Deep Whole-Genome Resequencing of Marek's Disease Virus Strains Reveals Intrastrain Variation

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Abstract

Current strategies to understand the molecular basis of Marek's disease virus (MDV) virulence primarily consist of cataloguing divergent nucleotides between strains with different phenotypes. However, each MDV strain is typically represented by a single low-coverage consensus genome despite the confirmed existence of mixed viral populations. To assess the reliability of consensus-only interstrain comparisons, we obtained two additional consensus genomes of vaccine strain CVI988 (Rispens) and two additional consensus genomes of the very virulent strain Md5 by sequencing viral stocks and cultured field isolates. In conjunction with the published genomes of CVI988 and Md5, this allowed us to perform 3-way comparisons between consensus genomes of the same strain. We found that consensus genomes of CVI988 can vary in as many as 240 positions involving 12 open reading frames (ORFs). We found that Md5 genomes varied only in 12 intergenic positions, with no variation within ORFs. Phylogenomic analyses showed all three Md5 consensus genomes clustered closely together, while also showing that CVI988-GenBank diverged from CVI988-UK and CVI988-USDA. Comparison of CVI988 consensus genomes with those of seven closely related isolates from China revealed 19 SNPs that appear to be unique to CVI988-GenBank. Finally, we evaluated the genomic homogeneity of CVI988 and Md5 populations by identifying positions with >2% read support for alternative alleles in two ultra-deeply sequenced samples. We were able to confirm that both populations of CVI988 and Md5 were mixed, exhibiting a total of 30 and 27 minor variant positions, respectively. We found no evidence of minor variants in the positions corresponding to the 19 unique SNPs in CVI988-GenBank. Taken together, our findings challenge prior claims of natural recombination between CVI988 and virulent strains and confirm the need to resequence MDV strains in order to ensure the accuracy of interstrain comparisons.

Keywords: Intrastrain; Diversity; Population; Genome; MDV; CVI988; Md5; Variation

Introduction

Marek's disease virus (MDV) is an oncogenic, lymphoproliferative and neuropathic alphaherpesvirus affecting chickens (*Gallid herpesvirus 2*, Genus *Mardivirus*; Family *Herpesviridae*). It was first reported in 1907 by Hungarian veterinarian József Marek (1). Successful isolation of MDV in culture was only realized six decades later, closely followed by the development of the very first vaccine, HPRS-16/Att (2–4). HPRS-16/Att was soon replaced by a better replicating, equally protective vaccine derived from strain FC126 of turkey herpesvirus (5). This vaccine, known as HVT, would go on to become the first widely used commercial vaccine for Marek's disease (MD) (6). HVT was successful in reducing MD incidence in commercial flocks until the late 1970s, when reports of strains with increased virulence that were able to bypass vaccine-induced immunity started to emerge across the USA (7, 8). A bivalent vaccine consisting of HVT alongside MDV serotype 2 strain SB-1 was introduced in response. However, a similar shift in virulence occurred in just over a decade (9, 10). Outside the USA, a vaccine known as CVI988 had been in use since the mid 1970s (11). Developed in the Netherlands by a group of veterinary scientists under Dr. Bart Rispens, CVI988 offered the best protection against the emerging variants, and it quickly became the "gold standard" vaccine around the world (12). CVI988 is colloquially known today as the "Rispens" vaccine for MDV. Though CVI988 has mostly kept MD losses at bay for the past 30 years, there are now reports of strains able to bypass CVI988-conferred immunity, and the most recent

estimate places MDV-associated losses in the poultry industry at around US $1-2 billion per annum (13–16).

Comparative genomics has been a driving force in efforts to understand the molecular basis of MDV-induced virulence ever since the first viral genomes were sequenced over two decades ago (17, 18). The current strategy involves cataloging variable nucleotide positions between the consensus genomes of different MDV strains. Comparisons are made between strains grouped on the basis of their ability to break through vaccine protection, or "pathotype," with the hope that this will help to identify the genes and polymorphisms associated with virulence (19, 20). However, despite MDV strains being known to exist as mixed populations, the degree to which the published consensus genomes represent the average for each strain has never been assessed (21–23). In addition, all available consensus genomes for MDV are low-coverage genomes that only account for the major variants (≥50%) present in the original viral population from which they were sequenced. The lack of information on minor variants (≤50%) can be problematic, due to previous reports suggesting that the diversity within a given viral population contributes to the overall pathotype of a strain (24–27). Although the extent of this contribution is still unknown, this nonetheless suggests that failing to account for minor variants can negatively impact our ability to correlate sequencing data to pathotype. Finally, many of the available reference genomes were not sequenced directly from viral stocks (i.e., a virus population grown in host cells). This includes the only available CVI988 consensus genome, which was sequenced from a bacterial artificial chromosome (BAC) clone, and the Md5 consensus genome, which was obtained from a set of five overlapping cosmid clones (28). By randomly selecting one viral haplotype from a population, these methods inherently reduce the chances of obtaining a

representative consensus genome. In addition, the use of BAC and cosmid approaches generates an artificially homogenous population of viral genomes, further moving away from the real-world biology of herpesvirus strains.

To address these issues, it is necessary to test the reliability of available MDV consensus genomes and assess the genomic homogeneity of MDV populations using next-generation sequencing (NGS) approaches. Whole-genome resequencing (WGR) is a simple yet powerful approach that consists of comparing newly sequenced individuals against a reference (29). Here, we have adapted this strategy for viruses, by sequencing multiple populations of the same strain and determining whether these yield a consistent consensus genome, under the assumption that large shifts in genomic variant frequencies will manifest as consensus-level differences. We independently sequenced two populations of the vaccine strain CVI988 and two populations of the very virulent prototype strain Md5, from a combination of viral stocks and cultured field isolates. The newly-sequenced genomes were compared with previously published consensus genomes using whole-genome pairwise alignments. Using this approach, we were able to detect variable positions between consensus genomes of each strain. We also evaluated the individual contribution of each consensus genome to overall viral diversity using phylogenomic approaches. We found 19 single nucleotide polymorphisms (SNPs) that were only present in CVI988-GenBank. Next, we assessed the genomic homogeneity of CVI988 and Md5 by analyzing ultra-deep Illumina sequencing data (>1,000x depth) and identified positions where at least 2% of the reads supported an alternative allele (30). This method enabled detection of minor variants and allowed us to confirm that the ultra-deep sequenced populations of CVI988 and Md5 were mixed. These findings challenge prior claims of natural recombination between

CVI988 and virulent strains, and highlight the need to resequence MDV strains in order to accurately link viral genotypes to virulence phenotypes.

Methods

Viral culture and DNA isolation of USDA CVI988 and Md5

Chicken embryo fibroblasts (CEFs) were used to culture viruses. Cultures were maintained in 1:1 mixture of Leibovitz's L-15 and McCoy's 5A (LM) media supplemented with fetal bovine serum (FBS), 200 U/ ml penicillin, 20 μg/ml streptomycin, and 2 μg/ml amphotericin B in a 37 °C, 5% CO_2 incubator. Cells were plated with 4% FBS LM media and maintained in 1% FBS LM media. For storage as viral stocks, infected cells were suspended in freezing media composed of 10% DMSO, 45% FBS, and 45% LM media and kept in liquid nitrogen.

DNA library preparation and sequencing of USDA CVI988 and Md5

Total DNA isolations for each MDV culture were quantified for total DNA by Qubit (Invitrogen) and either viral or vaccine DNA present by a previously described qPCR assay (30). Samples were then acoustically sheared using Covaris and high-throughput, deep sequencing DNA library preparations were produced using the Kapa HyperPrep Library Kit (Roche) as per manufacturer's protocol, with 14 PCR cycles. Then, samples were processed through a previously described oligo-enrichment procedure, where MDV-specific probes (31) (myBaits, Arbor Biosciences) were used with the myBaits Target Capture Kit (Arbor Biosciences) to enrich for MDV DNA (32). An additional PCR amplification (14 cycles) yielded libraries which were assessed for quality by Qubit, as well as a KAPA-specific qPCR, which allowed for accurate dilutions of libraries to 4pM before sequencing. Finally, libraries were sequenced using

v3 chemistry 300bp × 300bp paired-end sequencing on an Illumina Miseq, as per manufacturer's instruction.

DNA library preparation and sequencing of UK CVI988 and Md5

Total DNA isolations for each MDV culture were quantified for total DNA by Qubit (Invitrogen) and either viral or vaccine DNA present by a previously described qPCR assay (31). Samples were then acoustically sheared using Covaris and high-throughput, deep sequencing DNA library preparations were produced using the Kapa HyperPrep Library Kit (Roche) as per manufacturer's protocol, with 14 PCR cycles. Then, samples were processed through a previously described oligo-enrichment procedure, where MDV-specific probes (32) (myBaits, Arbor Biosciences) were used with the myBaits Target Capture Kit (Arbor Biosciences) to enrich for MDV DNA (33). An additional PCR amplification (14 cycles) yielded libraries which were assessed for quality by Qubit, as well as a KAPA-specific qPCR, which allowed for accurate dilutions of libraries to 4 pM before sequencing. Finally, libraries were sequenced using v3 chemistry 300 bp × 300 bp paired-end sequencing on an Illumina MiSeq, as per the manufacturer's instruction.

Processing of sequencing reads and genome assembly

Viral reads were identified by aligning raw reads in FASTQ files against a local database of 97 MDV genomes using BLASTN with an E-value cutoff of $1x10^{-2}$. The selected reads were then subjected to the quality control and preprocessing step (Step 1) of our published viral genome assembly (VirGA) workflow (34). These properly paired reads were then used for *de novo* assembly using metaSPAdes v3.14.0 (35). The resulting file containing the metaSPAdes scaffolds in FASTA format served as input for the remaining steps of VirGA (Steps 3-4), which include genome linearization, annotation and post-assembly quality assessments. For the

reference-guided contig-ordering step, we used a trimmed version (TRL and TRS regions removed) of the published consensus genome of each strain (**Table 1**). New consensus genomes were also trimmed for downstream analyses. Tandem repeats were identified in trimmed consensus genomes using Tandem Repeats Finder (TRF) v4.09 (36). Manual verification and masking of tandem repeats was conducted using Geneious Prime v2023.0.4.

Pairwise identity and genetic distance comparisons

Trimmed consensus genomes with masked tandem repeats were aligned using MAFFT v7.505 with default settings (37). The resulting alignment file in clustal format was imported into Geneious to visualize variable positions (SNPs and Indels) between the genomes. Published MDV consensus genomes with PubMed Identifiers (n = 29) and all consensus genomes included in the present study (n = 6) were aligned using MAFFT (see **Supplementary Table 5** for details). A neighbor-joining (NJ) tree was constructed from the multi-genome alignment file in clustal format using the Geneious Tree Builder tool with a Tamura-Nei genetic distance model and no outgroup.

Identification of minor variant positions

A custom script was used to identify positions with at least 2% read support for an alternative allele – i.e., a minor variant – in genomes with >1,000x coverage. Positions in tandem repeats and homopolymers were excluded. All remaining minor variant positions were verified through manual inspection of BAM alignment files using the Integrative Genomics Viewer (IGV) v2.12.3 (38).

Results

Resequencing viral stocks and field isolates of CVI988 and Md5 using Illumina whole-genome sequencing

For this study, we obtained complete genome sequences for two populations each of the vaccine strain CVI988 and the virulent strain Md5 (vv pathotype). For Md5, both the USDA and UK viral populations were derived from standard lab stocks (i.e., a virus population initiated from a prior viral stock and expanded on host cells). The UK CVI988 was likewise derived from lab stock. In contrast, the USDA Rispens was isolated from sample collected at a Pennsylvania farm, before isolation and expansion on host cells in the lab. All viral stocks were propagated similarly for viral DNA isolation and sequencing library preparation (see Methods for details). The UK viral genomes were additionally enriched using published methods for oligonucleotide-bait based enrichment in solution (32), which can enable a greater depth of coverage by enriching viral sequences from the overall milieu of DNA. Viral consensus genomes for each stock were determined using a previously published combination of *de novo* and reference-guided assembly methods (**Table 1**; see Methods for details).

The MDV genome has a double-stranded DNA (dsDNA) structure, with a full length of approximately 175-180 kb. The genomic layout is split into two large unique regions known as Unique Long (UL) and Unique Short (US), each flanked by inverted repeat sequences (IRL, IRS) and terminal repeat sequences (TRL, TRS) (**Figure 1A**, center). The α-type packaging sequences are located at the genomic termini and at the IRL/IRS junction. None of the new genomes contained any truncated or missing proteins. The average G+C content of all genomes was 44.0%. The coverage depth of Illumina sequencing data exceeded 100X for all viral genomes,

and it reached an average of 1,000X for the two genomes subjected to oligonucleotide-bait based enrichment (**Table 1**).

Pairwise alignment of same-strain consensus genomes reveals greater intrastrain variation in CVI988 than Md5

For each strain, trimmed consensus genomes with masked tandem repeats were aligned and visualized. Variable positions corresponding to SNPs, insertions and deletions (Indels) were identified and verified through manual inspection of sequence-read alignment files (i.e., BAM files). A total of 240 variable positions were detected in the three consensus genomes of vaccine strain CVI988, impacting a total of 12 ORFs. These include the genes UL22 / gH (MDV034), UL49 /VP22 (MDV062), MDV074, MDV075, Meq /RLORF7 (MDV076 / MDV005), vIL-8 / RLORF2 (MDV078 / MDV003), MDV080, MDV085, US2 (MDV091), US3 (MDV092), SORF4 / S4 (MDV093) and US6 /gD (MDV094) (**Figure 1A, Supplementary Table 1**). In contrast, only 12 variable positions were detected in the three consensus genomes of very virulent strains Md5 (**Supplementary Table 2**). No variable positions were found within ORFs in the Md5 consensus genomes.

The *meq* oncogene contributes significantly to overall intrastrain variation in consensus genomes of CVI988

Out of 240 variable positions detected in the CVI988 consensus genomes (**Supplementary Table 1**), 217 were found in IRL (n = 207 bp) and IRS (n = 10 bp). A large portion of these variable loci (n = 177 bp) corresponded to a 59 amino acid (AA) insertion in the *meq* oncogene, which was found in the CVI988-GenBank and CVI988-USDA consensus genomes but absent in

the CVI988-UK consensus genome (**Figure 2**). For *meq*, previous studies have suggested the existence of up to three isoforms: Meq, Long Meq (L-meq) and Short Meq (S-meq) (39–42). Meq is the standard isoform and is 339 AA in length. L-meq is a longer version of Meq that is characterized by a 59 AA insertion in the transactivation domain (TAD), which is often accompanied by a 3 bp CCA deletion immediately upstream from the insertion site. S-meq is a shorter version of Meq that exhibits a 41 AA deletion in the TAD. The CCA deletion that is typically associated with the presence of the L-meq isoform was confirmed to exist as a subpopulation of considerable size (~ 40%) in the UK CVI988 population, but it did not appear to be linked to the presence of the 177 bp insertion (43). All three Md5 genomes exhibited the standard isoform of *meq* (**Figure 2**), again indicating less diversity than found in the CVI988 genomes.

Increased intrastrain variation in structural repeat regions is associated with greater homopolymer instability

Outside of *meq*, 37 of the 40 remaining variable positions found in structural repeat regions of CVI988 consensus genomes were associated with differences in homopolymer lengths (**Supplementary Table 1**). By contrast, all variable positions found in the unique regions of CVI988 consensus genomes (n = 20) were SNPs, with an even distribution between synonymous (n = 10) and non-synonymous (n = 10) SNPs. In Md5 consensus genomes, all of the 12 variable positions were found in either IRL (n = 7) or IRS (n = 5) and were all associated with differences in homopolymer lengths (**Supplementary Table 2**).

Intrastrain variation in unique regions is associated with 19 unique SNPs in the published CVI988 consensus genome

Out of the 20 SNPs in unique regions of CVI988 consensus genomes, 19 were unique to CVI988-GenBank, including 3 SNPs in UL and 16 SNPs in US (**Figure 1B**). Phylogenomic analysis confirmed divergence of CVI988-GenBank from CVI988-UK and CVI988-USDA (**Figure 3**). To evaluate whether these 19 SNPs could be found in closely related strains, we aligned the three consensus genomes of CVI988 with the consensus genomes of seven co-clustering strains (HNLC503, HC/0803, SY/1209, Hrb/1504, DH/1307, DH/1504, CH/10; all from China) and compared the nucleotides present at these positions. All 19 SNPs remained exclusive to CVI988-GenBank. CVI988-UK, CVI988-USDA and all seven Chinese isolates shared the same nucleotides in all of these positions (**Figure 1B**). These nucleotides also matched the sequences of these 19 positions in the three Md5 genomes included in this study (**Figure 1B**).

Ultra-deep sequencing reveals presence of minor variants in CVI988 and Md5 populations

In addition to consensus-level comparisons, we performed minor variant analysis on the deeply sequenced UK genomes (**Figure 4**, see **Methods** for details). This included UK CVI988 (>10,000 ×) and UK Md5 (>8,500 ×; **Table 1**). A total of 30 minor variant positions were detected in the original UK CVI988 population across a combination of coding and regulatory regions. These minor variants impacted a total of 9 ORFs: MDV011, UL13 (MDV025), UL26 (MDV038), MDV069, MDV072, MDV077, ICP4 (MDV084), US3 (MDV092) and SORF4 / S4 (MDV093) (**Supplementary Table 3).** Similarly, a total of 27 minor variants were detected in

the UK Md5 population. These impacted the following ORFs: UL19 (MDV031), UL41 / VHS (MDV054), UL44 gC (MDV057), UL48 / VP16 (MDV061), UL52 MDV066, MDV069 and MDV072 (**Supplementary Table 4**). Twelve of the 14 minor variant positions found across MDV069 and MDV072 were common to both strains. No minor variants were identified in the US region of the UK Md5 population. Additionally, we verified that no minor variant positions coincided with the 19 unique SNPs in CVI988-GenBank (**Figure 1B**).

Discussion

The goal of comparative genomics is to provide a link between genotype and phenotype. The accuracy with which these two elements are represented is therefore directly proportional to our ability to make biologically relevant conclusions through the use of comparative genomic methods. DNA viruses have long been considered to be inherently stable, especially when compared to RNA viruses, which are now described as mutant swarms, or "quasispecies" (44, 45). This had led to the assumption that viral consensus genomes, which represent the majority nucleotide at any given position, can accurately represent the genotype of MDV strains such as vaccine strain CVI988 and the very virulent strain Md5. As demonstrated here and in prior studies, these and other herpesvirus populations can harbor minor variants both in culture and *in vivo* (46–49). Here, by resequencing and ultra-deep sequencing viral stocks and field isolates of these strains, we have sought to evaluate the impact of conducting interstrain comparisons without accounting for the underlying variation in MDV strains.

CVI988 is an attenuated and non-oncogenic vaccine strain that has long been known to exist as a mixed population due to undergoing multiple passages *in vitro*, in different laboratories and in

multiple vaccine manufacturing companies. There have also been reports suggesting that the effectiveness of CVI988 vaccines can vary depending on the manufacturer, and that even different batches from the same manufacturer can show differences in effectiveness (50, 51). In contrast, Md5 is a virulent field strain of MDV that is currently used as the prototype very virulent (vv) strain in the USDA-ARS-Avian Disease and Oncology Laboratory (ADOL) pathotype classification system (52). As such, the fact that we found 240 variable positions between consensus genomes of CVI988 while only finding 12 variable positions between consensus genomes of Md5 is consistent with their history and real-world biology. Whether the larger number of variant loci in CVI988 reflects the strain's attenuation through serial passage, or if it is a feature of this strain's genetic background, remains to be determined. In addition, the fact that we were able to detect 30 minor variant positions in CVI988-UK and 27 minor variant positions in Md5-UK confirms that both strains exist as mixed viral populations. Over time minor variant differences may expand during passage(s) in culture or during *in vivo* infections, leading to consensus level differences (46, 53). Minor variants may thus provide an explanation for the differences observed between consensus genomes of each strain.

Having access to the minor variant profiles of CVI988 and Md5 has the added advantage of providing insight into the potential effects of intrastrain variation in loci of high interest, such as pp38. Currently available qPCR assays to distinguish between CVI988 and virulent MDV strains are based on a single nucleotide polymorphism (SNP) located at position 320 of the pp38 gene (54). Our results show pp38 to be a highly conserved gene between different populations of CVI988 and Md5, with no minor variant positions coinciding with this gene. This not only confirms the robustness of qPCR assays based on SNP #320 of pp38, but also establishes pp38

as a locus with high levels of within-strain conservation. Overall, our study provides a solid starting point for assessing intrastrain diversity in MDV strains. However, access to additional consensus genomes of CVI988 and Md5 will still be required in order to obtain the complete picture of MDV intrastrain diversity, as well as resequencing of other virulent and attenuated MDV strains.

Among our most significant findings was the discovery of 19 SNPs present in the unique regions of CVI988-GenBank but absent in the two new CVI988 consensus genomes sequenced as part of this study. These 19 SNPs were also not present as minor variants in the CVI988-UK viral population, where these positions were sequenced at an average depth of >10,000 ×. CVI988-GenBank was sequenced by Spatz *et al.* in 2007 from a BAC clone of CVI988 at 6× coverage (**Table 1**). This BAC clone was derived by Petherbridge *et al.* in 2003 from a commercial vaccine supplied by Fort Dodge Animal Health (55). As part of the cloning process a BAC vector was inserted into US2, and the missing region was later reconstructed using PCR and wild-type CVI988 DNA. While explaining the origin of these 19 unique SNPs in CVI988-GenBank is beyond the scope of our current study, our results strongly suggest that consensus genomes of CVI988 stocks do not typically differ from virulent strains such as Md5 in any of these positions.

The implications of these findings are multiple. Firstly, these 19 SNPs have been reported as differences between CVI988 and virulent strains like Md5, RB-1B, GA and Md11 by many prior studies, starting with the original publication by Spatz *et al.* (56–58). Secondly, and perhaps most significantly, the 16 unique SNPs in US of CVI988-GenBank impacting US2, US3, SORF4 and

US6 / gD have been used as the main evidence of naturally occurring recombination between CVI988 and virulent strains, starting in 2018 by He *et al.* (59). Multiple studies have followed, resulting in a total of seven isolates from China being described as natural recombinants of CVI988 on the basis of these 16 SNPs (60–62). Our two new consensus genomes of CVI988 share the same nucleotides in all 16 positions with these seven isolates, implying that they are likely to be field isolates of CVI988 and not natural recombinants as previously described. Notably, the US regions of CVI988-UK and DH/1504 share 100% identity, with all other genomes sharing over 99.8% identity in US. These findings indicate that an overreliance on the published CVI988 consensus genome has led to a historical overestimation of genomic divergence between CVI988 and virulent strains. At the same time, these data challenge previous notions regarding the role of recombination in MDV evolution and highlight the urgent need to use genomic resequencing more widely in order to verify the actual instances of sequence divergence and/or recombination.

Finally, it is worth mentioning that current Illumina-based approaches do not provide insight into certain regions in MDV genomes where additional intrastrain variation is likely to be found. Specifically, tandem repeats have been suggested to be an important source of diversity in herpesviruses yet remain mostly inaccessible to NGS strategies both at the consensus and sub-consensus levels (48, 63). The use of long read technologies (e.g., Oxford Nanopore and Pacific Biosciences) could potentially help to overcome these limitations and make it possible to reliably assess intrastrain variation at tandem repeats and homopolymers (64, 65). In addition, the use of long reads could enhance our ability to link minor variant positions into proper haplotypes and help us to better understand the real-world biology of MDV strains.

In conclusion, by resequencing viral stocks and field isolates of the vaccine strain CVI988 and the very virulent strain Md5, we have shown that consensus genomes of these strains can differ in multiple positions across all genomic regions. We have also established that these differences are most likely made possible by fluctuations in a mixed population of viral genomes, the existence of which we have demonstrated using ultra-deep Illumina sequencing. In addition, we have shown how not accounting for underlying diversity in MDV strains has led to a historical overestimation of divergence between CVI988 and virulent strains and provided evidence that contradicts prior claims of naturally occurring recombination between the two. In doing so, we have demonstrated the value of resequencing MDV strains as part of comparative genomic studies and challenged prevailing notions regarding the magnitude and significance of diversity in DNA viruses.

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Tables

Table 1: Sequencing statistics for new and previously published CVI988 and Md5 consensus genomes

MDV Strain	# MDV-specific reads	# Reads used for assembly	Coverage	GenBank Accession	Genome length (bp)	Prior citation
CVI988	N/A	N/A	6x	DQ530348	178,311	(28)
CVI988-USDA	227,487	138,122	149x	TBD	176,089	-
CVI988-UK	11,997,583*	9,730,216	10,055x	TBD	177,063	-
Md5	N/A	N/A	6x	NC_002229	177,874	(18)
Md5-USDA	1,030,131	727,124	793x	TBD	174,996	-
Md5-UK	10,138,498*	8,296,132	8,530x	TBD	176,079	-

*The UK viral stock populations were subjected to oligo-bait enrichment to increase the yield of viral DNA (32, 33).

Figure Legends

Figure 1: Comparative analysis of CVI988 and Md5 consensus genomes

A) A total of three trimmed genomes (TRL and TRS removed) were obtained for each strain (CVI988 = Blue, Md5 = Red) and aligned using MAFFT. Variable positions including SNPs and Indels for each individual genome are highlighted (dark lines) based on their relative locations in the genome (UL = Unique Long, IRL = Internal Repeat Long, IRS = Internal Repeat Short, US = Unique Short). The total number of variable positions for each individual genome and the total for each strain are indicated on the left; these loci are listed in detail in **Supplementary Table 1**. For each strain, ORFs with variable positions in at least one of the three consensus genomes belonging to that strain are highlighted and labeled (with alternative names when possible). The total number of divergent ORFs for each strain is 12 for CVI988 and zero for Md5. **B)** A total of 19 unique SNPs (UL = 3, US = 16) were identified in CVI988-GenBank. These SNPs were not present in CVI988-UK or CVI988-USDA. They were also not present in seven closely related isolates from China (see **Figure 3**). These SNPs impacted a total of 5 ORFs, including VP22, US2, US3, SORF4 and gD. Md5-GenBank, Md5-UK and Md5-USDA shared nucleotides at these positions with all genomes except CVI988-GenBank.

Figure 2: Comparison of the *meq* oncogene in Md5 and CVI988 consensus genomes

Schematic representation of the location and structure of *meq* isoforms present at consensus level in Md5 (red) and CVI988 (blue) populations. The *meq* oncogene is comprised of the proline/glutamine (Pro/Gln) rich domain, the basic region (BR), the leucine zipper (ZIP) and the

transactivation domain (TAD) at the C-terminal region. Differences in the AA sequence of the two strains are highlighted and labeled (A = Alanine, K = Lysine, S = Serine, E = Glutamic acid). Meq is 339 AA in length. The L-Meq isoform contains a 59-amino acid insertion in the transactivation domain, resulting in a total length of 398 AA.

Figure 3: Neighbor-joining tree of previously published MDV strains and the new consensus genomes of CVI988 and Md5

A total of 29 MDV strains with published consensus genomes in GenBank (**Supplementary Table 5**) were used to infer a neighbor-joining tree using Geneious Prime v2023.0.4. The genomes of each strain used for our comparative analyses are highlighted alongside their respective branches (Md5 = Red, CVI988 = Blue).

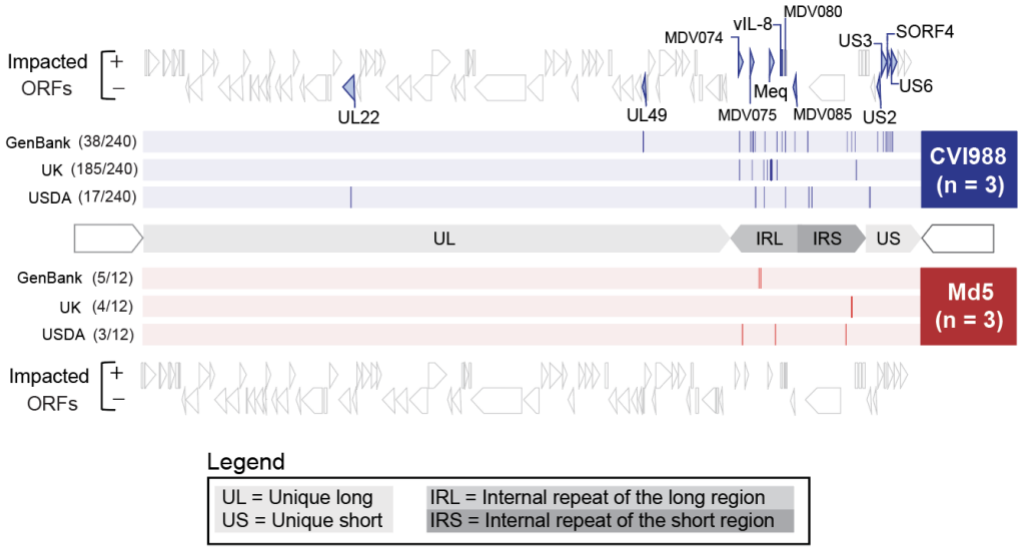
Figure 4: Minor variant distribution in CVI988 and Md5 UK populations

For each of the ultra-deep sequenced viral populations (see **Table 1**), positions where the minor allele was present in ≥ 2% of the reads were identified and classified depending on whether they fell on a coding or non-coding region and whether they resulted in a synonymous or non-synonymous substitution. ORFs associated with minor variant positions of all types are labeled and highlighted beneath the graph using the same criteria. Minor variants corresponding to tandem repeats and homopolymers were excluded. **(A)** Minor variant distribution in the UK CVI988 population. **(B)** Minor variant distribution in the UK Md5 population.

Figures

Figure 1: Comparative analysis of CVI988 and Md5 consensus genomes

A



B

Region	UL			US																
Gene	VP22			US2	US3			SORF4			gD									
Position (bp)	111,859	111,866	111,876	159,254	159,527	159,552	160,197	160,899	161,025	161,269	161,518	161,531	161,709	161,799	162,018	162,065	162,163	162,183	162,218	
CVI988-GenBank	C	C	G	G	C	T	T	C	G	G	T	C	T	A	T	G	C	C	A	
CVI988-UK	A	G	C	A	G	C	C	T	A	A	C	T	C	C	A	A	G	T	T	
CVI988-USDA	A	G	C	A	G	C	C	T	A	A	C	T	C	C	A	A	G	T	T	
DH/1504	A	G	C	A	G	C	C	T	A	A	C	T	C	C	A	A	G	T	T	
SY/1209	A	G	C	A	G	C	C	T	A	A	C	T	C	C	A	A	G	T	T	
Hrb/1504	A	G	C	A	G	C	C	T	A	A	C	T	C	C	A	A	G	T	T	
DH/1307	A	G	C	A	G	C	C	T	A	A	C	T	C	C	A	A	G	T	T	
HC/0803	A	G	C	A	G	C	C	T	A	A	C	T	C	C	A	A	G	T	T	
CH/10	A	G	C	A	G	C	C	T	A	A	C	T	C	C	A	A	G	T	T	
HNLC503	A	G	C	A	G	C	C	T	A	A	C	T	C	C	A	A	G	T	T	
Md5-USDA	A	G	C	A	G	C	C	T	A	A	C	T	C	C	A	A	G	T	T	
Md5-UK	A	G	C	A	G	C	C	T	A	A	C	T	C	C	A	A	G	T	T	
Md5-GenBank	A	G	C	A	G	C	C	T	A	A	C	T	C	C	A	A	G	T	T	

Figure 2: Comparison of the *meq* oncogene in Md5 and CVI988 consensus genomes

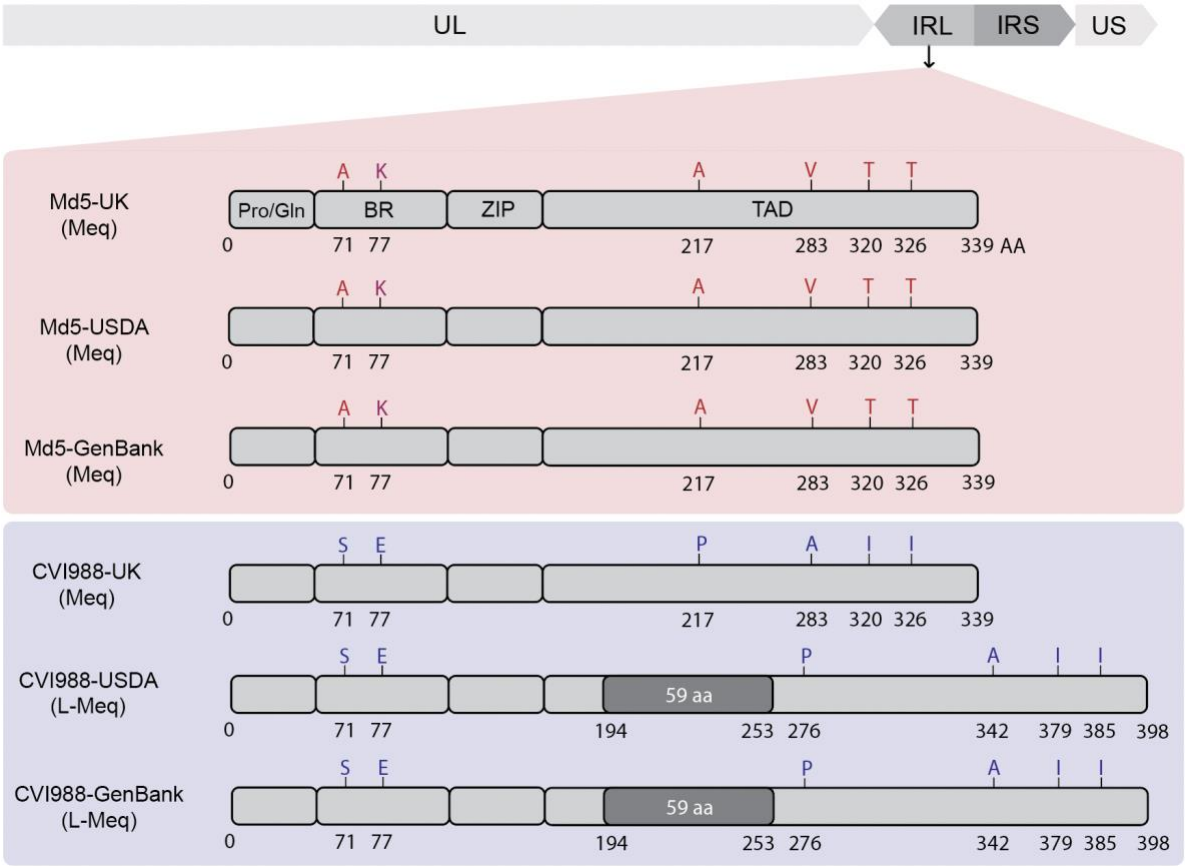


Figure 3: Neighbor-joining tree of previously published MDV strains and the new consensus genomes of CVI988 and Md5

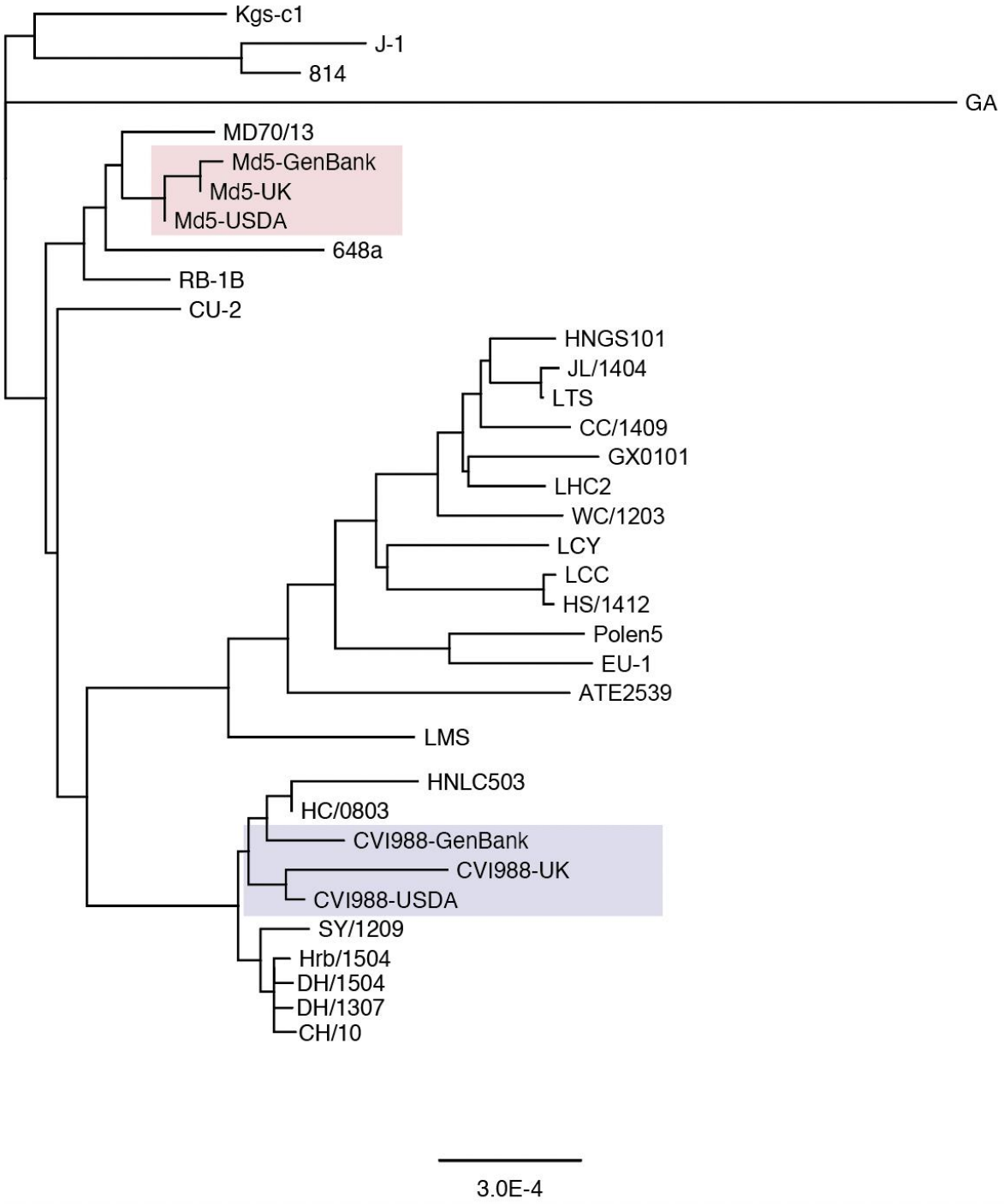
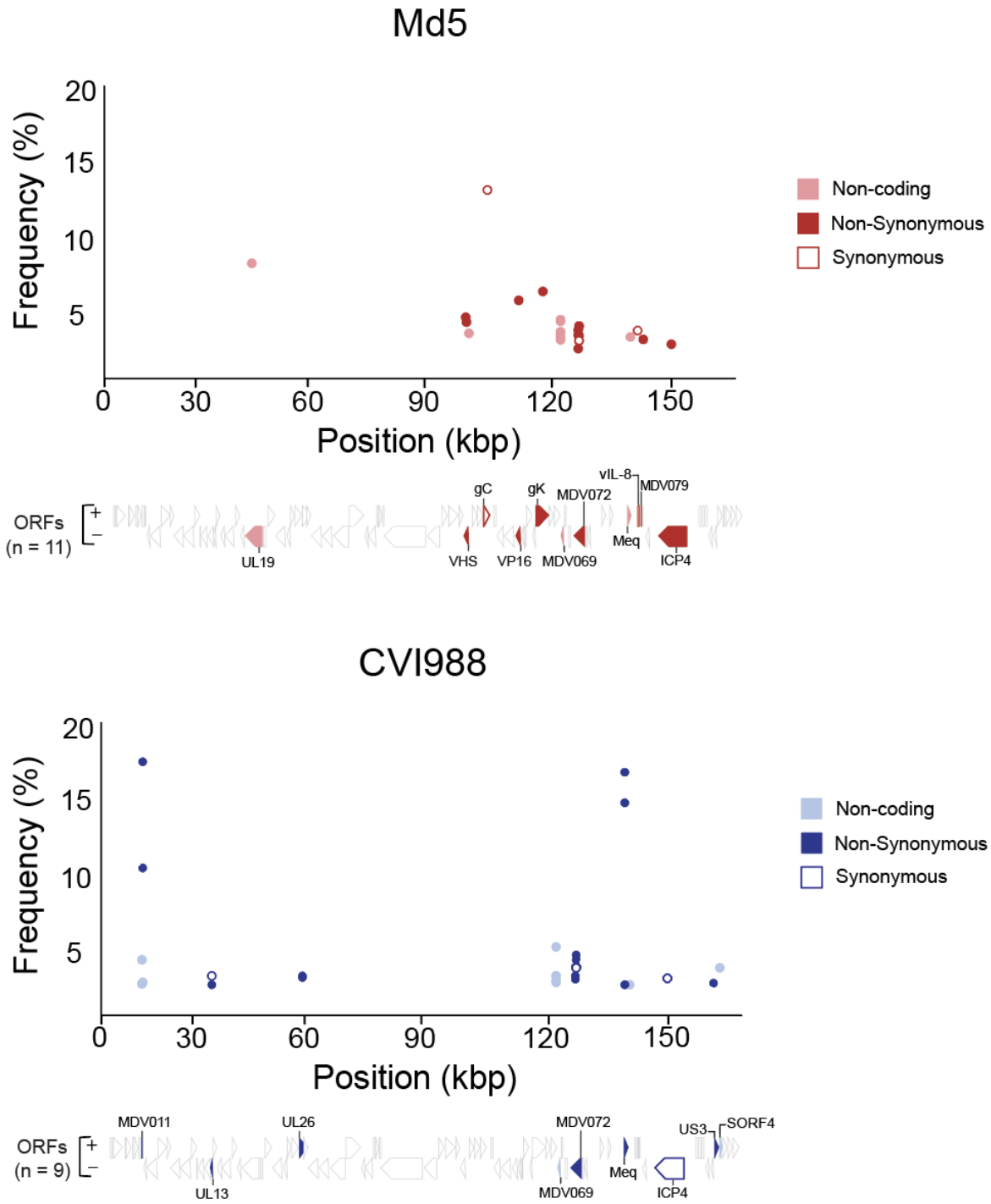


Figure 4: Minor variant distribution in CVI988 and Md5 UK populations



Supplementary Tables

Supplementary Table 1: Variable positions in CVI988 consensus genomes

Genome	ORF	Alternative name	Position	This CVI988 genome	Other CVI988 genomes	Description
USDA	MDV034	UL22 (gH)	52,546	T	C	Synonymous substitution
GenBank	MDV062	UL49 (VP22)	111,859	C	A	Synonymous substitution
GenBank	MDV062	UL49 (VP22)	111,866	C	G	Ser155 -> Cys155
GenBank	MDV062	UL49 (VP22)	111,876	G	C	Ala152 -> Pro152
USDA	MDV074	-	127,041	C	-	Frameshift
USDA	MDV074	-	127,042	G	-	Frameshift
USDA	MDV074	-	127,043	G	-	Frameshift
USDA	MDV074	-	127,044	C	-	Frameshift
USDA	MDV074	-	127,045	T	-	Frameshift
UK	MDV075	-	128,398	-	C	Homopolymer contraction, non-coding
GenBank	MDV075	-	130,655	C	-	Homopolymer extension, non-coding
GenBank	MDV075	-	130,656	C	-	Homopolymer extension, non-coding
USDA	MDV075	-	128,207	T	-	Homopolymer extension, non-coding
USDA	MDV075	-	128,208	T	-	Homopolymer extension, non-coding
USDA	MDV075	-	128,877	-	T	Homopolymer contraction, non-coding
GenBank	MDV075	-	131,742	A	T	Met48 -> Lys48
GenBank	MDV075	-	131,766	C	-	Homopolymer extension, frameshift
GenBank	MDV075	-	131,979	A	-	Homopolymer extension, non-coding
GenBank	MDV075	-	131,980	A	-	Homopolymer extension, non-coding
UK	MDV075	-	129,719	-	A	Homopolymer contraction, non-coding
GenBank	-	-	132,535	T	-	Homopolymer extension
GenBank	-	-	132,536	T	-	Homopolymer extension
GenBank	-	-	134,710	A	-	Homopolymer extension
UK	-	-	132,665	-	C	Homopolymer contraction
USDA	-	-	133,221	G	-	Homopolymer extension
UK	-	-	133,498	-	G	Homopolymer contraction
UK	MDV076	Meq	134,320	-	G…G	Meq isoform lacking 177 bp insertion
UK	-	-	135,147	-	G	Homopolymer contraction
GenBank	-	-	137,581	G	-	Homopolymer extension
UK	-	-	135,235	T	-	Homopolymer extension
GenBank	-	-	137,670	-	TT	Homopolymer contraction
GenBank	-	-	138,647	T	-	Homopolymer extension, frameshift
GenBank	MDV078	vIL-8	140,028	A	-	Homopolymer extension, frameshift
USDA	MDV080	-	138,538	-	T	Homopolymer contraction, frameshift
GenBank	-	-	141,642	A	G	Synonymous substitution
USDA	-	-	139,196	C	T	Synonymous substitution
GenBank	MDV085	-	152,350	C	-	Homopolymer extension, frameshift
GenBank	MDV085	-	152,351	C	-	Homopolymer extension, frameshift
USDA	-	-	150,420	A	-	Homopolymer extension
USDA	-	-	150,421	A	-	Homopolymer extension

Genome	ORF	Alternative name	Position	This CVI988 genome	Other CVI988 genomes	Description
USDA	-	-	150,422	A	-	Homopolymer extension
GenBank	MDV086	-	153,340	A	-	Homopolymer extension, frameshift
UK	-	-	151,727	-	T	Homopolymer contraction
GenBank	-	-	154,385	C	-	Homopolymer extension
USDA	-	-	151,853	C	A	Synonymous substitution
USDA	-	-	152,098	-	G	Homopolymer contraction
GenBank	MDV091	US2	159,254	G	A	Val51 -> Ala51
GenBank	MDV092	US3	159,527	C	G	Ser4 -> Thr4
GenBank	MDV092	US3	159,552	T	C	Synonymous substitution
GenBank	MDV092	US3	160,197	T	C	Synonymous substitution
GenBank	MDV093	SORF4	160,899	C	T	Leu22->Ser22
GenBank	MDV093	SORF4	161,025	G	A	Tyr64 -> Cys64
GenBank	MDV093	SORF4	161,269	G	A	Synonymous substitution
GenBank	MDV094	US6 (gD)	161,518	T	C	Synonymous substitution
GenBank	MDV094	US6 (gD)	161,531	C	T	Ile29 -> Thr29
GenBank	MDV094	US6 (gD)	161,709	T	C	Synonymous substitution
GenBank	MDV094	US6 (gD)	161,799	A	C	Synonymous substitution
GenBank	MDV094	US6 (gD)	162,018	T	A	Synonymous substitution
GenBank	MDV094	US6 (gD)	162,065	G	A	Glu207 -> Gly207
GenBank	MDV094	US6 (gD)	162,163	C	G	Gly240 -> Arg240
GenBank	MDV094	US6 (gD)	162,183	C	T	Synonymous substitution
GenBank	MDV094	US6 (gD)	162,218	A	T	Ile258 -> Asn258

Supplementary Table 2: Variable positions in Md5 consensus genomes

Genome	ORF	Position	Consensus	Call	Description
USDA	-	127,709	C	-	Homopolymer contraction
GenBank	-	129,507	-	A	Homopolymer extension
GenBank	-	129,508	-	A	Homopolymer extension
GenBank	-	129,588	-	T	Homopolymer extension
GenBank	-	130,253	-	T	Homopolymer extension
GenBank	-	134,602	-	G	Homopolymer extension
USDA	-	132,800	G	-	Homopolymer contraction
USDA	-	149,305	C	-	Homopolymer contraction
UK	-	152,067	C	-	Homopolymer contraction
UK	-	152,227	T	-	Homopolymer contraction
UK	-	152,227	G	-	Homopolymer contraction
UK	-	152,239	T	-	Homopolymer contraction

463 **Supplementary Table 3: Minor variants in UK CVI988**

Position in the genome	Major allele	Minor allele	Minor allele frequency	Major allele # forward reads	Major allele # reverse reads	Minor allele # forward reads	Minor allele # reverse reads	ORF	Type
3018	T	C	4.65%	5651	2646	275	130	MDV011	Upstream non-coding
3021	G	T	2.22%	5817	2636	126	66	MDV011	Upstream non-coding
3056	A	G	3.21%	5990	6705	199	222	MDV011	Upstream non-coding
3095	A	G	17.30%	4691	5465	919	1206	MDV011	Non-synonymous (Thr2 -> Ala2)
3204	A	G	10.06%	2353	2384	266	264	MDV011	Non-synonymous (Glu38->Gly38)
22747	A	T	2.65%	2845	3416	63	108	MDV025	Synonymous
22749	T	G	2.09%	2853	3418	46	88	MDV025	Non-synonymous (Thr265->Pro265)
45308	G	T	2.55%	4836	3741	72	153	MDV038	Non-synonymous (Ala239->Ser239)
45326	A	C	2.70%	4512	2768	61	141	MDV038	Non-synonymous (Thr245->Pro245)
108639	G	A	2.11%	6326	5563	138	119	MDV069	Upstream non-coding
108648	G	A	2.26%	6323	5494	151	123	MDV069	Upstream non-coding
108691	A	G	2.34%	5802	5540	145	128	MDV069	Upstream non-coding
108705	T	C	2.66%	3553	5549	127	122	MDV069	Upstream non-coding
108707	T	G	2.66%	3552	5519	122	126	MDV069	Upstream non-coding
109134	A	G	2.43%	4877	5946	116	154	MDV072	Non-synonymous (Leu875->Pro875)
109143	A	G	2.66%	4802	5647	123	163	MDV072	Non-synonymous (Val872->Ala872)
109186	G	A	3.76%	3676	4473	141	178	MDV072	Non-synonymous (His858->Tyr858)
109200	C	T	3.25%	3332	4093	81	169	MDV072	Non-synonymous (Cyst853->Tyr853)
109202	G	T	3.27%	3270	4023	80	167	MDV072	Synonymous
109263	T	G	4.08%	2886	2890	84	162	MDV072	Non-synonymous (His832->Pro832)
109298	C	T	3.28%	2684	2155	51	113	MDV072	Non-synonymous (Trp820->STOP)
121037	A	G	14.59%	25181	19947	5447	2316	MDV076	Non-synonymous (Thr180->Ala180)
121045	A	G	16.71%	23815	19819	6391	2431	MDV077	Non-synonymous (Phe111->Leu111)
121070	C	-	58.08%	29714	19086	14302	14301	MDV076	Deletion
121071	C	-	57.02%	30172	19826	14287	14286	MDV076	Deletion
121072	A	-	57.12%	29125	19029	14288	14287	MDV076	Deletion
124200	T	C	2.20%	3776	3761	86	84	MDV077	Upstream non-coding
131065	T	C	2.60%	11669	13033	441	219	MDV084	Synonymous
144079	A	G	2.16%	6886	5749	157	123	MDV092	Non-synonymous (Glu54->Gly54)
150529	A	G	3.79%	252	271	7	18	MDV093	Downstream non-coding

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Supplementary Table 4: Minor variants in UK Md5

Position in the genome	Major allele	Minor allele	Minor allele frequency	Major allele # forward reads	Major allele # reverse reads	Minor allele # forward reads	Minor allele # reverse reads	ORF	Type
36117	A	G	7.89%	3400	3374	278	303	MDV031	Non-coding
84011	T	G	4.15%	2275	1949	107	76	MDV054	Non-synonymous (His136->Pro136)
84098	G	T	3.82%	2575	1105	106	40	MDV054	Non-synonymous (Thr107->Asn107)
84812	T	C	3.09%	1091	3673	33	119	MDV054	Non-coding
88814	A	G	12.92%	3903	2728	590	397	MDV057	Synonymous
96655	C	T	5.32%	4509	2255	259	121	MDV061	Non-synonymous (Asp35->Asn35)
100749	G	A	5.96%	2422	4690	229	222	MDV066	Non-synonymous (Val192->Ile192)
108784	G	A	2.61%	4183	3701	53	158	MDV069	Non-coding
108793	G	A	2.80%	4122	3688	65	160	MDV069	Non-coding
108836	A	G	3.17%	3769	3694	68	177	MDV069	Non-coding
108850	T	C	3.90%	2128	3659	63	172	MDV069	Non-coding
108852	T	G	4.03%	2122	3624	63	179	MDV069	Non-coding
108914	G	T	3.05%	2982	3659	51	158	MDV069	Non-coding
109246	G	A	2.01%	3501	4391	92	70	MDV072	Non-synonymous (Ala886->Val886)
109279	A	G	2.97%	3503	4076	142	90	MDV072	Non-synonymous (Leu875->Pro875)
109288	A	G	3.29%	3495	3995	158	97	MDV072	Non-synonymous (Val872->Ala872)
109331	G	A	3.57%	2986	3374	151	85	MDV072	Non-synonymous (His858->Tyr858)
109345	C	T	2.55%	2852	3217	78	81	MDV072	Non-synonymous (Cyst853->Tyr853)
109347	G	T	2.57%	2803	3185	76	82	MDV072	Synonymous
109408	T	G	3.58%	2753	2417	110	82	MDV072	Non-synonymous (His832->Pro832)
109443	C	T	2.87%	2726	2034	90	51	MDV072	Non-synonymous (Trp820->STOP)
118716	C	T	2.68%	6309	4595	161	139	MDV076	Upstream non-coding
124098	G	A	3.12%	5261	3764	168	123	MDV078	Synonymous
125178	G	C	2.55%	158	301	4	8	MDV079	Non-synonymous (Ala107->Pro107)
131736	T	G	2.25%	6893	3297	137	98	MDV084	Non-synonymous (Glu1725->Ala1725)
134220	G	A	21.44%	3490	2598	963	699	MDV084	Non-synonymous (Ala897->Val897)
136722	T	C	31.19%	7233	6650	3294	3002	MDV084	Non-synonymous (Gln63->Arg63)

Supplementary Table 5: Published reference genomes for additional 29 strains used to construct NJ tree

Strain	Accession	Publication Date	Length	PMID	Region
Kgs-c1	LC589272	15-Oct-20	174,999	33228722	Asia
J-1	KU744555	18-Feb-17	176,118	27112385	Asia
814	JF742597	23-Feb-12	172,541	21984218	Asia
GA	AF147806	29-Jun-04	174,077	10823954	North America
MD70/13	MF431495	12-Dec-17	177,844	29151863	Europe
648a	JQ806361	4-Apr-13	176,080	22923089	North America
RB-1B	EF523390	29-Oct-07	178,246	17721813	North America
CU-2	EU499381	5-Aug-08	176,922	18351449	North America
HNGS101	MG432697	6-Mar-18	175,888	29407378	Asia
JL/1404	KU744559	18-Feb-17	176,083	27112385	Asia
LTS	KU744557	18-Feb-17	176,023	27112385	Asia
CC/1409	KU744560	18-Feb-17	175,561	27112385	Asia
GX0101	JX844666	3-Dec-12	178,101	23166235	Asia
LHC2	MW247189	19-Apr-22	177,327	35266322	Asia
WC/1203	KU744558	18-Feb-17	176,057	27112385	Asia
LCY	KX290013	12-Apr-17	175,319	28194622	Asia
LCC	KU744556	18-Feb-17	175,525	27112385	Asia
HS/1412	KU744561	18-Feb-17	175,532	27112385	Asia
Polen5	MF431496	12-Dec-17	177,821	29151863	Europe
EU-1	MF431494	12-Dec-17	177,828	29151863	Europe
ATE2539	MF431493	12-Dec-17	177,868	29151863	Europe
LMS	JQ314003	1-Aug-12	177,526	22476905	Asia
HNLC503	MG518371	29-Apr-19	178,195	31698116	Asia
HC/0803	MW531728	10-Jul-22	174,808	35266322	Asia
SY/1209	MW247208	19-Apr-22	177,240	35266322	Asia
Hrb/1504	MW247204	19-Apr-22	177,839	35266322	Asia
DH/1307	MW247201	19-Apr-22	177,235	35266322	Asia
DH/1504	MW247202	19-Apr-22	177,991	35266322	Asia
CH/10	MW247200	19-Apr-22	177,401	35266322	Asia

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