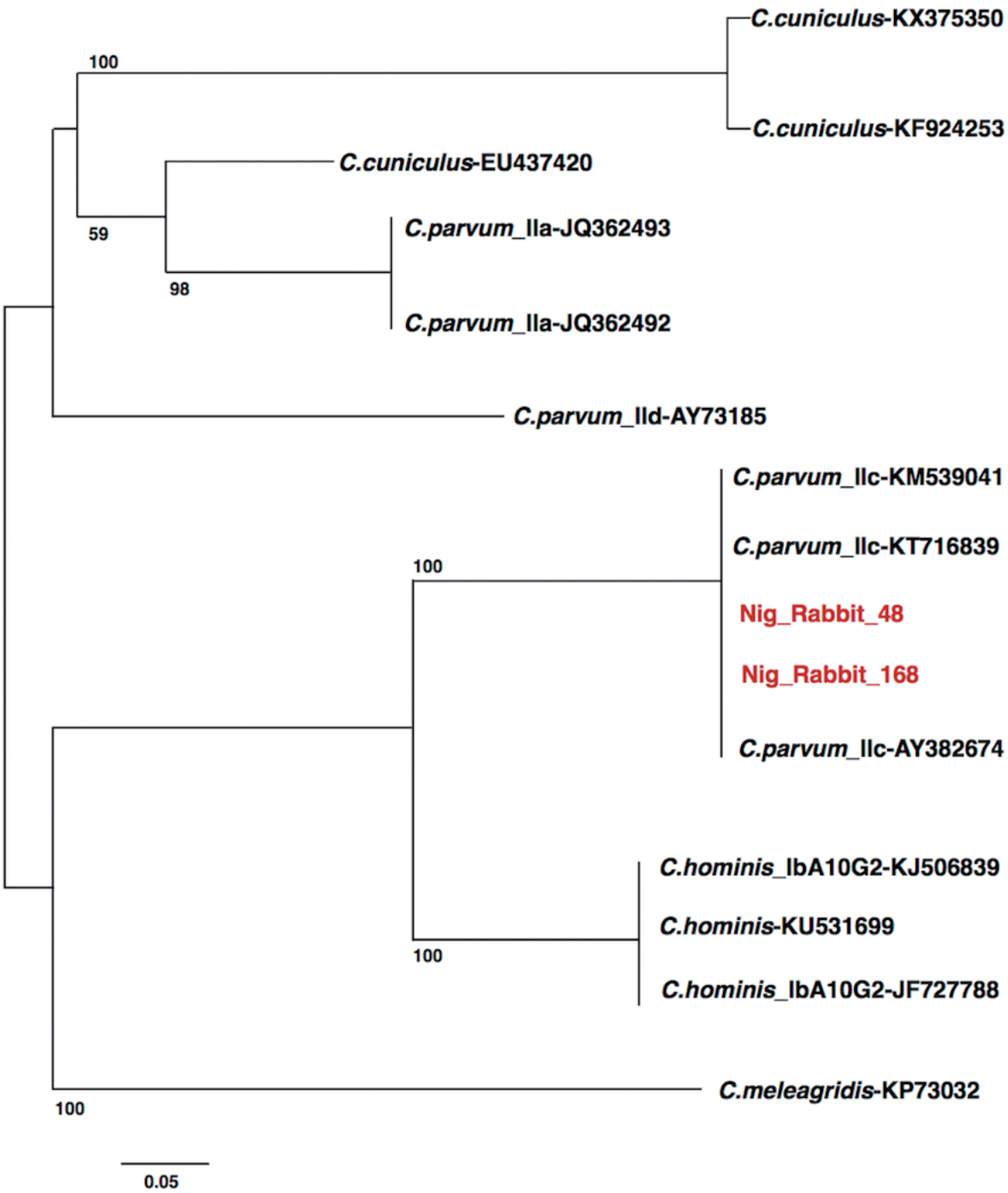

Fig. 3. showing the phylogenetic relationships obtained among *Cryptosporidium* species from the present study and those already deposited in the Genbank based on the neighbor-joining analysis of the partial sequences of the gp60 gene. The numbers at the nodes represent bootstrap values of 1000 replicates (> 50%).

sequences from the GenBank and relatedness was inferred by construction of phylogenetic trees using the Neighbour-Joining method [20].

Results

Cryptosporidium genotype

An overall prevalence of 3.7% (4/107) was obtained by acid-fast staining. Nested PCR amplification of the 18S rRNA gene of the positive samples yielded products of expected size (590 bp) in all the four samples. All four PCR products were successfully sequenced and had 99% homology with many reference *C. parvum* sequences at the 18S rRNA gene locus. The sequences of the obtained isolates had nucleotide substitution "C" for

"T" at position 362 and "A" for "T" at position 386 when compared to *C. hominis* and C. *cuniculus* (Fig. 1). Phylogenetic an analysis of the 18S rRNA genes showed that the obtained isolates clusterd closer to *C. parvum* sequences than to *C. hominis and C. cuniculus* sequences (Fig. 2).

***Cryptosporidium* subtype**

Sequence analysis of the gp60 gene yielded *C. parvum* subtype family IIc in two isolates with both having 100% homology with some *C. parvum* sequences, including KM539041, KT716839 and AY382674. Furthermore, sequence analysis of the gp60 gene showed that the two subtyped isolates were distinct from *C. hominis* and *C. cuniculus* with differences in nucleotide sequences differences at positions 1 to 6, 12, 21 and 62 (Fig. 1). Phylogenetic analysis of gp60 gene showed that the obtained isolates clusterd closer to *C. parvum* sequences than to *C. hominis and C. cuniculus* sequences (Fig. 3).

Discussion

The prevalence of 3.7% obtained for *Cryptosporidium* infection in farmed rabbits in this study is lower than the 6.8% and 19% obtained in previous studies from Australia and Japan, respectively. However, our finding is comparable to the 3.4% prevalence reported in Henan province, China [11], but higher than the 2.3% and 0.9% obtained in Heilongjiang province, China [12] and the UK [21], respectively. The discrepancies in prevalence results have been attributed in most cases to sensitivity and specificity of detection methods, sample size, management systems and study design [11,12], The variations in type of rabbits (pet, laboratory and wild), geographical locations and other factors make it difficult to adequately compare results of epidemiological studies.

BLAST search analysis of sequences obtained from 18S rRNA gene of positive samples in this study identified the isolates as *C. parvum*, with high level of similarity to some *C. hominis and C. cuniculus* sequences. Whereas previous reports [10,11], have showed that *C. parvum*, *C. hominis* and *C. cuniculus* shared sequence similarities in both the 18S rRNA and gp60 genes, identified nucleotide substitutions and the results of phylogenetic analysis showed that the isolates obtained in this study were *Cryptosporidium parvum*, and were distinct from *C. cuniculus* and *C. hominis*.

Analysis of the gp60 gene has increasingly proved useful for the determination of genetic diversity within *Cryptosporidium* genotypes and subtypes. Hence, the gp60 locus is often targeted for subtyping *Cryptosporidium* isolates obtained from rabbits [22,23]. In this study, analysis of the gp60 gene sequence identified two isolates as *C. parvum* subtype family IIc. This finding is rather unusual in rabbits since subfamily IIc is known to be a human pathogen that has been found to circulate among humans and designated as anthroponotic [24]. The subtype IIc identified in this study is one of the most common *C. parvum* isolates recovered exclusively from humans worldwide [24,25], with only few exceptions where it was isolated from hedgehog [26,27). Although experimental infection of rabbits with *C. parvum* has been reported, the natural infection has not been documented [28]. Our findings suggest that the *Cryptosporidium*-positive rabbits were actually infected by the pathogen and shed oocysts detectable by microscopy, rather than passive carriage of ingested oocysts. While it is not clear whether the positve rabbits maintained the organism as reservoirs or could probably transmit it to humans, the present finding coupled with previous reports might suggest that the host range of *Cryptosporidium* subtypes have expanded beyond humans. Further studies are therefore required to determine the significance of finding *C. parvum* IIcA5G3 in rabbits. The results of such investigation might be valuable towards the revision of the anthroponotic status of the subfamily IIc as earlier suggested by Krawczyk et al. [27].

In conclusion, our findings underscore the need to track the possible spread of human *Cryptosporidium* subtypes to in-contact livestock and pets that share the same geographical space with them. We advocate further investigation to clarify the probable role of domestic animals and livestock in the epidemiology of anthroponotic *Cryptosporidium* species. Necessary precautions should be taken in the handling rabbits in order to avoid possible human infection.

Conflict of interest. We declare no conflicts of interest.

Acknowledgements

The authors thank Prof. Giovanni Widmer of the Cummings' School of Veterinary Medicine, Tufts University, USA for his assistance during the laboratory work.

References

[1] Tyzzer E.E. 1912. *Cryptosporidium parvum* (sp.nov.), a *coccidium* found in the small intestine of the common mouse. *Archiv für Protistenkunde* 26: 394-412.

[2] Inman L.R., Takeuchi L.R. 1979. Spontaneous cryptosporidiosis in an adult female rabbit, *Veterinary Pathology* 16: 89-95.

[3] Xiao L., Sulaiman I.M., Ryan U.M., Zhou L., Atwill E.R., Tischler M.L., Zhang X., Fayer R., Lal A.A. 2002. Host adaptation and host-parasite co-evolution in *Cryptosporidium:* implications for taxonomy and public health, *International Journal of Parasitology* 32: 1773-1785.

[4] Ryan U.M., Xiao L., Read C., Zhou L., Lal A.A., Pavlasek I. 2003. Identification of novel *Cryptosporidium* genotypes from the Czech Republic. *Applied Environmental Microbiology* 69: 4302-4307. doi:10.1128/AEM.69.7.4302-4307.2003

[5] Robinson G., Wright Elwin S., K., Hadfield S.J., Katzer F., Bartley P.M., Hunter P.R., Nath M., Innes E.A., Chalmers R.M. 2010. Re-description of *Cryptosporidium cuniculus* Inman and Takeuchi, 1979 (Apicomplexa: Cryptosporidiidae): morphology, biology and phylogeny. *International Journal of Parasitology* 40: 539-1548. https://doi.org/10.1016/j.ijpara.2010.05.010

[6] Robinson G., Elwin K., Chalmers R.M. 2008. Unusual *Cryptosporidium* genotypes in human cases of diarrhea. *Emerging Infectious Diseases* 14: 1800-1802. https://doi.org/10.3201/eid1411.080239

[7] Chalmers R.M., Robinson G., Elwin K., Hadfield S.J., Xiao L., Ryan U.M., Modha D., Mallaghan C. 2009. *Cryptosporidium* rabbit genotype, a newly identified human pathogen. *Emerging Infectious Diseases* 15: 829-830. doi:10.3201/eid1505.081419

[8] Puleston R.L., Mallaghan C.M., Modha D.E., Hunter P.R., Nguyen-Van-Tam J.S., Regan C.M., Nichols G.L., Chalmers R.M. 2014. The first recorded outbreak of cryptosporidiosis due to *Cryptosporidium cuniculus* (formerly rabbit genotype), following a water quality incident. *Journal of Water and Health* 12: 41-50. https://doi.org/10.2166/wh.2013.097

[9] Koehler A.V., Whipp M.J., Haydo S.R., Gasser R.B. 2014. *Cryptosporidium cuniculus* - new records in human and kangaroo in Australia. *Parasites and Vectors* 7: 492. https://doi.org/10.1186/s13071-014-0492-8

[10] Nolan M.J., Jex A.R., Haydon S.R., Stevens M.A., Gasser R.B. 2010. Molecular detection of *Cryptosporidium cuniculus* in rabbits in Australia, *Infection, Genetics and Evolution* 10:1179-1187. https://doi.org/10.1016/j.meegid.2010.07.020

[11] Shi K., Jian F., Lv C., Ning C., Zhang L., Dearen T.K., Li N., Qi M., Xiao L. 2010. Prevalence, genetic characteristics and zoonotic potential of *Cryptosporidium* species causing infections in farm rabbits in China. *Journal Clinical Microbiology* 48: 3263-3266. https://doi.org/10.1128/JCM.00819-10

[12] Zhang W., Shen Y., Wang R., Liu A., Ling H., Li Y., Cao J., Zhang X., Shu J., Zhang L. 2012. *Cryptosporidium cuniculus* and *Giardia duodenalis* in rabbits: genetic diversity and possible zoonotic transmission. *PLoS One* 7: e31262. doi:10.1371/journal.pone.0031262

[13] Liu X., Zhou X., Zhong Z., Chen W., Deng J., Niu L., Wang Q. 2014. New subtype of *Cryptosporidium cuniculus* isolated from rabbits by sequencing the gp60 gene. *Journal of Parasitology* 100: 532-536. https://doi.org/10.1645/13-223.1

[14] ANOFEL *Cryptosporidium* National Network. 2010. *Cryptosporidium* National Network Laboratory-based surveillance for *Cryptosporidium* in France: 2006-2009. *Euro Surveillance* 15: 19642-19690.

[15] Molloy S.F., Smith H.V., Kirwan P., Nichols R.A., Asaolu S.O., Connelly L., Holland C.V. 2010. Identification of a high diversity of *Cryptosporidium* species genotypes and subtypes in a pediatric population in Nigeria. *American Journal of Tropical Medicine and Hygiene* 82: 608-613. https://doi.org/10.4269/ajtmh.2010.09-0624

[16] Onifade A.A., Abu O.A., Obiyan R.I., Abanikannda O.T. 1999. Rabbit production in Nigeria: Some aspects of current status and promotional strategies. *World Rabbit Science* 7: 51-58. https://doi.org/10.4995/wrs.1999.380

[17] Casemore D.P. 1991. Laboratory methods for diagnosing cryptosporidiosis. *Journal of Clinical Pathology* 44: 445-451. https://dx.doi.org/10.1136/jcp.44.6.445

[18] Wee S.H., Lee C.G., Kim B.S., Joo H.D., Kang S.W. 1994. Isolation of *Cryptosporidium parvum* oocysts from fecal samples: the combination of ether extraction and discontinuous sucrose gradients. *Korean Journal of Parasitology* 32 : 7-12 (in Korean with summary in English),

[19] Sulaiman I.M., Hira P.R., Zhou L., Al-Ali F.M., Al-Shelahi F.A., Shweiki H.M., Iqbal J., Khalid N., Xiao L. 2005. Unique endemicity of cryptosporidiosis in children in Kuwait. *Journal of Clinical Microbiology* 43: 2805-2809. doi:10.1128/JCM.43.6.2805-2809.2005

[20] Tamura K., Stecher G., Peterson D., Filipski A., Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* 30: 2725-2729. https://doi.org/10.1093/molbev/mst197

[21] Chalmers R.M. 1996. The distribution of *Cryptosporidium* in livestock and wild animal populations on a Warwickshire farm. PhD thesis, Coventry University, Coventry.

[22] Kaupke A., Kwit E., Chalmers R.M., Michalski

M.M., Rzezutka A. 2014. An outbreak of massive mortality among farm rabbits associated with *Cryptosporidium* infection. *Research in Veterinary Science* 97: 85-87. https://doi.org/10.1016/j.rvsc.2014.04.016

[23] Yang Z., Zhao W., Shen Y., Zhang W., Shi Y., Ren G., Yang D., Ling H., Yang F., Liu A., Cao J. 2016. Subtyping of *Cryptosporidium cuniculus* and genotyping of *Enterocytozoon bieneusi* in rabbits in two farms in Heilongjiang Province, China. *Parasite* 23: 52. https://doi.org/10.1051/parasite/2016063

[24] Xiao L., Feng Y. 2008. Zoonotic cryptosporidiosis. *FEMS Immunology and Medical Microbiology* 52: 309-323. https://doi.org/10.1111/j.1574-695X.2008.00377.x

[25] Bouzid M., Tyler K.M., Christen R., Chalmers R.M., Elwin K., Hunter P.R. 2010. Multi-locus analysis of human infective *Cryptosporidium* species and subtypes using ten novel genetic loci. *BMC Microbiology* 10: 213. https://doi.org/10.1186/1471-2180-10-213

[26] Dyachenko V., Kuhnert Y., Schmaeschke R., Etzold M., Pantchev N., Daugschies A. 2010. Occurrence and molecular characterization of *Cryptosporidium* sp. genotypes in European hedgehogs (*Erinaceus europaeus*). in Germany. *Parasitology* 137: 205-216. https://doi.org/10.1017/ S0031182009991089

[27] Krawczyk A.I., Docters van Leeuwen D., Jacobs-Reitsma W., Wijnands L.M., Bouw E.I., Jahfari S., van Hoek H.A., van der Giessen W.B., Roelfsema J.H., Kroes M., Kleve J., Dullemont Y., Sprong H., de Bruin A. 2015. Presence of zoonotic agents in engorged ticks and hedgehog faeces from *Erinaceus europaeus* in (sub) urban areas. *Parasites and Vectors* 8: 210. https://doi.org/10.1186/s13071-015-0814-5

[28] Robinson G., Chalmers R.M. 2010. The European rabbit (*Oryctolagus cuniculus*), a source of zoonotic cryptosporidiosis. *Zoonoses and Public Health* 57: e1-e13. doi:10.1111/j.1863-2378.2009.01308.x

Received 20 April 2019
Accepted 06 June 2019