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The landscape of persistent human DNA viruses in femoral bone

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Abstract

The imprints left by persistent DNA viruses in the tissues can testify to the changes driving virus evolution as well as provide clues on the provenance of modern and ancient humans. However, the history hidden in skeletal remains is practically unknown, as only parvovirus B19 and hepatitis B virus DNA have been detected in hard tissues so far. Here, we investigated the DNA prevalences of 38 viruses in femoral bone of recently deceased individuals. To this end, we used quantitative PCRs and a custom viral targeted enrichment followed by next-generation sequencing. The data was analyzed with a tailor-made bioinformatics pipeline. Our findings revealed bone to be a much richer source of persistent DNA viruses than earlier perceived, discovering ten additional ones, including several members of the herpes- and polyomavirus families, as well as human papillomavirus 31 and torque teno virus. Remarkably, many of the viruses found have oncogenic potential and/or are likely to reactivate in the elderly and immunosuppressed individuals. Thus, their persistence warrants careful evaluation of their clinical significance and impact on bone biology. Our findings open new frontiers for the study of virus evolution from ancient relics as well as provide new tools for the investigation of human skeletal remains in forensic and archeological contexts.

Introduction

DNA viruses commonly establish persisting infections in humans, remaining thus, their genetic material imprinted in the tissues throughout life. These DNAs exhibit phylogeographies resembling those of humans[1], pointing to shared evolutionary and dispersal paths that can, together, add to the understanding of human population history and migrations[2–8]. In addition, the specific geographical distributions of DNA viruses, in both global[2,9] and local[10–12] scales, may provide new insights into the origins of unidentified individuals.

In this regard, the most widely studied virus is JC polyomavirus (JCPyV), a highly prevalent pathogen with three main phylogenetic clusters spread across Africa, Asia, and Europe. JCPyV´s genotype-specific global spread has been proposed as an indicator of the provenances of both modern[12] and ancient humans[13–15]. Although its timescale of evolution has been debated[16–18], recent work by Forni et al.,[8] based on ~1100 worldwide strains, supports co-dispersal of this virus with major human migratory routes as well as its co-divergence with human mitochondrial and nuclear markers.

In addition to the spatial and temporal dimensions, viruses also display intriguing age-dependent distributions[19–21]. This is the case with genotype 2 of parvovirus B19, the traces of which went missing in Europe around 1970s. Thus, contemporary DNA findings of this virus variant in tissues are confined to elderly individuals or historic human remains[22].

Despite these encouraging data, the utility of the viral DNAs has not been fully addressed in forensic and anthropological settings. Reasons for this include the lack of a comprehensive picture of the overall distribution of viral DNAs in various organs, limiting the use of some human tissues in versatile scenarios, and most importantly, the fact that their prevalences in bones and teeth are almost entirely unknown.

Of the latter, we were the first to detect parvovirus B19 DNA in bones from soldiers of World War II[23], followed by findings of this same virus[24] and of hepatitis B[25,26] virus in archaeological remains dated from the Neolithic to medieval times. These singular discoveries confirm the presence and preservation of viral DNA in hard tissues and call for the investigation of the full landscape of viral DNAs that here persist.

In this study, we searched for 38 highly prevalent DNA viruses in the femoral bones of recently deceased individuals. To this end, we used in-house quantitative PCRs (qPCRs) and virus-targeted enrichment coupled with next-generation sequencing (NGS).

Our findings significantly expand the present knowledge on the virome of human bone, opening new frontiers for the study of virus evolution as well new tools for the investigation of forensic and anthropological cases.

Materials and methods

Study subjects

The study cohort consisted of 27 deceased individuals of Finnish origin, aged 36 to 85 years (mean 67.6) with a male:female ratio of 19:8. The postmortem interval ranged from 4 to 30 days (mean 8). The manners of death were disease (n=19), occupational disease (n=1), injury (n=5), and suicide (n=2). In none of the cases the medical records revealed the use of immunosuppressants or pre-conditions related to infectious-diseases, except for one with a history of herpes-zoster a few weeks before death. The mode and cause of death are presented in Figure 2. The study protocol was reviewed by the Ethics Committee of Helsinki and Uusimaa Hospital District (approval 164/13/03/00/2014).

Specimen collection and preparation

A 5-10 cm piece of the femoral diaphysis was collected and cut longitudinally with an oscillating tip saw. The bone was cleaned using a toothbrush and washed sequentially in distilled water, 0.1 % sodium hypochlorite, and 96.1 w/w ethanol. The bones were let to dry for 5 to 7 days at room temperature under flow in a laminar hood. From each individual, the external and internal surfaces of femoral bone were sampled using a dentist drill (Schick Qube) except for three cases, in which the bone was cryomilled (Spex 6775 Freezer/mill; Spex). The sample preparations and drillings were performed in enclosed dedicated facilities, using full-body protection suits.

DNA extraction

Approximately 0.1-0.4g of bone powder were lysed and extracted as specified before[23] and eluted in a final volume of 50-100 µl. The DNA extractions were performed in enclosed facilities, dedicated to the extraction of human DNA. No viral work is performed in these rooms.

The total DNA was quantitated with Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific).

The quality of the total DNA was evaluated with the Genomic DNA Reagent Kit in a LabChip GX Instrument (Perkin Elmer).

Quantitative PCRs

The quantifications of parvovirus B19, Merkel cell, JC and BK polyomaviruses, and nine human herpesviruses as well as of the human single-copy gene RNase P were performed with in-house quantitative PCRs (qPCRs) as described[27–30] [Pyöriä *et al.* in press]. The quantification of hepatitis B virus and human papillomavirus type 31 DNAs were performed with commercial kits (Hepatitis B Virus PCR Kit, GeneProof; Genesig Human papillomavirus 31 Standard kit, PrimerDesign) according to the manufacturer instructions.

For the quantification of torque teno virus, a qPCR was optimized[31,32] to amplify and detect the conserved untranslated region (UTR) of the virus using the following primers: AMTS fwd (5'-GTGCCGNAGGTGAGTTTA-'3), AMTAS rev (5'-AGCCCGGCCAGTCC-'3), AMTASgr4 rev (5'-AGCCCGGCCAGACC-'3) and AMTPTU probe (5'-FAM-TCAAGGGGCAATTCGGGCT-BHQ1-'3). The qPCR reaction consisted of 1x Maxima probe qPCR Master mix (Thermo Fisher Scientific) with 0.03 µM of ROX passive reference dye, 0.5 µM of each of the primers, 0.4 µM of the probe, 5 µl of the template, and nuclease-free water to a final volume of 25 µl. After initial denaturation at +95°C for 10 min, the qPCR cycles were +95 °C for 15 s and +55 °C for 1 min for 45 cycles.

The qPCR amplicons were 63 to 154 nucleotides in length.

Plasmid dilution series were used in all the qPCR runs as positive controls and to create standard curves for quantification. The plasmids of parvovirus B19, human herpesvirus 1-8, Merkel cell, JC and BK polyomaviruses, and RNaseP are described elsewhere[33][Pyöriä *et al.* in press]. For torque teno virus, a plasmid, named 10B, containing 1184 nucleotides of the virus was cloned from a healthy blood donor's plasma (GenBank MT448658).

The virus amplifications were completed with AriaMx Real-Time PCR System except for those of torque teno virus, RNaseP, and human papillomavirus 31 that were analyzed with Stratagene Mx3005P qPCR System (both Agilent).

The qPCR mixes, sample handling, plasmid dilutions, and amplifications, were performed each in completely separate rooms, following strict protocols and work-flows to prevent contamination. Negative controls (PCR-grade water) were included in every step starting from the DNA extraction.

PCR inhibition tests

PCR efficiency due to carryover of inhibitors following DNA extraction was controlled using DNA extracts from bone together with or in parallel to a pre-quantified RNaseP plasmid[33].

To evaluate the performance of different polymerases and the quantification accuracy, four different commercial master mixes were tested. From this, Maxima probe qPCR master mix was selected and used throughout the study.

The impact of residual EDTA on the qPCR performance was examined by testing varying EDTA concentrations in the lysis buffer (0.5 to 500 nM) and by adding excess EDTA (0.05 to 50 nM) to the qPCR reaction. In addition, pre-quantified plasmid dilutions were extracted following the bone extraction protocol (i.e. lysis buffer containing 500 nM EDTA).

The impact of residual Ca^{2+}-ions from bone in the extracts was investigated by addition of $MgCl_2$ to the qPCR reactions (in final concentrations of 4 to 7 mM).

Library preparation, viral enrichment, and sequencing

The sequencing libraries were prepared on 10 to 1000 ng of total DNA using the KAPA HyperPlus library preparation kit (Roche), following the manufacturer protocol with two modifications: 1) mechanical fragmentation with a Covaris E220 of the DNA with target fragments of 200 nt and 2) the use of xGen Dual Index UMI Adapters (Integrated DNA Technologies).

After sonication, the fragment length distributions were analyzed with the DNA High Sensitivity Reagent Kit in a LabChip GX Instrument (Perkin Elmer).

The viral enrichment was performed using two consecutive rounds of hybridization on individual samples following recommendations for low input DNA (MyBaits v4 kit; Arbor Biosciences). For each library, 200 ng per round of biotinylated RNA-baits were used. The baits were 100 nt in length and designed with 2X tiling (Supplementary Table 1 for a list of viruses). xGen Universal Blockers-TS Mix (Integrated DNA Technologies) were used to block unspecific binding to the adapters during hybridization.

During library preparation and viral enrichment, the libraries were amplified 3x13-25 cycles. The clean-up steps were performed with either KAPA Pure Beads (Roche) or MinElute PCR Purification Kit (Qiagen).

The enriched libraries were quantified with KAPA Library Quantification Kit (Roche) using Stratagene 3005P qPCR System (Agilent) and subsequently pooled for sequencing on NovaSeq 6000 (SP PE151 reagent kit; Illumina).

NGS data analysis

The viral genomic sequences were reconstructed after removal of PCR duplicates, using a customized bioinformatics pipeline (TRACESPipe; [Pratas *et al.* in revision; available for download at https://github.com/viromelab/tracespipe).

The consensus, as well as single sequences (when in low coverage), were confirmed by BLAST (NCBI). The highest similarity was used to classify the virus genotype. For parvovirus B19, the sequences covering >70 % of the viral genome (n=7) were aligned with previously published full or near-full length sequences in EMBL-EBI Clustal Omega and analyzed with Bioedit v.7.2.5 (Ibis Biosciences).

For the following viruses, the consensus sequences will be available in GenBank with respective accession numbers: parvovirus B19 (7 sequences; MT410184-MT410190); human papillomavirus type 31 (MT410191); hepatitis B virus (MT410192); Merkel cell polyomavirus (MT410193).

Statistical analysis

The differences in viral or cell copy numbers in the external and internal surfaces of femoral bone were calculated with Student's t-test in RStudio (version 1.0.153).

Results

Viral DNA prevalences in the external and internal surfaces of the femoral bone.

To determine the most optimal site for sampling, we first investigated the differences in prevalence and quantity of persisting viral DNAs in the external and internal surfaces of 27 femoral bones (Fig 1). To this end, we examined three ubiquitous viruses (torque teno, parvovirus B19, and Epstein-Barr virus) known to infect >70% of the global population and to persist in several soft tissues in the body. To control for bias by uneven amounts of DNA in the extracts, we performed in parallel a qPCR for the human single-copy gene RNAse P. We found no significant differences in the viral DNA prevalences ($p>0.1$) nor copy numbers (p values >0.5) between these two surfaces. Hence, subsequent analyses were performed only on samples taken from the external surface.

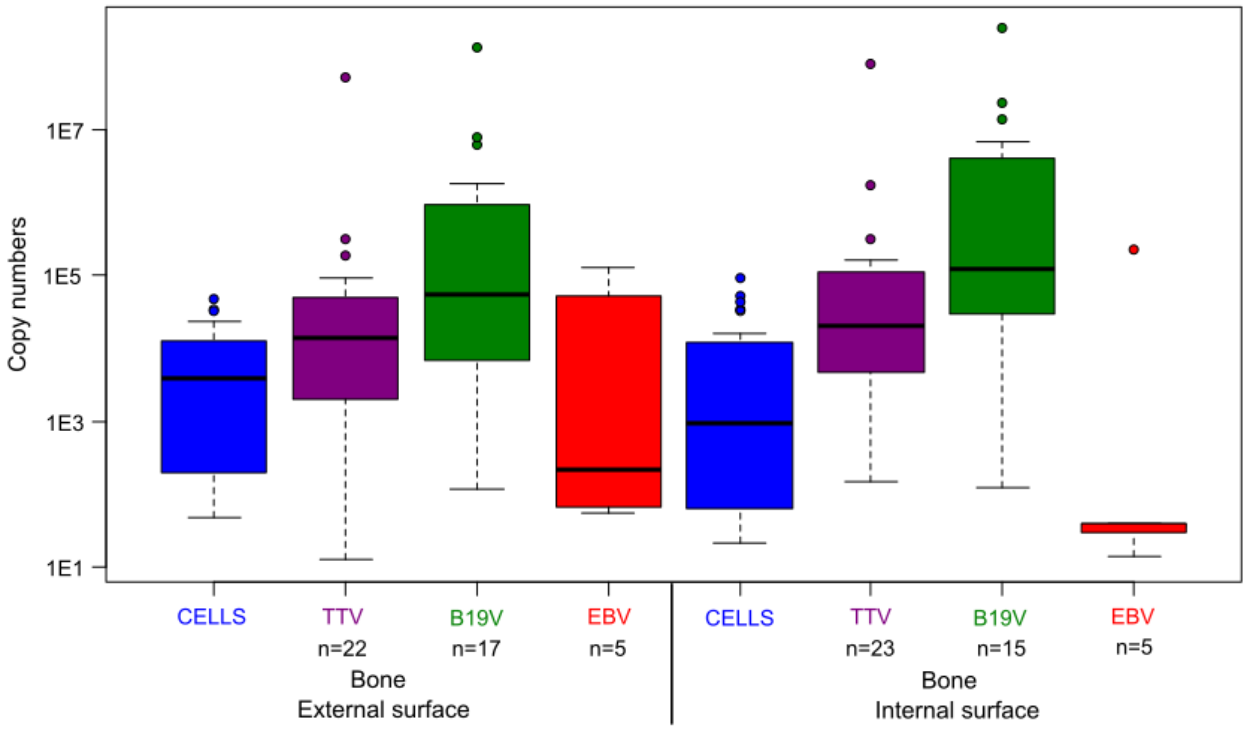


Fig 1. Viral loads in the external and internal surfaces of the femoral bone. The numbers of cells as well as the DNA copies of torque teno virus (TTV), parvovirus B19 (B19V), and Epstein-Barr virus (EBV) from the external and internal surfaces of femoral bone were determined. No significant differences between these two locations were observed ($p> 0.5$). The cell counts are expressed per 1 µl of DNA extract and the viral DNA copy numbers per 1E6 cells.

Viruses are highly prevalent in human femoral bone

We then investigated the prevalences of altogether 38 persistent virus genomes using targeted enrichment and confirmatory qPCRs. Overall, the 27 study subjects harbored on average 2.6 virus-

types in their femoral bones, with a maximum of seven in one individual. Altogether, we detected 12 different virus-types in 92.6 % of the bones, with only two individuals (> 60 years of age) being completely negative for all viruses tested. The viral findings are presented in Table 1 and Figure 2.

The viruses most prevalent in bone were torque teno virus and parvovirus B19 with genoprevalences of 81.5% and 74.1%, respectively (Table 1). The third most prevalent was Merkel cell polyomavirus at 33.3%, followed by Epstein-Barr virus (25.9%), human papillomavirus (22.2%), human herpesvirus 7 (18.5%) and JC polyomavirus (14.8%). Other viral sequences detected were of herpes simplex 1, varicella-zoster, cytomegalovirus, human herpesvirus 6B, and hepatitis B virus.

The median viral copy numbers per one million cells were 1.9E4 for torque teno virus, 2.4E3 for parvovirus B19, 8.7E3 for Merkel cell polyomavirus, and 2.1E1 for Epstein-Barr virus (Table 1).

The most common co-occurrences were of parvovirus B19 and torque teno virus, found in 70.3% of the samples. Interestingly, we detected in the bone of one individual three cancer-associated viruses: hepatitis B virus, human papillomavirus type 31, and Merkel cell polyomavirus.

Table 1. Viral DNA prevalence in human femoral bone

Family	Virus	Genome size (kb)	Geno-prevalence	NGS+ cases	qPCR+ cases	Breadth coverage (range or value of single sample)	Viral load (copies/ million cells)
Parvoviridae	B19V	5.6	74.1 %	17	16	4.5-100.0 %	2.4E3
Herpesviridae	HSV1	152	3.7 %	1	1	3.4 %	4.9E1
	VZV	125	3.7 %	1	1	13.6 %	2.0E0
	EBV	170	25.9 %	6	4	1.0-3.3 %	2.1E1
	CMV	236	3.7 %	0	1	-	9.8E1
	HHV6B	162	11.1 %	3	0	2.0-3.1 %	-
	HHV7	150	18.5 %	5	0	4.8-12.0 %	-
Polyomaviridae	JCPyV	5.1	14.8 %	3	2	3.7-14.8 %	2.7E1
	MCPyV	5.4	33.3 %	9	4	1.4-58.6 %	8.7E3
Papillomaviridae	HPV	8	22.2 %	6	1*	1.8-89.6 %	8.7E3
Hepadnaviridae	HBV	3.2	7.4 %	2	1	15.6-44.5 %	1.7E3
Anelloviridae	TTV	3.8	81.5 %	9	22	2.1-56.3 %	1.9E4

* Only HPV type 31 qPCR was performed. B19V: parvovirus B19; HSV1: herpes simplex virus-1; VZV: varicella-zoster virus; EBV: Epstein-Barr virus; CMV: cytomegalovirus; HHV6B: human herpesvirus 6B; HHV7: human herpesvirus 7; JCPyV: JC polyomavirus; MCPyV: Merkel cell polyomavirus; HPV: human papillomavirus; HBV: hepatitis B virus; TTV: torque teno virus

We verified the accuracy of the qPCR results by examining the patterns of DNA fragmentation in the extracts and by evaluating the impact of potential inhibitors carried over during lysis and extraction. The quality of the total DNA in each sample was analyzed with a LabChip GX Instrument, which revealed 100 to 500 nt fragments in addition to intact genomic DNA (≤40 kb; Fig 3). We found no PCR inhibition accountable to excess EDTA or Ca^{2+} in the extracts.

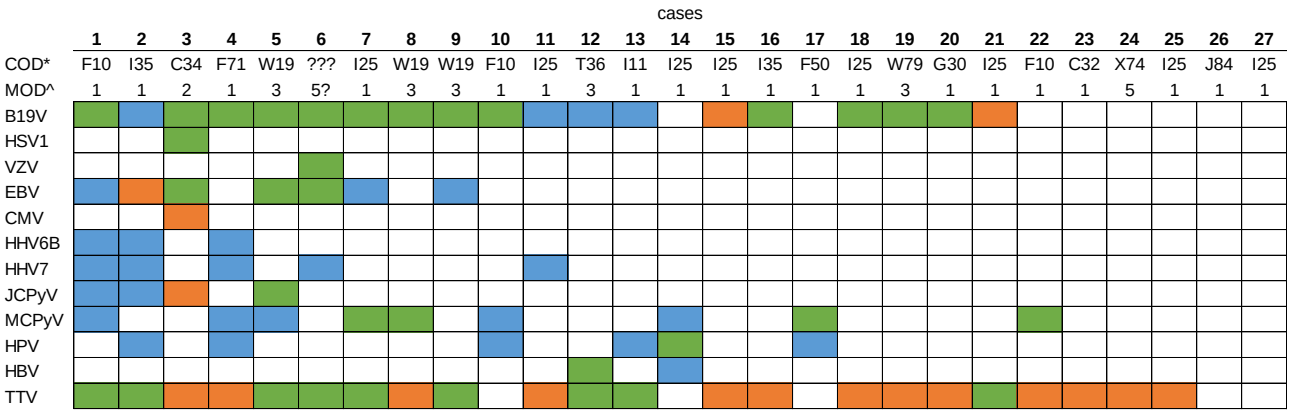


Fig 2. Viral DNA occurrence by NGS and qPCR in bone per study subject. The viral findings are presented as green (NGS+, qPCR+), yellow (NGS+, qPCR-), orange (NGS-, qPCR+), and white (NGS-, qPCR-). *The cause of death (COD) is given according to the WHO ICD10 classification. ^The manner of death (MOD) is presented as 1=disease, 2=occupational disease, 3=injury, 5=suicide. The results are presented from left to right according to the highest and lowest virus prevalences in bone. B19V= parvovirus B19; HSV1=herpes simplex virus-1; VZV= varicella-zoster virus; EBV= Epstein-Barr virus; CMV= cytomegalovirus; HHV6B= human herpesvirus 6B; HHV7= human herpesvirus 7; JCPyV= JC polyomavirus; MCPyV= Merkel cell polyomavirus; HPV= human papillomavirus; HBV= hepatitis B virus; TTV= torque teno virus.

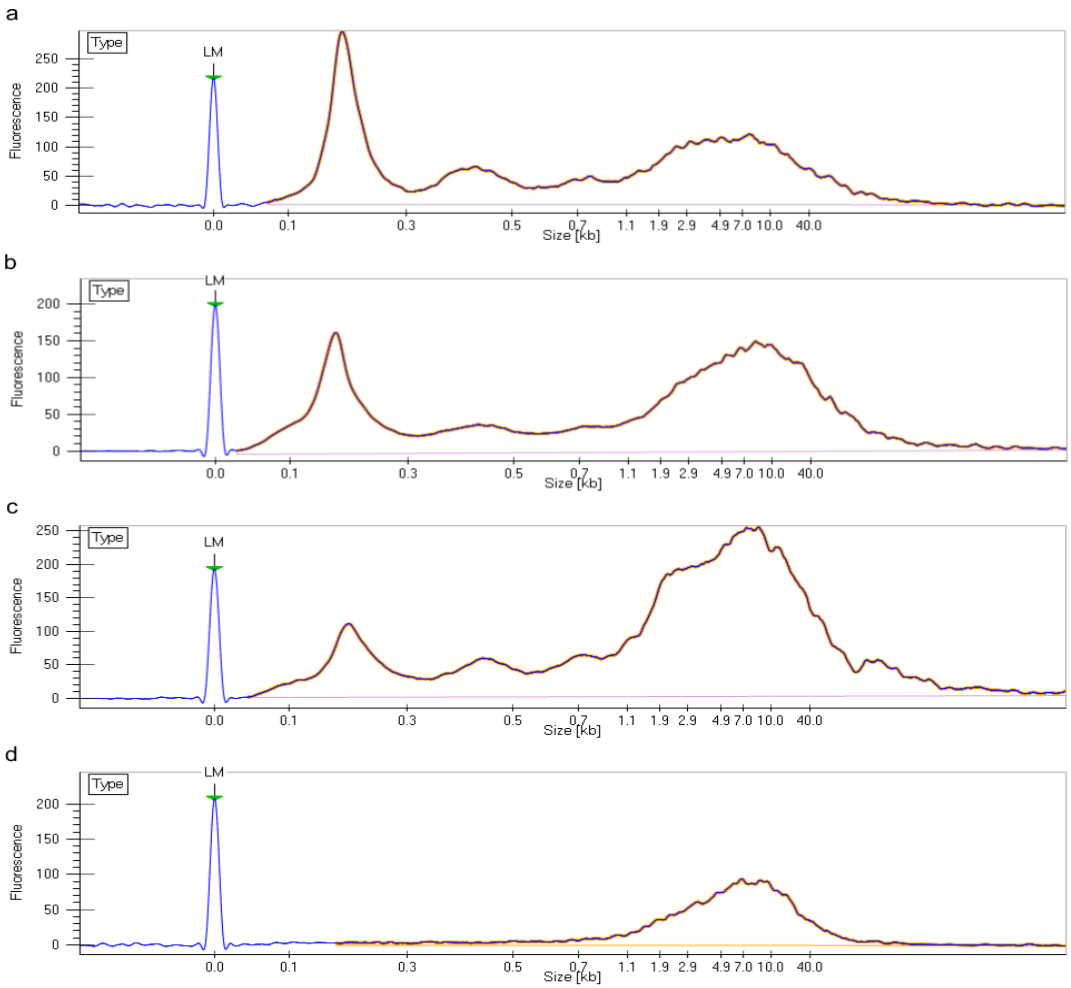
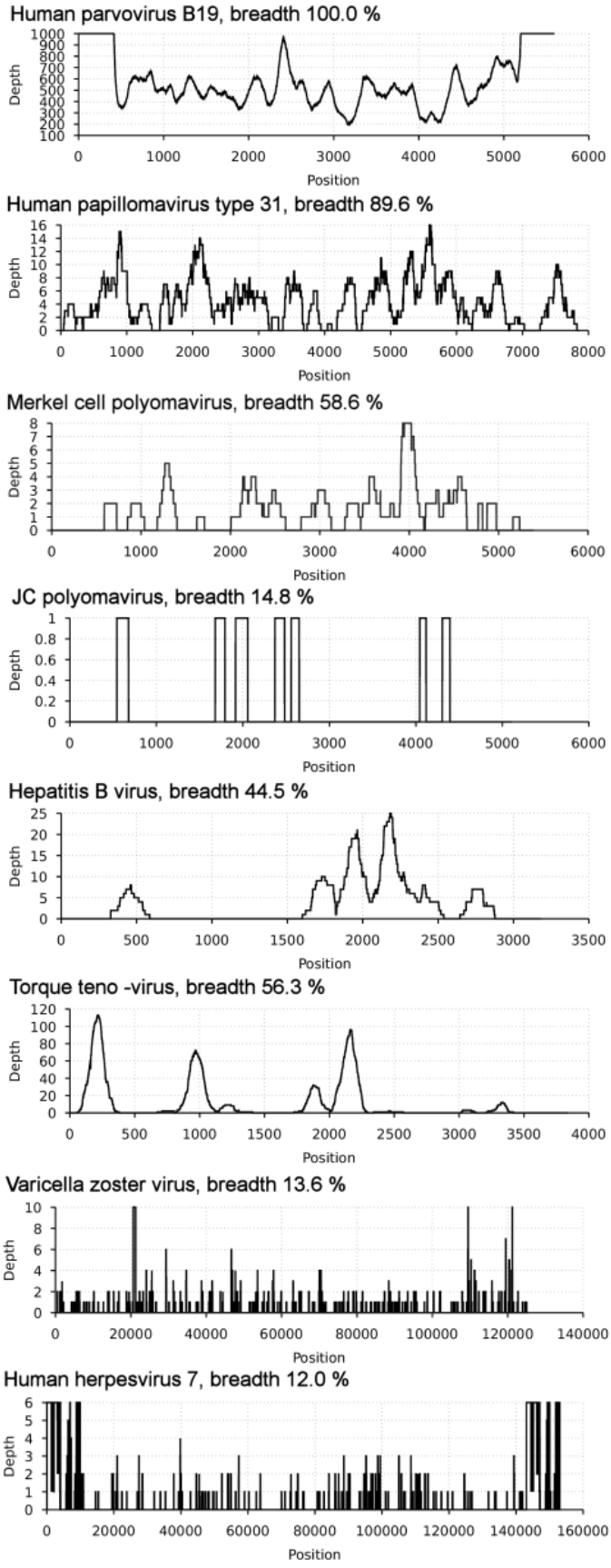


Fig 3. DNA fragment analysis of total DNA. Fragment length distribution of patterns of genomic DNA for four representative samples as established with LabChip GX.

Viral capture and sequence analysis

As established from the qPCR results, the persisting viral DNA quantities were far below 1% of total DNA present in a sample. Thus, to enrich this fraction, we performed in-solution capture with biotinylated RNA oligonucleotides prior to sequencing in Novaseq 6000. Subsequently, we analyzed the NGS data with a custom pipeline, TRACESPipe (Pratas *et al.* in revision), which reconstructs the viral sequences using both reference-based alignment and *de-novo* assembly.

We reconstructed a total of 15 viral genomic sequences, with a minimum of 15% breadth coverage. The highest qualities were attained for parvovirus B19 (n=7; average breadth coverage 86.1%), human papillomavirus (n=1; breadth coverage 89.6%), Merkel cell polyomavirus (n=1; breath coverage 58.6%), JC polyomavirus (n=1; breadth coverage 14.8%), hepatitis B virus (n=2; breadth coverages 44.5% and 15.6%), and torque teno virus (n=3; breadth coverages 56.3%, 30.6% and 24.9%). The breadth and depth coverages of representative viruses are presented in Figure 4.



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239 **Fig 4. Coverage profiles of reconstructed viral DNA sequences.** NGS coverage profiles (breadth and
240 depth) of representative viruses.

By NCBI BLAST, we found close relation of the reconstructed genomes to previously published sequences. We recovered seven full/near-full B19V genomic sequences, three of which were of genotype 1 (95.16% to 99.61% similarity to AY504945.1) and four of genotype 2 (90.1% to 98.6%, similarity to AB550331.1). The latter genotype represents an extinct form of this virus and was found in the present study exclusively in individuals older than 68 years. Of this genotype, only a single full-length sequence has ever been published (AB550331.1). We found that this genotype's hairpins present similar flip and flop configurations to those of genotype 1.

The human papillomavirus sequence showed 99.1% similarity to type 31 (KU298889.1); Merkel cell polyomavirus 98.7% to the European/Caucasian type (KF266963.1) and JC polyomavirus 100.0% to genotype 1 (MF662198.1). The two hepatitis B virus sequences matched 99.4% and 99.6% to genotypes D (JX898691.1) and A (MN507849.1), respectively; and the torque teno virus sequences showed 90.2%, 99.0% and 96.2% similarity to strains AF122920.1, KT163880.1 and FR751497.1, respectively.

Moreover, we confirmed by BLAST unique sequences (mean length 100 nt) mapping to the following viruses: herpes simplex 1 (1 case, 28 reads), varicella-zoster (1 case, 362 reads), Epstein Barr (6 cases, altogether 270 reads), human herpesvirus 6B (3 cases, total of 66 reads), human herpesvirus 7 (5 cases, total of 274 reads), JC polyomavirus (3 additional cases, total of 13 reads), Merkel cell polyomavirus (8 additional cases, total of 15 reads), human papillomavirus (5 additional cases, total of 14 reads), torque teno virus (6 additional cases, total of 26 reads), and parvovirus B19 (10 additional cases, total of 673 reads).

Discussion

The analysis of viral DNAs shows great potential as complementary markers for human identification as well as for estimation of provenance and migration. However, one limitation of their use in these contexts is insufficient knowledge of the landscape of viruses persisting in the host, in particular in human bone, with only parvovirus B19[23,24] and hepatitis B[25] virus having been detected in this tissue so far.

To this end, we systematically explored the prevalences of 38 ubiquitous viruses in human femoral bone. We discovered an unprecedented number of viral DNAs, detecting up to seven per individual. Besides the already known, we report here on ten new viruses including several members of the herpesvirus family (herpes simplex-1, varicella-zoster, Epstein-Barr, cytomegalovirus, human herpesviruses 6B and 7), JC- and Merkel cell polyomaviruses, human papillomavirus 31, and torque teno virus. Intriguingly, a common feature shared by these viruses (except for HHV-6B) is their persistence in soft tissues in episomal form[34]. Although the methods used in this study prevent us from confirming whether this is also the case in bone, the low copy numbers detected are indicative of a quiescent infection. Moreover, some of them (e.g. herpes-, polyoma-, and papillomaviruses) are

known to establish latency as densely packed nucleosomes, as mechanism to regulate gene expression[35,36]. Thus, the circular form and the tight histone packaging may confer DNA viruses superior preservation in relation to the host DNA (nuclear and mitochondrial)[37–39]. In this regard, the analysis of viral DNA could be of utmost value for forensic investigations or studies of ancient human remains dealing with highly compromised samples.

One challenging factor in the study of the human virome continues to be its extremely low proportion in relation to other sources of DNA. With this in mind, we carried out our screening using two approaches, qPCR and NGS. We found that the viral DNAs were exceeded on average a 1000-fold by the nuclear DNA alone, in line with the loads reported for persisting viruses in soft tissues[40–42]. By targeted enrichment and NGS we identified a higher number of viruses than by qPCR, an observation that can in part be explained by moderate DNA fragmentation in the samples. The only exception was torque teno virus (TTV), for which we found a significantly higher number of positive samples by qPCR. This circular ssDNA virus exhibits substantial heterogeneity[43], which was under-represented by the three reference sequences upon which our baits were designed. Hence, a better characterization of it could require a broader bait coverage of its five genogroups together with analysis at the amino acid level, as certain motifs are more likely to be shared by different TTVs. The qPCR on the other hand, targeted the conserved UTR region, to recognize a wide a repertoire of TT-viruses.

Unsurprisingly, larger genome coverages were recovered from the viruses with the highest copy numbers (~log3 copies/million cells). However, many of the viral loads fell below this threshold, whereby, follow-up singleplex enrichments may be beneficial to increase the analytical resolution of the data. Importantly, even for the low coverage genomes, we confirmed the sequences retrieved to be virus-specific and hence, genuine findings of the femoral bone.

In all, we unveil that the human femoral bone is a much richer source of persistent DNA viruses than earlier known. We propose that a “Human Virome Panel” could be built as an efficient tool for assessment of human provenance in conjunction with the standard human DNA profiling. Indeed, the phylogeographical distributions of JC[13,14,44], BK[2], varicella-zoster[7], and Epstein Barr[6] viruses have been examined in this context and shown to add power to forensic cases and anthropological studies. Undoubtedly, larger cohorts, both global and local, are required to validate the benefits and boundaries of such panel in multiple taphonomic conditions. This work could also help to identify patterns in the age distributions of certain viruses and their genotypes[19,20], to support biometric estimations.

Moreover, our data raise intriguing questions on the clinical significance of the long-term presence of these viruses – or their genomes – in the human skeleton. Indeed, many of the viruses we found can reactivate, and several have oncogenic potential. Among the latter, we detected Merkel cell

polyomavirus and human papillomavirus 31, two oncoviruses which, remarkably, are mucocutaneous. Certainly, these unexpected findings warrant further studies on the transcriptional, translational and reactivation potential of these pathogens as bone residents, particularly among the elderly and immune suppressed.

Conclusions

Our work substantially expands the current knowledge on the spectrum of DNA viruses persisting in human bone and opens new perspectives on their applicability in the investigation of human skeletal remains. It also supports the search for viruses from ancient relics, which can foreseeably remodel our understanding of virus evolution.

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Conflicts of Interest

The authors declare no conflict of interest.

Contributions

M.T., K.H., M.F.P and A.S designed the study. M.T. executed the experiments. MT, D.P. and M.F.P analyzed the data, E.V. and M.S.V contributed with a plasmid and qPCR optimization, M.T., M.F.P. and A.S. drafted the manuscript. All authors commented, edited and approved the final version of the manuscript.

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