The fecal virome of domesticated animals

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Abstract  Next-generation sequencing is a new research tool in our hands helping us to explore still unknown fields of human and veterinary virology. Metagenomic analysis has enabled the discovery of putative novel pathogens and the identification of the etiologic agents of several diseases, solving long-standing mysteries caused by divergent viruses. This approach has been used in several studies investigating fecal samples of livestock, and companion animal species, providing information on the diversity of animal fecal virome, helping the elucidation of the etiology of diarrheal disease in animals and identifying potential zoonotic and emerging viruses.

Keywords  Metagenomics · Virus · Viral gastroenteritis · Viral flora · Domestic animal · Next generation sequencing · High throughput sequencing

Introduction

Gastrointestinal flora of domestic animals has significant influence on the efficacy and the productiveness of animal breeding and food production. The microbiological flora is highly diversified across various animal species and individuals, though we have only scarce information about the normal or diseased intestinal micro-flora [14]. Bacteria, phages and eukaryotic viruses are important members of the intestinal flora, but their function, chiefly for orphan (i.e. viruses not associated to a specific disease) eukaryotic viruses, remains unknown [1]. It is well known that the majority of viruses in the gut are constituted by phages, which may have effects on bacterial properties by affecting their genetic variability. Our knowledge about eukaryotic viruses, with regard to virus ecology, the mechanisms of persistence in the animal populations, and viral pathogenicity appears rather limited. Discovering novel pathogens and determining the genome sequence and structure is a major breakthrough, mainly for eukaryotic viruses, and provide the bases for detailed and focused investigations. Identification of viruses by metagenomic approaches has been flourishing in the last decade. The sequence independent amplification, coupled with massive parallel sequencing on various next generation platforms, is a promising method in discovery of viruses, which may help estimate the diversity of microorganisms in clinical or environmental samples. Though several pathogens have been identified as causative agents of diarrhea, in numerous cases commonly used diagnostic methods proved to be inefficient, suggesting that in some cases novel, so far unknown pathogens might be responsible for the infection (even in epidemic outbreaks) [7]. Thus comprehensive molecular biological studies may help us describing novel virus types, species, genera and even families. In this review our goal was to summarize the results provided by metagenomic studies investigating the viral flora of the intestines of domesticated animals (Table 1).

The fecal virome of livestock and companion mammals

Horses

Investigation of the viral background of gastrointestinal diseases in domesticated horses is important because of the
high individual value of each animal. Equine feces had been subjected to metagenomic analysis of dsDNA viruses using sequence-independent cloning method [4]. With this method numerous phage sequences including *Siphoviridae, Myoviridae, Podoviridae* and a clone from a vertebrate Orthopoxvirus was obtained.

Swine

Pigs are one of the most common livestock animals worldwide, although the farming conditions are very diverse. Swine can be kept under intensive, almost industrialized conditions with high density, but they can easily re-adapt themselves to near-natural or even feral circumstances where they often live near or among the human population, or other domesticated and wildlife animals. All these conditions make swine an ideal host for the rapid change of viral genome via recombination and/or reassortment events, thus generating novel viruses that may pose a risk for animal and also for human health. Diarrhea has a considerable influence on the economy of the swine industry. Diarrhea in pigs can be associated with several known pathogens, e.g., rotaviruses, coronaviruses (transmissible gastroenteritis, porcine epidemic diarrhea), porcine reproductive and respiratory syndrome virus, African swine fever virus, classical swine fever virus, *Escherichia coli*, *Salmonella* sp., *Clostridium difficile*, *Bacteroides fragilis*, *Enterococcus durans*, *Chlamydia sp.*, *Strongyloides* sp., *Trichuris suis, coccidia*, etc. [21]. Although our knowledge is permanently expanding, several cases of diarrhea often remain undiagnosed. Comparing the virome of healthy and diarrheic pigs may help identifying unknown candidate causative agents.

In a recent study, fecal samples of healthy and diarrheic piglets have been processed and subjected to unbiased metagenomic analysis [19]. The specimens were collected in North Carolina, from a high-density commercial farm, with 1,000 sows. After the nucleic acid purification and library construction the samples were pyrosequenced in a Genome Sequencer FLX Titanium (GS-FLX Titanium). Sequence analysis revealed the presence of several porcine RNA viruses, such as kobuviruses, enteroviruses, sapeloviruses, teschoviruses, sapoviruses, astroviruses, and coronaviruses. The authors also obtained sequences from DNA viruses of the family *Circoviridae* and *Parvoviridae*. Beside the previously characterized viruses they detected new astrov-, and bocaviruses, as well as highly divergent RNA viruses, namely posavirus 1 and 2 (porcine stool associated RNA virus) distantly related to members of the order *Picornivirales*. The average number of detected viruses per sample was 4.2 among healthy and 5.4 in diseased animals. Bocavirus and coronavirus were more frequently detected in diarrheic animals, but otherwise no greater amount of any specific virus was amplified from the diarrheic fecal samples. In younger unweaned piglets only 1.5 virus was identified probably due to the maternal immunity. A higher rate of co-infections was evidenced, which could be a result of the intensive farming conditions, the young age and a yet unstable viral flora. It is unclear whether the presence of these viruses can be derived from chronic shedding, or short, frequent infection-reinfection periods.

Sachsenröder et al. [17] have designed and optimized a protocol for the preparation of fecal samples for deep sequencing. Three kinds of phages, T4, M13 and MS2 representing dsDNA, ssDNA, ssRNA viral genomes, respectively, were used in the process of the optimization. Known amount of each phage was added to the sample and their presence and load was monitored throughout the whole process by quantitative real-time PCR. Efforts were also made to minimize the amplification steps to avoid biasing during random amplification. Fecal samples from five pigs were pooled and used in this study, two of the animals had watery diarrhea. For deep sequencing 454 Life Sciences GS-FLX pyrosequencing platform was applied. Most of the generated sequences were from bacteriophages and 15 different mammalian viruses were identified in the stool samples. The RNA viruses included rotavirus group A and C, astrovirus, kobuvirus, enterovirus B, teschovirus, picornavirus, sapovirus, picobirnavirus (PBV). The DNA viruses included circovirus, bocavirus, poxvirus, parvovirus and herpesvirus. With some exceptions these results were similar to the observations reported by Shan et al. [19]. A virus similar to the novel Chimpanzee stool associated circular ssDNA virus (ChiSCV) was identified in this study and was tentatively named pig stool associated circular ssDNA virus (PigSCV).

In another study fecal specimens of healthy piglets were analyzed with high-throughput sequencing with an Illumina HiSeq-2000 sequencer, discovering a new member of the family *Picornaviridae*. This virus was tentatively named swine pasivirus 1 (SpaV1), and referred into a new clade of Pasivirus (Parecho sister), as it was most closely related to members of the *Parechovirus* genus, especially with Ljungan virus (LV) [18]. The SpaV1 infection seems to be asymptomatic in piglets. Several members of the large and heterogeneous family *Picornaviridae* (small, non-enveloped positive sense, single-stranded RNA viruses) are recognized in pigs, namely the encephalomyocarditis virus (genus *Cardiovirus*), the porcine enterovirus B (genus *Enterovirus*), porcine kobuvirus (genus *Kobuvirus*), porcine sapelovirus (genus *Sapelovirus*), and porcine teschovirus (genus *Teschovirus*).

Rabbit

Astroviruses are often associated with enteric infections in humans and animals. Stenglein et al. [22] reported an
### Table 1  Studies describing the fecal virome of domesticated animals

| Sampled species | Viruses founda | Sequencing method | Number of trimmed reads | Samples | Number of references |
|-----------------|----------------|-------------------|-------------------------|---------|---------------------|
| Horse           | Siphoviridae (λ-like phages) | Cloned and sequenced | No data | Feces | [4] |
|                 | Myoviridae (T4-like phages) | | | | |
|                 | Podoviridae (T7-like phages) | | | | |
|                 | Orthopoxvirus | | | | |
| Swine           | Kobuvirus | 454 Life Sciences GS-FLX pyrosequencing platform | 570,000 | Feces | [21] |
|                 | Enterovirus | | | | |
|                 | Sapovirus | | | | |
|                 | Sapelovirus | | | | |
|                 | Coronavirus | | | | |
|                 | Teschovirus | | | | |
|                 | Porcine astrovirus (PAstV) | | | | |
|                 | PAstV5-33 | | | | |
|                 | Porcine bocavirus (PBoV) | | | | |
|                 | Porcine circo-like virus | | | | |
|                 | Porcine picorna-like virus | | | | |
|                 | (posavirus) | | | | |
| Swine           | Kobuvirus | 454 Life Sciences GS-FLX pyrosequencing platform | 66,129 | Feces | [17] |
|                 | Rotavirus C and A | | | | |
|                 | ChiSCV | | | | |
|                 | Porcine astrovirus (PAstV) | | | | |
|                 | Enterovirus | | | | |
|                 | Sapovirus | | | | |
|                 | PBV | | | | |
|                 | Bocavirus | | | | |
|                 | Poxvirus | | | | |
|                 | Parvovirus | | | | |
|                 | Teschovirus | | | | |
|                 | Circovirus | | | | |
|                 | Herpesvirus | | | | |
|                 | Picornavirus | | | | |
| Swine           | SPaV1 | Illumina HiSeq-2000 sequencer | 27,146,966 | Feces | [18] |
| Rabbit          | Astrovirus rabbit/TN/2009/USA | Ion Torrent PGM | 3,000,000 | Feces | [22] |
| Dog             | Canine parvovirus 2 (CPV2) | 454 Life Sciences GS-FLX pyrosequencing platform | 276,000 | Feces | [10] |
|                 | Canine coronavirus (CaCV) | | | | |
|                 | Rotavirus | | | | |
|                 | CaKoV | | | | |
|                 | Canine sapovirus 1 and 2 (CaSaV1-2) | | | | |
| Ferret           | Ferret kobuvirus (MpKoV) | 454 GS Junior instrument | >253,000 | Rectal swab | [20] |
|                 | Ferret parechovirus (MpPeV1) | | | | |
|                 | Ferret papillomavirus (MpPV1) | | | | |
|                 | Ferret anellovirus (MpfTTV1) | | | | |
| Turkey           | Members of Picornavirales and picorna-like viruses | 454 Life Sciences GS-FLX pyrosequencing platform | 139,000,000 | Intestinal homogenate | [6] |
|                 | PBVs | | | | |
|                 | Sapovirus | | | | |
|                 | Lagovirus | | | | |
|                 | Avian reovirus | | | | |
|                 | Turkey astrovirus (TAsTV) | | | | |
outbreak of enterocolitis in domestic rabbits (*Oryctolagus cuniculus*) in Tennessee, USA, with extremely high mortality. Clinical signs included lethargy, bloating, mucoid diarrhea and death. Heavy intestinal coccidiosis was also observed. Electronmicroscopic examination and preliminary screening was performed with Virochip microarray, and suspected astrovirus in the background of the disease. To recover the whole genome sequence metagenomic analysis, Ion Torrent Personal Genome Machine instrument was applied. The sequenced astrovirus was closely related to rabbit astroviruses discovered previously in a large epidemiological investigation in rabbits with enteritis and in asymptomatic rabbits [13]. In this study published by Martella et al. [13], the average viral RNA copy number in the quantitative RT-PCR was 100-fold higher in the feces of rabbits with symptoms compared to the asymptomatic animals. Further experiments are needed to determine the role of the identified viruses in the enteric disease.

Dogs

Dogs are in close interaction with human and they are the most popular companion animals kept worldwide. The large number of dogs, the fact that they are co-habitating with people and can also be in contact with wild animals makes them potential risk factors, as the source and transmitter of zoonotic diseases, like rabies or rotavirus. In a recent study feces of diarrheic sheltered and privately owned dogs was examined in a sequence-independent manner to get an insight into the viral flora of the canine gut [10]. Besides the already characterized viruses causing diarrhea in dogs such as canine parvovirus 2 (CPV2), canine enteric coronavirus (CCoV) and rotavirus, sequence reads from novel viruses were also generated. Nucleotide sequences related to insect and plant viruses were also found referring to their diet or the contamination of food. In a few samples sequences related to kobuviruses were detected showing the greatest similarity with human Aichi virus and polyprotein organization typical for picornaviruses. These viruses were provisionally named canine kobuviruses (CaKoV). Sapoviruses are members of *Caliciviridae* family, causing diarrhea in humans, pigs and minks. Two novel sapoviruses were identified in this study and given the provisional name canine sapovirus 1 and 2 (CaSaV-1 and -2). These divergent viruses were classified into a new genogroup. Subsequent prevalence studies were performed using real-time PCR assay for CaSaV1 and CaKoV. The feces of 200 healthy and 200 diarrheic dogs were screened. CaSaV1 was detected only in one healthy and one diarrheic dog, whilst 14 healthy and six diarrheic samples were positive for CaKoV. No correlation was found between the detected viruses and the diarrhea.

Ferrets

Ferrets are susceptible to many human pathogen viruses therefore they are frequently chosen small animals for modeling viral infections, including influenza A virus and SARS coronavirus. They are also commonly kept as companion animals. In order to have a broader knowledge about viruses harbored by ferrets, a metagenomic study was performed recently. Information about new and so far unknown viruses were obtained [20]. Rectal swab samples were taken from farm and household animals with or without diarrhea, and from immuno-compromised laboratory ferrets. High throughput sequencing identified ferret coronavirus, ferret hepatitis E virus, Aleutian mink disease virus, murine astrovirus and several chicken viruses, likely due to the diet of the animals. In addition sequences were obtained from astroviruses, torque teno virus (family *Anelloviridae*), PBV (family *Picobirnaviridae*), kobu- and parechovirus (family *Picornaviridae*), and papillomavirus (family *Papillomaviridae*). Monitoring ferret hepatitis E virus and coronavirus using real-time PCR assay revealed...
that pet ferrets were shedding hepatitis E virus more often than farm animals. The ferret kobuvirus clustered with bovine and ovine kobuviruses in the Aichivirus B species. The ferret parechovirus-like virus obtained from an animal without diarrhea had similar genome organization but was highly diverse from human parechovirus and LV (genus Parechovirus), suggesting that the identified virus is a member of a new genus within the family Picornaviridae. Interestingly a papillomavirus was also detected in a sample. Papillomaviruses are not associated with gastro-enteritis and they are usually not found in the feces of mammals. Ferret anelloviruses belonging to a novel torque teno virus species, presumably within the genus Xitorque-virus, were also identified.

Fecal virome of poultry and exotic companion birds

Enteric diseases in the poultry industry generate remarkable losses for farmers all over the world. There are multifactorial enteric diseases, such as poult enteritis mortality syndrome (PEMS) and poult enteritis complex (PEC) of turkeys, and runting-stunting syndrome of chicken (RSS) [5]. The pathogenic agents of PEMS, PEC and RSS have not been clearly defined yet. Although several viruses, mainly avian reoviruses, rotaviruses, astroviruses, coronaviruses, enteroviruses and calciviruses were identified in the intestinal content of birds, their pathogenic role is henceforward poorly understood. These viruses can also cause asymptomatic infections in flocks. In addition to viruses, bacterial pathogens, parasite infections, as well as poor environmental conditions may exacerbate the disease. Metagenomic investigation could generate important data and help understand the etiology of multifactorial diseases of avian species.

Turkey and chicken

Intestinal samples collected from turkey were tested in sequence independent manner in a study focusing on RNA viruses in the feces. The samples were collected from farms with histories of enteric diseases. High-throughput nucleic acid sequencing was performed with GS-FLX Titanium pyrosequencer (Roche). The authors could identify sequences showing homology to members of the Picornaviridae family, mostly to kobuviruses. In connection with enteric disease of turkey, small (15–30 nm), round viruses were previously detected by electronmicroscopic examination, although their pathogenicity or transmission capability remained elusive. A unique novel turkey PBV was also partially characterized. Putative turkey-origin calcivirus sequences were detected showing the highest similarity to members of Sapovirus and Lagovirus genera. Among others, avian astrovirus and avian reovirus sequences were found showing similarity to the previously described turkey astrovirus type 2 frequently detected in turkey intestines and avian reoviruses, respectively. Results of this study may help us to design further metagenomic investigations and molecular diagnostic tests to detect novel viruses, determine their pathogenic role, analyze and further characterize the intestinal RNA virome of healthy and diarrheic birds [6].

In another work two parvoviruses, the chicken and turkey parvovirus was detected in intestinal homogenates of chicken with RSS and turkeys with PEMS using sequence independent amplification of particle-associated nucleic acids. Based on the amplified sequences, the parvoviruses were closely related to each other. It was presumed that these novel parvoviruses are capable of autonomous replication. The role of the chicken and turkey parvovirus in the etiology of poult enteric diseases has not been determined [24].

Novel picornaviruses with unexpected genome features had been characterized by pyrosequencing and were subsequently detected by RT-PCR in fecal samples collected from healthy and diseased turkey from several flocks in a Hungarian study. The phylogenetic analysis of the obtained sequences showed that although these viruses were most closely related to members of Avihepatovirus genus, it formed a distinct clade, provisionally named Avisivirus genus (Avihepatovirus sister-clade virus) [3].

Parrot

Proventricular dilatation disease (PDD) of psittacine birds is a usually fatal syndrome documented in both captive and wild living species. The disease was first described several decades ago and was believed to be infectious caused by an unknown virus. Infection mainly affects the autonomous nerves of the upper and middle digestive tract, central and peripheric nervous system. In correlation with the microscopic changes, birds exhibit gastrointestinal (regurgitation, crop impaction, poor appetite, weight loss, passage of undigested food in the feces) and/or neurologic (ataxia, abnormal gait, proprioceptive defects) symptoms. Studies applying Virus chip, a microarray analysis, were performed on samples originating from parrots that had been diagnosed with PDD in order to identify the etiologic agent. In the majority of the samples putative, genetically diverse avian bornaviruses (ABVs) were detected. Sequence independent ultra-high throughput sequencing was performed with positive samples to recover the whole genome of bornaviruses [9]. These novel ABVs showed remarkable heterogeneity from the previously identified bornaviruses. The presence of ABV was not confirmed in every sample, which could be explained by the different tissue tropism of
the viruses or stage of infection. It is also possible that PDD has multiple etiologic agents, and ABV does not play a role in all of the cases. Further analysis including high-throughput sequencing of positive samples is needed to gain more information on the viral flora of PDD.

Similar results were obtained in a research study also seeking for the causative agent of PDD using high-throughput sequencing. Based on the sequence of the new highly divergent avian strains of bornavirus real-time PCR-assay was developed for the detection of these viruses [8].

**The fecal virome of selected host species: the interface of domesticated and wild animals**

In developing countries the margin between livestock and wild living animals cannot be clearly distinguished, as farm animals are kept among extensive conditions allowing contact with wild animals; additionally they usually share the same environment with the human population. Wild animals can serve as reservoirs for numerous viruses pathogenic for humans and domestic animals. These circumstances may lead to pathogen crossovers, appearance and re-appearance of emerging zoonotic diseases. A viral metagenomic analysis was performed on sera of Ugandan bushpigs (Potamochoerus larvatus) to identify any potential pathogens of domesticated pigs they may carry. The study revealed novel variants of porcine parvovirus 4 and torque teno sus virus 1 and 2 [2]. Although latter viruses do not cause enteritis, they are important copathogens in other syndromes, as post-weaning multisystemic wasting syndrome, and both can be excreted with feces.

Rodents and bats are reservoirs for several known pathogens of humans and animals, they live in large numbers, mean a constant threat for livestock animals and sometimes for humans. Collecting information about viruses they can harbor and shed by feces is essential in preventing emerging viral infections. In a study fecal samples of 105 different rodents (mouse, rat, vole) were subjected to metagenomic analyses [15]. Sequences related to a high number of mammalian viruses families Circoviridae, Picobirnaviridae, Picornaviridae, Astroviridae, Parvoviridae, Papillomaviridae, Adenoviridae, and Coronaviridae were detected. Bat guano collected in California and Texas contained sequences showing homology to members of Parvoviridae, Circoviridae, Picornaviridae, Adenoviridae, Poxviridae, Astroviridae, and Coronavirus families [12]. In both studies a large portion of the detected sequences belonged to viruses of insects and plants, also fungi in the bat samples mirroring their and the insects’ variable diet. The number of amplified and detected novel viruses reflects the diversity of mammalian viruses.

The virome of the gut from wild pine martens (Martes martes) and European badgers (Meles meles) has been studied in the Netherlands using next-generation sequencing with a 454 GS Junior instrument (Roche) [23]. In the pine marten rectal swab samples sequence reads showing homology to bacteriophages (Myo-, Podo-, Siphoviridae, Microviridae), bocavirus from the Parvoviridae, kobavirus from the Picornaviridae and torque teno virus from the Anelloviridae, Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SSHADV-1) from the Geminiviridae-like family were found. In European badgers samples viruses homologue to Bombyx mori cytopivirus from the Reoviridae, cumbid circovirus from the Circoviridae, canine distemper virus from the Paramyxoviridae, SSHADV-1 from the Geminiviridae-like family, and torque teno virus from the Anelloviridae family were detected.

Sea lions (Zalophus californianus) can harbor a wide variety of viruses, in addition to their own pathogens they can be also infected with viruses of terrestrial animals. Fecal samples from sea lions of three age groups, pups, juveniles and adults under rehabilitation were collected and analyzed with metagenomic approach [11]. Several novel viruses of families Astroviridae, Picornaviridae, Caliciviridae, Reoviridae and Parvoviridae were described and partially characterized. In pups the number of detected viruses was the lowest compared to the other groups while in adults in rehabilitation it was the highest. This could be explained by the maternal immunity of pups and the poor immune status of diseased animals. Animals were usually infected with more than one virus in all group. As observed in other animal species viruses most probably related to their diet were also detected, like densoviruses of insects and crustaceans or nodavirus-like sequences from fishes and insects.

Wild pigeons can be in close contact with other domesticated sport and show pigeons, they can live in the same lofts, consume the same food and water; contaminate the habitat with their feces and other excreta. Due to these conditions pathogens can be easily transmitted among them. A recent metagenomic study investigated 51 fecal samples, collected in Hong Kong and Hungary and found several known and novel viruses [16]. Sequence reads were related to circoviruses, parvoviruses, picornaviruses, rotaviruses, adenoviruses, astroviruses, caliciviruses and tobamoviruses, as well as plant and insect viruses, apparently reflecting the diet of feral pigeons. A novel parvovirus was identified and characterized. The pigeon parvovirus was related to chicken and turkey parvoviruses previously found in feces of birds with clinical signs of enteric diseases. Data analysis revealed an unusually long middle ORF that resembled to an ORF with unknown function of the fowl adenovirus genome. Phan et al. [15] proposed a
new provisional genus, called *Aviparvovirus*, which would include the pigeon-, turkey and chicken parvoviruses. Two novel picornaviruses were described and tentatively named as mesivirus 1 and 2 (Megrivirus sister-clade virus) based on their relationship to the turkey hepatitis virus of the *Megrivirus* genus. A pigeon rotavirus was also identified and characterized as a novel member of species *Rotavirus G*. This study also highlights the importance of investigating and monitoring the fecal flora of wild animals, as important sources of human and animal viruses.

**Concluding remarks**

Viral discovery has accelerated in the last decade with the introduction of new techniques, such as high-density pathogen chips and next-generation sequencing. As NGS facilities became more prevalent the cost of sequencing runs had been substantially decreased. Though further development and reducing the costs are needed for using this method as a diagnostic tool for emerging infectious diseases, virus discovery or monitoring, the technique has a promising future. Until recently virus discovery was based on PCR often using general and degenerate primers or virus isolation and observing the cytopathic effects (CPE). Viruses difficult to propagate or with no CPE or highly divergent nucleotide sequence often demonstrated the pitfalls of these methods. Sequence similarity based searches resulted in discovery of numerous new divergent viruses. Investigation of viral metagenomes of different samples taken from healthy and diseased individuals is a powerful tool for veterinary sciences, it can help us understand the pathogen diversity underlying a multifactorial disease, can reveal the probable etiologic agent behind an epidemic, and can elucidate the viral evolution in connection with a zoonosis, or a virus shift between species. Viral metagenomics is a potend device referring also to wild animals. As wild animals can be reservoirs for numerous pathogenic agents, which can also infect domestic animals and humans, monitoring them is a substantial requirement. NGS is often performed using DNA sample obtained by sequence-independent methods, therefore the obtained data has to be analyzed carefully and the host and food origin viruses should be separated. Additionally, we are just at the beginning to understand the influence of food origin viruses on the flora of the host. Obviously further examinations are needed to determine if these agents are members of the normal flora or can be connected with any disease.

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