Common occurrence of divergent Cryptosporidium species and Cryptosporidium parvum subtypes in farmed bamboo rats (Rhizomys sinensis)

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Abstract

Background: Bamboo rats are widely farmed in southern China for meat, but their potential in transmitting pathogens to humans and other farm animals remains unclear.

Methods: To understand the transmission of Cryptosporidium spp. in these animals, 709 fecal samples were collected in this study from Chinese bamboo rats (Rhizomys sinensis) on nine farms in Jiangxi, Guangxi and Hainan provinces, China. They were analyzed for Cryptosporidium spp. using PCR and sequence analyses of the small subunit rRNA gene. Cryptosporidium parvum, C. parvum-like and C. ubiquitum-like genotypes identified were subtyped by sequence analysis of the 60 kDa glycoprotein (gp60) gene.

Results: Altogether, Cryptosporidium spp. were detected in 209 (29.5%) samples. The detection rate in samples from animals under two months of age (70.0%, 105/150) was significantly higher than in samples from animals above 2 months (18.6%, 104/559; χ² = 150.27, df = 1, P < 0.0001). Four Cryptosporidium species/genotypes were identified: C. parvum (n = 78); C. occultus (n = 1); a new genotype that is genetically related to C. ubiquitum (n = 85); and another new genotype that is genetically related to C. parvum (n = 44). Among them, C. parvum (27,610 ± 71,911 oocysts/gram of feces) and the C. parvum-like genotype (38,679 ± 82,811 oocysts/gram of feces) had higher oocyst shedding intensity than the C. ubiquitum-like genotype (2470 ± 7017 oocysts/gram of feces) and the C. occultus (1012 oocysts/gram of feces). The C. parvum identified belonged to three subtypes in two rare subtype families, including IpA9 (n = 43), IpP6 (n = 6) and IloA15G1 (n = 9), while the C. parvum-like and C. ubiquitum-like genotypes generated very divergent gp60 sequences.

Conclusions: Results of the present study suggest that bamboo rats on the study farms were infected with diverse Cryptosporidium species and divergent C. parvum subtypes, which probably had originated from their native habitats. As similar C. parvum subtypes have been recently detected in humans and farmed macaques, attentions should be paid to the potential role of these new farm animals in the transmission of zoonotic pathogens.

Keywords: Cryptosporidium, Zoonotic, Bamboo rat, Subtype, Molecular epidemiology
Background

Cryptosporidium spp. are protozoan parasites inhabiting the gastrointestinal epithelium of humans and other vertebrate animals [1]. They are ubiquitous in the environment; humans can be infected with Cryptosporidium spp. through contact with infected persons (anthroponotic transmission) or animals (zoontotic transmission) and ingestion of contaminated food (food-borne transmission) or water (water-borne transmission) [2].

To date, over 40 Cryptosporidium species have been recognized, together with almost an equal number of genotypes [3]. Among them, Cryptosporidium parvum has a broad host range and is the major Cryptosporidium species associated with the occurrence of diarrhea in farm animals [4]. As one of the two dominant Cryptosporidium species in humans, it is an important zoonotic pathogen [2]. Sequence analysis of the 60 kDa glycoprotein (gp60) gene has identified over 20 subtype families of C. parvum [5]. Among them, the common ones are host-adapted, such as Ila in dairy cattle, IIC in humans, and Ild in small ruminants [3]. Others, such as the newly identified subtype families IIp and IIo, were found mainly in bamboo rats and crab-eating macaques [6, 7]. Cryptosporidium ubiquitum is another zoonotic species with a broad host range. Sequence analysis of the gp60 gene has also identified host-adapted subtype families within the species, some of which have been found in humans and small ruminants in industrialized nations, while others have been found in rodents [8]. Therefore, genetic characterization is important in the assessment of the pathogenicity and public health potential of Cryptosporidium spp. in animals.

Bamboo rats are widely farmed in China since 1990 due to the high protein content and perceived medical values of the meat [9]. There were about 10,000 farms (households) of bamboo rats in China in 2011, with an annual production of 30 million animals, of which ~500,000 were exported to Southeast Asian countries [10]. However, in China, bamboo rats have long been known as a reservoir of the opportunistic pathogen Pneumocystis carinii [11, 12]. In recent years, other emerging pathogens such as Akabane virus, beta-lactam resistant Escherichia coli, Entero cytotoxin bieneusi and Giardia duodenalis have been detected in farmed bamboo rats [9, 13–15]. In a study of 92 fecal samples collected from a pet market in Sichuan Province, those from one asymptomatic and two diarrheic bamboo rats were positive for C. parvum [6]. Therefore, as recently domesticated rodents, bamboo rats have the potential of transmitting zoonotic pathogens to other farm animals and humans.

In this study, we examined the occurrence of Cryptosporidium spp. in farmed bamboo rats in southern China and identified the presence of diverse Cryptosporidium species and divergent C. parvum subtypes in these animals. We postulate that these unusual Cryptosporidium spp. probably originated from their native habitats.

Methods

Specimens

Between September 2017 and December 2018, 709 fecal samples were collected from Chinese bamboo rats (Rhizomys sinensis) on nine farms in Jiangxi, Guangxi, and Hainan provinces, China. Most of the farms sampled were newly established with predominantly adult animals and a small number of young animals. In contrast, Farms 1 and 4 were established facilities, had over 1,000 bamboo rats per farm, and provided animals to other farmers because of the availability of large numbers of young animals. On these farms, 5–10 bamboo rats were kept in the same pen, except for breeding pairs, which were kept in individual pens. For young animals under 6 months of age, 2–4 samples of fresh fecal pellets were collected from different locations in the pen to minimize repeated sampling of the same animal, while for older animals, only one sample was collected per pen. The animals in the study were divided into 6 convenient age groups: 1–2 months-old; 3–4 months-old; 5–6 months-old; 7–9 months-old; and 1–3 years-old; with a few of unknown age (Table 1). These fecal samples were stored in 2.5% potassium dichromate before DNA extraction.

Detection, genotyping and subtyping of Cryptosporidium spp.

Aliquots of 200 mg fecal samples were washed to remove potassium dichromate with distilled water by centrifugation at 2000×g for 10 min. DNA was extracted from washed fecal materials using the Fast DNA Spin Kit for Soil (MP Biomedical, Santa Ana, CA, USA) as previously described [16]. The extracted DNA was analyzed for Cryptosporidium spp. using a nested PCR targeting a ~830-bp fragment of the small subunit rRNA (SSU rRNA) gene [17]. Representative Cryptosporidium species/genotypes were characterized by restriction fragment length polymorphism (RFLP) analysis of the secondary SSU rRNA PCR products using restriction enzymes SspI (New England BioLabs, Massachusetts, USA) and VspI (Promega, Madison, WI, USA) [17]. The C. parvum, C. parvum-like genotype and C. ubiquitum-like genotype identified in this study were further subtyped by PCR and sequence analysis of the gp60 gene [18, 19]. The intensity of oocyst shedding was assessed by using a SYBR Green-based qPCR (18S-LC2) targeting a ~278-bp fragment of the SSU rRNA gene [20]. The master mix of the qPCR contained 10 μl of 2× SYBR Green real-time PCR master mix (Thermo Fisher Scientific, Waltham, MA, USA) in a 20 μl reaction. The qPCR
was performed on a LightCycler 480 II (Roche, Indianapolis, IN, USA) as described previously [7]. All qPCR analyses included one positive control and two negative controls. The number of oocysts per gram of feces (opg) was calculated based on the Cq values of the amplification obtained from the analyzed sample against a standard curve generated from qPCR analysis of fecal samples spiked with known numbers of oocysts of the *C. parvum* IOWA isolate (Waterborne, Inc., New Orleans, USA).

### Sequence analysis

All positive PCR products of the SSU rRNA and *gp60* genes were sequenced bi-directionally on an ABI 3730 Autosequencer (Applied Biosystems, Foster City, CA, USA) to identify the *Cryptosporidium* species and *C. parvum* subtypes presented, respectively. The nucleotide sequences generated were assembled using ChromasPro 2.1.5.0 (http://technelysium.com.au/ChromasPro.html), edited using BioEdit 7.1.3.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

### Table 1 Distribution of *Cryptosporidium* species/genotypes and *Cryptosporidium parvum* subtypes in bamboo rats on farms in Jiangxi, Hainan and Guangxi provinces, China

| Location | Farm | Animal age | n  | No. positive (%) | Cryptosporidium spp. |
|----------|------|------------|----|------------------|----------------------|
|          |      |            |    |                  | *C. ubiquitum*-like | *C. parvum*-like | *C. occultus* | *C. parvum* | *C. parvum* subtype |
| Guangxi  | 1    | 0–2 months | 9  | 7 (77.8)         | 4                    | 3                  | –            | –            | –                      |
|          |      | 7–9 months | 6  | 2 (33.3)         | 2                    | –                  | –            | –            | –                      |
|          |      | 1–3 years  | 88 | 16 (18.2)        | 5                    | 4                  | –            | 7            | *IloA15G1* (n = 6)     |
|          |      | Subtotal   | 103| 25 (24.3)        | 11                   | 7                  | –            | 7            | *IloA15G1* (n = 6)     |
|          | 2    | 0–2 months | 11 | 2 (18.2)         | 1                    | –                  | –            | 1            | *IloA6* (n = 1)        |
|          |      | 1–3 years  | 43 | 1 (2.3)          | –                    | –                  | –            | 1            | –                      |
|          |      | Subtotal   | 54 | 3 (5.6)          | 1                    | –                  | –            | 2            | *IloA6* (n = 1)        |
|          | 3    | 0–2 months | 4  | 1 (25.0)         | –                    | –                  | –            | 1            | *IloA6* (n = 1)        |
|          |      | 1–3 years  | 24 | 2 (8.3)          | 2                    | –                  | –            | –            | –                      |
|          |      | Subtotal   | 28 | 3 (10.7)         | 2                    | –                  | –            | 1            | *IloA6* (n = 1)        |
|          | 4    | 0–2 months | 50 | 34 (68.0)        | 2                    | 21                 | –            | 11           | *IloA9* (n = 7)        |
|          |      | 3–4 months | 25 | 12 (48.0)        | 10                   | –                  | –            | 2            | *IloA9* (n = 1)        |
|          |      | 4–6 months | 28 | 11 (39.3)        | 8                    | 3                  | –            | –            | –                      |
|          |      | 7–9 months | 16 | 5 (31.3)         | 2                    | 2                  | –            | 1            | –                      |
|          |      | 1–3 years  | 123| 6 (4.9)          | 4                    | 1                  | –            | 1            | *IloA9* (n = 1)        |
|          |      | Subtotal   | 244| 68 (27.9)        | 26                   | 28                 | –            | 14           | *IloA9* (n = 9)        |
|          | 5    | 1–3 years  | 30 | 2 (6.7)          | 1                    | –                  | –            | 1            | –                      |
|          |      | Subtotal   | 30 | 2 (6.7)          | 1                    | –                  | –            | 1            | –                      |
|          | 6    | 1–3 years  | 18 | 2 (11.1)         | –                    | 1                  | –            | 1            | –                      |
|          |      | Subtotal   | 18 | 2 (11.1)         | –                    | 1                  | –            | 1            | –                      |
| Jiangxi  | 7    | 0–2 months | 18 | 15 (83.3)        | 9                    | –                  | –            | 6            | *IloA9* (n = 3), *IloA6* (n = 3) |
|          |      | 3–4 months | 19 | 10 (52.6)        | 8                    | –                  | 1            | 1            | *IloA9* (n = 1)        |
|          |      | 4–6 months | 21 | 7 (33.3)         | 6                    | –                  | –            | 1            | *IloA9* (n = 1)        |
|          |      | 7–9 months | 13 | 3 (23.1)         | 3                    | –                  | –            | –            | –                      |
|          |      | 1–3 years  | 82 | 16 (19.5)        | 12                   | –                  | –            | 4            | *IloA9* (n = 4)        |
|          |      | Subtotal   | 153| 51 (33.3)        | 38                   | –                  | 1            | 12           | *IloA9* (n = 9), *IloA6* (n = 3) |
| Hainan   | 8    | 0–2 months | 17 | 13 (76.5)        | 5                    | 4                  | –            | 4            | *IloA6* (n = 1), *IloA15G1* (n = 2) |
|          |      | 3–4 months | 6  | 5 (83.3)         | –                    | 4                  | –            | 1            | *IloA15G1* (n = 1)     |
|          |      | 4–6 months | 3  | 2 (66.7)         | –                    | 1                  | –            | 1            | –                      |
|          |      | Subtotal   | 26 | 20 (76.9)        | 5                    | 9                  | –            | 6            | *IloA6* (n = 1), *IloA15G1* (n = 3) |
|          | 9    | 0–2 months | 41 | 34 (82.9)        | –                    | –                  | –            | 34           | *IloA9* (n = 25)       |
|          |      | Unknown    | 12 | 1 (8.3)          | 1                    | –                  | –            | –            | –                      |
|          |      | Subtotal   | 53 | 35 (66.0)        | 1                    | –                  | –            | 34           | *IloA9* (n = 25)       |
| Total    | –    |            | 709| 209 (29.4)       | 85                   | 45                 | 1            | 78           | *IloA15G1* (n = 9), *IloA9* (n = 43), *IloA6* (n = 6) |
Table 2 Occurrence of Cryptosporidium species/genotypes in farmed bamboo rats in Guangxi, Jiangxi and Hainan provinces, China, broken down by age

| Age       | n   | No. positive (%) | Cryptosporidium spp. | C. parvum subtype |
|-----------|-----|------------------|----------------------|-------------------|
|           |     |                  | C. ubiquitum-like    | C. parvum-like    | C. occultus | C. parvum |
| 0–2 months| 150 | 105 (70.0)       | 21                    | 28                | 0          | 56         | IIpA9 (n = 36), IIpA6 (n = 6) |
| 3–4 months| 50  | 28 (56.0)        | 18                    | 5                 | 1          | 4          | IIpA9 (n = 2), IIoA15G1 (n = 2) |
| 4–6 months| 53  | 20 (37.7)        | 14                    | 4                 | 0          | 2          | IIpA9 (n = 1), IIoA15G1 (n = 1) |
| 7–9 months| 36  | 10 (27.8)        | 7                     | 2                 | 0          | 1          | –          |
| 1–3 years | 408 | 45 (11.0)        | 24                    | 6                 | 0          | 15         | IIpA9 (n = 4), IIoA15G1 (n = 6) |
| Unknown   | 12  | 1 (8.3)          | 1                     | 0                 | 0          | 0          | –          |
| Total     | 709 | 209 (29.5)       | 85                    | 45                | 1          | 78         | IIpA9 (n = 43), IIpA6 (n = 6), IIoA15G1 (n = 8) |

Abbreviations: n, total number of samples; –, gp60 PCR negative

Statistical analysis
Cryptosporidium detection rates in bamboo rats were compared among age and reproduction groups using the Chi-square test implemented in SPSS v.20.0 (IBM Corp., New York, NY, USA). Differences were considered significant at P < 0.05.

Results
Cryptosporidium infection in bamboo rats
Of the 709 samples collected from bamboo rats on 9 farms, 209 (29.5%) were positive for Cryptosporidium spp. in PCR analysis of the SSU rRNA gene. The detection rates in bamboo rats ranged from 5.6% to 76.9% among the 9 farms (Table 1). Farms 8 and 9 in Hainan had significantly higher detection rates than other farms (χ² = 17.6, df = 1, P < 0.0001; χ² = 17.3, df = 1, P < 0.0001, respectively). Among the 6 farms in Guangxi, Farms 1 and 4 had slightly higher detection rates than other farms (χ² = 0.866, df = 1, P = 0.22; χ² = 1.62, df = 1, P = 0.121, respectively). Regarding rat age, the highest detection rate was 70.0% in the 0–2 month-old group, which was significantly higher than in older animals overall (18.6%; χ² = 165.2, df = 1, P < 0.0001), especially in 1–3 year-old animals (11.0%; χ² = 194.1, P < 0.0001; Table 2).

Cryptosporidium species/genotypes
All 209 Cryptosporidium-positive PCR products of the SSU rRNA gene were successfully sequenced. The results showed the presence of C. parvum (n = 78), Cryptosporidium occultus (n = 1), and two new Cryptosporidium genotypes. Of the latter, one was genetically related to C. ubiquitum (n = 85), while the other was related to C. parvum (n = 44). The nucleotide sequences generated from C. parvum were identical to each other and a nucleotide sequence (GenBank: KC885892) also obtained from bamboo rats [6]. The latter had one A to T substitution from the SSU rRNA sequences of C. parvum commonly found in humans, cattle and other animals (Fig. 1). Similarly, the nucleotide sequence from C. occultus had two nucleotide substitutions compared with the GenBank sequence MH807493 obtained from humans. The C. ubiquitum-like genotype had 17 nucleotide substitutions compared with the partial SSU rRNA gene sequence obtained previously from C. ubiquitum (GenBank: KY596691) in Chinchilla lanigera [21], while the C. parvum-like genotype had 11 nucleotide differences from a partial SSU rRNA gene sequence of C. parvum reported from dairy cattle (GenBank: MF074700) [22]. As expected, in the phylogenetic analysis of the SSU rRNA nucleotide sequences, the C. parvum-like genotype clustered together with C. parvum, while the C. ubiquitum-like genotype clustered with C. ubiquitum (Fig. 2).

The Cryptosporidium species and genotypes identified in the present study produced different banding patterns in a RFLP analysis of the SSU rRNA PCR products using
the SpI and Vsi restriction enzymes. The RFLP profile of *C. occultus* was similar to that of *C. suis*. Similarly, the *C. ubiquitum*-like genotype produced a RFLP profile similar to *C. ubiquitum*. In contrast, the banding pattern for the *C. parvum*-like genotype was different from *C. parvum* due to the presence of a G to A substitution in the hypervariable region of the SSU rRNA gene, leading to the creation of an additional Vsi restriction site. This led to the cleavage of the upper Vsi band in *C. parvum* into two smaller fragments (Fig. 3).

### Distribution of *C. parvum*, *C. parvum*-like and *C. ubiquitum*-like subtypes

The 78 *C. parvum*, 45 *C. parvum*-like and 85 *C. ubiquitum*-like isolates were further subtyped by sequence analysis of the *gp60* gene. Among them, 59 of the *C. parvum*, 30 of the *C. parvum*-like and 44 the *C. ubiquitum*-like isolates were successfully subtyped. Three subtypes of two rare subtype families were identified for *C. parvum* samples: IlpA9 (*n* = 43); IlpA6 (*n* = 6); and IloA15G1 (*n* = 9). One subtype each was identified for *C. parvum*-like and *C. ubiquitum*-like genotypes (Fig. 2). The nucleotide sequences of IlpA9, IlpA6 and IloA15G1 were identical to the GenBank reference sequence KC885904 obtained from bamboo rats, KC885904 obtained from bamboo rats and JN867335 obtained from humans, respectively [6, 23]. The sequences from the *C. parvum*-like genotype were identical to each other and had a nucleotide identity of 87% to LC270810 obtained from camels [10]. Similarly, the sequences from the *C. ubiquitum*-like genotype had a nucleotide identity of 86% to KX698306 obtained from a water sample [24].

### Oocyst shedding intensity of *Cryptosporidium* spp.

The intensity of oocyst shedding in infected bamboo rats was assessed using 18S-LC2 qPCR. The numbers of oocysts per gram of feces were 27,610 ± 71,911 (*n* = 27), 38,679 ± 82,811 (*n* = 32), 2470 ± 7017 (*n* = 37) and 1012 (*n* = 1) for *C. parvum*, *C. parvum*-like genotype, *C. ubiquitum*-like genotype and *C. occultus*, respectively (Table 1).
Discussion

Results of this study suggest that Cryptosporidium spp. are common in bamboo rats in Jiangxi, Guangxi and Hainan provinces, China. The overall detection rate of 29.5% for Cryptosporidium spp. is much higher than the 3.3% (3/92) in the only other study of cryptosporidiosis in bamboo rats conducted from a pet market in Sichuan Province [6]. The intensive nature of animal farming could have contributed to the high prevalence of Cryptosporidium spp. in bamboo rats in the present study. As often seen with cryptosporidiosis in other farmed animals, the detection rate was significantly higher in bamboo rats under two months of age (70.0%) than those above 2 months (18.6%). Among the nine farms, Cryptosporidium detection rates were higher on the two farms in Hainan, probably because of the sampling of only young animals on these farms. Farms 1 and 4 are leading breeders of bamboo rats in Guangxi. The large size of the farm and frequent animal trade could be responsible for the higher Cryptosporidium detection rates (24.3% and 27.9%, respectively) than on the other 4 farms (5.6–11.1%). The higher detection rate of Cryptosporidium spp. in breeding animals (13.1%) than in other adults (0–6.7%) could also be attributed to co-housing of animals from different cages.

Altogether, four Cryptosporidium species or genotypes were found in bamboo rats in this study. Of these, C. parvum has been detected in three bamboo rats previously [6]. The detection of C. occultus in one bamboo rat was also expected, as this species is mostly parasitizing rats to which bamboo rats are genetically related [25]. In addition to the two known Cryptosporidium species, we showed a common occurrence of two new Cryptosporidium genotypes in the studied animals, one genetically related to C. parvum and the other related to C. ubiquitum. Bamboo rats could be natural hosts of these two new Cryptosporidium genotypes, as indicated by their high occurrence in these animals.

The C. parvum-like and C. ubiquitum-like found in the present study appear to be genetically unique. Although C. parvum, C. ubiquitum-like and C. parvum-like were common in bamboo rats, C. parvum and the C.
parvum-like genotype were mainly detected in animals under two months of age, while the C. ubiquitum-like genotype was found in all age groups. In addition, C. parvum and the C. parvum-like genotype had much greater oocyst shedding intensity than the C. ubiquitum-like genotype. This observation is similar to the occurrence of C. parvum and C. ubiquitum in ruminants [2]. Further studies are needed to understand the host range of the new C. parvum-like and C. ubiquitum-like genotypes.

Cryptosporidium parvum found in bamboo rats in this study belongs to several rare subtypes. This is the most important zoonotic species with a broad host range, including ruminants, equine animals, rodents and primates [26]. However, genetic diversity and host adaptation are known to be present in C. parvum, with over 20 subtype families being described by sequence analysis of the gp60 gene [3]. The Ilp subtype family detected in our study was previously reported from only in a few bamboo rats in China [6]. Similarly, the rare C. parvum Ilo subtype family was first found in diarrheal patients with a history of travel to Thailand [23] and subsequently found in bamboo rats and crab-eating macaques in China [6, 7].

The public health significance of Cryptosporidium spp. in bamboo rats is not entirely clear. As mentioned above, the Ilo subtype family of C. parvum found in the present study appears to be a minor human pathogen that has been found in only a few cryptosporidiosis cases [23]. However, it has recently been reported in 18 farmed crab-eating macaques in China [7]. Therefore, precaution should be taken to prevent the spread of this unique C. parvum subtype in farm animals. Similarly, although C. occultus has only been found in a few human cases [27], it appears to have a broad host range, including cattle, yak and Tanezumi rats [28–30]. As the new Cryptosporidium genotypes identified in this study are genetically related to C. parvum and C. ubiquitum, two well-known zoonotic Cryptosporidium species [3, 8], there is a need to examine their potential as causative agents of human infection.

Conclusions
Several Cryptosporidium species and genotypes, namely C. parvum, a C. parvum-like genotype, and a C. ubiquitum-like genotype, appear to be common in farmed bamboo rats in southern China. The C. parvum Ilp and Ilo subtype families may have initially originated from native rodents, but have recently expanded to humans and non-human primates in China and Southeast Asia. Attention should be paid to monitoring the dispersal of these emerging C. parvum subtypes in farm animals.
Abbreviations
PCR: polymerase chain reaction; qPCR: a quantitative PCR; gp60: 60 kDa glycoprotein gene; SSU rRNA: the small subunit rRNA; RFLP: restriction fragment length polymorphism.

Acknowledgements
We thank the farm owners for the assistance in sample collection from bamboo rats.

Authors’ contributions
YF and LX conceived and designed the experiments. FL and ZZ and SH performed the experiments. FL, ZZ, SH, WZ, JZ, MK, YG and NL analyzed the data. FL, YF and LX wrote the paper. All authors read and approved the final manuscript.

Funding
This study was supported by the National Natural Science Foundation of China (31820103014 and 31630078), and the 111 Project (D20008).

Availability of data and materials
Data supporting the conclusions of this article are included within the article.

Ethics approval and consent to participate
The study was approved by the Research Ethics Committee of the South China Agricultural University.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 3 August 2019 Accepted: 16 March 2020
Published online: 24 March 2020

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