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Equine hepacivirus persistent infection in a horse with chronic wasting

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Short title: Hepacivirus persistent infection in a horse

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Summary

Equine hepacivirus is the closest homolog of hepatitis C virus. Limited data on the clinical features of this infection are available. We report the identification of a horse with high-titer viremia by equine hepacivirus. Over a 15-month follow up, the clinical signs and the viremic status persisted, suggesting a chronic evolution.

Key words: Hepacivirus, horse, hepatitis, viraemia.

Introduction

Recently, several new hepaciviruses (HVs), Flaviviridae family, have been discovered in different animal species. Among those, equine HV (EqHV), originally described as canine HV, or non-primate HV, represents the closest known relative of hepatitis C virus (HCV) identified to date. Moreover, in terms of viral tropism and course of infection, EqHV also resembles HCV infection in humans as its RNA is detectable predominantly within hepatocytes and the infection evolves trough two stages (acute and chronic) in horses (Pfaender et al., 2014, 2015).

EqHV has been identified in horses from United States, Brazil, Japan, New Zealand, and Europe (Pronost et al., 2016) with antibodies being detected in 30-40% and viremia in 3% of the animals, but its association with liver disease remains uncertain. A mild elevation of liver enzymes was observed at seroconversion in some of the affected horses (Pfaender et al., 2015), thus suggesting that adaptive immunity may contribute to acute liver damage by EqHV, as observed for HCV acute infection in humans (Maasoumy and Wedemeyer, 2012). However, in most of the infected horses, analyses of the liver functionality did not reveal hepatic impairment since the serum levels of hepatic markers (GGT, AST and ALT) were mainly within the reference ranges (Lyons et al., 2014; Pfaender et al., 2015; Ramsay et al., 2015). Only a few reports describe severe hepatitis in EqHV-infected horses (Reuter et al., 2014; Pfaender et al., 2015). Post-mortem analysis of tissues from a highly viremic horse revealed histopatologically mild-to moderate hepatitis (Pfaender et al., 2015), suggesting EqHV hepatotropism. However, the horse was also co-infected by pegivirus (Flaviviridae family) and it was not possible to assess the contribution of each virus to the observed liver disease. Yet, a causative relationship between EqHV and hepatitis needs to be addressed thoroughly. This study reports the detection of a natural infection by EqHV in a horse with apparent liver disease and high-level viraemia.

Case report

On October 2015, a 16-year old male horse from a farm located in Locorotondo, prefecture of Bari, Apulia, required medical attention. The animal displayed weight loss and reluctance to work although the appetite was preserved. The veterinarian collected whole blood and serum samples which tested negative for a panel of horse pathogens (panel HP), including equine infectious anaemia virus, Anaplasma, Ehrlichia and Babesia spp. in conventional PCR assays (Nagarajan et al., 2001; Kim et al., 2003; Nagore et al., 2004). The hepatic markers were altered (Table 1) whilst the hematocrit was normal.

In spite of changes in feed management, the animal did not gain weight and after one year, on November 2016, the veterinarian collected serum and blood samples for virological and bacteriological investigations. Biochemical screening of the hepatic functionality was repeated, revealing altered levels of the liver markers (Table 1), with the hematocrit being normal.

The animal again tested negative to the diagnostic panel HP and to bacteriological investigations. As we suspected an infectious hepatic disease, we also included EqHV and two equine pegiviruses (Theiler's disease associated virus, TDAV, and equine pegivirus, EPgV) in the diagnostic panel HP, using a TaqMan assay developed for detection and quantification of hepacivirus (Burbelo et al., 2012) and a conventional RT-PCR (Chandriani et al., 2013) for pegiviruses. The animal tested negative for TDAV and EPgV whilst EqHV RNA was detected at high titers (3,51 x 10^6 RNA copies per ml) in the blood. We therefore re-tested the serum sample collected in 2015 and also a serum sample collected on January 2017. Both the samples tested positive for EqHV (Table 1), indicating high-level viraemia in the animal, at least since October 2015.

RNA extracts of the 2015 and 2017 sera samples were subjected to conventional RT-PCR, using specific oligonucleotides (Table 2) designed on conserved regions of the NS3, NS5B and

5'UTR (Figure 1). The amplicons (of 187 nucleotide [nt], 308 nt and 293 nt in size, respectively) were purified, cloned and sequenced (GenBank accession numbers KY554652, KY554653 and KY554654). Sequence editing, multiple codon-based (translation) alignments and phylogenetic analysis of detected viruses were performed using Geneious software v9.1.5 (Biomatters, Auckland, New Zealand). Phylogenetic trees were inferred on the basis of the 5'UTR, NS3 and NS5B, using the Unweighted Pair Group Method with Arithmetic Mean. The evolutionary distances were computed using the Maximum Composite Likelihood method. Upon sequence comparison, the 2015 and 2017 EqHV strains displayed 100% identity to each other. These results are consistent with the literature, reporting a very low genetic variation for EqHV (Burbelo et al., 2012; Simmonds, 2013). By Fasta (http://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html) interrogation of the sequence database, the equine virus shared the highest nt identity to strain KU771/JPN/2015 (LC030428) in the NS3 (92.7%), B82/BGR/2015 (KX421286) in the NS5B (94.5%) and H3-011/USA/2011 (JQ434008) in the 5' UTR (98.1%). Upon phylogenetic analysis using a set of sequences from European and non-European countries, two distinct clusters were observed in the NS5B, NS3 and 5'UTR trees, and the sequences of the EqHV strain identified in this study segregated within subtype 1 (Figure 2).

Discussion

Although EqHV has been described repeatedly in horses worldwide, its association with liver disease has not been determined firmly. In several studies on EqHV-positive horses, the liver markers fell in the reference ranges with only a few exceptions (Lyons et al., 2014; Pfaender et al., 2015). It has been shown that, in contrast to HCV infection, at least 60% of the horses are able to clear EqHV within two months. So it could be speculated that this high rate of viral

clearance among horses together with their average lifespan (25-30 years) may result in less extensive liver injury during infection (Pfaender et al., 2015).

An interesting finding of our case was the long-term viremia by EqHV in a horse with clinical signs of a wasting condition and with hematochemical parameters suggestive of hepatic disease. Consecutive sampling from the animal revealed EqHV viremia for at least 15 months, with the viral load higher than 10^5 RNA copies/ml. As reported by the owner, during the sampling period, the horse clinical conditions (weight loss and reluctance to work) remained stable. In parallel, biochemistry analyses indicated that the liver enzymes remained altered but the animal was not icteric, with the bilirubin levels being normal. This is not unusual, as several horses with chronic liver disease do not become icteric. In a five-month follow up of EqHV infection in a horse, a correlation was suggested between decreasing of EqHV titers in blood and normalization of the hematochemical parameters (Reuter et al., 2014). This correlation (persistence of clinical signs/altered liver markers and high-titer viremia) was also apparent in our case, although virus persistence was much longer.

Our study had some limitations. We could not perform a liver biopsy to confirm the hepatic inflammation and to demonstrate the presence of EqHV antigen in the hepatic tissues. Also, we only based our observations and speculations on a single case report.

Gathering information on the disease patterns associated with EqHV will be helpful to understand better if and to which extent EqHV is able to affect liver functionality. Also, additional information on the epidemiology and genetic diversity of EqHV will be helpful to understand if there are differences in terms of virulence (i.e. progression of liver disease) and geographical distributions among the various strains, as observed for HCV in humans (Irshad et al., 2013).

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Figure legend

Fig.1. Genomic regions analysed for characterization of EqHV. Oligonucleotides' position (nt) is referred to the genome sequence of the EqHV strain AK-2012 NPHV-NZP-1 (GenBank accession no. JQ434001).

Fig. 2. Phylogenetic trees based on partial NS3, NS5B and 5'UTR genomic portions. HCV subtype 1a H77/USA/1997 (GenBank accession number AF00960) was used as outgroup. The samples detected in our study are indicated by black arrows. The scale bar indicates the number of substitution per site.

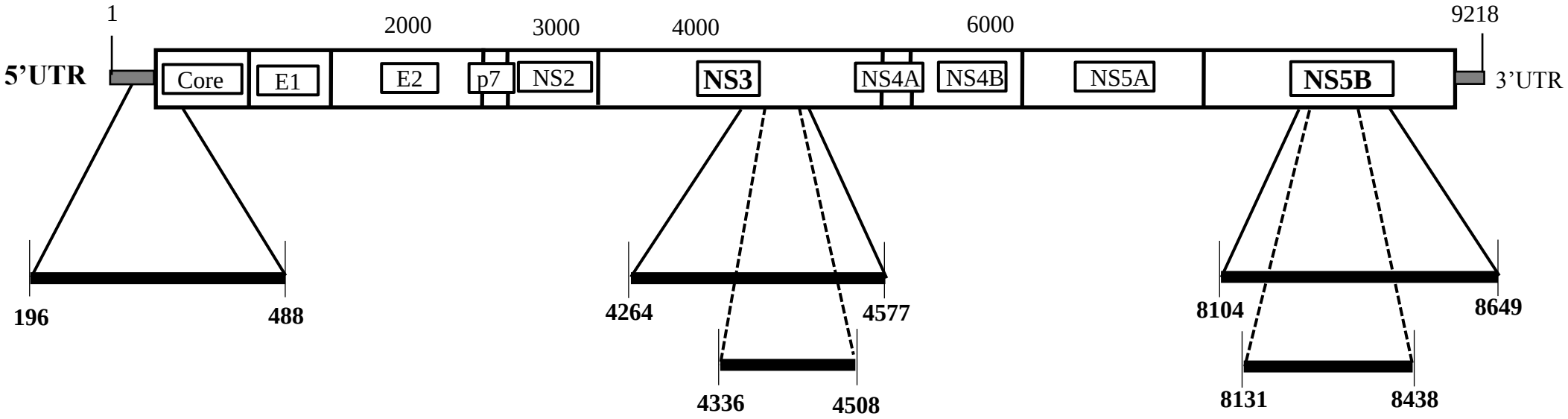
Table 1: Biochemical parameters and EqHV quantification in serum samples collected form a naturally infected horse

	Horse 826/15			Reference range in horse
	Serum October 2015	Serum November 2016	Serum January 2017	
Total bilirubin	0.95	0.71	0.86	0.50-3.00
AST	609*	861	878*	170-330
ALT	18*	19*	19*	4-16
ALP	534*	274	311	160-380
GGT	20.8	18.6	20.3	8-25
LDH	850*	1105*	1110*	100-600
CPK	254	256	251	100-260
Real time RT-PCR [1]	$1.04x10^5$	$3.51x10^6$	$1.07x10^6$	-

AST, aspartate aminotransferase; ALT, Alanine transaminase; ALP, alkaline phosphatase; GGT, Gamma-glutamyltransferase; LDH, Lactate dehydrogenase; CPK, Creatine phosphokinase; *, altered values; [1] (copies/ml).

Table 2. List of oligonucleotides used in PCR protocols for sequencing of genomic fragments. Amplicon sizes are referred to the sequence of Hepacivirus strain AK-2012 NPHV-NZP-1 (GenBank accession no. JQ434001).

Gene target	Primer	Sequence 5'-3'	Amplicon size	Reference
5'UTR	Quanti5UF1	GAGGGAGCTGRAATTCGTGAA	293nt	*Burbelo et al., 2012*
	492R	GCGCCGGAMGGGAATACTAC		This study
NS3	EQNS3OS	ATWTGTGATGARTGCCAYAGYAC	314nt	*Lyons et al., 2012*
	EQNS3OAS	TAGTAGGTBACAGCRTTAGCYCC		
	EQNS3IS	TCYAARGGTGTDAAGCTTGTTGT	187nt	
	EQNS3IAS	TGGCAGAAGYTAAGRTGYCTYCC		
NS5AB	EQNS5BIS	AARTGYTTTGACTCYACBGTCACTC	413nt	*Lyons et al., 2012*
	EQNS5BOIS	ACTRTGACTRATYGTYTCCCAACTCG		
	EQNS5BIS2	CAYGATATAGAHACTGAGAGRGA	308nt	
	EQNS5BIAS2	TCRTCTTCCTCRACGCCYTTRCTGG		


1
2000
3000
4000
6000
9218
5'UTR
Core
E1
E2
p7
NS2
NS3
NS4A
NS4B
NS5A
NS5B
3'UTR
196
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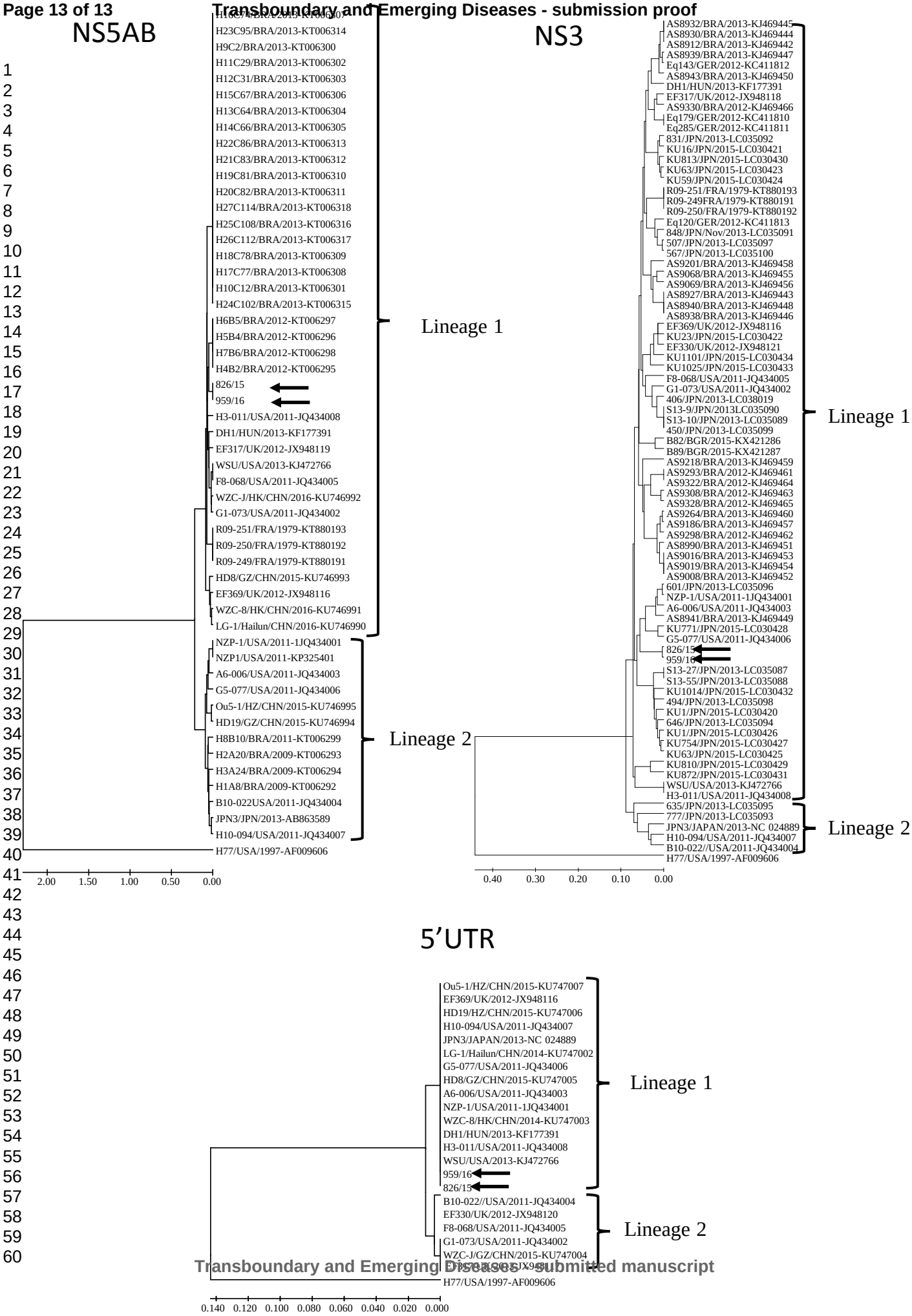
4264
4577
4336
4508
8104
8649
8131
8438


NS5AB
H10C74/BRA/2013-KT006307
H23C95/BRA/2013-KT006314
H9C2/BRA/2013-KT006300
H11C29/BRA/2013-KT006302
H12C31/BRA/2013-KT006303
H15C67/BRA/2013-KT006306
H13C64/BRA/2013-KT006304
H14C66/BRA/2013-KT006305
H22C86/BRA/2013-KT006313
H21C83/BRA/2013-KT006312
H19C81/BRA/2013-KT006310
H20C82/BRA/2013-KT006311
H27C114/BRA/2013-KT006318
H25C108/BRA/2013-KT006316
H26C112/BRA/2013-KT006317
H18C78/BRA/2013-KT006309
H17C77/BRA/2013-KT006308
H10C12/BRA/2013-KT006301
H24C102/BRA/2013-KT006315
H6B5/BRA/2012-KT006297
H5B4/BRA/2012-KT006296
H7B6/BRA/2012-KT006298
H4B2/BRA/2012-KT006295
826/15
959/16
H3-011/USA/2011-JQ434008
DH1/HUN/2013-KF177391
EF317/UK/2012-JX948119
WSU/USA/2013-KJ472766
F8-068/USA/2011-JQ434005
WZC-J/HK/CHN/2016-KU746992
G1-073/USA/2011-JQ434002
R09-251/FRA/1979-KT880193
R09-250/FRA/1979-KT880192
R09-249/FRA/1979-KT880191
HD8/GZ/CHN/2015-KU746993
EF369/UK/2012-JX948116
WZC-8/HK/CHN/2016-KU746991
LG-1/Hailun/CHN/2016-KU746990
Lineage 1
NZP-1/USA/2011-1JQ434001
NZP1/USA/2011-KP325401
A6-006/USA/2011-JQ434003
G5-077/USA/2011-JQ434006
Ou5-1/HZ/CHN/2015-KU746995
HD19/GZ/CHN/2015-KU746994
H8B10/BRA/2011-KT006299
H2A20/BRA/2009-KT006293
H3A24/BRA/2009-KT006294
H1A8/BRA/2009-KT006292
B10-022USA/2011-JQ434004
JPN3/JPN/2013-AB863589
H10-094/USA/2011-JQ434007
Lineage 2
H77/USA/1997-AF009606
2.00 1.50 1.00 0.50 0.00
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AS8912/BRA/2013-KJ469442
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Eq143/GER/2012-KC411812
AS8943/BRA/2013-KJ469450
DH1/HUN/2013-KF177391
EF317/UK/2012-JX948118
AS9330/BRA/2012-KJ469466
Eq179/GER/2012-KC411810
Eq285/GER/2012-KC411811
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KU16/JPN/2015-LC030421
KU813/JPN/2015-LC030430
KU63/JPN/2015-LC030423
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R09-250/FRA/1979-KT880192
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507/JPN/2013-LC035097
567/JPN/2013-LC035100
AS9201/BRA/2013-KJ469458
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AS8938/BRA/2013-KJ469446
EF369/UK/2012-JX948116
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EF330/UK/2012-JX948121
KU1101/JPN/2015-LC030434
KU1025/JPN/2015-LC030433
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S13-10/JPN/2013-LC035089
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AS9308/BRA/2012-KJ469463
AS9328/BRA/2012-KJ469465
AS9264/BRA/2013-KJ469460
AS9186/BRA/2013-KJ469457
AS9298/BRA/2012-KJ469462
AS8990/BRA/2013-KJ469451
AS9016/BRA/2013-KJ469453
AS9019/BRA/2013-KJ469454
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A6-006/USA/2011-JQ434003
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KU771/JPN/2015-LC030428
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Lineage 2
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959/16
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