

What has molecular epidemiology ever done for wildlife disease research? Past contributions and future directions

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Abstract The increasing availability of novel molecular techniques has transformed the study of human health and disease epidemiology. However, uptake of such approaches has been more conservative in the field of wildlife disease epidemiology. We consider the reasons for this and discuss current and potential applications of molecular techniques in a variety of relevant areas within the field of wildlife disease research. These include conducting wildlife disease surveillance, identifying sources of pathogen emergence, uncovering host-pathogen dynamics and managing current outbreaks, including the development and monitoring of wildlife vaccines. We highlight key examples of applications of molecular epidemiological approaches to wildlife disease scenarios and draw parallels from human disease research to suggest potential future directions. The potential value of next generation sequencing technologies to the field of wildlife disease research is discussed, and initial applications are highlighted, balanced against consideration of the challenges involved. Using a wide range of examples drawn from research into human, livestock and wildlife diseases, we demonstrate the value of using molecular epidemiological approaches at all

scales of wildlife disease research, from pathogen strains circulating at a global scale to intra-individual host-pathogen dynamics. The potential future contribution of these technologies to the field of wildlife disease epidemiology is substantial. In particular, they are likely to play an increasingly important role in helping us to address a principal challenge in the management of wildlife diseases which is how to tease apart the transmission dynamics of complex multi-host systems in order to develop effective and sustainable interventions.

Keywords Disease surveillance · Next generation sequencing · Wildlife vaccines · Wildlife reservoirs · Host-pathogen evolution · Disease transmission

Introduction

Since its emergence in the 1970s, the term ‘molecular epidemiology’ has appeared in a vast number of publications in a wide range of scientific disciplines. Initially, the term was used primarily in the study of human cancer to describe the process of identifying biomarkers within populations which improved identification of subgroups at greater risk of developing disease (Vineis and Perera 2007). However, the term is now widely used in the field of infectious disease biology, where it has been defined as involving ‘the various techniques derived from immunology, biochemistry and genetics for typing and sub-typing pathogens’ (Tibayrenc 1998). A broader definition that goes some way to capturing the breadth of the subject is ‘a science which utilises molecular biology to define the distribution of disease within a population and relies heavily on integration of traditional epidemiological approaches to identify the etiological determinants of this distribution’ (Snow 2011). The influence of molecular epidemiology in the field of human health research has been extensive. Ongoing surveillance of the spatio-temporal distribution of

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disease strains has helped uncover drivers of disease transmission (Liu et al. 2008), infer the geographic origin of pathogens (Hemelaar et al. 2011) and provided a baseline against which changes can be detected (Koopmans et al. 2000). In addition, transmission routes of zoonotic pathogens have been identified (Feng and Xiao 2011; Salyer et al. 2012), and the evolutionary provenance of pathogenic strains (Byrnes et al. 2010) and antibiotic resistance mechanisms (Kumarasamy et al. 2010; Hoffmann et al. 2007) have been pinpointed. Molecular technologies have also been widely used in the development of vaccines against human pathogens (Serruto et al. 2009; Santolaya et al. 2012; Seib et al. 2009) and in responsive investigations of disease outbreaks (Grad et al. 2012; Gardy et al. 2011; Rasko et al. 2011). In this review, we will explore similar applications of molecular technologies in the field of wildlife diseases and suggest directions for future applications.

Bibliometrics

A comprehensive literature search for journal articles published since 1980 using the term ‘molecular epidemiology’ revealed over 111,000 results. In order to consider the growth of molecular approaches in epidemiology, the proportion of molecular epidemiology articles within ‘epidemiology’ articles for the fields of clinical, livestock, zoonoses and wildlife research was calculated. Time series analysis and forecasting of publication trends illustrate the proportion of epidemiology papers incorporating molecular epidemiological approaches for each of the four categories (Fig. 1). When the overall numbers of molecular epidemiology publications within each field are considered, unsurprisingly, the vast majority fall within the field of human clinical research (Fig. 2). The application of molecular epidemiological techniques in livestock, zoonoses and wildlife disease research has steadily grown from the mid 2000s onwards, but such studies still represent only a small proportion of the total observed. The origin of the term in human clinical research is indicated by its earlier appearance in this field, filtering into the other fields shortly afterwards (Fig. 1). Time series forecasting indicates that the proportion of molecular epidemiology publications is predicted to increase relatively steeply in the fields of livestock and zoonotic research, but the trend is less certain in the field of wildlife disease research as indicated by the wider confidence intervals (Fig. 1).

The bias towards human disease is perhaps not surprising or unexpected given that molecular approaches were first developed in this field and human diseases naturally attract a greater level of attention and funding than diseases of animals. It may also reflect the greater focus on non-infectious diseases related to genetic or environmental factors in human systems, for example the large number of studies which employ

molecular epidemiological approaches to identify biomarkers associated with cancer. In general, diseases of livestock or wildlife have only been considered important when agriculture or human health is potentially threatened (Daszak et al. 2000), which is consistent with the smaller number of publications and conservative uptake of molecular epidemiological techniques in the field of non-zoonotic wildlife disease. There are also a range of practical challenges involved in any study of disease epidemiology in wild populations, including access to individuals, absence of validated diagnostic tests, logistics and costs of sampling, poor baseline surveillance and inherent uncertainty surrounding species ecology and behaviour (Delahay et al. 2008). Nevertheless, as we draw parallels with studies using molecular techniques in other fields, we will see that there are many potentially valuable applications of molecular methods to the epidemiology of disease in wildlife populations. In this review, we consider how molecular epidemiological approaches can help wildlife managers address key questions about disease dynamics, and we suggest directions and opportunities for their wider application in this field.

Molecular methods

Although DNA-based techniques have been in use for less than 50 years (Medini et al. 2008), during recent decades, a wide range of molecular techniques have emerged for the study of pathogens. One example is typing based on 16S ribosomal RNA (rRNA), in which the percentage of sequence similarity of rRNA molecules between samples is used to classify species (99 % sequence identity is used as the cut-off between separate species) (Medini et al. 2008). In alternative techniques, other genomic elements are sequenced and used as the basis of classification such as housekeeping gene fragments in multi-locus sequence typing (MLST) and enzyme profiles in multi-locus enzyme electrophoresis (MLEE) in bacteria. However, these systems can struggle to distinguish amongst very similar strains (Achtman 2001), such as members of the *Mycobacterium tuberculosis* complex (Frothingham 1995; Köser et al. 2012) or strains of *Bacillus anthracis* (Keim et al. 2001), the causative agent of anthrax.

The choice of an appropriate molecular typing technique requires an understanding of the genomic structure of the pathogen in question. Bacterial genomes consist of a core genome, common to all strains, dispensable genes which are not present in all strains and genomic islands, clusters of contiguous genes with a specialised function (e.g. virulence) (Relman 2011). Classical methods of classifying bacterial pathogens are based on phenotypic characteristics such as cellular structure (Medini et al. 2008), colony morphology (Baron 1996) and antibiotic susceptibility (Harwood et al. 2000). In contrast, viral pathogens contain small genomes, are highly diverse and some can evolve very rapidly (Fierer

Fig. 1 Time series analysis and forecasting of the proportion of ‘epidemiology’ journal articles that are ‘molecular epidemiology’ in the fields of clinical research, livestock research, zoonoses research and wildlife research. Based on ISI Web of Science search within topic field conducted in April 2014. Time series forecasting carried out using the ‘forecast’ package in R (Hyndman and Khandakar 2007)

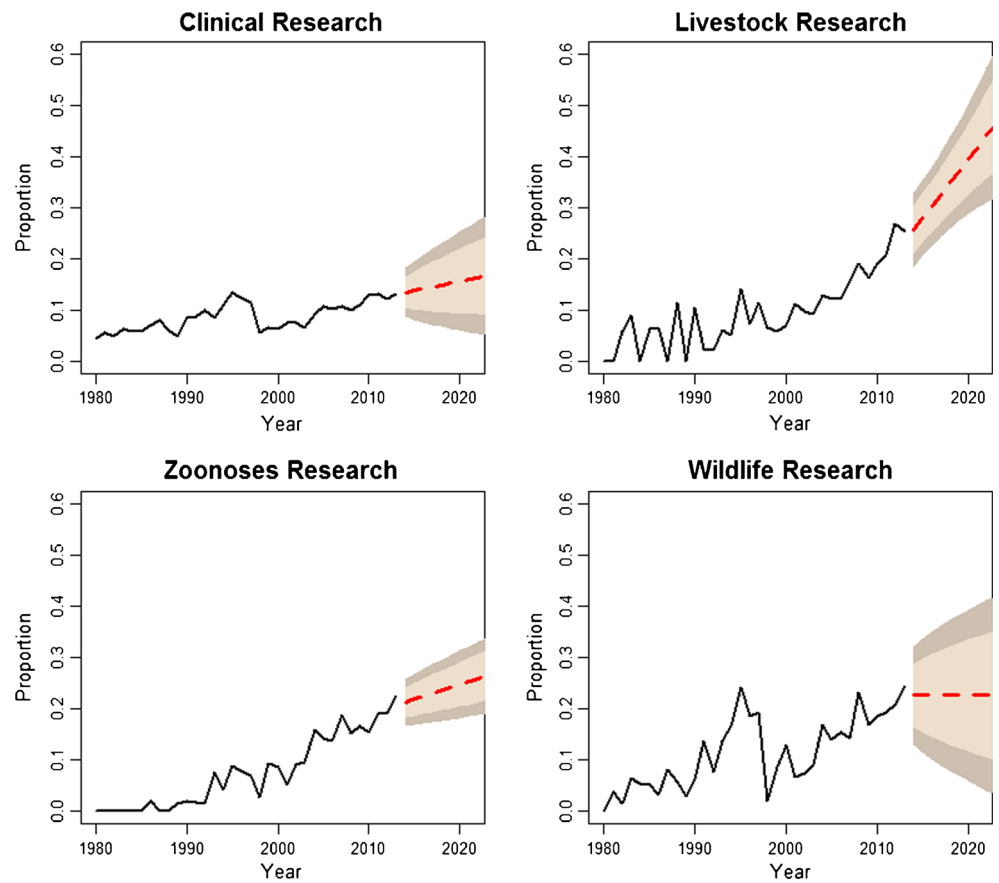
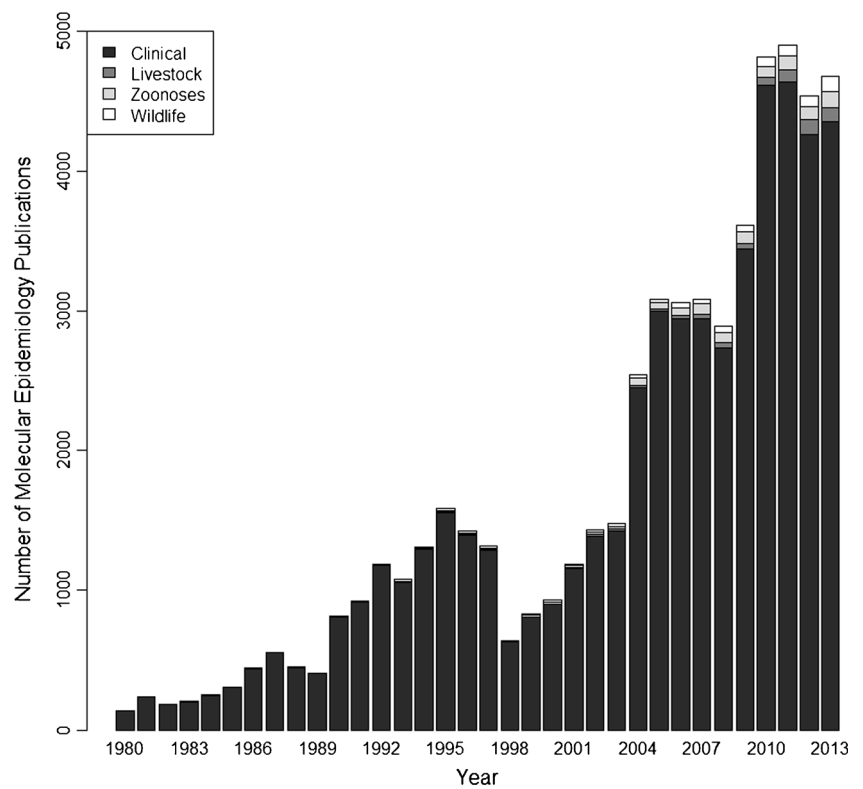


Fig. 2 Number of ‘molecular epidemiology’ journal articles published in the fields of clinical research, livestock research, zoonoses research and wildlife research. Search conducted using ISI Web of Science in April 2014



et al. 2007). For example, the ability of the influenza virus to rapidly change its antigenic profile requires continual development of vaccines (Relman 2011). Genetic diversity in fungal pathogens depends on (a) the mode of reproduction of the species, sexual or asexual, and (b) the presence of ‘transposable elements’, mobile genetic elements which can insert themselves within genes, changing their structure and function (Daboussi 1997). Protozoans such as the human pathogens *Trypanosoma brucei* and *Leishmania major* have genomes with species-specific surface antigens and variable strategies of invading hosts and evading immune responses. Conservation of gene order between species is high, indicating the presence of a strong selection pressure to conserve certain gene clusters and their associated function (El-Sayed et al. 2005).

The underlying genomic structure and diversity within a particular pathogen will influence the choice of typing method. All the typing techniques described above are fundamentally limited as they only examine a small section of the host genome. Depending on the biology of the pathogen, the section under examination will vary in the degree to which it is representative of the whole genome. For example, the typing methods traditionally used to categorize strains of *Mycobacterium bovis* (the causative agent of bovine TB) are spoligotyping (spacer-oligonucleotide typing) and variable number tandem repeat (VNTR) typing, both of which are based on small, hyper-variable genomic regions that are generally evolving at a higher rate than the rest of the genome (Joshi et al. 2012). Such methods are therefore potentially more useful for differentiating between species than detecting finer scale intra-specific variation, although this will depend largely on the genetic diversity within the particular pathogen complex under examination. Rapidly evolving viruses may generate sufficient diversity for intra-specific strains to be differentiated on the basis of more restricted areas of the genome than bacterial pathogens, but only by examining the complete genome of a species can finer genetic structuring be uncovered (Medini et al. 2008). The development of ‘next generation’ sequencing approaches, which base classification on the identification of single nucleotide polymorphisms (SNPs—single base substitutions); insertions or deletions that vary between individual genomes have facilitated the rapid sequencing of whole genomes, opening the door for studies which were previously impossible. In order to construct a phylogenetic tree from a group of sequenced isolates, phylogenetically informative SNPs (i.e. those shared by two or more isolates) are identified through the examination of the maximum sequenceable genome of each isolate. SNPs occur in both coding and non-coding regions, but those in the latter are less likely to exert a phenotypic effect and therefore are less likely to be affected by selective pressures. Hence, whole genome sequencing infers phylogenetic relationships from the maximum amount of genetic information available.

The current next generation sequencing (NGS) technologies are based on breaking the original genome into fragments, which are then extensively sequenced to yield short read sequences. These reads, which can then either be mapped to a reference genome (where present) or assembled against each other (‘*de novo*’ assembly) in order to identify potential SNPs. SNPs which successfully pass the required quality checks can then be used to produce phylogenetic trees and inform transmission models. The cost and required infrastructure for these technologies have so far limited their widespread uptake (Metzker 2010). However, costs are rapidly falling (Köser et al. 2012), uptake is increasing and the incorporation of NGS into routine human disease surveillance (Roetzer et al. 2013) and clinical diagnostics (Boyd 2013) appears to be imminent. Full sequence data has all the potential applications of strain typing but at a far higher resolution and gives the opportunity to determine the extent of the differences amongst strains rather than to simply distinguish them from one another. However, there are challenges in these approaches in relation to the storage, quality control and manipulation of the enormous amounts of data that they generate (Pop and Salzberg 2008). Also, lack of standardisation in bioinformatics protocols limits the extent to which sequences can be compared across laboratories (Köser et al. 2012) and technologies (Metzker 2010). Nevertheless, NGS has enormous potential to uncover fine-scale disease transmission dynamics, which may otherwise remain hidden to epidemiologists.

Disease surveillance

The importance of surveillance for wildlife diseases is well established (Delahay et al. 2008). Ongoing surveillance can act as an early warning system for outbreaks of new or emerging diseases, allowing pre-emptive management interventions and potentially helping to inform assessment of risk related to conservation interventions such as translocations of endangered populations (Artois et al. 2009).

It is important to assess the extent of genetic diversity within a pathogen population as this has implications for how refined molecular tools need to be in order to investigate disease transmission events. For example, with a low-diversity pathogen such as *Bacillus anthracis*, the most divergent strains are thought to be 99.99 % similar in terms of nucleotide sequencing (Rasko et al. 2011), and therefore, most isolates will appear homogenous, no matter how rigorous the sequencing method. In contrast, within the HIV-1 virus, there is a wealth of genetic diversity which is organised into ‘sub-types’ within which genetic variation can range between 8 and 17 % and can be as much as 35 % amongst sub-types (Hemelaar et al. 2011). Genetically diverse populations of the virus, termed ‘quasi-species’, can be harboured by a single host individual (Pandit and Sinha 2010). Only through

ongoing surveillance of circulating strains can the intrinsic genetic diversity of a pathogen be captured.

Ongoing surveillance of strain diversity can inform the development of diagnostic tests which may be employed for the identification and potentially selective removal of infected individuals in livestock and wildlife populations. The removal of test positive individuals could potentially exert a selection pressure on pathogen populations, with selection favouring strains that produce a weak or negative diagnostic test response. Hence, diagnostic test development is ideally an ongoing process, which seeks to keep one step ahead of such selection pressure. An example from human health is that of *Neisseria meningitidis*, the bacterial cause of meningitis A. Molecular approaches indicate that within this bacterial complex, there are a number of clonal groups, some of which cause disease and others which live commensally within human hosts (Achtman 2001). Horizontal gene transfer between group members can generate genetic diversity. The identity of the most frequent genotype in a population can vary as changing forces of selection favour different strains (Achtman 2001) with clear implications for the design of effective vaccines.

Comparison of pathogen genotypes in regions where infection is endemic versus those where infections only occur sporadically may uncover genetic differences associated with the two scenarios, related for example to pathogenicity factors. Molecular typing may also have important applications in detecting the emergence of new pathogenic strains in populations. In the case of bacteria, the jump from benign to pathogenic could potentially occur relatively rapidly, through the acquisition of a genomic island which codes for a pathogenicity factor (Hacker and Carniel 2001) and molecular typing may aid the detection of such events.

Phylogeography

Molecular techniques are widely used to describe the spatio-temporal distribution of variant pathogen strains. For example, the characteristic home ranges of *M. bovis* genotypes in cattle have been mapped across the affected areas of the UK (Smith et al. 2003). Routine mapping of this kind may identify the appearance of atypical strains in an area; this may indicate that a 'novel' transmission event has occurred (e.g. the translocation of an infected host animal from another region). Geographic differences in virulence between pathogen strains may also occur, as has been identified for the fungal pathogen *Cryptococcus gatti* (Byrnes et al. 2010). Spatial mapping exercises can also tell us something about the evolution of pathogen strains as geographically dispersed genotypes may be considered more likely to be ancestral strains than those with a restricted home range (Smith et al. 2003). Examining the prevalence of disease in a region can also be used to infer

risk factors which could inform management strategies. Incorporating molecular information into these investigations can provide greater insight into possible causes than simply comparing populations with and without disease (Cowled and Ward 2012).

By examining the strains that are appearing at the moving edge of an epidemic front, it may be possible to gain insights into the factors that are driving disease spread. For example, molecular epidemiology may be a useful tool in determining the proximate causes of new cases of bovine tuberculosis infection in UK cattle at the fringes of the endemic areas, helping to distinguish whether infection is seeded from livestock movements or the presence of infected wildlife. A very different example, focused on conservation of a highly threatened species, is provided by devil facial tumour disease (DFTD) in Tasmanian devils (*Sarcophilus harrisii*), where identifying the location of the disease front has informed management options. Geographic differences have been noted in the epidemiology and population effects of DFTD on devils. Genotyping techniques could be applied to suggest whether this variation is a result of functional sequence variations between strains or differences in disease resistance alleles between populations (Hamede et al. 2012).

When phylogenetic trees of a particular pathogen are overlaid with epidemiological data (such as geographic location of outbreaks), they can be used to map spatial disease spread. This can help epidemiologists infer where transmission events have occurred and therefore potentially to predict and manage future disease risks. For example, examining the geographic localisation of strains of *Mycobacterium leprae*, the bacterial cause of leprosy in humans indicated that global disease spread was most likely linked with historic human migration patterns and trade routes (Monot et al. 2009). Epidemiological linkages between particular populations or geographic locations can be identified if shared genotypes are recorded more often than would be expected by chance (Archie et al. 2009). Host geography has also been found to play a role in rates of pathogen evolution. In the case of *Lyssavirus* (rabies) in bat populations, rates of viral evolution by nucleotide substitution vary depending on whether the host species is in a temperate or tropical environment, which may be related to differences in the seasonality of bat activity and the influence of climate on rates of virus transmission (Streicker et al. 2012). Examining pathogen phylogenies can provide an understanding of rates of new strain emergence, helping epidemiologists to predict and prepare for new disease outbreaks. Also, where transmission rates vary between strains of the same pathogen, either due to differences in infectivity amongst strains or the availability of susceptible hosts, this could be identified through considering rates of spread. Phylogeographic investigations have been conducted on a wide range of human pathogens, including the zoonotic bacteria *Yersinia pestis* (Vogler et al. 2011), dengue virus (Nunes et al. 2012) and

influenza (Cheng et al. 2012). In the case of vector-borne diseases, the same approach can be used to investigate vector distributions, as carried out in a study of *Triatoma infestans*, the primary insect vector of Chagas disease (Perez de Rosas et al. 2011). Phylogeographic approaches have also been used, albeit to a lesser extent, in wildlife and livestock diseases, for example to consider the ecological drivers behind foot and mouth disease (FMD) in cattle (de Carvalho et al. 2013), rates of viral evolution driving infectious bursal disease virus in farmed poultry (Cortey et al. 2012) and the role of the global expansion of fish farming in the spread of salmonid proliferative kidney disease (Henderson and Okamura 2004).

Roots of emergence

The construction of pathogen phylogenetic trees has made an enormous contribution to the study of human disease, leading to the emergence of the field of evolutionary medicine (Bull 1994). Virulence is known to differ amongst pathogen strains, and this variation is the result of evolutionary processes. Genetic signatures in pathogen phylogenies allow us to look back at the underlying ecological selection pressures which have previously been exerted on a pathogen and shaped its evolution (Biek and Real 2010). Correct inference of ancestry (i.e. determining which strains of a particular pathogen are ancestral and which are descendant) is key to building a clear picture of pathogen population structures (Medini et al. 2008). For example, the population structure of *Mycobacterium bovis* genotypes in the UK suggests a ‘clonal expansion’ of genotype evolution from a common ancestor, through a combination of selection and ‘ecological opportunity’ as invasion into new geographic areas occurred (Smith et al. 2003). Inferring ancestry is also extremely valuable for dating disease transmission events and tracing cross-species transmission in multi-host disease complexes, such as SIV/HIV and hepatitis B in humans and non-human primates (Neel 2010; Starkman et al. 2003). If a pathogen has been transmitted from one species to another, the phylogeny within the recipient species should be nested within that of the source species (Archie et al. 2009). Disease introduction through migration or translocation events can be suggested where there is a genetic mismatch with resident strains, as was recently been inferred for some species of blood parasites in wild birds in Japan where strains of *Leucocytozoon* from migratory and resident birds were phylogenetically separated (Yoshimura et al. 2014). Hence, phylogenetic investigation can be used to identify risk factors for future disease outbreaks.

A substantial body of work exists where whole genome sequencing has been applied to the study of human viral pathogens, such as influenza (Holmes et al. 2005) and HIV (Henn et al. 2012), and in recent years, this approach has also been applied to investigations of viral pathogens in wildlife,

including the detection of highlands J virus in a critically endangered species of crane (Ip et al. 2014), the development of a genome database of orbiviruses (Maan et al. 2013) and an investigation into encephalitis cases in captive polar bears (Szentiks et al. 2014). Work is currently underway to determine the phylogeny of the pathogenic fungus *Geomyces destructans*, the causative agent of white-nose disease in bats (Blehert 2011), with a draft sequence recently published (Chibucos et al. 2013). A number of pathogens of veterinary importance have had at least one isolate sequenced, including African swine fever virus (Chapman et al. 2011), *Mycoplasma haemofelis* (the causative agent of feline infectious anaemia) (Barker et al. 2011) and *Streptococcus equi* (Paillot et al. 2010), although these studies have focused primarily on describing pathogenicity factors, rather than epidemiological outcomes.

The potential for next generation sequencing to infer the origin and population structure of veterinary and wildlife pathogens is substantial. We may expect uptake to be initially greater in the fields of zoonotic and livestock diseases, where the potential human ‘cost’ is perceived to be higher. As illustrated above, phylogenetic approaches offer so much more than an opportunity to delve backwards into the evolutionary history of a pathogen. They can also help us to understand the drivers of the current distribution of pathogens and help us predict their likely distribution in the future.

Routes of transmission

When investigating the dynamics of infection in a given host population, we reasonably assume that transmission is more likely to have occurred between individuals infected with the same strain of a pathogen than amongst those infected with different strains (Wylie et al. 2005). Pathogen genotyping can therefore help to rule out or implicate particular transmission pathways, which may be valuable in tracing the initial source of infection and preventing further disease spread. The availability of next generation sequencing technologies has allowed contact networks and transmission pathways to be inferred with greater confidence and accuracy (Gardy et al. 2011). Given the relatively recent availability of these technologies, and their decreasing cost, their full potential in the field of human health has yet to be realised (Walker et al. 2013), and to date, their use in relation to livestock and wildlife diseases has been limited. However, there are notable examples such as studies of TB in cattle and badgers in the UK (Biek et al. 2012), brucellosis in livestock and wildlife (Foster et al. 2009) and MRSA in livestock (Price et al. 2012). In studies of human pathogens such as *M. tuberculosis* (Cook et al. 2007; Gardy et al. 2011; Walker et al. 2013), MRSA (Harris et al. 2013), *Clostridium difficile* (Eyre et al. 2013) and *Chlamydia trachomatis* (Wylie et al. 2005), genotyping of pathogenic isolates has informed contact tracing, suggested

the existence of undetected carriers and helped to both construct and verify the conclusions of social network analysis of disease outbreaks. Clinical disease outbreaks in human populations are often treated on a ‘case by case’ basis, on the understanding that no two events are epidemiologically identical. On the other hand, wildlife disease managers are often called upon to use simple management strategies to tackle disease in multiple socially structured populations, without information on the particular transmission dynamics in each situation. The overlaying of data on pathogen strain diversity onto ecological information could be used in wildlife populations to assess transmission rates in relation to population structure (e.g. social groups and herds). In the case of the European badger, the prevailing social structure in high-density populations has been associated with the clustering of infection within social groups (Delahay et al. 2000). Disruption of this social structure, as observed following culling, leads to a reduction in this clustering, as surviving individuals range more widely (Jenkins et al. 2007). Further information on the role of social behaviour in the spread of infection may be achievable by investigating the genetic diversity of *M. bovis* strains in badger populations. If social structure acts as a barrier to disease spread, then we would expect the degree of relatedness amongst *M. bovis* strains within badger social groups to be greater than that observed amongst social groups. Wherever wildlife is implicated as a reservoir of zoonotic and/or livestock disease, such approaches may be valuable in identifying chains of disease transmission between species and could potentially indicate the direction of disease transmission.

In order to make meaningful inferences about transmission dynamics, a pathogen must be acquiring mutations within an epidemiologically meaningful timeframe, and the genotyping method applied must have the ability to detect this variation (Grad et al. 2012). Epidemiologists studying pathogens with very little variation between strains will require a typing method that is able to detect small differences between isolates. Where discrimination between isolates is not possible using conventional methods, whole genome sequencing (WGS) may be the only tool suitable for looking at fine-scale transmission dynamics. The exceptionally high level of genetic resolution achievable using WGS means that even sequencing a restricted number of isolates can reveal a wealth of epidemiologically valuable information. Where access to long-term wildlife studies is possible, a ‘phylogenetic’ approach (Grenfell et al. 2004) of overlaying pathogen phylogenies onto well-documented epidemiological systems is potentially very powerful. Novel molecular approaches are not a replacement for traditional epidemiological investigations but are complementary, allowing a finer scale approach. For this reason, long-term, well-studied epidemiological systems are the ideal scenarios in which to explore the contribution of cutting edge sequencing to uncovering the drivers of disease

transmission. Examples of such well-studied systems include TB infection in wild badgers (Delahay et al. 2000) and meerkats (Drewe 2010), chronic wasting disease in white-tailed deer (Williams et al. 2002) and DFTD in Tasmanian devils (Hamede et al. 2009). It is important to note, however, that, even with the added resolution provided by WGS, there are considerable challenges to identifying pathogen transmission chains. The point at which mutations are acquired in a given transmission sequence is unknown, and when mutation rates are slow compared to pathogen generation time, closely related isolates may appear genetically identical as they lack informative mutations (Kao et al. 2014).

Host-pathogen dynamics

Pathogens can have widely differing effects in different host species, as is the case for the squirrel parapox virus which causes severe disease in the European Red Squirrel (*Sciurus vulgaris*) but has no observed effects on the North American Grey Squirrel (*Sciurus carolinensis*) (Sainsbury et al. 2000). Variability in the observed costs of pathogen infection has also been observed amongst individuals of the same species. Heterogeneities in susceptibility to infection among individuals can affect the estimation of the transmission parameter R_0 (the basic reproductive number) (Hudson et al. 2002). In such instances, molecular techniques may allow us to distinguish between differences in pathogenicity which arise from strain variation and those which reflect heterogeneity in host immune responses. Scaling up these effects can impact on host population dynamics as regulation by a pathogen requires its *per capita* impact to outweigh the intrinsic population growth rate (Hudson et al. 2002). If the *per capita* impact on host fitness is widely variable amongst individuals, then inferring population regulation is more complicated. Variation in how a pathogen physiologically affects individuals within a population has implications for onward transmission and persistence of disease (Cross et al. 2005). For example, inter-individual variation in the amount or concentration of pathogenic material excreted and the duration over which this occurs is likely to affect the number of secondary cases observed. Ignoring this individual heterogeneity and assuming that each infected individual contributes to the same number of secondary infections can lead to highly inaccurate estimations of R_0 . Molecular techniques can also allow us to examine individual variation in susceptibility and resistance within a host population by assessing the genetic basis of the immune response: an approach known as immunogenetics. Individuals with greater allelic diversity within immune genes such as the major histocompatibility complex (MHC) are able to mount an appropriate immune response against a greater variety of pathogens (Castro-Prieto et al. 2012). Accounting for heterogeneity in individual susceptibility and for differential strain

pathogenicity is likely to allow R_0 to be estimated with a greater degree of accuracy.

It has been suggested that immunogenetic data should be used more widely to complement wildlife management decisions, particularly where populations are restricted or fragmented with a limited gene pool, as is often the case for highly endangered species (Acevedo-Whitehouse and Cunningham 2006). A key example of this approach is investigation into the spread by biting of a contagious cancer amongst Tasmanian devils which is thought to have caused a 90 % population decline (Siddle et al. 2010). The absence of an immune response in infected devils is thought to be linked to the limited genetic diversity within their MHC complex (Siddle et al. 2007). Examination of MHC genetic diversity within devil populations in other areas of Tasmania, where the disease is absent or at low prevalence, have identified some unique profiles which may confer disease resilience. If this were the case, then selective breeding and translocation of resilient individuals has been suggested as a means of controlling disease spread (Hamede et al. 2012). In contrast, as MHC profiles are likely to be adapted to local pathogenic selection pressures, a poor choice of origin or destination could leave a translocated individual unable to cope with a different pathogen community and so at a selective disadvantage (Castro-Prieto et al. 2012). Hence, the application of sequence-based approaches to assess the immunogenetic characteristics of populations of endangered species may have the potential to increase the likelihood of successful translocation (Boyce et al. 2011).

Molecular epidemiology also allows us to zoom in to an even finer scale than that of inter-individual variation, and to consider intra-individual host-pathogen dynamics. Infectious pathogens persist in the context of a co-evolutionary arms race with the host (Acevedo-Whitehouse and Cunningham 2006) which can be considered as a habitat ‘patch’ occupied by a parasite ‘community’ (Hudson et al. 2002). Where an individual host is infected with multiple strains of the same pathogen, strain competition can occur, with certain strains favoured owing to their faster growth rate or ability to grow in a certain tissue (Bull 1994). It is interesting to consider, however, that the traits which allow a particular strain to dominate within the host environment may not necessarily optimise onward transmission although they may increase pathogen virulence (Bull 1994). However, outcomes of multiple infection on evolution of virulence and subsequent effects on individual host fitness are variable (Rankin et al. 2007). The application of suitable molecular techniques to detect multiple strains of a pathogen within a host and to detect within-host pathogen strain evolution or strain competition may have important implications, as for example the scale of competition between bacterial strains is thought to influence the evolution of virulence (Griffin et al. 2004). Comparative genomics studies, in which the aim is to link genetic sequence differences between strains with

phenotypic differences in the host (e.g. differential pathogenicity), have acquired valuable additional resolution from the development of next generation sequencing technologies (Hu et al. 2011).

Understanding variations in the impact of pathogens both amongst and within individuals may be critical to achieving effective management at the population level. Furthermore, disregarding heterogeneity in host responses and failing to acknowledge within host-pathogen dynamics (such as the role of multiple infection) may result in unexpected, potentially adverse, outcomes of management interventions. Molecular approaches have much to offer at both scales of analysis.

Vaccine development and monitoring

Vaccination is currently being used or considered as a management option in several high profile wildlife disease scenarios, including the control of bovine TB in badgers in the UK (Chambers et al. 2010; Carter et al. 2012), chlamydia in koalas in Australia (Carey et al. 2010), haemorrhagic disease and myxomatosis in rabbits in Europe (Spibey et al. 2012). Molecular epidemiology has a key role to play in the development of effective vaccines for wildlife and monitoring their impacts on disease epidemiology. Vaccine development against human pathogens has greatly benefited from technological advances in gene sequencing, and now, the sequences of many pathogens are available. This has led to the emergence of the field of ‘reverse vaccinology’ which typically involves mining the pathogen sequence for antigens that may be suitable as vaccine targets (Serruto et al. 2009). In pan-genome reverse vaccinology, multiple isolates of a pathogen species are considered. This is based on the idea of the existence of a ‘pan-genome’, which acknowledges that any single isolate of a pathogen does not exhibit all the genetic diversity within that species, especially if they are capable of generating genetic diversity through recombination or horizontal gene transfer. Consequently, it is necessary to sequence multiple genomes in order to get a better measure of the entire genomic repertoire of a species (Tettelin et al. 2008). In comparative reverse vaccinology, sequences of pathogenic strain isolates are compared with those of non-pathogenic isolates of the same species, in order to identify antigens associated with pathogenicity. The first human pathogen for which a vaccine has been developed and recently licensed using this approach is serogroup B meningitis (*N. meningitidis*), responsible for 80 % of meningitis cases in Europe (Santolaya et al. 2012).

As well as informing the development of vaccines, genome sequencing technologies also have applications for monitoring the effectiveness of vaccine deployment. As an increasing proportion of a population is immunised, the selection pressure favouring strain variants that are unaffected by vaccination will grow. The emergence of these strains, known as

‘escape mutants’, could be monitored by sequencing isolates before and after vaccination to look for new mutations related to immunity in the targeted proteins (Seib et al. 2009). This sort of approach could be extremely valuable in monitoring the impacts of vaccination in wildlife populations. Comparing the genetic diversity of pathogen populations before, during and after vaccine deployment could provide valuable information on the potential emergence of resistant strains or differential vaccine performance against variant strains. Strain typing could also help monitor reversion to virulence of live vaccines, as vaccination has on occasion been observed to result in clinical disease. This was recently reported in a red fox (*Vulpes vulpes*) in which strain typing was able to identify the live rabies vaccine as the aetiological agent (Hostnik et al. 2014). Live vaccines, such as the oral rabies vaccine, may be derived from multiple strains. Genetic characterisation of these strains can uncover the genetic basis for their attenuation (Geue et al. 2008). Population coverage of vaccines which may be horizontally transmissible within a population (Angulo and Juan 2007) could also be monitored using molecular diagnostics.

Understanding the antigenic diversity of a pathogen is key in vaccine design and is only possible through ongoing surveillance as the most frequent antigenic strain of a pathogen in a population may change in response to the selection pressure of immunisation (Achtman 2001), favouring new antigenic types which are able to evade the acquired immunity of the host (Bull 1994). Pathogens such as HIV, malaria and influenza have particularly high antigenic diversity (Buckee et al. 2011). A vaccine must either induce cross-immunity to all antigenic strains of a pathogen circulating within a population, or different vaccines may be required according to which antigenic strain is predominant in a given situation. Different strains of a pathogen may also vary in terms of the magnitude or type of immune response invoked (Wedlock et al. 2007). In the case of human seasonal influenza, it has been suggested that it is the changing immune response within the host population which creates the conditions for the emergence of the next dominant strain (Recker et al. 2007). Molecular typing approaches offer powerful tools for furthering our understanding of antigenic diversity in wildlife populations and the role of vaccination in disease control.

Identifying reservoirs

Another key challenge for wildlife managers is identifying populations that may act as reservoirs of infection for livestock, humans or other wildlife of conservation or economic importance. Assessment of the risks of onward spread requires a clear understanding of transmission dynamics within and amongst the species concerned (Hudson et al. 2002). Disease reservoirs can potentially be composed of one or more

epidemiologically connected populations or environments where the pathogen can be permanently maintained (Haydon et al. 2002). Molecular techniques may be of value in inferring transmission routes amongst multiple host species, although confusion can arise if pathogens are capable of remaining infectious in the environment. Inter-specific transmission has been inferred through strain comparison of *Giardia* (Feng and Xiao 2011) and *Cryptosporidium* in human and animal hosts (Xiao and Ryan 2004). Transmission between wildlife and commercially important livestock can also be inferred through comparing pathogen genotypes, as demonstrated for bovine TB in cattle and badgers in the UK (Goodchild et al. 2012; Biek et al. 2012; Woodroffe et al. 2005) and for *Babesia bovis* and *B. bigemia*, the bacteria responsible for cattle tick fever in white-tailed deer in the USA (Holman et al. 2011).

Molecular typing techniques provide valuable insights into multi-host systems when considering populations of conservation concern. Examples include hookworm and feline leukaemia virus transmission from domestic cats (*Felis catus*) to the critically endangered Iberian lynx (*Lynx pardinus*) (Millan and Blasco-Costa 2012; Meli et al. 2009) and transmission of canine parvovirus and rabies from domestic dogs (*Canis lupus familiaris*) to endangered African wild dogs (*Lycaon pictus*) (Woodroffe et al. 2012). Strain typing of pathogens can also indicate the presence of an undetected wildlife reservoir, or even multiple reservoirs, where strain diversity appears too high to have been generated by mutation alone. However, in order to make such assessments, a sufficient number of samples should ideally be available from all host species in the system under study, and any host-related variation in pathogen mutation rates should be known (Kao et al. 2014).

Management strategies

One of the greatest challenges faced by wildlife disease managers is unpredictability in the outcome of interventions. This is in part due to the fundamental challenges of working with free-ranging wildlife, but is exacerbated by a lack of reporting when unintended outcomes occur, which has limited the degree to which we can learn from past interventions (Lloyd-Smith et al. 2005). Coupling genetic information from hosts and pathogens, with ecological factors, can help to predict patterns of disease emergence, spread and control (Biek and Real 2010). Employing molecular approaches can help managers to monitor the epidemiological impacts of interventions with a potentially high degree of resolution and hence allow a more informed approach to refining management actions. Where a novel or re-emerging pathogen appears and wildlife populations are implicated, either as the reservoir or target of disease, managers may be called on to advise on potential interventions. Rapid molecular typing can quickly reveal a wealth of information about a disease outbreak and help to

identify true transmission events, trace individual contacts and identify the true source of a particular pathogen. In the field of public health, molecular strain typing has been used to trace the source for outbreaks of a wide range of pathogens including *E. coli* (Grad et al. 2012), TB (Gardy et al. 2011), pneumonia (Snitkin et al. 2012) and even deliberately introduced pathogens associated with bioterrorism (Rasko et al. 2011). Molecular epidemiological investigations during an outbreak can also suggest the existence of undetected carriers through using pathogen phylogenies in association with social network analysis, as conducted in investigations of human TB outbreaks (Walker et al. 2013) and can help identify super-spreading individuals who make a disproportionately large contribution to secondary infections (Woolhouse et al. 1997). Through the comparison of multiple isolates from the same host individual over time, pathogen micro-evolution can be examined (Gardy et al. 2011). Understanding the rate at which a pathogen can acquire mutations has important implications for choosing appropriate diagnostic tests, predicting the emergence of new strains and informing potential intervention strategies, such as vaccination. Comparison of pathogen strains prior to and during an outbreak can indicate whether the epidemic is due to a genetic change in the pathogen or rather to some social or environmental trigger (Gardy et al. 2011). The genetic diversity amongst isolates associated with a particular disease outbreak can also provide information about the size of the initial infection; limited diversity among isolates may indicate that a population bottleneck has occurred, suggesting that the outbreak could have been caused by few initially infected individuals (Grad et al. 2012). However, this requires pre-existing knowledge regarding what level of diversity is typical for that pathogen, which highlights the importance of ongoing disease surveillance.

Molecular epidemiological investigations have been carried out on a wide range of disease outbreaks in livestock, including Newcastle disease in poultry (Gould et al. 2001), FMD in cattle (Cottam et al. 2006) and bluetongue virus in sheep (Maan et al. 2004; Barros et al. 2007). Of these, only the investigation of FMD employed complete genome sequencing. Examples of molecular epidemiological investigations in wildlife include studies on outbreaks of phocine distemper in seals on the Danish coast (Line Nielsen et al. 2009), salmonella in passerines (Hernandez et al. 2012), viruses of anthropogenic origin in protected ape populations (Köndgen et al. 2008) and the source of DFTD in Tasmanian devils (Murchison et al. 2012).

A major wildlife disease outbreak which represents a real threat to global biodiversity is the recent emergence of amphibian chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis*. This pathogen has been isolated from all continents where its hosts are found (Fisher et al. 2009) and is thought to be the principal cause of decline in over 200 species of amphibian. There is substantial variation

in observed host responses to infection with some species appearing to be resistant whilst others succumb quickly to its lethal effects, and virulence has been found to vary amongst strains (Blaustein et al. 2005). The full genomes of two geographically diverse chytrid isolates were sequenced and used to identify areas of variation within the genome. Low sequence diversity was observed between the two isolates, but genomic areas with some variation were targeted by multi-locus sequence typing of a global set of chytrid isolates which were used to create a phylogenetic tree, illustrating the geographic origin of each isolate and its host species (James et al. 2009). From examining the tree, it was apparent that all the isolates could feasibly have originated from a single clonal lineage as, in one host animal, the same chytrid sequence diversity existed as was found in the whole global sample (James et al. 2009). This molecular signature is consistent with the rapid spread of a novel pathogen, and hence, movement of animals for trade purposes has been suggested as a potential explanation for its current global distribution (Fisher et al. 2009).

Combining molecular epidemiological approaches, in particular high resolution sequencing, with traditional epidemiological techniques may be a powerful approach in disease outbreak investigation. This is made even more powerful where data is also available from background pathogen surveillance.

Discussion

The value of molecular epidemiology in the study of human disease is well-established. We now have at least one complete genomic sequence for nearly all bacteria responsible for human disease. An extraordinary amount of genetic diversity has been uncovered, including variation from within clonal cultures (Medini et al. 2008). Phylogenetic tools can be applied to genetic sequence data within open source packages such as BEAST (Drummond et al. 2012) providing powerful insights into pathogen spread within host populations. Also, pathogen sequence data can improve the performance of disease transmission models by reducing the number of candidate transmission trees (Kao et al. 2014), as pathogen genetic data is integrated with epidemiological data to inform transmission model construction within a Bayesian framework (Jombart et al. 2014). Despite the particular challenges involved in applying molecular technologies in the field of wildlife disease, the emergence of a number of high profile zoonotic diseases and dramatic declines in the abundance of some wildlife populations in recent years have raised awareness of this area of study (Daszak et al. 2004) and the application of cutting edge molecular tools is increasing.

The application of molecular technologies poses significant challenges even when used in tandem with traditional

Table 1 Summary of applications of molecular epidemiology to wildlife disease research, including key examples

Application	Summary	Examples of use in wildlife
Disease surveillance	Ongoing surveillance of circulating pathogen strains can help to capture the intrinsic genetic diversity of the pathogen, informing the use and development of appropriate diagnostics	Avian influenza viruses in wild birds (Hoye et al. 2010) Bovine tuberculosis in wildlife (Romero et al. 2008)
Phylogeography	Spatio-temporal mapping of variant pathogen strains can highlight the appearance of atypical strains in an area, identify geographic differences in pathogen virulence and help to infer risk factors	Devil facial tumour disease (Hamede et al. 2012) Rabies virus (Streicker et al. 2012) Foot & Mouth Disease (de Carvalho et al. 2013)
Roots of emergence	The construction of phylogenetic trees from genotype data can indicate which pathogen strains are ancestral and which are descendant. This can be useful in dating disease transmission between populations and multiple host species.	<i>Leucocytozoon</i> blood parasites in wild birds (Yoshimura et al. 2014) White Nose Disease in bats (Chibucos et al. 2013) Orbiviruses (Maan et al. 2013)
Routes of transmission	Pathogen genotyping can help to rule out or implicate particular transmission pathways, which may be valuable in tracing the initial source of infection and preventing further disease spread	Bovine TB in badgers and cattle (Biek et al. 2012) Brucellosis (Foster et al. 2009)
Host-pathogen dynamics	Molecular techniques can allow us to examine individual variation in susceptibility and resistance within a host population by assessing the genetic basis of the immune response	Devil facial tumour disease (Siddle et al. 2010) MHC differentiation in Namibian cheetahs (Castro-Prieto et al. 2012) Bovine TB resistance in wild boar (Acevedo-Whitehouse and Cunningham 2006)
Vaccine development and monitoring	Molecular epidemiology has a key role to play in the development of effective vaccines for wildlife and monitoring their impacts on disease epidemiology	Reversion to virulence of rabies vaccine strain (Hostnik et al. 2014) Oral rabies vaccine strains in wildlife (Geue et al. 2008)
Identifying reservoirs	Molecular techniques may be of value in inferring transmission routes amongst multiple host species. Strain typing of pathogens can also indicate the presence of an undetected wildlife reservoir, or even multiple reservoirs, where strain diversity appears too high to have been generated by mutation alone	Rabies in African wild dogs (Woodroffe et al. 2012) Feline Leukaemia Virus in Iberian lynx (Millan and Blasco-Costa 2012), (Meli et al. 2009) Cattle Tick Fever (Holman et al. 2011)
Management strategies	Employing molecular approaches can help managers to identify the sources of disease outbreaks and to monitor the epidemiological impacts of interventions with a potentially high degree of resolution	Phocine Distemper in seals (Line Nielsen et al. 2009) Salmonella in passerines (Hernandez et al. 2012) Viruses in protected ape populations (Köndgen et al. 2008)

epidemiological approaches. Techniques such as whole genome sequencing produce huge amounts of data which can be expensive to store and computationally costly to handle. However, the availability of online ‘cloud’ storage provides a potential solution (Baker 2010), and as uptake of these technologies increases, we can expect further developments in data storage and handling capabilities. DNA amplification required for next generation sequencing can introduce sequencing errors and a lack of standardised quality control procedures between laboratories can add uncertainty to sequence data (Kao et al. 2014). Particular challenges for the application of molecular approaches in wildlife populations include the presence of multiple hosts and the possibility of environmental persistence of the pathogen. Such circumstances mean that even whole genome sequencing cannot pick out individual transmission pathways as there will often be multiple routes by which the same pattern of genotypes could have arisen.

Future developments in molecular technology could have exciting applications in the field of wildlife disease. Rapid, field sequencing of isolates from populations and their environment (for example using hand-held

sequencers) could allow a ‘forensic’ approach to investigating disease outbreaks, in which localised management might be tailored to the particular source of infection. This could be useful for example in the case of bTB in UK cattle, where the source of infection is likely to vary widely between herds and geographic locations. In the field of human health, interest is growing in ‘precision medicine’ whereby the entire human genome of a patient is sequenced and a tailored health plan produced based on the patient’s particular genetic composition. It is conceivable that human genome sequencing will become a routine procedure at birth, allowing the development of ‘personalised programmes of lifelong health promotion’ (Tonellato et al. 2011). As we have seen by considering a variety of examples above, technological advances first developed in the field of human health are subsequently employed in livestock and wildlife. It is plausible therefore that as sequencing costs fall, individual level, genome tailored approaches may become attractive for the management of disease in wildlife species of very high conservation value. The management of DFTD in individual Tasmanian devils might be a case in point (Table 1).

Developments in the field of metagenomics, in which multiple microbe genomes could be sequenced directly from environmental samples (Doolittle and Zhaxybayeva 2010), may provide valuable tools for wildlife disease management and research. Such an approach could be used to screen for pathogens prior to translocation of threatened species or for clarifying transmission routes where a pathogen can persist in the environment. The latter would for example be useful in studies of bTB transmission amongst wildlife and livestock, where there is a potential role for environmental contamination with *M. bovis* (Duffield and Young 1985; Vicente, in press).

In summary, molecular technologies allow us to consider pathogens at a wide range of spatial and temporal scales: from individual host-pathogen dynamics, to global patterns of strain diversity. Following their emergence in the field of human health, they have begun to be adopted for the purposes of investigation and management of disease in wildlife. At the present time, these tools have a range of applications in wildlife disease research from the local investigation of disease outbreaks to unearthing the evolutionary history and global spread of pathogens. The potential future contribution of these technologies to the field of wildlife disease epidemiology is substantial. In particular, they are likely to play an increasingly important role in helping us to address a principal challenge in the management of wildlife diseases which is how to tease apart the transmission dynamics of complex multi-host systems in order to develop effective and sustainable interventions.

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