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Molecular and histopathological features of *Cryptosporidium ubiquitum* infection in imported chinchillas *Chinchilla lanigera* in Japan

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ABSTRACT

Long-tailed chinchillas *Chinchilla lanigera* are popular rodent species kept both in households, where they are hand-raised as pets, and in zoological facilities. From January 2016 to February 2017, 13 juvenile chinchillas from five facilities in Japan were diagnosed with cryptosporidiosis at the animal hospital. Eight of the cases were fatal. All of the animals were imported from the Czech Republic by the same vendor. Histopathological and multilocus sequence analyses using 18S ribosomal RNA, actin, 70-kDa heat shock protein, and 60-kDa glycoprotein genes confirmed *Cryptosporidium ubiquitum* of subtype XIId as the etiological agent. Multilocus analysis demonstrated the presence of two new sequence types closely related to the *C. ubiquitum* Xlld strain isolated from a human in the USA. This study indicated that potentially zoonotic *Cryptosporidium* is widespread and may have caused a high number of deaths among imported juvenile chinchillas.

1. Introduction

The long-tailed chinchilla *Chinchilla lanigera* is a small nocturnal rodent belonging to the family Chinchillidae. The historic distribution of this species extends throughout the arid, barren, and rugged areas connecting the coastal mountain ranges and the Andes of South America. Since their population continues to decline due to the activities of humans, the international trade of wild chinchillas is prohibited [1]. Thus, all chinchillas available in the legal pet trade are bred and raised on farms, or by private breeders. Nearly every year, thousands of captively bred chinchillas are imported from other counties into Japan without quarantine. The growing international trade of these animals is contributing to the emergence and spread of infectious diseases via transmission of the pathogens carried by the imported animals.

Cryptosporidium is a genus of coccidian protozoan parasite of medical and veterinary importance and is the etiological agent for cryptosporidiosis. Currently, > 30 *Crypsotporidium* species are recognized and are capable of infecting a wide range of vertebrate animals, and causing mild to severe gastrointestinal or respiratory diseases in their host species [2–4]. One species of interest is *Cryptosporidium ubiquitum* Fayer, Santín and Macarisin, 2010 has previously been identified as the *Cryptosporidium* cervine genotype, less frequently as cervid or W4 genotype, and is considered to be an emerging zoonotic pathogen [5]. This species has been reported in various countries to infect both wild and domestic ruminants; rodents; carnivores; marsupials; and primates, including many human cases [5–7].

In the present study, we report the clinical, histopathological, and molecular features of cryptosporidiosis caused by *C. ubiquitum* in juvenile chinchillas imported into Japan. Our results serve to alert veterinarians; workers in the pet trade; animal care and service workers at zoos, animal shelters, and pet stores; and pet owners who may be exposed to imported chinchillas regarding the threat of *C. ubiquitum* infection and the development of cryptosporidiosis.

2. Materials and methods

2.1. Animals and specimens

The animals in the study included 13 juvenile chinchillas (IDs: 1604–1611, 1702–1706) taken to Banquet Animal Hospital, Tokyo, Japan from January 2016 to February 2017. The animals were all imported from the Czech Republic by the same vendor with the intent to be sold as pets and had been housed in five different facilities (Table 1). Fresh feces were collected from each animal and placed in separate vials containing 70% ethanol or 2.5% aqueous potassium dichromate ($K_2Cr_2O_7$) solution, and stored at 4 °C. In order to investigate the

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Table 1
Characteristics of thirteen *Cryptosporidium* infections in juvenile chinchillas.

Case IDs	locality	Date of diagnosis	Initial and subsequent clinical signs	Duration of illness	Outcome
1604	Saitama	Jan, 2016	Anorexia and diarrhea	several days	N/A
1605	Saitama	Jan, 2016	Rectal prolapse, diarrhea, and intussusception	several days	Death
1606	Saitama	Oct, 2016	Loss of vigorous prostration, diarrhea	several days	Death
1607	Saitama	Oct, 2016	Asymptomatic (health check)	–	N/A
1608	Tokyo A	Oct, 2016	Diarrhea	several days	Death
1609	Saitama	Oct, 2016	Loss of vigorous prostration, and diarrhea	8 days	Death
1610	Saitama	Nov, 2016	Sudden death	–	–
1611	Tokyo A	Nov, 2016	Sudden death	–	–
1702	Saitama	Nov, 2016	Anorexia, diarrhea	4 days	N/A
1703	Saitama	Dec, 2016	Asymptomatic (health check)	–	N/A
1704	Kanagawa A	Dec, 2016	Rectal prolapse and diarrhea	2 days	Death
1705	Tokyo B	Jan, 2017	Anorexia and diarrhea	10 days	N/A
1706	Kanagawa B	Feb, 2017	Anorexia, diarrhea, and rectal prolapse	2 days	Death

N/A: not available.

prevalence of *Cryptosporidium* infection among captively bred chinchillas imported from other geographical areas, 50 fecal specimens collected from 45 chinchillas (30 juvenile, 15 adult) imported from the USA, and five adult chinchillas from the Netherlands were placed in separate vials containing 70% ethanol and stored at 4 °C. No ethical approval was obtained because this study did not involve animal experiments and only used clinical specimens, obtained non-invasively from animals.

2.2. Pathological examination

For histopathological analyses, two dead animals (animal IDs: 1605 and 1608) were autopsied. The brain, esophagus, intestines, kidney, heart, lung, liver, spleen, and adrenal glands were dissected from each animal, fixed in 10% neutral-buffered formalin, embedded in paraffin, cut at 4 μm-thick sections using a microtome, and stained with hematoxylin and eosin (HE). Specimens were analyzed by light microscopy.

Intestinal tissues were also analyzed by immunofluorescence assay (IFA). For IFA, *Cryptosporidium* oocysts in the formalin-fixed paraffin-embedded sections of intestines were stained with EasyStain (BTF, North Ryde, Australia), which contained fluorescein isothiocyanate (FITC)-labeled antibodies specific for *Cryptosporidium* and *Giardia*. Conventional methods were used for staining. Fluorescence signals in the sections were visualized using a BZ-X700 fluorescence microscope (Keyence, Osaka, Japan).

2.3. DNA extraction, PCR, and sequencing

The fecal specimens preserved in ethanol or $K_2Cr_2O_7$ were used for molecular analyses. Oocysts collected from the feces were washed with double-distilled water, purified using sucrose centrifugal flotation, and subjected to three freeze-thaw cycles in transfers between a − 70 °C freezer and a 56 °C heat block in order to ensure efficient lysis. Total genomic DNA was extracted using a PowerSoil DNA Isolation Kit (Mo Bio Laboratories, USA) according to the manufacturer's instructions. The isolated DNA was used as template for PCR analysis.

PCR analysis included amplification of the 18S ribosomal RNA (18S), actin, and the 70-kDa heat shock protein (HSP70) genes using nested PCR as previously described for identification of *Cryptosporidium* species [8–11]. In general, PCR amplification was performed in a 25 μl volume containing 1 μl of each forward and reverse primers (10 μM), 12 μl of PCR mixture (OnePCR, GeneDirex, USA), 10 μl of double distilled water, and 1 μl of template. For the nested amplification, 1 μl of the first PCR product was added to 49 μl of a second PCR containing 25 μl of OnePCR mixture, 22 μl of double distilled water, and 1 μl of each of the internally nested forward and reverse primers. Cycling for both rounds of amplification was performed at 94 °C for 5 min for initial template denaturation, followed by 30 or 35 cycles of 94 °C for 45 s, the specific annealing temperature for each primer set, and 72 °C for 1 min for product extension. A final extension was performed at the end of the cycling profile at 72 °C for 7 min. Detailed information regarding the primers, amplification cycling conditions, and the appropriate references are shown in Supplemental table 1. The PCR products from the second amplification were purified and sequenced at Macrogen Japan (Kyoto, Japan) using the same primers that were used during the secondary PCR amplification.

The DNA samples were also subjected to multilocus sequence typing (MLST) of the 60-kDa glycoprotein (GP60) gene, and the four genetic loci of cgd6_1590, cgd6_60, cgd2_3690, and cgd4_370 [12–14]. The sequencing results were used for detecting intra-subtype variability of *Cryptosporidium* species.

2.4. Sequence analyses

Sequence data obtained for the 18S, actin, HSP70, and GP60 genes were aligned using the ClustalW with initial fixed parameter values in Molecular Evolutionary Genetics Analysis (MEGA) (https://megasoftware.net/home), version 7.0 software [15]. Sequence similarity was determined using BLAST analysis from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/Blast.cgi).

Naming of the four genetic loci cgd6_1590, cgd6_60, cgd2_3690, and cgd4_370 for MLST analysis was done in accordance with the nomenclature used for the allelic profile of *C. ubiquitum* [14] and the MLST sequence type was then determined by the combination of alleles identified. The gene sequences obtained for the four loci were concatenated and aligned using MEGA. Phylogenetic trees were constructed using neighbor-joining (NJ) [16] and maximum likelihood (ML) methods [17] by MEGA. A substitution model and optional parameter sets were determined using the Tamura-Nei model [18], and selected according to the Akaike information criterion (AIC), respectively. Since there was no sequence for use as an out-group sequence for the four genetic loci, midpoint rooting was used to estimate phylogenetic relationships. The phylogenetic tree was evaluated using bootstrap methodology based on 1000 replicates for both trees. Representative nucleotide sequences generated in the study have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers LC3340000 (18S), LC3340001 (actin), LC3340002 (HSP70), LC3340004 (GP60), LC3340005 (cgd2_3690), LC3340006- LC3340007 (cgd4_370), LC3340008 (cgd6_60), and LC3340009 (cgd6_1590).

3. Results

3.1. Diagnosis

Following delivery to the animal hospital, the chinchillas were

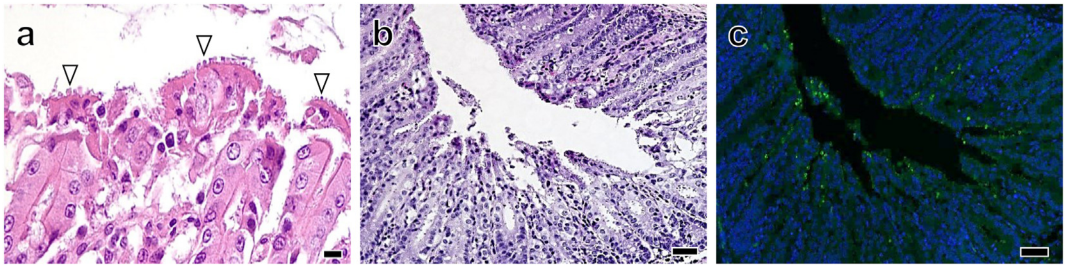

Fig. 1. Ileum of chinchillas. (a & b) HE staining. *Cryptosporidium* spp. appear as rounded purple organisms on the microvilli of epithelial cells (arrow heads). (c) Fluorescent antibody staining. The nuclei of the cells were stained with DAPI and the *Cryptosporidium* oocysts wall with FITC-conjugated fluorescent antibodies. Bar = 10 μm (a), and 40 μm (b & c).

diagnosed as cryptosporidiosis based on the presence of oocysts in their stool specimens. Of the 13 chinchillas, two had already died. Of the other live chinchillas, nine presented with anorexia, diarrhea, and vigorous prostration; six of the animals succumbed to their disease and died within a few days. The remaining two animals were asymptomatic, despite living in proximity of the affected animals. Detailed information regarding the animals and their symptoms is shown in Table 1. Clinical findings for the two dead chinchillas evaluated showed rectal prolapse, diarrhea, and intussusception for one animal (ID: 1605), and only diarrhea for the other (ID: 1608).

Histopathological examination revealed numerous tiny round structures on the mucosal surface of jejunum and ileum of both animals (Fig. 1a & b). IFA using FITC-conjugated antibodies demonstrated a pattern of *Cryptosporidium* antigen-staining almost identical to the structures found in the HE sections (Fig. 1b & c). Both animals developed lesions consistent with bacterial pneumonia. No significant abnormalities were observed in the other organs examined.

3.2. Sample analysis and phylogenetic analysis

3.2.1. 18S, actin and HSP70 genes

A 454-bp fragment of the 18S gene was amplified and sequenced for all DNA samples except for one (ID: 1607). The sequences were identical to each other upon comparison and showed 100% identity (454/454) to the standard sequence of *C. ubiquitum* (Accession no. HM209366) from cattle *Bos taurus*. PCR targeting the actin gene produced positive results for eight of the specimens (IDs: 1604, 1605, 1606, 1608, 1609, 1611, 1702, and 1704) and the 945-bp fragments were successfully sequenced. The sequences for these eight study specimens were identical to each other, demonstrating 100% identity (940/940) with *C. ubiquitum* isolated from the feces of an Eastern gray squirrel *Sciurus carolinensis* from the USA (Accession no. KT027512). The 1699 bp fragment of HSP70 gene was amplified and sequenced in two overlapping fragments (F2a-R2a and F2b-R2b) of approximately 880 and 890 bp, respectively for six of the specimens (IDs: 1604, 1605, 1606, 1608, 1611 and 1704). Sequence analysis of these revealed that they were identical to each other, with 100% identity (1699/1699) to *C. ubiquitum* isolated from a human in Canada (Accession no. DQ389176). Based on these results, we concluded that the microorganisms present in the study chinchillas were *C. ubiquitum*.

The additional samples from chinchillas imported from the USA and the Netherlands were negative for all PCR. This suggested that these imported chinchillas were not infected with *Cryptosporidium* at the time of analysis.

3.2.2. GP60 subtype and MLST sequence types

The partial sequences of the GP60 gene was amplified and sequenced from seven (IDs: 1605, 1606, 1608, 1609, 1611, 1704, and 1706) of eight deceased animals showed 100% identity (875/875) with *C. ubiquitum* belonging to subtype XIId (Accession no. KR260679).

MLST results are summarized in Table 2. The sequences of two loci, cgd6_60 and cgd2_3690, shared 100% identity with allele 3 (Accession no. KX286380) at a 933/933 nucleotide match, and allele 2 (Accession no. KX286385) at a 658/658 match. On the other hand, the sequences of locus cgd4_370 were divided in two known alleles. The first of these alleles was detected in the DNA for two of the animals (IDs: 1608 and 1609) and had 100% identity (526/526) with allele 1 (Accession no. KX286354). DNA samples from the remaining animals showed 100% identity (526/526) with allele 4 (Accession no. KX286357). Furthermore, the partial sequences of locus cgd6_1590 from the seven deceased animals (IDs: 1605, 1606, 1608, 1609, 1611, 1704, and 1706) were successfully sequenced and were found to be identical to each other. This sequence is not registered with the DDBJ/EMBL/NCBI databases and showed 99% identity (652/653) with alleles 12 (Accession no. KX286371) and 13 (Accession no. KX286372). We therefore propose that the *C. ubiquitum* isolated from these chinchillas constitute a new cgd6_1590 sequence, making it allele 19.

Based on concatenating data across multiple genes of the four genetic loci, the phylogenic relationships among the GP60-subtype families of *C. ubiquitum,* showed similar topologies, except for bootstrap values. For simplicity, we present only the NJ tree with bootstrap values obtained from both trees (Fig. 2). Midpoint rooting trees showed two major clades: one containing the Xlla-subtype families detected in ruminants, and the second containing XIIb, Xllc, and XIId subfamilies detected from rodents (porcupine, chipmunk, and chinchilla) and primates (human and sifaka). The sequences from the chinchillas from the current study formed monophyletic groups with the Xlld subtype isolated from a human reported in the USA.

4. Discussion

There are only a few reports of *Cryptosporidium* spp. infection of chinchillas. Yamini and Raju [19] reported the case of cryptosporidiosis with severe diarrhea in an 8-month-old chinchilla from a pet shop, and found several spherical microorganisms in the intestinal epithelial cells that are characteristic of *Cryptosporidium* spp. Investigation of protozoan parasites in the fecal specimens of chinchillas bred in captivity in Brazil between 2002 and 2003 did not reveal any *Cryptosporidium* spp. among the 250 individuals sampled [20]. Qi et al. [21] investigated the prevalence of *Cryptosporidium* infection in 140 chinchillas in China from 2012 to 2013, and found one animal positive for *C. parvum* and 13 animals testing positive for *C. ubiquitum* subtype Xlld. Recently, *C. ubiquitum* subtype Xlla was detected in two chinchillas from farms in Poland [22]. The present investigation, for the first time, demonstrated the histopathological features and molecular characterization of *C. ubiquitum* subtype XIId infection among imported juvenile chinchillas in Japan. Clinical features indicated that the juvenile chinchillas were susceptible to infection with this protozoan.

Cryptosporidium ubiquitum is known to infect a wide range of mammalian species. It has been found in various countries in domesticated and wild ruminants (sheep, goats, deers, blesbok, nyala, ibex, and cattle), rodents (squirrels, chipmunks, woodchucks, beavers,

Table 2
Hosts, countries, and molecular features of *Cryptosporidium ubiquitum*.

Host	Country	GP60-subtype	MLST allels				Sequence type (ST)
			cgd6_1590	cgd6_60	cgd2_3690	cgd4_370	
Chinchilla[a]	Japan	XIId	19	3	2	4	19
Chinchilla[b]	Japan	XIId	19	3	2	1	20
Sheep	P.R.China	XIIa	4	1	1	3	1
Impala	South Africa	Xlla	4	1	1	4	2
Buffalo	South Africa	Xlla	5	1	1	4	3
Swamp deer	Nepal	Xlla	2	1	1	4	4
Swamp deer	Nepal	Xlla	3	1	1	4	5
Sheep	Spain	Xlla	1	1	1	4	6
Human	USA	Xllb	7	2	2	1	7
Verreaux's sifaka	USA	Xllb	6	2	2	1	8
Human	USA	Xllb	6	2	3	5	9
Human	USA	Xllb	8	2	3	5	10
Human	USA	Xllc	9	2	2	1	11
Porcupine	USA	Xllc	10	2	2	1	12
Human	USA	Xllc	9	2	3	5	13
Human	USA	Xllc	11	2	2	1	14
Human	USA	XIId	13	3	2	1	15
Human	USA	XIId	14	2	2	1	16
Eastern chipmunk	USA	XIId	12	2	2	1	17
Human	USA	XIId	13	2	3	5	18

[a] IDs 1605, 1606, 1611, 1704, and 1706.
[b] IDs 1608, and 1609.

porcupines, deer mice, house mice, and gerbils), carnivores (raccoons), and primates (lemurs and humans) [5–7]. It is also found in sources of drinking water and wastewater in various geographic locations [5,13,23]. In Japan, the *Cryptosporidium* sp. isolated from the large Japanese field mouse *Apodemus speciosus*, was identified as *C. ubiquitum* [11]. To our knowledge, our study is the first report of C. *ubiquitum* infection being detected in imported pets in Japan.

Among *Cryptosporidium* species, *C. hominis* and *C. parvum* are responsible for > 90% of human cases of cryptosporidiosis [4,24]. However, recently *C. ubiquitum* has been noted as another species that may be a major zoonosis pathogen capable of infecting humans [5,7,13,14]. For *C. parvum, C. hominis,* and some related species, sequence analysis of the GP60 gene has been used for subtyping markers in order to investigate genetic diversity, infection sources, and transmission dynamics [12]. Li et al. [13] developed a subtyping tool targeting the GP60 gene, and identified six family subtypes (Xlla–Xllf) among *C. ubiquitum.* Two new subtypes (Xllg and XIIh) were recently detected in wastewater and sewer overflow samples [23]. These subtypes differ in host range and geographical distribution. For instance, the Xlla subtype was found in ruminants worldwide, subtypes Xllb–Xllf were found in rodents in the USA and Slovak Republic, subtypes Xllb and Xlld had been detected in drinking water in the USA, and subtypes Xllg and XIIh were found in China. Prediger et al. [25] reported subtype Xllb from Eastern gray squirrels *Sciurus carolinensis* in Italy, which suggests the possibility of the introduction of this subtype from the North America. However, subtyping using a single locus is not a reliable marker for analysis of population structures of these species due to genetic recombination that may occur. Recently, using MLST as a tool for typing *C. ubiquitum,* 18 sequence types were detected among 19 specimens [14]. The present study demonstrated the presence of two new sequence types in chinchillas. Phylogenetic analysis using the MLST data indicates that the ruminant Xlla subtype formed a monophyletic group genetically distant from the other family subtypes Xllb, Xllc, and Xlld. Furthermore, the two new sequence types detected from the imported chinchillas were closely related to each other. In this study, *Cryptosporidium* samples taken from different facilities shared the

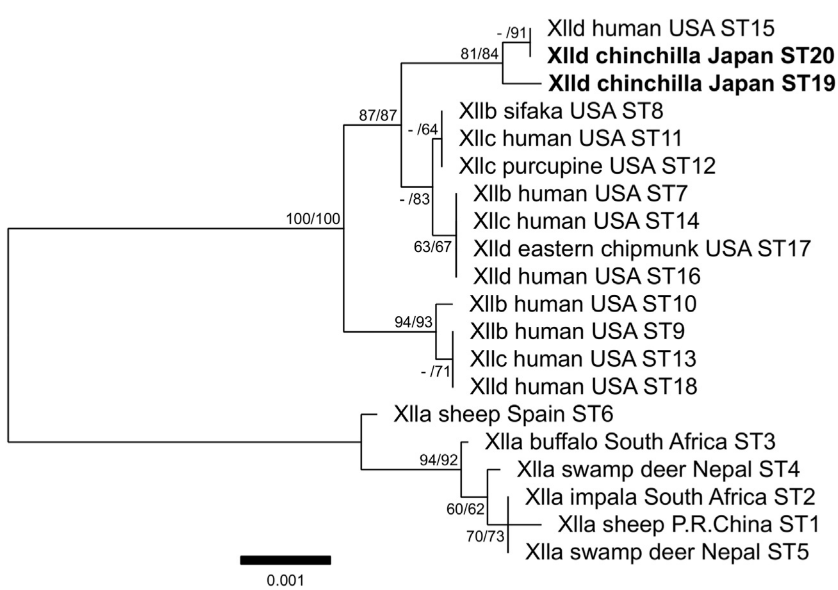

Fig. 2. Phylogenetic relationships of *Cryptosporidium ubiquitum* subtypes based on partial sequences of the four genetic loci cgd6_1590, cgd6_60, cgd2_3690, and cgd4_370) of *C. ubiquitum.* The tree was generated with the neighbor-joining (NJ) method using distances calculated by the Tamura-Nei model. Numbers at the nodes represent bootstrap of 1000 replicates (> 50%) for maximum likelihood (ML; left) and NJ (right). The scale bar represents the number of nucleotide substitutions per site.

same or closely related sequence types, suggesting that the infections may have occurred before or during importation by the vendor from Czech Republic, and that the infected animals were subsequently introduced into the different facilities. Furthermore, the two new sequence types formed a cluster with *C. ubiquitum* subtype XIId, which was isolated from a human in the USA. This indicated that *C. ubiquitum* shedding from the imported juvenile chinchillas found in the present study could have potentially infected human beings as well. Actually, a suspected case presenting with severe diarrhea was reported by one of the owners who had taken one of the affected chinchillas. Unfortunately, microbial examination for that owner was not carried out; however, it is likely that *Cryptosporidium* infection occurred in this case.

Most emerging infection diseases (EIDs) are caused by zoonotic pathogens and are significant burdens to the world economy and public health [26]. Most of these diseases originate in wildlife and spread to other species including humans, and their incidence has increased significantly over the past few decades. To prevent the emergence and future outbreak of EIDs, disease surveillance efforts should focus on activities that are associated with close relationships between humans and wildlife. Statistics by the Japanese Ministry of Health, Labor and Welfare show that ten million individual animals were imported into Japan every year without inspection from 2015 to 2017, including about 9000 chinchillas that were mainly imported from the Czech Republic (http://www.mhlw.go.jp/, access 2018.1.30). Therefore, because of the import practices and regulations, several pathogens including *Cryptosporidium* may be transmitted by these imported animals and have the potential to cause emerging infectious diseases in the future.

In summary, we present the histopathological and molecular features of cryptosporidiosis among juvenile chinchillas imported into Japan. Based on our findings, *Cryptosporidium* infection should be considered as a differential diagnosis for intestinal disease in pet chinchillas. Furthermore, since a few individual animals may be asymptomatic reservoirs for infection, it is desirable to have adequate quarantine periods, even when no symptoms are initially observed.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2018.09.002.

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