

Chapter 35

Technical Advances in Veterinary Diagnostic Microbiology

Dongyou Liu

Introduction

Forming a significant part of biomass on earth, microorganisms are renowned for their abundance and diversity. From submicroscopic infectious particles (viruses), small unicellular cells (bacteria and yeasts) to multinucleate and multicellular organisms (filamentous fungi, protozoa, and helminths), microorganisms have found their way into virtually every environmental niche, and show little restraint in making their presence felt. While a majority of microorganisms are free-living and involved in the degradation of plant debris and other organic materials, others lead a symbiotic, mutually beneficial life within their hosts. In addition, some microorganisms have the capacity to take advantage of temporary weaknesses in animal and human hosts, causing notable morbidity and mortality. Because clinical manifestations in animals and humans resulting from infections with various microorganisms are often nonspecific (e.g., general malaise and fever), it is necessary to apply laboratory diagnostic means to identify the culprit organisms for treatment and prevention purposes.

Veterinary diagnostic microbiology is devoted to the identification and detection of microorganisms that cause diseases in animals. Considering the close similarity among microorganisms causing diseases in humans and animals, many laboratory techniques that have been developed for the identification and detection, subtyping and phylogenetic analysis, virulence determination, and drug resistance assessment of human pathogens, have been thus readily adopted for the investigation of animal pathogens, or vice versa. Furthermore, apart from zoonotic pathogens that occur in both human and animals, animals of different classes and categories often have unique pathogens of their own. Therefore, veterinary diagnostic microbiology faces

D. Liu (✉)

Royal College of Pathologists of Australasia Biosecurity Quality Assurance Program,
Virology Research Laboratory, Prince of Wales Hospital, Randwick, NSW 2031, Australia
e-mail: liu@cvm.msstate.edu

even greater challenges than its medical counterpart in achieving accurate, sensitive, and rapid identification and detection of pathogenic microorganisms in animals.

In view of the fact that many human pathogens have originated/evolved from microorganisms commonly occurring in animals, accurate identification and tracking of animal pathogens are crucial for the control and prevention of zoonotic infections in human populations. The threat of zoonotic pathogens (e.g., *Bacillus anthracis*) being used in bioterrorism attacks, and the emergence of rapidly evolving antibiotic-resistant microorganisms and animal pathogens causing severe diseases in humans (e.g., SARS coronaviruses, and avian influenza viruses), have made the development and application of improved diagnostic methods for animal pathogens increasingly important.

Identification and Detection

Accurate identification and detection of pathogenic microorganisms in animals have been and will remain the primary objective for veterinary diagnostic microbiology.

Similar to its medical counterpart, veterinary diagnostic microbiology has traditionally relied on various phenotypic procedures for microbial characterization. These procedures assess the morphological, biological, biochemical, serological, in vitro and in vivo characteristics as well as other phenotypic properties of microorganisms, and have played an essential role in the identification and detection of microbial pathogens affecting humans and animals. More recently, molecular techniques have been increasingly applied for identification and detection of microbial pathogens (Table 35.1) [1].

Morphological characterization is based on the premise that various classes of microorganisms often show distinct morphological features (e.g., size, shape, internal and external components, colony morphology) which allow their initial identification upon macroscopic and microscopic examination. Application of light microscopy or transmission and scanning electron microscopy (EM) together with stains/dyes helps reveal additional morphological details. Hematoxylin and eosin (H&E), Gram, Giemsa, crystal violet stains are common stains used to enhance the contrast of microbes to their background. Additionally, Gimenez' and Pinkerton's stains are useful for detection of rickettsial organisms in tissue sections; Ziehl-Nielsen, Kinyoun, or auramine O stains for initial detection of mycobacteria; KOH, lactophenol cotton blue, India ink, and Southgate's mucicarmin stains for detection of fungi; periodic acid-Schiff (PAS), Grocott's methenamine silver (GMS), Fontana-Masson, Gridley's, and H&E stains for detection of mycotic elements in tissue biopsies. In general, morphological characterization is rapid and inexpensive, but has relatively low sensitivity and specificity, and its result interpretation is somewhat subjective. To improve the sensitivity and specificity of microscopic detection of pathogenic microorganisms (especially viruses), fluorescently labeled antibodies may be utilized. Application of highly sensitive and specific fluorescent sensor molecules in electron microscopy, fluorescence microscopy, or time-lapse microscopy

Table 35.1 Common laboratory techniques for identification and detection of microbial pathogens

Technique	Key features
Morphological	Macroscopic and microscopic examination of morphological features (e.g., size, shape, internal and external components, colony morphology) allows for rapid, inexpensive identification of microorganisms. Use of general or specialized stains/dyes further enhances the contrast of microbes to their background. Nonetheless, morphological characterization often lacks desired sensitivity and specificity
Biochemical	Examination of metabolic or enzymatic products of microorganisms (e.g., carbohydrate, protein, amino acid, fat, and enzyme) using biochemical techniques permits their discrimination at genus- and species-levels. However, the performance of biochemical tests is impacted by factors that affect microbial growth and metabolism
Serological	Detection of specific interactions between host antibodies and microbial antigens (e.g., protein or carbohydrate) by serological techniques provides indirect evidence for causal relationships between diseases and microbial pathogens. Serological tests have a relatively high sensitivity, specificity and quick turn-around time, but may show cross-reactivity with closely related microbial species
Biological, in vitro and in vivo	Assessment of biological features (e.g., host range, transmission pattern, pathological effects, geographical origin) of microorganisms helps diagnose microbial infections in cases where other relevant data are scarce In vitro culture techniques using laboratory media and cell lines facilitate isolation and propagation of target microorganisms for subsequent morphological, biochemical, serological, and molecular characterization In vivo assays using laboratory animals and chicken embryos allow for recovery of microorganisms that fail to grow on culture media or cell lines, and help determine host susceptibility and immune response to, and pathogenic effects of microorganisms
Molecular	Detection of nucleic acids using molecular techniques offers direct evidence on the presence of microorganisms. Application of nucleic acid amplification technologies further improves the speed, sensitivity, and specificity of microbial identification and detection

has further enhanced morphological characterization of microorganisms. Besides unraveling paradigms of pathogen entry and pinpointing the exact intracellular location, these new techniques permit direct monitoring of the intracellular lifestyle of microbial pathogens and yield insights into the underlying mechanisms of their pathogenicity [2, 3]. Furthermore, atomic force microscopy (AFM) techniques offer a powerful platform for analyzing the structure, properties and functions of microbial pathogens as well as the localization, mechanics, and interactions of the individual cell wall constituents, contributing to the elucidation of the molecular bases of cell adhesion (nanoadhesome) and mechanosensing (nanosensosome) [4, 5].

Biochemical characterization focuses on the metabolic or enzymatic products of microorganisms, including distinct patterns of carbohydrate, protein, amino acid, fat metabolisms and production of particular enzymes. Biochemical tests are often conducted to distinguish between aerobic and anaerobic breakdown of carbohydrates, to show carbohydrates that can be attacked, to detect specific breakdown products of

carbohydrates (e.g., the formation of acids, alcohols and gases when grown in selective liquid or solid media), to determine the ability of carbohydrates to utilize substrates such as citrate and malonate, to examine the metabolism of protein and amino acids (e.g., gelatine liquefaction, indole production, amino acid decarboxylase test, and phenylamine deaminase test) as well as of fats (e.g., hydrolysis of tributyrin), and detect production of enzymes (e.g., catalase test, oxidase test, urease test, ONPG test, and nitrate reduction). Assessment of fungal primary metabolites such as ubiquinones (coenzyme Q) is useful for the taxonomy of black yeasts and filamentous fungi, whereas examination of fungal secondary metabolites (e.g., steroids, terpenes, alkaloids, cyclopeptides, and coumarins) by chromatographic techniques provides another means for fungal identification. A recent approach for biochemical characterization of microorganisms centers on the characteristic outer surface charges of microbes that contribute to their distinct migration under a direct-current electric field such as capillary electrophoresis (CE), leading to rapid and efficient separation, identification, quantitation, and characterization of intact microorganisms (i.e., bacteria, viruses, and fungi) [6]. Another useful technique for biochemical characterization of microbes is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), which has been shown to be useful for specific identification of *Francisella* and other microbial pathogens [7].

Serological characterization on the basis of specific reactions between host antibodies and microbial antigens (usually protein or carbohydrate) provides highly sensitive, specific, and rapid identification of microorganisms. Detection of rising levels of specific IgA, IgM, and IgG antibody titers or seroconversion in blood, urine, and fecal materials offers indirect evidence for causal relationships between diseases and microbial pathogens [8]. An interesting development in the serological characterization of disease-causing microorganisms is the use of chemically synthesized peptides. Generated by chemical approaches, these peptides are composed of two or more amino acids linked together by peptide bonds. By mimicking naturally occurring peptides or segments of proteins, these peptides serve as synthetic antigens in peptide microarrays as potential diagnostic tools in high-throughput immunoassays [9]. Other new developments in serological characterization of microorganisms include biosensors and nanotechnology (nanoarrays and nanochips). Biosensors involve the use of a microbe-specific antibody and a transducer (e.g., electrochemistry, reflectometry, interferometry, resonance, and fluorimetry) to convert a biological interaction into a measurable signal. In the particle concentration fluorescence immunoassay (PCFIA) for brucellosis, submicron polystyrene particles are coated with antigen and placed in a 96-well vacuum plate. After addition of fluorescent conjugate followed by vacuum filtration to remove unbound conjugate, the total particle-bound fluorescence is measured by front surface fluorimetry. Nanotechnology (nanoarrays and nanochips) offers small scale platforms to identify an array of infectious agents or serotypes on a single chip.

Biological characterization focuses on the issues related to the host susceptibility, transmission patterns, pathological effect(s), and geographical origin of microbial pathogens, which are critical in helping achieve correct diagnosis of microbial infections in cases where other relevant data are scarce [10]. In vitro isolation and

propagation on laboratory media and cell lines offers a valuable tool for identification and diagnosis of microbial infections. The size, color, shape and form of colonies formed by microorganisms on nutritional agar and other selective media are diagnostically informative. In case of viral pathogens, the formation of a region of dead cells resulting from viral growth (called a “plaque”) suggests their cytopathogenic effects (CPE). Parasitic protozoa may also be cultivated as a means of identification [11]. However, because not all microorganisms will grow in laboratory media and cell lines, embryonated eggs, insect vector, and laboratory animals (e.g., rodents) may be utilized. For example, *Trypanosoma cruzi*, the causal agent for Chagas disease, is grown in the guts of its vector triatomine bug for confirmation and diagnosis. The availability of cultured isolate/strain permits further antigenic studies, antibiotic susceptibility testing, and genetic studies. Despite their relatively high expense and length of time required, in vitro and in vivo techniques have contributed to the studies of microbial taxonomy, biology, epidemiology, pathogenesis, and treatment response. A recent development in the use of in vivo techniques for microbial characterization relates to the in vivo bioluminescence imaging or biophotonic imaging (BPI). Based on genetically engineered bioluminescent/fluorescent microorganisms, this technique enhances the study of microbial infections and host immune responses [12]. Application of genetically engineered mice with luciferase reporters for specific microbial or host genes helps overcome the limitations of in vivo bioluminescence imaging for assessment of microbial replication, activation of key genes in host immunity, and response to tissue damage in vivo [13].

Because of their time-consuming, occasionally variable nature, and/or their limited sensitivity and specificity, phenotypic approaches (e.g., morphological, biochemical, serological, and biological characterization) to the identification of microorganisms are increasingly supplemented with molecular techniques. Progresses in the areas of genetic target selection, template preparation, transition from nonamplified to amplified approaches, and product detection over the past two decades have made molecular methods an indispensable tool in the laboratory diagnosis of microbial pathogens in veterinary medicine [1].

With regard to the selection of genetic targets, the following three types may be considered: nonspecific, shared, and specific genetic targets. Nonspecific genetic targets include the guanine and cytosine composition (or G+C content), short random primer sites, randomly dispersed repetitive extragenic palindromes (REP), enterobacterial repetitive intergenic consensus sequences (ERIC), variable-number tandem repeats (VNTR) (also known as simple sequence repeats (SSRs), or microsatellites), restriction enzyme sites, and so on. Shared genetic targets include ribosomal RNA (rRNA) genes (e.g., 16/18S rRNA, 23/25/28S rRNA), internal transcribed spacer (ITS) regions, mitochondrial DNA (mtDNA), and housekeeping genes, etc. Specific genes are uniquely present and represent ideal targets for the identification of pathogenic bacteria, fungi and parasites.

Preparation of nucleic acid templates from cultured isolates and clinical specimens represents an important initial step for molecular identification and detection of microorganisms. This often involves (1) disruption of cell walls, (2) denaturation of nucleoprotein complexes, (3) inactivation of endogenous DNase/RNase, and

(4) removal of contaminating proteins, polysaccharides, polyphenolic pigments, and other compounds [14]. While enzymatic digestion (e.g., using lyticase, zymolase, chitinase, glucucase, and/or proteinase K) and occasionally acid and alkali treatments may be effective for breaking up bacterial and yeast cells, mechanical grinding, sonication or bead-beating is often necessary to disrupt the mycelial and helminth cell walls. Following extraction with organic solvents (e.g., phenol/chloroform) and detergents (e.g., sodium dodecyl sulfate, SDS; hexadecyltrimethylammonium bromide, CTAB; and *N*-lauroylsarcosine), which denatures cytosolic proteins and lipid membranes and inactivates endogenous DNase/RNase, nucleic acids of high purity are obtained after precipitation with ethanol or isopropanol. The recent development of various easy-to-use commercial kits has negated the need to use hazardous organic solvents in the isolation of microbial DNA/RNA. Furthermore, automated nucleic acid extraction systems have become increasingly sophisticated and affordable, contributing to the streamlining of template preparation and reduction of potential cross-contamination during manual handling.

The early generation molecular procedures rely on nonamplified, hybridization approaches, such as DNA–DNA hybridization (for estimation of guanine–cytosine ratio or G-C content), and use of gene probes in dot blot, Southern blot, and fluorescence in situ hybridization (FISH), etc. [15]. A more recent development in the DNA hybridization-based approach is DNA microarray, in which high-density oligonucleotide probes (or segments of DNA) are immobilized on a solid surface, and used to hybridize (catch) any complementary sequences (labeled with fluorescent nucleotides) in a test sample. Subsequent detection and quantification of fluorescence signal permits identification and determination of the relative abundance of nucleic acid sequences in a sample [16]. Although these nonamplified procedures have adequate specificity, they are relatively insensitive, often requiring large quantity of starting materials for reliable detection. Nonetheless, some of these nonamplified techniques remain valuable for comparison of microbial genomes and for identification of species- and virulence-specific gene regions. For example, dot blot hybridization was employed for screening genomic DNA libraries of *Dichelobacter nodosus* strains causing virulent and benign footrot, and several virulent- and benign-specific gene regions for subsequent differentiation of virulent and avirulent *D. nodosus* strains were identified as a result [17, 18]. This approach was also applied for identification of novel virulence-specific gene regions in zoonotic bacterial pathogen *Listeria monocytogenes* and novel species-specific gene in animal bacterial pathogen *Listeria ivanovii* [19, 20].

The mid-1980s witnessed the advent of a novel, highly efficient in vitro nucleic acid amplification technique known as polymerase chain reaction (PCR). This technique has the capacity to synthesize billions of copies from a single nucleic acid template within 3–4 h, and demonstrates superior sensitivity, exquisite specificity, rapid turnover time and amenableness to automation for high throughput testing. Since then, PCR and its variants (e.g., nested PCR, multiplex PCR, real-time PCR, quantitative PCR, reverse transcription PCR (RT-PCR), arbitrarily primed PCR (AP-PCR), random amplified polymorphic DNA (RAPD), degenerate oligonucleotide primed PCR (DOP-PCR), sequence-independent single primer amplification (SISPA), and rolling circle amplification (RCA)) have been widely applied in research and clinical laboratories for identification and phylogenetic analysis of microorganisms

[21–24]. Apart from PCR, other nucleic acid amplification procedures include nucleic-acid-sequence-based amplification (NASBA), ligase chain reaction (LCR), strand displacement amplification, Q- β replicase-mediated amplification, linear-linked amplification, and loop-mediated isothermal amplification (LAMP), etc.

Conventional methods for detection of nucleic acid products are based on electrophoretic separation followed by staining with ethidium bromide, gelstar, or SYBR Green. Whereas agarose gel electrophoresis provides a convenient, inexpensive way for separation and semiquantitation of DNA and RNA, polyacrylamide gel electrophoresis (PAGE) is useful for separating small nucleic acid fragments (<500 bp). Among the various PAGE-based procedures, single strand conformational polymorphism analysis (SSCP), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are widely applied. SSCP is capable of detecting single nucleotide variations, and in combination with capillary electrophoresis (CE), SSCP-CE provides an automated system for rapid separation of nucleic acid products. Recent advances in instrument automation and fluorescent dye chemistry permit real-time monitoring of PCR amplicons (so-called real-time PCR). Besides the use of double-stranded DNA intercalating dye (e.g., SYBR Green), specifically designed probes such as hydrolysis dual-labeled probes (TaqMan[®]), hybridization probes (LightCycler), molecular beacons, peptide nucleic acid (PNA) probes, TaqMan minor groove binding (MGBTM) probes, locked nucleic acid (LNA[®]) primers and probes, and scorpionsTM may be utilized [25].

Other nucleic acid detection approaches include DNA microarray (also known as DNA chip, gene or genome chip, or gene array), biochips (biosensors), line probe assay (LiPA), enzymatic signal amplification (e.g., ELISA and flow cytometry), and DNA sequencing. Biochips (biosensors) are small analytical devices designed for nucleic acid-based electrical/optic detection (fluorescence or chemiluminescence) [26]. DNA sequencing analysis provides a most accurate way to determine the identity of microbial organisms. While the classic Sanger method (also known as “chain termination method” or “dideoxy sequencing”) can read up to 900 bp per and produce 100 kb of sequence data per run, the “next generation sequencing” technologies (e.g., the 454 pyrosequencing-based instrument (Roche Applied Sciences), genome analysers (Illumina), and the SOLiD system (Applied Biosystems)) show improved efficiency for DNA sequencing analysis. Although Illumina and 454 sequencing technologies read 76–106 bp and 250–400 bp, they have the capacity to generate 20 Gb and 400 Mb of sequence data per run, respectively.

Subtyping and Phylogenetic Analysis

Microbial pathogens are noted for the diversity and their ability to adapt and survive in challenging environments. The ability to identify and track microbial strains and varieties involved in disease outbreaks is crucial for their control and prevention. For this reason, a number of phenotypic and molecular procedures have been developed and applied for subtyping and phylogenetic analysis of microbial strains and varieties causing animal diseases (Table 35.2) [27].

Table 35.2 Common laboratory techniques for typing and phylogenetic analysis of microbial pathogens

Technique	Key features
Biotyping	Biotyping separates microbial strains into “biotypes” on the basis of their metabolic and enzymatic activities (e.g., sugar fermentation, amino acid decarboxylation/deamination, urease activity, hydrolysis of compounds, hemagglutination, and hemolysis), colonial morphology, and environmental tolerances (e.g., tolerance to pH, chemicals, dyes, and heavy metals). Biotyping is generally reproducible and easy to perform and interpret. However, it has poor discriminatory power due possibly to variation in gene expression and point mutation
Phage typing	Phage typing distinguishes microbial strains into “phage types” by their patterns of resistance or susceptibility to a standard set of bacteriophages, depending on the presence or absence of particular receptors on the bacterial surface for phage (virus) binding. Phage typing shows good reproducibility, discriminatory power and ease of interpretation, but requires maintenance of biologically active phages and demands technical skills. In addition, many strains are nontypeable
Serotyping	Serotyping differentiates microbial strains into serotypes (serovars) according to the antigenic variations present on the surface structures (e.g., lipopolysaccharides, membrane proteins, capsular polysaccharides, flagella and fimbriae). Agglutination, latex agglutination, coagglutination, or fluorescent and enzyme labeled assays may be used for serotyping. Serotyping has good reproducibility, and ease of interpretation and performance. However, serotyping depends on the availability of good quality reagents, and some autoagglutinable (rough) strains are nontypeable. Additionally, the technique tends to have poor discriminatory power due to cross-reactive antigens
Bacteriocine typing	Bacteriocine typing assesses microbial strains for their susceptibility to a set of bacterial peptides (bacteriocine), and has been employed to type stains of <i>Pseudomonas aeruginosa</i> , <i>Escherichia coli</i> , and <i>Yersinia pestis</i> , etc. The technique has good reproducibility, discriminatory power, and ease of interpretation, but it is technically demanding and many strains are nontypeable
Multilocus enzyme electrophoresis (MLEE) typing	MLEE typing separates strains into “electromorphs” (typically reflecting amino acid substitution that alters the charge of the protein) in accordance with their distinct electrophoretic mobilities of a set of metabolic enzymes. The technique has excellent reproducibility and ease of interpretation, but shows moderate discriminatory power, and requires expensive equipments
Antibiogram typing	Antibiogram typing compares different microbial isolates in their susceptibility to a set of antibiotics. The technique has ease of performance and interpretation and reasonable reproducibility. However, it has poor discriminating power
Restriction endonuclease analysis (REA) or restriction fragment length polymorphism (RFLP)	Digestion of chromosomal DNA with certain restriction endonuclease produces various fragments whose number and sizes (from 0.5 to 50 kb) are distinct among microbial strains and varieties. This technique has good reproducibility, but generates complex profile of hundreds of bands that may be difficult to interpret

(continued)

Table 35.2 (continued)

Technique	Key features
Pulse-field gel electrophoresis (PFGE)	Based on restriction fragment length polymorphism (RFLP), PFGE uses selected restriction enzymes to yield between 8 and 25 large DNA bands of 40–600 kb in size, and alternating currents to cause DNA fragments to move back and forth, resulting in a higher level of resolution of large fragments. This technique has good reproducibility, and ease of interpretation. However, it requires costly reagents and equipment
Ribotyping	Ribotyping uses a ribosomal RNA (rRNA) probe derived from the <i>Escherichia coli</i> to detect the restriction fragment patterns of 16S rRNA, 23S rRNA, and tRNAs after digestion of chromosomal DNA with appropriate restriction enzymes. Microorganisms are classified as separate species if their sequences show <98 % homology and are classified as different genera if their sequences show <93 % identity. As a derivative of RFLP, this technique is reproducible and is easy to interpret. However, it requires costly reagents and equipment
Amplified fragment length polymorphism (AFLP)	AFLP is a modification of RFLP through the addition of adaptors to restriction enzyme-digested DNA followed by PCR amplification and electrophoretic separation of PCR products, generating highly informative, polymorphic patterns of 40–200 bands for individual microbial strains. An obvious shortcoming of AFLP is its requirement for the ligation of linkers and indexers to enzyme-digested DNA from individual strains
PCR-restriction fragment length polymorphism (PCR-RFLP)	PCR-RFLP involves PCR amplification of one or more microbial housekeeping or virulence-associated genes followed by digestion with selected restriction enzymes and separation by agarose gel electrophoresis. The resultant band patterns allow differentiation of microbial subtypes. The technique obviates the need to ligate linkers and indexers before PCR amplification (as in AFLP), and represents a sensitive, discriminatory, and reproducible method for tracking and epidemiological investigation of microbial strains and varieties
Multilocus sequence typing (MLST)	In MLST, multiple DNA segments are amplified by PCR and examined by DNA sequencing analysis, leading to phylogenetic comparison of multiple isolates, and definitive identification of microbial strains and subtypes. MLST is reliable and easy to interpret
Mobile genetic element-PCR (MGE-PCR)	MGE-PCR uses a single primer in PCR to amplify particular MGEs followed by electrophoresis to discriminate amplicon profiles. This technique has been utilized to characterize different isolates of <i>Trypanosoma brucei</i> by targeting RIME which has a relatively high copy number in the genome

The common phenotypic subtyping procedures include biotyping, phage typing, serotyping, bacteriocine typing, multilocus enzyme electrophoresis (MLEE) typing, and antibiogram typing (Table 35.2). The genotypic subtyping (fingerprinting) approach targets the microbial chromosome and plasmid DNA such as their composition, homology, and presence or absence of specific genes. The genotypic subtyping techniques consist of two categories: nonamplified techniques (e.g., restriction endonuclease analysis (REA) or restriction fragment length polymorphism

(RFLP), pulse-field gel electrophoresis (PFGE), and ribotyping) and amplified techniques (e.g., amplified fragment length polymorphism (AFLP), PCR-restriction fragment length polymorphism (PCR-RFLP), and multilocus sequence typing (MLST), and mobile genetic element-PCR (MGE-PCR)) (Table 35.2) [28].

Virulence Determination

Many microbial species encompass a diversity strains with varied virulence potential. The availability of laboratory techniques to accurately assess the pathogenic potential of these microorganisms is vitally important to their control and prevention. For example, gram-negative bacterium *Dichelobacter nodosus* harbors strains that cause virulent, intermediate or benign footrot in sheep. As virulent and some intermediate footrot induces lameness and severe pain in affected sheep, leading to ill-thrift and reduced weight gain, it is necessary to apply control measures to stem the economic losses. On the other hand, benign footrot causes minimal harm to affected sheep and has the tendency to self-cure, it is unnecessary and indeed wasteful to treat benign footrot. Traditionally, the virulence of *D. nodosus* strains is determined by elastase test and gelatin gel test, which may take up to 4 weeks to complete, and often demonstrate notable variability. After comparative analysis of recombinant DNA libraries from *D. nodosus* virulent and benign strains, a panel of virulent- and benign-specific genes was identified. Use of gene probes and primers derived from these genes facilitate rapid and sensitive determination of *D. nodosus* virulence [17, 18].

Gram-positive bacterium *L. monocytogenes* is a zoonotic pathogen that encompasses a spectrum of strains with various pathogenic inclination. While some *L. monocytogenes* strains are highly pathogenic and sometimes deadly, others are relatively avirulent and cause little harm in the host. The current laboratory techniques for assessing the virulence of *L. monocytogenes* strains include the mouse virulence assay and in vitro cell assays. While the mouse virulence assay is capable of providing an in vivo measurement of all virulent determinants, its high expense limits its application. Representing a low-cost alternative to the mouse virulence assay for assessing *L. monocytogenes* virulence, in vitro cell culture techniques measure the ability of *L. monocytogenes* to cause cytopathogenic effects in the enterocyte-like cell line Caco-2, to form plaques in the human adenocarcinoma cell line HT-29, or to cause death in chicken embryos. Several other cell lines (e.g., hepatocyte Hep-G2, macrophage-like J774, epithelial Henle 407 and L2) are also useful for studies on *L. monocytogenes* ability to adhere, invade, escape from vacuoles, grow intracellularly and spread to neighboring cells. However, these techniques are time-consuming, and occasionally variable. Following recent identification of novel virulence-specific genes (e.g., *inlJ*), the virulence of *L. monocytogenes* strains can be rapidly and specifically determined by PCR [19, 29].

Drug Resistance Assessment

Microorganisms have the ability to acquire resistance to drugs that are used for their treatment. As drug are often used in animals (e.g., such as cows, pigs, chickens, fish, etc.) that provide an important source of human food, microorganisms exposed to these drugs can develop antibiotic resistance through horizontal gene transfer events (e.g., conjugation, transduction, or transformation) and point mutations [30]. The resistant bacteria in animals due to antibiotic exposure can be transmitted to humans through the consumption of meat, from close or direct contact with animals, or through the environment. For example, use of fluoroquinolone in poultry production has been linked to the emergence of fluoroquinolone resistant campylobacter infections in humans. Some bacteria (e.g., *Staphylococcus aureus*, enterococci, gonococci, streptococci, salmonella, and *Mycobacterium tuberculosis*) have developed multidrug resistance [31]. For example, methicillin-resistant *S. aureus* (MRSA) are resistant to non-beta-lactam antimicrobial drugs as well. Identification of MRSA has implications not only for treatment of infected animals, but for potential zoonotic transmission [32, 33]. While application of in vitro culture technique facilitates determination of MIC (medium inhibition concentration) of the strains, detection of specific gene mutations provides an alternative approach for assessment of antimicrobial drug resistance in microorganisms such as *B. hyodysenteriae* [34].

Conclusion

Given the diversity of animal hosts that are susceptible to a wide variety of microbial infections, veterinary diagnostic microbiology faces a greater challenge than its medical counterpart in achieving a correct and timely identification of culprit microorganisms causing significant economic losses in agricultural production. Although phenotypic procedures are useful for microbial identification, their time-consuming nature and occasional variability have provided the impetus for the development of nucleic acid detection methodology. With a high sensitivity, exquisite specificity and speed, molecular procedures especially those involving nucleic acid amplification (e.g., PCR) have been widely adopted in clinical and research laboratories for identification, typing, virulence determination, and drug resistance assessment of microorganisms of veterinary and medical importance. Further improvement through miniature and multiplexing will help reduce the cost of conducting molecular testing in diagnostic microbiology.

References

1. Rao SS, Mohan KV, Atreya CD (2010) Detection technologies for *Bacillus anthracis*: prospects and challenges. *J Microbiol Methods* 82:1–10

2. Ray K, Bobard A, Danckaert A et al (2010) Tracking the dynamic interplay between bacterial and host factors during pathogen-induced vacuole rupture in real time. *Cell Microbiol* 12:545–556
3. Bobard A, Mellouk N, Enninga J (2011) Spotting the right location-imaging approaches to resolve the intracellular localization of invasive pathogens. *Biochim Biophys Acta* 1810: 297–307
4. Dupres V, Alsteens D, Andre G, Dufrêne YF (2010) Microbial nanoscopy: a closer look at microbial cell surfaces. *Trends Microbiol* 18:397–405
5. Alsteens D, Dupres V, Andre G, Dufrêne YF (2011) Frontiers in microbial nanoscopy. *Nanomedicine (Lond)* 6:395–403
6. Simonet BM, Ríos A, Valcárcel M (2008) Capillary electrophoresis separation of microorganisms. *Methods Mol Biol* 384:569–590
7. Seibold E, Maier T, Kosrzewa M, Zeman E, Spletstoeser W (2010) Identification of *Francisella tularensis* by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry: fast, reliable, robust, and cost-effective differentiation on species and sub-species levels. *J Clin Microbiol* 48:1061–1069
8. Prickett JR, Zimmerman JJ (2010) The development of oral fluid-based diagnostics and applications in veterinary medicine. *Anim Health Res Rev* 11:207–216
9. Saravanan P, Kumar S (2009) Diagnostic and immunoprophylactic applications of synthetic peptides in veterinary microbiology. *Microbiol Res* 1:1e
10. Houpiikian P, Raoult D (2002) Traditional and molecular techniques for the study of emerging bacterial diseases: one laboratory's perspective. *Emerg Infect Dis* 8:122–131
11. Wilkins W, Raji A, Parker S et al (2010) Examining heterogeneity in the diagnostic accuracy of culture and PCR for *Salmonella* spp. in swine: a systematic review/meta-regression approach. *Zoonoses Public Health* 57:S121–S134
12. Andreu N, Zelmer A, Wiles S (2011) Noninvasive biophotonic imaging for studies of infectious disease. *FEMS Microbiol Rev* 35:360–394
13. Luker KE, Luker GD (2010) Bioluminescence imaging of reporter mice for studies of infection and inflammation. *Antiviral Res* 86:93–100
14. Liu D (2008) Preparation of *Listeria monocytogenes* specimens for molecular detection and identification. *Int J Food Microbiol* 122:229–242
15. Gibson W (2009) Species-specific probes for the identification of the African tsetse-transmitted trypanosomes. *Parasitology* 136:1501–1507
16. Banér J, Gyarmati P, Yacoub A et al (2007) Microarray-based molecular detection of foot-and-mouth disease, vesicular stomatitis and swine vesicular disease viruses, using padlock probes. *J Virol Methods* 143:200–206
17. Liu D (1994) Development of gene probes of *Dichelobacter nodosus* for differentiating strains causing virulent, intermediate or benign ovine footrot. *Br Vet J* 150:451–462
18. Liu D, Webber J (1995) A polymerase chain reaction assay for improved determination of virulence of *Dichelobacter nodosus*, the specific causative pathogen for ovine footrot. *Vet Microbiol* 43:197–207
19. Liu D, Ainsworth AJ, Austin FW, Lawrence ML (2003) Characterization of virulent and avirulent *Listeria monocytogenes* strains by PCR amplification of putative transcriptional regulator and internalin genes. *J Med Microbiol* 52:1065–1070
20. Liu D, Ainsworth AJ, Austin FW, Lawrence ML (2004) PCR detection of a putative N-acetylmuramidase gene from *Listeria ivanovii* facilitates its rapid identification. *Vet Microbiol* 101:83–89
21. Drozd M, Kassem II, Gebreyes W, Rajashekara G (2010) A quantitative polymerase chain reaction assay for detection and quantification of *Lawsonia intracellularis*. *J Vet Diagn Invest* 22:265–269
22. Tarlinton RE, Dunham SP (2011) Pushing the envelope: advances in molecular techniques for the detection of novel viruses. *Vet J* 190:185–186

23. Tasker S (2010) The polymerase chain reaction in the diagnosis of infectious diseases. *Vet Clin Pathol* 39:261–262
24. Bexfield N, Kellam P (2011) Metagenomics and the molecular identification of novel viruses. *Vet J* 190:191–198
25. Clothier KA, Jordan DM, Thompson CJ, Kinyon JM, Frana TS, Strait EL (2010) *Mycoplasma bovis* real-time polymerase chain reaction assay validation and diagnostic performance. *J Vet Diagn Invest* 22:956–960
26. Gabig-Ciminska M (2006) Developing nucleic acid-based electrical detection systems. *Microb Cell Fact* 5:9
27. Belák S, Thorén P, LeBlanc N, Viljoen G (2009) Advances in viral disease diagnostic and molecular epidemiological technologies. *Exp Rev Mol Diagn* 9:367–381
28. Johansson A, Petersen JM (2010) Genotyping of *Francisella tularensis*, the causative agent of tularemia. *J AOAC Int* 93:1930–1943
29. Liu D, Lawrence ML, Austin FW, Ainsworth AJ (2007) A multiplex PCR for species- and virulence-specific determination of *Listeria monocytogenes*. *J Microbiol Methods* 71:33–40
30. Weese JS (2010) Methicillin-resistant *Staphylococcus aureus* in animals. *ILAR J* 51:233–244
31. Morgan M (2008) Methicillin-resistant *Staphylococcus aureus* and animals: zoonosis or humanosis? *J Antimicrob Chemother* 62:1181–1187
32. Cohn LA, Middleton JR (2010) A veterinary perspective on methicillin-resistant staphylococci. *J Vet Emerg Crit Care (San Antonio)* 20:31–45
33. Smout MJ, Kotze AC, McCarthy JS, Loukas A (2010) A novel high throughput assay for anthelmintic drug screening and resistance diagnosis by real-time monitoring of parasite motility. *PLoS Negl Trop Dis* 4:e885
34. Hidalgo A, Carvajal A, Vester B, Pringle M, Naharro G, Rubio P (2011) Trends towards lower antimicrobial susceptibility and characterization of acquired resistance among clinical isolates of *Brachyspira hyodysenteriae* in Spain. *Antimicrob Agents Chemother* 55:3330–3337