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A diarrheic chicken simultaneously co-infected with multiple picornaviruses: Complete genome analysis of avian picornaviruses representing up to six genera

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ABSTRACT

In this study all currently known chicken picornaviruses including a novel one (chicken phacovirus 1, KT880670) were identified by viral metagenomic and RT-PCR methods from a single specimen of a diarrheic chicken suffering from a total of eight picornavirus co-infections, in Hungary. The complete genomes of six picornaviruses were determined and their genomic and phylogenetic characteristics and UTR RNA structural models analyzed in details. Picornaviruses belonged to genera *Sicinivirus* (the first complete genome), *Gallivirus*, *Tremovirus*, *Avisivirus* and "Orivirus" (two potential genotypes). In addition, the unassigned phacoviruses were also detected in multiple samples of chickens in the USA. Multiple co-infections promote and facilitate the recombination and evolution of picornaviruses and eventually could contribute to the severity of the diarrhea in chicken, in one of the most important food sources of humans.

Introduction

Members of family Picornaviridae are small viruses with positive sense, single-stranded RNA genomes whose length range between 7.2 and 9.7 kb with the predominant presence of a single Open Reading Frame (ORF). The general picornavirus genome layout contain the 5′UTR-[ORF: L (not always present)-VP0 (or cleaved into VP4 and VP2)-VP3-VP1-2A-2B-2C-3A-3B-3C-3D]-3′UTR-polyA-tail (Palmenberg et al., 2010). A few picornaviruses have slightly modified genome plans such as the cadiciviruses (genus *Dicipivirus*) with two ORFs or the unassigned aaliviruses with up to six 2A regions (Woo et al., 2012; Wang et al., 2014). The 5′ and 3′ un-translated regions (UTRs) contain several structurally conserved motifs, generally one of the five different types of internal ribosomal entry sites (IRES) and the apical "8" structure at the 5′ end and s2m, "barbell-like" structure or repeated sequences similar to the 'Unit As" of megriviruses at the 3′ end (Palmenberg et al., 2010; Boros et al., 2014c). The family Picornaviridae currently consists of 50 species grouped into 29 genera and a continuously increasing number of novel, currently unassigned picornaviruses (Knowles et al., 2012; Adams et al., 2015; www.picornaviridae.com). The picornaviruses have been identified from various vertebrate species, including birds. The majority of the currently known avian picornaviruses belong to five different phylogenetic clusters: the megrivirus-, the passerivirus-, the avihepatovirus-, the tremovirus-, and the avian sapelovirus clusters of which the last cluster is not known to contain chicken picornaviruses (Boros et al., 2014c). At least six different picornaviruses: the chicken megrivirus (genus *Megrivirus*, megrivirus cluster), sicinivirus A1 (*Sicinivirus*, passerivirus cluster), chicken gallivirus 1 (*Gallivirus*, passerivirus cluster), chicken picornavirus 2, 3 (Unassigned, avihepatovirus cluster), Orivirus A1 ("Orivirus", avihepatovirus cluster) and avian encephalomyelitis virus 1 (*Tremovirus*, tremovirus cluster) have been identified from chickens. Among these the complete genome of sicinivirus has not been reported before (Farkas et al., 2012; Boros et al., 2014a, 2014b; Bullman et al., 2014; Lau

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et al., 2014; Tannock and Shafren, 1994). These avian picornaviruses have been frequently detected in cloacal specimens collected from both healthy and affected chickens (Lau et al., 2014; Bullman et al., 2014; Farkas et al., 2012; Boros et al., 2014a), but little information is available with regard to co-infections with these viruses. There are only a few studies describing the presence of galli-, and avisiviruses; as well as galli-, and sicinivirus present as co-infections in turkeys (Boros et al., 2013) and chickens (Bullman et al., 2014). Poultry, including chickens are one of the most important food sources of humans, and gastro-intestinal infections of these birds are known to negatively impact meat and egg production and cause veterinary, economic, and even human health concerns (Guy, 1998; Chan et al., 2015). Therefore, the discovery of further avian viruses which could be involved in gastro-intestinal infections potentially bears on veterinary care and medical importance. Metagenomic approaches give us the opportunity to simultaneously detect large number of sequences from different microbes including viruses which could contribute to the development of diarrheic syndromes (Day et al., 2015, Finkbeiner et al., 2008). Although due to the short sequence reads and sometimes poor coverage, the assembled metagenomic sequences of field samples usually hold the risk of containing artificial chimeric contigs and unnoticed sequence variants especially when closely related species or multiple genotypes with uneven copies are present simultaneously (Teeling and Glöckner, 2012; Vázquez-Castellanos et al., 2014). Therefore in this study the complete genomes of members of the enteric picornavirome identified by viral metagenomic approach of a single, diarrheic chicken were determined and analyzed. Using different RT-PCR types and metagenomic sequences, the complete genomes of six different picornaviruses were determined, which belong to 4 known (*Sicinivirus*, *Gallivirus, Tremovirus*, *Avisivirus*) and one novel ("Orivirus") genera. The taxonomic position of one picornavirus, chicken phacovirus 1 strain Pf-CHK1/PhV cannot be resolve unambiguously. Furthermore, the prototype strain chicken/Pf-CHK1/2013/HUN (GenBank Accession number: KM203656) of Orivirus A1 (genus "Orivirus") previously described by our research group (Boros et al., 2014a) was also identified from the same cloacal sample.

Results

Viral metagenomic overview

The *in silico* analysis of viral metagenomic sequences of the single specimen Pf-CHK1 identified a total of 13,016 unique viral reads of which 10,906 likely originated from viruses infecting bacteria (*Podoviridae*: $N=10$, *Siphoviridae*: $N=43$ and *Microviridae*: $N=7178$), plants (*Phycodnaviridae*: $N=40$) and unclassified ($N=3635$) virus families based on BLASTx *E* scores $< 10^{-5}$. The rest of the sequence reads ($N=2110$) belong to different eukaryotic viruses, with reads from RNA virus families predominating ($N=1715$, $\approx 80\%$ of the eukaryotic viral reads). The largest group of RNA viruses represented are picornaviruses ($N=1032$ reads), followed by the group of picobirnavirus-related sequences with considerably fewer reads (Fig. 1). The detailed BLASTx analyses of the picornavirus-related sequences suggested the presence of at least seven different picornaviruses in the analyzed sample (Fig. 1).

A total of six complete genomes from the seven picornaviruses identified by BLASTx analyses of metagenomic reads were verified and determined in this study using RT-PCR methods. The 4 sequence reads related to megriviruses were not detectable by RT-PCR, which could suggest the initially low copy number of megriviruses in the sample.

Genome analysis of the Sicinivirus-related picornavirus

The sicinivirus-related reads covered $\approx 74\%$ of the 9243-nt-long genome of Sicinivirus A1 (SiV-A1) strain UCC001 (KF741227) with variable depth of coverage ranging 1–17 (Fig. 2). Due to the variable depth of coverage and the unknown 5′ and 3′ ends, multiple RT-PCR reactions were used to verify all of the covered genome regions and to acquire the complete genome. The 9883-nt-long picornavirus genome of strain Pf-CHK1/SiV (KT880665) is the longest picornavirus genome reported to date. The Pf-CHK1/SiV show an overall 82% nt identity to the partial genome (5′ end is

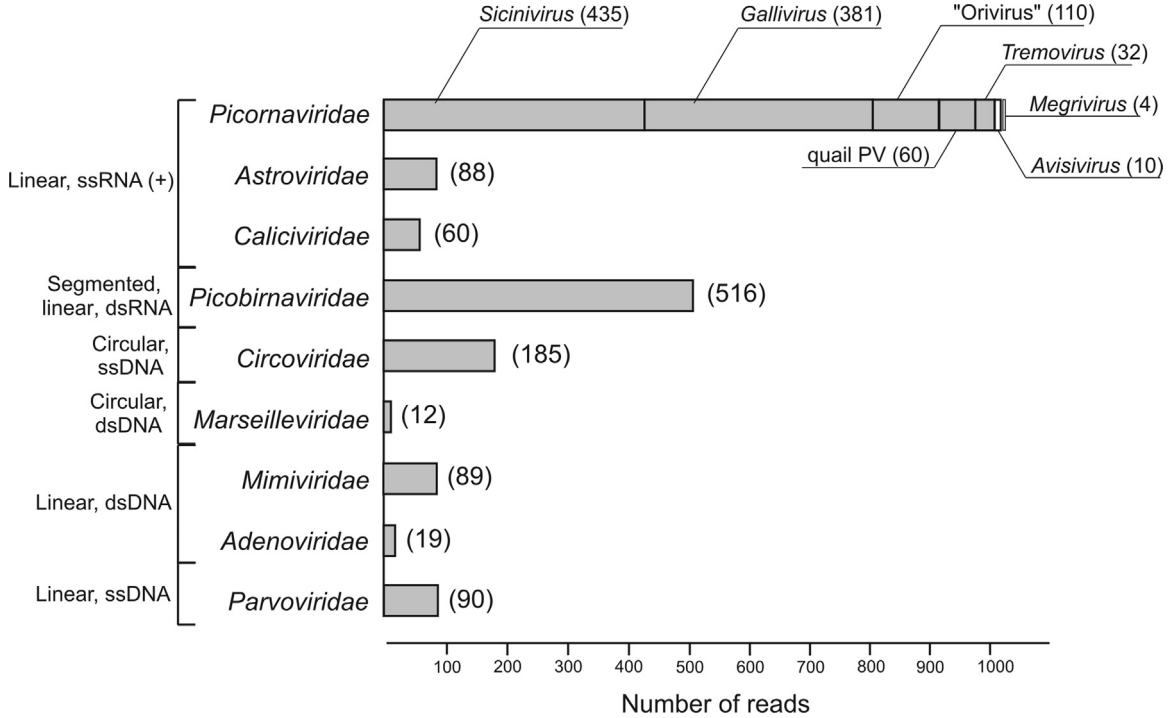

Fig. 1. Virus family-level categorization of eukaryotic viral metagenomic reads is based on the best BLASTx-scores (*E*-value $\leq 10^{-5}$). The picornavirus-related reads are further characterized based on the highest sequence identity to the members of the different genera. quail PV: quail picornavirus 1 (currently unassigned). Numbers in brackets represent the number of reads.