Chapter 1

Pathogenomics and Molecular Advances in Pathogen Identification

Rosa Estela Quiroz-Castañeda

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.73695

Abstract

Today exists a spread spectrum of tools to be used in pathogen identification. Traditional staining and microscopic methods as well as modern molecular methods are presented in this chapter. Pathogen identification is only the beginning to obtain information related to pathogenicity of the microorganism in the near future. Once the pathogen is identified, genome-sequencing methods will provide a significant amount of information that can be elucidated only through bioinformatics methods. In this point, pathogenomics is a powerful tool to identify potential virulence factors, pathogenicity islands, and many other genes that could be used as therapeutic targets or in vaccine development. In this chapter, we present an update of the molecular advances used to identify pathogens and to obtain information of their diversity. We also review the most recent studies on pathogenomics with a special attention on pathogens of veterinary importance.

Keywords: pathogenomics, pathogen identification, phylogeny, genome sequencing, infectious diseases

1. Introduction

Infectious diseases not only represent one of the biggest threats to public health but also to animal health and welfare. A significant number of pathogenic microorganisms can be transmitted by vectors; among these, vector-borne pathogens are considered important since they can spread easily pathogens to previously pathogen-free livestock areas [1].

Nowadays, we can identify cultured or non-cultured organisms with molecular techniques and even reconstruct a phylogeny to propose a new species or reclassify reported microorganisms.

Molecular identification methods offer some advantages as being more sensitive and quicker than traditional culture methods at relatively low cost. Many microorganisms are difficult to culture or noncultured which difficult their study *in vitro,* this is overcome by using genome sequencing as an alternative [2].

Currently, the high-throughput next generation sequencing (HT-NGS) technologies have provided a huge amount of information in genomics researches [3]. Genome sequencing and new omics studies, such as pathogenomics, reveal a new landscape of study of microorganisms and reveal unexpected aspects of pathogen biology. These studies have brought a re-evaluation of definitions of pathogens and virulence factors [4].

In order to understand the complex interaction established between host-pathogen, several genomes of farm animals are sequenced (http://www.ensembl.org/info/about/species.html): cat (*Felis catus*), chicken (*Gallus gallus*), cow (*Bos taurus*), dog (*Canis lupus familiaris*), horse (*Equus caballus*), pig (*Sus scrofa*), sheep (*Ovis aries*), turkey (*Meleagris gallopavo*), and duck (*Anas platyrhynchos*).

However, a scarce number of reports are focused on studying pathogenomics of microorganisms affecting farm animals, a field with a high potential to provide new insights to understand pathogen-host interaction from an omic point of view. The new omics techniques applied in veterinary studies provide a new landscape for research in order to elucidate the mechanisms that pathogens employ to develop infection, and then try to develop new mechanisms of control and treatment.

2. Pathogenomics

Molecular identification methods afford for culturable and non-culturable pathogens' identification; however, the entire genome information remains unknown. High-throughput sequencing technologies have opened the possibility to get access to valuable information contained in the genome [5].

Pathogenomics is a discipline that seeks to mark out virulence factors and their contributions to overall pathogenesis by comparing gene repertoires of pathogenic and non-pathogenic strains/species [4].

Today, sequencing and comparing genomes of several strains of a single pathogen is a relatively short time process [6]. One of the crucial genomic analyses is driven to understand microorganisms' pathogenicity and virulence through intensive and refined bioinformatics tools.

Over the years, the genomics information has changed the concept of a static microbial genome and has demonstrated that bacterial genomes are in a dynamic process. The bacterial genome dynamics is driven basically by three forces: gene gain, gene loss, and gene change, and these three forces comprise of several factors affecting bacterial genome dynamics, such as gene duplication, single-nucleotide polymorphism (SNPs), horizontal gene transfer (HGT), recombination and rearrangements, among others (**Figure 1**) [4, 6].

http://dx.doi.org/10.5772/intechopen.73695

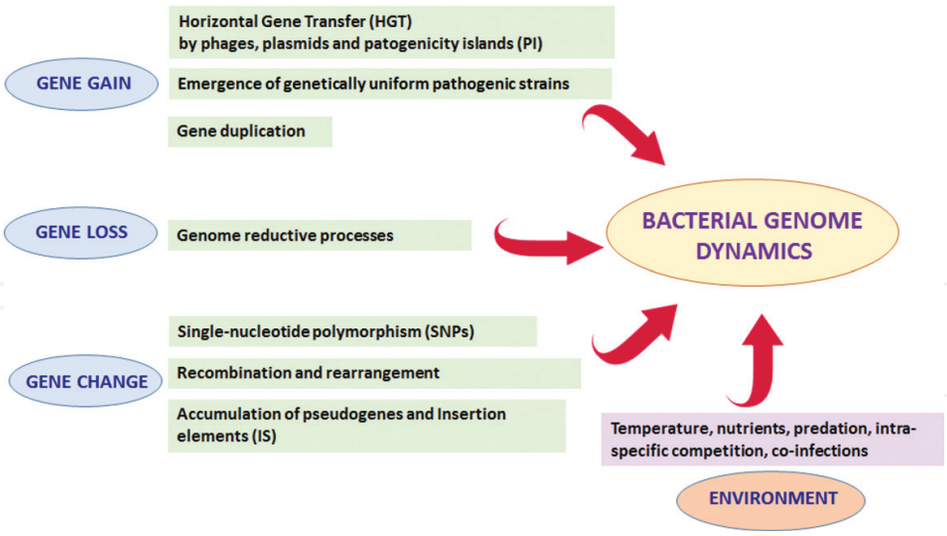

Figure 1. Gene gain, gene loss, gene change, and the environment are the main factors influencing on bacterial genome dynamics.

The smallest variation in bacterial genomes is the SNPs, which have been detected more recently by whole-genome sequencing. Sondgeroth et al. [7] used SNPs of five genes to monitor potential changes in the *B. bovis* population composition before and after passage through the tick vector. A substantial polymorphism among *F. hepatica* isolates was observed by Cwiklinski et al. [8], and they found that 48% of genes exhibited at least one non-synonymous SNP, and these genes were associated with biological processes as axonogenesis and chemotaxis.

2.1. Pathogenomics studies

In order to address what makes bacteria pathogenic is important to know the functional differences between pathogenic and non-pathogenic strains or species. Nowadays, the number of sequenced genomes increases constantly and this has made feasible comparative analyses between pathogenic and non-pathogenic bacterial genomes.

A significant variation in size and content of genomes between different genera and species, and even in strains of the same species has been reported [6].

Pathogenicity is an ability of an organism to cause disease and microorganisms possess several factors that enable them to increase their virulence or degree of pathogenicity.

Toxicity and invasiveness are the two properties of pathogens to cause disease; the first one refers to degree to which a substance causes harm and the latter is the ability to penetrate into the host and then spread [9]. Host and pathogens have co-evolved over millions of years and in this relationship, pathogens have modified their virulence to adapt to the host immune system.

Due to gain or loss of genes pathogens adapting to the changing environments, in this sense, genomic studies are indispensable to identify differences between genomes to provide invaluable insights into virulence and pathogenesis [10].

2.1.1. Mobile genetic elements (MGE)

The term MGE encompasses specialized genetic elements that play a role in genomic instability including plasmids, bacteriophages, transposons, genomic islands (GI), inteins, introns, retroelements and integrons, and many other specialized genetic elements such as insertion sequence elements (IS), miniature inverted-repeat transposable elements [MITEs], repetitive extragenic palindromic [REP] sequences, and bacterial interspread mosaic element [BIMEs] [11]. Among bacterial strains exist a particular interest due to the presence of virulence factors that may be introduced to a new host genome. As MGE are involved in genomic rearrangements and virulence acquisition they are considered important elements in bacterial genome evolution [12].

MGE encode proteins that are involved in cell surface structures (capsular polysaccharides, O-antigen, S-layer, flagella, pilli, and porins) and toxins [10]. Type II and type III toxin-antitoxin systems (TASs) belong to the class of bacterial MGEs that are spread by horizontal gene transfer and they appear to behave like selfish elements contributing to the stable maintenance and dissemination of plasmids and genomic islands in bacterial populations [13]. Here, we will focus on explaining some of the features of IS, GIs, and PAIs.

2.1.1.1. Insertion sequences (IS)

IS elements represents an important component of most bacterial genomes; they usually have a size of ranging from 0.7 to 3.5 kB, including a transposase gene encoding the enzyme that catalyzes IS movement. ISs are the smallest and simplest autonomous mobile genetic elements that contribute massively to HGT and have an important role in genome organization and evolution. Many ISs are delimited by short terminal inverted repeat (IR) and are flanked by direct repeat (DR). More than 3500 ISs from bacteria and archaea have been described. These DNA segments are capable of transposing within and between prokaryotic genomes causing insertional mutations and chromosomal rearrangements.

They cause gene inactivation and have strong polar effects or activation or alteration of the expression of adjacent genes [14–17].

Although IS elements are genomics parasites that harm their host by increasing the rate of deleterious mutations, they generate beneficial mutations trough their transposition and recombination. Indeed, IS elements are considered important elements for the adaptive evolution of their host [15, 18].

2.1.1.2. Genomic islands (GEIs)

There exists several ways how bacterial genomes can evolve, including mutations, rearrangements or HGT, contributing to diversification, and adaptation of microorganisms to environmental niches. GEIs are large DNA sequences specifically present in the genomes of certain bacteria strains but not in the genomes of closely related variants. These are non-self-mobilizing integrative and excisive elements that encode diverse functional characteristics; usually, they are integrated in bacterial chromosome but also can be found in plasmids or phages [11]. Recent information on GEIs suggests that these elements have become strongly selective for adaptive

and auxiliary functions (pathogenicity, symbiosis, aromatic compound metabolism, mercury resistance, and siderophore synthesis) [19–21].

2.1.1.3. Pathogenicity islands (PAIs)

PAIs are a group of GEIs that carry one or more virulence-associated genes and mobility genes and occupy chromosome regions as large as 10 kb to more than 100 kb; "pathogenicity islets" are smaller fragments of DNA ranging from 1 to 10 kb. PAIs are part of a flexible gene pool that contain mobility genes so that they can be integrated into the host genome including genes encoding to integrases, transposases, phage genes, and origins of replication [22]. **Table 1** shows the main features of PAIs.

Virulence factors (VFs) are encoded by genes found in pathogenic microorganisms. Pathogenic bacteria possess various VFs that allow them colonize a variety of niches, cause infection, and survive in the hosts [23]. In order to combat infectious diseases, it is absolutely necessary to discover virulence factors of pathogenic microbes to identify targets for novel drugs and design of new vaccines [24].

A special interest has emerged on VFs study, mainly due to the constant and persistent antimicrobial resistance observed in pathogenic microorganisms, because they have modified

Genomic Islands	**Ref.**
Large segments of DNA, 10-200 kb	[22]
GC content different from the rest of the chromosome	[23]
Often inserted at tRNA genes	[23]
Usually flanked by 16-20 bp perfect or almost perfect Direct Repeats (DR)	[23]
Contain genes encoding integrases or factors related to plasmid conjugation or phages involved in GEI transfer	[23]
Often carry Insertion Elements or transposons to mobilize genetic material	[24]
Offer a selective advantage for hos bacteria and often described as pathogenicity, symbiosis, metabolic, fitness or resistance islands	[23]
Pathogenicity Islands	
Clusters of contiguous genes present in some related strains or species, but absent in non-pathogenic bacteria.	[4]
They confer on the host bacterium a complex and distinctive virulence phenotype by the presence of virulence factors.	[4]
Presence of mobility genes (integrases, transposases)	[5]
High percentage of hypothetic proteins	[5]
Different genomic sequence signature	[5]

Table 1. Characteristics of genomic and pathogenicity islands.

their virulence mechanisms to adapt to host defense system [9]. Today, complete genome sequences of different microbial species either pathogenic or non-pathogenic enable comparative studies to identify specific VFs in species through bioinformatics analyses.

As in other bacteria of clinical importance, some MGE have been identified in farm animals' pathogens. The intraerythrocytic parasite of cattle *B. bovis* has mechanisms to protect their cytoadhesion from the host adaptive immune response, and this function is mainly accomplished through antigenic variation of a virulence factor called VESA1 protein (Variant Erythrocyte Surface Antigen-1) [25]. VESA1 is a size-polymorphic, heterodimeric protein that comprises of two subunits of 105–115 and 120–135 kDa in mass approximately, depending on the isolate and clonal line [26]. Genomic and transcriptomic analyses reveal that sMORF could have a significant role for a rapid antigenic variation. However, experimental evidence is necessary [27].

In *F. hepatica*, some proteins are virulence-associated factors. These proteins are cathepsin L cysteine peptidase (FhCL) and have functions in parasite virulence including tissue invasion and suppression of host immune responses. Among the functions are degradation of red blood cells, a vital process when the parasite is located in the bile duct and needs to digest a large quantity of host cells to support the enormous production of progeny (30–50,000 eggs/day/worm) [28]. Through phylogenetic analyses, Robinson et al. [28] classified cathepsin L gene family into three clades (Clades 1, 2, and 5) expressed by tissue-migrating adult worms and two clades (Clades 3 and 4) expressed by early infective juvenile stage. Each of these cathepsins is expressed in different larvae stages. Collagenolytic activities have been reported in FhCL2 and FhCl3, suggesting that this activity is essential to the parasite in order to degrade the connective tissue matrix of the organs that break through during migration [29].

In Gram-negative pathogens, type IV Secretion System (T4SS) has a conserved structured and function that is crucial for virulence and intracellular survival. The importance of this system in *Anaplasmataceae* is its possibility as functional virulence factors due to its retention and protein conservation among rickettsial species [30]. Recently, the high complexity of the Rickettsia T4SS was revealed. Gillespie et al. [31] focus on the components of the Rickettsiales vir homolog (rvh), a collection of VirB and VirD protein-encoding genes. They found that these genes are comprised of unprecedented gene family expansion. Three families of gene duplication are contained in rvh genes: rvhb9, rvhB8, and rvhB4, and some genes are equivalent in other T4SS. This study shows the need to characterize Rickettsia rvh components.

Some molecules have been investigated because of their physiological importance in microorganisms. Aminopeptidases have been used as therapeutic and prophylactic targets in many parasitic infections and other diseases [32]. In *B. bovis*, a member of the methionine aminopeptidase (MAP) family was characterized and expressed in *E. coli*. The results showed that the construction aminopeptidase (MAP)-glutatione-S-tranferase (GST) was antigenic by inducing high levels of cytokines and immunoglobulin IgG titers in the host, and importantly, inhibitors of MAP inhibit the growth of *Babesia* parasites both *in vitro* and *in vivo* [32]. Methionine aminopeptidases have an important role in N-terminal methionine excision from the polypeptide in ribosome during protein synthesis; their physiological importance relies on the lethality of its absence in bacteria and yeast [33, 34]. MAPs in malaria play an important role in parasite biology due to their role in parasite hemoglobin during peptide catabolism [35].

Database	Website	Information	Ref.
VFDB Virulence Factor Database	http://www.mgc.ac.cn/VFs/	Curating information about virulence factors of bacterial pathogens	[33]
PAIDB v2.0 Pathogenicity Islands Database	http://www.paidb.re.kr	Pathogenicity islands (PAIs), candidate PAIs and resistance islands (REIs) in prokaryotic genomes.	[35]
PHIDIAS-Pathogen-Host Interaction Data Integration and Analysis System	http://www.phidias.us/victors/intro.php	Comprises genes experimentally observed to be necessary for virulence, including bacteria, viruses, parasites, and fungi.	[39]
MvirDB	http://mvirdb.llnl.gov/	Microbial database of protein toxins, virulence factors, and antibiotic resistance genes	[34]
PIPS Pathogenicity Island Prediction Software	http://www.bioinformatics.org/groups/?group_id=1063	Prediction of pathogenicity islands in an integrative manner.	[40]
PredictBias	http://www.bioinformatics.org/sachbinfo/predictbias.html	Identification of genomic and pathogenicity	[41]
MP3 Software	http://metagenomics.iiserb.ac.in/mp3/index.php.	Prediction of pathogenic proteins in genomic and metagenomic data	[42]
ProtVirB	http://bioinfo.icgeb.res.in/protvirdb	Database of protozoan virulent proteins	[43]
GIPSy Genomic Island Prediction Software	http://www.bioinformatics.org/groups/?group_id=1180	Prediction of GEIs including PAIs, REIs, and Symbiotic Islands (SIs)	[40]
Island Viewer 3	http://www.pathogenomics.sfu.ca/islandviewer/	Web-base resource for the prediction and analysis of GIs in bacterial and archaeal genomes	[44]
OASIS Optimized Annotation System for Insertion Sequences	https://github.com/dgrtwo/OASIS	Program that uses a library of IS to identify IS in bacteria	[45]
ISfinder	http://www-is.biotoul.fr/	Database for bacterial IS	[46]
ISsaga	http://issaga.biotoul.fr/ISsaga/issaga_index.php	Web application. Computational tools and methods for high quality IS annotation	[17]
ISQuest Software	https://omictools.com/isquest-tool	Identifies bacterial IS elements in raw read data or contigs	[47]

Table 2. Selection of online resources for analysis and search of virulence factors, toxins, GEIs, and PAIs in microorganisms [39–47].

2.2. Bioinformatics tools in pathogenomics

As mentioned before, despite the recent advances of modern medicine based on genomic data, still infectious diseases are considered as one of the biggest threats to public and animal health [36]. Comparative genomic analysis of pathogenic and non-pathogenic bacteria can reveal horizontally transferred genes between bacteria, thus conferring new properties. PAIs have some detectable properties, like genomic signatures and mobility genes helping in integration into the host genome, as genomic signatures helps to identify pathogens, functional signatures provide information about what a pathogen is capable of [5, 37].

Recently, an updated database has been reported, the Virulence Factor Database or VFDB (http://www.mgc.ac.cn/VFs/) that provide the latest information about virulence factors of various bacterial pathogens, especially those obtained by next generation sequencing technologies (NGS) [36]. The Pathogenicity Islands Database, PAIDB (http://www.paidb.re.kr), is a database that contains comprehensive information on all reported PAIs and candidate PAIs in prokaryotic genomes; additionally, information of Resistance Islands (REIs) is considered.

The importance of PAIs, a subset of GIs, is that genomes of pathogenic bacteria mediate the horizontal transfer of genes encoding a significant number of virulence factors [38].

PAIDB also contains information of antimicrobial resistance islands, REIs. This, another, class of GIs is linked to pathogenesis by conferring resistance to multiple antibiotics and thus facilitating the emergence of multidrug–resistance pathogens. PAIDB contain 223 types of PAIs and 1331 GenBank accessions of complete or partial PAI and 88 types of REIs from 108 accessions [38]. Several database and software are available for *in silico* analysis of PAIs, VFs, and IS (**Table 2**).

3. Methods used to identify pathogens

3.1. Staining and microscopic methods

Many methods are available to identify bacteria, and microscopy has an important role in microorganism identification. Especially, when an urgent diagnosis is required, fast microscopic methods are the first option. Many bacterial pathogens are identified by staining methods, and among these, differential stains are common in microbiology and provide some information about the species and many times can be compared to automated species differentiation methods [48].

Gram-stain is an old differential technique, but very popular to distinguish between Gram-positive and Gram-negative bacteria. This method is based on the different cell wall structures and components of both bacteria types. The bacterial cell wall of Gram-positives is stained by crystal violet and iodine, which form an insoluble blue dye complex while Gram-negatives are counterstained by fuchsin or safranin. This staining is also applied to some fungi, such as *Candida* spp., *Nocardia* spp., or *Actinomyces* spp. When cells walls are damaged or even cells are dead, false Gram-negatives may result [48, 49].

Another common technique is Giemsa staining, which was primarily developed for the visualization and histopathological diagnosis of *Plasmodium* spp. at the end of nineteenth century. Now, this staining method is used to identify many other parasites including *Babesia bovis*, *B. bigemina*; *Leishmania* spp., *Trypanosoma* spp.; *Toxoplasma gondii*, and others, and bacteria as *Anaplasma marginale*, *A. phagocytophilum*, and fungi (**Figure 2**).

The Giemsa's solution is a composition of methylene blue and oxidation products of methylene blue (Azure A and B) that stain primary proteins and nucleic acids [48].

Even natural herbal dyes as curcuma, alizarin, and henna have been used to stain *Fasciola hepatica* without the carcinogenic effect of traditional synthetic dyes [50, 51]. Fluorescent dyes such SYBR Green 1, YOYO-1, and ethidium homodimer-2 could be detected using fluorescent microscopy in combination with Giemsa staining, this method has been proposed to improve microscopic diagnosis of *Plasmodium falciparum* [52]. According to these results, the combinations of fluorescent and non-fluorescent dyes could be applied to enhance other microorganisms' identification.

Oocysts parasites identification is an issue that has been resolved through microscopic observation. In avian coccidiosis, most of the oocysts have a very similar morphological appearance with size differences that allow distinguish them. Castañón et al. [53] reported an approach based on image recognition by algorithms to identify *Eimeria* spp. oocysts; the authors extracted morphological information by using computer vision techniques in order to perform an automatic species differentiation of oocysts. The parameters considered in the identification process were: (1) multiscale curvature, (2) geometry, and, (3) texture to construct a 13-dimensional future vector for each oocyst image. With this powerful tool, molecular diagnosis based on PCR using the ribosomal ITS1 or multiplex PCR can be complemented with the use of the *Eimeria* Image Database [53].

With the recent development of fluorescent techniques and imaging tools, farm animals' pathogen identification has become a more efficient and reliable process.

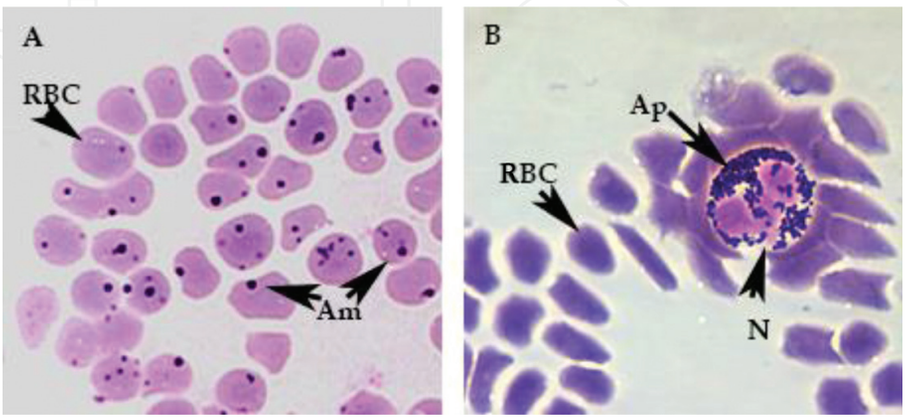

Figure 2. Visualization of (A) Giemsa staining of *A. marginale* (Am) inside bovine red blood cells (RBC), (B) Giemsa staining of *A. phagocytophylum* (Ap) inside a human neutrophil (N). (Anaplasmosis Unit, CENID-PAVET, INIFAP, Human blood was obtained with patient's consent).

3.2. Genotypic methods

Conventionally, cultural and biochemical techniques are the primary methodology for identifying most pathogens; however, Koch's postulates are critical for fulfilling most of the times [2, 54]. The culture-based testing usually yield results in several days or even up to weeks after sampling without success guaranteed, because of the unsuitable culturing conditions and the special requirements for the bacterial species [55].

Besides, pathogen identification can be a hard task when these cannot be cultured. With the development of molecular techniques and sequencing technologies, many non-cultured microorganisms, as *Mycoplasma haemobos* and *M. wenyonii* have been identified and many microorganisms already known have been reorganized phylogenetically [56].

3.2.1. Polymerase chain reaction (PCR)

Molecular methods based on nucleic acid amplification have circumvented the culturing problem with some benefits. In the molecular detection by PCR, the pathogen is first detected by PCR product amplification and then identified by sequencing, resulting in more rapid diagnoses [55]. Several pathogens of veterinary importance have been detected successfully by PCR, including *A. marginale*, *B. bovis*, *Mycobacteria* spp., *F. hepatica*, and *F. gigantica*, *Theileria* spp., among others [57–61]. Ribosomal RNA (rRNA) genes have emerged as the most prominent target in microbial detection mainly due to fact that the region represents a versatile mix of highly conserved and moderately to highly variable segments [62]. In bacteria, the rRNA genes are firstly transcribed from the ribosomal operon as 30S rRNA and then cleaved into 16S, 23S, and 5S rRNA by RNase III. The ribosomal operon size, nucleotide sequences, and secondary structures of 16S, 23S, and 5D rRNA are well conserved within bacterial species [63]. Since rRNA genes are evolving more slowly than protein encoding genes they have a particular importance for identification and phylogenetic analysis of distant related species [64].

3.2.1.1. Molecular markers

During the last two decades, the 16S rRNA sequences have been widely used for the identification and classification of bacteria, the main uses of 16S sequences are: identification and classification of isolated pure cultures and estimation of bacterial diversity in environmental samples without culturing through metagenomic approaches [65].

The rRNA operon in bacteria comprises 16S, 23S, and 5S, spaced by intergenic spacer regions (ITS, also called internal transcribed spacer) which have been used also to detect and differentiate pathogens [62]. Amplified PCRs products based on ITS sequences have distinguished 55 bacterial species, including 18 *Clostridium* and 15 *Mycoplasma* [66]. More recently, rRNA ITS, specifically 16S–23S, has been used in *Vibrio* identification [67], *Mycoplasma* from cattle [68], *Brucella* [69, 70], and Mycobacteria [71, 72].

Similarly to 16S rRNA, 18 s rRNA is a sequence commonly used for eukaryotic identification, such as parasites. Actually, there exists several molecular markers used to identify *B. bovis* and *B. bigemina* using 18S rRNA, cytochrome b gene, antigenic protein encoding genes msa-1 and msa-2, EF-1a, beta-tubulin, among others [73].

Alternatively, other genes less commonly used that can help in bacterial identification are chaperonin-60 (*cpn60*) [74], chaperonine GroEl [75], recombination and repair protein (*recN*), and DNA polymerase III subunits γ, τ (*dnaX*) [76], the β-subunit of RNA polymerase (*rpoB*) [76], and esterase (*est*) [77]. Among all genes used to identify bacteria, still 16S rRNA is the most used when the bacterial pathogen is non culturable, this is mostly because there exists a significant number of 16S rRNA sequences available in databases that can be used to compare and then identify [Ribosomal Database Project (http://rdp.cme.msu.edu) and Greengenes (http://greengenes.lbl.gov)]. These databases are not always complete because 16S rRNA sequences are constantly reported and many are still missing, besides, many times the species can only be identified at genus level and analyses with other genes are necessary [78].

Amplification of 16S sequence have allowed identification and phylogenetic reconstruction of several *Anaplasma* species in China, including *A. marginale y A. ovis* [79]; a comparison between *A. marginale* and *A. centrale* 16S rRNA revealed that both sequences have 98.08% identity, even with this level of identity *A. centrale* was identified by PCR primers based on 16S rRNA [80]. Bovine hemoplasmas "*Candidatus* Mycoplasma haemobos" and *Mycoplasma wenyonii* has also been detected by 16S rRNA PCR and RT-PCR in Brazil and Switzerland [81–83]. Detection of rickettsia *A. marginale*, causal agent of bovine anaplasmosis, using genomic DNA as template for PCR is an alternative diagnostic tool. Singh et al. [84] used a semi-nested PCR assay for the detection of *A. marginale* in carrier cattle in India. The PCR was optimized to identify the major surface protein 5 (Msp5) based on primers previously reported [85]. The nested PCR (nPCR) employing *msp5* primer sequences were able to detect as few as 30 infected erythrocytes per ml of the blood and then detect low levels of rickettsiaemia in cattle [85].

Noaman and Shayan [86] employed 16S ribosomal RNA (rRNA, GenBank M60313) gene of *A. marginale* on DNA isolated from blood samples of cattle. The nucleotide sequence of 16S rRNA is highly conserved in *Anaplasma* spp., and is use to amplify fragments of the gene in all known *Anaplasma* species.

B. bigemina, one of the several *Babesia* species known to cause bovine babesiosis has also been detected by PCR, besides, the amplified product is parasite and species specific [87]. From sensitivity studies, the authors showed that the 278-bp fragment amplified by PCR and visualized in reactions could contain as little as 10 pg. of parasite template DNA.

3.3. Multiplex PCR

A variant of the PCR is the multiplex PCR (mPCR) that detects more than one species at a time in a very effective way using a mixture of locus-specific primers in a single reaction [88]. mPCR offers an important advantage over single-species PCR because co-infections can be detected, for instance, detection of swine, avian and equine viruses [89–91], bacteria in fish [92], cattle bacteria and parasites, including *Mycobacterium bovis*, *T. annulata*, *F. hepatica*, and *B. bovis* [93–97], and nematodes [98] have been reported.

Multiplex PCR for detecting multiple pathogens has not been widely used in animal health diagnostic laboratories because this assay is difficult to optimize and validate [2].

3.4. PCR-restriction fragment length polymorphism (PCR-RFLP)

PCR-RFLP is an approach based on the fact that the genomes of closely related pathogen have variations in sequence, thus, a different length can be obtained from enzymatic digestion of a PCR fragment [99]. Due to that digested DNA represents a unique pattern, and this method offers a much greater sensitivity for the identification of pathogens, especially when culture is difficult. Using PCR-RFLP, *Theileria annulata* was identified in ticks (Ixodidae), showing that parasite has a preference for *Hyalomma anatolicum anatolicum* which suggest that its major role in transmission of parasite [100]. Identification of parasites as *Fasciola* species also employs PCR-RFLP analysis. The region between 18S and 28S (which includes ITS1, 5.8S, ITS2) of ribosomal RNA was amplified by PCR and then digested with restriction endonucleases, in this analysis, 90 *Fasciola* samples from different geographical regions were identified as *F. hepatica* or *F. gigantica* [58].

3.5. DNA microarrays

DNA microarrays are a viable platform for detection of pathogenic organisms. This detection has a cost lower than multiplex PCR and technologies like high-throughput sequencing [101]. A microarray is a miniaturized device that contains short single-stranded DNA oligonucleotides (25- to 70-mers) probes attached to a solid substrate. These probes would be complementary to segments of one or more target organism genome. El-Ashker et al. [59] identified *Babesia*, *Theileria*, and *Anaplasma* species in cattle using DNA microarray. This novel DNA microarray system was compared with microscopy and PCR assay for the diagnosis of bovine piroplasmosis and anaplasmosis. All samples positive by PCR for *Babesia*/*Theileria* spp. also were positive in the microarray analysis, which supports this technique as a valuable improvement in veterinary diagnoses. Another microarray developed for *Mycoplasma* spp. consist of probes for 55 different cattle pathogenic bacteria including *M. mycoides* subs. *Mycoides* [102]. To date, no microarray has been developed for diagnosis of bovine tick-borne diseases.

Acknowledgements

This work was supported by Project PN-CONACYT No. 248855.

Author details

Rosa Estela Quiroz-Castañeda

Address all correspondence to: quiroz.rosa@inifap.gob.mx

National Center for Disciplinary Research in Veterinary Parasitology, National Institute for Research in Forestry Agriculture and Livestock (CENID-PAVET, INIFAP), Jiutepec, Morelos, México

References

[1] Tomley FM, Shirley MW. Livestock infectious diseases and zoonoses. Philosophical Transactions of the Royal Society of London. Series B. The Royal Society. 2009;**364**(1530):2637-2642

[2] Cai HY, Caswell JL, Prescott JF. Nonculture molecular techniques for diagnosis of bacterial disease in animals: A diagnostic laboratory perspective. Veterinary Pathology. 2014;**51**(2):341-350

[3] Pareek CS, Smoczynski R, Tretyn A. Sequencing technologies and genome sequencing. Journal of Applied Genetics. 2011;**52**(4):413-435

[4] Pallen MJ, Wren BW. Bacterial pathogenomics. Nature. 2007;**449**(7164):835-842

[5] Che D, Hasan MS, Chen B. Identifying pathogenicity islands in bacterial pathogenomics using computational approaches. Pathogens (Basel, Switzerland). 2014;**3**(1):36-56

[6] Sudheesh PS, Al-Ghabshi A, Al-Mazrooei N, Al-Habsi S. Comparative pathogenomics of bacteria causing infectious diseases in fish. International Journal of Evolutionary Biology. 2012;**2012**:457264

[7] Sondgeroth KS, McElwain TF, Ueti MW, Scoles GA, Reif KE, Lau AOT. Tick passage results in enhanced attenuation of *Babesia bovis*. Infection and Immunity. 2014;**82**(10): 4426-4434. Adams JH, editor. 1752 N St., N.W., Washington, DC: American Society for Microbiology

[8] Cwiklinski K, Dalton JP, Dufresne PJ, La Course J, Williams DJL, Hodgkinson J, et al. The *Fasciola hepatica* genome: Gene duplication and polymorphism reveals adaptation to the host environment and the capacity for rapid evolution. Genome Biology. London: BioMed Central. 2015;**16**(1):71

[9] Beceiro A, Tomás M, Bou G. Antimicrobial resistance and virulence: A successful or deleterious association in the bacterial world? Clinical Microbiology Reviews. 2013; **26**(2):185-230

[10] Lehmann J, Matthias M, Vinetz J, Fouts D. Leptospiral pathogenomics. Pathogens. 2014; **3**(2):280-308

[11] Darmon E, Leach DRF. Bacterial genome instability. Microbiology and Molecular Biology Reviews. 2014;**78**(1):1-39

[12] Tobes R, Pareja E. Bacterial repetitive extragenic palindromic sequences are DNA targets for Insertion Sequence elements. BMC Genomics. 2006;**7**(1):1-12

[13] Van Melderen L. Toxin–antitoxin systems: Why so many, what for? Current Opinion in Microbiology. 2010;**13**(6):781-785

[14] Charlesworth B, Sniegowski P, Stephan W. The evolutionary dynamics of repetitive DNA in eukaryotes. Nature. 1994:215-220

[15] Schneider D, Lenski RE. Genome plasticity and the evolution of microbial genomes. Dynamics of insertion sequence elements during experimental evolution of bacteria. Research in Microbiology. 2004;**155**(5):319-327

[16] Mahillon J, Chandler M. Insertion sequences. Microbiology and Molecular Biology Reviews. 1998;**62**

[17] Varani AM, Siguier P, Gourbeyre E, Charneau V, Chandler M. ISsaga is an ensemble of web-based methods for high throughput identification and semi-automatic annotation of insertion sequences in prokaryotic genomes. Genome Biology. 2011;**12**(3):1-9

[18] Blot M. Transposable elements and adaptation of host bacteria. Genetica. 1994;**93**(1):5-12

[19] Sullivan JT, Trzebiatowski JR, Cruickshank RW, Gouzy J, Brown SD, Elliot RM, et al. Comparative sequence analysis of the symbiosis island of *Mesorhizobium loti* strain R7A. Journal of Bacteriology. United States. 2002;**184**(11):3086-3095

[20] Larbig KD, Christmann A, Johann A, Klockgether J, Hartsch T, Merkl R, et al. Gene islands integrated into tRNA(Gly) genes confer genome diversity on a *Pseudomonas aeruginosa* clone. Journal of Bacteriology. United States. 2002;**184**(23):6665-6680

[21] Gaillard M, Vallaeys T, Vorholter FJ, Minoia M, Werlen C, Sentchilo V, et al. The clc element of *Pseudomonas* sp. strain B13, a genomic island with various catabolic properties. Journal of Bacteriology. United States. 2006;**188**(5):1999-2013

[22] Gal-Mor O, Finlay BB. Pathogenicity islands: A molecular toolbox for bacterial virulence. Cellular Microbiology. 2006;**8**(11):1707-1719

[23] Yang J, Chen L, Sun L, Yu J, Jin Q. VFDB 2008 release: An enhanced web-based resource for comparative pathogenomics. Nucleic Acids Research. 2008;**36**(SUPPL. 1):539-542

[24] Wu H-J, Wang AH-J, Jennings MP. Discovery of virulence factors of pathogenic bacteria. Current Opinion in Chemical Biology. 2008;**12**(1):93-101

[25] Xiao Y-P, Al-Khedery B, Allred DR. The *Babesia bovis* VESA1 virulence factor subunit 1b is encoded by the 1β branch of the ves multigene family. Molecular and Biochemical Parasitology. 2010;**171**(2):81-88

[26] Allred DR, Hines SA, Ahrens KP. Isolate-specific parasite antigens of the *Babesia bovis*-infected erythrocyte surface. Molecular and Biochemical Parasitology. Netherlands. 1993;**60**(1):121-132

[27] Brayton KA, Lau AOT, Herndon DR, Hannick L, Kappmeyer LS, Berens SJ, et al. Genome sequence of *Babesia bovis* and comparative analysis of apicomplexan hemoprotozoa. PLOS Pathogens. United States. 2007;**3**(10):1401-1413

[28] Robinson MW, Tort JF, Lowther J, Donnelly SM, Wong E, Xu W, et al. Proteomics and phylogenetic analysis of the cathepsin L protease Family of the Helminth pathogen *Fasciola hepatica*: Expansion of a repertoire of virulence-associated factors. Molecular & Cellular Proteomics. 2008;**7**(6):1111-1123

[29] Robinson MW, Corvo I, Jones PM, George AM, Padula MP, To J, et al. Collagenolytic activities of the major secreted cathepsin L peptidases involved in the virulence of the Helminth pathogen, *Fasciola hepatica*. PLOS Neglected Tropical Diseases. Public Library of Science. 2011;**5**(4):e1012

[30] Sutten EL, Norimine J, Beare PA, Heinzen RA, Lopez JE, Morse K, et al. *Anaplasma marginale* type IV secretion system proteins VirB2, VirB7, VirB11, and VirD4 are immunogenic components of a protective bacterial membrane vaccine. Infection and Immunity. 2010;**78**(3): 1314-1325

[31] Gillespie JJ, Phan IQH, Driscoll TP, Guillotte ML, Lehman SS, Rennoll-Bankert KE, et al. The Rickettsia type IV secretion system: Unrealized complexity mired by gene family expansion. Pathogens and Disease. 2016;**74**(6):pii: ftw058

[32] Munkhjargal T, Ishizaki T, Guswanto A, Takemae H, Yokoyama N, Igarashi I. Molecular and biochemical characterization of methionine aminopeptidase of *Babesia bovis* as a potent drug target. Veterinary Parasitology. 2016;**221**:14-23

[33] Li X, Chang YH. Amino-terminal protein processing in *Saccharomyces cerevisiae* is an essential function that requires two distinct methionine aminopeptidases. Proceedings of the National Academy of Sciences of the United States of America. 1995;**92**(26):12357-12361

[34] Chang SY, McGary EC, Chang S. Methionine aminopeptidase gene of *Escherichia coli* is essential for cell growth. Journal of Bacteriology. United States. 1989;**171**(7):4071-4072

[35] Naughton JA, Nasizadeh S, Bell A. Downstream effects of haemoglobinase inhibition in *Plasmodium falciparum*-infected erythrocytes. Molecular and Biochemical Parasitology. Netherlands. 2010;**173**(2):81-87

[36] Chen L, Zheng D, Liu B, Yang J, Jin Q. VFDB 2016: Hierarchical and refined dataset for big data analysis—10 years on. Nucleic Acids Research. 2016;**44**(D1):D694-D697

[37] Zhou CE, Smith J, Lam M, Zemla A, Dyer MD, Slezak T. MvirDB—A microbial database of protein toxins, virulence factors and antibiotic resistance genes for bio-defence applications. Nucleic Acids Research. Oxford University Press. 2007;**35**(Database issue):D391-D394

[38] Yoon SH, Park Y-K, Kim JF. PAIDB v2.0: Exploration and analysis of pathogenicity and resistance islands. Nucleic Acids Research. 2014;**21**

[39] Xiang Z, Tian Y, He Y. PHIDIAS: A pathogen-host interaction data integration and analysis system. Genome Biology. 2007;**8**(7):1-15

[40] Soares SC, Abreu VAC, Ramos RTJ, Cerdeira L, Silva A, Baumbach J, et al. PIPS: Pathogenicity Island Prediction Software. PLoS One. Public Library of Science. 2012;**7**(2):e30848

[41] Pundhir S, Vijayvargiya HKA. PredictBias: A server for the identification of genomic and pathogenicity islands in prokaryotes. In Silico Biology. 2008;**8**(3–4):223-234

[42] Gupta A, Kapil R, Dhakan DB, Sharma VK. MP3: A software tool for the prediction of pathogenic proteins in genomic and metagenomic data. PLoS One. Public Library of Science. 2014;**9**(4):e93907

[43] Ramana J, Gupta D. ProtVirDB: A database of protozoan virulent proteins. Bioinformatics. 2009;**25**(12):1568-1569

[44] Dhillon BK, Laird MR, Shay JA, Winsor GL, Lo R, Nizam F, et al. IslandViewer 3: More flexible, interactive genomic island discovery, visualization and analysis. Nucleic Acids Research. 2015;**43**(W1):W104-W108

[45] Robinson DG, Lee M-C, Marx CJ. OASIS: An automated program for global investigation of bacterial and archaeal insertion sequences. Nucleic Acids Research. 2012;**40**(22):e174

[46] Siguier P, Perochon J, Lestrade L, Mahillon J, Chandler M. ISfinder: The reference centre for bacterial insertion sequences. Nucleic Acids Research. 2006;**34**(Suppl. 1):D32-D36

[47] Biswas A, Gauthier DT, Ranjan D, Zubair M. ISQuest: Finding insertion sequences in prokaryotic sequence fragment data. Bioinformatics. 2015;**31**(21):3406-3412

[48] Makarewicz O, Stein C, Pfister W, Loffler B, Pletz M. Identification methods—An overview. In: Popp J, Bauer M, editors. Modern Techniques for Pathogen Detection. Jena, Germany: Wiley Blacwell; 2015. p. 350

[49] Beveridge TJ. Use of the Gram stain in microbiology. Biotechnic & Histochemistry. Taylor & Francis. 2001;**76**(3):111-118

[50] Daryani A, Sharif M, Meigouni M. Staining of *Fasciola hepatica* by natural herbal dyes. Comparative Clinical Pathology. 2011;**20**(4):305-308

[51] OIE. Bovine anaplasmosis. In: OIE WO for AH, editor. OIE Terrestrial Manual 2015. OIE, World Organisation for Animal Health; 2015. Chapter 2.4.1, pp. 1-15

[52] Guy R, Liu P, Pennefather P, Crandall I. The use of fluorescence enhancement to improve the microscopic diagnosis of Falciparum malaria. Malaria Journal. 2007;**6**(1):1-8

[53] Castañón CAB, Fraga JS, Fernandez S, Gruber A, da F. Costa L. Biological shape characterization for automatic image recognition and diagnosis of protozoan parasites of the genus *Eimeria*. Pattern Recognition. 2007;**40**(7):1899-1910

[54] Cheng C, Sun J, Zheng F, Wu K, Rui Y. Molecular identification of clinical "difficult-to-identify" microbes from sequencing 16S ribosomal DNA and internal transcribed spacer 2. Annals of Clinical Microbiology and Antimicrobials. 2014;**13**(1):1

[55] Järvinen A-K, Laakso S, Piiparinen P, Aittakorpi A, Lindfors M, Huopaniemi L, et al. Rapid identification of bacterial pathogens using a PCR- and microarray-based assay. BMC Microbiology. 2009;**9**:161

[56] Martínez-Ocampo F, Rodríguez-Camarillo SD, Amaro-Estrada I, Quiroz-Castañeda RE. Draft genome sequence of "*Candidatus* Mycoplasma haemobos," a hemotropic mycoplasma identified in cattle in Mexico. Genome Announcements. 2016;**4**(4):e00656-16

[57] Figueroa JV, Chieves LP, Johnson GS, Buening GM. Multiplex polymerase chain reaction based assay for the detection of *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* DNA in bovine blood. Veterinary Parasitology. 1993;**50**(1):69-81

[58] Mahami-Oskouei M, Dalimi A, Forouzandeh-Moghadam M, Rokni M. Molecular identification and differentiation of Fasciola isolates using PCR-RFLP method based on internal transcribed spacer (ITS1, 5.8S rDNA, ITS2). Iranian Journal of Parasitology. 2011;**6**(3):35-42

[59] El-Ashker M, Hotzel H, Gwida M, El-Beskawy M, Silaghi C, Tomaso H. Molecular biological identification of *Babesia*, *Theileria*, and *Anaplasma* species in cattle in Egypt using PCR assays, gene sequence analysis and a novel DNA microarray. Veterinary Parasitology. Elsevier BV. 2015;**207**(3–4):329-334

[60] Lopez JE, Palmer GH, Brayton KA, Dark MJ, Leach SE, Brown WC. Immunogenicity of *Anaplasma marginale* Type IV secretion system proteins in a protective outer membrane vaccine. Infection and Immunity. American Society for Microbiology. 2007;**75**(5, 5):2333-2342

[61] Carelli G, Decaro N, Lorusso a, Elia G, Lorusso E, Mari V, et al. Detection and quantification of *Anaplasma marginale* DNA in blood samples of cattle by real-time PCR. Veterinary Microbiology. 2007;**124**(1–2):107-114

[62] Sachse K. PCR detection of microbial pathogens: Specificity and performance of Diagnostic PCR assays. In: Methods in Molecular Biology. Vol. 126. Totowa, New Jersey, USA: Humana Press; 2012. pp. 3-29

[63] Maidak BL, Cole JR, Lilburn TG, Parker CT, Saxman PR, Farris RJ, et al. The RDP-II (ribosomal database project). Nucleic Acids Research. Oxford, UK: Oxford University Press. 2001;**29**(1, 1):173-174

[64] Srinivasan R, Karaoz U, Volegova M, MacKichan J, Kato-Maeda M, Miller S, et al. Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. PLoS One. Public Library of Science. 2015;**10**(2):e0117617

[65] Rajendhran J, Gunasekaran P. Microbial phylogeny and diversity: Small subunit ribosomal RNA sequence analysis and beyond. Microbiological Research. 2011;**166**(2): 99-110

[66] Scheinert P, Krausse R, Ullmann U, Söller R, Krupp G. Molecular differentiation of bacteria by PCR amplification of the 16S–23S rRNA spacer. Journal of Microbiological Methods. 1996;**26**(1):103-117

[67] Hoffmann M, Brown EW, Feng PCH, Keys CE, Fischer M, Monday SR. PCR-based method for targeting 16S-23S rRNA intergenic spacer regions among *Vibrio* species. BMC Microbiology. 2010;**10**(1):1-14

[68] Tamiozzo PJ, Estanguet AA, Maito J, Tirante L, Pol M, Giraudo JA. Detection of *Mycoplasma canadense* and *Mycoplasma californicum* in dairy cattle from Argentina. Revista Argentina de Microbiología. 2014;**46**(2):119-121

[69] Yu WL, Nielsen K. Review of Detection of Brucella sp. by Polymerase Chain Reaction. Croatian Medical Journal. Croatian Medical Schools. 2010;**51**(4):306-313

[70] Rijpens NP, Jannes G, Van Asbroeck M, Rossau R, Herman LM. Direct detection of *Brucella* spp. in raw milk by PCR and reverse hybridization with 16S-23S rRNA spacer probes. Applied and Environmental Microbiology. 1996;**62**(5):1683-1688

[71] Park H, Jang H, Kim C, Chung B, Chang CL, Park SK, et al. Detection and identification of mycobacteria by amplification of the internal transcribed spacer regions with genus- and species-specific PCR primers. Journal of Clinical Microbiology. American Society for Microbiology. 2000;**38**(11):4080-4085

[72] Sevilla IA, Molina E, Elguezabal N, Pérez V, Garrido JM, Juste RA. Detection of mycobacteria, *Mycobacterium avium* subspecies, and *Mycobacterium tuberculosis* complex by a novel tetraplex real-time PCR assay. Journal of Clinical Microbiology. 2015;**53**(3): 930-940

[73] Ríos TS, Ríos OL. Principal molecular markers used to identify *Babesia bovis* and *Babesia bigemina*. Revista MVZ Córdoba. Universidad de Córdoba, Montería, Colombia. 2011;**16** (2):2470-2483

[74] Links MG, Dumonceaux TJ, Hemmingsen SM, Hill JE. The chaperonin-60 universal target is a barcode for bacteria that enables *de novo* assembly of metagenomic sequence data. PLoS One. Public Library of Science. 2012;**7**(11):1-10

[75] Ybañez AP, Sivakumar T, Battsetseg B, Battur B, Altangerel K, Matsumoto K, et al. Specific molecular detection and characterization of *Anaplasma marginale* in Mongolian cattle. The Journal of Veterinary Medical Science. 2013;**75**(4):399-406

[76] Zeigler DR. Gene sequences useful for predicting relatedness of whole genomes in bacteria. International Journal of Systematic and Evolutionary Microbiology. 2003;**53**(6): 1893-1900

[77] Miranda KR, Neves FPG, dos Santos-Filho J, de Paula GR, Lobo LA, Oelemann WMR, et al. Application of DNA sequence analysis based on five different conserved genes (16S rDNA, rpoB, gdh, est and pgm) for intra-species discrimination of *Bacteroides fragilis*. Anaerobe. 2013;**19**:58-61

[78] Yarza P, Spröer C, Swiderski J, Mrotzek N, Spring S, Tindall BJ, et al. Sequencing orphan species initiative (SOS): Filling the gaps in the 16S rRNA gene sequence database for all species with validly published names. Systematic and Applied Microbiology. 2013;**36**(1):69-73

[79] Liu Z, Luo J, Bai Q, Ma M, Guan G, Yin H. Amplification of 16S rRNA genes of *Anaplasma* species in China for phylogenetic analysis. Veterinary Microbiology. 2005; **107**(1–2):145-148

[80] Inokuma H, Terada Y, Kamio T, Raoult D, Brouqui P. Analysis of the 16S rRNA gene sequence of *Anaplasma centrale* and Its phylogenetic relatedness to other *Ehrlichiae*.

Clinical and Diagnostic Laboratory Immunology. American Society for Microbiology. 2001;**8**(2):241-244

[81] Girotto A, Zangirolamo AF, Bogado ALG, Souza ASLE, da Silva GCF, Garcia JL, et al. Molecular detection and occurrence of "*Candidatus* Mycoplasma haemobos" in dairy cattle of Southern Brazil. Revista Brasileira de Parasitologia Veterinária. 2012;**21**(3):342-344

[82] Meli ML, Willi B, Dreher UM, Cattori V, Knubben-Schweizer G, Nuss K, et al. Identification, molecular characterization, and occurrence of two bovine hemoplasma species in Swiss cattle and development of real-time TaqMan quantitative PCR assays for diagnosis of bovine hemoplasma infections. Journal of Clinical Microbiology. 2010; **48**(10):3563-3568

[83] McAuliffe L, Lawes J, Bell S, Barlow A, Ayling R, Nicholas R. The detection of *Mycoplasma* (formerly *Eperythrozoon*) *wenyonii* by 16S rDNA PCR and denaturing gradient gel electrophoresis. Veterinary Microbiology. 2006;**117**(2–4):292-296

[84] Singh H, Jyoti HM, Singh NK, Rath SS. Molecular detection of *Anaplasma marginale* infection in carrier cattle. Ticks and Tick-Borne Diseases. Elsevier GmbH. 2012;**3**(1):55-58

[85] Torioni de Echaide S, Knowles DP, McGuire TC, Palmer GH, Suarez CE, McElwain TF. Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5. Journal of Clinical Microbiology. American Society for Microbiology. 1998;**36**(3):777-782

[86] Noaman V, Shayan P. Comparison of microscopy and PCR-RFLP for detection of *Anaplasma marginale* in carrier cattle. Iranian Journal of Microbiology. Tehran University of Medical Sciences. 2010;**2**(2):89-94

[87] Figueroa JV, Chieves LP, Johnson GS, Buening GM. Detection of *Babesia bigemina*-infected carriers by polymerase chain reaction amplification. Journal of Clinical Microbiology. 1992;**30**(10):2576-2582

[88] Markoulatos P, Siafakas N, Moncany M. Multiplex polymerase chain reaction: A practical approach. Journal of Clinical Laboratory Analysis. John Wiley & Sons, Inc. 2002; **16**(1):47-51

[89] Lee E, Kim E-J, Shin Y-K, Song J-Y. Design and testing of multiplex RT-PCR primers for the rapid detection of influenza A virus genomic segments: Application to equine influenza virus. Journal of Virological Methods. 2016;**228**:114-122

[90] Zeng Z, Liu Z, Wang W, Tang D, Liang H, Liu Z. Establishment and application of a multiplex PCR for rapid and simultaneous detection of six viruses in swine. Journal of Virological Methods. 2014;**208**:102-106

[91] Yao LN, Ruan W, Zeng CY, Li ZH, Zhang X, Lei YL, et al. Pathogen identification and clinical diagnosis for one case infected with *Babesia*. Chinese Journal of Parasitology & Parasitic Diseases. 2012;**30**

[92] Mata AI, Gibello A, Casamayor A, Blanco MM, Domínguez L, Fernández-Garayzábal JF. Multiplex PCR assay for detection of bacterial pathogens associated with warm-water Streptococcosis in Fish. Applied and Environmental Microbiology. American Society for Microbiology. 2004;**70**(5):3183-3187

[93] Picozzi K, Carrington M, Welburn SC. A multiplex PCR that discriminates between *Trypanosoma brucei brucei* and zoonotic *T. b. rhodesiense*. Experimental Parasitology. 2008; **118**(1):41-46

[94] Santín M, Zarlenga DS. A multiplex polymerase chain reaction assay to simultaneously distinguish *Cryptosporidium* species of veterinary and public health concern in cattle. Veterinary Parasitology. 2009;**166**(1–2):32-37

[95] Junlong L, Li Y, Liu A, Guan G, Xie J, Yin H, et al. Development of a multiplex PCR assay for detection and discrimination of *Theileria annulata* and *Theileria sergenti* in cattle. Parasitology Research. 2015;**114**(7):2715-2721

[96] Araujo CP, Osorio ALAR, Jorge KSG, Ramos CAN, Filho AFS, Vidal CES, et al. Detection of *Mycobacterium bovis* in bovine and bubaline tissues using nested-PCR for TbD1. PLoS One. Public Library of Science. 2014;**9**(3):e91023

[97] Bilgiç HB, Karagenç T, Simuunza M, Shiels B, Tait A, Eren H, et al. Development of a multiplex PCR assay for simultaneous detection of *Theileria annulata*, *Babesia bovis* and *Anaplasma marginale* in cattle. Experimental Parasitology. 2013;**133**(2):222-229

[98] Zarlenga DS, Barry Chute M, Gasbarre LC, Boyd PC. A multiplex PCR assay for differentiating economically important gastrointestinal nematodes of cattle. Veterinary Parasitology. 2001;**97**(3):201-211

[99] OIE WO for AH. Biotechnology in the diagnosis of infectious diseases. In: Manual for Diagnostic Test and Vaccines for Terrestrial Animals. 7th Editio ed. OIE, World Organisation for Animal Health; 2015. pp. 1322-1337

[100] Tavassoli M, Tabatabaei M, Nejad BE, Tabatabaei MH, Najafabadi A, Pourseyed SH. Detection of *Theileria annulata* by the PCR-RFLP in ticks (Acari, Ixodidae) collected from cattle in West and North-West Iran. Acta Parasitologica. 2011;**56**(1):8-13

[101] McLoughlin KS. Microarrays for pathogen detection and analysis. Briefings in Functional Genomics. 2011;**19**

[102] Tonelli A, Sacchini F, Krasteva I, Zilli K, Scacchia M, Beaurepaire C, et al. One test microbial diagnostic microarray for identification of *Mycoplasma mycoides* subsp. mycoides and other *Mycoplasma* species. Molecular Biotechnology. 2012;**52**(3):285-299