"Next generation" surveillance: An epidemiologists' perspective on the use of molecular information in food safety and animal health decision making.

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The increasing use of molecular typing is transforming surveillance and the control of diseases in people and animals (Kao et al. 2014; Struelens & Brisse 2013). Molecular and genomic data are becoming increasingly available and affordable and completely new technologies have emerged in recent years, all with their specific advantages and disadvantages (Sabat et al. 2013). Furthermore established technologies such as pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST), which itself has only emerged in the last decade, are being replaced by whole genome sequencing (WGS). Advances in genetic and genomic characterisation create remarkable possibilities for health, in particular where typing data are combined with other, epidemiological data such as individual characteristics including demographic data, exposures, time, and geographic information (Goering et al. 2013). Overall molecular typing complements traditional epidemiological data (EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards) 2013) and can support both control- and strategy-focussed surveillance, i.e. surveillance aimed at the detection of outbreaks that require a specific response or surveillance providing information for preventive public health action (Muellner et al. 2013). In outbreak investigation, for instance, the use of molecular markers can substantially improve the precision of case definition. This, in return, can reduce misclassification bias and increase the likelihood of identifying controllable risk factors, thus improving power of the study (Höfler 2005). The routine collection of molecular typing data has also been the driver behind recent advances in source attribution of food-borne pathogens (Muellner et al. 2013). In particular for multi-host pathogens, where the contribution of individual sources of infection can be difficult to evaluate, these tools can make an important contribution by enhancing the ability to detect shared or separated transmission pathways in different host species (Mather et al. 2013). With the growing availability of typing tools, opportunities are plentiful to increase, for example, the resolution of surveillance data to advance both control- and strategy-focussed approaches. Advances in sampling designs could substantially increase the added value obtained by extending surveillance efforts to the molecular level. However to date, few recommendations for good practice in the design of studies or continuous, systematic sampling efforts using molecular-level pathogen data and feedback mechanisms to inform action (e.g. Döpfer et al. 2008; Muellner et al. 2013; EFSA 2014).

Although molecular typing offers many opportunities and costs (e.g. per per unit of DNA) continue to drop, the costs of typing still have to be justified in times of ever-increasing resource constraints. The value of the additional information gained by using methods with higher specificity has to be weighed against the benefits of utilizing the same resources to gather a larger number of samples or for other types of analyses. In particular, if observations become too few, the epidemiological analysis of the data generated can become very difficult, affecting for example the ability to detect disease trends. Also, the need to purchase laboratory equipment must be balanced against the need for acquisition of other assets such as IT infrastructure. Further, the use of molecular typing is no substitute for sound surveillance design. As for any surveillance approach, information value is highly dependent on system attributes such as data quality and representativeness (Drewe et al. 2012). The value of specific molecular surveillance activities yet needs to be fully and formally evaluated, including an assessment of attributes such as usefulness, simplicity, data quality, flexibility, acceptability, representativeness, timeliness and value for money (e.g. Drewe et al. 2012). Examples of such evaluation of ongoing molecular surveillance programmes would be highly desirable.

We believe that an accurate and agreed terminology is an important first step towards better integration of molecular surveillance into existing surveillance standards, protocols and working principles. This article therefore aims to propose a formal definition of molecular surveillance. Furthermore we provide an epidemiologists' perspective on the use of molecular tools in disease and hazard surveillance to complement previous work that focuses on technical, pathogen or disease specific aspects of surveillance (European Centre for Disease Prevention and Control (ECDC) - Health Comunication Unit - Eurosurveillance Editorial Team 2013; Nadon et al. 2013).

In accordance with the most up-to-date definition of surveillance provided by the RISKSUR Consortium (The RISKSUR Project 2014) we propose that molecular surveillance is defined as "the systematic, continuous or repeated, measurement, collection, collation, analysis, interpretation and timely dissemination of molecular-level information about micro-organisms. These data are then used to describe health hazard occurrence and to contribute to the planning, implementation, and evaluation of risk mitigation actions'. For completeness this definition

could also be extended to monitoring and the term molecular monitoring used, where molecular-level information is generated by monitoring activities.

In molecular surveillance, conventional surveillance approaches are utilised, with molecular typing added where considered necessary to improve the resolution of the data (Denisuik et al. 2013). Such surveillance often involves molecular typing of selected isolates only. This is currently the most common form. In human clinical microbiology, which encompasses both patient diagnostics and public health microbiology, efforts are underway to implement next generation sequencing (NGS) as part of routine diagnostics and surveillance. Use of NGS is envisaged for all clinical isolates, rather than a subset of isolates, but the availability of isolates would be driven by presentation of clinical cases and not by pre-determined study designs (Didelot et al. 2012). Where possible molecular information should form an integral part of the surveillance design, rather than an added component. In such an approach, sample collection is informed by aspects of both host and pathogen populations or sources under investigation. Molecular information is also explicitly relevant for the surveillance objective. For example, the heterogeneity of the pathogen population, the molecular clock speed of various markers (i.e. their pace of evolution) relative to the spatio-temporal scale of the investigation, and the aim of the investigation would inform the study design, including sample collection strategy, choice of isolates to characterize within a sample, and molecular marker selection. Such surveillance would be based on both macro- and molecular-level characteristics and would make full use of molecular information.

In molecular surveillance, just like in any epidemiological and evolutionary analyses, the molecular tools deployed need to be fit-for-purpose and ideally are optimised during their development for the outcome in mind (Muellner et al. 2013). Of particular importance in the context of molecular surveillance is the link between sensitivity and specificity of the surveillance system as a whole and the sensitivity and specifity of the molecular method utilised. Formal evaluation of not only the analytical, but alo the diagnostic sensitivity and specificity of the molecular methods and detection protocol used in the context of surveillance is therefore highly recommened to meet the minimum standard of performance required. For example a molecular test might lack the level of sensitivity or specificity provided by a non-molecular test,

such as a serological essay. If not accounted for in the design, this might negatively affect the ability of the surveillance system to meet its objective, albeit a high level of resolution is achieved at the micro-organism level.

Just like any other population-focused approach, molecular surveillance needs to be supported by sound epidemiological concepts and designs, as bias affects molecular studies as well as studies that do not include molecular data. One important advantage of molecular surveillance is the ability to reduce of misclassification bias resulting from improved, more precise, case definitions. A simple example of this in anoutbreak setting has been described by Muellner et al. (2011). However this is not always the case and will be highly dependent on the level of discrimination of the typing methods and how it matches the speed of evolution of the pathogen. Where the method applied is too discriminatory, another type of missclassification can be introduced and in consequence related cases may not be not recognised as such (Petersen et al. 2011).

It is worth noting that all bias commonly reported in epidemiological research (i.e. confounding, misclassification of exposures, and selection and collider-stratification biases) can still affect molecular surveillance activities. In some instances, bias can even be created or increased by the integration of molecular methods. This would be the case, for example, when only a subset of the isolates collected is submitted for further characterization. In such case, a planned selection of the isolates using randomized and, if relevant, stratified sampling procedures is essential to maintain the original sampling fractions and avoid introducing selection bias. Molecular typing is no substitute for sound surveillance design, and sample size, unit of analysis and sources of bias have to be carefully considered.

We need to be aware of the fact that currently molecular strain typing, regardless of the degree of sophistication of the technology, is commonly applied to an incomplete data set since not all relevant clinical or non-clinical isolates may be available and, even when next generation sequencing (NGS) is used, all isolate characteristics, e.g. repeat regions that occur in multiple copies throughout the genome, may not have been analysed (Goering et al. 2013). In the absence of epidemiological metadata, typing is at risk of being no more than an inefficient use of

laboratory and bioinformatics resources producing data of mediocre quality and potentially leading to inappropriate disease control decisions. Although the amount of molecular data is expected to increase, e.g. as traditional multi-locus-sequence-typing (MLST) is replaced by whole genome sequencing (WGS) for bacterial pathogens (Spratt 2012) and in-silcio typing, where molecular typing results are computed from WGS data (Carrillo et al. 2012), the information utility will continue to be highly dependent on the epidemiological metadata. This has recently been illustrated using sequence information of influenza strains (VonDobschuetz et al. 2015). In this example, the lack of metadata severely limited the utility of sequencing to provide information for early warning of zoonotic influenza strain emergence.

The objectives of molecular surveillance can be diverse and include the detection of a specific pathogenic strain or virulence trait, such as toxin production or antimicrobial resistance, the assessment of species or strain abundance or richness, or the investigation of transmission chains. As in traditional surveillance, the surveillance objective should drive the study design to assure the generation of relevant molecular data. Sampling in the context of molecular surveillance can be particularly problematic. When using molecular methods, it is possible to create a substantial number of observations based on a single sample. For example, a faecal sample may contain many bacterial species as well as multiple strains within these species, and in addition to this the observed species and strain composition will vary over time. Potential sources of strains of interest may be missed if a limited number of isolates per sample is characterized (Döpfer et al. 2008). Furthermore, an understanding of the within-host or within-sample heterogeneity is needed because incomplete sampling of this heterogeneity may result in erroneous interpretations regarding epidemiological relatedness. For example, quasispecies clouds of viruses infecting epidemiologically independent individuals may show some overlap due to within-host heterogeneity, making it difficult to rule out epidemiological links based on molecular data alone (Smith & Waterman 1992). Similarly, within-host hetereogeneity of genome sequences for bacterial species affects our ability to infer directionality in transmission chains between individuals, including people and animals (Harris et al. 2013; Harrison et al. 2013).

Despite the sophistication and information density offered by genomic or metagenomic analysis and recent advances in computational biology (Pybus et al. 2013), selection of biological samples will continue to affect the outcome of molecular analyses. Current sequence collections are likely to be heavily biased, e.g. by host species or geographic origin, and provide a very limited basis to inform decision making when epidemiological principles used to inform the collection of different isolates are not reported. This is of particular importance where typing results are included in openly accessible databases. In this later case, epidemiological data are often very scarce and sampling strategies used are not reported and also can be heterogeneous within a given database (van den Borne et al. 2010). Due to its shortcoming the data is generally of limited use to risk assessment, trend analysis or source attribution or other population-based approaches. Furthermore the content of openly accessible databases, while certainly of value, is often wrongly referred to as 'surveillance data'. In an attempt to strengthen the reporting of results of molecular epidemiological studies an extension of the STROBE statement has recently been published (Field et al. 2014).

Care should also be taken when interpreting molecular data in the context of surveillance as the evolutionary mechanisms that underlie genetic polymorphism may not necessarily relate to epidemiological processes. Epidemiological concordance should be of primary concern when developing molecular surveillance activities, and typing concordance should be seen as very valuable, but adjunct information, rather than the other way around. Different markers within a species may evolve by different biological mechanisms, which may result in the almost independent evolution of the core genome of a pathogen and clinically relevant characteristics such as antimicrobial resistance, as recently shown for *Salmonella* DT104 (Mather et al. 2013). The choice of markers (molecular or genomic) used in surveillance is crucial and attention should be given to the 'clock-speed' i.e. does the marker selected evolve at the appropriate pace to provide tracking over the spatiotemporal scale of interest (Struelens et al. 1998). With the increasing availability of Next Generation Sequencing (NGS), understanding of the association between markers and epidemiological processes will continue to be critical in molecular surveillance. For example, in the analysis of the molecular epidemiology of methicillin resistant *Staph. aureus* (MRSA) in humans and animals, separate analyses were conducted for the core

and accessory genome of the pathogen because they evolve in different ways, and consequently correlate with epidemiological processes in different ways (Price et al. 2012).

A key need of the discipline is also the development of combined molecular and epidemiological criteria that define the "sameness" of isolates for example in the context of outbreak investigation, such as the Tenover criteria for Pulsed-Field Electrophoresis Profiles (PFGE) used for the investigation of outbreaks in hopsital settings. While concepts like similarity of strains, host-association and relatedness of strains provide a first decision-basis for molecular epidemiology (Muellner et al. 2013), more standardised criteria are much needed in particular for WGS data.

Molecular epidemiology should be integrated into routine surveillance activities rather than conducted *ad hoc*. This was also one of the main conclusions following a major outbreak of foodborne disease caused by *E. coli* 0104:H4 in Germany (STEC Workshop Reporting Group 2011). Ideally, performance and utility of molecular tools should be considered for surveillance already during their development (Muellner et al. 2013). The addition of WGS data from high through-put sequencing platforms could potentially improve currently available typing tools for source attribution (Anonymous 2014), and this could also be of value for molecular surveillance. The work on influenza virus is currently among the most advanced in this area including the EMPRES-i genetic surveillance module, which links epidemiological and genetic information and thus supports risk assessments of human-animal influenza threats (Claes et al. 2014). Bias can easily be introduced by population characteristics and the spatial and temporal characteristics of the samples. This is of particular importance when isolates are compared across large spatial or temporal scales. Spatial and temporal scales are often interpreted as proxies for the number of pathogen replication cycles and hence the likelihood of accumulation of mutations. However our understanding of this important relationship still suffers from serious shortcomings e.g. little is known about the impact of latent versus active infection on mutation and fixation rates, about the impact of transfer to a new host species, or about the difference between short- and long-term evolution (Muellner et al. 2011). A recent Scientific Opinion published by the European Food Safety Authority ((EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards) 2013)) provides an in-depth evaluation of molecular typing methods for major food-borne microbiological

hazards and their use for attribution modelling, outbreak investigation and scanning surveillance and further discusses some of the points introduced in this manuscript.

Risk-based surveillance serves as a good example as to how surveillance and other epidemiological approaches can be integrated. Following their successful application, including their use during the bovine spongiform encephalopathy (BSE) epidemic, risk-based approaches are increasingly seen as an efficient design to conduct disease surveillance (The RISKSUR Project 2014). The basis of this approach is the combined application of risk assessment methods with traditional design approaches to ensure appropriate and cost-effective data collection (Stärk et al. 2006). Similarly, molecular epidemiology tools could be integrated in risk-based surveillance to create a new generation of designs combining the benefits of both approaches. This would be of particular value where a link could be made between disease risk and, for example, changes in virulence markers or pathogen evolution (Delannoy et al. 2014).

In conclusion**,** molecular and genomic typing technologies can add value to infectious disease surveillance and will be key to timely tracing of future disease outbreaks and for progress on important public health issues. However, typing is not a substitute for sound surveillance design. Further, it should not be seen as a parallel form of surveillance but as an integral part of the combined analysis. There is a need to further investigate the interface between molecular surveillance and more established epidemiological approaches. More work is necessary to develop good practice principles for molecular surveillance. Since the nineties, microbiology has been completely transformed by the development of molecular diagnostic methods. Likewise, greater availability of these molecular methods has massively changed epidemiological research, more particularly research in food safety and animal health surveillance. We now also need concepts and methods for “next generation surveillance”. This will require a deeper understanding of both the potential and limitations of molecular techniques among epidemiologists and the development of accepted standards. This could be achieved by specific training and by increased interaction and deep collaboration for example between epidemiologists and diagnosticians or bioinformaticians.

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