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Research paper

Multilocus sequence typing and population genetic structure of *Cryptosporidium cuniculus* in rabbits in Heilongjiang Province, China

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ABSTRACT

Multilocus sequence typing (MLST) tools have been used widely to characterize population genetic structure of some *Cryptosporidium* species. To understand MLST subtypes and population genetic structure of *Cryptosporidium cuniculus* from rabbits in Heilongjiang Province, China, 34 *C. cuniculus* DNA specimens were collected including VbA21 (n = 6), VbA28 (n = 2), VbA29 (n = 18) and VbA32 (n = 8). They were analyzed by nested-PCR amplification and sequencing at seven microsatellite, minisatellite and polymorphic loci including CP47, CP56, ML2, DZ-HRGP, MSC6–5, MSC6–7 and RPGR. The CP47, CP56, MSC6-5 and MSC6-7 loci were monomorphic. The remaining loci were polymorphic, with two, three and two subtypes being found at ML2, DZ-HRGP and RPGR loci, respectively. Six MLST subtypes were obtained based on sequence information of 29 DNA specimens successfully amplified at all eight loci including *gp60* locus. Linkage disequilibrium (LD) analysis showed a clonal population structure of *C. cuniculus* in the investigated areas. STRUCTURE, neighbor-joining and network analyses indicated the presence of two distinct groups, corresponding to VbA21 subtype and VbA28, VbA29 and VbA32 subtypes. This is the first report of MLST analysis of *C. cuniculus*. A clonal population structure of *C. cuniculus* suggested the prevalence of *C. cuniculus* in Heilongjiang Province is not attributed to the introduction of rabbits. Thus, prevention and control strategies should be focused on making stricter measures to avoid occurrence of cross-transmission and re-infection among rabbit individuals. Based on the previous findings of VbA21 subtype only in rabbits and VbA28, VbA29 and VbA32 subtypes both in rabbits and humans, the results of subpopulation analyses might be used to assess zoonotic potential of *C. cuniculus* subtypes in Vb family. These data will be helpful to explore source attribution of infection/contamination of *C. cuniculus* and understand its transmission dynamics in humans and rabbits in the investigated areas.

1. Introduction

Cryptosporidium spp. are common intestinal protozoa occurring in humans and many animal species and have been reported to be associated with diarrhea, with self-limiting diarrhea in immunocompetent individuals, but severe diarrhea and dissemination to extra-intestinal sites in some high-risk individuals, particularly those with HIV infection (Ryan et al., 2014). Extensive genetic variation has been confirmed within the genus *Cryptosporidium*. Among 37 species and more than 40 genotypes identified, 21 species/genotypes have been found in humans (Ryan et al., 2016; Kváč et al., 2018; Čondlová et al., 2018; Zahedi et al., 2017; Jezkova et al., 2016; Yang et al., 2016; Ebner et al., 2015). Epidemiological data have revealed that the vast majority of human cases of cryptosporidiosis are caused by *C. hominis* and *C. parvum* (Ryan et al., 2016). Other *Cryptosporidium* species such as *C. cuniculus* appears to be the third most commonly identified species in patients with diarrhea in the UK during the period 2007–2008, lying behind *C. parvum* and *C. hominis* (Chalmers et al., 2011). To date, more than 70 human cases of *C. cuniculus* infection have been reported worldwide, distributing in the UK, Nigeria, Australia, France, Spain, and New Zealand (Chalmers et al., 2009, 2011; Molloy et al., 2010; ANOFEL *Cryptosporidium* National Network, 2010; Koehler et al., 2014; Martínez-Ruiz et al., 2016; Garcia-R et al., 2017).

DNA sequence analysis of the 60 kDa glycoprotein (*gp60*) gene is the

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most common subtyping tool, aiming at identifying infection sources and investigating transmission dynamics as well as their taxonomy. However, limitations of a single locus scheme have been identified including low *gp60* subtype diversity in certain countries. In recent years, multilocus sequence typing (MLST) methods have been developed to provide higher discrimination between *Cryptosporidium* spp. than gp60 single locus analysis. A variety of microsatellite, minisatellite and polymorphic markers loci have been used in global, regional and local parasite population studies and in epidemiological investigations of some *Cryptosporidium* species, including *C. hominis*, *C. parvum*, *C. meleagridis* and *C. ubiquitum* as well as *C. muris* and *C. andersoni* (Feng et al., 2011, 2013, 2014; Wang et al., 2014b; Tang et al., 2016). These MLST data will deepen our understanding of transmission dynamics of cryptosporidiosis. However, to date, no MLST data of *C. cuniculus* have been reported.

In the present study, rabbit-derived *C. cuniculus* DNA specimens were obtained from Heilongjiang Province, China. Genetic characterizations of *C. cuniculus* were analyzed by PCR amplification and sequencing of eight MLST loci including the *gp60* locus and population genetic structure was assessed by genetic diversity tests and measurements of linkage disequilibrium. Based on characterization of population genetic structure, efficient control strategies can be made to intervene and prevent occurrence of rabbit cryptosporidiosis among rabbit individuals. Meanwhile, these data will be helpful to explore source attribution of infection/contamination of *C. cuniculus* and understand its transmission dynamics in humans and rabbits in the investigated areas.

2. Materials and methods

2.1. C. cuniculus DNA specimens

A total of 34 *C. cuniculus* DNA specimens were obtained from rabbit feces in Heilongjiang Province, China during October 2008–June 2017—VbA21 (n = 6) in 2008; VbA28 (n = 2) in 2016; VbA29 (n = 18) in 2009, 2015 and 2017; VbA32 (n = 8) in 2010, 2015 and 2017. All the *gp60* gene sequences of the same subtype were identical to each other. Genomic DNA of *C. cuniculus* was extracted from 200 mg of each fecal sample using a QIAamp DNA Mini Stool Kit (Qiagen, Hilden, Germany) according to the manufacturer recommended procedures. A negative control was added during DNA extraction to check whether cross-contamination occurred or not. All of them were identified and subtyped by PCR and sequencing of the small subunit (SSU) rRNA and the *gp60* genes, respectively (Xiao et al., 1999; Alves et al., 2003).

2.2. Multilocus sequence typing

C. cuniculus is genetically closely related to *C. hominis*, sharing 99.5%, 99.9%, 100% and 99.5% nucleotide sequence identity at the SSU rRNA, actin, *Cryptosporidium* oocyst wall protein (COWP) and 70 kDa heat shock protein (HSP-70) loci, respectively (Robinson et al., 2010). Thus, these target genes were selected based on their polymorphic nature and a wide application in *C. hominis*, including the genes coding for 47 kDa protein (CP47), a 56 kDa trans-membrane protein (CP56), a possible sporozoite cystein-protein (ML2), a hydroxyproline-rich glycoprotein (DZ-HRGP), a hypothetical trans-membrane protein (MSC6–5), a serine repeat antigen (MSC6–7), and a hypothetical retinitis pigmentosa GTPase regulator (RPGR), all in chromosome 6. All the 34 *C. cuniculus* DNA specimens were analyzed at seven MLST loci by nested PCRs. The primers and amplification conditions in nested PCR analysis were described previously (Gatei et al., 2006; Wang et al., 2014a). TaKaRa Taq DNA polymerase (TaKaRa Bio Inc., Tokyo, Japan) was used for all the PCR reactions. A negative control with no DNA added was included in all PCR tests. All the PCR products were subjected to electrophoresis in a 1.5% agarose gel and visualized by staining the gel with GelStrain (TransGen Biotech., Beijing, China).

2.3. DNA sequence analysis

All nested PCR products were sequenced using their respective secondary PCR primers on an ABI PRISM™ 3730xl DNA Analyzer using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). Sequence accuracy was confirmed by sequencing in both directions. If DNA preparations of *C. cuniculus* were positive at one gene locus and negative at the other loci, they were subjected to two more repeated PCR amplifications accordingly at the locus negative for *C. cuniculus*. Nucleotide sequences obtained in the present study were subjected to BLAST searches (http://www.ncbi.nlm.nih.gov/blast/) and then aligned and analyzed with each other and those from GenBank database using the program Clustal X 1.83 (http://www.clustal.org/).

2.4. Phylogenetic relationship

A phylogenetic analysis of MLST subtypes of *C. cuniculus* was performed by constructing a neighbor-joining tree of concatenated sequences of eight MLST loci including the *gp60* locus using the program Mega 6 (http://www.megasoftware.net/) based on the evolutionary distances calculated by Kimura 2-parameter model. The reliability of these trees was assessed using the boot strap analysis with 1,000 replicates.

2.5. Population genetic analysis

Multilocus linkage disequilibrium (LD) in allelic data with and without *gp60* gene was assessed by calculating the standardized index of association (I_A^S) using LIAN 3.5 (http://guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl). LD and linkage equilibrium (LE) would be indicated by a positive value and by 0 or a negative value, respectively. In addition, the population genetic structure was assessed by calculating the variance of pairwise differences (V_D) and the 95% critical value (L) for V_D. If the V_D is less than L, the population is in LE, indicating a panmictic population structure; otherwise, the population is non-panmictic and a degree of LD exists. Based on the fact that gp60 is the most polymorphic gene marker identified so far in the *Cryptosporidum* genome, intragenic LD and recombination rates were calculated based on concatenated sequences with and without *gp60* gene by using DnaSP 5.10.01 (http://www.ub.es/dnasp/).

2.6. STRUCTURE and network analyses

Subpopulation structure of *C. cuniculus* was analyzed by using Bayesian analysis tool STRUCTURE version 2.2 (http://pritch.bsd.uchicago.edu/structure.html). Several analyses of allelic data were performed using K (likely populations) ranging from 2 to 10, with 50,000 iterations after a burn-in of 50,000 iterations. The output at K = 2 provided the best fit to the MLST data and was used in further analyses. Meanwhile, network version 4.6.1.0 (http://www.fluxus-engineering.com/sharenet_rn.htm) was also used to identify a subpopulation structure of the *C. cuniculus* by a median-joining algorithm analysis.

3. Results

3.1. PCR amplification rates and MLST subtypes

All the 34 *C. cuniculus* DNA specimens were subjected to nested PCR amplification of seven MLST genes. The highest PCR amplification rate 97.1% (33/34) was found at the MSC6-5 locus, followed by 94.1% (32/34) at the MSC6-7 locus, 91.2% (31/34) at the RPGR locus, 88.2% (30/34) at the CP56, ML2 and DZ-HRGP loci and 85.3% (29/34) at the CP47 locus.

Table 1
Allele composition of multilocus sequence types of *C. cuniculus* with sequence data at eight MLST genetic loci.

gp60 subtype (n)	Allele composition							MLST subtype (n)
	CP47	CP56	ML2	DZ-HRGP	MSC6–5	MSC6–7	RPGR	
VaA21 (6)	1	1	1	1	1	1	1	1 (5)
VaA28 (2)	1	1	2	2	1	1	1	2 (2)
VaA29 (18)	1	1	2	2	1	1	1	3 (14)
	1	1	2	3	1	1	1	4 (1)
	1	1	2	2	1	1	2	5 (1)
VaA32 (8)	1	1	1	1	1	1	1	6 (6)

By sequence analysis, *C. cuniculus* showed monomorphic nature at the CP47, CP56, MSC6–5 and MSC6-7 loci (MG273690-MG273693). Polymorphic nature was observed at the other three loci, with two, three and two subtypes being found at ML2 (MG273694 and MG273695), DZ-HRGP (MG273696-MG273698) and RPGR (MG273699 and MG273700) loci, respectively. A total of 29 *C. cuniculus* DNA specimens were successfully amplified at the seven loci, including VbA21 (n = 5), VbA28 (n = 2), VbA29 (n = 16), VbA32 (n = 6). Six MLST subtypes (MLST-1 to MLST-6) were obtained, with MLST-3 being dominant (Table 1).

3.2. LD and population genetic structure

A total of 29 *C. cuniculus* DNA specimens subtyped successfully at all the eight loci were included in LD analysis. Multilocus LD was assessed by calculating I_A^S between alleles for all pairwise combinations of all the loci including *gp60* gene by using LIAN 3.5. The value of I_A^S (0.1926) was positive and the V_D (1.8633) was more than L (0.9399). By a Monte Carlo analysis, a significant P_{MC} value < 0.01 was generated. These results indicated the existence of LD and a clonal population structure. When the same multilocus genotype (MLG) was treated as one individual, LD was reduced based the fact that the value of I_A^S (0.0497) was positive and the V_D (1.1143) was less than L (1.4000). As gp60 contributes more genetic heterogeneity than other loci in *Cryptosporidium* genome, the analysis was also conducted on allelic data excluding *gp60* gene. Similar results were obtained (Table 2). The results indicated gp60 gene sequences had little influence on the result of LD analysis.

Recombination analysis using DnaSP revealed no recombination events (Rm) at these loci. It was also observed that the concatenated sequence data of eight gene loci including the *gp60* gene (4055 bp long) had 17 polymorphic sites and five haplotypes. LD was also estimated using the *ZnS* statistics. These *ZnS* values and complete intragenic LD (|D′| Y = 1.0000 + 0.0000X) indicated the existence of LD and a clonal population structure (Table 3).

3.3. Subpopulation structure

Subpopulation structure was inferred by Bayesian analysis using STUCTURE at K = 2. Two ancestral lineages of *C. cuniculus* were produced, corresponding to VbA21 subtype in Lineage I and VbA28, VbA29 and VbA32 subtypes in Lineage II (Fig. 1). The result was also supported by neighbor-joining (Fig. 2) and network analyses (Fig. 3) of the entire concatenated multilocus sequences.

4. Discussion

In the present study, differences in PCR amplification rates of *C. cuniculus* DNA specimens were observed at seven MLST loci, ranging from 85.3% to 97.1%, which were mostly lower than those from *C. hominis* (Li et al., 2013; Feng et al., 2014) and *C. parvum* (Wang et al., 2014a). PCR amplification rates at MLST loci differ by *Cryptosporidium* species. In general, *C. hominis* has higher amplification rates than *C. parvum* and *C. meleagridis* (Gatei et al., 2007, 2008; Feng et al., 2013, 2014; Wang et al., 2014a,b; Li et al., 2013). This might be related to the fact that current primers used for MLST analysis were designed according to complete genomes of *C. hominis* TU502 isolate as well as *C. parvum* Iowa isolate (Gatei et al., 2006; Wang et al., 2014a). Meanwhile, numerous molecular data have confirmed inter- or intra- species genetic variations of *Cryptosporidium* species/genotypes (Ryan et al., 2014). These facts above may result in the failure of PCR amplification for different *Cryptosporidium* species, even within the same species. Thus, it is necessary to develop an MLST technique for high-resolution subtyping of *C. cuniculus* from humans and animals by selecting polymorphic markers established on the basis of the whole genome of *C. cuniculus*.

In the present study, *C. cuniculus* showed monomorphic nature at the CP47, CP56, MSC6–5 and MSC6-7 loci. DZ-HRGP had relatively higher sequence polymorphism among seven MLST loci excluding *gp60* locus. Similar results were also observed in a study of population genetic structure of *C. parvum* IId family (Wang et al., 2014a). However, the DZ-HRGP gene showed monomorphism in MLST studies of *C. hominis* from India, Kenya, and the USA (Gatei et al., 2006). Here, a low degree of polymorphism was observed at ML2 and RPGR loci, accounting for only one base difference between two subtypes at either of two loci.

Six MLST subtypes (MLST-1 to MLST-6) were obtained in the present study. Previous studies indicated that distinct MLST subtypes of *C. hominis* were observed to be linked to continental proximity of *C. hominis* populations (Gatei et al., 2006). Likewise, MLST subtypes of *C. parvum* from China, Egypt, and Sweden differed from each other, and even in the same area in Sweden, several MLST subtypes were detected

Table 2
Multilocus linkage disequilibrium analysis based on allelic data of *C. cuniculus*.

Population group	Number	H	I_A^S	P_{MC}	V_D	L	$V_D > L$	LD or LE
gp60 included	29	0.2158 ± 0.1000	0.1926	< 0.01	1.8633	0.9399	Yes	LD
gp60 excluded	29	0.1552 ± 0.0919	0.1285	< 0.01	0.9975	0.6321	Yes	LD
gp60 included[a]	6	0.3000 ± 0.1234	0.0497	0.14	1.1143	1.4000	No	LE
gp60 excluded[a]	4	0.2286 ± 0.1163	0.0405	0.39	0.8286	1.2571	No	LE

H: mean genetic diversity; I_A^S: standardized index of association; P_{MC}: significance of obtaining this value in 1,000 Monte Carlo simulations; V_D: variance of pairwise differences; L: 95% critical value for V_D; $V_D > L$ indicates linkage disequilibrium.

[a] Considering each group of specimens with the same multilocus genotype (MLG) as one individual.

Table 3
Genetic diversity in 29 *C. cuniculus* isolates based on intragenic analysis of concatenated multilocus gene sequences.

Concatenated sequence	No. of site (bp)	No. of polymorphic sites	No. of segregating sites	No. of haplotypes, H	Haplotype diversity, Hd	Intralocus genetic association (*ZnS*)	95% CI (*ZnS*)	Intragenic linkage disequilibrium(LD) |D′|	Recombination events (Rms)
gp60 included	4055	17	17	5	0.6429	0.6481	0.16171–0.67029	Y = 1.0000 + 0.000X	0
gp60 excluded	3271	4	4	4	0.5690	0.1814	0.15433–0.69298	Y = 1.0000 + 0.000X	0
gp60 included[a]	4055	17	17	5	0.9333	0.6682	0.15824–0.70309	Y = 1.0000 + 0.000X	0
gp60 excluded[a]	3271	4	4	4	1.0000	0.2593	0.15119–0.68641	Y = 1.0000 + 0.000X	0

|D′|: linkage disequilibrium (LD), where Y is the LD value, and X is the nucleotide distance in kb.
[a] Considering each group of specimens with the same concatenated sequence as one individual.

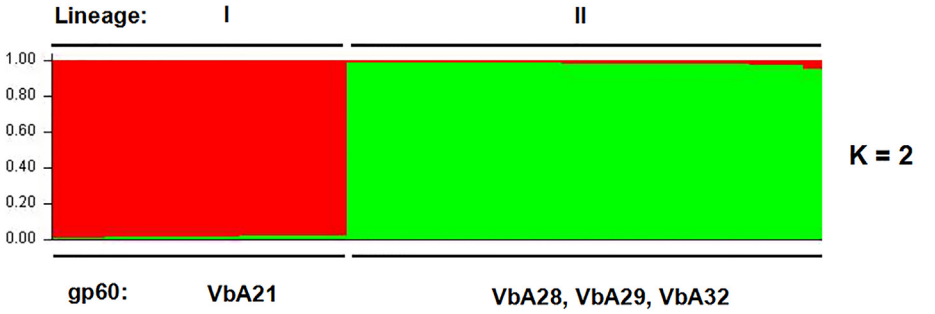

Fig 1. Subpopulation structure of *C. cuniculus* isolates by Bayesian analysis of MLST allelic data. A subpopulation pattern is shown for K values of 2 used in the analysis. Colored regions indicate major ancestral contributions. Lineages and *gp60* subtypes of *C. cuniculus* isolates are shown over and under the color pattern, respectively.

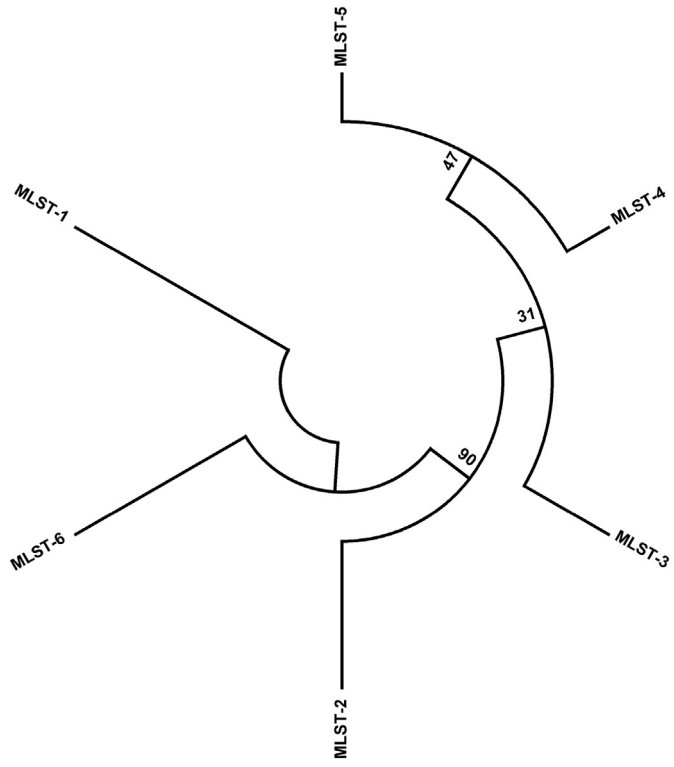

Fig 2. Phylogenetic relationship of MLST subtypes of *C. cuniculus* at eight genetic loci (including *gp60*) as inferred by a neighbor-joining analysis of the concatenated nucleotide sequences based on genetic distance by the Kimura 2-parameter model. MLST-1 is in Lineage I containing VbA21 isolates. MLST-2 to MLST-6 are in Lineage II containing VbA28, VbA29 and VbA32 isolates.

(Wang et al., 2014a). Due to the lack of data of MLST subtypes of *C. cuniculus*, we were unable to compare the present data with those from other geographical areas. However, sequence information of MLST subtypes of *C. cuniculus* deposited in GenBank database can be used in the future comparative study of the allelic profiles of *C. cuniculus* DNA specimens from different geographical areas and hosts, which will also improve our understanding of the epidemiology and evolution of *C. cuniculus*.

The results of *LD* analysis indicated that *C. cuniculus* had a clonal population structure, implying the absence of recombination among lineages of *C. cuniculus* in the investigated rabbits, which was supported by DnaSP analysis (Rm = 0). Since multilocus subtypes of *C. cuniculus* are relatively stable in time and in place, MLST analysis can be used effectively in the longitudinal tracking of the transmission in the surveys of cryptosporidiosis/outbreaks caused by *C. cuniculus* in the investigated areas. The characterization of a clonal population structure of *C. cuniculus* also indicates that the prevalence of *C. cuniculus* in rabbits in Heilongjiang Province is not attributed to the introduction of rabbits. However, when the same multilocus genotype (MLG) was treated as one individual, the result of I_A^S value ($I_A^S = 0.0497$, $V_D < L$) suggested that *C. cuniculus* had an epidemic population structure, which might result from rapid expansion of two dominate MLST subtypes (MLST-3 and MLST-6).

In the present study, it was observed that VbA29 DNA specimens presented two subtypes at DZ-HRGP and RPGR loci, respectively. Similar results were also described in MLST subtyping analysis of *C. parvum*, in which IIaA15G2R1 produced different subtypes at other MLST loci, especially ZPT, CP47 and RPGR (Feng et al., 2013). Feng et al. (2013) attributed this phenomenon to occurrence of genetic recombination within some *gp60* subtypes. However, no recombination was detected in the present study, which might be related to a low degree of genetic heterogeneity of two subtypes at either of two loci (DZ-HRGP and RPGR).

Currently, two subtype families have been identified out of *C. cuniculus* based on sequence analysis of *gp60* gene. Va family is mostly found in humans and occasionally seen in rabbits; in contrast, Vb family is more commonly found in rabbits than in humans (Yang et al., 2016). The existence of identical *C. cuniculus* subtypes in humans and rabbits suggests zoonotic potential (Zhang et al., 2012). In the present study, by STRUCTURE, neighbor-joining and Network analyses, four *gp60* subtypes were clustered into two different lineages, with VbA21 in Lineage I and VbA28, VbA29 and VbA32 in Lineage II. To date, VbA21 is only found in rabbits and the other three subtypes are found both in humans and rabbits (Yang et al., 2016). These results above indicate subpopulation analysis might be used to further assess zoonotic potential of

Fig 3. Median-joining network for various subtypes of *C. cuniculus*. Circles are proportional to the frequency of each multilocus genotype (MLG) (six MLGs on the basis of segregating sites of concatenated sequences including *gp60* gene). Length of lines connecting MLGs is proportional to the number of single nucleotide polymorphisms. The number of each MLST subtype is present in brackets.

subtypes in Vb family of *C. cuniculus*. Due to relatively small number of *C. cuniculus* DNA specimens from rabbits and the lack of *C. cuniculus* DNA specimens from humans, we cannot drew definitive conclusions about the relationship between subpopulation differentiation and zoonotic potential. It needs to be confirmed by analyzing sequence characterizations of a large number of *C. cuniculus* DNA specimens from different hosts at more polymorphic marker loci in the future.

5. Conclusion

This is the first report of MLST analysis of *C. cuniculus*. Six MLST subtypes were obtained. A clonal population structure of *C. cuniculus* suggests the prevalence of *C. cuniculus* in the investigated rabbits is not attributed to the introduction of rabbits. Thus, prevention and control strategies should be focused on making stricter measures to avoid occurrence of cross-transmission and re-infection among rabbit individuals. Two ancestral lineages of *C. cuniculus* were observed, indicating subpopulation analyses might be used to assess zoonotic potential of subtypes in Vb family of *C. cuniculus*. The present study provides useful data for exploring the source attribution of infection/contamination and assessing the transmission dynamics of *C. cuniculus* in the future.

Conflict of interest

The authors declared no conflict of interest. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

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