

Chicken skin virome analyzed by high-throughput sequencing shows a composition highly different from human skin

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Abstract Recent studies show that human skin at homeostasis is a complex ecosystem whose virome include circular DNA viruses, especially papillomaviruses and polyomaviruses. To determine the chicken skin virome in comparison with human skin virome, a chicken swabs pool sample from fifteen indoor healthy chickens of five genetic backgrounds was examined for the presence of DNA viruses by high-throughput sequencing (HTS). The results indicate a predominance of herpesviruses from the *Mardivirus* genus, coming from either vaccinal origin or presumably asymptomatic infection. Despite the high sensitivity of the HTS method used herein to detect small circular DNA viruses, we did not detect any papillomaviruses, polyomaviruses, or circoviruses, indicating that these viruses may not be resident of the chicken skin. The results suggest that the turkey herpesvirus is a resident of

chicken skin in vaccinated chickens. This study indicates major differences between the skin viromes of chickens and humans. The origin of this difference remains to be further studied in relation with skin physiology, environment, or virus population dynamics.

Keywords Metagenomics · DNA viruses · Skin · Chicken · Herpesviruses

Introduction

For all vertebrates, skin represents a major interface and physical barrier between the body and the environment. In mammals and birds, skin is constituted of an epidermis, a keratinized stratified squamous epithelium, and a dermis, separated by a basal membrane. Bird skin differs from that of mammals by the thinness of the epidermis, the presence of feathers instead of hair, the absence of sebaceous glands, and a high lipid content in the epidermis [1, 2].

By using high-throughput sequencing (HTS), recent studies have revealed that human skin hosts a complex microbiome including a viral component, termed skin virome [3, 4]. Papillomaviruses and Polyomaviruses are the predominant viruses comprising the human virome in healthy skin. Other DNA viruses from the *Circoviridae* and the *Poxviridae* family (*Molluscum contagiosum*) have also been described [4, 5]. In the *Circoviridae* family, we recently identified the first human gyrovirus (hGyV1) from a skin swab of a healthy donor. This new hGyV1 shares 70 % genome identity with the chicken anemia virus (CAV), a chicken immunosuppressive virus [5, 6] and belongs to a set of viruses found in chicken meat and in human stools and blood [7, 8]. Interestingly, to date, all

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virus genomes detected at the skin level in healthy humans belong to DNA virus families.

The purpose of the present study was to compare the skin virome of chickens to that of humans. To this end, we determined the types of DNA viruses present at the surface of the chicken skin by HTS, using a pool of swabs sampled from 15 healthy vaccinated adult chickens (5.5- to 12-month old) of five genetic backgrounds [Fayoumi, Rhode Island R+, Gavora ev0, commercial Red layer, and “Noire du Berry” (a French breed)], reared in 2 independent houses (Table 1).

Materials and methods

Animals and skin swabs sampling

Fayoumi, Rhode Island R+, and Gavora ev0 chickens were between 11- and 12-month old. Commercial Red layer chickens were 23-week old, and “Noire du Berry” chickens were 34-week old. These chickens had received multiple vaccines, between the time of hatching and egg production, according to prophylaxis schedules commonly used in France in breeders or layers. Commercial Red layers were vaccinated against Marek’s disease (MD; Marek’s disease virus, *Herpesviridae*) and infectious bronchitis (infectious bronchitis virus, *Coronaviridae*). The vaccines administered to the “Noire du Berry” have not been documented. The three other breeds were vaccinated against MD, infectious bronchitis, Gumboro disease (infectious bursal disease virus, *Birnaviridae*), swollen head syndrome (avian metapneumovirus, *Paramyxoviridae*), Newcastle disease (Newcastle disease virus, *Paramyxoviridae*), infectious anemia (CAV, *Circoviridae*), avian encephalomyelitis (Avian encephalomyelitis virus, *Picornaviridae*), and egg drop syndrome (egg drop syndrome virus, *Adenoviridae*). For each of the eight vaccines, a live vaccine was used at least once. Two types of MD vaccines

were used at hatch: the herpesvirus of turkey (HVT) vaccine alone for the breeders, or HVT combined with an attenuated GaHV-2 Rispens/CVI988 for the commercial Red layers. In addition, to prevent infectious diseases, good sanitation practices were followed, including temperature (heating and pad cooling system) and air control (extract ventilation by fans), the use of commercial food (crumbles) and drinking water, entry restriction to poultry workers and rare visitors, and change of footwear and clothing before entering the houses (protective clothing wear, hair cover, and gloves). The chickens were not specific pathogen free (SPF), except for *Salmonella* (which was monitored every 8 weeks from hatch).

Fayoumi, Rhode Island, and Gavora ev0 chickens have been breeders at the INRA facility for more than 20 years. Commercial Red layer and “Noire du Berry” chickens were introduced in our animal facility (Building B) 4 and 22 weeks before skin swabs collection, respectively. Birds from each line were reared in groups from hatch to 18 weeks and subsequently in adult individual cages.

The swabs were collected from healthy animals without any clinical signs of disease. Swabs were collected from the side of the body in an area well protected by feathers in order to limit fecal contamination, by rubbing the surface of the skin with a sterile cotton swab moistened with water. Each cotton was then dipped into 250 μ L of sterile water at 4 °C. The swabs pool was generated within 1-h post-sampling by pooling 50 μ L of each individual skin swab and was frozen at –80 °C until DNA extraction.

DNA sample extraction and library preparation for HTS

One hundred fifty microliters of sample was treated with a cocktail of nucleases at 37 °C for 2 h. Enzymes were inactivated with a final concentration of 3 mM EDTA and heating for 10 min at 65 °C. Nucleic acid extraction was conducted with the QIAamp *Cador* Pathogen Mini Kit

Table 1 Skin swabs samples pooled

Chicken line	Number of animals	Age (weeks)	Building/room	Vaccines	Origin
Fayoumi	3	49	A/31	MD (HVT), IB, IBD, SHS, ND, IA, AE, EDS ^a	INRA breeding
Rhode Island R+	3	50	A/11	MD (HVT), IB, IBD, SHS, ND, IA, AE, EDS	INRA breeding
Gavora ev0	3	51	A/12	MD (HVT), IB, IBD, SHS, ND, IA, AE, EDS	INRA breeding
Red layer (commercial)	3	23	B/1	MD (Rispens, HVT-IBD), IB	External
“Noire du Berry”	3	34	B/7	not documented	External

^a MD Marek’s disease, IB infectious bronchitis, IBD infectious bursal disease, SHS swollen head syndrome, ND newcastle disease, IA infectious anemia, AE avian encephalomyelitis, EDS egg drop syndrome

(Qiagen). The DNA library was obtained by multiple displacement amplification assay (MDA) as follows: nucleic acid was amplified by the bacteriophage phi29 polymerase using random primers, so as to obtain high molecular weight DNA, according to the manufacturer's instructions (REPLI-g Mini Kit, Qiagen). Sample extraction and random amplification procedures were carefully performed to prevent cross-contamination, using the best precautionary PCR standards. After MDA, around 10 µg of DNA was obtained at a concentration of 216 ng/µL.

HTS and bioinformatic analysis

Reads were generated from the DNA library (prepared as described above) on an Illumina® HiSeq-2000 sequencer (DNAMVision, Gosselies, Belgium) with a sequencing depth of 2.4×10^8 paired-end reads of 101 nucleotides (nt) in length. Sequences were trimmed and filtered according to their quality score. Note that sequencing library preparation may introduce residual sample cross-contamination. After chicken genome sequence subtraction (Gallus gallus complete genome, galGal4, ICGSC Gallus_gallus-4.0, GCA_000002315.2) with Cushman2 and BlastN, reads were assembled in contigs using CLC Genomics Assembly Workbench (Cambridge, USA), and contigs and singletons were assigned a given taxonomy using the Blast algorithm. Criteria for taxonomic assignment have been described previously [9]. Sequences of the main contigs are available upon request.

Mardivirus PCR

PCR for chicken mardiviruses was used in order to validate HTS results. For that, we used primers specific for GaHV-2, GaHV-3, and HVT already described by Cortes and colleagues [10] with 2 and 10 ng of amplified DNA extracted from skin swabs. PCR conditions used were as follows: 3 min at 94 °C, then 30 cycles with 15 s at 94 °C, 15 s at 60 °C, and 15 s at 72 °C, followed by 10 min at 72 °C. One ng of the following positive control DNA was used for each PCR: a Bac20 bacmid for GaHV-2, a HPRS-24 DNA extracted from infected cells for GaHV-3, and a HVT DNA extracted from cells infected with a lyophilized vaccine preparation. Amplicons of 68, 66, and 62 bp were expected for GaHV-2, GaHV-3, and HVT, respectively.

Re-sampling feather follicles from the bird farm for Mardiviruses isolation and viral DNA detection by PCR

Three growing feathers were sampled from 3 progeny chickens of 4 lines randomly (Fayoumi, Gavora WL, Rhode Island, and “Noire du Berry”). Feather pulps were

extracted mechanically, chopped, and either frozen until DNA extraction or used for virus isolation. For virus isolation, the pulp was subsequently treated with collagenase for 10 min at 37 °C, and the cell suspension was filtrated on a 70-µm pore nylon membrane, pelleted, and resuspended in the ad hoc volume. The cell suspension was next co-cultivated in 12-well plates with a monolayer of primary chicken embryonic skin cells (CESCs) prepared as previously described [11]. Four days post-infection, cells were observed under an inverted microscope to score the cytopathic effect. All cultures were passaged once to amplify possible undetected infection and re-observed 5 days later.

Feathers pulp DNA extractions were performed as previously described [12].

Reticuloendotheliosis virus (REV) PCR

PCR was used to confirm the presence of a REV sequence in the DNA extracted and amplified from the skin swabs pool. For that, two couples of primers were designed based on known REV sequences (Genbank GI: 671183996, CY1111 isolate) and on the contig having homologies with REV: REV1 set, 654_REVinF (5'CAATGCGCGTACTGTAAGGA3') with 655_REV226R (5'TAGACATAGGCCCCACAGGT3'), and REV2 set, 656_REVinR (5'CTTCTTCCAATGTCCCTCCT3') with 657_REV197F (5'TTGCCCAGAAGGTTTTTCGAC3'). PCR conditions were 3 min at 94 °C and then 30 cycles with 45 s at 94 °C, 45 s at 55 °C, and 45 s at 72 °C, followed by 10 min at 72 °C. The PCR was performed on 10 ng of DNA. The amplicons expected for REV were of 225 bp with REV1 set and 196 bp with REV2 set.

Results

HTS output result analysis

Sequencing generated 8.0×10^7 (80,174,428) reads. After filtering steps including for the chicken host genome, the remaining 2.4×10^7 reads were assembled into contigs and assigned to the closest taxonomy. An average of 95.13 % of the 100 nt reads could be assembled into contigs. Contigs and singletons identified as close to known vertebrate viruses represented about 36,000 reads, whereas 18,106 contigs and singletons were not assigned to known species. Among the viral sequences, 21,594 corresponded or were close to the rep gene of TN4 circovirus. In our experience, similar sequences are often detected in viral metagenomic studies independently of the source of samples, like described for some parvovirus-like DNA [13]. Moreover, if real, these viral reads would most likely be of fecal origin; therefore, despite their numbers, these

sequences were not considered. The data corresponding to other viral sequences are reported in Table 2.

Herpesviruses sequences

Herpesviridae-related sequences from the Mardivirus genus represented 94.95 % of the reads assigned to viruses of vertebrates (Fig. 1). Sequences from the Gallid herpesvirus type 3 (GaHV-3) were predominant, with 121 contigs and more than 14 000 reads. These contigs showed a nucleotide identity between 98 and 100 % with the reference sequence (SB-1 strain, GI: 336091060), with 29 contigs over 700 nt and the longest being 9122 nt. The sequenced contigs covered about 61 % of the GaHV-3 complete genome (165 994 bp).

Sequences related to HVT were also detected, with 46 contigs and 485 reads. These sequences had 99.55 to 100 % nt identity with the reference sequence of the vaccine strain (FC126 strain, GI: 12025107) and covered 8.9 % of the total genome. After careful examination, we did not detect any sequence corresponding to Gallid herpesvirus type 2 (GaHV-2) or to other avian herpesviruses like GaHV-1.

These data demonstrate the presence of the HVT and GaHV-3 DNA at the chicken skin surface. In order to verify these results, PCR assays were performed from extracted amplified DNA, with three different sets of primers specific for GaHV-2, GaHV-3, and HVT species [10]. PCR analysis confirmed the presence of GaHV-3 DNA (Fig. 2a). HVT DNA was also detected by PCR but with a signal of lower intensity. GaHV-2 DNA was not detected, corroborating the HTS results. In order to assess the persistence of GaHV-3 virus in the farm, 3–5 growing feathers were sampled from the progeny flock, 8 months after collecting the skin swabs, for all breeds (except the commercial Red layer, which was no more present). Growing feathers were used to obtain enough pulp for DNA extraction. For each animal, the feather pulp was extracted and co-cultivated with CESC in order to isolate mardiviruses in culture as previously described [12]. Overall, 82 % of the co-cultures showed a cytopathic effect. DNA extracted from these co-cultures was positive by PCR for HVT only but not for GaHV-3. Of note, PCR on DNA directly extracted from the feather tips of the same animals were negative for HVT and GaHV-3, indicating that, for HVT, co-culture is more sensitive than direct PCR on feather pulps. Our attempt to detect the GaHV-3 strain from the farm in feather pulps of adults from the next generation was therefore unsuccessful, in contrast to the vaccinal HVT. Taken together, these results show that HVT is detectable on healthy chicken skin by HTS or from feathers by co-culture at two different samplings. This suggests that HVT might be a chicken skin resident in vaccinated chickens.

Table 2 Identification of viral species in chicken swab pool

Family	Main species/GI	Number of contigs/ Number of reads in contigs	Number of close contigs	Average contig length/maximum contig length	Average/maximum match length in contigs	Number of singletons	Number of close singletons	Total number of reads	Average identity % of contigs/ singletons
Herpesviridae	Gallid herpesvirus 3/336091060	121/14,064	120	874/9122	869/9061	175	155	14,146	99.76/98
Herpesviridae	Meleagrid herpesvirus 1/12025107	46/485	46	333/1231	325/1231	119	119	604	99.55/100
Single-strand circular virus group (SCV)	Turkey stool associated circular ssDNA virus/ 605039169	0/0	0	0/0	0/0	776	1	776	0/73.71
Retroviridae	Avian leukosis virus/ 308569771	0/0	0	0/0	0/0	1	1	1	0/93.88
Retroviridae	Avian endogenous retrovirus EAV-HP/ 12709849	1/3	0	0/0	0/0	3	2	3	0/87.85
Retroviridae	Reticuloendotheliosis virus/671183996	1/4	1	0/0	0/0	0	0	4	0/85

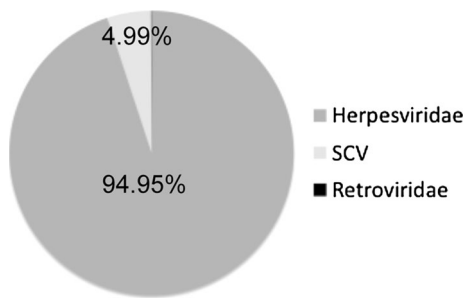
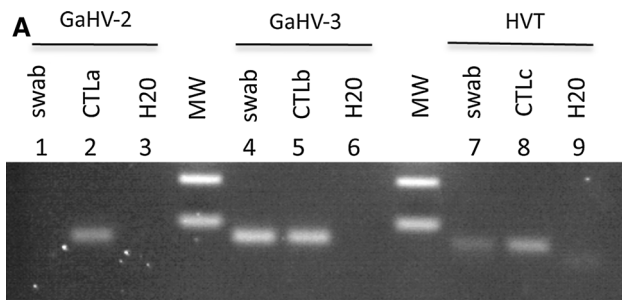


Fig. 1 Percentage of the total identified vertebrates viral reads in the chicken skin swabs pool. Herpesviridae sequences from the mardivirus genus predominated with 94.95 %. Circular single-strand DNA virus (SCV) and *Retroviridae* sequences represented 4.99 and <0.1 % of the reads, respectively



B
 AATCCGCGCCGATATTAGGAAGAAAATCCAAAAGATAGATGGGTTCCAGG
 GGAAATCTCTCCGAGTTAGTAGCTATTTGCCAGAAAGGTTTTTCGACCAACG
 GGAAGACCCAGCCAAGGCAACTCACGAATCACTCAGAAGATGGCCAAGG
 TACTACTCGCCAGGAGAGTAGAGCGGAGAGAGGAAGTAAGAAGACGCCT
 CCGGGTAAGGGACGCCCGCTTTGGGGAAAAACCAATGCCTACTGTAA
 GGAGGAGGGGACATTGGGAAGAAGAACTGTCCAAAACCTCGTAAGCGGGGCA
 ACCCCAGTGTGGTAGAAGAATTACAATAGGGCCGTCAGGGTTCTTCCGCC
 CTCCGTGAACCCAGGCTAAAAGTTAAAGTAGGGGGGCAATAATAGATTTT
 CTAGTAGATACGGGAGCGACCCATTCTGTAGTGCAGAAACCTGTGGGGCCCT
 ATGTCTAAAGAATCTGTAGCAATTATCGGGGCTACTGGGAACATACGGAATT
 ACCCTAAGTCTGAAGGGC

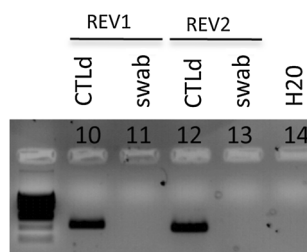


Fig. 2 PCR validated the presence of mardiviruses sequences but not REV sequences. **a** DNA (2 ng) extracted from skin swabs and amplified by phi were next amplified using three sets of primers in parallel, with proper positive (*lanes 2, 5, and 6*) and negative controls (*lanes 3, 6, and 9*). GaHV-2, GaHV-3, and HVT PCR products were 68, 66, and 62 bp respectively. **b** The sequence showed corresponds to the sequence detected by HTS (in *gray and italics*) and the flanking region of the REV CY1111 strain. The two sets of primers used are indicated: REV1 (*underlined sequences*) and REV2 (sequences in *bold*). REV1 and REV2 PCR products were 226 and 197 bp, respectively. Positive (*lanes 10 and 12*) and a negative (*lane 14*) controls are shown. CTLa, Bac20 bacmid; CTLb, GaHV-3 vDNA; CTLc, HVT vDNA; CTLd, CU91 cells genomic DNA

Retroviruses sequences

We identified 2 contigs (contig_39693, 171nt, 3 reads; contig_154933, 164 nt, 4 reads) and 4 singletons related to *Retroviridae*. One contig and 3 singletons corresponded to endogenous avian retroviruses of the EAV-HP/evJ family (GI:496350531 and GI: GI:50880255). One singleton was located in the 3'UTR of avian leukosis virus type E (ALV-E) sequence (94 % identity on 97 nt with GI:308569771). Lastly, one small contig contained 52 nt that corresponded to a gamma-retrovirus sequence located in the gag/pol junction, showing 85 % identity with reticuloendotheliosis viruses (REV) (GI: 671183996, CY1111 isolate) and 80 % identity with a mammalian galidia ERV sequence (GI: 544141755). In order to distinguish between a REV sequence or a Galidia ERV [14], we designed two sets of REV primers around the short REV region identified by HTS, with one primer of each set inside the sequenced region and another in the corresponding flanking region of the REV CY1111 strain (Fig. 2b). Although these primers amplified a REV-positive DNA control, no signal was detected by PCR with the DNA subjected to HTS, suggesting that this unique sequence was probably not a REV sequence but more likely contamination with a mammalian sequence with only a 68 % identity with REV in this region (Fig. 2b). Therefore, all retroviral sequences identified were presumably endogenous chicken sequences or from contaminant mammalian DNA from other samples treated in parallel in the sequencing platform.

Other viral sequences

A total of 776 singletons (4.99 % of the total reads) (Fig. 1) shared 73.7 % identity with the Turkey stool-associated circular ssDNA virus (TuSCV; gi:605039169), whose genome is 2749-nt long. The reads covered about half of the genome, with 724 reads on a small region of rep ORF.

Lastly, we analyzed contigs sequences that did not correspond to any known sequences, for the presence of new viral sequences. Thirty-three contigs over 700-nt long (with a median of 849 nt; composed from 19 to 22,934 reads, with a median at 72 reads per contig) were examined for the presence of open-reading frames (ORFs) with the NCIB “ORF finder” software. When the ORF encoding products measured over 70 amino-acid in length, they were subsequently analyzed with blastp programs for the presence of protein sequences having even distant similarities with viral proteins. Among these contigs, we did not detect any sequence that might indicate the presence of new vertebrate viruses. Therefore, we did not find any sequence from unknown small DNA viruses from the *Papillomaviridae*, the *Polyomaviridae*, or the *Circoviridae* families.

Discussion

We describe herein for the first time the skin virome of chickens, from healthy vaccinated adults of five genetic backgrounds raised indoor in cages, either individually or in group. The birds were multi-vaccinated with 3–8 different live vaccines, from DNA (Marek's disease, chicken anemia, egg drop syndrome) or RNA (infectious bronchitis, Gumboro disease, swollen head syndrome, avian encephalomyelitis, Newcastle disease) virus families. We show the presence of numerous viral sequences, most of them identified as Mardivirus members of the *Herpesviridae*, a family of large enveloped DNA viruses.

Among the *Herpesviridae*, two species were detected by HTS, the GaHV-3 and the HVT, with 27 times more reads for GaHV-3 than for HVT. The higher proportion of GaHV-3 sequences in the DNA extract detected by HTS was in accordance with the PCR results. These two Mardivirus species are non-pathogenic (non-oncogenic) for chickens [15–17]. In addition, the DNA genome of both viruses has previously been shown to be present in feather tips and dust collected from the environment by PCR techniques [18, 19]. The presence of HVT was expected because 80 % of the chickens sampled in this study were vaccinated against Marek's Disease with a HVT strain. In experimental conditions, Islam et al. reported that HVT DNA sequences were detectable up to 8 weeks from danders of chicks vaccinated at hatch [19]. There is no study reporting the duration of vaccine shedding or of an apathogenic virus under natural infection in the field, even though it is suspected to occur after 8 weeks. Here, we found that HVT DNA, presumably encapsidated, is detectable for at least 18 weeks post vaccination on the skin. To our knowledge, it is the first time that HVT DNA sequences have been detected from skin swabs and not from feather tips or dust.

The presence of GaHV-3 DNA sequences showing 98 to 100 % identity with SB-1 was striking and unexpected. Indeed, MD vaccines based on GaHV-3 SB-1 strain have no marketing authorization in France. In addition, a laboratory DNA contamination before sequencing is very unlikely because the swabs pool and the DNA extraction/amplification were performed in rooms in which mardiviruses nucleic acids were not previously manipulated. Therefore, a natural asymptomatic infection of the flock with a GaHV-3 field strain is the most probable origin of these sequences. The high identity with SB-1 sequence is compatible with a field strain. Indeed, Mardiviruses genomes are considered relatively stable. A molecular study performed on 85 virulent GaHV-2 strains isolated in Poland over 40 years showed 99 to 100 % similarities in Meq gene, one of the most variable gene in this species

[20]. In addition, GaHV-3 (like GaHV-2 and HVT) is also shed from feathers and present in dust [21]. Finally, GaHV-3 asymptomatic infections occur occasionally and have been previously suspected in chicken farms in the United Kingdom in relationship with positive qPCR on feather tips (S. Baigent, Personal communication). Among the five chicken lines studied herein, two (Red layers and "Noire du Berry") were introduced in Building B of the INRA farm a few weeks earlier (4 and 22 weeks before skin swabs). It is likely that one of these lines was infected at its arrival with a GaHV-3 strain and that the infection was still present at the time of the swab sampling. As GaHV-3 was not re-isolated from feather tips sampled on the progeny birds (unlike the HVT vaccine), 8 months after the skin swabs, it is probable that this infection was only transient. This is compatible with the fact that all birds were eliminated between the two samplings, the absence of known vertical transmission of GaHV-3, and the extensive cleaning of the chicken houses between the breeding of parental and progeny groups.

No GaHV-2 sequences were detected by HTS, although some birds sampled were vaccinated with the GaVH2 Rispens/CVI988 strain (commercial Red layers). The replication of CVI988 virus in the feather follicle has been previously reported [22, 23] and its shedding attested by the presence of viral DNA in dust [23]. Baigent and al. reported high levels (10^6 to 10^8) of CVI988 genome copy numbers per 10^6 feather tip cells, from 10- to 28-day post-vaccination in maternal antibody-free SFP chickens [22]. Other authors reported titers that never exceeded 10^6 and was below 10^2 at 56 days in maternal antibody-free White leghorn vaccinated at 1 day [23]. Considering that Rispens may be less shed than HVT (especially over time), and that only 20 % of the birds had received Rispens, the absence of GaHV-2 in this analysis is not totally surprising.

Interestingly, no human herpesviridae DNA sequences have been detected to date on healthy human skin by HTS, although some of these viruses, like Varicella-Zoster virus and Herpes Simplex Virus (HSV-1/HHV-1 and HSV-2/HHV-2) [24–27], are known to replicate in this tissue and/or in the mucosa, and being shed, after primary infection and reactivation. One possible explanation of the absence of herpesviridae from healthy human skin virome could be the localized shedding and rapid virus clearance after reactivation. Indeed, asymptomatic herpes simplex virus reaction episodes were shown to occur frequently at genital or oral level, but are usually rapidly controlled within 6–12 h in immunocompetent adults [28] by skin-resident memory CD8 + T cell [29].

We also detected sequences sharing homologies with the turkey stool associated circular single-strand DNA virus, a virus initially identified in turkey feces [30]. Because the

identity is only of 73 %, it is likely that these sequences belong to a novel species of circular single-strand DNA virus. As these viruses have been detected in the stools of various species (pig, chimpanzee, bovine, and turkey) [30–33], the presence of this virus on chicken skin presumably can be attributed to a fecal contamination (even if we were cautious to sample a relatively clean region of the body).

Interestingly, we did not detect any DNA sequences of *Papillomaviridae*, *Polyomaviridae*, or *Circoviridae*. The technique used herein to amplify nucleic acid before HTS is based on amplification with the polymerase of phage phi 29, which is particularly efficient for small circular DNA genomes due to its capability to do rolling circle amplification [34]. Therefore, the absence of reads from these virus families is not due to a lack of sensitivity, but reflects a true absence of these families as resident viruses on the chicken skin, in contrast to human skin. It is remarkable that only four *Papillomaviridae* have been described in birds to date (*Psittacus erithacus* PePV1, *Fringilla coelebs* FcPV1, *Francolinus leucosceptus** FIPV1, and *Pygoscelis adeliae* papillomavirus 1 PaCV1), none of which infecting the *Gallus gallus* species [35, 36], whereas more than 160 have been found in humans. Regarding *Polyomaviridae*, five have been identified in birds but not in chickens, whereas thirteen have been found in humans, eleven of which were found since 2007, mostly by HTS methods [37]. Polyomaviruses usually induce inflammatory acute disease in birds but only benign infections in non-immunocompromised humans. Our skin virome analysis therefore reinforces previous observations that Papillomaviruses and Polyomaviruses, if they exist in chickens, are not “commensal viruses” in chicken skin. In chickens, several *Circoviridae* exclusively from the gyrovirus genus have been identified to date: the well-known CAV, the avian gyrovirus type 2 (AGV2) [38], the hGyV1, and the closely related gyrovirus 2, 3, 4, and 7 (GyV2, GyV3, GyV4, GyV7-SF) [8, 39]. In chickens, gyroviruses have been predominantly detected from chicken feces and muscle [8, 39], except for the CAV which has been detected previously in feathers [40]. The absence of gyroviruses in our study, including CAV for which 60 % of the chickens were vaccinated, indicates that gyroviruses seem not to be skin “resident” in chickens.

The comparison of chicken and human skin virome shows strong differences and suggests that skin viromes may be species specific. Although we cannot totally exclude that these results are due to the limited number of chicken skin swabs examined, data published on human skin virome suggest that a single pool is sufficient to identify most skin-resident viruses by HTS. Indeed, it is important to highlight that papillomaviruses, polyomaviruses, and gyroviruses DNA are detectable from all human skin swabs, even if the virus species and their proportions vary between individuals [3]. In addition, we

observed a low diversity of virus sequences at the skin surface of live chickens compared to humans. Finally, the sampling and HTS could have missed rare and/or transient viruses associated or not to cognate diseases or environmental factors, and which might occur more frequently in free-range chickens (backyards or outdoor large farms) not tested in our study.

The factors that determine the virome composition at the skin level in an animal species are still unknown. Several parameters might explain the diversity of viruses in the skin, including the environment, the skin physiology and composition, the immune response at the skin level and also the vaccinations, as here in chickens where HVT vaccine is highly detected. Interestingly, in this study, the DNA viruses detected from chicken skin swabs are not zoonotic, and therefore, the skin itself is probably not a major source of human infections, either for consumers, butchers, and farm workers.

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Authors Contribution D. Gourichon was in charge of the chicken sanitary state and housing, and helped with skin swab samplings. M. Dumarest prepared the viral DNA for HTS. S. Rémy performed the experiments to verify the presence of herpesviruses and REV DNA. C. Denesvre and M. Eloit designed the project, conducted the sequence analyses, and wrote the manuscript.

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