Identification and genetic characterization of a novel picornavirus from chickens

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A novel picornavirus from commercial broiler chickens (Gallus gallus domesticus) has been identified and genetically characterized. The viral genome consists of a single-stranded, positive-sense RNA genome of >9243 nt excluding the poly(A) tail and as such represents one of the largest picornavirus genomes reported to date. The virus genome is GC-rich with a G+C content of 54.5 %. The genomic organization is similar to other picornaviruses: 5′ UTR–L–VP0–VP3–VP1–2A–2B–2C–3A–3B–3C–3D–3′ UTR. The partially characterized 5′ UTR of 373 nt appears to possess a type II internal ribosomal entry site (IRES), which is also found in members of the genera Aphthovirus and Cardiovirus. This IRES exhibits significant sequence similarity to turkey ‘gallivirus A’. The 3′ UTR of 278 nt contains the conserved 48 nt ‘barbell-like’ structure identified in ‘passerivirus’, ‘gallivirus’, Avihepatovirus and some Kobuvirus genus members. A predicted large open reading frame (ORF) of 8592 nt encodes a potential polyprotein precursor of 2864 amino acids. In addition, the virus contains a predicted large L protein of 462 amino acids. Pairwise sequence comparisons, along with phylogenetic analysis revealed the highest percentage identity to ‘Passerivirus A’ (formerly called turdivirus 1), forming a monophyletic group across the P1, P2 and P3 regions, with >40, <40 and <50 % amino acid identity respectively. Reduced identity was observed against ‘gallivirus A’ and members of the Kobuvirus genus. Quantitative PCR analysis estimated a range of 4×10^5 to 5×10^8 viral genome copies g⁻¹ in 22 (73 %) of 30 PCR-positive faeces. Based on sequence and phylogenetic analysis, we propose that this virus is the first member of a potential novel genus within the family Picornaviridae. Further studies are required to investigate the pathogenic potential of this virus within the avian host.

INTRODUCTION

Members of the family Picornaviridae are small, non-enveloped, icosahedral viruses with a positive-sense RNA genome. They comprise a large group of human and animal pathogens, including human poliovirus (family prototypic virus), hepatitis A virus and foot-and-mouth disease virus. At present, the family is composed of 17 genera and a number of proposed genera (http://www.picornaviridae.com).

Generally, the genomic organization of picornaviruses is conserved. For example, the genes encoded by kobuviruses are as follows: L, VP0, VP3, VP1, 2A, 2B, 2C, 3A, 3B, 3C and 3D. The size of the RNA genome of picornaviruses ranges from 7.2 to 9.1 kb and although some Picornaviridae

The GenBank /EMBL/DDBJ accession number for the ‘sicinivirus 1’ genomic sequence determined in this study is KF741227.

Five supplementary tables are available with the online version of this paper.

To date, only a small number of picornaviruses have been reported in bird species. Known avian picornaviruses members, including the genus Diciviruses, encode more than one polyprotein (Woo et al., 2012), picornaviruses typically encode a single ORF flanked by a 5′ and 3′ untranslated region (UTR). Translation produces a single polyprotein that can be functionally divided into three regions: P1, P2 and P3. The P1 region encodes the capsid proteins and the P2 and P3 regions encode proteins involved in proteolytic processing and virus replication (Giachetti et al., 1992). A number of picornaviruses such as ‘Passerivirus A’ and kobuviruses encode a leader protein (L), flanking the P1 region (Lau et al., 2011; Reuter et al., 2010; Woo et al., 2010). Additionally, the VP0 protein of some picornaviruses is proteolytically cleaved to produce individual VP4 and VP2 proteins. In general, the L protein along with the 2A viral protease, show the greatest variation among picornavirus proteins (Lau et al., 2011).

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include duck hepatitis A virus (DHAV-1, genus Avihepatovirus) (Kim et al., 2006), avian sapelovirus (Duck picornavirus TW90A, genus Sapellovirus) (Tseng & Tsai, 2007) and turkey hepatitis virus (Melegrivirus A, genus Melegrivirus) (Honkavuori et al., 2011). Three viruses recently identified in tracheal and cloacal swabs of dead turkeys form two proposed new picornavirus genera, ‘Passeriviruses’ containing ‘Passerivirus A’ and ‘Osciviruses’ containing ‘oscivirus A1’ and ‘oscivirus A2’ (formerly known as turdivirus 2 and 3) (Woo et al., 2010). Additionally, two pigeon picornaviruses, pigeon picornavirus A and B (Kofstad & Jonassen, 2011) and a quail picornavirus (Pankovics et al., 2012) have recently been identified. Furthermore, two candidate novel genera of picornaviruses, ‘gallivirus A’ and ‘Turkey avitisivirus’ (TuASV), were recently detected in healthy turkeys and commercial turkeys with enteric and/or stunting syndrome: strains turkey/M176/2011/HUN, and turkey/M176-TuASV/2011/HUN, respectively (Boros et al., 2012, 2013; Farkas et al., 2012).

Viruses known to infect chickens include avian influenza and avian encephalomyelitis virus (AEV, genus Tremovirus; Marvil et al., 1999). Chicken anaemia virus (CAV, genus Gyrovirus of the Circoviridae) has been reported to promote immune suppression (Schat, 2009), as has infectious bursal disease virus (IBDV, genus Avibirnavirus, family Birnaviridae; Mahgoub et al., 2012). Furthermore, infectious bronchitis virus (IBV) is a coronavirus that causes a highly contagious respiratory and reproductive tract disease in chickens (Jackwood, 2012). Additionally, avian hepatitis E virus (genus Hepevirus) causes hepatitis by the faecal–oral route of transmission (Huang et al., 2004). Recently, the partial nucleotide sequences of chicken ‘galliviruses’ detected in the faeces of chickens have been reported (Farkas et al., 2012).

This study reports the molecular identification and characterization of the genome of a novel chicken picornavirus. Initial screening studies of chicken faecal samples, using human norovirus RNA polymerase consensus primers, identified a PCR product with homology to members of the genus Kobuvirus. The genome of this picornavirus has subsequently been sequenced. Based on sequence and phylogenetic analysis we propose that this virus, termed ‘sicinivirus 1’ (sicini is the Gaelic word for chickens), is the first member of a potentially novel genus within the family Picornaviridae.

**RESULTS**

The identification of this novel picornavirus has its origins in a project designed to investigate the presence of norovirus-like viruses in domesticated farm animals (cattle, pigs, sheep and chicken faecal samples). Reverse transcriptase (RT)-PCR performed using the broadly reactive norovirus specific Ni/E3 primers (Green et al., 1995) identified an approximate 240 bp fragment in 30% (124 of 773 samples) of chicken samples. This PCR fragment was larger than expected for norovirus-related viral species (predicted size 113 bp). Subsequently, the PCR fragment was sequenced and a translational reading frame of 30 amino acids was identified, exhibiting 67% identity to the RNA polymerase protein (3D protein) of aichivirus B (bovine kobuvirus). An additional RT-PCR using a sense primer to this DNA sequence, along with an oligo(dt) primer, was successfully performed to amplify the 3’ region of the virus. Subsequent sequencing established that the virus has a 3’ UTR of 278 bp (excluding the poly(A) tail). A new investigation was then undertaken on 30 chicken cloacal samples obtained from approximately 8-week-old chickens slaughtered at a commercial chicken processing farm in Ireland. In total, 73% of samples were positive by PCR using primers 23F/23R (3’ region of the 3D gene of ‘sicinivirus 1’). Quantitative realtime PCR identified one sample (sample 23) with relatively high viral RNA numbers and this sample was used to generate cDNA that was sequenced using an Illumina MiSeq sequencing platform. The metagenomic sequencing data returned 13 contig sequences with a total length of 30185 bp and an average GC content of approximately 55%. BLASTP analysis revealed that 11 of the 13 contigs encoded components of viral RNA genomes and six of these contigs encoded regions of the ‘sicinivirus 1’ genome, which were further confirmed by genomic walking PCR.

**Genomic organization**

The genome of ‘sicinivirus 1’ is >9243 nt in size, excluding the poly(A) tail (Fig. 1). The virus genome is GC-rich with a G+C content of 54.5%. The genomic organization is similar to other picornaviruses: 5’ UTR–L–VP0–VP3–VP1–2A–2B–2C–3A–3B–3C–3D–3’ UTR. A large ORF of 8592 nt is predicted to encode a potential polyprotein precursor of 2864 aa. The partially characterized 5’ UTR contains >373 nt while the 3’ UTR is 278 nt in length. The sequenced ‘sicinivirus 1’ genome is predicted to encode a large L protein followed by P1, P2 and P3 regions potentially encoding the capsid proteins VP0, VP3 and VP1, the 2A, 2B and 2C and the 3A, 3B, 3C and 3D proteins respectively (Fig. 1). Furthermore, putative protease cleavage sites of the polyprotein were mapped using the NetPicoRNA prediction program and alignment of the ‘sicinivirus 1’ amino acid sequence to ‘Passeriviruses A’. Glutamine (Q) was located at the C-terminal of all 10 protease cleavage sites.

**Analysis of the 5’ UTR and 3’ UTR**

The 5’ UTR of ‘sicinivirus 1’ is incomplete as the domains A–I of the potential type II internal ribosomal entry site (IRES) are missing (Fig. 2). Sequence homology and Mfold RNA secondary structure analysis of the available 5’ UTR sequence indicates the virus contains domains J, K and L of a type II IRES, with strong sequence and structure homology to that of ‘gallivirus A’ (Boros et al., 2012). The J domain of ‘sicinivirus 1’ shares 67% nucleotide sequence homology with ‘gallivirus A’ (Boros et al., 2012).
Fig. 1. Predicted genome organization of 'sicinivirus 1'. The P1 region represents the structural proteins VP0, VP3 and VP1 and contains 847 aa (2541 nt). The P2 region represents the 2A, 2B and 2C proteins and contains 697 aa (2091 nt). The P3 region represents the proteins 3A, 3B, 3C and 3D and contains 842 aa (2526 nt). The protein name along with nucleotide (upper numbers) and amino acid (lower numbers) lengths are indicated within each gene box. The genomic region is indicated below each gene box within the square brackets. The predicted protein cleavage sites are indicated above each gene junction (one letter amino acid code). A partial 5' UTR region of 373 nt and a 3' UTR region of >278 nt are presented at the corresponding ends of the gene box.

Fig. 2. Predicted RNA secondary structure of the 'sicinivirus 1' IRES. The partial structure of the 5' UTR is missing regions A–I but includes domains J, K and L (labelled), in addition to a pyrimidine-rich region located between domains K and L. These domains correspond to a type II IRES structure and share conserved nucleotide sequences to other picornaviruses such as the turkey 'gallivirus A' (NC_018400) and turkey TuASV (KC465954) (Boros et al., 2012, 2013). Labels indicate specific nucleotides.
structure of the stem–loop is almost identical to that of 'gallivirus A' with the K domain protruding at a 90 °C angle from the right side of the J domain stem. In addition, two tetraloop GNRA motifs are present in 'sicinivirus 1' domains J–L. A 19 nt (position 153–171) pyrimidine-rich tract p(Y) is present between domains J/K and L. This p(Y) tract is very similar in structural location to that of 'gallivirus A' (Boros et al., 2012). The 5′ UTR of type I and type II IRESs usually contain a conserved 3′ terminal sequence Y_n–X_m–ATG motif, where the Y_n defines the size of a pyrimidine tract (n; 8 to 10 nt) and X_m is the distance it is spaced from the AUG; 'sicinivirus 1' has a potential Y_n–X_m–ATG motif of Y_7–X_45–ATG.

The 3′ UTR of 'sicinivirus 1' exhibits homology to kobuviruses and other chicken and turkey picornaviruses (Fig. 3). This region has a number of secondary RNA stem–loop structures including a 48 nt 'barbell-like' structure identified in 'Passerivirus A', 'gallivirus', avihepatovirus and some Kobuvirus genus members (Boros et al., 2012). The strongest sequence homology between these viruses is observed in the lower loop of the barbell, two regions of 15 nt and 10 nt identity respectively (Fig. 3). The upper loop of the barbell contains a shorter p(Y) region to that found in kobuviruses and 'gallivirus A' (Boros et al., 2012). The location of this barbell structure in 'sicinivirus 1' is towards the 3′ end of the 3′ UTR in contrast to

**Fig. 3.** Conserved motif analysis of the 3′ UTR of 'sicinivirus 1'. Nucleotide alignment of the 3′ UTR of several picornaviruses (CLUSTAL W). The grey boxes identify the regions which are conserved; an asterisk is located below sequences that are completely conserved across the seven virus strains analysed. The arrows demonstrate that the nucleotides which form this 'barbell-like' structure in 'sicinivirus 1' are highly conserved across picornaviruses (Boros et al., 2012). The presence of a poly(Y) tract, which is characteristic of this structure, is noted. The numbers correspond to the nucleotides within the 3′ UTR of the following viruses; SCN-1 ('sicinivirus 1'), TV-1 ('Passerivirus A'; NC_014411), AICHI (aichivirus A, aichi virus 1; GQ927711), CaKV (canine kobuvirus 1; JN088541), M176 (‘gallivirus A’; JQ691613), TRK91 (turkey/TRK91/USA/2010; JF424830) and CHK175 (CHK175/USA/2010; JF424827).
'Passerivirus A' and aichivirus A where it is located at the 5’ end of the 3’ UTR.

**Analysis of coding regions**

The first in-frame AUG codon is located at nucleotide 374 and it contains the optimal Kozak consensus sequence (AxxAxxUGG). Translation from the first in-frame AUG predicts a 462 amino acid L protein in 'sicinivirus 1'. This predicted L protein does not show homology to any other picornavirus L protein and it does not appear to possess protease activity (Gorbalenya et al., 1991). The predicted capsid proteins are in the following order as predicted by NetPicoRNA and alignment with members of the genera *Kobuvirus* and *Passerivirus*: VP0, VP3, VP1. There is no evidence of proteolytic cleavage of the putative VP0 to VP4 and VP2 proteins. A number of conserved picornavirus motifs are present in the predicted proteins of 'sicinivirus 1'. The myristylation site GSISST is present in the VP0 protein. The predicted 2A protein contains a motif TWAL and NT analogous to the observed HWAL and NC motifs in 'Passerivirus A'. Furthermore, the potential 2C protein contains two conserved motifs, the NTPase motif (GXXGXGKS) as GPPGCGKS and the helicase motif (DDLXQ) as DDVGQ. The catalytic triad (H-D/E-C) is present in the putative 3C protein as H-D-C, the RNA binding domain (KFRDI) as QFKDL and the GXCG motif as GLCG. The predicted 3D protein contains the conserved motifs KDE[LI]R as KDELR, GG[LMN]PSG as GGNPSG, YGDD as YGDD and FLKR as FLKR.

**Comparative genomic and phylogenetic analysis**

BLAST and phylogenetic analysis of the predicted polyprotein of 'sicinivirus 1' establishes that it exhibits the greatest identity to 'Passerivirus A' (Table 1 and Fig. 4a, b, c). 'sicinivirus 1' shares an overall amino acid identity of 40.9% (P1, P2 and P3 regions) to 'Passerivirus A'. Furthermore, phylogenetic analysis of the P1, 2C and 3CD regions reveals that 'sicinivirus 1' and 'Passerivirus A' consistently form monophyletic groups. Although phylogenetic analysis reveals that 'gallivirus A' forms a sister clade with this group, in terms of amino acid identity shared with 'sicinivirus 1', it is surpassed by aichivirus C.

Additionally, in relation to 'sicinivirus 1', 'Passerivirus A' has the least number of amino acid substitutions per site across the P1, 2C and 3CD regions (Tables S2, S3 and S4, available in the online Supplementary Material). The maximum amino acid identity of the 'sicinivirus 1' 2A and 2B proteins against other picornavirus members is 30%, in both cases with 'Passerivirus A', while the predicted L protein shows no significant homology to any other L protein.

Analyses of codon usage for amino acids that use either NNC or NNT indicate a slight preference for the codon NNC (0.54) in the genome of 'sicinivirus 1' (Table S5). 'Passerivirus A' and 'oscivirus A1' are 0.71 and 0.33 respectively. This tends to be reflected in their GC content with 'sicinivirus 1' at 54.5% and 'Passerivirus A' at 58%. 'Oscivirus A1' has a lower GC content of 47%, possibly reflecting cytosine deamination.

**Viral load**

Quantitative PCR analysis was applied to quantify the levels of 'sicinivirus 1' genome in chicken faecal samples. In the 73% (22 out of 30) positive samples, viral genome copies ranged from $3.9 \times 10^5$ to $5.2 \times 10^8$ per gram of cloacal content.

**DISCUSSION**

In this study we report the identification of a novel picornavirus from chicken faeces. The use of norovirus consensus primers in an attempt to identify norovirus-like viruses in farm animals lead to the serendipitous detection

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**Table 1.** Comparison of genomic features of 'sicinivirus 1' and nine species of picornaviruses

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>GenBank accession no</th>
<th>Size (nt)</th>
<th>G + C (%)</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>Whole genome (P1, P2, P3)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Passerivirus'</td>
<td>'Passerivirus A' (turdivirus 1)</td>
<td>NC_014411</td>
<td>8025</td>
<td>58.00</td>
<td>30.5</td>
<td>38.1</td>
<td>46.8</td>
<td>40.9</td>
</tr>
<tr>
<td><em>Kobuvirus</em></td>
<td><em>Aichivirus C</em> (porcine kobuvirus)</td>
<td>NC_011829</td>
<td>8210</td>
<td>52.40</td>
<td>24.9</td>
<td>33.1</td>
<td>39.9</td>
<td>35.7</td>
</tr>
<tr>
<td>'Gallivirus'</td>
<td>Turkey 'gallivirus A'</td>
<td>NC_018400</td>
<td>8496</td>
<td>48.30</td>
<td>17.7</td>
<td>31.6</td>
<td>45.6</td>
<td>35.5</td>
</tr>
<tr>
<td><em>Kobuvirus</em></td>
<td><em>Canine kobuvirus 1</em></td>
<td>JN088541</td>
<td>8289</td>
<td>58.20</td>
<td>26.5</td>
<td>34.5</td>
<td>41.4</td>
<td>33.9</td>
</tr>
<tr>
<td><em>Kobuvirus</em></td>
<td><em>Aichivirus B</em> (bovine kobuvirus)</td>
<td>NC_004421</td>
<td>8374</td>
<td>54.60</td>
<td>24.9</td>
<td>32.1</td>
<td>40.6</td>
<td>33.9</td>
</tr>
<tr>
<td><em>Salivirus</em></td>
<td><em>Salivirus A</em></td>
<td>GQ179640</td>
<td>7982</td>
<td>56.70</td>
<td>22.9</td>
<td>31.6</td>
<td>38.1</td>
<td>31.7</td>
</tr>
<tr>
<td>'Oscivirus'</td>
<td>'Oscivirus A1' (turdivirus 2)</td>
<td>NC_014412</td>
<td>7641</td>
<td>46.92</td>
<td>21.9</td>
<td>23.2</td>
<td>35.7</td>
<td>28.7</td>
</tr>
<tr>
<td>'Oscivirus'</td>
<td>'Oscivirus A2' (turdivirus 3)</td>
<td>NC_014413</td>
<td>7678</td>
<td>46.60</td>
<td>20.3</td>
<td>23.9</td>
<td>35.2</td>
<td>27</td>
</tr>
<tr>
<td><em>Aphthovirus</em></td>
<td><em>Foot-and-mouth disease virus O</em></td>
<td>NC_004004</td>
<td>8134</td>
<td>55.30</td>
<td>11.8</td>
<td>18.3</td>
<td>21.4</td>
<td>20.4</td>
</tr>
</tbody>
</table>

*Percentages are reflective of the amino acid identity against the entire corresponding sequence of 'sicinivirus 1'.
of a PCR fragment which when sequenced demonstrated homology to aichivirus B. Others have employed consensus primers as an initial screening tool to identify novel viruses. For example, conserved primers to the 3D\textsuperscript{pol} gene of picornaviruses identified three new picornaviruses: ‘Passerivirus A’, ‘oscivirus A1’ and ‘oscivirus A2’ (Woo et al., 2010), and novel ‘galliviruses’ in chicken and turkey faecal swabs (Farkas et al., 2012).

A virus enrichment and metagenomic approach was applied to identify the genome of ‘sicinivirus 1’. A number of sample preparation steps greatly enhanced the enrichment of virus-specific RNA. The treatment of the faecal extract with nuclease, while the virus RNA remained protected within viral particles, greatly decreased subsequent contamination of the RNA preparation with enteric bacterial and chicken nucleic acid. The subsequent enrichment of polyadenylated RNA by oligo(dT) capture along with the use of sequence-independent priming and specific reverse priming from the 3D region of ‘sicinivirus 1’, resulted in a very significant enrichment of RNA virus genomes. This enrichment is reflected in the metagenomic sequencing data which returned 13 contig sequences, 11 of which were from viruses with polyadenylated RNA genomes and six of these contigs encoded regions of the ‘sicinivirus 1’ genome.

Phylogenetic analysis across the P1, 2C and 3CD regions of ‘sicinivirus 1’ indicates that it forms a monophyletic group with the recently identified ‘Passerivirus A’. However, the amino acid identities of the P1, P2 and P3 regions of these two viruses are below the 40%, 40% and 50% thresholds respectively, which is required for members of the same genus. Furthermore, the L, 2A, 2B and 3A proteins of ‘sicinivirus 1’ share low percentage identity with other picornaviruses. Given that the closest picornavirus currently within the NCBI database, ‘Passerivirus A’, is below the amino acid threshold for genus continuation, we propose that ‘sicinivirus 1’ is the first member of a potentially novel picornavirus genus, suggestively ‘Sicinivirus’. According to the ICTV’s (International Committee of Taxonomy of Viruses) definition of a picornavirus genus (http://www.picornastudygroup.com/definitions/genus_definition.htm), members of a genus should normally share phylogenetically related P1, P2 and P3 genomic regions, with >40%, >40% and >50% amino acid identity, respectively. The predicted P1, P2 and P3 genomic regions of ‘sicinivirus 1’ share, 30.5%, 38.1% and 46.8% amino acid identity, respectively, to its closest relative ‘Passerivirus A’, with an overall identity of 40.9% (Table 1). Interestingly, ‘gallivirus A’ forms a sister clade with both ‘sicinivirus 1’ and ‘Passerivirus A’. It is likely that these viruses may have shared a common ancestor.

The partial 5’ UTR of ‘sicinivirus 1’ is missing regions A–I but contains domains J, K and L of a type II IRES; regions H–L constitute a complete type II IRES. Five structurally distinct classes of IRES have been reported in picornaviruses. Type II IRESs contain five main domains (H, I, J, K and L) and have been reported in aphthovirus, par echovirus, cardiovirus, erbovirus, cosavirus, ‘gallivirus’ and ‘hunnivirus’ (Reuter et al., 2012; Sweeney et al., 2012). They are typically 400–500 nt in size and possess a 3’ end Yn–Xm–AUG motif which can act as the initiator codon. The ‘sicinivirus 1’ IRES shares a strong structural resemblance to ‘gallivirus A’. A fifth class of picornavirus IRES, an Aichi-like IRES, is present in members of the Kobuvirus, Salivirus and ‘Oscivirus’ genera (Sweeney et al., 2012). Interestingly, the ‘Passerivirus A’ 5’ UTR remains to be characterized (Sweeney et al., 2012). L proteins are the most diverse among picornavirus proteins, sharing little sequence homology, as observed with the ‘sicinivirus 1’ L protein. The size of L proteins can vary from 70 amino acids in cardiovirus, to 450 amino acids for avian sapelovirus. ‘Sicinivirus 1’ is predicted to encode a very large L protein of 462 amino acids, similar in size to the 451 amino acid L protein of avian sapelovirus (Tseng & Tsai, 2007).

At the time of slaughter, no grading scheme was employed to assess the health status of the chickens that were included in this study. However, ‘sicinivirus 1’ RNA genome concentrations of 3.9 × 10\textsuperscript{4} to 5.2 × 10\textsuperscript{8} were observed per gram of cloacal faeces, indicating that significant ‘sicinivirus 1’ replication is likely to be taking place in the cloacae of these chickens by 8 weeks of age. The question as to how virus shedding may relate to disease, severity of symptoms and clinical presentation, remains to be established. Also, semiquantitative PCR indicated that 9 of 30 cloacae were jointly positive for ‘sicinivirus 1’ and chicken ‘gallivirus’ (CHK1) and 93 % (27/30) were positive for Campylobacter jejuni (data not show).

The two recently identified turkey picornaviruses, ‘galli virus A’ and TuASV, were detected in both healthy and diseased turkeys (Boros et al., 2012, 2013). ‘Gallivirus A’ was detected in 88% of farms where flocks exhibited symptoms of enteritis and/or stunting syndrome. Interestingly, co-infection with ‘gallivirus A’ and TuASV was detected in 63% of specimens and 75% of farms examined (Boros et al., 2012). Other disease-associated viruses, such as turkey rotavirus and astroviruses, have been reported in both healthy and diseased poultry (Pantin-Jackwood et al., 2007). Moreover, chickens are noted as the main reservoir for human C. jejuni infection (Rosenquist et al., 2006), one of the most common bacterial causes of gastroenteritis in the developed world (Moore et al., 2005). The possibility that multiple viruses and bacteria could in combination contribute to symptoms and disease in the animal host has been proposed (Smith et al., 2013). Such investigations would require controlled infection studies along with detailed pathological and immunological analysis.

In summary, we report the molecular identification of a novel picornavirus in chickens. Further studies will establish its distribution in chicken flocks and its potential role as an avian pathogen.
METHODS

Virus nucleic acid isolation and enrichment. Faeces were recovered from the cloacal content of broiler chickens immediately after normal commercial slaughter. A faecal stool sample (20% in PBS) was rigorously mixed and allowed to settle at room temperature for 1 h, 2 ml was removed and clarified at 5000 g for 10 min. The supernatant was removed and filtered through a 0.45 µm filter (Millipore). A total of 95 µl of this sample was added to 14 µl of 10× Turbo DNase buffer with the addition of 14 units of TURBO DNase (Ambion), 20 MBU Baseline-ZERO DNase (Epicentre), 20 units RNase A (Fermentas) and incubated at 37 °C for 90 min (final volume 140 µl). One volume of Baseline-ZERO Stop solution (EDTA) was added. Viral RNA was extracted from viral particles using a QiAamp Viral RNA Mini kit (Qiagen), and no carrier RNA was added to the viral sample. The viral RNA was eluted in 60 µl elution buffer (Qiagen) containing 40 U RiboLock RNase Inhibitor (Thermo Scientific). Polyadenylated RNA was isolated using Dynabes Oligo (dT)₃₅ according to the manufacturer’s instructions (Dynal).

cDNA synthesis and PCR amplification. cDNA was generated usingSuperscript III Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. Polyadenylated RNA was primed using two different strategies. A sequence-independent amplification of faecal mRNA was performed as described by Victoria et al. (2009), primed with 50 pmol of primer RAN (GCCGGAGCTCTGGAGATA-TCNNNNTTNNNNN). Another RT reaction was primed with a ‘sicinivirus 1’ specific reverse primer (23R; GTACTGCAAGACCTCGTCN). One volume of Baseline-ZERO Stop solution (EDTA) was added. Viral RNA was extracted from viral particles using a QiAamp Viral RNA Mini kit (Qiagen), and no carrier RNA was added to the viral sample. The viral RNA was eluted in 60 µl elution buffer (Qiagen) containing 40 U RiboLock RNase Inhibitor (Thermo Scientific). Polyadenylated RNA was isolated using Dynabes Oligo (dT)₃₅ according to the manufacturer’s instructions (Dynal).

Phylogenetic and homology analysis. Nucleic acid and deduced amino acid sequences corresponding to the ‘sicinivirus 1’ genome (9243 nt and 2864 aa) and the individual P1, P2 and P3 regions were compared using BLASTN and BLASTP, against accessible sequence data of various picornaviruses: ‘Passerivirus A’, aichiivirus A, aichiivirus B, aichiivirus C, salivirus A, ‘gallivirus A’, ‘oscriivirus A1’, ‘oscriivirus A2’, human rhinovirus B14, enterovirus A, enterovirus J, human poliovirus 1, hepatitis A virus, foot-and-mouth disease virus type O and chicken ‘gallivirus’ CHK1. For phylogenetic analysis, the amino acid sequences of the P1, 2C and 3CD regions of the picornaviruses were compared using CLUSTAL X and CLUSTAL W (Larkin et al., 2007). Polyprotein cleavage sites were predicted using the NetPicoRNA 1.0 server (Blom et al., 1996). Pfam (Punta et al., 2012) and BLASTP tools were used to predict conserved protein domains/families within the ‘sicinivirus 1’ polyprotein (Blom et al., 1996). RNA secondary structures were predicted using the MFold web server (Zuker, 2003).

Quantification of virus levels in chicken stool samples. SYBR Green (Sigma-Aldrich) based quantitative PCR, was performed on cDNA generated from faecal RNA extracted samples using a Roche LightCycler 480II thermocycler. A PCR generated product of 197 bp from the 3’ end of the 3D gene of ‘sicinivirus 1’ was generated using primers 23F and 23R (Table S1). Serial dilutions were performed to generate a series of standards ranging in concentration from 10⁻⁷ to 10⁻⁷ molecules. Unknown faecal samples were quantified from the standard curve generated by the second derivative maximum method.
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