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Metagenomics and Diagnosis of Zoonotic Diseases

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Abstract

Zoonotic diseases represent a public health problem worldwide, since approximately 60% of human pathogens have a zoonotic origin. A variety of methodologies have been developed to diagnose zoonosis, including culture-dependent and immunological-based methods, which allow the identification of a huge range of pathogens. However, some of them are not detected easily with these approaches. Additionally, molecular tests have been developed, and they are designed to identify a single pathogen or mixtures of them. In this context, metagenomics comes as an alternative to get genome sequences of different microorganisms, which comprise a microbial community. Metagenomics have been used to characterize microbiomes and viromes, which are not cultivable under laboratory conditions. This methodology could be a powerful tool in the diagnosis of zoonotic diseases because it allows not only identification of genus and species, but also detection of some proteins in specific conditions on specific tissues, through structural and functional metagenomics, respectively.

Keywords: zoonosis, metagenomics, virome, microbiome, diagnostic

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1. Introduction

Zoonotic diseases represent a public health problem worldwide, since approximately 60% of human pathogens have a zoonotic origin. Many of the most important human pathogens are either zoonotic or originated as zoonosis before adapting to humans. Consequently, humans are continuously being exposed to novel animal pathogens [1, 2].

In recent years, the epidemiological safety has been threatened with new emerging zoonotic diseases such as Zika, Ebola, H1N1 influenza and severe acute respiratory syndrome. Several risk assessment studies have estimated that 75% of emerging pathogens are zoonotic in origin (OPS 2016). The rise of these emerging diseases might be related to the increase in population,

the growth of cities, the destruction of natural habitats, the modernization of agricultural practices and the climate change, among others [3, 4].

Zoonotic diseases are pathologies that can be distributed between animals and humans. Fungi, parasites, bacterias and viruses, being bacteria and viruses the zoonotic agents more prevalent can cause different zoonosis are very common and have a high frequency in the population. They are derived from the interactions with animals during the daily activities. Taylor and coworkers reported that of 1415 pathogens known to infect humans, 61% were or are zoonotic [5].

Animals are important elements in our daily lives. They inhabit our houses, some as pets; we have a close contact with them in the zoo; animals are essential part of the agricultural practices around the world. The saliva, blood, urine or feces of animals, which are infected, transmit zoonotic agents. Several animals are only carrier of different pathogens for human, but they do not develop the illness. These animals are defined as vector. Probably, the most famous vector is the mosquito *Aedes aegypti*. This mosquito is the causal agent of dengue, an important viral disease in tropical zones in America, Africa and Asia. Another way to get a zoonotic disease is through food consumption. It is very frequent in our countries to consume unpasteurized milk, undercooked meat or fish, unwashed fruits and vegetables, which can be contaminated with urine and feces from infected animals. Zoonoses can be dangerous, and some of them can cause death if not diagnosed and treated on time. Thus, zoonotic can be acquired if we work with animals, have pets, practice hobbies involving animals or consume water or food contaminated with pathogens from animals.

The World Organization for Health recognizes as the most common zoonotic disease as:

1. Lyme disease and Rocky Mountain spotted fever, both transmitted by a tick bite.
2. West Nile virus (WNV) transmitted by a mosquito bite.
3. Dengue, malaria and chikungunya transmitted by an infected mosquito.
4. *Salmonella* infections transmitted by baby chick, chicken, duck, turtle or snake.
5. *Escherichia coli* infections transmitted by infected animals, such as cows.

With this in mind, zoonoses are a considerable risk to human health. Derived of this, important research projects are being developed to understand and study the epidemiology, dynamics, distribution and infection of zoonotic agents. However, the diagnosis of zoonotic agents, and the description and distribution of new zoonotic microorganisms and viruses remain a challenge for international public health. Therefore, the study of the microbiota of free-living animals as well as pets provides useful knowledge for prediction and treatment of new zoonoses. Different areas of knowledge are included in this purpose, such as molecular biology, immunology and epidemiology, among others.

2. Conventional diagnosis

A variety of methodologies have been developed to diagnose zoonoses. For example, the culture of microorganisms allows the easy identification of a huge range of pathogens. However,

some of them are not detected easily with this approach. In this case, these microorganisms are underestimated or frequently misdiagnosed. Some of them are predominant and can be highly prevalent and important in the environment. Parallel with the culture methods, microscopy techniques to identify pathogens from tissues have provided an important support in the diagnosis of zoonotic diseases. The sampling of some tissues is usually very invasive, this being an important disadvantage. These approaches are complementary.

Important advances in the diagnosis of zoonotic diseases have been achieved over time. Various antigen-based assays and methods are used for the diagnosis of these diseases, optimizing handling and security of samples and decreasing the time in reporting the results. Serological assays are used for the detection of antibodies in serum samples from humans. Indirect fluorescent antibody (IFA), indirect hemagglutination assay, complement fixation, direct agglutination test (DAT) and enzyme-linked immunosorbent assay (ELISA) are some of these assays with different specificities and sensitivities [6, 7]. But up to date, the antibody availability is a huge problem. The pathogen classification considering several serotypes and other pathogenic characteristics is limited to the existence of specific antibodies.

The development of test such as Western blotting and immunochromatographic test using antigens produced by genetic engineering have enabled the confirmation of serological tests to differentiate strains within a species and identify some virulence factors involved in the pathogenic disease process. Given the new developments at the genomic level from the 50s, several technologies for nucleic acid manipulation of infectious agents have been developed, such as the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), pulse field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), ribotyping, spoligotyping, high-throughput sequencing and qPCR for use in clinical samples from humans and animals. These techniques have been successfully used to diagnose animal pathogens. Although molecular methods provide high specificity and sensitivity, they have been used mainly in research studies and not in clinical diagnosis. Their expensive costs can explain the above. The molecular diagnosis is designed to identify a single pathogen or mixtures of them, but considering a limited number of them. This scope is very simple if we want to study the microbial interactions between pathogens and the resident microbiota in any organism. It is known that these interactions are epidemiologically very important and can influence in the illness [8]. Thus, it is essential to develop new methodologies capable to detect simultaneously multiple pathogens and allow to take in context with the resident microbiota. With this in mind, the clinical diagnosis demands new approaches to study the presence of any pathogens and, at the same time, their interaction with other microorganisms, microbial populations and communities.

Particularly, viruses are the most abundant form of life on the Earth. However, few groups of them can be cultivated in laboratory conditions. They can be identified using several methods, such as electron microscopy, cell culture, inoculation and serology, among others. All these techniques require a previous knowledge of the virus because they are based on comparisons with known viruses. Those viruses that cannot be cultivated in the laboratory can be identified by molecular methods such as microarray, subtractive hybridization-based and PCR-based methods [9]. To apply molecular methods to characterize viruses also requires previous information about the target virus. However, some new methods have overcome this limitation: sequence-independent single primer amplification, degenerate oligonucleotide primed

PCR, random PCR and rolling circle amplification [9]. But, all these techniques do not allow the complete understanding of the virome in both animals and humans.

Next-generation sequencing (NGS) techniques are definitively new tools to get a complete understanding of the viral composition in humans and animals. These sequencing tools have many applications in research and diagnosis in humans and animals, and they are allowing the high-throughput prospecting to identify pathogen viruses in animals [10]. Next-generation sequencing techniques offer thousands to millions of reads able to detect any virus in few copies. Thus, global studies to prospect the viral composition of any organism are necessary to advance in our understanding of zoonoses.

3. Metagenomics as a diagnostic tool

Metagenomics is an alternative to get genome sequences from different microbial community. This approach have been used with diagnostic proposes (**Figure 1**). Metagenomics-based approaches have over the past few decades been developed in efforts to assess, analyze and exploit biodiversity in a wide variety of different environmental niches. Metagenomics approaches have gained importance in clinical studies, even with diagnostic proposes. Conventional diagnostic (cultivation-dependent methods) identifies pathogen species, strains and serotypes of interest in independent colonies through isolation of microorganisms and obtaining axenic cultures. For this reason, diagnostic investigation of pathogen microbes through culture-independent methods has become invaluable, with metagenomics being employed to study microbiomes and viromes, which are not cultivable under laboratory conditions.

There are two main areas in metagenomics: structural and functional metagenomics. The first of them is relevant to analyze, identify and describe the microbiomes and viromes in animals and humans. Structural metagenomics studies the composition of microbial populations and communities describing the major genera and species that colonize tissues or

Figure 1. Metagenomics for zoonotic diseases.

organisms. It also provides relevant information about the ecological niches of microorganisms and hypothesizes about relationships established between pathogens, hosts and native microbiomes/viromes.

On the other hand, functional metagenomic would be attractive in the diagnostic field, since some proteins would be detected in specific conditions on specific tissues. The best contribution to knowledge of the structural metagenomic approach has been to identify truly novel species and genera. Some of these have no close relatives and even form deeply branched lineages.

The extraction of high-quality DNA is the first critical step in the metagenomic analysis. Frequently, a huge quantity of human or animal DNA is isolated when microbiomes and viromes are studied from humans or animal samples. This approach was originally developed to analyze microbial genomes contained in environmental samples, but in the last decade its application has been extended to describe new animal and human pathogens. Also, metagenomics has been used to characterize microbiomes and viromes from different tissues and organisms, being relevant in clinical microbiology with a great impact on public health [2, 11]. Other global studies are also important to study animal pathogens, such as metatranscriptomics, metaproteomics and lipidomics, among others. The high-throughput sequencing technologies allow getting a huge sequence database including genes, transcripts and proteins and also allow establishing metabolic networks to understand the relationship between pathogens and hosts [11, 12].

Microorganisms colonize a wide variety of hosts, including animal and humans. They have very specialized ecological niches, even colonize and sicken tissues and whole organisms. Prokaryotic organisms show the highest metabolic diversity and they have been extensively studied as animal pathogens. Viruses are the most abundant in the nature and they are considered as important zoonotic agents.

Metagenomics and high-throughput sequencing technologies are allowing an increase of the studies related to zoonotic diseases and microbiota in animals. These technologies generate millions of short sequence reads (approximately 150 pb) and facilitate the analysis, since cloning procedures are not required. Metagenomics is a powerful and useful tool to describe the diversity and dynamic of bacteria, virus and fungal species in tissues and samples obtained from different animals.

In addition to the findings of viral genomes, metagenomics has contributed to the characterization of microbiomes in different samples, such as canine oral cavity healthy dogs and gastrointestinal tract of several organisms (e.g., feline, canine, human, mouse and chicken). These studies have found taxonomic units with zoonotic potentialities. On the other hand, the previous works revealed a closely phylogenetic relationship between microbiomes from different organisms [13, 14].

Infectious viral diseases, both emerging and reemerging, remain a threat to human and animal health. The increase in these infections appears to be related to human activities and climatic changes which cause outbreaks and pandemics. Some viruses related to these outbreaks are the influenza A viruses, Ebola, Middle East Respiratory Syndrome (MERS) coronavirus and new viruses belonging to the family Bunyaviridae, as the Schmallenberg virus. It is possible that Ebola virus was introduced into the human population through zoonotic

transmission by fruit bat (*Pteripodidae*). It is known that Schmallenberg virus was the agent of outbreaks in ruminants in the European Union. The virus was propagated from the Middle East to the Republic of Korea, causing 186 confirmed human cases with 36 deaths in July 2015 [2]. Moreover, zoonotic viruses, bacteria and parasites can be transmitted to humans from livestock production chain or wild animals, which are used as food (e.g., domestic vertebrates and invertebrates). This situation represents a serious infection risk for humans. The infection transmission and its amplification in the population may occur when the causative agents in wildlife are mobilized and introduced into new hosts like cattle, causing outbreaks that amplify the pathogen transmission to humans [1].

3.1. Metagenomics for viral diagnostics?

Viral metagenomics is a culture-independent approach that is used to investigate the complete viral genetic populations of a biological sample. This methodology becomes a powerful tool for identifying new and emerging viruses, considering that animals remain a reservoir for the virus that can cause zoonosis. Increased knowledge of the viral flora in healthy and diseased individuals is important for both animal and human health [15]. In this regard, the metagenomic assays for the discovery of viruses are based mainly on the sequence-independent amplification of nucleic acids from clinical samples, in combination with next-generation sequencing platforms and bioinformatics tools for sequence analysis. They are relatively simple and fast and allow detection of hundreds of viruses simultaneously, even unknown viruses that might be highly divergent from those that are already described [2]. These platforms offer different throughputs, as mentioned by [15]. High-throughput sequencing technology, Roche 454, is based on pyrosequencing; its throughput is 0.4–0.6 Gb/run, with reads of 400 nt. Solexa/Illumina uses a system with reversible terminators and has a higher throughput (7.5 Gb–1.8 Tb/run) with a read length of 75–150 nt depending on the sequencing system. SOLiD system is based on ligation and cleavable probes; its throughput is 80–320 Gb/run, but it produces reads of only 50–75 nt, making sequence analysis more difficult. These technologies have allowed the detection of new and known viruses from diverse samples such as animal tissues (e.g., brain, lymph nodes), insects (bees), fecal stools and oral swabs. Identified viruses by this approach are among astrovirus, bornavirus, torovirus i, circovirustipo 2, parvovirus, coronavirus and herpesvirus [15].

In addition, some protocols for the detection, purification and enrichment of virus from organ tissue have also been developed. Kohl and coworkers proposed a method called tissue-based universal virus detection for viral metagenomics (TUViD-VM) [16]. This approach was used in chicken tissues inoculated with one of four viruses: poxvirus (vaccinia virus) representing DNA virus with envelope, Reovirus (Orthoreovirus) nonenveloped viruses, orthomyxoviruses (influenza viruses), paramyxoviruses (Sendai virus) and RNA enveloped viruses. Viruses were specially selected for their potential to cause viral emerging diseases. The developed protocol considers several steps as tissue homogenization, ultracentrifugation for the separation of viral particles, RNA extraction, amplification and finally random next-generation sequencing (NGS). The established protocol allowed the quick and reliable purification and enrichment of virus, and an increase in the amount of detectable viral nucleic acids with a sensitivity of 100–1000 virus copies/mL of homogenized organ material. This TUViD-VM

protocol can be used in metagenomic and virome studies to increase the likelihood of detecting viruses from any biological source (**Figure 2**).

A workflow was developed for recuperation of complete genomes of new virions from metagenome projects. Several phases were considered, starting with the assembly of the reads into long fragments with assignment of specific contigs (named seed) from the desired virus. The analysis can then continue in linkage of other fragments to the seed contig to raise a tentative genome. Finally, a full-length viral genome is obtained (**Figure 3**).

Metagenomics is a relevant method in identification of virus causing gastrointestinal diseases in animals. Several viromes have been studied by metagenomics approaches, which have been related to zoonosis. For example, it has been reported that horses have different phages, such as *Siphoviridae*, *Myoviridae* and *Podoviridae*. These viral particles can control bacterial populations inhabiting into the gastrointestinal tract. On the other hand, pigs contain viral sequences corresponding to kobuviruses, enteroviruses, sapeloviruses, teschoviruses, sapoviruses, astroviruses, coronaviruses, also the families *Circoviridae* and *Parvoviridae*, and bocaviruses and posavirus 1 and 2 (RNA virus). Some of them have been related to illness in different animals. Additionally, a described case in rabbit revealed a great number of Astrovirus sequences related to enteric disease. Other study reported in diarrheic dogs, the presence of canine parvovirus 2 (CPV2), canine enteric coronavirus (CcoV), rotavirus, insect and plant viruses, canine kobuviruses and sapoviruses (canine sapovirus 1 and 2). Finally, studies from bird feces (turkey and chicken with enteric disease) showed kobuviruses, calicivirus (*Sapovirus* and *Lagovirus*), avianastrovirus and avian reovirus [17].

Metagenomics profiles have exhaustively allowed to know the associated arboviruses to the principal hematophagous arthropods with medical importance. *Flaviviridae* (TBEV, OHFV, SREV and WNV), *Bunyaviridae* (KKV, CCHFV and SOLV), *Reoviridae* (CTFV), *Hepadnaviridae* (HBV), *Rhabdoviridae* and *Togoviridae* (CHIKV and ONNV) are viruses detected in blood-feeding

Figure 2. Clinical relevance of disease ecology. Transmission of infection and amplification in people (bright red) occurs after a pathogen from wild animals (pink) moves into livestock to cause an outbreak (light green) that amplifies the capacity for pathogen transmission to people (figure and legend were taken from [1]).

Figure 3. Steps in recovery of full-length viral genomes from metagenomes. Figure and legend were taken with permission from [2]. http://www.frontiersin.org/files/Articles/165597/fmicb-06-01069-HTML/image_m/fmicb-06-01069-g003.jpg.

arthropods by serological or molecular techniques [18]. Metagenomic studies have found animal viruses in mosquitoes, which can infect to human and/or transmit zoonosis. *Anelloviridae*, *Circoviridae*, *Herpesviridae*, *Poxviridae* and *Papillomaviridae* have been detected in mixed-species female mosquitoes [19]. In other arthropod species as *Anopheles* sp., *Ochlerotatus* sp., *Culex* sp. and *Aedes* sp., several animal viruses are reported: *Reoviridae* (*Orbivirus*), *Rhabdoviridae*, *Bunyaviridae*, *Flaviviridae* and *Togaviridae* [20–22]. The virome of arthropods is very important because humans and arthropods share a common habitat and they cause serious diseases, even epidemic. Metagenomic has revealed a large number of known and unknown insect-specific or zoonotic agents associated with arthropods [23, 24]. RNA virome of arthropods is under study; however, mosquitoes largely transmit RNA viruses.

Metagenomic studies conducted in mosquitoes in Australia revealed the presence of animal viruses as Edge Hill virus and Walla virus, and other virus able to infect marsupials [20]. In the same metagenomic profile could be identified viruses that infect humans, such as Ross River virus and *Alphavirus*. *Alphavirus* belongs to the *Togaviridae* family, and it is the main etiologic agent in Australia of the influenza-like illness and/or polyarthritis [20]. In this metagenomic study has also reported a novel virus, a dipteran-mammal-associated rhabdovirus: dimarhadbovirus.

These new methodologies that increase the ability to detect different species of viruses are of great interest in the diagnosis of many zoonoses. New adenoviruses have been discovered over the past 3 years, and some have been implicated as pathogens for humans. These findings show that many viruses of this kind can be discovered in the future. The detection of these viruses from rodent samples, its main host, would establish control measures to prevent or reduce the proportion of zoonotic diseases caused by them, whose manifestations in

humans are known to cause a severe hemorrhagic fever, acute central nervous system disease, congenital malformations, and infection in organ transplantation recipients [25].

Other important advances in determining viruses have been shown by Dacheux and coworkers. In this study using some above-mentioned technologies, they determined the viral diversity of five different species of insectivorous bats French, who are in close contact with humans. The viromes described in this work revealed the presence of families of known viruses that infect bacteria, plants/fungi, insects or vertebrates. The most relevant groups were those that potentially infect mammals (e.g., Retroviridae, Herpesviridae, Bunyaviridae, Poxviridae, Flaviviridae, Reoviridae, Bornaviridae and Picobirnaviridae). The data revealed the detection of new viruses of mammals, including rotaviruses, gammaretroviruses, bornaviruses and bunyaviruses with the identification of the first bat nairovirus (**Figure 4**) [26].

These findings are of great interest because they demonstrated that bats naturally harbor viruses, which can infect mammals. The identification of known and unknown viruses in these natural hosts also allows to determine the role played by bats in the spread of zoonotic viral infections [26].

The first evidence of viral metagenomics was published by Breitbart et al. [27]. In this chapter, authors concluded that the viral diversity has been totally underestimated. Viruses are considered as the most abundant and diverse form of life in the nature [28], with more than 7000 different viral genotypes found in the marine ecosystems. Viral metagenomics has studied the viral composition associated with different body sites, and DNA virus communities are the mainly studied [29]. Viral metagenomic approaches in animals bring the opportunity to describe novel antibiotic resistance genes, new virulence factors and new genotypes in specific animal species [30]. For example, it surely recovers novel anellovirus sequences from animal as have been found from blood samples in humans [29].

On the other hand, bacteriophages are ubiquitously and widely distributed in any ecosystem, with estimation between 10^{13} and 10^{15} particles in the human body [31].

For example, several works have reported bacteriophage populations in salivary [32], respiratory tract [33], gastrointestinal tract [34] and oropharyngeal samples [35]. It is known that viruses of bacteria play an important role on the dynamic of bacteria populations in human

Figure 4. Distribution of contig sequences after BLASTx analysis. Percentage of sequences related to the main categories of existing viruses: vertebrate (blue), plant/fungal (green), invertebrate (brown), protozoan (yellow) viruses and bacteriophages (gray), and unassigned viral sequences (no data available concerning the taxonomic family, indicated in red). The total number of viral contigs is indicated below each pie chart (figure and legend were taken with permission from [17]). <http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0087194>.

and have influence on the horizontal gene transfer processes, among others [36, 37]. In this sense, Breitbart and Rohwer in 2005 published that bacteriophages can also play important roles on healthy and disease states in humans. They can confer new pathogenic phenotype in pathogenic bacteria. To study this phenomenon in animals can be interesting because we allow to develop new diagnostic assays, identify new pathogenic factors and design new therapies for zoonosis. Metagenomic profiles of viral compositions have suggested that the human oropharynx is an important reservoir of virulence genes [33]. There are strong evidences of horizontal gene transfer from bacteriophages to bacteria in humans, because antibiotic resistance genes have been found in bacteriophages studied in cystic fibrosis patients [38]. These studies not only allow to identify the viral composition in humans and animals, but also allow to confirm the presence of known and unknown virulence genes, the dynamic between viruses and bacteria and design new diagnostic tools.

Metagenomic approaches have been successfully used to study emergent viruses. These global tools were applied to study the yellow fever virus in the hemorrhagic fever in Uganda and the flu virus, both in 2010. Metagenomics allowed to elucidate the complete genome of the flu virus, when the information about this virus was totally missing [39, 40]. Thus, metagenomics has important implications to study new emergent virus and its genome and consequently take in advance controls to prevent their dissemination. The results obtained from metagenomic studies in humans and animals have also positive impact in the development of new and robust molecular techniques with diagnostic interest.

The collection of a good and representative sample is necessary with metagenomic proposes. Viral enriched sample can be obtained by filtration and ultracentrifugation, and particles are purified with sucrose, glycerol or cesium chloride density gradient [41]. Because viral genome is shorter than those in prokaryotic and eukaryotic cells, the filtration is essential to remove bacteria and host cells. However, if we use filters of 0.2 μm , large viruses are totally missing in the sample and the viral fingerprinting will be underestimated. So, methodological details should be adjusted with our particular interest.

The amplification of viral genome is usually recommended before nucleic acid extractions. Linker amplified shotgun library method is frequently used to amplify viral genomes. Viral DNA or cDNA obtained from RNA viruses should be fragmented, ligated and PCR-amplified [27]. But this technique has an important disadvantage because ssDNA viral genomes cannot be amplified and they are missing in the final metagenome [42]. The isothermal amplification of the DNA or cDNA obtained from RNA viruses is also recommended by using random hexamer and the phi29 DNA polymerase. This methodology is called multiple displacement amplification, and it is an alternative technique to linker amplified shotgun library method. Multiple displacement amplification is preferentially used to amplify ssDNA [42, 43]. It is important to note that the used amplification method will have a significant influence on the metagenome preparation and consequently on downstream analyses and comparisons.

After metagenome preparation, a bioinformatics workflow is necessary to make good and accurate interpretations. This workflow includes in general four steps: preprocessing, annotation, assembly and, finally, the estimation of genotypes, abundances, community, structure and diversity. During the annotation, several databases are specifically used for viruses, such as ProVide [44], MGTAXA [<http://mgtaxa.jcvi.org>], MetaVir [45], VIROME [Bhavsar et al. in preparation] and VMGAP [46].

The taxonomic classification is defined as an active field in viral metagenomics [30]. Two main methods are used to classify the sequence with taxonomic purposes: similarity-based methods and composition-based methods.

Viral metagenomics has really allowed us to describe pathogen viral agents for diverse diseases [10, 15, 47–49]. Metagenomic tools have also been conducted to characterize the baseline viral diversity for humans and animals [18].

3.2. Bacterial metagenomics

Bacteria are an important microbial group that frequently causes zoonosis. Many bacteria are zoonotic agents involved in gastrointestinal diseases, which affect a wide group of animals. An important microbiome is contained inside the gastrointestinal tract of animals as a proof of selection process of microbes by host gut and specific feed. Complete knowledge about gastrointestinal tract microbiome is not possible with conventional culture, but metagenomics supports a great amount of biological data that reflect the gastrointestinal tract microorganisms and their potential [50, 51]. For example, *Campylobacter jejuni* colonizes the ceca of chickens without causing disease approximately at 3 weeks of age and this remains present throughout the chicken life.

A metagenomics analysis of chicken cecal microbiome using both free-pathogen and *C. jejuni*-infected individuals revealed a high distribution of *Actinobacteria*, *Bacteroides*, *Chlorobi*, *Deferribacteres*, *Firmicutes*, *Fusobacteria*, *Proteobacteria* and *Verrucomicrobia*. *Firmicutes* is the most important phyla independent of chicken type, and it was dominant in all chicken ceca. *Bacteriodes* phyla had high abundance in free-pathogen chicken. *Campylobacter*-like sequences were found in the chicken infected with *C. jejuni*. There were not identified archaea sequences, and some Eukarya sequences were determined in this study [52].

The metagenomic has allowed the description of microbiomes from samples obtained of a limited number of mammalian species. The study of microbiome from wild and domestic animals brings an important knowledge about resident and pathogen microorganisms that can be transmitted to the human and to cause several diseases. For example, there is an increase interest to study the bat-associated microbiota because bats are an important reservoir and vector of zoonotic pathogens [53]. Bats are widely distributed in the world, being the second diversity species of mammals [54]. They inhabit forests, gardens, orchards and agricultural areas, among other ecosystems. Thus, it is very important as zoonotic control to know the microbiome in bat, particularly pathogenic bacteria and viruses [53]. Some previous studies focused on virus have reported the presence of Rabies virus [55], Nipah virus [56, 57], Hendra virus [58], and European and Australian bat lyssaviruses [59], among others. We have to note that the study of pathogenic bacteria in bats has been poorly considered. Few works have reported the presence of *Salmonella* spp. [60] and *Clostridium* spp. [61] isolated from bat samples.

The metagenomics brings new possibilities to describe extensively the pathogenic microbiota inhabiting in bats since culture-based methods are very limited. Hatta and coworkers studied the rectal microbiota in bats using high-throughput sequencing of V3-V4 region of the 16S rRNA. They found the presence of 103 genera of bacteria. *Campylobacter* was detected as a prevalent genus being identified *C. jejuni* and *C. coli* in rectal samples from bats (*Rousettus amplexicaudatus*). *C. jejuni* is defined as a serious agent for diarrheic diseases in humans, and

bats are an important reservoir for this species. This study revealed that the predominant phyla was Firmicutes, and the authors identified 66 families, *Clostridiaceae*, *Campylobacteraceae* and *Enterobacteriaceae* as being predominant. Moreover, 103 genera were classified and *Clostridium* and *Campylobacter* were the majority. Other studies have described as dominant genera to *Leuconostoc*, *Betaproteobacteria* and *Enterobacter*.

Brucellosis is other zoonosis extensively found in humans, causing 500,000 human infections per year around the world. *Brucella melitensis* affects humans through consumption of infected milk, meat or animal contact, leading to spreading into reticuloendothelial tissue or osteoarticular effects. Shotgun metagenomics is a useful option to detect Brucellosis in historical human material. A study on a skeleton of a ~60-year-old male with features of diffuse idiopathic skeletal hyperostosis and 32 calcified nodules in the pelvic girdle was carried up in order to identify *Brucella*'s sequences. It was obtained 10,000 sequences related with *B. melitensis* genomes, providing approximately 0.7-fold coverage of a medieval *Brucella* genome from the strain Geridu-1. Sequences showed abundant CT and GA base conversions, a signal of the damage in ancient DNA. A phylogenetic analysis provides evidences that the *B. melitensis* Geridu-1 is closely connected with four *B. melitensis* strains. Additional tests such as deletions and locations of insertion sequences confirm the assignment of the Geridu-1 strain within *B. melitensis* [62].

Many studies have been performed to describe the oral flora in dogs and cats, since these animals are very frequently found as pets. Microorganisms, especially the bacteria in the oral cavity, play important physiological roles. They provide protection against opportunist pathogens and are an essential barrier with the host immune system [63]. But, the oral flora also can cause dental caries, periodontitis and systemic infections, among others [64]. Considering that dogs are the most common companion animals, Oh and coworkers published a work describing the composition of the canine oral microbiome [65]. They found in the dog's microbiome human pathogen bacteria, and at the same time, the authors concluded that its relationships with the owners are largely unclear. In this study, 246 operational taxonomic units were detected in 10 samples from dogs and their owners, where Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria and Actinobacteria were the predominant phyla in human oral cavity. On the other hand, Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes and Fusobacteria were predominant in oral sample of the sampled dogs. Related studies have been developed in order to clarify and understand the dynamic of oral-to-oral transfer of zoonotic bacteria. Oh and coworkers concluded that the oral microbiomes of dogs and their owners were different. Regarding the oral-to-oral transfer, the authors recovered evidence that *Neisseria shayegani*, *Porphyromonas canigingivalis*, *Tannerella forsythia* and *Streptococcus minor* from dogs to human can be possible. Thus, the canine oral microbiome can be zoonotic and oral-to-oral transfer from dogs to human is a possible cause of oral diseases and a risk for the public health. Periodontal diseases have a high prevalence in dogs [66], and it has been demonstrated that *Pasteurella multocida* and *Tannerella forsythia* can be transmitted from animal to human [67–69].

4. Reverse zoonotic disease transmission

Zoonotic diseases have been related with the infection transmission from animals to humans, but some pathogens can be transmitted from humans to animals. Zooanthroponosis, bidirectional

zoonosis, anthroponosis, anthroozoonosis, human-to-animal disease transmission and reverse zoonosis are the terms to refer when any human pathogen infects animals. The first transmission of human parasites to animals was published in 2000 [70]. In general, anthroozoonosis and its ecology are poorly studied; however, they are defined as an important health trouble in the world. *Mycobacterium tuberculosis*, *M. bovis*, *Staphylococcus aureus*, Methicillin-resistant bacteria, *Streptococcus pneumoniae*, *Campylobacter* sp., *Salmonella* sp., *Shigella sonnei*, *S. boydii*, *S. flexneri*, *Escherichia coli*, Oxacillin-resistant bacteria and *Helicobacter pylori* are some bacteria that have caused reverse zoonosis in livestock, wildlife and companion animals.

Viruses also have been reported as reverse zoonosis agents, for example, hepatitis E, measles, human metapneumovirus, influenza A (H1N1), rotavirus, human herpesvirus 1 and 4, and human adenovirus A-F have been transmitted from humans to animals. Parasites as *Chilonastix mesnili*, *Endolimax nana*, *Strongyloides fuelleborni*, *Trichuris trichiura*, *Encephalitozoon intestinalis*, *Giardia duodenalis*, *C. parvum*, *Blatocystis* sp., *Ascaris lumbricoides*, *T. trichiura* and *Isospora* sp., and fungi as *Microsporum* sp., *Trichophyton* sp., *Tricophyton rubrum*, *Candida albicans* and *Microsporum gypseum* have been also reported in animal pathogenesis obtained from ill humans [70].

Bacteria (38%) are the prevalent agents of reverse zoonosis and other as viruses (29%), parasites (21%) and fungi (13%) are also involved in human-to-animal disease transmission. This type of transmission has been studied and conducted in all the continents except Antarctica, North America being the region with highest prevalence (**Figure 5**) [70]. The main transmission ways include fomite, oral contact, aerosols and inoculation.

Two patterns of transmission have been defined to describe the transmission from wildlife to humans [71]. In the first one, a viral disease from wildlife is rarely transmitted to humans

Figure 5. Timeline and frequency of reverse zoonoses publications included in this review shown by pathogen type.

and then that can be horizontally transmitted from humans-to-humans. In this pattern, the virus maintains its cycle in humans. Simian Immunodeficiency virus is the major example of this pattern [72]. The second pattern involves two or more animals and humans, with any arthropod as mediator. In this case, the transmission humans-to-humans is very rare (e.g., West Nile virus) [73].

With the previous background, the diagnosis of reverse zoonosis via metagenomics would bring novel information about the transmission routes, ecology of these zoonosis and anthro-pozoonosis and serotypes that cause infections in animals. Pathogen bacteria, parasites, viruses and fungi, including those available to produce reverse zoonosis, can be identified by metagenomic profile analysis of ill animals and humans. Metagenomics also can provide novel and useful information about the dynamic and ecology of pathogen populations. Other global studies as transcriptomics can suggest the differential transcriptional levels of pathogens in different hosts. This research will bring important information to design specific therapies for different hosts.

Viruses approximately comprise 200 human pathogen species, and novel pathogen viruses are discovered each year in the rate of two per year [74]. The viral tropism is extensively discussing because some viruses can rapidly jump between species such as avian [75] and swine [76] influenza epidemics. These points are attractive to study viral reverse zoonosis and identify mutations and viral new properties involved in anthro-pozoonosis. Some authors report that between 36 and 562 viral pathogens remain to be discovered [74]. Probably some of them can cause animal illness.

5. Metagenomic and surveillance programs

As mentioned in the chapter, metagenomics provides a powerful approach to study viromes and microbiomes from different wild, domestic animals and humans. Detection of new and reemerging infectious agents in such hosts not only is a source of information relevant to public health, regarding the ecology and epidemiology of infectious disease, but also allows the establishment of appropriate surveillance programs; mainly in developing countries, to prevent transmission of infectious diseases in humans still remains a threat worldwide.

The increasing of the population represents a large risk to facilitate the zoonotic diseases. Also, the distribution of human settlements to regions previously inhabited influence the incidence, geographical distribution and the incorporation of infectious agents, favoring the transmission of infectious diseases. Haagmans and coworkers showed the role of camels in the transmission of Middle East Respiratory Syndrome Coronavirus (MERS-CoV)-to-humans [77]. These dates show the importance on metagenomics studies in animal species that have not been considered as a reservoir of zoonotic agents. In addition, it could improve surveillance programs in infectious diseases, which may include new host such as arthropods, wild and domestic animals.

Epidemiological surveillance programs must be accompanied by economic capacity, infrastructure, research and interdisciplinarity allowing adequate and timely response, to

address emerging and reemerging infectious diseases in all countries, especially in developing countries where the incidence of these diseases is increasing. Recent study by Pan American Health Organization (PAHO 2016), which involved the participation of ministries of health in different countries of Latin America, showed that the greatest need in these countries regarding emerging infectious diseases lies in the diagnosis and laboratory capabilities for specific diseases such as rabies, leptospirosis, brucellosis, West Nile virus (WNV), Bovine Spongiform Encephalopathy (BSE), and conditions for surveillance of Ebola virus disease (EVD) and avian influenza (AI) [78].

In this context, metagenomics would be a potential tool for the detection of new species that could potentially be a threat for human health; furthermore, it is used for surveillance of emerging diseases.

6. Conclusions

As analyzed, metagenomics provides a powerful and useful approach to study viromes and microbiomes in animals and humans. The relevant information derived from metagenomic studies provides new highlights about zoonotic diseases and its relationships with human. Due to, metagenomic should be extensive to diagnostic activities in order to identify the presence of new viruses and other zoonotic agents, even human pathogens. Metagenomics also provides information about the structural composition of the microbial populations and communities, and how can change them during different zoonosis. Thus, culture-independent methods open new opportunities in the zoonotic diagnostics because these allow to work with complex samples and describe in detail the associated microbiota and virome.

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