Ferrets exclusively synthesize Neu5Ac and express naturally humanized influenza A virus receptors

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Mammals express the sialic acids N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) on cell surfaces, where they act as receptors for pathogens, including influenza A virus (IAV). Neu5Gc is synthesized from Neu5Ac by the enzyme cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH). In humans, this enzyme is inactive and only Neu5Ac is produced. Ferrets are susceptible to human-adapted IAV strains and have been the dominant animal model for IAV studies. Here we show that ferrets, like humans, do not synthesize Neu5Gc. Genomic analysis reveals an ancient, nine-exon deletion in the ferret CMAH gene that is shared by the Pinnipedia and Musteloidia members of the Carnivora. Interactions between two human strains of IAV with the sialyllactose receptor (sialic acid—α2,6Gal) confirm that the type of terminal sialic acid contributes significantly to IAV receptor specificity. Our results indicate that exclusive expression of Neu5Ac contributes to the susceptibility of ferrets to human-adapted IAV strains.
Influenza A virus (IAV) remains the most serious infectious disease threat to human health. Seasonal IAV kills 250,000–500,000 people each year worldwide. However it is the potential for the emergence of highly virulent pandemic IAV strains, such as the H1N1 strain that killed 20–40 million people, which illustrates the grave risk posed by this pathogen. IAV is a member of the family Orthomyxoviridae and has a negative-sense, single-stranded and segmented RNA genome. IAV antigenic diversity is high, with mutations accumulating during viral replication (antigenic drift) and by exchange of genomic material between IAVs co-infecting the same cell (antigenic shift). Therefore IAVs are further subtyped based on antigenic differences in the two membrane glycoproteins: haemagglutinin (HA) and neuraminidase (NA). HA is responsible for the initial attachment of the virus to the host cell membrane by binding to sialic-acid (SA) receptors, while NA ensures mobility of virus in the respiratory tract and release of new viral progeny by its sialic-acid cleavage activity. Sequence variations in these proteins may alter IAV host range and virulence by changing their specificity for the spectrum of distinct HA3 receptor structures and NA substrates on the cells, tissues and organs of vertebrate hosts. This continual and rapid IAV evolution results in the emergence of new strains from animal reservoirs to infect humans; the lack of protective immunity from previous IAV infections; the requirement for regular human IAV with fully characterized IAV receptor structures and NA substrates on the cells, tissues and organs of vertebrate hosts. Therefore IAV–receptor interaction10,20.

Results

Ferrets do not express Neu5Gc. We developed the hypothesis that a contributing factor to the susceptibility of ferrets to human strains of IAV may be the type of sialic acid they express. To explore this hypothesis, initial studies were conducted using serum samples from ferret and other species known to express either Neu5Gc or Neu5Ac14. Western blot with Neu5Gc–specific immunoglobulin (Ig)Y antibody revealed reactivity in murine and bovine serum, but not human or ferret samples (Fig. 1a). Western blots of samples from these species were probed with Sambucus nigra (SNA), a lectin that does not discriminate between these two types of sialic acid (Fig. 1b), demonstrating that sialic-acid residues are present on serum glycoproteins in all species. The same serum samples were analysed by high-performance liquid chromatography to quantitate the amount and type of sialic acid present. Both ferret and human serum had Neu5Ac, but no detectable Neu5Gc, while mouse serum contained predominantly Neu5Gc (Fig. 1c). Further investigation was conducted using cryopreserved tissue sections from brain, lung, kidney, liver and spleen prepared from healthy ferrets and mouse. These sections were probed with Neu5Gc–specific IgY antibody, and also with SubAB, an AB5 toxin with a B subunit that selectively binds Neu5Gc carbohydrate structures10,21. These studies revealed abundant staining of Neu5Gc in all mouse tissues using both methods (Fig. 1d; Supplementary Fig. 2a–g), but no staining was observed in the equivalent ferret tissue samples (Fig. 1d; Supplementary Fig. 2h,i). Taken together, these data show that, like humans, ferrets do not express Neu5Gc.

The ferret CMAH gene is deleted. To determine the molecular basis for the lack of Neu5Gc expression in a ferret, we investigated the ferret CMAH gene. Synteny in the CMAH region is well conserved in mammalian genomes, with the same genes present in the CMAH flanking regions of eukaryotes (Fig. 2a) and the ferret (Fig. 2b). The coding sequence of CMAH is also well conserved. Primer sets to amplify exons from all mammalian CMAH genes were designed based on the most conserved exons (exons 3, 5, 8, 11 and 12; Fig. 2c). All of the exons amplified from the carnivore species cat and dog genomic DNA. All except exon 3 amplified from human genomic DNA. This region corresponds to the deletion event that inactivated the human CMAH gene resulting in the loss of Neu5Gc biosynthesis18. Only exons 11 and 12 amplified from ferret DNA, suggesting that there may be a large deletion in ferret CMAH. A ferret bacterial artificial chromosome (BAC) clone library was screened using probes specific for conserved regions flanking CMAH in related carnivore genomes (Fig. 2b), resulting in the isolation of the BAC clone 182P23. Sequence analysis of this clone facilitated design of a probe that was used to isolate BAC clone 446P7. These two BAC clones were sequenced using single-molecule real-time (SMRT) sequencing technology, resulting in two complete sequences that overlapped and covered the entire CMAH region. Sequence analysis identified a large deletion that results in loss of the first nine coding-sequence exons of CMAH in the ferret genome, and multiple stop mutations in exon 11. The deletion is consistent with the exon PCR amplification data (Fig. 2c). Primers were designed at the boundaries of the deleted region and used in PCR to confirm that the deletion exists in independent individual ferrets. Recent data available from the Broad ferret genome project (http://www.broadinstitute.org/annotation/genome/ferret, accessed 9 July 2014) are consistent with data presented here, but do not currently annotate the CMAH deletion. We conclude that the lack of Neu5Gc expression in ferrets is due to deletion of the majority of the CMAH gene.

The ferret CMAH deletion is an ancient mutation. To determine the evolutionary origin of the CMAH-deletion event in ferret, we used the same CMAH exon primer set to examine genomic DNA from 15 Mustelidae species selected to cover all genera. All showed the same profile as ferret (Supplementary
Fig. 3a–c), suggesting that the CMAH-deletion event occurred prior to the divergence of the Pinnipedia and Musteloidea lineages. The analysis was then widened to include the other members of the Arctoid lineages, Ursidae and Pinnipedia\textsuperscript{22}, and revealed that all members of the Pinnipedia tested also shared the same CMAH deletion as the Musteloids (Supplementary Fig. 3b). The Ursidae species Ursus americanus (American black bear) contained all CMAH exons tested as did Urocyon cinereoargenteus (grey fox), which is the basal species of the Canidae family\textsuperscript{23} (Supplementary Fig. 3a,b). We propose that the

| Analyte | Ferret serum | Normal human serum | Bovine serum | Mouse serum |
|---------|-------------|-------------------|--------------|-------------|
| Neu5Ac  | 139.3±1.7   | 151.5±2.5         |              | 28.1±0.8    |
| Neu5Gc  | ND          | ND                | 1.836±45     |             |

ND = not detected; n = 3, *concentration ± s.d.
CMAH mutation occurred in the infraorder Arctoidea after divergence of the Ursidea from the Pinnipedia and Musteloidea lineages, dating the mutation to between 38 and 40 million years ago. Our data are consistent with and support studies that propose that the Ursidea descended from an Arctoidea ancestor separate from Pinnipedia and Musteloidea. The absence of Neu5Gc expression has also been observed in chickens, reptiles, various species of birds (with the exception of ducks), the platypus, in western dog breeds and recently in new world monkeys. An inactive CMAH allele results in loss of CMAH expression that generates blood group antigen differences in cats. It has been proposed that the loss of Neu5Gc expression in humans may have resulted from selective pressure from pathogens that utilize sialic-acid-containing receptors. Our finding that two major families of carnivores also lack Neu5Gc expression, and that this event preceded the human CMAH mutation, which occurred only ~3 million years ago, suggests that this selective pressure has been present throughout evolutionary history of vertebrates. This finding is also consistent with the hypothesis that inactivation of CMAH is a crucial speciation event, as this mutation may generate reproductive incompatibility.

Terminal sialic-acid type and linkage affect IAV interactions. To a large extent, IAV host range has been seen through the prism of IAV receptor type and distribution in the host. The location of these receptors in host organs and tissues dictate the type of pathology. Sialic-acid receptor recognition by both HA and NA play an important role in maintaining a balance for successful infection. In addition to the linkage type, that is SAa2,3Gal or SAa2,6Gal, the type of sialic acid has also been
suggested to have an influence in IAV host range. This is supported by erythrocyte agglutination assays using red blood cells expressing distinct sialic-acid types, IA V-binding assays with Neu5Ac or NeuGc receptors, modification of sialic-acid cleavage rates with Neu5Ac or NeuGc receptors, amino-acid modifications of HA and NA. A fundamental difference between non-Neu5Gc-expressing IAV hosts, such as human and ferret, and the Neu5Gc-expressing IAV reservoirs, such as pig and duck, is that the latter two species express both Neu5Gc- and Neu5Ac-terminated receptors. Selective pressure for adaptation in humans is therefore restricted to Neu5Ac receptor, however, in pigs and ducks IAV can adapt to either Neu5Ac and Neu5Gc receptors.

To determine receptor-binding preference of human-adapted whole IAV and to resolve the relative roles of sialic-acid linkage type and sialic-acid species, we have conducted a series of saturation transfer difference (STD) nuclear magnetic resonance (NMR) experiments. STD NMR is a versatile technique to investigate protein–ligand binding by saturating the protein resonances without affecting ligand signals. In the study presented here, STD NMR experiments were performed with intact influenza virus particles of two recently isolated human viruses (A/Perth/16/2009 H3N2 and A/California/04/2009 pH1N1), complexed with a mixture of 6-sialyllactose (6SL) synthetic ligands terminating in Neu5Ac (6SLAc) and Neu5Gc (6SLGc). We and others have previously reported about using STD NMR to investigate ligand binding to intact virions and virus-like particles. The results presented in this study are the first using whole intact influenza virus particles. In the current study, the interaction between HA displayed on whole IAV particles and sialyllactose ligands were analysed in the presence of a low concentration of oseltamivir carboxylate (OC; 50μM) to completely block the NA active site to prevent sialyllactose ligand binding to the NA and consequently sialic-acid cleavage (Fig. 3b, Supplementary Fig. 4).

Figure 3b shows 1H NMR and STD NMR spectra of an equimolar mixture of 6SLAc and 6SLGc in complex with whole IAV. Strong STD NMR signals for the 6SLAc-specific protons are observed, especially for the methyl protons of the N-acetamido group, for both H3 and 09H1 viruses. In contrast, 6SLGc shows only very weak interactions with both viruses under identical experimental conditions. For both virus strains (pH1N1 virus (A/California/04/2009, left panel) and H3N2 virus (A/Perth/16/2009, right panel)) strong STD NMR signals are observed for the protons of the N-acetamido group (NHa) (6SLAc), while the STD NMR signal intensities of the methylene protons (NGGc, 6SLGc) were generally very weak. Similarly, the STD NMR signal intensities of the H3eq and H3ax protons are stronger for 6SLAc compared with 6SLGc (magnified at the top). The results clearly demonstrate that pH1N1 and H3N2 IAVs show a strong preference for Neu5Ac-containing sialosides. To confirm that 6SLGc has a very weak affinity to HA, we also conducted STD NMR experiments of 6SLGc in the absence of 6SLAc under otherwise identical experimental conditions (Supplementary Fig. 5). STD NMR control experiments of heat-treated virus and of OC in the absence of sialosides were performed to affirm that all observed STD NMR signals describe specific HA binding (Supplementary Fig. 6). Our NMR data with pure, fully characterized synthetic receptor structures confirm a profound preference for 6SL terminating in Neu5Ac for two currently circulating human strains, consistent with adaptation of human IAV strains to Neu5Ac-terminated receptors. Data mining of glycan array results from the Consortium for Functional Glycomics (CFG) database (http://www.functionalglycomics.org) also revealed preferential binding by human IAV HA to Neu5Ac compared with Neu5Gc-terminating structures. Comparison of the binding of whole-human-adapted IAV strains or purified HA to glycan arrays, displaying sialyllactose or sialyllactosamine structures, with identical spacer structures, terminating in either Neu5Ac or Neu5Gc also indicate a strong preference for Neu5Ac-terminating structures (Supplementary Figs 7 and 8). The cleavage of four distinct sialyllactose substrates by whole influenza virus-associated NA was followed by a time-course 1H NMR study. Figure 3c shows substrate conversion of 3SLAc, 3SLGc, 6SLAc and 6SLGc using identical virus preparations as used in the STD NMR experiments (Supplementary Fig. 9). Both substrates cleave 2,3-sialosides more efficiently than 2,6-sialosides. In case of the pH1N1 virus, the N-acetyl-containing substrates get converted slightly faster than the N-acetyl counterparts of the same linkage. On the contrary, N-acetyl-containing substrates are significant better substrates for the NA of the H3N2 virus compared with the N-acetyl-containing substrates. Both linkage type and sialic-acid species contribute significantly towards NA specificity. For the H3N2 strain, the Neu5Ac-containing 3SLAc and 6SLAc are preferred over the equivalent substrate with Neu5Gc regardless of the linkage.

**Discussion**

Our study reveals that ferrets are a naturally humanized model system with respect to IAV receptor biology. Previous studies have shown that ferrets have similar IAV receptors with respect to the SAx2,6-linkage and anatomical distribution. Here we show that ferrets, like humans, exclusively express Neu5Ac on these receptors. Our STD NMR analysis of whole human IAV with fully characterized IAV receptor structures, and NA activity assays, confirm the importance of Neu5Ac in both HA and NA functions. Sub-optimal interactions of human-adapted IAV with Neu5Gc-terminated viral receptors, may explain why other dominant rodent animal models (mouse, rabbit, rat and guinea pig) are not optimal for studies with human-adapted IAV strains. We conclude that exclusive expression of Neu5Ac in ferrets is a contributing factor to their unique suitability as a model for human-adapted IAV. Recently, a CMAH mutation has been reported in new world monkeys, and this mutation differentiates them from old world monkey species, which express Neu5Gc. This new finding supports the importance of exclusive expression of Neu5Ac-terminated receptors in human-adapted IAV model systems, as marmosets, a new world monkey species, cannot transmit human-adapted IAV. The implications of our discovery of the exclusive expression of Neu5Ac in ferrets extend beyond the IAV field. Ferrets may serve as a natural model system for other human pathogens that utilize sialic-acid receptors such as rotavirus, and for studies on the emerging role of the Neu5Gc xeno-auto-antigen in inflammatory autoimmune and neoplastic human disease.

**Methods**

**Western blot analysis of sialic-acid expression in serum.** Serum samples were purchased from commercial suppliers: ferret (Joram Bioscience), human (H4S22, Sigma), bovine (B6655, Sigma) and mouse (M9095, Sigma). Serum samples were diluted 1:10 in a 50-μl volume of 1× NA buffer (N3786, Sigma) and incubated at 37°C for 3 h before SDS-polyacrylamide gel electrophoresis of 10 μg of each sample (NuPage 4–12% Bis-Tris gel). For detection of Neu5Gc, primary antibody (1/2,000 dilution) and blocking solution (0.5% v/v in PBS) was used was supplied by Sialix (formerly GC-Free Inc., San Diego, CA, USA); Secondary antibody used was anti-chicken IgG (IgG) alkaline phosphatase conjugate produced in rabbit (A9171, Sigma) at 1/10,000 dilution. For detection of sialic acid (Neu5Ac and Neu5Gc), lectin SNA-alanine phosphatase conjugate (LA-6002-1, EY Laboratories) was used at 1/1,000 dilution in 1% bovine serum albumin (w/v) in PBS. All membranes were washed in 1× tris-buffered saline, 0.05% Tween 20. Detection of bands with anti-Neu5Gc-specific sera or SNA that were present in NA (+) sample and absent in the NA (+) are interpreted as binding to serum proteins, with glycosylations terminated with sialic acid.
Figure 3 | Molecular analysis demonstrating the importance of sialic-acid (SA) type on HA and NA specificity. (a) Chemical structures of SA₂₂,₃Gal and SA₂₂,₆Gal in which SA is either Neu5Ac (3’S'LAc, 6’S'LAc) or Neu5Gc (3’S'LGc, 6’S'LGc). (b) Haemagglutinin receptor specificity of human influenza A viruses pH1N1 and H3N2. ¹H NMR (bottom row) and STD NMR (above) spectra were obtained of an equimolar mixture of 2 mM 6’S'LAc (Neu5Ac₂₂,₆Galβ₁,₄Glc) and 6’S'LGc (Neu5Gc₂₂,₆Galβ₁,₄Glc) with pH1N1 virus (A/California/04/2009, left panel) and H3N2 virus (A/Perth/16/2009, right panel), respectively. All NMR samples also contained a low concentration of oseltamivir carboxylate (50 μM, OC), a very potent nanomolar inhibitor of the viral neuraminidase to inhibit sialic-acid cleavage (Supplementary Fig. 4). Shown are only the axial and equatorial H₃ protons (H₃ax, H₃eq) and the N-acetamido methyl (NHAc) and methylene (NHGc) protons of the sialic-acid moiety that are clearly distinguishable between 6’S'LAc and 6’S'LGc. The entire spectra are shown in Supplementary Fig. 5. (c) Neuraminidase substrate specificity of human influenza A viruses pH1N1 and H3N2. ¹H NMR spectroscopy was employed to follow the cleavage of sialosides (6’S'LAc, 6’S'LGc, 3’S'LAc, 3’S'LGc) upon addition of pH1N1 virus (A/California/04/2009, left panel) and H3N2 virus (A/Perth/16/2009, right panel), respectively. The conversion rate was calculated using the absolute peak intensity of the sialyllactose H₃eq-signals (± 7.5% error) based on substrate depletion by a successive series of ¹H NMR spectra over 20 min at 37 °C (Supplementary Fig. 9).
Sugar analysis of serum for the detection of sialic acids. A 20-µl subsample of the stock samples was subjected to mild acidic conditions using 0.1 M trifluoroacetic acid. The pungent odor of the reaction mixture was neutralized by drying under vacuum and the residue was reconstituted in MilliQ water (100 µl). The analysis was carried out using a high-performance anion-exchange chromatograph with pulsed amperometric detection (HPAEC-PAD) fitted with a PA1 guard column (4 × 50 mm) connected to a CarboPac PA1 column (4 × 250 mm) held at 30°C (ref. 65). The sample (10 µl) was injected into the HPAEC-PAD and analysed using a basic solvent (NaOH), at a flow rate of 1 ml min⁻¹. The analytes detected were quantified using external calibration66. Samples were analysed in triplicate and the data averaged.

Isolation of ferret CMAH region BAC clones. Comparison of regions shown in Fig. 2a was performed using genome sequences for mouse (accession code NC_000076.9, human (NC_000060.12) and cat (NC_018727.1)), respectively. Outer probes indicated on Fig. 1b (sequences numbered as in GenBank accession codes KJ027518 and KJ027519) were designed in conserved regions flanking CMAH that were 100% homologous to other vertebrate species. These regions were amplified with primers (Supplementary Table 1) using ferret DNA (Zyagen GF-237, CA) as template using the Roche RealTime PCR kit (Roche Diagnostics, Mannheim, Germany). PCR product was pooled, purified and quantitated prior to being used as a probe.

PCR analysis of the CMAH-deletion region in mammalian species. PCR primers CMAH_F0R and CMAH_REV were designed to span the entire deleted region of ferret CMAH (see accession code JX064862 for primer sequences). These primers amplify a 7-kb PCR product from ferret genomic DNA (Zyagen). Primer walking and Sanger sequencing using BigDye v3.1 and BigDye v1.1 and deaza dGTP (Applied Biosystems) were used in combination to determine the sequence of this repeat-rich 7-kb region of the ferret genome (the sequence has been deposited in the GenBank database accession code JX064862). The entire sequencing process was used to sequence the fragment from a second, independently verified ferret sample. DNA from this second ferret sample was extracted (DNeasy Blood and Tissue Kit, Qiagen) from ferret kidney tissue provided by the Institute of Medical and Veterinary Science. Animal tissue for various research purposes has been collected from the Institute of Medical and Veterinary Science, South Australia, in accordance with the institute's animal ethics guidelines.

Propagation and purification of IAV. Adherent Madin Darby canine kidney (MDCK) cells were obtained from the WHO Collaborating Centre for Reference and Research on Influenza (Vidrl, Melbourne). Cells were grown in Eagle’s minimum essential medium supplemented with 1% penicillin/streptomycin, 1% GlutaMAX and 10% fetal calf serum at 37°C. The HA and NA activities of the IAV strains used in these experiments were previously characterized29–31.

MuniNA inhibition assay. The activity of IAV was quantitatively assessed using the fluorescent substrate 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (MUN, Sigma-Aldrich)69–71. The 10-kd reaction mixture containing 0.1 mM MUN, 1 µM OC and 1 µl of the purified ultraviolet-inactivated virus in reaction buffer (50 mM sodium acetate, 6 mM CaCl₂, pH 5.5) was prepared in a black 96-well plate on ice. Different dilutions of virus in triplicate were used to quantify the virus concentration, which is still completely inhibited by 1 µM MUN. The purified virus was inactivated by 20-min exposure to ultraviolet light. The virus stock was then sequentially buffer exchanged to 20 mM deuterated phosphate buffer pH 7.1 and 70 mM NaCl.

1H NMR-based NA activity assay. All enzyme reactions were performed at 310 K in 20 mM sodium acetate buffer containing 6 mM CaCl₂, pH 5.5, the optimal pH for NA activity. In a standard 1H NMR experiment, a spectrum of each individual reaction mixture containing 1 mM of one of the four sialylactose substrates (3′SLAc, 3′SLGaC, 6′SLAc and 6′SLGaC) was acquired at t = 0 min. After addition of purified ultraviolet-inactivated virus (A/California/04/2009 pH1N1, A/Pert/16/2009 pH1N1) and NA to each reaction mixture every 30 min for up to 20 min with 8 samples of substrate concentrations could be calculated based on the decrease of the absolute peak intensities of the sialylactose H3eq signals. Field variations, differences in baseline correction and background noise have been taken into consideration by applying an error of 7.5% to the obtained rates.

The NA activity of pH1N1 and H3N2 purified virus samples was measured with a substrate mixture of 1 mM 3′SLAc, 3′SLGaC, 6′SLAc and 6′SLGaC in the absence and presence of 50 µM OC at 310 K. The virus concentration was
identical as in STD NMR experiments to ensure a complete blocking of the NA’s active site under STD NMR conditions.

**STD NMR experiments.** All STD NMR spectra were acquired in Shigemi Tubes (Shirakaba, Tokyo, Japan) with a Bruker Avance spectrometer at 283 K using 3H, 13C, or 15N gradient cryoprobe equipped with z-gradients and a STD NMR set-up similar to previous experiments using whole-rotavirus particles.48,49 The virus was saturated on resonance at −1.0 p.p.m. and off-resonance at 300 p.p.m. with a cascade of 60 selective Gaussian-shaped pulses of 50-ms duration. A 100-μis delay between each pulse was applied, resulting in a total saturation time of 3 s. A relaxation delay of 4 s was used. A total of 1,024 scans per STD NMR experiment were acquired and a WATERGATE sequence was used to suppress the residual HDO signal. Spin-lock filter with 5-kHz strength and duration of 10 ms was applied to suppress protein background. Substrate concentrations of 2 mM for 3SLGc, 3SLc, 6SLc, and 6SLGc were used in all STD NMR set-ups in the presence of 50 μM OC. OC was preincubated with the virus for 10 min at room temperature before adding the various substrates. Control STD NMR experiments were performed with an identical experimental set-up of virus with 50 μM OC, but in the absence of the sialyllactose ligands, to exclude any potential STD NMR signals derived from OC binding to NA. For a second control experiment, the virus sample was incubated at 70°C for 20 min prior to the STD NMR experiment to identify any unspecified binding of the sialyllactose substrates to the virus particle.

Data mining of publicably available glycan array experiments using IAV.

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Author contributions
P.S.K.N. and H.W. conducted analysis of SA expression. P.S.K.N. and M.P.J. designed and conducted molecular and phylogenetic studies in the investigation of the ferret CMAH mutation in ferret and animal samples, tested and contributed to writing these sections of the manuscript. R.B., T.H. and M.v.I. designed and conducted NMR analysis of HA and NA specificity and contributed to writing these sections of the manuscript. L.E.H.-T. designed, conducted and wrote the section on data mining. J.A.S., S.W.L., P.L.H. and P.S.K.N. contributed to sequence analysis. M.v.I., S.M.G., A.E.O.T., J.C.P., P.J.C., A.W.P., P.S.K.N. and M.P.J. contributed to design of the study. I.C.P., P.J.C., A.W.P. and M.P.J. conceived the study. M.P.J., P.S.K.N., R.B. and T.H. wrote the manuscript.

Additional information
Accession codes: Nucleotide sequence data from this manuscript have been deposited in the GenBank database with the following accession codes: ferret CMAH-deleted region (JX036482), BAC clone CH237-182P23 (KJ027518) and BAC clone CH237-446P7 (KJ027519).

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