Bulgarian Journal of Veterinary Medicine, 2022, **25**, No 4, 665–671
ISSN 1311-1477; DOI: 10.15547/bjvm.2399

Short communication

MULTILOCUS GENOTYPING ANALYSIS OF 114 *GIARDIA DUODENALIS* ISOLATES FROM DIFFERENT POPULATIONS OF DOMESTIC DOGS IN JAPAN

N. ITOH, Y. IIJIMA, S. KAMESHIMA & Y. KIMURA

Laboratory of Small Animal Internal Medicine, School of Veterinary Medicine, Kitasato University, Japan

Summary

Itoh, N., Y. Iijima, S. Kameshima & Y. Kimura, 2022. Multilocus genotyping analysis of 114 *Giardia duodenalis* isolates from different populations of domestic dogs in Japan. *Bulg. J. Vet. Med.*, **25**, No 4, 665–671.

To determine the genotypes and zoonotic potentials of *G. duodenalis* isolates from different populations of domestic dogs in Japan, a total of 114 *Giardia* positive samples were examined using multilocus genotyping analysis at the 3 loci of glutamate dehydrogenase (*gdh*), beta-giardin (*bg*), and triose phosphate isomerase (*tpi*). Although the dog-adapted assemblages C and D were dominant, the zoonotic assemblage A was also demonstrated at a percentage of 23.7% (27/114). The results suggest that canine *G. duodenalis* isolates in Japan have the potential for zoonotic transmission.

Key words: domestic dogs, *Giardia duodenalis*, multilocus genotyping, zoonotic potential

Giardia duodenalis is one of the most common enteric protozoa in the world and can cause diarrhoea in mammalian hosts including humans and dogs (Certad *et al*., 2017; Ryan *et al*., 2019; Tangtrongsup & Scorza, 2010). *G. duodenalis* has been divided into at least 8 different assemblages (= genotypes) from A to H based on molecular analysis, and the host adaptability is different depending on each assemblage (Feng & Xiao, 2011; Ryan & Cacciò, 2013; Ryan *et al*., 2019). Although assemblages of C to H are host-adaptive, assemblages A and B are recognised as zoonotic genotypes and have a wide host range (Feng & Xiao, 2011; Ryan & Cacciò, 2013; Ryan *et al*., 2019). Considering the close contact with humans and the high-prevalence levels in dogs such as pet shop puppies and breeding kennel dogs (Itoh *et al*., 2011; 2015; Xu *et al*., 2016), it is important to evaluate the genotype and potential for zoonotic transmission of *G. duodenalis* in the isolates obtained from these domestic dogs. Recently, multilocus genotyping (MLG) analysis has been developed as one of the most informative tools for *G. duodenalis* genotyping, as some isolates demonstrate different results depending on the target-

ing loci (Feng & Xiao, 2011; Ryan & Cacciò, 2013). The current MLG analysis employs housekeeping genes such as glutamate dehydrogenase (*gdh*), beta-giardin (*bg*), and triose phosphate isomerase (*tpi*) (Xu *et al*., 2016; Julien *et al*., 2019; Li *et al*., 2019). However, there are no available large-scale (over 100 samples) reports regarding the MLG analysis of canine *G. duodenalis* isolates in Japan. The present study aimed to evaluate the genotypes and zoonotic potential of *G. duodenalis* isolates from different populations of domestic dogs in Japan using MLG analysis.

Between August 2015 and July 2018, a total of 114 DNA samples were submitted for the present study. All DNA samples were obtained from canine faeces in which *Giardia* cysts were detected by a zinc-sulfate floatation technique (specific gravity of 1.20). Faecal specimens were collected randomly from different populations of domestic dogs in Japan under the permission of their owners. Out of 114 samples, 20 isolates were from private household dogs (2 months to 10 years of age) that presented at 6 veterinary clinics in 3 different regions (3 clinics in Tohoku, 2 clinics in Kanto, and 1 clinic in Kinki), 45 isolates were from puppies (≤ 3 months of age) kept in 4 pet shops in East Japan (2 shops in Tohoku and 2 shops in Kanto), 26 isolates were from dogs (7 months to 8 years of age) kept in 6 breeding kennels located in 3 different regions (2 kennels in Tohoku, 1 kennel in Kanto, and 3 kennels in Chubu), 12 isolates were from dogs (7–15 years of age) kept in a veterinary nursing school (Kanto), and 11 isolates were from dogs (2 months to 4 years of age) kept in a training school (Tohoku). DNA extraction, after the collection of *Giardia* cysts by a sucrose gradient concentration method with a specific gravity of 1.26, was performed using a QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The obtained DNA samples were stored at −20 °C prior to analysis.

Nested PCR targeting the 3 loci of *gdh*, *bg*, and *tpi* was performed to determine *G. duodenalis* assemblages according to the previously reported protocols (Cacciò *et al*., 2002; 2008; Sulaiman *et al*., 2003; Lalle *et al*., 2005). The PCR mixtures were comprised of 1× buffer with final concentrations of 2.0 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer, 1.25 units of GoTaq DNA polymerase (Promega Corporation, Madison, WI, USA), and 3.0 μL of template DNA in a total reaction volume of 25 μL. Only the PCR mixture for *bg* had a final concentration of 3.0 mM $MgCl_2$. For the secondary reactions, each PCR mixture was the same as the primary reaction, except that the primary PCR amplicons were used as a template.

All secondary PCR products were identified by electrophoresis on 1.5% agarose gels. The fragments were visualized after staining with AtlasSight DNA Stain (Bioatlas, Tartu, Estonia) under UV light using a transilluminator. PCR amplicons of the predicted size were purified using a QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany). Sequencing analysis was performed in a commercial laboratory (FASMAC Co., Ltd., Atsugi, Kanagawa, Japan). Sequence alignment and compilation were carried out using the MEGA 7.0.26 (www. megasoftware.net) program. To determine the assemblages and sub-assemblages (AI or AII) of assemblage A at *gdh* and *tpi*, the obtained DNA sequences were compared to GenBank data of *G. duodenalis* by BLAST searches (http://www.ncbi.nlm nih.gov/).

All 114 samples successfully amplified specific fragments at 2 or more loci. The whole isolates obtained here showed 99–100% similarity to the corresponding reference sequences in GenBank. The assemblages of *G. duodenalis* isolates detected at the different loci are summarised in Table 1. Overall, the results of the MLG analysis demonstrated that 73 isolates (64.0%; 73/114) were determined as single assemblages (e.g. all identified assemblages at different loci corresponded to one genotype) and that the remaining 41 isolates (36.0%; 41/114) were determined as mixed assemblages (identified assemblage were different depending on analysed locus). In single assemblages, the predominant one was assemblage D (43.8%; 32/73), followed by assemblage C (42.5%; 31/73), assemblage A (12.3%; 9/73) and assemblage B (1.4%; 1/73). Regarding mixed assemblages (including duplicate assemblages within a single locus cause of double peaks in sequencing chromatograms), assemblages C and D (hereinafter referred to as "C+D") were common (75.6%; 31/41), followed by assemblages A+D (9.8%; 4/41), assemblages A+C (7.3%; 3/41), assemblages A+C+D (4.9%; 2/41), and assemblages B+D (2.4%; 1/41). The determination of sub-assemblages for assemblage A at gdh and/or tpi exhibited that all of the isolates were classified into the sub-assemblage AI.

The obtained assemblages, according to the populations, are summarised in Table 2. Including mixed assemblages, both assemblages of C and D were always demonstrated in all populations. Assemblage A was also demonstrated in all populations except for private household dogs and assemblage B was observed in household dogs and pet shop puppies.

Regarding the relationship between the characterised assemblages and the analysed loci, assemblages A and C were identified at all 3 loci used here, while assemblage B was demonstrated at *bg* and *tpi*, and assemblage D was demonstrated at *gdh* and *bg*. The identification of duplicate assemblages within a single locus was shown at all analysed loci, and the coexistence of assemblage C with D (C/D) was the most common.

This research is the first report to evaluate the genotypes and zoonotic potentials regarding large numbers of *G. duodenalis* samples (over 100 samples) from different populations of domestic dogs in Japan using MLG analysis. In the present study, the mixed assemblages, including duplicate assemblages within a single locus, both of which were interpreted as a mixed infection consisting of different assemblages (Feng & Xiao, 2011; Ryan & Cacciò, 2013), were demonstrated in approximately 40.0% of the isolates. In addition, the zoonotic assemblages were also demonstrated in mixed assemblages with non-zoonotic assemblages, and we could not observe those inclusions without MLG analysis. The results suggested that a portion of the canine *G. duodenalis* infections were mixed infections with a diversified combination of different assemblages, and this situation was not influenced by the populations of dogs, because the mixed assemblages were detected from all of the populations. Previous studies also commonly reported that canine *G. duodenalis* infections were composed with mixed assemblages (Itagaki *et al*., 2005; Xu *et al*., 2016; Adell-Aledón *et al*. 2018; Li *et al*., 2019). To recognise the mixed infections, especially those including the zoonotic assemblages, MLG analysis for *G. duodenalis* isolates is obviously a required technique, and its availability should be reemphasised, con-

Table 1. Determined assemblages of *Giardia duodenalis* isolates from domestic dogs in Japan

Determined assemblage	*gdh*	*bg*	*tpi*	No. of isolates
Single assemblage (n=73)				
assemblage A (n=9)				
	A(AI)*	–	A(AI)	2
	A(AI)	A	A(AI)	7
assemblage B (n=1)				
	–	B	B	1
assemblage C (n=31)				
	C	C	C	25
	C	–	C	2
	–	C	C	4
assemblage D (n=32)				
	D	D	–	32
Mixed assemblages (n=41)				
assemblages A+C (n=3)				
	A(AI)	C	A(AI)	1
	C	C	A(AI)/C[#]	1
	–	A	C	1
assemblages A+D (n=4)				
	D	D	A(AI)	2
	D	–	A(AI)	1
	–	D	A(AI)	1
assemblages B+D (n=1)				
	D	D	B	1
assemblages C+D (n=31)				
	C	C/D	C	3
	C/D	C	C	1
	C/D	D	C	5
	C/D	C/D	C	4
	C/D	–	C	2
	D	D	C	5
	D	C/D	C	1
	–	D	C	5
	D	–	C	1
	C	D	C	2
	–	C/D	C	2
assemblages A+C+D (n=2)				
	C/D	C/D	A(AI)	1
	C/D	A/D	C	1

* ( ): Sub-assemblage; [#] Duplicate assemblages within single locus. Corresponding reference sequences in GenBank were as followed: Assemblage A: KJ027433.1 at *gdh*, MF497409.1 at *bg*, EU041756.1, KM190791.1 at *tpi;* Assemblage B: KJ888980.1 at *bg*, MF095053.1, AY368171.1 at *tpi;* Assemblage C: KJ027432.1, KY979489.1, MF990014.1 at *gdh*, JF422718.1, JX867769.1, KJ027413.1 at *bg*, AY228641.1, KP258397.1, KR855639.1 at *tpi;* Assemblage D: EF507619.1, EF507638.1 at *gdh*, KJ027418.1, KY979501.1 at *bg*.

Table 2. Determined assemblages of *Giardia duodenalis* isolates from different populations of domestic dogs in Japan

Populations of dogs	Examined No.	Determined assemblages								
		Single assemblage				Mixed assemblages				
		A	B	C	D	A+C	A+D	B+D	C+D	A+C+D
Private household dogs	20	0	1	8	8	0	0	0	3	0
Pet shop puppies	45	0	0	13	17	0	3	1	10	1
Breeding kennel dogs	26	0	0	3	5	0	0	0	17	1
Veterinary nursing school dogs	12	9	0	1	0	1	1	0	0	0
Training school dogs	11	0	0	6	2	1	0	0	1	1

sistent with previous studies (Feng & Xiao, 2011; Ryan & Cacciò, 2013).

Overall, whether single assemblage or mixed assemblages, the predominant detected genotype was assemblage D, followed by assemblage C, and both assemblages were demonstrated in all of the populations. Considering assemblages D and C are dog-adapted genotypes (Feng & Xiao, 2011; Ryan & Cacciò, 2013; Ryan *et al*., 2019), these results are non-contradictory compared to previous reports (Itagaki *et al*., 2005; Itoh *et al*., 2011; Xu *et al*., 2016; Adell-Aledón *et al*., 2018; Li *et al*., 2019). In contrast, it was surprising that the zoonotic assemblage A was determined at the total percentage of 23.7% (27/114), a level that should not be ignored, in overall single and mixed assemblages. Moreover, assemblage A was isolated from all populations of examined dogs except for private household dogs. According to the sub-assemblage analysis at *gdh* and *tpi*, every isolate of assemblage A corresponded to the sub-assemblage AI, which has been demonstrated in both humans and other mammals, including dogs (Feng & Xiao, 2011; Ryan & Cacciò, 2013). Even though the examined isolates in each population are limited in numbers, these findings suggest that the zoonotic assemblage A (AI) is likely to invade the plural managed populations of domestic dogs in Japan, and we cannot neglect the risk of canine *G. duodenalis* transmission to humans. The population of private household dogs in the present study had no isolates of assemblage A. However, a previous study in Japan has shown the isolation of assemblage A from private household dogs (Itagaki *et al*., 2005). In addition, the dogs kept in the veterinary nursing school and training school have the potential to contact humans frequently. Therefore, the risk of *G. duodenalis* transmission from domestic dogs to humans should be observed in Japan. Assemblage B, which is also considered a zoonotic genotype, was determined in only 2 isolates, one of which was derived from the population of private household dogs and the other of which was from the population of pet shop puppies. In general, assemblage B is frequently isolated from human patients, but cases of dogs infected with assem-

blage B are rare (Itagaki *et al*., 2005; Feng & Xiao, 2011; Ryan & Cacciò, 2013; Xu *et al*., 2016; Certad *et al*., 2017; Skhal *et al*., 2017; Li *et al*., 2019). The above facts indicate that the risk of zoonotic transmission via assemblage B from domestic dogs in Japan is much lower than that of assemblage A.

Although the recommended 3 loci (*gdh*, *bg*, and *tpi*) were used for MLG analysis here, there were some deviations among the analysed loci in regard to PCR amplification and genotyping, as pointed out in earlier articles (Feng & Xiao, 2011; Ryan & Cacciò, 2013). The amplicons at *bg* were able to distinguish all assemblages, but of course, not perfectly. In contrast, *gdh* and *tpi* could not amplify assemblage B and D, respectively. However, *tpi* evidently has an advantage for the evaluation of assemblage A. In addition, *gdh* and *bg* have a predominance for determining the duplicate mixture assemblages within a single locus. Thus, these 3 loci act complementarily and are recommended to use for MLG analysis of *G. duodenalis* isolates. Moreover, the deviation of the obtained results and the characteristics that depended on the loci used are the reasons for the necessity of MLG analysis (Feng & Xiao, 2011; Ryan & Cacciò, 2013).

CONCLUSION

The present study suggests that *G. duodenalis* isolates from different populations of domestic dogs in Japan have the potential for zoonotic transmission at considerable level. In particular, assemblage A, which is one of the zoonotic genotypes, is likely to be common in dogs. In addition, to discuss the zoonotic transmission of canine *G. duodenalis* isolates, the MLG approach (using at least 3 loci: *gdh*, *bg*, and *tpi*) is essentially required.

REFERENCES

Adell-Aledón, M., P. C. Köster, A. de Lucio, P. Puente, M. Hernández-de-Mingo, P. Sánchez-Thevenet, M. A. Dea-Ayuela & D. Carmena, 2018. Occurrence and molecular epidemiology of *Giardia duodenalis* infection in dog population in eastern Spain. *BMC Veterinary Research*, **14**, 26.

Cacciò, S. M., R. Beck, M. Lalle, A. Marinculic & E. Pozio, 2008. Multilocus genotyping of *Giardia duodenalis* reveals striking differences between assemblages A and B. *International Journal for Parasitology*, **38**, 1523–1531.

Cacciò, S. M., M. De Giacomo & E.Pozio, 2002. Sequence analysis of the β-giardin gene and development of a polymerase chain reaction–restriction fragment length polymorphism assay to genotype *Giardia duodenalis* cysts from human faecal samples. *International Journal for Parasitology*, **32**, 1023–1030.

Certad, G., E. Viscogliosi, M. Chabé & S. M. Cacciò, 2017. Pathogenic mechanisms of *Cryptosporidium* and *Giardia*. *Trends in Parasitology*, **33**, 561–576.

Feng, Y & L. Xiao, 2011. Zoonotic potential and molecular epidemiology of *Giardia* species and giardiasis. *Clinical Microbiology Reviews*, **24**, 110–140.

Itagaki, T., S. Kinoshita, M. Aoki, N. Itoh, H. Saeki, N. Sato, J. Uetsuki, S. Izumiyama, K. Yagita & T. Endo, 2005. Genotyping of *Giardia intestinalis* from domestic and wild animals in Japan using glutamate dehydrogenase gene sequencing. *Veterinary Parasitology*, **133**, 283–287.

Itoh, N., T. Itagaki, T. Kawabata, T. Konaka, N. Muraoka, H. Saeki, K. Kanai, S. Chikazawa, Y. Hori, F. Hoshi & S. Higuchi, 2011. Prevalence of intestinal parasites and genotyping of *Giardia intestinalis* in pet shop puppies in east Japan. *Veterinary Parasitology*, **176**, 74–78.

Itoh, N., K. Kanai, Y. Kimura, S. Chikazawa, Y. Hori & F. Hoshi, 2015. Prevalence of intestinal parasites in breeding kennel dogs

in Japan. *Parasitology Research*, **114**, 1221–1224.

Julien, D. A., J. M. Sargeant, R. A. Guy, K. Shapiro, R. K. Imai, A. Bunce, E. Sudlovenick, S. Chen & J. Li, 2019. Prevalence and genetic characterization of *Giardia* spp. and *Cryptosporidium* spp. in dogs in Iqaluit, Nunavut, Canada. *Zoonoses Public Health*, **66**, 813–825.

Lalle, M., E. Pozio, G. Capelli, F. Bruschi, D. Crotti & S. M. Cacciò, 2005. Genetic heterogeneity at the beta-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. *International Journal for Parasitology*, **35**, 207–213.

Li, J., X. Dan, K. Zhu, N. Li, Y. Guo, Z. Zheng, Y. Feng & L. Xiao, 2019. Genetic characterization of *Cryptosporidium* spp. and *Giardia duodenalis* in dogs and cats in Guangdong, China. *Parasites & Vectors*, **12**, 571.

Ryan, U. & S. M. Cacciò, 2013. Zoonotic potential of *Giardia*. *International Journal for Parasitology*, **43**, 943–956.

Ryan, U., N. Hijjawi, Y. Feng & L. Xiao, 2019. *Giardia*: An under-reported foodborne parasite. *International Journal for Parasitology*, **49**, 1–11.

Skhal, D., G. Aboualchamat, A. AI Mariri & S. Al. Nahhas, 2017. Prevalence of *Giardia duodenalis* assemblages and subassemblages in symptomatic patients from Damascus city and its suburbs. *Infection, Genetics and Evolution*, **47**, 155–160.

Sulaiman I. M., R. Fayer, C. Bern, R. H. Gilman, J. M. Trout, P. M. Schantz, P. Das, A. A. Lal & L. Xiao, 2003. Triosephosphate isomerase gene characterization and potential zoonotic transmission of *Giardia duodenalis*. *Emerging Infectious Diseases*, **9**, 1444–1452.

Tangtrongsup, S. & V. Scorza, 2010. Update on the diagnosis and management of *Giardia* spp infections in dogs and cats. *Topics in Companion Animal Medicine*, **25**, 155–162.

Xu H, Y. Jin, W. Wu, P. Li, L. Wang, N. Li, Y. Feng & L. Xiao, 2016. Genotypes of *Cryptosporidium* spp., *Enterocytozoon bieneusi* and *Giardia duodenalis* in dogs and cats in Shanghai, China. *Parasite & Vectors*, **9**, 121.

Paper received 12.10.2020; accepted for publication 17.12.2020

Correspondence:

N. Itoh
Laboratory of Small Animal
Internal Medicine,
School of Veterinary Medicine,
Kitasato University, Higashi 23-35-1,
Towada, Aomori 034-8628, Japan
e-mail: naoitoh@vmas.kitasato-u.ac.jp