Chickens host diverse picornaviruses originated from potential interspecies transmission with recombination

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While chickens are an important reservoir for emerging pathogens such as avian influenza viruses, little is known about the diversity of picornaviruses in poultry. We discovered a previously unknown diversity of picornaviruses in chickens in Hong Kong. Picornaviruses were detected in 87 cloacal and 7 tracheal samples from 93 of 900 chickens by reverse transcription-PCR, with their partial 3Dpol gene sequences forming five distinct clades (I to V) among known picornaviruses. Analysis of eight genomes from different clades revealed seven different picornaviruses, including six novel picornavirus species (ChPV1 from clade I, ChPV2 and ChPV3 from clade II, ChPV4 and ChPV5 from clade III, ChGV1 from clade IV) and one existing species (Avian encephalomyelitis virus from clade V). The six novel chicken picornavirus genomes exhibited distinct phylogenetic positions and genome features different from related picornaviruses, supporting their classification as separate species. Moreover, ChPV1 may potentially belong to a novel genus, with low sequence homologies to related picornaviruses, especially in the P1 and P2 regions, including the predicted L and 2A proteins. Nevertheless, these novel picornaviruses were most closely related to picornaviruses of other avian species (ChPV1 related to Passerivirus A, ChPV2 and ChPV3 to Avisivirus A and Duck hepatitis A virus, ChPV4 and ChPV5 to Melegrivirus A, ChGV1 to Gallivirus A). Furthermore, ChPV5 represented a potential recombinant picornavirus, with its P2 and P3 regions possibly originating from Melegrivirus A. Chickens are an important reservoir for diverse picornaviruses that may cross avian species barriers through mutation or recombination.

INTRODUCTION

Picornaviruses are widely distributed in various animals, including humans, causing respiratory, cardiac, hepatic, neurological, mucocutaneous and systemic diseases of varying severity (Tracy et al., 2006). They are small, positive-sense, single-stranded RNA viruses with icosahedral capsids and an ~7–9 kb genome. The family Picornaviridae is currently divided into 26 genera (Aphthovirus, Aquamavirus, Avihepatovirus, Avisivirus, Cardiovirus, Cosavirus, Dicippivirus, Enterovirus, Erbovirus, Gallivirus, Hepatovirus, Hennivirus, Kobuvirus, Megrivirus, Mischivirus, Mosavirus, Oscivirus, Parechovirus, Pasivirus, Passerivirus, Rosavirus, Salivirus, Sapelovirus, Senecavirus, Teschovirus and Tremovirus) (Adams et al., 2013; Knowles et al., 2012). Picornaviruses are known for their ability to evolve through mutation and recombination, which may allow them to evade immunity and adapt to new ecological niches (Benschop et al., 2010; Yip et al., 2010, 2013; Boros et al., 2012a, b; Huang et al., 2012).

Approximately three-quarters of all emerging infectious disease agents in humans are believed to be zoonotic in
origin (Woo et al., 2006b). Since the SARS (severe acute respiratory syndrome) epidemic, there has been an increase in interest in studying novel zoonotic viruses, including picornaviruses (Chiu et al., 2008; Drexler et al., 2010; Kapoor et al., 2008a, b; Lau et al., 2005; Woo et al., 2006a). Several novel human picornaviruses, including human rhinovirus C, Cosavirus, Saffold virus and Salivirus/klassevirus, have been discovered in the past few years (Drexler et al., 2008; Holtz et al., 2009; Jones et al., 2007; Kapoor et al., 2008a; Lau et al., 2007, 2009; Li et al., 2009; McErlean et al., 2008). Recently, we have also described the identification of novel picornaviruses from wild and domestic animals, including cats, dogs, bats and birds (Lau et al., 2011, 2012; Woo et al., 2010, 2012). The identification of previously undescribed picornaviruses in animals is important for a better understanding of their host range, genetic diversity, evolution and potential for cross-species transmission.

While birds are well known as an important reservoir for emerging pathogens, only a small number of picornavirus species have been identified from avian sources compared with their enormous species diversity. Turdiviruses were first discovered in Hong Kong from dead wild birds. They were of the genus Turdus in the family Turdidae, which may potentially belong to three new species within two new genera (Woo et al., 2010). Duck hepatitis A virus (DHAV), in the genus Avisiviridae, and the more recently identified Melegrivirus A (formerly turkey hepatitis virus), in the genus Megiviridae, circulate among ducks and turkeys respectively, and may cause significant economic losses in poultry farms (Honkavuori et al., 2011). Recently, two novel picornaviruses, Gallivirus A (formerly turkey gallivirus) and Avisivirus A (formerly turkey avisivirus), have been identified in faecal samples of turkeys in Hungary (Boros et al., 2012a, 2013). Although chickens are the most important poultry and food animals worldwide, the diversity and significance of picornaviruses in these birds are not well understood. Avian encephalomyelitis virus (AEV) is the only picornavirus in chickens well studied in recent decades. Recently, viral sequences closely related to Gallivirus A were identified in faecal swabs of both chickens and turkeys from the United States (Farkas et al., 2012). A novel picornavirus, most closely related to Passeriviridae A (formerly turdivirus), has also recently been reported in chickens in Ireland (Pullman et al., 2014), suggesting that the diversity of chicken picornaviruses has been underestimated.

In this study, we describe a molecular epidemiological study and the discovery of a previously unknown diversity of picornaviruses in chickens, including six novel picornavirus species. These chicken picornaviruses are closely related to their counterparts in other avian species. A potential recombinant picornavirus related to Melegrivirus A was also identified, suggesting that chickens are an important reservoir for diverse picornaviruses that may overcome the species barrier through recombination.

### RESULTS

#### Chicken surveillance and identification of diverse chicken picornaviruses

Screening of 900 tracheal and 900 cloacal samples from 900 chickens by reverse transcription-PCR (RT-PCR) for a 155 bp fragment of the 3Dpol gene was positive in 87 cloacal and 7 tracheal samples from 93 chickens. These sequences fell into five clades, I to V, representing diverse picornaviruses (Fig. 1). The sequences from 57 samples fell into clade I, being most closely related to Passeriviridae A, with <71 % nt identity. The sequences from seven samples fell into clade II, being most closely related to Avisiviridae A, with <66 % nt identity. The sequences from 20 samples fell into clade III, with 73–86 % nt identity to Melegrivirus A. The sequences from eight positive samples fell into clade IV, with 72–76 % nt identity to Gallivirus A. The sequences from two positive samples fell into clade V, with 80–81 % nt identity to AEV. The positive detection rates of picornaviruses among chickens were 3.2 % (22/680 samples) in the winter (January to March) and 6.4 % (72/1120 samples) in the summer (June to October) seasons during the study period.

#### Genome organization and coding potential of chicken picornaviruses

Eight genomes (two from each of clades I, II and III, and one from each of clades IV and V) were sequenced directly from cloacal samples and characterized. The mol% G+C contents were 55, 45–48, 44–45, 45 and 44 % for clades I (ChPV1_55C, ChPV1_100C), II (ChPV2_44C, ChPV3_45C), III (ChPV4_5C, ChPV5_27C), IV (ChGV1_518C) and V (AEV_C204C), respectively, with genome sizes of 8287–8331, 7167–7310, 9564–9591, 8432 and 6955 bp, respectively (excluding polyadenylated tract) (Table S1, available in the online Supplementary Material). The genome sizes of some strains may be larger, as further sequencing of the ends may have been hampered by secondary structures.

The eight sequenced strains have genome organization typical of picornaviruses, with untranslated regions (UTRs) at both 5' and 3' ends, and a large ORF that encodes potential polyprotein precursors known to be cleaved by virus-encoded proteases (Fig. 2). The hypothetical protease cleavages in the P1 (encoding capsid protein), P2 and P3 (both encoding non-structural proteins) regions are shown in Table 1. Differences in genome organization were observed between the different clades: (i) ChPV1_55C and ChPV1_100 (clade I), and ChGV1_518C (clade IV) contain leader sequences; (ii) 2A of ChPV2_44C and ChPV3_45C (clade II) encodes a protein that may be processed into several smaller fragments (Fig. 3); (iii) ChPV4_5C and ChPV5_27C (clade III) contain an additional, hypothetical protein between P1 and P2; and (iv) AEV_C204C (clade V) possesses a predicted cleavage site in VP0 (VP4/VP2) (Fig. 2). Other distinct genome features, as compared with known picornaviruses, are discussed below (Table 2).
Fig. 1. Phylogenetic analysis of partial 3D\textsuperscript{pol} gene sequences of chicken picornaviruses detected in this study. The present strains were numbered, with ‘C’ representing cloacal sample and ‘T’ representing tracheal sample. Asterisks indicate the chicken picornavirus strains with complete genomes sequenced in this study. Bootstrap values expressed as percentages are shown at nodes and only those >70% are shown. Bar, estimated number of substitutions per 10 nt. AEV, Avian encephalomyelitis virus (NC\textunderscore 003990); AIV, Aichivirus A (NC\textunderscore 001918); ChGV/CHK175/USA/2010, chicken gallivirus strain CHK175/USA/2010 (JF424827); CPDV-209, Cadicivirus A (JN819202); DHAV-1, duck hepatitis A virus 1 (NC\textunderscore 008250); DPV, Avian sapelovirus (NC\textunderscore 006553); EMCV, Encephalomyocarditis virus (NC\textunderscore 001479); EV-C, Enterovirus C (NC\textunderscore 002058); ERBV2, equine rhinitis B virus 2 (NC\textunderscore 003077); FMDV-C, foot-and-mouth disease virus type C (NC\textunderscore 002554); HAV, hepatitis A virus (NC\textunderscore 001489); HCoSV-A1, Cosavirus A strain HCoSV-A1 (NC\textunderscore 012800); HPcV, Human parechovirus (NC\textunderscore 001897); PTV1, porcine teschovirus 1 (NC\textunderscore 003988); SalVNG-J1, Salivirus strain NG-J1 (NC\textunderscore 012957); SePV1, seal aquamavirus A1 (NC\textunderscore 009891); SVV, Seneca Valley virus (NC\textunderscore 011349); TV1, Passerivirus A (NC\textunderscore 014411); TV2, Oscivirus A (formerly turdivirus 2) (NC\textunderscore 014412); TuASV, Avisivirus A (KC465954); TuGV, Gallivirus A (NC\textunderscore 018400); THV, Melegrivirus A (HM751199).

**Phylogenetic analyses**

Phylogenetic trees of the P1, P2 (excluding 2A) and P3 (excluding 3A) regions of the chicken picornaviruses are shown in Fig. 4. The 2A and 3A regions were excluded to avoid bias due to poor sequence alignment. ChPV1\textunderscore 55C and ChPV1\textunderscore 100C (clade I) form a distinct cluster, distantly
related to *Passerivirus A* in all three regions. Their genomes share 80.8% nt identity, representing two different strains of novel chicken picornavirus 1, ChPV1. ChPV2_44C and ChPV3_45C (clade II), together with *Avisivirus A*, form another distinct cluster, distantly related to DHAV in all three regions. Their genomes share only 53.8% nt identity to each other, representing two novel chicken picornaviruses, ChPV2 and ChPV3. ChPV4_5C and ChPV5_27C (clade III) share 80.3% nt identity to each other, representing two novel chicken picornaviruses, ChPV4 and ChPV5. They are closely related to *Melegrivirus A* in the P2 and P3 regions. However, in the P1 region, ChPV5_27C is only distantly related to ChPV4 and *Melegrivirus A*, with 38.6% aa identity to the P1 region of ChPV4, compared with 99.3 and 99% aa identities in the P2 and P3 regions, respectively, between ChPV5_27C and ChPV4. ChGV1_518C (clade IV) forms a distinct cluster with *Gallivirus A* in all three regions. Its genome shares only 66% nt identity with that of *Gallivirus A*, representing a novel chicken gallivirus 1, ChGV1. AEV_204C (clade V) is closely related to AEV (NC_003990), with 83.1% nt identity, suggesting that it belongs to the species AEV.

**Genome features of ChPV1 distantly related to *Passerivirus A* (clade I chicken picornaviruses)**

ChPV1_55C and ChPV1_100C share similar genome sequences and features, except a different predicted VP1/2A cleavage site (Table 1, Fig. 4). A leader (L) protein is present, with ≤18.4% aa identity to those of known picornaviruses. Moreover, it represents the shortest L protein (21 aa) among known picornaviruses (49–2451 aa). No predicted proteolytic activity or zinc-finger motifs were found (Table 2) (Chen et al., 1995; Dvorak et al., 2001; Gradi et al., 2004; Hinton et al., 2002; Tseng & Tsai, 2007).

The predicted 2A of ChPV1_55C and ChPV1_100C exhibit ≤28.4% aa identity to those of known picornaviruses. While they do not possess the conserved H-box/NC motif involved in cell proliferation control (Hughes & Stanway, 2000; Tseng et al., 2007b; Woo et al., 2010), the predicted 2C of ChPV1_55C and ChPV1_100C possess the GxxGxGKS motif for NTP-binding (Gorbalenya et al., 1989b), and the DDLxQ motif for putative helicase activity (Gorbalenya et al., 1990), although Leu was substituted by Val as in *Passerivirus A* and some picornaviruses (Cohen et al., 1987; Lindberg & Johansson, 2002; Racaniello & Baltimore, 1981; Tseng et al., 2007b; Woo et al., 2010; Yamashita et al., 1998).

Similarly to *Passerivirus A*, the predicted 3CPro of ChPV1_55C and ChPV1_100C contain the catalytic triad H-E-C (Bazan & Fletterick, 1988). They also contain the conserved GxxG motif in the protease active site, and the GxH motif (Gorbalenya et al., 1989a; Hammerle et al., 1992), although Gly was replaced by Ala. The predicted
Table 1. Coding potential and putative proteins of the genomes of chicken picornaviruses analysed in this study

<table>
<thead>
<tr>
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<th>Clade I</th>
<th>Clade I</th>
<th>Clade II</th>
<th>Clade II</th>
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<td>VP3</td>
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</table>

3D\textsuperscript{pol} of ChPV1_55C and ChPV1_100C contain the conserved KDE[LI]R, GG[LMMN]PSG, YGDD and FLKR motifs (Kamer & Argos, 1984).

**Genome features of ChPV2 and ChPV3 distantly related to Avisivirus A (clade II chicken picornaviruses)**

The conserved sequence Yₙ-Xₘ-AUG is present in the 5' UTR of ChPV2_44C and ChPV3_45C (Table 2). The putative translation initiation sites are contained by an optimal Kozak context (RNAUGG), with in-frame AUG at positions 493 (ChPV2_44C) and 458 (ChPV3_45C). The 5' UTR of many picornaviruses possesses an internal ribosomal entry site/segment (IRES) that is responsible for directing the initiation of translation in a cap-independent manner and requires both canonical translation initiation and IRES\textsuperscript{trans}-acting factors (Fitzgerald & Semler, 2011; Shih et al., 2011). While no definite IRES structure was observed in clade I viruses, ChPV2_44C and ChPV3_45C share 93 and 85% nt identity at a region of the 5' UTR corresponding to positions 497–554 and 474–554, respectively, in Avisivirus A (KC614703). Moreover, they contain a type II-like IRES with stem–loops, major domains (I and
J-K) and conserved motifs (Fig. 5). However, domain H, with a polypyrimidine tract-binding protein binding site, UCUUU, was noted in ChPV3_45C but not ChPV2_44C or Avisivirus A. Similar to Encephalomyocarditis virus (EMCV), Gallivirus A and Avisivirus A, the pyrimidine-rich region is located near the 3' end of 5' UTR.

The predicted 2A of ChPV2_44C and ChPV3_45C exhibit ≤31.1 % aa identity to those of known picornaviruses. They share only 24.8 % aa identity to each other. Moreover, based on the presence of proteolytic cleavage sites predicted by the NetPicoRNA program and/or multiple sequence alignment, they may be processed into several smaller proteins, as in some picornaviruses, including Avisivirus A and DHAV (Figs 2 and 3). They possess the H-box/NC motif and the Asn-Pro-Gly-Pro (NPGP) motif required for co-translational cleavage at 2A/2B or cleavage into small 2A proteins (Ryan & Flint, 1997). The two in-tandem aphthovirus 2A-like sequence repeats with DxExNPG/P found in Avisivirus A were noted in ChPV2_44C, but not ChPV3_45C (Boros et al., 2013). The predicted 2A2 belongs to the P-loop-containing nucleoside triphosphate hydro-lases superfamily, with the conserved nucleotide phosphate-binding motif GxxxxGK[ST]. The predicted 2A3 contains the conserved H-Box and NCET motifs (Hughes & Stanway, 2000; Tseng & Tsai, 2007). The predicted 2C possesses the GxxGxGKS motif and the DDLxQ motif, where Leu is substituted by Met in ChPV3_45C instead of Phe as in avipathoviruses. The conserved motifs in the predicted 3C\textsuperscript{pro} and 3D\textsuperscript{pol} are also present.

**Genome features of ChPV4 and ChPV5 related to Melegrivirus A (clade III chicken picornaviruses)**

In contrast to Melegrivirus A, in which no IRES was reported (Honkavuori et al., 2011), ChPV4_5C and ChPV5_27C possess a predicted type IV or hepacivirus/pestivirus (HP)-like IRES with stem–loop domains II to III, conserved G loop in domain IIIId and apical loop, UGAUAG, in domain IIId (last G substituted by A) (Table 2, Fig. 5) (Hellen & de Breyne, 2007).

The putative novel protein (324 aa) between VP1 and 2A, not found in other picornaviruses, is shorter than that in Melegrivirus A (401 aa), and shares 92 % aa identity between ChPV4_5C and ChPV5_27C. Differences in predicted cleavage sites between VP1, hypothetical protein and 2A of ChPV4_5C and ChPV5_27C were noted (Table 1).

The predicted 2A of ChPV4_5C and ChPV5_27C display 88.4–91.5 % aa identity to that of Melegrivirus A, with the conserved H-box/NC motif. The predicted 2C possess the GxxGxGKS motif and the DDLxQ motif where Leu is substituted by Phe as in megriviruses. The conserved motifs in the predicted 3C\textsuperscript{pro} and 3D\textsuperscript{pol} are also present.

**Genome features of ChGV1_518C distantly related to Gallivirus A (clade IV chicken picornaviruses)**

The 5' UTR of ChGV1_518C shares 83 % nt identity at a region corresponding to nt 295–671 in Gallivirus A (NC_018400). It contains the conserved sequence Yn-Xm-AUG and a type II-like IRES consisting of five major domains, H to L (Fig. 5). While one stem–loop structure representing domain L was found in EMCV and Gallivirus A (Yu et al., 2011), two stem–loop structures are present between the pyrimidine-rich region and the start codon of polyprotein in ChGV1_518C. The pyrimidine-rich region of EMCV is located downstream of domain L, while those of ChGV1 and Gallivirus A are located upstream of this domain.

The predicted L protein of ChGV1_518C exhibits ≤32.6 % aa identity to those of known picornaviruses. No predicted proteolytic activity and zinc-finger motif were found. Two myristoylation sites Gxxx[ST] are present in the N-terminus of VP0 downstream of L. The predicted 2A shares 71.9 % aa identity to that of Gallivirus A, and possesses the H-box/NC motif. The predicted 2C possesses the GxxGxGKS motif and the DDLxQ motif where Leu is substituted by Ile. However, the catalytic triad of the predicted 3C\textsuperscript{pro} is H-E-C, instead of H-Q-C in Gallivirus A. Conserved motifs in the predicted 3D\textsuperscript{pol} are also present (Table 2).

**Genome features of AEV_204C (clade V chicken picornaviruses)**

The genome of AEV_204C shares similar features to that of AEV under Tremovirus (Table 2).

**Viral culture**

No cytopathic effect was observed in cell lines inoculated with positive samples. RT-PCR of culture supernatants, cell lysates and chick embryo allantoic fluid for monitoring viral replication also showed negative results.

**DISCUSSION**

We report the discovery of a previously unknown diversity of picornaviruses, including six novel picornaviruses, from chickens in Hong Kong, indicating that these poultry animals are an important reservoir for diverse picornaviruses. Picornaviruses were detected in 7 (0.7 %) of 900 tracheal and 93 (10.3 %) of 900 cloacal samples, with their...
Table 2. Comparison of genomic features of the five clades of chicken picornaviruses to those of different genera in the Picornaviridae family

<table>
<thead>
<tr>
<th>Region</th>
<th>Function, conserved motif or feature</th>
<th>Genus</th>
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<tbody>
<tr>
<td></td>
<td>Avihepato-</td>
<td>Hepato-</td>
</tr>
<tr>
<td>5' UTR</td>
<td>Pattern</td>
<td>Y7 × 65 (DHV1)</td>
</tr>
<tr>
<td></td>
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<td>L</td>
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<tr>
<td>VP0</td>
<td>Cleaved into VP4 and VP2; Myristylation site; Chow et al. (1987); GxxRxKGS</td>
<td>N</td>
</tr>
<tr>
<td>2A</td>
<td>NTase motif; Gorbalenya et al. (1989b); GxxRxKGS</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>RNA-binding domain motif; Hammerle et al. (1992); KRIDJ</td>
<td>Y</td>
</tr>
<tr>
<td>3D†††</td>
<td>Motif: KDE[ELI]; Kamer &amp; Argos (1984)</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Motif: GGM/CG</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Motif: GG[LMNP]SG; Kamer &amp; Argos (1984)</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Motif: YGIDD; Kamer &amp; Argos (1984)</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Motif: FLKR; Kamer &amp; Argos (1984)</td>
<td>Y</td>
</tr>
</tbody>
</table>

*Porcine kobuvirus SUN-1-HUN was predicted to have type IV IRES.
†YN, Presence of L but L is not protease.
§2A of HAV and Oscivirus A does not contain the characteristic catalytic amino acid residues with chymotrypsin-like proteolytic activity, the NPGP motif or the H-box/NC motif.
§Y, Motif DDLxQ is present.
Fig. 4. Phylogenetic analyses of the (a) P1, (b) P2 (excluding 2A) and (c) P3 (excluding 3A) of the eight chicken picornaviruses with complete genomes sequenced in this study (shaded in grey). Chicken picornaviruses and other avian picornaviruses are indicated by a pictogram of a typical ordinal host (chicken, duck, turkey, wild birds). Bootstrap values expressed as percentages are shown at nodes and only those >70% are shown. Bar, estimated number of substitutions per 2 (P1) and 5 (P2 and P3) aa.
(a) GNRA tetraloop motif

Clade II
ChPV2_44C

13
115
320
492

Pyrimidine-rich region

Clade II
ChPV3_45C

150
210
17
110
457
300

370
Diverse chicken picornaviruses

Clade III
ChPV4_5C

Clade IV
ChGV1_518C

Pyrimidine-rich region

GNRA tetraloop motif
motif. Moreover, a G substitution was observed in the DDLxQ motif of the predicted 2C protein (Fig. S1). However, more genome sequences from different strains of ChPV5 are required to determine whether this is a general phenomenon in ChPV5 or an incidental recombination event in strain 27C. Nevertheless, the finding suggests that chickens may serve as a reservoir for diverse picornaviruses in fostering recombination to generate new viruses.

ChGV1, belonging to clade IV, represents a novel picornavirus species that may belong to the genus Gallivirus, and ChPV5 could possibly be a recombinant virus strain. Both ChPV4 and ChPV5 were predicted to have a type IV IRES. Interestingly, while ChPV4 exhibits 89.5 % aa identity to Melegrivirus A in the P1 region, ChPV5 only displays 38 % aa identity to Melegrivirus A in the P1. When compared with ChPV4, ChPV5 displays 38.6, 99.3 and 99 % aa identities in the P1, P2 and P3 regions, respectively. The discordant phylogenetic relationship may suggest potential recombination events between ChPV4 or Melegrivirus A and an unknown picornavirus with a distinct P1 region, leading to the generation of strain ChPV5_27C. This is also supported by similarity plot analysis showing a potential recombination event at a position between VP1 and the hypothetical protein (Fig. S1). However, more genome sequences from different strains of ChPV5 are required to determine whether this is a general phenomenon in ChPV5 or an incidental recombination event in strain 27C. Nevertheless, the finding suggests that chickens may serve as a reservoir for diverse picornaviruses in fostering recombination to generate new viruses.

Fig. 5. Predicted IRES structures of (a) clade II viruses, ChPV2_44C and ChPV3_45C (type II-like), (b) clade III viruses, ChPV4_5C and ChPV6_27C (type IV), and (c) clade IV virus, ChGV1_518C (type II-like). The AUG start codon is in bold and underlined.

partial 3Dpol gene sequences falling into five different clades among known picornaviruses. Higher detection rates were consistently observed during the summer months than in the winter months, suggesting a higher prevalence of picornaviruses in chickens in summer. Analysis of the complete genomes of eight different strains from the five clades confirmed that they represented distinct picornaviruses with different genome features. Phylogenetic trees constructed using the P1, P2 (excluding 2A) and P3 (excluding 3A) regions revealed seven different picornavirus species including six novel picornavirus species (ChPV1 from clade I, ChPV2 and ChPV3 from clade II, ChPV4 and ChPV5 from clade III, and ChGV1 from clade IV) and one existing species, AEV, from clade V. In addition to their distinct phylogenetic positions, the novel chicken picornaviruses also exhibit genome features different from related picornaviruses, supporting their classification as separate species.

ChPV1, belonging to clade I, represents a novel picornavirus species different from Passerivirus A and may belong to a novel genus. Although it is most closely related to Passerivirus A of the genus Passerivirus (formerly genus ‘Orthoturdivirus’), its P1, P2 (excluding 2A) and P3 (excluding 3A) regions possess only 33.1–33.9, 38.5–38.7 and 55.8–55.96 % aa identities, respectively, to the corresponding regions in Passerivirus A. Its predicted L and 2A proteins also display very low homologies to those of Passerivirus A. In contrast to Passerivirus A, the predicted 2A of ChPV1 does not possess the H-box/NC motif. Moreover, a G→A substitution was observed in the GxH motif of ChPV1. These distinct features from Passerivirus A support the classification of ChPV1 as a separate species. Although the P3 region of ChPV1 exhibits >50 % aa identity to that of Passerivirus A (which slightly exceeds the 50 % cut-off for defining a novel genus according to International Committee on Taxonomy of Viruses criteria), the low homologies to known picornaviruses in the L protein, and the P1 and P2 regions, including 2A, suggest that ChPV1 may potentially belong to a separate genus. Genome sequence analysis of more ChPV1 viruses should help determine the genetic diversity of ChPV1 and whether it should be placed under a novel genus within the Picornaviridae family. The novel picornavirus identified in chickens from Ireland is also phylogenetically related to Passerivirus A, but it is probably a different virus from ChPV1. While a type II-like IRES was reported in the former virus (Bullman et al., 2014), no predicted IRES was found in ChPV1. Further genomic and phylogenetic analysis will help better delineate their relationship when genome sequences are available from the Irish strains.

While ChPV2 and ChPV3, belonging to clade II, represent two novel picornavirus species, it is arguable whether they belong to the same genus as or a different genus from Avisivirus A. They are most closely related to Avisivirus A, with 44.6, 53.9 and 50.5 % aa identities between ChPV2 and ChPV3, and 42.4–44.1, 53.5–53.6 and 50.8–53.4 % aa identities to Avisivirus A in the P1, P2 (excluding 2A) and P3 (excluding 3A) regions, respectively. However, their predicted 2A proteins display very low homologies to that of Avisivirus A and to each other. ChPV3 possesses an L→M substitution in the DDLxQ motif of the predicted 2C proteins different from Avisivirus A and avipapoviruses. These distinct genomic features contrast with the observed high aa identities to Avisivirus A in the P1, P2 and P3 regions, and therefore it is not certain whether ChPV2 and ChPV3 should be classified in the same genus as or a different genus from Avisivirus A.
pyrimidine-rich region. Moreover, they share low homologies in the predicted I proteins and exhibit a different catalytic triad in the predicted 3Cpro protein, supporting the classification of ChGV1 as a separate species from Gallivirus A. Besides the other seven potential strains of ChGV detected in this study, a potential ChGV strain, CHK175, has also been detected in the United States (Farkas et al., 2012). However, no genome sequence was available for comparison. Since Gallivirus A has been identified in turkeys in Hungary and the United States, ChGV1 may also be circulating in chicken populations worldwide.

Our finding of at least seven picornavirus species potentially belonging to five different genera, and the distant phylogenetic relationship of clade I, II, III and IV viruses to wild bird, turkey and duck counterparts (Fig. 4), suggest that chickens are susceptible to diverse picornaviruses with potential cross-species transmission from other avian species. This may reflect the frequent contact of chickens with other avian species, which may facilitate interspecies viral transmission, analogous to the emergence of avian influenza viruses in poultry (Chen et al., 2013; Peiris et al., 1999; Yuen et al., 1998). Moreover, the detection of a potential recombinant chicken picornavirus, ChPV5_27C, also suggests that picornaviruses may overcome the species barrier to infect chickens through recombination. Interspecies transmission of picornaviruses has been reported among different bat genera or species, which may be explained by their species diversity, ability to fly and tendency for roosting (Lau et al., 2011). A natural intraspecies recombinant bovine/porcine enterovirus has also been identified in sheep (Boros et al., 2012b), suggesting that picornaviruses may infect different animals sharing close habitats. In contrast, only a single picornavirus species was identified among domestic cats, having a more limited habitat (Lau et al., 2012). As chickens are often exposed to other poultry animals or avian species, it is important to monitor the genetic evolution of chicken picornaviruses and study their potential for interspecies transmission.

The pathogenicity of the novel chicken picornaviruses remains to be determined. The only previously documented picornavirus in chickens, AEV, is known to infect young chickens, as well as pheasants, quail and turkeys (Marvil et al., 1999). It causes slight reduction in egg production in susceptible laying hens, and can be transmitted to embryos, resulting in reduced hatching and neurological diseases in chicks. In this study, chickens positive for the different picornaviruses were apparently healthy. Some of the viruses, including viruses from clades I, II, III and IV, were also detected in the tracheal samples of seven chickens, which may suggest possible respiratory in addition to enteric tropism. Although further studies are required to examine their possible disease association in chickens, their phylogenetic relationship with pathogenic viruses in other avian species may suggest potential pathogenicity. For example, Passerivirus A, the closest virus to ChPV1 belonging to clade I, was first discovered in tracheal and cloacal swabs of dead wild birds, although its disease association is yet to be determined. ChPV4 and ChPV5, belonging to clade III, are distantly related to Avisivirus A, which was discovered in faecal samples of both healthy and diseased turkeys, and to DHAV, which causes a highly lethal, contagious infection characterized by ataxia and opisthotonos in young ducklings (Boros et al., 2012a; Honkavuori et al., 2011). Melegrivirus A, closely related to ChPV4 and ChPV5 belonging to clade III, has been associated with turkey viral hepatitis, a highly infectious disease associated with high mortality in young turkey poult s that causes substantial economic losses (Honkavuori et al., 2011). The virus was discovered in liver samples, bile, intestine, serum and cloacal swabs of diseased turkey poult s (Honkavuori et al., 2011). Gallivirus A, phylogenetically related to ChGV in clade IV, has been detected in both healthy and diseased (with enteric and/or stunting syndrome) commercial turkeys (Boros et al., 2013). Further studies are required to determine the virulence and economic impact of the present novel picornaviruses in chicken populations.

Chickens are well known as an important reservoir for a wide range of pathogens, including viruses of different families, some of which may emerge in humans, causing epidemics (Chen et al., 2013; Peiris et al., 1999; Yuen et al., 1998). For example, the emergence of avian influenza virus epidemics in the Asian population may be related to their cultural preference for freshly killed poultry prepared domestically or at live poultry markets. Since the avian influenza A H5N1 virus epidemic in 1997, the Hong Kong government has set up policies for local poultry surveillance, including regular testing for avian influenza and Newcastle disease viruses. Based on our present findings, continuous surveillance of picornaviruses in chickens and other poultry animals is important in understanding their potential for emergence.

METHODS

Chicken surveillance and sample collection. Chicken samples from commercial chicken farms in Hong Kong were provided by the Food and Environmental Hygiene Department (FEHD), the government of the Hong Kong Special Administrative Region (HKSAR), as part of an animal surveillance programme from September 2008 to August 2012. Samples were collected from 60- to 80-day-old chickens at retail live poultry markets from the whole territory, during the winter (January to March) and summer (June to October) seasons every year of the study period. Tracheal and cloacal swab samples were collected using procedures described previously (Lau et al., 2005, 2008).

RNA extraction. Viral RNA was extracted from tracheal and cloacal swab samples using the EZ1 Virus Mini kit v2.0 (Qiagen). The RNA was eluted in 60 μl AVE buffer (elution buffer provided by Qiagen) and was used as the template for RT-PCR.

RT-PCR of 3Dpol gene of picornaviruses using conserved primers and DNA sequencing. Picornavirus screening was performed by amplifying a 3Dpol gene fragment of picornaviruses of around 155 bp using conserved primers (5′-AAGGTGGVATGCK-TCHGG-3′ and 5′-ATAACAGCATCATGCRCRATARA-3′) designed by multiple alignment of the nucleotide sequences of the 3Dpol genes of

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various picornaviruses, using previously described protocols (Lau et al., 2011, 2012; Woo et al., 2010). Reverse transcription was performed using the SuperScript III kit (Invitrogen), and the reaction mixture (10 µl) contained RNA, first-strand buffer (50 mM Tris/HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂), 5 mM dTT, 50 ng random hexamers, 500 µM each dNTP and 100 U Superscript III reverse transcriptase. The mixtures were incubated at 25 °C for 5 min, followed by 50 °C for 60 min and 70 °C for 15 min. The PCR mixture (25 µl) contained cDNA, PCR buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl, 2 mM MgCl2 and 0.1% gelatin), 200 µM each dNTP and 1.0 U Taq polymerase (Applied Biosystems). The mixtures were amplified in 40 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 10 min, in an automated thermal cycler (Applied Biosystems). Standard precautions were taken to avoid PCR contamination and no false positive was observed in negative controls.

All PCR products were gel-purified using the QIAquick gel extraction kit (Qiagen). Both strands of the PCR products were sequenced twice with a 3730xl DNA Analyser (Applied Biosystems), using the two PCR primers. The sequences of the PCR products were compared with known sequences of the 3Dpol genes of picornaviruses in the GenBank database.

**Genome sequencing.** Eight genomes of different chicken picornaviruses (two genomes each from clades I, II and III, and one genome each from clades IV and V) were amplified and sequenced using previously published strategies (Lau et al., 2011, 2012), with the RNA extracted from the cloacal samples as templates. RNA was converted to cDNA by a combined random-priming and oligo(dT) priming strategy. The cDNA was amplified by degenerate primers designed by multiple alignment of the genomes of the phylogenetically closely related picornaviruses, including *Passerivirus A* (formerly turdivirus 1), *Oscivirus A* (formerly turdivirus 2 and 3), *Aichivirus A*, bovine kobuvirus, duck hepatitis A viruses 1 and 3 (GenBank accession no. GU182406, GU182408, GU182410, NC_001918, NC_004421, NC_008250 and DQ256132), and additional primers designed from the results of the first and subsequent rounds of sequencing. These primer sequences are available on request. The 5’ ends of the viral genomes were confirmed by RACE using the SMARTer RACE cDNA Amplification kit (Clontech). Sequences were assembled to produce final sequences of the viral genomes.

**Phylogenetic and genome analysis.** The nucleotide sequences of the genomes and the deduced amino acid sequences of the ORF were compared to those of other picornaviruses by multiple sequence alignments. The hypothetical protease cleavage sites of the poly-proteins were predicted by the NetPicoRNA 1.0 program and/or multiple alignments by using available complete genome sequences of related picornaviruses. The unrooted phylogenetic tree of 3Dpol gene fragments was constructed using the neighbour-joining method for aligned nucleotide sequences in CLUSTAL_X 2.0. The maximum-likelihood phylogenetic trees of the P1, P2 (excluding 2A) and P3 (excluding 3A) regions were constructed using MEGAS (Tamura et al., 2011), with bootstrap values calculated from 1000 trees. Secondary structure prediction in the 5’ UTR was performed using Mfold (Zuker, 2003).

**Viral culture.** A total of 12 cloacal samples positive for picornaviruses (3 from clade I, 4 from clade II, 3 from clade III, 1 from clade IV and 1 from clade V) were cultured in Vero (African green monkey kidney; ATCC CCL-81), Vero E6 (African green monkey kidney; ATCC CRL-1586), CrFK (Crandell feline kidney; ATCC CCL-94), LMH (chicken liver; ATCC CRL-2117) and DF-1 (chicken embryonic fibroblast; ATCC CRL-12203) cells and chick embryos.

**Nucleotide sequence accession numbers.** The nucleotide sequences of the eight genomes of chicken picornaviruses studied have been lodged in the GenBank sequence database under accession numbers KP979331–KP979338.

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**REFERENCES**


Diverse chicken picornaviruses


