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Zoonotic and genetically diverse subtypes of *Blastocystis* in US pre-weaned dairy heifer calves

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Abstract

Blastocystis is an emerging zoonotic pathogen with global distribution. However, limited data exist on the prevalence and genetic diversity of *Blastocystis* in the USA and in food animals. We conducted the first large-scale molecular investigation of *Blastocystis* in the USA by testing 2539 fecal samples from dairy heifer calves from 13 states. *Blastocystis* was detected in 73 (2.9%) fecal samples and in 10 of the 13 tested states. Molecular characterization showed a wide diversity of subtypes. Eleven subtypes were identified, seven previously reported (ST-3, ST-4, ST-5, ST-10, ST-14, ST-17, and ST-21) and four potentially novel subtypes (named ST-23 to ST-26). Zoonotic subtypes 3, 4, and 5 were found in 67% (49) of the positive specimens in this population. Our results suggest that cattle could serve as reservoirs of infection for humans and other domestic animals highlighting the potential risk of zoonotic transmission for *Blastocystis*.

Keywords *Blastocystis* · Zoonotic · Subtypes · Cattle

Introduction

Blastocystis is a parasitic protist which causes intestinal infection in humans and many other animals (Clark et al. 2013). It is perhaps the most common human intestinal parasite in the world, with an estimated one billion infections globally (Andersen and Stensvold 2016). Recent studies have found *Blastocystis* infections associated with a variety of gastrointestinal symptoms including diarrhea, nausea, and abdominal pain (Ajjampur and Tan 2016). *Blastocystis* infection has also been linked to irritable bowel syndrome, chronic spontaneous urticaria (hives), and in rare instances, invasive infection (Janarthanan et al. 2011; Fréalle et al. 2015; Ajjampur and Tan 2016). However, *Blastocystis* pathogenicity remains a topic of some controversy, as asymptomatic carriage is common.

Infection occurs via the fecal-oral transmission route through the consumption of cysts in contaminated food or water (Tan 2008). *Blastocystis* has been reported in sewage and surface and irrigation water (Suresh et al. 2005; Lee et al. 2012b; Moreno et al. 2018; Koloren et al. 2018). Additionally, molecular evidence supporting waterborne transmission of a zoonotic *Blastocystis* subtype was reported in a study in Nepal (Lee et al. 2012a, 2012b). Because water quality and sanitation practices likely play a role in *Blastocystis* transmission, the World Health Organization has recently included this parasite on the list of potential pathogens of importance in drinking water quality (WHO 2011). *Blastocystis* has also been recently detected in ready-to-eat package salads in Italy (Caradonna et al. 2017).

Specimens of *Blastocystis* are morphologically indistinguishable by microscopy, yet *Blastocystis* displays remarkable genetic diversity. In fact, *Blastocystis* is currently divided into 22 subtypes (STs) based on polymorphism at the small subunit (SSU) rDNA gene (Alfellani et al. 2013b; Zhao et al. 2017). *Blastocystis* appears to have little host specificity and is considered a zoonotic pathogen (Noël et al. 2005). Ten of the 22 subtypes, ST-1 to ST-9 and ST-12, have been identified in human samples, and all but ST-9 have also been reported in animals (Ramírez et al. 2016; Stensvold and Clark 2016). ST-1 and ST-8 were found in primates and their caretakers in zoos in Australia and the UK (Parkar et al. 2007; Stensvold et al. 2009). ST-2 was found in both monkeys and local children in Nepal (Yoshikawa et al. 2009). ST-5 was found in pigs and their caretakers in China and Australia (Yan et al. 2007; Li et al. 2007; Wang et al. 2014).

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In the USA, data on *Blastocystis* is limited. In a 2002–2004 survey of human fecal samples from 48 states, *Blastocystis* was the most common parasite identified with 16% of samples being *Blastocystis*-positive solely by visual examination (Amin 2006). The few molecular studies conducted in humans in the USA have identified *Blastocystis* in 7 to 43% of the samples tested (Jones et al. 2009; Whipps et al. 2010; Scanlan et al. 2016; Nash et al. 2017). Yet, no large-scale human or animal studies have attempted to characterize *Blastocystis* prevalence and subtype diversity in the USA using molecular techniques. The limited data available on *Blastocystis* in food animals have demonstrated that cattle can harbor both zoonotic and enzootic subtypes of *Blastocystis* (Abe et al. 2003; Abe 2004; Stensvold et al. 2009; Santín et al. 2011; Fayer et al. 2012; Alfellani et al. 2013b; Ramírez et al. 2014; Badparva et al. 2015; Zhu et al. 2017; Wang et al. 2018). Therefore, the aims of this investigation were to estimate the sample-level prevalence of *Blastocystis* in dairy calves from across the USA and to molecularly identify *Blastocystis* subtypes to better understand the potential role of dairy heifer calves in human infection.

Methods

Specimens Fecal samples from 2539 pre-weaned (< 2 months of age) dairy heifer calves were collected as part of a nationwide study in collaboration with National Health Monitoring System (NAHMS) of the USDA's Animal and Plant Health Inspection Service (APHIS). Specimens collected for this study consisted of a convenience sample of dairy operations from the following 13 US states with number of farms per state shown in parentheses: California (12), Colorado (≤ 5), Iowa (6), Michigan (8), Minnesota (11), Missouri (≤ 5), New York (20), Ohio (≤ 5), Pennsylvania (9), Vermont (≤ 5), Virginia (≤ 5), Washington (10), and Wisconsin (29). States with five or fewer farms sampled are given as ≤ 5 to preserve the confidentiality of study participants. Individual specimens were collected directly from the rectum into a plastic cup, capped, labeled, and immediately placed in an insulated container packed with ice or cold packs. Specimens were shipped to the USDA, ARS (Beltsville, MD) and processed within 1–3 days of collection. All samples were collected between April 2014 and July 2015.

Detection and identification To concentrate parasites, each specimen was sieved and concentrated using a cesium chloride gradient (Santín et al. 2004). For DNA extraction, the DNeasy Tissue Kit (Qiagen, Valencia, CA) was used per instructions of the manufacturer with minor exceptions. Modifications included overnight incubation with proteinase K and elution in 100 μl of AE buffer to increase the quantity of recovered DNA. To detect *Blastocystis*, an approximately 500 base pair fragment of the *Blastocystis* SSU rDNA gene, which contains a variable region suitable for subtyping, was amplified by PCR (Santín et al. 2011). PCR products were analyzed using a QIAxcel (Qiagen, Valencia, CA). All positive PCR products were purified using Exonuclease I/Shrimp Alkaline Phosphatase (Exo-SAP-IT™, USB Corporation, Cleveland, OH), and sequenced in both directions using the same PCR primers in 10 μl reactions, Big Dye™ chemistries, and an ABI 3130 sequencer analyzer (Applied Biosystems, Foster City, CA). Sequence chromatograms of each strand were aligned and examined with Lasergene software (DNASTAR, Inc., Madison, WI), and nucleotide sequences were compared with sequences in GenBank database by BLAST analysis to identify *Blastocystis* subtypes.

Cloning When the sequence trace of a specimen had the appearance of a mixed infection, the SSU rDNA product was cloned using the TOPO TA cloning kit (Invitrogen Corp. Carlsbad, CA). Transformants were screened by PCR and sequenced in both direction using M13 forward and reverse primers per the sequencing protocol previously described. Up to 16 clones per specimen were sequenced.

Phylogenetic analysis All sequences were assigned a *Blastocystis* subtype based on the best match by BLAST search in the GenBank database using the consensus subtype terminology (Stensvold et al. 2007). Novel subtypes were assigned when 5% sequence divergence from the SSU-rDNA of known subtypes was observed (Clark et al. 2013). The nucleotide sequences obtained in this study have been deposited in GenBank under accession numbers MH634436-MH634478.

Sequences obtained in this study as well as nucleotide sequences from *Blastocystis* subtypes previously identified (Appendix Table 4) were aligned with the Clustal W algorithm using MEGA X (Kumar et al. 2018). Phylogenetic analyses were made by the Neighbor-Joining (NJ) method of Saitou and Nei (Saitou and Nei 1987), and genetic distance was calculated with the Kimura 2-parameter model using MEGA X (Kumar et al. 2018).

Results

Of the 2539 fecal samples tested in this study, 73 (2.9%) were positive for *Blastocystis* by PCR and sequencing. Positive samples from calves were present in 10 of the 13 states surveyed, and sample-level prevalence in those states with positive samples varied considerably (Table 1).

All PCR amplicons were sequenced to determine subtypes. Seventy-two of the 73 PCR amplicons were sequenced successfully. The PCR primers used in this study were selected because they have been shown to be highly sensitive compared to other published *Blastocystis* primers while also containing a highly variable region that allows for subtyping of PCR amplicons (Santín et al. 2011). Nucleotide sequence analysis revealed the

Table 1 Prevalence and subtypes of *Blastocystis* in pre-weaned dairy calves in the USA

State	No. of farms sampled*	No. of tested	No. of positive (%)	Subtype (no. observed)
California	12	241	3 (1.2)	ST-3 (1), ST-17 (1), ST-24 (1)
Colorado	≤ 5	208	1 (0.5)	ST-14 (1)
Iowa	6	64	0 (0.0)	None
Michigan	8	98	5 (5.1)	ST-5 (3), ST-14 (1), ST-10/ST-14/ST-26 (1)
Minnesota	11	205	4 (2.0)	ST-4 (4)
Missouri	≤ 5	23	0 (0.0)	None
New York	20	324	11 (3.4)	ST-3 (3), ST-5 (1), ST-10 (1), ST-14 (3), ST-25 (1), ST-10/ST-24 (1), ST-23/ST-26 (1)
Ohio	≤ 5	55	1 (1.8)	ST-14 (1)
Pennsylvania	9	119	7 (5.9)	ST-5 (6), ST-10 (1)
Vermont	≤ 5	58	15 (25.9)	ST-4 (14), not subtyped (1)
Virginia	≤ 5	16	0 (0.0)	None
Washington	10	462	14 (3.0)	ST-5 (12), ST-26 (2)
Wisconsin	29	666	12 (1.8)	ST-5 (5), ST-10 (1), ST-14 (1), ST-17 (2), ST-21 (2), ST-26 (1)
Total		2539	73 (2.9)	

*States with five or fewer farms sampled are given a value of ≤ 5 farms to preserve the confidentiality of study participants

presence of 11 distinct subtypes, seven previously reported subtypes (ST-3, ST-4, ST-5, ST-10, ST-14, ST-17, and ST-21) and four novel subtypes (named ST-23 to ST-26). Heterogeneity in nucleotide sequences was observed within subtypes for all subtypes for which more than one sequence was obtained. A phylogenetic analysis of all distinct nucleotide sequences obtained in this study was carried out together with reference sequences (Appendix Table 4) obtained from GenBank. The resulting phylogenetic tree supported the presence of 26 different clades that corresponded with subtypes ST-1 to ST-26 (Fig. 1).

Novel subtype nucleotide sequences obtained by direct sequencing and cloning were compared to the most similar nucleotide sequences available in the GenBank database. The three different nucleotide sequences identified as ST-23 displayed a similarity of 95.6 to 96.2% with ST-10 (JQ9963690); the three different nucleotide sequences identified as ST-24 displayed a similarity of 99.8 to 100% with sequences available in GenBank (HF569209 and HF569219); the only nucleotide sequence identified as ST-25 displayed a similarity of 99.5 to 100% with sequences available in GenBank (HF569213 and HF569221); and the eight nucleotide sequences identified as ST-26 displayed a similarity of 97.5 to 99.8% with sequences available in GenBank (HF569204 and HF569225). The GenBank sequences with similarity to ST-24, ST-25, and ST-26 were all from an unpublished study, and subtype designations were not provided.

Mixed infection was suspected in 15 animals based on nucleotide sequence traces and was confirmed by cloning in three. In the other 12 samples, only one subtype was identified in each of the samples by cloning. Mixed infections were observed in 4.1% (3/73) of the positive animals and identified in two states, Michigan and New York (Table 1). In mixed infections, subtypes ST-10, ST-14, ST-23, ST-24, and ST-26 were found in the following combinations, ST-23/ST-26, ST-10/ST-24, and ST-10/ST-14/ST-26. Among the STs observed in this study, the zoonotic subtypes ST-4 and ST-5 had the highest prevalence, 24.7% (18/73) and 37% (27/73) of positive samples, respectively (Table 2). ST-5 was more widely distributed than ST-4 and was detected in five states, while ST-4 was found in two states (Table 1). ST-14, identified in eight animals from five states, was the third most commonly identified subtype (Tables 1 and 2).

Discussion

Blastocystis is an emerging pathogen commonly detected in humans and animals. Its presence and distribution in the USA is largely unexplored. Most epidemiological studies of *Blastocystis,* in the USA and globally, have focused on small sample sets from geographically restrained regions. Here, we tested 2539 calf fecal samples from across the USA for *Blastocystis*. This study represents the first large-scale attempt to characterize the prevalence and subtype distribution of *Blastocystis* in the USA. The prevalence of *Blastocystis* in this study was 2.9%. In the only other study conducted in cattle in the USA, 19% of dairy cattle samples from a single operation were *Blastocystis*-positive (Fayer et al. 2012). However, all the samples in our study came from pre-weaned calves. In the other study, samples were tested from cattle ranging from less than 1 month to 7 years of age, and *Blastocystis* was not reported in any of the pre-weaned calves examined (Fayer et al. 2012). Similarly, a study in China found *Blastocystis* prevalence in dairy calves less than 3 months to be 5%, while prevalences of 15.4% and 15.2% were reported in calves 3–12 months and > 12 months, respectively (Zhu et al. 2017). Our findings support that age is likely a factor influencing

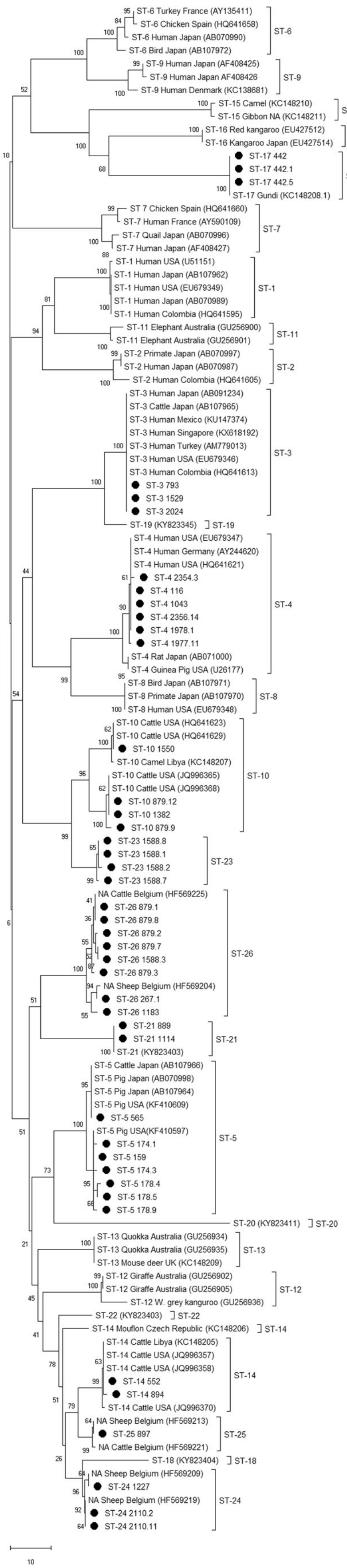

Fig. 1 Phylogenetic relationships among *Blastocystis* subtypes identified in this study and known *Blastocystis* subtypes, as inferred by a neighbor-joining analysis of the SSU rDNA gene sequence, based on genetic distances calculated by the Kimura two-parameter model (MEGA X software). Reference sequences have host and location information with the GenBank accession number in parenthesis. Nucleotide sequences determined in this study are identified with black filled circles before the subtype name. Bootstrap proportions (in percent) are attached to the internal branches (1000 replicates)

prevalence of this parasite with detection in pre-weaned calves being lower overall compared to older animals. This trend is also observed in humans where age is associated with infection and infants have the lowest infection rates (Pandey et al. 2015). Pre-weaned calves, like human infants, may have a lower risk for exposure due to the controlled manner in which their food is often provided (e.g., directly from a breast or bottle).

A wide genetic diversity was found for *Blastocystis* in calves with 11 distinct subtypes identified, seven previously reported subtypes (ST-3, ST-4, ST-5, ST-10, ST-14, ST-17, and ST-21) and four novel subtypes (named ST-23 to ST-26). Mixed infections with different subtypes of *Blastocystis* were detected in three calves, and nucleotide sequence heterogeneity within the same subtype was quite common. Genetic heterogeneity at the SSU rDNA gene has been reported before in *Blastocystis* and other protozoans such *Cryptosporidium* (Santín and Fayer 2007; Fayer et al. 2014). This genetic heterogenicity has been associated with the presence of multiple copies of the SSU rDNA gene in the parasites genome. Three subtypes considered zoonotic, ST-3, ST-4, and ST-5, were identified in 67% of the positive pre-weaned calves from eight of the ten states with positive calves. These findings are on par with the small-scale studies of humans in the USA, where ST-1, ST-2, ST-3, ST-4, and ST-8 were found (Table 3) (Jones et al. 2009; Whipps et al. 2010; Scanlan et al. 2016). ST-4 and ST-5 were the most commonly observed subtypes in this study (Table 2). A study from

Table 2 Percentage of *Blastocystis* subtypes infecting pre-weaned dairy calves in the USA

Subtype	No. of samples	Percentage of infected animals
ST-3	4	5.5
ST-4	18	24.7
ST-5	27	37
ST-10	5	6.8
ST-14	8	11
ST-17	3	4.1
ST-21	2	2.7
ST-23	1	1.4
ST-24	2	2.7
ST-25	1	1.4
ST-26	5	6.8
Not subtyped	1	1.4

Table 3 Molecular studies of *Blastocystis* in humans and animals in the USA

Host	Location	No. tested	Prevalence	Subtype (no. observed)	Reference
Humans	Oregon	21	43%	ST-1 (1), ST-3 (6), not subtyped (2)	(Jones et al. 2009)
Humans	Oregon	19	26%	ST-1 (1), ST-2 (1), ST-3 (1), ST-4 (1), ST-8 (1)	(Whipps et al. 2010)
Humans	Colorado	139	7%	ST-1 (2), ST-2 (3), ST-3 (5)	(Scanlan et al. 2016)
Humans	Not reported	44	25%	ST-1 (3), ST-2 (2), ST-3 (5), ST-1/ST-3 (1)	(Nash et al. 2017)
Cattle	Maryland	7	Not reported	ST-10 (7)	(Santín et al. 2011)
Swine	Maryland	6	Not reported	ST-5 (6)	(Santín et al. 2011)
Cattle	Maryland	47	19%	ST-10 (6), ST-14 (1), ST-10/ST-14 (2)	(Fayer et al. 2012)
Swine	Maryland	32	34%	ST-5 (11)	(Fayer et al. 2014)

Iran also reported that ST-5 was commonly found in cattle (Badparva et al. 2015). ST-5 has previously been reported in the USA in swine (Santín et al. 2011; Fayer et al. 2014). In China and Australia, ST-5 was not only found in pigs but also in their caretakers demonstrating its zoonotic potential (Yan et al. 2007; Li et al. 2007; Wang et al. 2014). While ST-4 has previously been reported in cattle in China, it has never been found in such high numbers in a population other than humans (Stensvold 2015; Zhu et al. 2017). It has been suggested that ST-4 is only rarely encountered outside of Europe (Alfellani et al. 2013a; Stensvold 2015). However, the ST-4 detections reported here in combination with the report of a human case of ST-4 in Oregon suggests this subtype may also be common in the USA (Whipps et al. 2010). These results are especially of interest as ST-4 has been associated with chronic diarrhea, IBD, and IBS in human infections (Mattiucci et al. 2016). ST-3, identified in four calves in California and New York, is the most common subtype reported in humans in the USA (Table 3) and has been associated with chronic gastrointestinal illness in patients from Oregon (Jones et al. 2009; Whipps et al. 2010; Scanlan et al. 2016; Nash et al. 2017). Studies from the USA, Denmark, and China have shown that ST-10 and ST-14 are the most commonly reported subtypes in cattle (Stensvold et al. 2009; Santín et al. 2011; Fayer et al. 2012; Zhu et al. 2017; Wang et al. 2018). However, ST-10 and ST-14 were not dominant in this study and only represented 6.8% and 11% of the positive samples (Table 2). The identification of ST-17 in three calves from California and Wisconsin and ST-21 in two calves from Wisconsin expands host and geographic ranges for these subtypes and supports their classification as unique subtypes. ST-17 has previously only been reported in a gundi from Africa and ST-21 in a waterbuck in China (Alfellani et al. 2013b; Zhao et al. 2017). Four novel subtypes, ST-23, ST-24, ST-25, and ST-26 were found in one, two, one, and five animals, respectively.

Sampling location and strategy may also be factors influencing both prevalence and subtype observations. Although these should not be generalized as estimates of the true animal-level *Blastocystis* prevalence in the state, in this study, the sample-level *Blastocystis* prevalence varied widely by state from 0 to 25.9%. The states with the most extreme prevalences (including the three states which had no positive specimens and the state with the greatest prevalence) had low sampling numbers with two of them having fewer than 25 calves tested (Table 1). Samples from Vermont had the highest prevalence (25.9%) and along with Minnesota (2%) were the only states from which samples had a single subtype, ST-4, present in tested calves. In comparison, the New York samples had a prevalence of 3.4% but eight subtypes (ST-3, ST-5, ST-10, ST-14, ST-23, ST-24, ST-25, and ST-26) were found in calves from this state. Most studies of *Blastocystis* subtype distribution are conducted over relatively constrained areas. However, in this study, we sampled dairy calves from 13 states across the USA. The approach used in this study better captures the true diversity of subtypes present in the USA.

This study represents the first major inroad to understanding *Blastocystis* subtype distribution in the USA. We have demonstrated that dairy calves harbor zoonotic subtypes of *Blastocystis*, and that this parasite is found across the USA. However, more work should be done to clarify the role of age and location in *Blastocystis* exposure risk. Given the prevalence and broad geographic distribution of zoonotic subtypes of *Blastocystis* in calves, our results highlight that cattle could serve as reservoirs of infection for humans and other animals and suggest that cattle could play a role in the transmission of these parasites to humans through contamination of ground and surface water with cysts of zoonotic *Blastocystis* subtypes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Disclaimers "USDA is an equal opportunity provider and employer."

Appendix

Table 4 *Blastocystis* reference sequences used for phylogenetic analysis in this study

Subtype	GenBank accession no.	Host	Location
ST-1	U51151	Human	USA
	AB107962	Human	Japan
	EU679349	Human	USA
	HQ641595	Human	Colombia
	AB070989	Human	Japan
ST-2	AB070997	Primate	Japan
	HQ641605	Human	Colombia
	AB070987	Human	Japan
ST-3	AB091234	Human	Japan
	AB107965	Cattle	Japan
	KU147374	Human	Mexico
	AM779013	Human	Turkey
	KX618192	Human	Singapore
	HQ641613	Human	Colombia
	EU679346	Human	USA
ST-4	U26177	Guinea pig	USA
	AB071000	Rat	Japan
	HQ641621	Human	USA
	AY244620	Human	Germany
	EU679347	Human	USA
ST-5	AB070998	Pig	Japan
	AB107964	Pig	Japan
	KF410609	Pig	USA
	KF410597	Pig	USA
	AB107966	Cattle	Japan
ST-6	AY135411	Turkey	France
	HQ641658	Chicken	Spain
	AB107972	Bird	Japan
	AB070990	Human	Japan
ST-7	AY590109	Human	France
	AF408427	Human	Japan
	HQ641660	Chicken	Spain
	AB070996	Quail	Japan
ST-8	AB107971	Bird	Japan
	EU679348	Human	USA
	AB107970	Primate	Japan
ST-9	AF408425	Human	Japan
	KC138681	Human	Denmark
	AF408426	Human	Japan
ST-10	HQ641629	Cattle	USA
	JQ996368	Cattle	USA
	HQ641623	Cattle	USA
	JQ996365	Cattle	USA
	KC148207	Camel	Libya
ST-11	GU256900	Elephant	Australia
	GU256901	Elephant	Australia
ST-12	GU256936	Kangaroo	Australia
	GU256902	Giraffe	Australia
	GU256905	Giraffe	Australia
ST-13	GU256934	Quokka	Australia
	GU256935	Quokka	Australia
	KC148209	Mouse deer	UK
ST-14	KC148206	Mouflon	Czech Republic
	KC148205	Cattle	Libya
	JQ996357	Cattle	USA
	JQ996370	Cattle	USA
	JQ996358	Cattle	USA
ST-15	KC148210	Camel	Libya
	KC148211	Gibbon	Not available
ST-16	EU427512	kangaroo	Japan
	EU427514	kangaroo	Japan
ST-17	KC148208	Gundi	Libya
ST-18	KY823404	Alpaca	China
ST-19	KY823345	Macaque	China
ST-20	KY823411	Ostrich	China
ST-21	KY823403	Waterbuck	China
ST-22	KY823402	Guanaco	China
NA (ST-24)	HF569209	Sheep	Belgium
NA (ST-24)	HF569219	Sheep	Belgium
NA (ST-25)	HF569213	Sheep	Belgium
NA (ST-26)	HF569225	Cattle	Belgium
NA (ST-25)	HF569221	Cattle	Belgium
NA (ST-26)	HF569204	Sheep	Belgium

NA subtype not previously assigned a subtype name in first report in GenBank

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