Diversity of zoonotic enterohepatic *Helicobacter* species and detection of a putative novel gastric *Helicobacter* species in wild and wild-born captive chimpanzees and western lowland gorillas

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ABSTRACT

A number of *Helicobacter* species cause gastrointestinal or hepatic disease in humans, including *H. pylori*, gastric non-*H. pylori* helicobacters from animal origin and enterohepatic *Helicobacter* species. Little is known on the presence of *Helicobacter* species in great apes, our closest living relatives and potential reservoirs of microorganisms that might emerge in humans. The aim of the present study was to investigate the presence of gastric and enterohepatic *Helicobacter* species in African chimpanzees and gorillas. Fresh fecal samples were collected from wild endangered chimpanzees and critically endangered western lowland gorillas from different African National Parks, as well as wild-born captive animals from primate sanctuaries. Intact *Helicobacter* bacteria were demonstrated in feces by fluorescence *in situ* hybridization. Screening using a *Helicobacter* genus-specific PCR revealed the presence of *Helicobacter* DNA in the majority of animals in all groups. Cloning and sequencing of 16S rRNA gene fragments revealed a high homology to sequences from various zoonotic enterohepatic *Helicobacter* species, including *H. cinaedi*

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and *H. canadensis*. A number of gorillas and chimpanzees also tested positive using PCR assays designed to amplify part of the *ureAB* gene cluster and the *hsp60* gene of gastric helicobacters. Phylogenetic analysis revealed the presence of a putative novel zoonotic gastric *Helicobacter* taxon/species. For this species, we propose the name '*Candidatus Helicobacter homininae*', pending isolation and further genetic characterization. The presence of several *Helicobacter* species not only implies a possible health threat for these endangered great apes, but also a possible zoonotic transmission of gastric and enterohepatic helicobacters from these primate reservoirs to humans.

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1. Introduction

A number of *Helicobacter* species have been reported or suggested to cause gastrointestinal or hepatic disease in humans, including *H. pylori*, gastric non-*H. pylori* helicobacters from animal origin (comprising *H. suis*, *H. felis*, *H. bizzozeronii*, *H. salomonis* and *H. heilmannii*), *H. hepaticus*, *H. pullorum*, *H. cinaedi* and *H. fennelliae* (Kusters et al., 2006; Haesebrouck et al., 2009; Karagin et al., 2010; Hansen et al., 2011). Apart from humans, members of the genus *Helicobacter* have also been detected in a wide variety of other animal species, both domesticated and non-domesticated (Fox et al., 2007; Haesebrouck et al., 2009; Schrenzel et al., 2010; Lertpiriyapong et al., 2014). These include various non-human primates, such as rhesus macaques, mandrills and crab-eating macaques (O'Rourke et al., 2004; Schrenzel et al., 2010). However, little is known on the presence of *Helicobacter* species, both gastric and enterohepatic, in great apes. One report has described the presence of *Helicobacter* DNA in feces of a limited number of western gorillas, bonobos and orangutans in zoological gardens (Schrenzel et al., 2010). In another study, attempts were made to amplify a 1200 bp *Helicobacter* 16S rRNA gene fragment from gastric biopsies from a number of captive wild-born chimpanzees, however without success (Moodley et al., 2012). Therefore, no evidence of gastric colonization by *Helicobacter* bacteria nor a possible co-evolution of *Helicobacter* species in our closest relatives has been demonstrated so far.

According to the IUCN list Red List of Threatened Species, chimpanzees (*Pan troglodytes*) and western lowland gorillas (*Gorilla gorilla gorilla*) are classified as endangered and critically endangered, respectively. Disease transmission from humans to these endangered species is of great concern, because human infectious pathogens have the potential to be transmitted to primates, in particular those animals habituated to human presence (Ferber, 2000). Great apes have been habituated to human presence for the purposes of ecotourism and research; during the habituation process animals become accustomed to human presence and are thought eventually to accept a human observer as a neutral element in their environment.

On the other hand, great apes may serve as a reservoir for the transmission of a wide range of pathogenic microorganisms to humans, especially since close relationships with humans have been established, for instance in primate sanctuaries or during the habituation process of great apes. Identification of such infectious agents in these

animals is an important step for predicting and preventing emerging infectious diseases in humans (Leveck et al., 2010; Menezes-Costa et al., 2013; Sak et al., 2013).

The aim of the present study was to investigate the presence of gastric and enterohepatic *Helicobacter* species in wild western lowland gorillas and chimpanzees as well as wild-born animals housed in African sanctuaries.

2. Methods

2.1. Animals and sampling

Fresh fecal samples from wild great apes were collected during follows of the animals or from/under their night nests. For animals in captivity within sanctuaries, fecal samples were collected by staff during routine cleaning. All fecal samples were immediately preserved in 96% ethanol for transport and until further processing. Different groups of animals were studied: (1) unhabituated ($n = 10$) as well as human-habituated ($n = 10$) western lowland gorillas (*G. gorilla gorilla*) from the Dzanga-Ndoki National Park, Central African Republic; (2) unhabituated western chimpanzees (*P. troglodytes verus*) from Cantanhez National Park, Guinea Bissau ($n = 19$) as well as eastern chimpanzees habituated to human presence (*P. troglodytes schweinfurthii*) from Kalinzu Forest Reserve in Uganda ($n = 20$); (3) wild-born captive chimpanzees ($n = 14$) and gorillas ($n = 15$) from Limbe Wildlife Centre, Cameroon as well as wild-born captive chimpanzees ($n = 15$) from the Sweetwaters Chimpanzee sanctuary, Kenya. Animals from sanctuaries were in daily contact with their keepers. Habituated apes were followed on a daily basis by researchers, local trackers and assistants, and observers aim to maintain a minimum distance of 7 m or more. Unhabituated groups can be accidentally encountered by the habituation or research team, although there were no systematic attempts to locate them. All fecal samples were collected noninvasively and did not cause any observable distress to the animals.

2.2. DNA extraction, PCR assays, cloning and sequencing

Prior to DNA extraction, evaporation of ethanol and homogenization of samples were performed as described by Sak et al. (2013). Subsequently, DNA extraction from fecal samples was performed using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) or the PSP Spin Stool DNA Kit (STRATEC Molecular GmbH, Berlin, Germany) and DNA extracts were eluted and frozen at -20°C in elution

buffers provided by the manufacturer. Isolated DNA was used for several PCR assays, of which amplification conditions can be found in Table 1.

Samples were screened for the presence of *Helicobacter* DNA using a previously described *Helicobacter* genus PCR, amplifying ~389 bp of the 16S rRNA gene (Choi et al., 2001). PCR products from all *Helicobacter*-positive samples were cloned using a TOPO® TA Cloning® Kit with pCR® 2.1-TOPO® Vector and TOP10 *E. coli*, according to the manufacturer's guidelines (Life Technologies, Carlsbad, CA, USA). For each successful cloning reaction, 5–10 clones were analyzed using universal M13 primers. Amplicons of the expected size were purified using a MSB Spin PCRapace kit (STRATEC Molecular GmbH). Purified PCR products were sequenced using the BigDye Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA) and sequences were determined on an automatic DNA sequencer (ABI Prism 3100 Genetic analyzer, Applied Biosystems). The electropherograms were exported to Kodon software (Applied Maths, Sint-Martens-Latem, Belgium), which was used for curating DNA sequences, including the removal of primer sequences.

For a number of samples from chimpanzees, a PCR was performed targeting part of the *hsp60* gene (~179 bp, depending on the species involved), in an attempt to confirm the putative detection of *H. cinaedi/fennelliae*, which were by far the most prevalent species in all groups when analyzing 16S rRNA gene sequences in this study. The obtained PCR fragments were purified and sequenced as described above.

In order to detect DNA from gastric *Helicobacter* spp., all samples were subjected to a PCR assay designed to amplify part of the *urease* A and B genes of gastric helicobacters (~250 bp, depending on the *Helicobacter* species involved). Samples positive for the presence of gastric *Helicobacter* DNA were also subjected to a PCR assay designed to amplify part (~198 bp) of the *hsp60* gene from gastric helicobacters. These primer pairs were designed based on multiple alignments of known *ureAB* and *hsp60* gene sequences from gastric helicobacters. The obtained PCR fragments were purified and sequenced as described above.

Table 1
PCR reactions performed in this study to identify *Helicobacter* spp.

Target gene (amplicon size in bp)	Primers	Variable parameters used			Reference
		MgCl ₂ conc. (mM)	Annealing temp. (°C)	No. of cycles	
16S rRNA (389 bp)	Hcom1: 5'-GTAAAGGCTCACCAAGGCTAT-3' Hcom2: 5'-CCACCTACCTCTCCCACTC-3'	1.5	63	35	Choi et al., 2001
<i>ureAB</i> (~250 bp)	NHPHkortF: 5'-CDGTRMGNTTTGARCNGG-3' NHPHkortR: 5'-GTDGTDGGDCRTACATWGA-3'	2.5	57	40	This study
<i>hsp60</i> gastric <i>Helicobacter</i> spp. (~198 bp)	hspGF: 5'-BACYATYTCDGCHAAATCYGATSA-3' hspGR: 5'-KGRGYGTCATTTTBTCTNGCRIT-3'	3.0	54	40	This study
<i>hsp60</i> gastric/ enterohepatic <i>Helicobacter</i> spp. (~179 bp)	Apehsp60F: 5'-GARGCWAARGGNATYRAYGA-3' Apehsp60R: 5'-CRCYYTKCATDGTNGMYTC-3'	3.0	52	40	This study
<i>ureAB</i> of <i>H. pylori</i> (217 bp)	BFHpyl_F1: 5'-AAAGAGCGTGGTTTTCATGGCG-3' BFHpyl_R1: 5'-GGGTTTACCGCCACCGAATTAA-3'	2.5	59	40	This study

For animals harbouring 16S rRNA clones with a high homology to the 16S rRNA from *H. pylori* (see Supplementary Table 1), a *H. pylori*-specific PCR was performed as well (Table 1).

2.3. Phylogenetic analysis of gastric *Helicobacter* species

For phylogenetic analysis, the intergenic region between *ureA* and *ureB* and the stop codon of *ureA* were removed, followed by concatenation of the two gene fragments. Alignments of *hsp60* and concatenated *ureAB* genes were first generated at the amino acid level using MAFFT-FFT-NS-i version 7 (Katoh and Standley, 2013) and then back-translated to nucleotide sequences using the TranslatorX perl script (Abascal et al., 2010) (Figs. S1 and S2). Distance matrices were generated using SplitsTree 4, applying the Kimura-2 parameter model, and phylogenetic networks were calculated using the Neighbor-Net algorithm (Bryant and Moulton, 2004). The phylogenetic trees were reconstructed using Bayesian phylogenetic inference. Two independent analyses of four MCMC chains run for 10 million generations with a tree sample each 5000 generations were conducted for each gene using MrBayes v 3.2.1 (Ronquist et al., 2012). The substitution model space was sampled during Bayesian MCMC to avoid a priori model testing. The number of discrete categories used to approximate the gamma distribution was set to 6.

2.4. Fluorescent *in situ* hybridization

The following probes were used for fluorescent *in situ* hybridization (FISH): EUB338-TEX615 (eubacterial 16S rRNA probe; 5'-GCTGCCTCCGTAGGAGT-3'; fluorescence emission maximum at 615 nm) (Amann et al., 1992), HRhShort-6FAM (*Helicobacter* genus-specific probe based on 16S rRNA gene sequences of known *Helicobacter* species; 5'-TCTCAGCGCCGATACCGTCATAG-3'; fluorescence emission maximum of 6-FAM: 521 nm) (this study), HEL717-6FAM (*Helicobacter* genus-specific probe based on 16S rRNA gene sequences of known *Helicobacter* species; 5'-AGGTCGCCTTCGCAATGAGTA-3') (Chan et al., 2005). As

mentioned above, fecal samples were stored in 96% ethanol immediately after collection. An aliquot was resuspended in 4% paraformaldehyde and incubated for 1 h at room temperature. After centrifugation (15 min, 2500 × g), the pellet was washed and resuspended in Hank's balanced salt solution (HBSS; Life Technologies). Ten microliter of this sample was applied onto a poly-L-lysine coated slide and dried for 5 min at 95 °C. Subsequently, slides were dehydrated in a series of ethanol (50, 80, 96%) and left to dry. Samples were incubated in hybridization buffer (100 mM Tris–HCl pH 7.2; 0.9 M NaCl; 0.1% SDS) containing 200 µg/mL of the HEL717-6FAM probe and 1000 µg/mL of the HRhShort-6FAM and EUB338-TEX615 probes in a dark, sealed moist chamber at 44 °C overnight. Slides were washed 3 times for 15 min in prewarmed (44 °C) hybridization buffer and incubated 2 times for 15 min in prewarmed (44 °C) washing solution (100 mM Tris–HCl pH 7.2; 0.9 M NaCl). Finally, slides were rinsed with HPLC-grade water, air dried in the dark, mounted with VECTASHIELD HardSet Mounting Medium (Vector labs, Burlingame, USA) and analyzed using a Leica DMRB fluorescence microscope.

3. Results

3.1. Detection of *Helicobacter* DNA and bacteria in fecal samples from gorillas and chimpanzees

Helicobacter DNA was detected in the majority of samples from animals in all groups tested. Subsequent cloning and sequencing revealed nucleotide sequences (European Nucleotide Archive accession numbers HG737359–HG737545) with a high homology to known 16S rRNA gene sequences from various, mostly enterohepatic *Helicobacter* species.

When comparing nucleotide sequences from different clones present in one sample and showing the highest similarity to the same *Helicobacter* species, minor differences could nevertheless be observed. An overview of the number of positive animals in each group, as well as the putative species, is shown in Table 2. A substantial number of animals harboured 16S rRNA gene fragments from several *Helicobacter* spp., suggesting multiple species can colonize the same animal. Detailed information for each animal can be found in supplementary Table 1.

FISH was performed on fecal samples from a number of *Helicobacter*-positive gorillas and chimpanzees from Limbe Wildlife Centre, revealing the presence of intact *Helicobacter* bacteria (Fig. 1).

3.2. Identification of a putative novel gastric *Helicobacter* species in great apes

A total of 14 samples also revealed a PCR amplicon of the expected size using the primer set amplifying part of the *urease* A and B genes of gastric helicobacters (1 habituated and 2 unhabituated gorillas from the Central African Republic, 1 habituated chimpanzee from Uganda as well as 7 unhabituated chimpanzees from Guinea-Bissau and 3 chimpanzees from African sanctuaries). Successful sequence analysis of PCR products from 7 animals (2 gorillas from the Central African Republic, 1 chimpanzee from Uganda as well as 3 chimpanzees from Guinea Bissau and 1 chimpanzee from the Kenyan sanctuary) revealed a low percentage of query coverage or identity with *urease* gene sequences of known valid gastric *Helicobacter* species (European Nucleotide Archive accession numbers HG737546–HG737552). However, all obtained sequences shared 97–98% identity with “*Helicobacter heilmannii*”-like

Table 2

Results of the 16S rRNA-based *Helicobacter* genus-specific PCR, cloning and sequencing performed on DNA prepared of fresh fecal samples.

Animal group	Origin	Animals positive for <i>Helicobacter</i> spp./total no. of animals	Putative species detected* (maximum identity; no. of positive animals (n); number of clones/total number of <i>Helicobacter</i> clones in this group)
Unhabituated gorillas	CAR [#]	10/10	<i>H. mesocricetorum</i> –“ <i>H. apodemus</i> ” (100%; n = 4; 8/29)/ <i>H. trogonum</i> (99–100%; n = 3; 6/29)/ <i>H. ganmani</i> (99–100%; n = 3; 5/29)/ <i>H. typhlonius</i> (99–100%; n = 2; 2/29)/ <i>H. cinaedi-fennelliae</i> (99%; n = 2; 4/29)/ <i>H. pylori-macacae</i> (99–100%; n = 2; 2/29)/ <i>H. rodentium</i> (96%; n = 1; 1/29)/ <i>H. pullorum-equorum-canadensis</i> (99%; n = 1; 1/29) ⁺
Habituated gorillas	CAR	8/10	<i>H. cinaedi-fennelliae</i> (99%; n = 2; 7/19)/ <i>H. pylori-macacae</i> /Wolinella sp. canine oral taxon 173 (100%; n = 2; 2/19)/ <i>H. rodentium</i> (97–100%; n = 2; 4/19)/ <i>H. pylori-cetorum-bizzozeronii</i> (100%; n = 1; 1/19)/ <i>Helicobacter</i> sp. MIT 01-3238 (99%; n = 1; 2/19)/ <i>H. rappini</i> (99%; n = 1; 3/19) ⁺
Gorilla in sanctuary	Cameroon	5/15	<i>H. cinaedi-fennelliae</i> (99%; n = 1; 14/19)/ <i>H. rodentium</i> (99–100%; n = 2; 5/19) ⁺
Unhabituated chimpanzees	Guinea-Bissau	15/19	<i>H. cinaedi-fennelliae</i> (98–99%; n = 8; 25/33)/ <i>H. pylori</i> (100%; n = 3; 3/33)/ <i>H. typhlonius</i> (99–100%; n = 2; 3/33)/ <i>H. suis</i> (98%; n = 1; 1/33)/ <i>H. trogonum</i> (99%; n = 1; 1/33) ⁺
Habituated chimpanzees	Uganda	14/20	<i>H. cinaedi-fennelliae</i> (96–99%; n = 10; 28/33)/ <i>H. pylori-macacae</i> (95–100%; n = 2; 2/33)/ <i>H. bizzozeronii-felis</i> (99%; n = 1; 1/33)/ <i>H. ganmani</i> (99%; n = 1; 1/33)/ <i>H. mastomyrinus</i> (99%; n = 1; 1/33) ⁺
Chimpanzee in sanctuary	Cameroon	7/14	<i>H. cinaedi-fennelliae</i> (99%; n = 3; 11/16)/ <i>H. pylori</i> (96–99%; n = 2; 2/16)/ <i>H. typhlonius</i> (99%; n = 1; 2/16)/ <i>H. rodentium</i> (100%; n = 1; 1/16) ⁺
Chimpanzee in sanctuary	Kenya	14/15	<i>H. cinaedi-fennelliae</i> (92–99%; n = 4; 17/39)/ <i>H. typhlonius</i> (100%; n = 2; 4/39)/ <i>H. apodemus-mesocricetorum</i> (100%; n = 2; 3/39)/ <i>H. ganmani</i> (99–100%; n = 2; 5/39)/ <i>H. canadensis</i> (100%; n = 1; 7/39)/ <i>H. pylori-macacae</i> (100%; n = 1; 1/39)/ <i>H. mastomyrinus</i> (99%; n = 1; 1/39)/ <i>H. cinaedi-canis-pullorum</i> (99%; n = 1; 1/39) ⁺

* Results of putative species detected and maximum identity were obtained by searching NCBI nucleotide databases using the BLASTN algorithm.

[#] Central African Republic.

Fig. 1. Fluorescent in situ hybridization performed on a fecal sample from a Chimpanzee, Cameroon. (A) Shown are curved, rod-shaped bacteria hybridizing with green fluorescent *Helicobacter* genus-specific probes HRhShort-6FAM and HEL717-6FAM. (B) Shown are all bacteria hybridizing with the red fluorescent eubacterial 16S rRNA probe EUB338-TEX615.

organism strain SH6, isolated *in vivo* by mouse passing homogenized gastric tissue from a 70-year-old Japanese male human patient (Matsumoto et al., 2009).

In addition, samples positive in the *ureAB*-based PCR were subjected to another PCR, targeting part of the *hsp60* gene of gastric *Helicobacter* species (primer set hspGF/hspGR, for details: see Table 1). For 1 chimpanzee from Guinea Bissau and 1 chimpanzee from Uganda, a clear PCR product was obtained and nucleotide sequences (European Nucleotide Archive accession numbers

HG737553–HG737554) showed 97% and 91% identity, respectively, with *H. felis hsp60*.

We further investigated the phylogenetic relationship between the *ureAB* and *hsp60* of these ape-associated gastric helicobacters and those of formerly described species. To determine whether the data sets support conflicting phylogenies or a single tree, Neighbor-Net networks were generated for pairwise sequence distances between *Helicobacter* spp. *ureAB* or *hsp60* genes (Fig. 2A and B; amplification using primer sets NHPHkortF/NHPHkortR and

Fig. 2. Phylogenetic networks of *Helicobacter* species based on partial *ureAB* (A) and *hsp60* (B) genes. Amplification was performed using primer sets NHPHkortF/NHPHkortR and hspGF/hspGR, respectively. The networks were built with SplitsTree4 (version 4.11.3) by the Neighbor-Net method. The clones from wild great apes, as well as strain SH6 are written as white letters on a black background. The *ureAB* network clearly indicates clustering of clones from wild great apes with “*Helicobacter heilmannii*”-like organism strain SH6, isolated from a Japanese human patient, suggesting these bacteria constitute a new *Helicobacter* taxon/species. HabGorCAR: clone from a habituated gorilla, Central African Republic; HabChimpUg: clone from a habituated chimpanzee, Uganda; UnhabChimpGuinea: clone from an unhabituated chimpanzee, Guinea-Bissau.

Fig. 3. Bayesian tree based on partial *Helicobacter ureA* and *ureB* genes. Shown is a 50% majority rule consensus tree among 3002 pooled trees from two independent Bayesian MCMC runs. The average standard deviation of split frequencies was 0.006893 and average Potential Scale Reduction Factor (PSRF) for parameter values was 1.000. Credible sets of trees included 268 trees; the 50% credible set contained 5 trees; the 90% credible set contained 73 trees; the 95% credible set contained 130 trees; the 99% credible set contained 238 trees. The numerals at internal nodes or branches indicate Bayesian posterior probabilities (shown as percentage for values above 50%). The clones from wild great apes as well as strain SH6 show a monophyletic relationship and are written as white letters on a black background.

hspGF/hspGR, respectively). The analysis showed conflicting phylogenetic signals, represented as parallel paths in the network, for both datasets. In particular, the nest-like structure of the split graph generated for *hsp60* hampered a correct reconstruction of the phylogeny of gastric *Helicobacter* species for this gene (data not shown). However, despite conflicting signals, partial *ureAB* gene sequences exhibit a significant “tree-like” structure and the clones obtained from great apes and “*Helicobacter heilmannii*”-like organism strain SH6 from a human patient form a distinct cluster, separated from the other species. A monophyletic relationship among the helicobacters from great apes and the human strain SH6 was confirmed by Bayesian phylogeny of *ureAB* (Fig. 3; 98.8% of posterior probability). In addition, the Bayesian analysis positioned the great apes clade in the larger monophyletic clade including all the different *H. heilmannii* s.l. species (Haesebrouck et al., 2011) with a posterior probability of approximately 78%.

For 1 chimpanzee from Guinea-Bissau (identification number T2484/10), an amplicon of the expected size was obtained using primers targeting part of the *ureAB* gene cluster from *H. pylori*. Subsequent nucleotide sequencing of this 217 bp fragment revealed an identity of 100% to known *H. pylori ureAB* gene sequences.

4. Discussion

Besides humans, very little information is available on the prevalence of *Helicobacter* spp. in other extant members of the Hominidae family. One report described the presence of *Helicobacter* 16S rRNA gene fragments,

with a high homology to the 16S rRNA gene from *H. cinaedi*, in fecal samples of 4 western gorillas (*Gorilla gorilla*), 1 bonobo (*Pan paniscus*), 1 Bornean orangutan (*Pongo pygmaeus*) and 4 Sumatran orangutans (*Pongo abelli*), all kept in captivity in zoos (Schrenzel et al., 2010). Most great ape species are endangered or even critically endangered, which demonstrates the need for further investigation into the presence of potential pathogens in these animals, including *Helicobacter* spp. The present study aimed at evaluating the presence of gastric and enterohepatic *Helicobacter* species in the gastrointestinal tract of wild (both unhabituated and habituated) western lowland gorillas and chimpanzees as well as in wild-born animals housed in African sanctuaries, by detecting and analyzing *Helicobacter* DNA, as well as intact bacteria, in fecal samples of these animals.

Helicobacter DNA was detected in the majority of fecal samples from wild animals and animals from African sanctuaries. Sequencing of the obtained clones revealed that most fragments showed a high homology to 16S rRNA gene sequences of several, mainly enterohepatic, zoonotic *Helicobacter* species. Fluorescence *in situ* hybridization performed on a number of positive fecal samples demonstrated the presence of intact bacteria belonging to the genus *Helicobacter*, confirming that these bacteria indeed colonize the gastrointestinal tract of great apes.

Both in chimpanzees and gorillas, the majority of clones shared 99% identity with *H. cinaedi/fennelliae*. *H. cinaedi* is known to colonize various animal species, including dogs, cats and hamsters, as well as humans, in which it is considered a strong candidate causative agent for

gastrointestinal disease (Kiehlbauch et al., 1995; Hansen et al., 2011). Moreover, *H. cinaedi* and other, closely related *Helicobacter* species such as *H. westmaedii*, have been described to cause bacteremia and concurrent systemic disease, especially in patients suffering from AIDS or other immunocompromising diseases (Tee et al., 1996; Trivett-Moore et al., 1997; Rimbara et al., 2013). Similar reports have been published for *H. fennelliae*-like organisms (Kemper et al., 1993). Other putative species detected in the present study include '*H. macacae*', previously isolated from rhesus monkeys with and without chronic idiopathic colitis (Fox et al., 2007), *H. pullorum* and *H. canadensis*, as well as several species mainly detected in rodents, such as *H. troglodytes* (only detected in unhabituated animals), *H. rodentium*, *H. ganmani*, *H. mesocricetorum*, *H. mastomyrinus* and *H. typhlonius*. In addition to *H. cinaedi* and *H. fennelliae*, several of these species (*H. rodentium*, *H. canadensis*, *H. pullorum*) have been associated with diarrhoea, colitis or chronic liver diseases in humans (Fox et al., 2000; Ceelen et al., 2005; Nakonieczna et al., 2010; Hansen et al., 2011). Although the exact pathogenic significance of these enterohepatic *Helicobacter* species remains to be investigated in great apes, it should be kept in mind that their transmission to these endangered species might be of concern (Ferber, 2000; Sak et al., 2013). Not only direct contact between humans and apes, but also the more frequent presence of humans in the vicinity of habituated apes or those under habituation may account for transmission of pathogens, for instance through contamination of the environment (Sak et al., 2013; Hasegawa et al., 2014).

The presence of these infections in chimpanzees and gorillas having contact with humans, for instance in primate sanctuaries or in groups of apes habituated to the presence of humans, indicates that *Helicobacter* species should be considered to be added to the list of infectious microorganisms transmittable to humans by great apes. Great apes should therefore be considered as a non-human reservoir of *Helicobacter* species for a potentially highly susceptible human population, since prevalences of HIV are high in Sub-Saharan countries, exceeding 5% in countries like Cameroon, Kenya and Uganda (World Health Organization, 2011).

However, caution is advisable with regards to *Helicobacter* species designation. First, the cloned and sequenced 16S rRNA fragments only cover about 350 bp. Attempts to amplify larger fragments using previously described and validated *Helicobacter* genus-specific PCR's (Moyaert et al., 2008) were in general unsuccessful. Degradation of DNA into smaller fragments is the most likely cause. Second, the use of 16S rRNA gene sequences does not always provide conclusive evidence for *Helicobacter* identification to the species level (Vandamme et al., 2000). Our attempt to confirm the putative detection of *H. cinaedi/fennelliae* by amplifying and sequencing part of the *hsp60* gene sequence (Mikkonen et al., 2004) for the sample from one chimpanzee from Guinea-Bissau and one from Kenya resulted in nucleotide sequences (European Nucleotide Archive accession numbers HG737555–HG737556) showing the highest homology to known *H. cinaedi* strains, although with an identity of only 87–89%. In addition, we observed the presence of slightly different sequences

apparently belonging to the same *Helicobacter* species in samples from the same animal, which suggests the presence of genetic variation in *Helicobacter* species colonizing individual animals. Future research, including bacterial isolation attempts, should elucidate the exact species and strains involved. Possibly, *H. cinaedi* strains colonizing great apes differ from those in other animal species, for instance due to coevolution with the host. Alternatively, these differences may reflect the presence of a hitherto unknown, new *Helicobacter* species.

Besides the superfluous presence of DNA from enterohepatic helicobacters, some 16S rRNA clones, both in chimpanzees and gorillas, showed a high homology to gastric helicobacters (including *H. pylori*, *H. bizzozeronii*, *H. felis*). This somewhat contrasts to what has been described by Moodley et al. (2012), who attempted to amplify a 1200 bp *Helicobacter* 16S rRNA gene fragment from gastric biopsies from a number of captive wild-born chimpanzees, however without success (Moodley et al., 2012). Interestingly, the use of *H. pylori ureAB*-specific primers in the present study confirmed the presence of *H. pylori* DNA in the gastrointestinal tract of at least 1 chimpanzee. This was an unhabituated animal from Guinea-Bissau, suggesting that the infection may have been contracted in the absence of close contact with humans. The fact that all other animals were negative when using this primer set, together with the absence of *H. pylori* in the animals investigated by Moodley et al., indicates that *H. pylori* infection in great apes is not common. Whether *H. pylori* truly colonizes the stomach of some great apes remains to be investigated, but nevertheless, it has been shown to be able to persistently colonize the stomach of chimpanzees after experimental infection (Hazell et al., 1992).

In order to identify gastric helicobacters to the species level, the analysis of (partial) *ureAB* and *hsp60* gene sequences has been shown to be far more informative compared to 16 rRNA sequence analysis (O'Rourke et al., 2004; Mikkonen et al., 2004). In our study, this indeed revealed the presence of gastric *Helicobacter* DNA. Interestingly, partial *ureAB* gene sequences from animals in all groups (unhabituated and habituated gorillas and chimpanzees from different geographical locations) were very similar. They showed a relatively high homology to only one so-called "*Helicobacter heilmannii*"-like organism, namely strain SH6 isolated from a 70-year-old Japanese patient suffering from chronic gastritis (Matsumoto et al., 2009). Phylogenetic analysis, especially of the partial *ureA* and *ureB* genes, strongly suggests that these ape-associated helicobacters constitute a new taxon/species belonging to the *H. heilmannii* sensu lato group (Haesebrouck et al., 2011) and also that these helicobacters colonizing chimpanzees and gorillas probably share a recent common ancestor with other gastric non-*H. pylori Helicobacter* species. Members of this group of gastric non-*H. pylori Helicobacter* species are known to cause gastric disease in their natural hosts, including pigs and non-human primates (Mackie and O'Rourke, 2003; Haesebrouck et al., 2009; De Bruyne et al., 2012). Future isolation attempts, for instance from the stomach mucosa of deceased chimpanzees/gorillas, may provide additional data regarding the identity and diversity of gastric

Helicobacter taxa, although isolation of gastric non-*H. pylori* helicobacters in general is extremely difficult and time-consuming (Baele et al., 2008). Until further confirmation, we propose the name '*Candidatus Helicobacter homininae*', capable of colonizing great apes, as shown in the present study, as well as humans suffering from gastric disease, as shown for "*Helicobacter heilmannii*"-like organism strain SH6 (Matsumoto et al., 2009).

In conclusion, we demonstrated for the first time the presence of *Helicobacter* DNA as well as intact bacteria belonging to the *Helicobacter* genus in feces of two endangered primate species, i.e. chimpanzees and western lowland gorillas, regardless of geographical location or level of contact with humans. Several enterohepatic, as well as a putative new gastric *Helicobacter* species, were detected. Most species have previously been described to infect humans and to cause gastrointestinal or systemic disease, both in healthy and immunocompromised individuals. Therefore, future research should not only focus on the possible health threat for these endangered great apes, but also on a possible zoonotic transmission of gastric and enterohepatic helicobacters, which eventually may lead to new emerging diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetmic.2014.08.032>.

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