CURATED COLLECTION

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Molecular Epidemiology of Infectious Zoonotic and Livestock Diseases*

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ABSTRACT Zoonotic and livestock diseases are very important globally both in terms of direct impact on human and animal health and in terms of their relationship to the livelihood of farming communities, as they affect income generation and food security and have other, indirect consequences on human lives. More than two-thirds of emerging infectious diseases in humans today are known to be of animal origin. Bacterial, viral, and parasitic infections that originate from animals, including hypervirulent and multidrug-resistant (MDR) bacterial pathogens, such as livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA), invasive nontyphoidal *Salmonella* of animal origin, hyperviruent *Clostridium difficile*, and others, are of major significance to public health. Understanding the origin, risk factors, transmission, prevention, and control of such strains has been a challenge for various reasons, particularly due to the transdisciplinary partnership between and among human, environment, and animal health sectors. MDR bacteria greatly complicate the clinical management of human infections. Food animal farms, pets in communities, and veterinary hospital environments are major sources of such infections. However, attributing such infections and pinpoint sources requires highly discriminatory molecular methods as outlined in other parts of this curated series. Genotyping methods, such as multilocus sequence typing, pulsed-field gel electrophoresis, restriction fragment length polymorphism, and several others, have been used to decipher sources of foodborne and other zoonotic infectious diseases. In recent years, whole-genome-sequence-based approaches have been increasingly used for molecular epidemiology of diseases at the interface of humans, animals, and the environment. This part of the series highlights the major zoonotic and foodborne disease issues. *This article is part of a curated collection.

INTRODUCTION

Molecular epidemiology and its applications to veterinary medicine and zoonotic diseases began in early 1970s. In 1973, using molecular biology tools available at the time, Edwin Kilbourne reported on the investigation of the 1918 influenza pandemic (also known as Spanish flu) that linked swine and human type A influenza virus H1N1 (1). This approach opened the door for a wider application of molecular biology tools to address population health problems in various animal health,

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zoonotic, and foodborne diseases. Molecular biology techniques that evolved from DNA restriction digestion analysis, hybridization, and amplification of genes by PCR to targeted gene sequencing and, most recently, high-throughput whole-genome sequencing (WGS) have become indispensable tools in epidemiologic investigations of veterinary and livestock infectious diseases.

As mentioned in Part 1 of this curated collection series, it should be noted that molecular epidemiology is one evolutionary step in progression of the epidemiology discipline and not a distinct discipline in itself. Molecular biology tools provide additional information useful to make epidemiological decisions that classical epidemiology methods (descriptive or analytic) cannot, whether for investigating outbreaks, establishing surveillance systems, or performing rapid diagnostic assays. The methods complement statistics and other approaches to more accurately decipher and respond to epidemiologic issues in a timely manner. The reader should also be mindful that genotyping of microbes is not the same as molecular epidemiology. Genotyping methods refer to a set of molecular biology tools used to characterize microbes, also commonly referred to as "DNA fingerprinting," Genotyping is a type of taxonomic classification method (strain classification). In order for an investigation to be epidemiologic in nature, it needs to fulfill the classical epidemiology dimensions of addressing etiologic agents and their hosts in their environment, disease distribution and determinants of disease distribution or transmission by time and place, and disease prevention and control, all at the population level.

This chapter highlights key veterinary medicine-associated issues focusing on contemporary priority zoonotic disease problems. It provides examples of applications of genotyping methods and how such methods are used to characterize epidemiology of leading global veterinary and public health issues such as multidrug-resistant (MDR) organisms, emerging and endemic viral diseases, and highly endemic and often neglected parasitic zoonotic diseases. It demonstrates that all of the principles of molecular epidemiology discussed in the previous parts of this series also apply to investigating zoonotic and livestock diseases.

OVERVIEW OF MAJOR ZOONOTIC AND LIVESTOCK DISEASES

Zoonotic and livestock diseases are very important globally both in terms of direct impact on human and animal health and in terms of their relationship to the livelihood of farming communities, as they affect income generation and food security and have other, indirect consequences on human lives. Below, we highlight some examples of leading bacterial and viral diseases of significance in livestock and public health. While we focus mainly on current emerging and reemerging issues, the reader should note that these are meant to be examples and models.

Hypervirulent *Clostridium difficile*

One of the increasingly important community-acquired infections in humans is *Clostridium difficile*-associated diarrhea (CDAD), particularly those cases caused by hypervirulent strains of *C. difficile*. *Clostridium* spp. are anaerobic spore-forming organisms. The spores are known to be present and survive in various environmental conditions. Once ingested, the spores survive the stomach acid pH and convert to vegetative forms in the small intestine after exposure to bile salts. As any anaerobic organisms do, *C. difficile* establishes itself in the lowest-oxygen-tension area of the large intestine (2). In a typical healthy adult animal or human, it does not cause any infection and survives symbiotically in the colon. However, when an infection is caused by a hypervirulent strain that produces high levels of enterotoxin and cytotoxins, it leads to colitis. This condition is common in animals, including horses, dogs, and some food animals.

In humans, CDAD is typically marked by pseudomembraneous colitis. CDAD has traditionally been associated with nosocomial infections, mainly in patients with underlying medical conditions exposed to oral antimicrobial drugs or proton pump inhibitors and in the elderly. However, the recent outbreaks of community-acquired infections caused by hypervirulent strains reported in Europe and North America indicate that that they do not conform with the classical risk factors listed above. Hypervirulent *C. difficile* has been reported to be among the leading causes of community-acquired infections that potentially include food and environmental sources (3). Understanding the origin, risk factors, and transmission of hypervirulent *C. difficile* has been a challenge in the last decade. One hypothesis is that hypervirulent strains have livestock reservoirs as a source of infections for humans. Several molecular epidemiology studies using various genotyping approaches have been conducted to provide or disprove this hypothesis, and they are highlighted in the next section.

MDR *Salmonella*

Despite major advances and improvements in hygiene, quality of food and water, and detection of foodborne pathogens, *Salmonella* remains the leading cause of

foodborne diseases worldwide. *Salmonella enterica* is one of the most important causes of bacterial foodborne illness in humans, with over 90 million cases worldwide each year (4, 5). In the United States, there are approximately 1 million cases of nontyphoidal salmonellosis reported every year (6). According to a European Union (EU) report, 88,715 human salmonellosis cases were confirmed from EU countries in 2014 (7). The outbreaks of *Salmonella* infection in humans are commonly associated with the consumption of contaminated food products of animal origin.

Antimicrobial agents are commonly used for disease treatment and prevention in humans and livestock animals. The total of global antimicrobial drug consumption in livestock animals, including pigs, poultry, and cattle, was approximately 63,151 tons in 2010, and it will increase up to 67% by 2030 (8). In the United States, approximately 14.9 million kilograms of total sales and distribution of antimicrobial drugs approved for food-producing animals (domestic and export data) was reported in 2013 (9). In Asia in 2010, China and India were ranked among the top five users of antimicrobial drugs in food animal production in the world (8). The extensive or indiscriminate use of antimicrobial drugs in livestock animals has contributed to increased frequency of antimicrobial resistance (AMR) in *Salmonella* and other bacterial pathogens through their selective pressure. Furthermore, the situation of AMR in *Salmonella enterica* is getting more serious because this pathogen has developed resistance to multiple antimicrobial agents. These MDR bacteria (resistant to three or more classes of antimicrobial drugs) greatly complicate the clinical management of human salmonellosis. In addition, the dissemination of AMR *Salmonella* through distribution of contaminated retail meat products is a global public health concern.

The AMR mechanisms associated with either chromosomes or plasmids play an important role in the spread of MDR *Salmonella*. Several plasmid families carry antibiotic resistance genes such as bla_{TEM} and $bla_{\text{CTX-M}}$, conferring resistance to β-lactam antibiotics, and *qnr* genes, conferring resistance to quinolones (10). These resistance genes spread by horizontal gene transfer among bacterial pathogens, resulting in the emergence of MDR *Salmonella* worldwide. Cephalosporins and fluoroquinolones are recommended for treatment of complicated salmonellosis in humans. Increasing resistance to these antibiotic classes has been reported for *Salmonella* strains from food products, food-producing animals, and humans (11–13). β-Lactam antibiotics are widely used in both humans and animals (14). TEM-type enzymes are the predominant family of plasmid-encoded β-lactamases in Gram-negative bacteria (15) and have spread worldwide. Importantly, most TEM-type variants are extended-spectrum β-lactamases (ESBL) that render bacteria resistant to broader-spectrum cephalosporins (16). In addition, the tetracycline resistance in strains is predominantly mediated by the *tet*(A) gene, which has been frequently identified in Gram-negative bacteria, including *Salmonella* (17, 18). Tetracycline is the most commonly used antibiotic in food animal husbandry.

MRSA in Veterinary Hospitals

In the last 20 years, veterinary hospitals have changed dramatically, emulating every day more their human counterparts; they include extended hospitalizations of animal patients, major surgical procedures, and other invasive diagnostic and therapeutic practices. Parallel to this growth is the increased incidence of hospital-acquired infections and nosocomial outbreaks, many of them produced by MDR bacteria such as ESBL-producing *Escherichia coli*, *Salmonella* serovars, enterococci, and methicillin-resistant *Staphylococcus aureus* (MRSA), among many others (19–21). Eighty-two percent of 38 accredited veterinary teaching hospitals reported multiple outbreaks during a 5-year window associated with these MDR pathogens. MRSA was the second most frequently recorded pathogen (21), which was particularly concerning due to its zoonotic potential.

MDR MRSA is a Gram-positive bacterium that emerged in the early 1970s in human medicine (discussed elsewhere in this series). This pathogen has typically caused infections in hospitalized patients and has been designated hospital-associated MRSA (HA-MRSA). It is one of the top three leading causes of hospital-associated infections in humans (22, 23). Although *S. aureus* is a commensal of several mammalian species, until recently animal reservoirs were considered to have negligible significance for human *S. aureus* infections. Since 2004, however, multiple reports worldwide have linked animal-to-human transmissions of MRSA and have heightened concerns about the risks of animals as potential reservoirs of zoonotic MRSA infections, especially in veterinary settings (24).

MRSA is frequently reported circulating in veterinary hospital environments worldwide (25–27). MRSA strains become resistant to all β-lactam antimicrobial drugs through alteration in the penicillin binding protein (PBP2a) that has a low affinity for β-lactams. The protein is encoded by the *mecA* gene and in some cases by *mecC*; these genes reside on a mobile genetic element

called a staphylococcal chromosomal cassette (SCC*mec*) (28). MRSA represents a major clinical challenge in veterinary medicine, especially in small-animal practice, due to the fact that over 70% of the antimicrobial drugs used are β-lactams (29), increasing the chance of treatment failure.

Until recently, MRSA strains have been classified as healthcare associated or community associated (CA), based on the patient's clinical epidemiology, as well as distinct phenotypic (antimicrobial resistance) and genotypic (e.g., SCC*mec* type, clonal/sequence type, *spa* type, and pulsed-field gel electrophoresis [PFGE] type, among others) characteristics (30) (see chapter x). A more recent classification has been added—healthcare-associated community onset *S. aureus*—but because the relevance of this type of strain in veterinary practice is still unknown, it is not further discussed in this chapter. In any case, in small-animal veterinary hospitals, the most common strains circulating are generally reported as HA-MRSA strains (e.g., USA100). They are frequently resistant to many classes of antimicrobial drugs, including aminoglycosides, quinolones, tetracyclines, and macrolides, among others (25, 31). In contrast, in equine veterinary practices, CA-MRSA strains (e.g., USA500) with less MDR frequency than in small-animal practices are more often reported (27).

Molecular epidemiological studies have shown that MRSA strains are constantly introduced into veterinary hospitals throughout the year, especially in those veterinary settings that deal with tertiary or specialized health care (25). Once introduced, some of these strains can survive in the environment from 2 up to 6 consecutive months on the same surfaces. Additionally, circulation throughout several areas and different contact surfaces of the hospital over time is frequently observed (25, 27). Finally, the same MRSA clones detected from admitted patients and the clinical environment have been isolated from hospital personnel, both during outbreaks and in routine practice, clearly showing the potential of zoonotic transmission in such settings. MRSA has been reported as the 2nd most common zoonotic pathogen causing occupation-related outbreaks in veterinary hospitals (21). This occupational transmission of MRSA is apparently of importance, as many studies frequently reported colonization rates of veterinarians and veterinary personnel to be higher than those observed in the general population in which they live (32).

Livestock-Associated MRSA

Genotyping techniques such as the multilocus sequence typing (MLST) are used to differentiate clonal complexes of *S. aureus*. In the early 2000s, a newly described livestock-associated MRSA (LA-MRSA) strain, identified as CC398, emerged in some European countries and spread from there worldwide, including to South America and the United States (33). This CC398 lineage has been shown to colonize humans, although invasive infections are rare (22, 34–36).

LA-MRSA CC398 has primarily been associated with pigs, and people in close proximity to the swine population (swine workers) have been shown to be at higher risk of being infected than other individuals (37, 38). A study conducted in the Netherlands showed that pig farmers were 760 times more likely to be colonized with MRSA than the general population (39). LA-MRSA transmission from pigs to humans has been shown to be dependent upon exposure duration in swine farms, inhalation of contaminated dust, and the density of pigs on the farm (34, 38, 40–44). Garcia-Graells et al. have shown that extent of exposure to the pigs plays an important role in LA-MRSA transmission to humans. The authors reported that even on swine farms with high MRSA carriage rates among pigs, transmission to the farmers was dependent on the exposure level and the acquisition was greatly reduced by restricting access to these farms (38).

Fluoroquinolone-Resistant (FQ-R) *Campylobacter*

Campylobacter is responsible for causing the second highest incidence of bacterial foodborne-associated infections in humans in the United States (45). The impact of foodborne illness on consumers and the food industry can be devastating. Campylobacteriosis can have severe consequences in humans, including Guillain-Barré syndrome, which is an autoimmune ascending paralytic disorder. Other long-term health outcomes associated with campylobacteriosis are reactive arthritis (Reiter's syndrome), irritable bowel syndrome, carditis, endocarditis, cholecystitis, pancreatitis, meningitis, and septicemia (46). Chickens are natural reservoirs for *Campylobacter* organisms, which reside in the birds' digestive tract and are spread by cross-contamination. Infection is established at around 2 to 3 weeks of age in broilers (47), and the chickens are mostly asymptomatic even when carrying high levels of campylobacters in their guts (48). The majority of human cases are attributed to *Campylobacter jejuni*, although human infections due to *Campylobacter coli* are estimated to be underreported (49). Exposure to poultry meat is an important risk factor for illness (50), and contaminated chicken is considered an important vehicle for human *Campylobacter*

infections. Among other common sources of infection are contaminated water and raw milk (51).

FQ-R *Campylobacter* is prevalent on poultry farms and in retail meat outlets (52). Although fluoroquinolone use for treatment of broilers has been discontinued since 2005 (9), the FQ-R *Campylobacter* strain in the retail poultry industry has remained persistent. One of the main reasons posited for the successful persistence of this strain in the absence of any FQ selection pressure is the increased fitness of this strain compared to that of their susceptible counterparts (53). In addition, FQ-R *Campylobacter* organisms have been identified as persistent contaminants of poultry products, and the current ban on FQ use in the poultry industry may not be sufficient to reduce its prevalence (54). FQ-R *Campylobacter* organisms have been shown to persist quite well in campylobacter biofilms, which is concerning for the retail meat industry (55). In a recent study conducted on poultry farms in the United Kingdom, the risk factors for the presence of FQ-R *Campylobacter* on 89 poultry meat farms include on-farm hygiene, cleaning and disinfection between multiple batches of birds, and control of wildlife (56).

Viral Diseases Associated with Livestock

There are many diseases caused by viruses that affect livestock. Some of the viral diseases are relatively innocuous, while others can be quite economically devastating due to production losses from morbidity and mortality. The more notable diseases are reportable to state and federal agencies and to the World Organisation for Animal Health (OIE). These diseases include exotic Newcastle disease, rabies, bluetongue, classical swine fever, equine encephalitis (Eastern and Venezuelan), foot-and-mouth disease, infectious avian encephalomyelitis, infectious hematopoietic necrosis, influenza, pseudorabies, and viral hemorrhagic fever. Although this is not a complete list of reportable viral diseases, the viruses that cause these diseases are a significant concern and should be monitored closely by animal health professionals.

In addition to the reportable viral diseases, there are many economically important viral diseases of livestock that are not reportable. Some are much more common and consequently of greater importance to the livestock industry. In general, respiratory and enteric diseases are of the highest concern. Often these diseases in livestock are multiagent syndromes. An excellent example is the respiratory disease syndrome seen in cattle, which can be caused by a mixture of viral and bacterial agents (57).

Although multiagent respiratory and enteric diseases are frequently observed, there are single-agent viral diseases that should be monitored by animal health professionals. One example in poultry is infectious bursal disease virus (IBDV). This virus causes an immunosuppressive disease resulting in secondary/opportunistic infections and poor immunologic responses to vaccines (58). It can be the underlying cause of the multiagent respiratory and enteric disease observed in poultry. Other poultry viral diseases that are important include infectious bronchitis, Newcastle disease, Marek's disease, and chicken anemia. Some of the more notable viral diseases in swine include porcine reproductive and respiratory syndrome, porcine epidemic diarrhea, porcine parvovirus infections, and transmissible gastroenteritis. Important viral diseases in cattle include bovine viral diarrhea, rotavirus and coronavirus infections, parainfluenza 3, and bovine respiratory syncytial virus. In fish there are several viral diseases that can affect aquaculture, including infectious pancreatic necrosis and viral hemorrhagic septicemia.

There are also emerging viral diseases that may or may not be fully appreciated for their economic effect on livestock. In many cases the RNA viruses are responsible for emerging or remerging diseases (59). This is due in part to the lack of proofreading capabilities of RNA-dependent RNA polymerases, which allows more genetic mutations to occur by natural selection. Diagnosis of these emerging viral diseases requires comprehensive herd and flock health records, regular health checks, and perceptive diagnostic skills. Morbidity and increased mortality in a herd or flock are often the first signs of an emerging or remerging disease outbreak. However, monitoring for new viral disease outbreaks is not always as simple as observing clinical signs. In some cases, the disease may be asymptomatic and only identifiable by reduced feed efficiency, lower growth rates, and/or reduced meat, egg, or milk quality. An emerging viral disease can also be complicated by secondary or opportunistic bacterial infections. Thus, diagnosis of the viral agent that initiates a disease outbreak can be challenging.

Influenza Viruses Associated with Animals

Among viral causes of livestock infections, influenza virus deserves a separate discussion. In particular, influenza A virus is a highly contagious pathogen that continues to evolve in many avian and mammalian species, causing threats to both animal and human health. As an important poultry and swine disease, both avian and swine influenza viruses have caused consid-

erable economic losses around the world. In humans, influenza virus typically infects 10 to 20% of the global population during seasonal epidemics, resulting in 3 million to 5 million cases of severe illness and 250,000 to 500,000 deaths per year (60). Based on the antigenic relationships of hemagglutinin (H) and neuraminidase (N), influenza A viruses are classified into subtypes. Currently, 18 H subtypes (H1 to H18) and 11 N subtypes (N1 to N11) have been identified (61).

According to its pathogenicity in poultry, avian influenza virus is divided into low-pathogenicity avian influenza and high-pathogenicity avian influenza (HPAI) viruses (62). Many avian influenza virus strains, including HPAI H5N1 and H7N7 and low-pathogenicity H7N9 and H9N2 viruses, are known to have potential to transmit directly from poultry to humans. The HPAI H5N1 outbreak is unprecedented in terms of its geographical distribution and involvement of wild birds in epidemiology. The HPAI H5N1 virus resulted in the deaths of millions of poultry in more than 60 countries on three continents and still circulates in domestic poultry and wild birds especially in Eurasia and Africa. There are also significant zoonotic implications of this panzootic, with more than 500 documented human cases, resulting in more than 300 deaths in 15 countries since 2003 (63). Between December 2014 and May 2015, 132 outbreaks of highly pathogenic H5Nx (multiple N subtypes) were reported in the United States (64). Most have occurred in turkeys and chicken layers in the Midwest. Minnesota lost more than 5 million turkeys and Iowa lost more than 20 million layers within a few months. This unique H5Nx virus continues to evolve, and we still do not know much about the enormous epidemic capacity of this virus.

Humans are periodically infected with swine origin influenza viruses, which may gain the ability for sustained transmission from human to human and lead to global pandemics, as highlighted by the 2009 H1N1 pandemic (65). More recently, a novel H3N2 virus termed "H3N2 variant (H3N2v)" with a matrix gene segment from the 2009 pandemic H1N1 virus has caused a number of human infections in the United States (66). In the United States, influenza viruses of at least six distinct antigenic H subtypes currently circulate in the swine population (67). Since the late 1990s, swine influenza viruses have evolved with increased speed and complexity. New strains of diverse origin, strains with novel combination of H and N subtypes, strains that have undergone extensive reassortments, and antigenic variants continue to emerge; this has complicated the selection of vaccine seed strains and consequently prevented development of effective vaccines in a timely manner. In addition to swine, influenza virus affects many other mammalian species, including pet animals. Until recently, dogs have not been considered as natural or intermediate hosts for influenza A viruses. However, establishment of two new subtypes of influenza viruses in dogs in the last decade and frequent reports of human and avian influenza virus infections highlight the increasing importance of influenza in canine and pet animal health and the potential role in transmission and spread of influenza viruses among humans and pet animals (68–70).

Because of herd immunity due to natural infection or vaccination in humans, and the increasing frequency of influenza immunization in animals, this immune selection pressure together with the high mutation rate of the viral genome facilitates the generation of genetic and antigenic variants. Together with their vast natural host reservoirs within which influenza viruses undergo myriad genomic restructuring "experiments," the emergence of novel strains is inevitable. The 2009 H1N1 pandemic is a good example demonstrating that predicting when and where the next pandemic strain will arise is almost impossible, even in 21st century. This poses a number of problems in designing timely preventive measures and emphasizes the importance of continued monitoring and better understanding of epidemiology in different animal species.

GENOTYPING METHODS FOR MAJOR ZOONOTIC AND LIVESTOCK PATHOGENS

Genotyping methods are specifically used to subtype organisms beyond phenotypic classifications. These molecular biology methods are also commonly referred as "DNA fingerprinting," as the starting material is DNA. These methods are used for various applications besides epidemiology, including forensic medicine and evolutionary biology studies. Genotyping methods often have superior discriminatory power compared to phenotyping approaches. In addition, these approaches are expected to have higher reproducibility and typeability as well as high throughputness, although in some cases these may not always be true. Genotyping methods often use a combination of one or more of the core molecular tools, including amplification, restriction digestion, hybridization, and/or sequencing. While there is a plethora of genotyping methods currently used for various purposes (Parts 2 to 4), some of the genotyping methods are more commonly used in zoonotic and livestock diseases. Table 1 shows a summary of the most common methods used in veterinary medicine.

TABLE 1 Overview of common genotyping methods used in veterinary medicine and key features

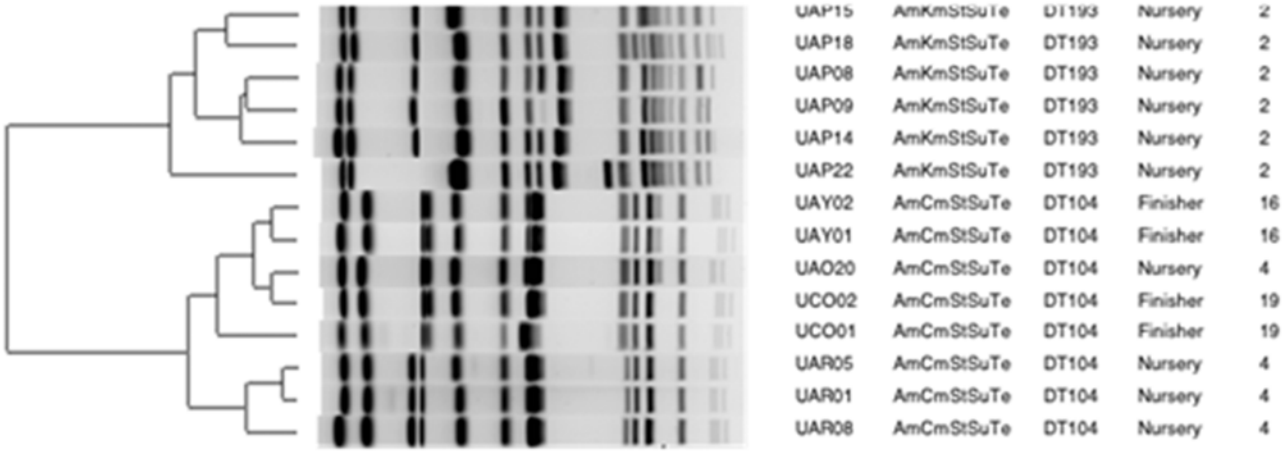
Name of genotyping method	Genome size limit	Unique factors	Fragment size	Electrophoresis system	Comment
PFGE	Bacteria or larger	Intact/rare cutter RE[a]	30 kb to >10 Mb	CHEF	Common/bacterial surveillance
RFLP	Used for broad genome size ranges: viruses/bacteria commonly	Any RE Repeated element probe OR targeted regional amplification	Variable	Regular horizontal agarose	Broad use in various genome sizes; named differently when targeted PCR and RE used (e.g., toxinotyping)
AFLP	Bacteria or larger	Double (rare and frequent) RE	50 to 500 bp	Long range (PolyAc or capillary)	High resolution/high throughput
REP-PCR	Bacteria or larger	Repeated element (known)	Variable	Regular horizontal agarose	Variable reports of discriminatory power
RAPD	Any	Useful for no genome information; limited resources	Variable	Regular horizontal agarose	Also for phenotype genotype correlation
MLST	Bacteria	HKGs/hypervariable genome or wide spatial or temporal	Sequence based	Capillary system	Mostly used for population genetics purposes
MLVA	Bacteria+	Tandem repeats			
Spoligotyping	Tuberculosis specific/ *Mycobacterium bovis*	Defined spacer oligonucleotide sequences	Hybridization based	None/hybridization chamber	

[a]RE, restriction enzyme.

As depicted in the table, each genotyping method has unique characteristics in terms of the genome size it can be used for: the core molecular approach(es) it utilizes, the level of sophistication, and other factors.

PFGE, a macrorestriction genotyping method that has been used since 1984 (71), is among the most common methods used to type livestock-associated bacterial pathogens, particularly foodborne pathogens. This method was originally developed to characterize *Saccharomyces cereviseae*, a fungal organism. The principle of this method is based on restriction digestion of an intact genome using a rare-cutter restriction endonuclease (Part 2). Consequently, it results in several relatively large restriction fragments of the genome of the targeted organism ranging between 30 kb and 10 Mb. While this is a major advantage, it also creates a challenge in terms of setting up an effective electrophoresis system to be able to separate such large DNA fragments. Currently, the contour-clamped homogeneous electric field system is used to separate large DNA fragments (Fig. 1). PFGE is currently the primary system used by Centers for Disease Control and Prevention (CDC) and regulatory agencies in the United States known as the PulseNet system. As described in Part 2, PulseNet is widely used to

FIGURE 1 An example of a PFGE macrorestriction profile of *Salmonella* serovar Typhimurium subtypes.



Q12 **TABLE 2** Comparison of discriminatory powers of selected genotyping and phenotyping methods as tested for a set of *Salmonella* serovar Typhimurium isolates and measured by the Simpson's index of diversity

Subtyping method	No. of types	Size(%) of largest type	DI[a]
AFLP	16	12	0.939
PFGE	14	15	0.925
REP-PCR	4	72	0.421
Antibiotyping	12	56	0.579
Phage typing	9	55	0.628

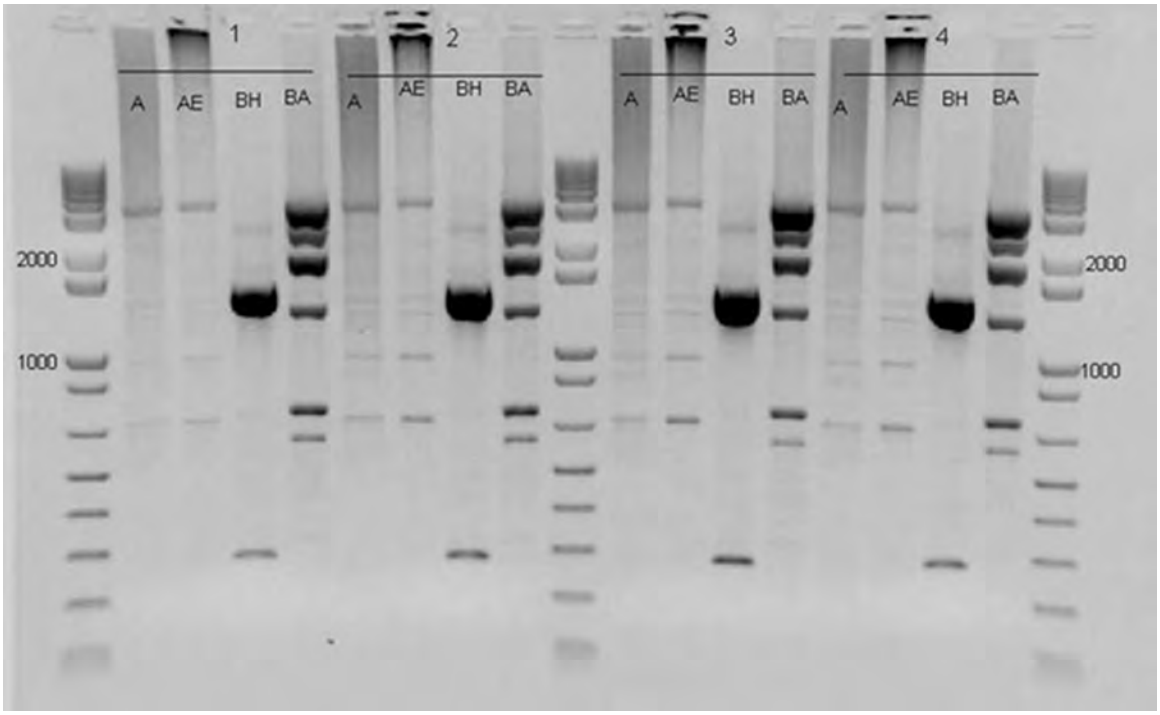
[a]Adopted from reference 76. DI, Simpson's index of diversity.

investigate outbreaks of *Salmonella*, *E. coli*, and other bacterial pathogens.

Restriction fragment length polymorphism (RFLP) is another genotyping method commonly used in veterinary medicine for a wide variety of genomes and purposes. As the name indicates, RFLP also depends on restriction DNA fragment profiling. However, a key component of the classical RFLP system is also the use of probe hybridization based on repeated elements present in the genome of the organism. An example is insertion sequence IS*6110* in *Mycobacterium tuberculosis* as described in other parts of this series. Another variation of the RFLP system, more and more commonly used in veterinary medicine and for other pathogens at the human-animal interface, is PCR-RFLP. This genotyping test is designed to amplify DNA segments of interest, often at some defined gene loci. Then the amplified products (amplicons) are digested with one or more restriction enzymes to create restriction profiles. One scheme of the PCR-RFLP (Fig. 2) commonly used in recent years is toxinotyping of *Clostridium difficile* (72), which targets the pathogenicity locus (PaLoc) of the organism that encodes enterotoxin and cytotoxins. Therefore, PCR-RFLP can also provide functional significance data besides subtyping isolates. Other advantages of RFLP analysis are that it can be used for analysis of any type of genome and does not necessitate expensive equipment setup in the laboratory.

Random amplified polymorphic DNA (RAPD) (also referred as arbitrarily primed PCR) is another genotyping method commonly used in livestock and zoonotic disease investigations. The method was first described in 1990 (73). RAPD, as the name indicates, is designed to amplify random targets within a genome of the organism. This is a relatively simple technique that can be conducted without prior knowledge of the nucleic acid sequence of the organism being queried. This method is also simple for training and among the least expensive to set up in laboratories. Therefore, it is more commonly used in low-resource settings. Overall, the method is expected to have moderate discriminatory

Q9 **FIGURE 2** PCR-RFLP (toxinotyping) profiles of *Clostridium difficile*.



power. However, its reproducibility is a limitation, since imperfect annealing could result in electrophoretic band variations among isolates. It can be used as a screening test to identify gene loci of interest for further investigation and cloning when a specific phenotype of interest is identified among a set of isolates. Using unique amplified fragments found among isolates with a specific phenotype, such as drug resistance or toxin, the DNA fragments of interest can be excised from the electrophoresis gel and further characterized either by cloning them into a plasmid or directly sequencing them.

Repetitive palindromic PCR (REP-PCR) is a method relatively less frequently used than the above-desribed methods. The subtyping method is based on known repeated elements that exist in various eukaryotic and prokaryotic genomes. As a method applied in infectious disease investigation, REP-PCR was first described based on repeated elements published in 1984 (74). Further, these elements were used as a genotyping targets by Versalovic et al. (75), and REP-PCR has been included among the early genotyping methods used in livestock disease investigations. Arguably, the most common repeated element targeted for subtyping in bacterial organisms, particularly foodborne enteric pathogens, is the enterobacterial repetitive intergenic consensus sequence. While this method is known to have broad species applicability, its reproducibility due to nonspecific amplification and discriminatory power has been questionable (76).

Amplified fragment length polymorphism (AFLP) is another genotyping method, among the ones more recently developed (77). This method has been used for genome mapping of eukaryotic organisms. A modified version of the AFLP has been used in various infectious disease studies of food safety and zoonotic significance. The AFLP test is based on amplifying genome fragments digested by two restriction enzymes, one rare (often EcoRI) and one frequent (often MseI) cutter. Thus, this method allows amplification of a random subset of DNA fragments representative of the genome. Often, the fragments range between 50 and 500 bp, with a total number of 200 fragments. Therefore, separation of such a large number of fragments had previously been accomplished by a long-range polyacrylamide gel and more recently by a capillary electrophoresis system. The main advantages of this method include its high discriminatory power, and it is also known for its high resolution and throughput. However, conducting AFLP in low-resource settings has been a challenge mainly since a genetic analyzer machine, such as the capillary system or even the long-range gel system, is expensive. As discussed in later sections, this method has been used for a wide variety of genomes of pathogens associated with livestock diseases.

MLST is another genotyping method that is increasingly used in veterinary medicine and associated zoonotic disease investigations. This method compares sequences of segments of housekeeping genes (HKGs). MLST is currently used for a specific set of organisms for which global databases have been established (e.g., http://www.mlst.net/ and http://pubmlst.org/; see Part 2). As HKGs are essential genes, this genotyping method has a very high typeability. However, its discriminatory power depends highly on the stability of the genome. The more stable the genome is, the less useful MLST would be for molecular epidemiology purposes since HKGs in such organisms would be highly conserved, with little or no variation even if the isolates are nonclonal. In stable genomes such as that of the Shiga toxin-producing *E. coli* O157:H7, MLST may be used for population genetics and evolutionary biology studies based on isolates from wide geographic or temporal sources. On the other hand, when the organism has a hypervariable genome, such as the case with *Campylobacter* spp., this method is preferable for epidemiologic studies when the isolates are indeed clonal. MLST is commonly used for investigation of LA-MRSA. The following HKGs are targeted for MRSA MLST genotyping: *arcC*, *aroE*, *glpF*, *Mgk*, *pta*, *tip*, and *yqiL*. Another foodborne organism, *Campylobacter*, is also frequently genotyped (subtyped) by MLST. The HKGs targeted include *aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt*, and *uncA*. Once the targeted regions of these genes are sequenced, the data can be analyzed and compared using various approaches, such as establishing allelic profiles that get annotated into sequence types or clonal complexes.

Whole-genome sequencing (WGS) is the newest addition to the repertoire of genotyping methods recently gaining momentum for epidemiologic investigations. However, its utility in veterinary epidemiology remains at an infant stage, as this approach is used only in a few cases in highly developed countries and only in cases of high-value foodborne disease outbreaks for trade enforcement and other regulatory purposes. Two examples of such application are in the *E. coli* O104:H4 outbreak in Germany (78) and, more recently, in the investigation of *Salmonella* serovar Bareilly outbreaks in two states in the United States that were traced to scrapped tuna imported from India (79). The principles behind different platforms used to perform WGS and pipelines used to analyze the vast amount of sequence data are discussed in detail Part 5 of this series.

APPLICATION OF GENOTYPING METHODS FOR EPIDEMIOLOGIC INVESTIGATION OF ZOONOTIC AND LIVESTOCK DISEASES

Genotyping methods are increasingly and more commonly used to address epidemiology of livestock and zoonotic diseases of significance, including those caused by unique strains such as MDR bacteria, hypervirulent infectious agents, and emerging zoonotic viruses. In this section, we present examples of molecular epidemiology studies of such disease agents associated with veterinary medicine in companion animal, food animal, and other livestock settings.

Molecular Epidemiology of Hypervirulent *C. difficile* and Tracking in Animals

As described earlier, one of the emerging issues of significance in terms of infectious diseases at the interface of humans and animals is the increasing prevalence of hypervirulent *C. difficile*, which is hypothesized to have animal reservoirs serving as major sources of CDAD in humans. As outlined in the earlier section, while *C. difficile* is known to cause gastrointestinal tract infections in various animals, including horses, pigs, dogs, and others, the occurrence of hypervirulent strains circulating in the animal population that are responsible for epidemic infections in humans had not been reported. Since 2004, an emerging hypervirulent strain has caused several community-acquired infections with severe clinical manifestations, including death. This particular strain, commonly known as NAP-1 (North American pulsed-field type 1), was reported by the CDC (80) to cause a multistate outbreak in the United States. It has also been recognized in other nations, particularly in Europe. As the risk factors for this epidemic did not conform with the classical toxigenic *C. difficile*, the need for investigating animal reservoirs using molecular epidemiologic methods was found to be of paramount importance. Therefore, several molecular epidemiologic studies targeting various livestock systems and companion animals were launched.

The genotyping methods utilized to investigate *C. difficile* focus on two major approaches: (i) genotyping based on the pathogenicity locus (PaLoc) region of the organism and (ii) genotyping to determine the overall clonality of isolates shared between animals and humans. In addition, various phenotyping approaches, particularly antimicrobial susceptibility testing, are used to supplement these genotyping approaches and make appropriate epidemiologic decisions.

The PaLoc region of *C. difficile* is a chromosomal locus composed of five genes within a 19.6-kb region (81). This locus carries genes of key significance for *C. difficile* toxigenicity. They include *tcdD* (a positive regulator), *tcdB* (cytotoxin gene/toxin B), *tcdE* (a putative gene with holin function), *tcdA* (enterotoxin gene/toxin A), and *tcdC* (a negative regulator gene). Besides its high functional significance, this region is also crucial as a target for characterizing the molecular epidemiology of toxigenic strains. Using three key genotyping methods, one can assess significance of the *C. difficile* strains of animal origin in human epidemics. These approaches include (i) a direct PCR amplification of three of the PaLoc genes, including the two toxin effector genes (*tcdA* and *tcdB*) and *tcdC* (the negative regulator gene); (ii) a PCR-RFLP approach known as toxinotyping (72) based on the scheme to determine the toxinotype of each isolate; and (iii) DNA sequencing of the amplicon product of the *tcdC* (negative regulator) gene to determine the presence and length of any deletion within the gene, which may indicate the malfunctioning negative regulator and thus hyperexpression and translation of enterotoxin and cytotoxin. It should be noted that the recent epidemic of hypervirulent *C. difficile* is caused by a toxinotype III strain with a characteristic 18-bp deletion within the *tcdC* gene.

The fourth genotyping approach used in *C. difficile* epidemiologic studies is based on determination of the overall clonality of isolates recovered from livestock and humans. In the United States, the standard system used for this purpose is PFGE. In other regions of the world, other methods, in particular, ribotyping (RFLP that uses ribosomal operons as target for probe hybridization), are used. The recent epidemic of hypervirulent *C. difficile* was caused by a NAP-1 strain, which was designated O27 by the ribotyping scheme. Various subtypes have since been reported in the United States and other nations (82).

A molecular epidemiologic study to determine the role of pigs in the dissemination of hypervirulent NAP-1 in the United States was conducted between 2007 and 2010 (83). This study targeted 30 swine farms in two states: Ohio and North Carolina. As expected, a high prevalence of *C. difficile* was reported among piglets within the first 2 weeks of age, with a much lower prevalence in nursery and negligible occurrence in finishing herds. This report indicated that more than 70% of the isolates recovered were toxigenic, with a majority carrying both toxins A and B. Few isolates were found to carry toxin B but not A, and a relatively small proportion (about 25%) was found to be nontoxigenic. The most striking finding was the fact that all the toxigenic (with both toxins A and B) strains also had a long deletion mutation (39 bp) within the negative regulator

gene *tcdC*, indicating their potential to be functionally hypervirulent. However, this deletion is markedly different from the 18-bp deletion reported among the epidemic NAP-1 isolates. PCR-RFLP toxinotyping indicated that the majority of the toxigenic isolates were toxinotype V, distinct from the epidemic strain, which was reported to be toxinotype III. Overall, the molecular epidemiologic study findings clearly indicated that while the study was not representative of the U.S. swine production system, the findings showed that there was no evidence that pigs served as the reservoirs of the epidemic hypervirulent NAP-1. It should also be noted that the potential hypervirulent strain of toxinotype V/39-bp deletion of *tcdC* had been reported previously in other studies of *C. difficile* isolated from pigs as well as human patients (84), indicating its high prevalence and potential for causing community-acquired infections. At this time, the reservoir of hypervirulent NAP-1 strains remains unknown.

MDR *Salmonella*

There are multiple approaches available to target MDR *Salmonella* resistance gene determinants and to assess their clonality, including PCR, RT-PCR, MLST, and AFLP, which are preferred by laboratories depending on ease of use, robustness of the technique, reproducibility, and the availability of resources and supplies as described in the previous section. PFGE has remained the "gold standard" method for genotyping *Salmonella* for many decades now and is used by multiple public health (PH) agencies to track the dissemination of drug-resistant *Salmonella* strains as well as to conduct outbreak investigations to prevent them from spreading (85). PFGE is also used by PH agencies to track multiple other bacterial pathogens and parasites. PFGE continues to assist PH agencies in trace-back investigations of drug-resistant bacterial pathogens and reduce PH burden.

PFGE is also used to determine the genotypic clonality or dissimilarity of the emerging MDR *Salmonella* strains and their epidemiology. The information is then used to monitor these emerging strains and design interventions to prevent their spread. Recently, a national outbreak of *Salmonella enterica* serotype Heidelberg resistant to multiple antimicrobial drugs was traced back to a single poultry farm in the United States (86). This was a multistate outbreak that infected 634 patients, with a 38% hospitalization rate and no deaths. MDR *S.* Heidelberg-contaminated chicken was implicated as the source after trace-back and epidemiologic investigations. The source was identified based on a PFGE-generated dendrogram which directly implicated three California production establishments. PFGE been used to study the emergence of MDR *S.* Typhimurium in other parts of the world, including Malaysia (87), *S.* Typhi and Paratyphi A in India (88), and *S.* Typhi in Bangladesh, Indonesia, Taiwan, and Vietnam (89).

The advent of WGS and the decreasing cost of sequencing have the potential in the near future to make sequencing the preferred method for genotyping microbes. Direct sequencing of drug resistance determinants has been known to help understand the evolution of different resistance mechanisms, including those associated with ESBL plasmids. As technology progresses, a faster turnaround time, robustness, and reduced costs will make WGS a more favorable approach to target MDR and virulent veterinary enteric bacterial pathogens.

Methicillin-Resistant *Staphylococcus aureus*

There are multiple genotyping techniques that are frequently used, individually or in combination, to reliably, accurately, and with high resolution type MRSA. Among the most common techniques are MLST, PFGE, *spa* typing, REP-PCR, and SCC*mec* typing. MLST has a superior discriminatory power compared with the other techniques listed in this chapter; therefore, it is an excellent method to study this pathogen causing infections in hospitals, cities, regions, and even countries. However, because it is labor-intensive, has a long turnaround time, and is costly, this technique is used mostly in epidemiologic studies and research, rather than as a regular tool during routine surveillance in hospital settings or "local" outbreak investigations.

The benchmark genotyping method for MRSA surveillance is PFGE, which in combination with the SCC*mec* typing and phenotyping (antimicrobial resistance profile) tests is used to determine strain clonality. The analysis of DNA banding patterns generated by PFGE allows highly discriminatory separation of MRSA isolates based on a standardized set of criteria (90). However, it is important to highlight that some MRSA strains, such as LA-MRSA (CC398), are nontypeable with the standard PFGE procedure that uses SmaI (the restriction enzyme most frequently used in PFGE typing *S. aureus*). Due to the methylation site present in the LA-MRSA DNA, other restriction enzymes, such as *Cfr*9I, have been recommended. A similar situation has been observed when characterizing other methicillin-resistant *Staphylococcus* spp., such as *S. pseudintermedius*, which requires their own unique restriction enzymes. If the proper restriction enzyme and protocol are used, the discriminatory power of this typing method is very high, which explains why PFGE remains today

the reference genotyping test for infection control and outbreak investigations.

SCC*mec* carrying the *mecA* gene is frequently used to complement the genotypic characterization of this pathogen. SCC*mec* typing is basically a multiplex PCR that targets different components or elements present in the SCC (*mec* gene complex, cassette chromosome recombinase [*ccr*] gene complex, and the integration site sequence). The SCC was recently reviewed extensively by Liu et al. (28), who captured the role played by this gene cassette in the global epidemiology, antimicrobial resistance, and the evolution of MRSA. This technique has, in general, low resolution, and therefore, it is used not as a stand-alone method but as a supporting tool for the other techniques described here.

spa typing is a sequence-based technique that targets the specific region of the gene for *S. aureus*-specific staphylococcal protein A (*spa*) that contains a variable-number tandem repeat locus. Based on the repeats identified, a unique code is provided (e.g., t001). This single-locus typing technique appears to have a resolution similar to those of MLST and PFGE, but it is significantly less expensive (<$15 per sample) and has a quick turnaround time, which explains why it has become more popular in hospital surveillance. However, its results must be analyzed with caution, as this method can misclassify particular MRSA types (91). It is expected that in the future, WGS will become the reference standard for MRSA surveillance and outbreak investigation, as the core MLST, *spa* type, SCC*mec* type, and resistome (combination of antimicrobial-resistant genes presents in the isolate) could all be extrapolated from the WGS data.

Molecular Epidemiology of Environmental MRSA at a Veterinary Teaching Hospital

It is considered that the veterinary hospital environment might play a role as a reservoir and a possible source of MRSA for patients and personnel. Therefore, to determine the regular presence, distribution, and characteristics of this pathogen in a large veterinary teaching hospital (with over 27,000 patients per year), as well as to establish patterns of contamination over time, active MRSA surveillance programs were established. Antimicrobial susceptibility testing, SCC*mec* typing, PFGE typing, and phylogenetic analysis were used to characterize and analyze isolates obtained from the environment, incoming patients, and veterinary personnel (25, 27, 31).

Over 8 years, MRSA was detected in 11.8% of high-contact surfaces, with the highest prevalence observed in the small-animal clinic (SAC), which was as high as 29.6%, followed by 8.6% in the equine clinic (EC). In the SAC, the predominant strain was SCC*mec* type II/USA100 (over 98% of the isolates), which is considered an HA-MRSA strain. All these isolates were classified as MDR strains (with resistance to 4 or 5 classes on average) with very complex resistance profiles. By PFGE analysis, these isolates were found to be closely related, which reflects a low diversity of MRSA strains circulating in this environment.

In contrast, in the EC, the predominant circulating strain was SCC*mec* type IV/USA500 (47.9%). Over 90% of all these isolates were classified as CA-MRSA, and they had a relatively low drug resistance profile (<70% were considered MDR but to only 3 or 4 classes at a time). As in the SAC, the diversity index of the EC isolates was also low. Interestingly, when the isolates from both sections were compared side by side in the same dendrogram, there was very little evidence of circulation of common strains between the two sections of the same veterinary hospital (Fig. 3). This indicated that each section has its own ecology, even though the sections share students and personnel. Q4

Genotyping analysis found that incoming MRSA-positive animals are capable of introducing new clones into the hospital environment on a regular basis. In many cases, some of these clones that got established in the environment were maintained there for up to 3 months. It was also possible to track the movement of some unique pulsotypes across different medical services.

During the surveillance period, there were multiple opportunities where the genotyping tests allowed identification of potential nosocomial exposures. In one case, one equine patient (admitted for colic) developed a severe MRSA postoperative infection in its surgical incisions. The isolate from this patient was indistinguishable from the predominant pulsotype circulating in the EC, including the surgical room, which indicated a likely nosocomial transmission at the time of the surgery or indirect contact with contaminated surfaces postsurgery.

More recently, a multipoint cross-sectional sampling of the hospital personnel was performed parallel to the environmental and animal surveillance. It was observed that 3.5% of the personnel were colonized by MRSA, with the majority of the strains found to be clonal or closely related to those detected circulating in the hospital environment. This clearly shows that occupation-related transmission is occurring in this population.

In summary, regular surveillance supported by genotyping tools can aid in understanding the ecology and transmission dynamics of MRSA infections in human and veterinary clinical settings. This type of

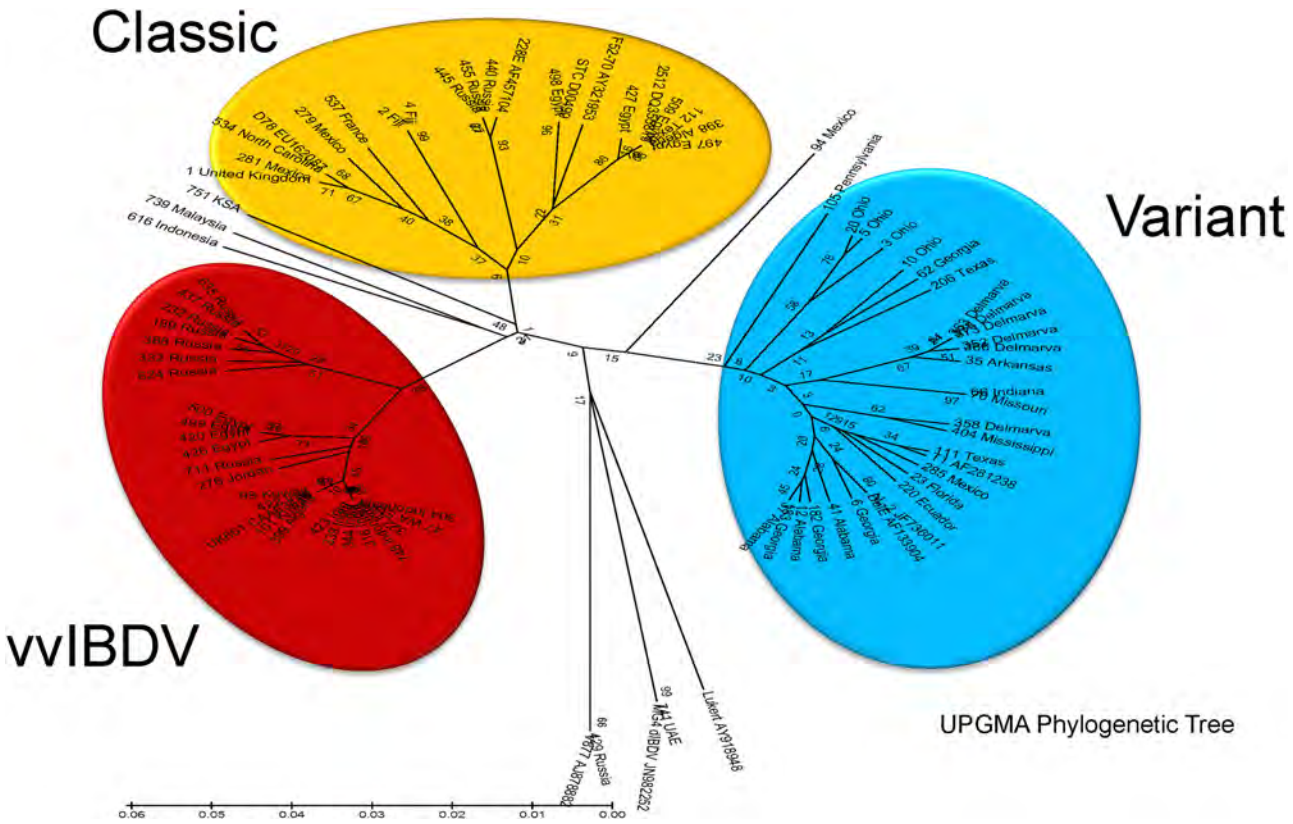


FIGURE 3 Phylogenetic analysis of MRSA from veterinary hospitals.

observation could be used soon to develop biosecurity and biocontainment protocols aimed at reducing environmental contamination and potential transmission of MRSA among hospital staff, clients, and patients. Finally, no matter the molecular method(s) used, the final interpretation of the results obtained for MRSA, and arguably for most pathogens, needs to be accompanied by the so called "shoe leather" epidemiology. It means that genotyping data should always be considered as just part of larger epidemiological investigation or study, to determine the epidemiologic association or relation of cases infected with a particular genotyped strain with a risk factor or a transmission pathway.

Influenza Virus

Initial influenza screening tests are performed to determine if influenza A virus is present in the samples or not. If positive for type A influenza virus, selected H and N subtyping is conducted, depending on the animal species involved. For poultry, because of the potential for H5 and H7 subtypes to mutate into highly pathogenic strains, all samples that test positive for type A influenza virus are further examined for H5 and H7 subtype viruses. Those H5- or H7-positive samples are subjected to confirmatory testing by the avian influenza reference laboratories (e.g., National Veterinary Service Laboratories, USDA) recognized by the OIE.

Real-time reverse transcription-PCR (RRT-PCR) targeting the matrix (M) gene is commonly used to screen for influenza A virus. Positive samples are then subjected to H5 and H7 tests and selected N subtyping RRT-PCR. Additional confirmatory testing includes partial genome sequencing and/or WGS. Typically, partial sequencing of the H protein gene is conducted first to compare with known HPAI virus sequences. If the genetic sequence matches that of a known HPAI virus, the sample is considered an HPAI virus and reported to the OIE. Depending on the importance of the sample and also with the recent advances in sequencing technology, WGS is routinely conducted, followed by phylogenetic analysis for epidemiologic tracking purposes.

Molecular Epidemiology of HPAI Virus

Most developed countries conduct routine surveillance of avian influenza virus in poultry and wildlife. In the United States, 45 USDA-approved laboratories in the National Animal Health Laboratory Network (NAHLN) and the U.S. Department of the Interior's

National Wildlife Health Center perform the surveillance in collaboration with the National Veterinary Services Laboratory (NVSL) and Southeast Poultry Research Laboratory, USDA. In recognition of the potential benefits of coordinated effort on surveillance, the OIE-Food and Agriculture Organization global network of experts on animal influenza (OFFLU) established a working group on wildlife influenzas in 2014 (92).

Phylogenetic analysis based on partial- or full-genome sequencing has become the choice of epidemiologic study of influenza. The two primary surface proteins, H and N, determine the important properties of avian influenza viruses, including the pathogenicity, response to vaccine, and zoonotic potential. For this reason, sequencing has been focused mainly on the H gene and to a lesser extent on the N gene. However, with recent advances in sequencing technology, its decreased cost, and relatively small viral genome size (about 14,000 nucleotides), more full-genome sequencing is conducted. Through genetic characterization and analysis of the influenza virus isolates, scientists can make informed assumptions on (i) the relatedness among influenza viruses, (ii) evolutionary trends, (iii) virulence of the virus, (iv) response to influenza vaccine, (v) resistance to antiviral drugs, and (vi) potential for interspecies transmission or adaptation of the virus to a new host. Examples of applying genetic information for epidemiologic investigation of HPAI viruses are described below.

As mentioned above, the HPAI H5N1 outbreak is unprecedented in terms of its geographical distribution and has continuously evolved since the isolation of prototype virus in 1996 (93). Thousands of gene sequences from HPAI H5N1 viruses are compiled in databases for use by researchers, such as GenBank (https://www.ncbi.nlm.nih.gov/genbank) and the Global Initiative on Sharing Avian Influenza Data (GISAID; http://platform.gisaid.org/). Because H5N1 virus continues to evolve in different avian species and different geographical locations, many unique genetic clades exist and continue to diverge within the H5 gene. To minimize the confusion and expedite the comparison of isolates of diverse origin, the WHO/OIE/FAO H5N1 Evolution Working Group (2008) was formed, and it recommended a unified nomenclature system for the HPAI H5N1 lineage (e.g., the H5 viruses isolated in the United States in 2014 and 2015 belong to clade 2.3.4.4).

Phylogenetic analysis of chronological H5N1 isolates explains the rapid spread of HPAI H5N1 into different countries and continents both by poultry and human movement and by migratory birds (94). For certain H5N1 clades (e.g., 2.3.2), phylogenetic studies indicate that the virus initially established in southern China spread northward along the poultry trading routes. In addition, the introduction of the HPAI H5N1 viruses into Africa or many other countries can be traced to the legal and illegal poultry trades. Most of the nationwide transmission within the country after the introduction of new virus has been associated with poultry/human movements (64).

On the other hand, phylogenetic analysis in combination with satellite tracking and remote sensing on early H5N1 viruses provide evidence that wild birds are implicated in the spread of HPAI H5N1 viruses from southern China to Africa along the migratory flyways. For example, the movement of clade 2.2.2 between Qinghai Lake (western China) and Tibet coincided with the migration route of bar-headed geese along the Central Asian flyway (95). Another good example of migratory-bird involvement in influenza virus dissemination is the recently observed sudden global spread of H5N8 virus. Based on outbreak information in Asia, Europe, and North America during 2014 and phylogenetic network analysis (median-joining network constructed based on HA gene sequences) in combination with waterfowl migration patterns, the H5N8 virus (clade 2.3.4.4) likely emerged in late 2013 in China, spread in early 2014 to South Korea and Japan, and reached Siberia and Beringia by summer 2014 via migratory birds. Genetically distinct subgroups emerged and subsequently spread along different flyways during fall 2014 into Europe and also North America (96, 97). Q5

Phylogenetic analysis has also been used to track the origin of the viruses involved in outbreaks in humans, swine, and pet animals. Since the first report of human H5N1 cases in Hong Kong in 1997, many human outbreaks that involved pure avian origin viruses with genotypic matching in all eight gene segments have been reported (98). Genetic analysis of specific amino acid mutations in the viral genome has provided insights into the adaptation or interspecies transmission of H5N1 virus (99). With the advent of the reverse genetics system for influenza virus, which is the creation of a virus from a full-length cDNA copy of the viral genome, manipulating the viral genome became possible, and many pioneering studies have been done to identify key amino acids involved in interspecies transmission. Using the reverse genetics system, the D627K amino acid substitution in the PB2 protein was determined to increase the virulence of H5N1 viruses in mice (100), and later, the same mutation was identified in human isolates (101). Since then, several key amino acids have been identified throughout the influenza virus genome and used as

molecular markers for assessing the risk of specific H5N1 viruses or clades for humans. However, recently, there has been extensive debate about the benefits and risks of dual use research concern, particularly with the use of gain-of-function experiments with potentially pandemic pathogens (102), which delays identifying additional molecular markers that can be useful for epidemiologic investigation.

Phylogenetic analysis became a standard way to describe the molecular epidemiology of many viral disease outbreaks, including influenza. However, the influenza sequence databases are getting larger each day, and typical phylogenetic analysis, data processing, and visualization of the outputs have become challenging. More importantly, sequences are often determined and stored out of context of other key data, including clinical, epidemiologic, or immunologic data. With progress in "big data" analysis and the integration of other epidemiological and functional data through worldwide collaboration and coordination led by the FAO and OIE, our understanding of influenza epidemiology and evolution will be enhanced. Other innovative genomic tools, such as proteotyping, microRNA expression profiling, and microarray-based analysis, will help to identify emergence of novel strains of increased virulence and host tropism, and also to develop new preventive measures, including vaccines.

Epidemiology of Zoonotic Viral Infections (Noninfluenza)

Zoonotic viral disease outbreaks are usually first observed in the human population. Most are inconsequential because transmission is from an infected animal to a human. Those zoonotic diseases that become a significant problem are the ones where transmission from an animal to a human is followed by the virus developing the capability to transmit between humans. Two recent zoonotic disease outbreaks where the virus was transmitted between humans involved severe acute respiratory syndrome (SARS) (103) and Middle East respiratory syndrome (MERS) (104). Both zoonotic diseases are caused by coronaviruses (CoVs), and although there are other animal hosts, the natural host reservoir for both CoVs seems to be bats (105). The CoVs have a single-stranded RNA genome and are found in humans and many animal species. Mutations frequently occur in these viruses because their RNA-directed RNA polymerase does not have proofreading activity. Thus, like influenza viruses, CoVs have the potential to evolve in ways that could change their virulence and/or host range. In the case of MERS-CoV and SARS-CoV, they did just that.

Molecular epidemiology investigations conducted to determine the most likely source of these CoVs were relatively simple in design. The viruses were isolated from humans and RT-PCR fragments of their spike protein gene were sequenced and compared to those of other known CoVs. The spike protein gene was selected because this protein has the most sequence variability among CoVs. It protrudes from the viral envelope and is responsible for attachment and antigenicity of CoVs (106). In the case of MERS, the sequence of a human MERS-CoV most closely matched that of a CoV detected in the Egyptian tomb bat (*Taphozous perforates*) (107). Further investigation indicated that the sequences of CoVs in bats from Asia and South Africa were also similar to that of MERS-CoV.

Initially, the Asian palm civet (*Paradoxurus hermaphroditus*) was thought to be the animal host reservoir for SARS-CoV because this cat was found in live-animal markets and restaurants in the Guangdong province of southern China, where the outbreak began, and was infected with the SARS-CoV (105). However, further investigation and surveillance studies demonstrated that the horseshoe bat (*Rhinolophus*) was the true animal reservoir. Sequence comparison of human SARS-CoV and horseshoe bat isolates demonstrated nearly identical sequences and some similar sequences that were designated SARS-like CoVs (108).

In both diseases, the region of the CoV genome that was RT-PCR amplified and sequenced was selected because previous work with other CoVs demonstrated that frequent mutations in the spike protein gene can lead to antigenic and tropism changes (109). Selecting a variable region of the viral genome to sequence was important because SARS-CoV and MERS-CoV had to be distinguished from other CoVs that are very common in the human and animal populations. Selecting a conserved sequence region would not have provided the information necessary to identify the animal reservoirs of these diseases.

Genotyping of Other Viruses

Molecular biology techniques can be used to track viral disease agents as they are transmitted through a susceptible host population. There are many ways to do this, but most start with the collection of an appropriate sample. It is important to collect samples at the appropriate time that are known to contain the viral pathogen. For example, testing samples collected within days of administering a live-attenuated vaccine to the herd or flock will likely detect only the vaccine virus. Timing of the sample collection is important because viruses spread

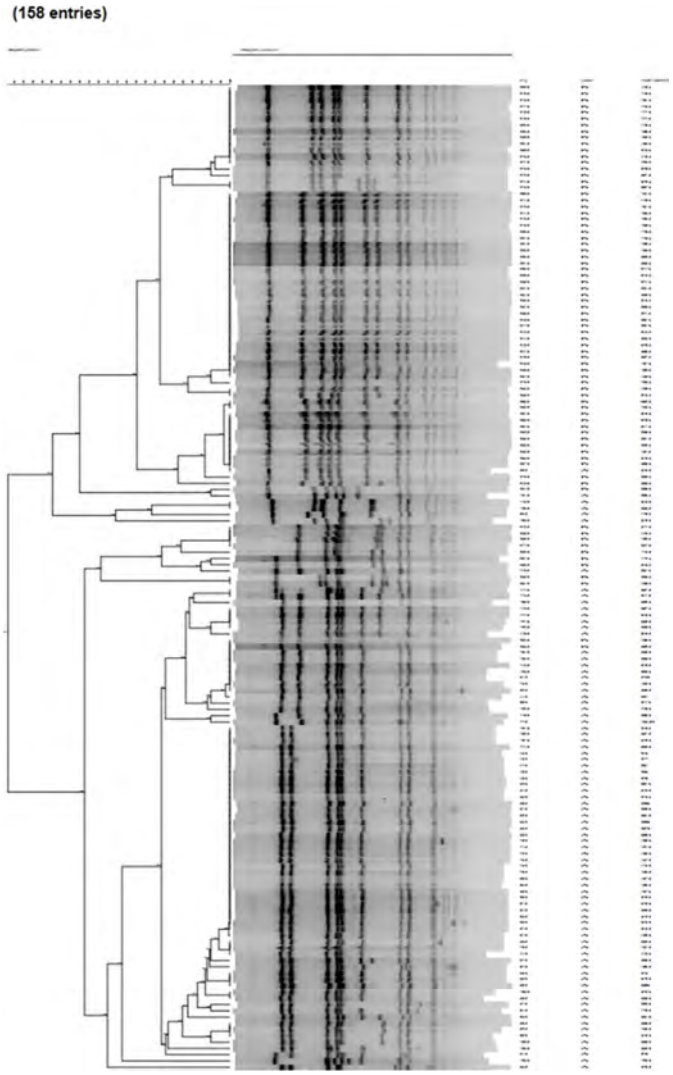
through a host population at different rates. The spread of a virus through a host population is dependent on many factors. The most obvious is the pathogenicity of the pathogen for its host. However, other host factors also can affect the ability of a virus to spread through a population. These include the susceptibility of the host, density of the host population, other disease agents in the population, and predisposing factors like stress and immune suppression.

Once the samples are collected, the next step is to determine what molecular biology assay should be used to detect the virus (see Part 3). The sensitivity of the assay is always important, but most diagnostic assays begin with a PCR or RT-PCR amplification procedure. The more important decision is selection of the appropriate region of the viral genome to amplify. A region of the genome should be selected for detection that will not only identify the pathogen but also answer important questions about its characteristics, such as antigenic type, pathogenicity, or epidemiologic origin (110). For example, selecting a conserved region of the genome may identify all viruses in a large group, but it may tell us nothing about the particular outbreak strain. If specific regions of the genome are known to contribute to important features of the virus like virulence or transmissibility, they could be useful in determining the epidemic potential of that virus. They also might provide some information on the trend in pathogenicity as the virus evolves during its spread through a population. Regions of the genome that seem to tolerate a significant amount of genetic change are usually subject to more environmental selection (110). These variable regions often control antigenicity of a virus, and their detection can be useful in determining the correct vaccine to use to protect future herds and flocks.

IBDV as an example

Infectious bursal disease virus (IBDV) is a birnavirus that has two genome segments of double-stranded RNA (111–113). This virus causes immune suppression when it infects young chicken (114). Two serotypes are recognized, but only one serotype (serotype 1) causes disease. Thus, it is important to design a molecular diagnostic test that will identify only the serotype 1 viruses. Furthermore, the serotype 1 viruses exist in multiple antigenic subtypes, and it has been shown that vaccines to one subtype do not fully protect chickens from a different subtype (115–117). A hypervariable sequence region in the capsid surface protein VP2 is known to control the antigenic subtype of IBDV. Targeting this region for a diagnostic assay has provided important information on the virus strain and potential vaccines that could be used to control the disease (118). There are several strategies that can be used to identify the hypervariable VP2 sequence. One is to use RT-PCR primers that are specific for each antigenic strain of IBDV. This is practical when only a few antigenic types exist and the virus evolves slowly. However, IBDV, like most RNA viruses, evolves quickly, and many antigenic subtypes have been identified. Using primers specific for each antigenic type is impractical. A negative result could indicate a true negative, or it could result from a mutation in the hypervariable region that no longer allows the primers to bind. Primers designed to target conserved regions on either side of the hypervariable region have been developed to amplify all serotype 1 IBDV (119). Q6

FIGURE 4 Phylogenetic tree of IBDV strains generated using sequences from the hypervariable region of VP2.



The different antigenic types can then be identified by sequence analysis of the variable region. The virulence of an IBDV strain has also been identified by this technique. Specific amino acids that are associated with high morbidity and mortality in the very virulent IBDV (vvIBDV) strains have been identified. Phylogenetic trees produced by this information have been valuable in tracking virus types as they spread from one region to another. The vvIBDV strains were initially detected in Northern Europe and quickly spread through Europe, Asia, the Middle East, and eventually into the Americas (120). Tracking these viruses was possible by RT-PCR amplification of the VP2 variable region. Phylogenetic trees clearly show a vvIBDV branch that is distinguished from the less pathogenic classic and variant strains.

Although the vvIBDV and variant and classic IBDV strains fall into specific branches on the phylogenetic tree, there are outliers that do not fit into these groups (Fig. 4). These viruses are clearly identified with primers in the conserved region of the viral genome and might have been missed if the primers were designed for specific IBDV strains based on the variable sequence region of VP2. It is also interesting to note the multiple branches within the three major groups. These minor branches are an indication that the IBDV strains are continuing to evolve.

CONCLUDING REMARKS

The use of molecular epidemiology has revolutionized population medicine and prevention and control efforts in foodborne, zoonotic, and livestock-associated diseases. The use of phenotype-based approaches such as serotyping, which has been instrumental in subtyping infectious agents into categories, has now largely evolved to the use of genotype-based approaches. As discussed in this review using several key examples, molecular biology tools are now regularly used in livestock and zoonotic disease outbreak investigations and surveillance systems as well as for research purposes. While molecular epidemiology was adopted slowly and late in veterinary settings, the increasing recognition of the discriminatory power of the tools and the improved cost of processing samples are expediting its utility. Such approaches have also enabled comparative assessment between isolates of human and animal origin, enabling rapid and effective tracking of outbreaks. Such investigations are crucial to track sources and make effective decisions in the prevention and control of zoonotic and livestock diseases.

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