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Use of molecular and genomic data for disease surveillance in aquaculture: Towards improved evidence for decision making

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Abstract

Diagnostic tools for the identification and confirmation of animal diseases have been evolving rapidly over the last decade, with diseases of aquatic animals being no exception. Hence, case definitions used in surveillance may now include molecular and genomic components and ultimately be based on the entire genome of a pathogen. While the opportunities brought on by this change in our ability to define and differentiate organisms are manifold, there are also challenges. These include the need to consider typing tool characteristics during sampling design, but also the re-thinking of diagnostic protocols and standards for the meaningful interpretation of the increasingly complex data presented to surveillance managers. These issues are illustrated for aquaculture using the example of multi-country surveillance of antimicrobial resistance of *Aeromonas* spp. strains isolated from rainbow trouts (*Oncorhynchus mykiss*) in Europe. In order to fully exploit the opportunities of molecular and genomic information, a multi-disciplinary approach is needed to develop harmonised diagnostic procedures and modified surveillance designs for aquaculture as well as for terrestrial animal production. This will require adjustments in the relevant standards applicable to assess food safety and trade risks.

Introduction

Diagnostic tools for the identification and confirmation of animal diseases have been evolving rapidly over the last decade, with diseases of aquatic animals being no exception. While polymerase chain reaction (PCR) has been implemented for the detection of antigens since the last millennium (Cunningham, 2002), new tools have been emerging more recently and are soon likely to be part of our standard diagnostic repertoire. These include kits based on sequencing certain parts of the genome of micro-organisms and – ultimately – diagnostics considering the information available on the entire genome of a bacterium, virus or parasite

(van Borm et al., 2015; Bayliss et al., 2017). DNA sequencing platforms can already offer accurate whole genome sequences (WGS) for bacteria in less than a day (Reuter et al., 2013). Generally, molecular surveillance – and genomic surveillance in analogue – can be 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" (Muellner et al., 2016). While molecular and genomic surveillance is relevant for all livestock and animal-derived foods, this article focuses on aquaculture specifically.

Surveillance is used for a range of objectives in aquaculture. These objectives include the confirmation of suspected disease cases in an aquaculture facility, pond, waterway or catchment area, i.e. surveillance with a focus on control (Muellner et al., 2016). This is particularly relevant after an outbreak has occurred. An alternative objective with a more strategic focus is to provide feedback on ongoing disease control efforts (e.g. an eradication programme) or the demonstration of absence of disease or infection, again for a defined population (Peeler & Otte, 2014). The latter is particularly relevant in the context of trade. Fish and shellfish are the world's most-traded agricultural goods (Bellmann et al., 2016). Most fish and shellfish are traded internationally leading to complex global food systems within which the quality and safety, but most importantly the biosecurity of products strongly depends on rigorous documentation of the disease-free status. For example, import bans have been called for to prevent the introduction of Tilapia Lake Virus from Asia to Zambia following an international alert (FAO, 2017). Another example is the risk of introduction of White Spot disease through shrimp import, which recently led to trade disruption and outbreaks of the disease in Australia (*Anonymous*, 2017). The trade of ornamental fish is also

affected by health status of the country of origin. Surveillance systems are therefore a critical prerequisite for the sustainable growth of the aquaculture sector.

Outbreaks of infectious diseases of economic relevance are being reported in all intensive aquaculture production regions (Rodger, 2016). Surveillance can help detect outbreaks early and is essential for documenting the progress of disease control, but surveillance also incurs costs which need to be balanced against the potential benefits (Stärk & Oidtmann, 2015). The minimum requirements for animal health surveillance are set out in the Aquatic Animal Health Code by the World Organisation of Animal Health (OIE, 2017). This reference document sets out the standards for trade as agreed by participating countries and details procedures for risk assessment and surveillance.

Within a surveillance programme, diagnostic tests are a major cost item. More efficient diagnostic methods are therefore attractive and should be considered when evaluating surveillance, provided that the quality of test result is equivalent to alternative tests. Higher costs could also be justified if outweighed by other advantages such as improved pathogen characterization, more rapid availability of results or practical advantages of sampling and transport. Surveillance evaluation - based on the experience from the human and terrestrial animal health field - is now starting to be applied to the aquaculture and marine environment in recognition of the need to create programmes that are as fit-for purpose and as cost-efficient as possible (Muellner et al., 2017). Increased precision of surveillance can be reached e.g. by including elements of risk assessment to develop risk-based surveillance designs. Likewise molecular and genomic tools can improve surveillance programs and bring many new opportunities, e.g. improved resolution of surveillance through improved case definitions. Case definitions are typically based on either clinical features of a disease

(syndromic surveillance) or – preferably – on the outcomes of diagnostic tests. The latter can be based on antibody or antigen detection. Furthermore, the use of genomic and molecular tools allows for improved precision of case definitions for exampling by being able to define specific genetic variants.

Surveillance based on molecular and genomic tools has a range of strength and weaknesses, and offers opportunities as well as threats as shown in Figure 1. These are applicable to surveillance for terrestrial animal diseases as well as for those conditions affecting aquatic animals. Their strengths are mostly related to increased resolution and precision as well as to the rapid availability of test results. However, weaknesses such as initial investment costs, complexity of the bioinformatics processing and – perhaps most importantly – the current lack of standardization and quality assurance severely hinder the general recognition and adoption of these methods as a basis for policy and trade decision making. At the same time, opportunities for outbreak detection, increased efficiency in the use of laboratory capacity and additional information gained from whole genome sequencing provide considerable value to disease detection and control. However the threats can be overcome provided that trans-disciplinary teams design and operate such surveillance programmes, and that relevant standards keep pace with the evolution of diagnostic tools.

While the opportunities provided by molecular and genomic tools are transforming epidemiology (Kao, 2014), and in consequence disease surveillance, interpretation of results remains a challenge (Muellner 2016; Kao, 2014). It has previously been noted that advances in typing methods are no substitute for adequate surveillance or study design, and best practices for molecular surveillance are much needed to successfully implement the vision (Muellner 2016).

In molecular surveillance, conventional surveillance approaches are utilized, with molecular typing added where considered necessary to improve the resolution of the data. This also means that we are moving from an environment of binary testing outcomes (positive vs. negative result) to increasingly complex results that require re-thinking of epidemiological response and explanatory variables (Muellner 2013). It is the objective of this article to systematically discuss the advantages and disadvantages of this "next generation" surveillance with specific emphasis on aquatic animals farmed or harvested for food.

Surveillance design: Where does typing data make a difference?

Similar to surveillance conducted in other sectors, surveillance of aquatic populations can be described by a series of elements including the surveillance objective, the case definition, the sampling strategy and intensity, the diagnostic tools used, the results and their communication to inform decision making. Among these elements of the disease surveillance cycle, the change from conventional to molecular diagnostic approaches affects mostly the data collection, sampling design, interpretation and communication of laboratory data. A broad range of molecular typing methods exits including very well established methods such as polymerase chain reaction (PCR). But increasingly, methods are extending to DNA-sequence based approaches including partial or whole genome sequencing (EFSA, 2013, 2014). The latter allow the accumulation of vast amounts of information from the genome of a single pathogen. Several authors have compiled reviews of available techniques (e.g. Sabat 2013, EFSA 2013, 2014) relevant to specific objectives or pathogen groups. Importantly, once sequence data is available it can be fitted to evolutionary and epidemiological models, allowing new insights into pathogen evolution, the nature of associations between strains of pathogens and host species, as well as aspects of between-host transmission (Muellner et al. 2013).

However, the interpretation of typing outcomes has become increasingly challenging in a research context alone; but even more so when the information provided needs to be linked to surveillance outcomes. Due to the complexity of the data, interpretation is no longer black and white but consists of continuous measures of relatedness and similarity which require novel analytical methods and new thinking. While the molecular information is highly useful in epidemiology, for example when molecular markers are used as risk factors, the analysis is not without challenges. For example, the time sequence between temporal occurrence of genetic changes and the spatio-temporal scale of a surveillance objective need to be aligned to ensure the scales of the investigations match (Muellner 2011). Also, sequence data as currently stored in public databases often lack accompanying information regarding the sampling location, clinical signs and other relevant characteristics that are essential for correct epidemiological interpretation. Therefore, such information should be added, for example to existing databases for fish pathogens (Fish Pathogen Database).

The latter might be further complicated by the occurrence of clustering (i.e. several strains may have originated from the same sample) which challenges correct epidemiological interpretation. Also, when genomic data is obtained subsequent to a culturing step, the culture(s) selected for analysis may not fully represent the heterogeneity within the sample. Several samples may need to be taken to fully capture within sample heterogeneity (Döpfer et al, 2008) and depending on the sampling design, the heterogeneity within the targeted host population may also be biased.

Aspects of bias are likely widened when molecular methods are used as the underestimation of the presence of rare genes, detection limits of diagnostic approaches, impact of using correction factors or the normalisation of metagenomics results are currently not quantified.

For example, the emergence of the *mcr-1* resistance gene in bacteria initially led to a world-wide alert only to reveal later that this gene had already been circulating for many years (Chen et al., 2017). These issues complicate the interpretation of standard surveillance terms such as "sensitivity" and "specificity", but also "coverage" and "accuracy". Infectious disease epidemiologists – and probably also others – should be aware of this.

An illustrated example: Antimicrobial resistance surveillance in aquaculture in Europe

While much focus in aquaculture is on economically devastating viruses such as salmon infectious anaemia, the occurrence of antimicrobial resistance (AMR) genes in aquatic animals is of increasing concern, particularly if considered as part of the human food chain (Done et al., 2015). Along with policy interventions aiming to reduce the frequency of resistance, the need for accompanying surveillance is highlighted by all relevant organisations, including the OIE. However, due to the large number of relevant resistance genes and their occurrence across numerous relevant species of micro-organisms, the design of effective and efficient surveillance systems is challenging and the subject of ongoing research.

The following difficulties in terms of classifying resistant isolates are commonly encountered when using conventional microbiological approaches. First, a lack of defined breakpoints to classify isolates into susceptible, intermediate and resistant hinders the analysis and interpretation of the sampling data. The definition of breakpoints depends on the availability of suitable isolates, which are often missing. Additionally, for some species such as *Aeromonas* spp., the classification to the species level is challenging because of their large taxonomic diversity (Kozińska, 2007). It is known that *Aeromonas* species contain 3–5 hybridization groups, and at least 14 phenotypically described separate *Aeromonas* species

are recognized (Carnahan & Joseph 2005). Therefore, information concerning resistance of particular *Aeromonas* spp. is unprecise. This can be improved by the use of molecular tools. For example, additional sequencing of 16S rDNA gene fragment can be used to determine particular species, and to link it with MIC values; thus illustrating the value of sequencing to refine and improve surveillance case definitions.

A number of challenges related to the use of whole genome sequencing can also be encountered in the context of AMR surveillance for aquaculture as well as terrestrial animals. Particularly the lack of standardised DNA extraction protocols introduces variability into surveillance results. It is known that DNA extraction yield, quality and composition varies significantly according to the protocol applied (Knudsen et al., 2016). To address this, a proposed standard operating procedure is required that allows for the valid comparison of results between laboratories as well as between studies (Munk et al., 2017). Recent experience also emphasises the need for benchmarking of methods and analytical approaches when using genome-based resistance data (Clausen et al., 2016).

We therefore argue that further work is urgently needed to integrate and compare AMR surveillance data originating from culture- *vs.* genome-based methods within antimicrobial resistance surveillance activities (Ellington et al., 2017). Regarding the latter, first progress has been made in terms of proposing protocols that integrate both epidemiological and microbiological aspects (Munk et al., 2017). However, further improvements including the integration of basic concepts of quality assurance are needed to harmonise results. The adoption and further development of guidance is important as impaired diagnostic interpretation is carried forward into full risk assessments where they can threaten the validity of the overall surveillance outcomes and their interpretation. Such methodological

harmonisation is an essential requirement before molecular methods can be included in international surveillance standards such as the Aquatic Animal Health Code.

From the micro to the macro

At present, molecular and genomic information are already used in a range of surveillance settings (Muellner et al., 2011). The rapid availability of results, the ability to investigate outbreaks and to establish links between related isolates as well as the possibility to ship extracted DNA without a cool chain are all contributing factors to the attractiveness of molecular and genomic typing tools (Figure 1). As with any novel technology, there are clearly also challenges to be overcome. In the context of food safety and international trade, the recognition of molecular and genomic information in the relevant standards will be a key requirement for their acceptance as a reference method.

Overall the strengths of molecular-based surveillance (and in extension genomic surveillance) include the ability to create high-resolution case definitions, ease of transport of samples in the absence of a cool chain, the rapid availability of results, international comparability and decreasing costs due to a high level of automatization. In addition, sequencing allows for the discovery of novel, yet unidentified hazards of any kind, including viruses, bacteria, or parasites. A further strength is the comprehensiveness of the information that can be obtained, including quantitative measures of pathogen occurrence and the ability to include evolutionary information into the data analysis which can super-charge the investigation of epidemiological links (Kao, 2015).

The latter can at the same time pose a challenge as the generation and the interpretation of such complex results requires staff that are competent in specialist methods (e.g.

bioinformatics), and the surveillance team's ability to successfully integrate different discipline such as epidemiology and molecular biology to allow for meaningful inferences that account for both pathogen and host population traits. This demand can delay the final interpretation of results despite the rapid availability of primary test outputs (Hasman et al., 2014). Further results can easily be misinterpreted and over-inferred from if underlying assumptions are not well understood and communicated As in any diagnostic method, bias is regarded a shortcoming and needs to be minimised where possible, and acknowledged in the interpretation of results. Bias in the context of molecular or genomic data has many new faces not only relating to sample size or the representativeness of the sample, but also extending to – among others – the typability of a micro-organism, the reproducibility of the typing or sequencing technique as well as the match of the scale of the typing methods or analysis with the surveillance objective (Muellner 2011). Sensitivity can now relate to either analytical or diagnostic sensitivity which can be strongly impacted by the culture step. Some specific bacterial strains may be harder to culture than others which will introduce bias. So-called viable but non-culturable (VBNC) bacteria are found in aquatic animals as well as elsewhere (Li et al., 2014). Despite the low cost of analysing a sample, substantial investment in equipment is required. This could potentially be mitigated by creating analytical hubs within geographical regions, rather than each country developing its own specialist capacity.

Further, at the operational level, regulatory weaknesses currently challenge next-generation surveillance. These include in particular gaps in quality assurance and standard setting. The latter may impact severely on trade, for example through 'false-positive' signals that point to associations, and therefore can create substantial hurdles to the implementation. However international and global efforts are currently being made to address this for example by the Global Microbal Identifier Working Group (Brisse et al., 2014). A problem is also the

mutation from avirulent to virulent strains, as can be the case – for example – for infectious salmon anaemia.

Due to their high resolution and the absence of the need of previous knowledge of the targeted agent, genome-based methods provide opportunities for early detection of agents, including pathogen discovery. Depending on the protocol, a culture step may not be required thus providing opportunities to reduce bias created by unculturable micro-organisms. The sequencing step itself is often outsourced to specialised companies that can provide rapid turn-around and high-quality readings as a service. The high cost of equipment is likely to encourage collaboration around centralised laboratories, thus not only increasing diagnostic efficiency but fostering inter-agency relationships. In addition, the inherent comparability of results and the increasing trend to share sequences on open platforms creates exciting opportunities for comparative analyses across regions and agents. Sequencing of the entire genome of an organism will also provide information that may not (yet) be in the focus of the analysis. Such additional information on virulence, resistance and other relevant genes adds value to the sample collected and increases the benefits from the sampling effort as several surveillance or research objectives can be investigated at once. However, this also requires a large research effort to help identify the relevant genetic markers.

It is critical to note that molecular typing and genomic data, however advanced and detailed they may be, cannot compensate for sound epidemiological design of surveillance. Samples need to be representative of the population about which conclusions will be drawn and of a sufficient sample size. When samples are collected, additional epidemiological information needs to be captured (so-called meta-data) to allow for an appropriate interpretation at population level. Single isolates – even if analysed at nucleotide level – provide very limited

epidemiological value in the absence of information on the target population from which they were drawn. Therefore, in order to benefit from the plentiful opportunities provided by the use of molecular and genomic data in surveillance, all experts involved need to collaborate closely to assure that the results derived with are valid throughout the process and provide a robust basis for veterinary and public health decision-making. Integrative rather than parallel analysis should be promoted at all steps of the surveillance process. Good practice guidelines should be developed and promoted for adoption and an intensified dialogue between epidemiologists and microbiologists as well as bioinformatics experts is much needed.

Finally, to ensure surveillance outputs provide "information for action", communication of technical surveillance outputs to end-user is critical in the context of molecular surveillance, e.g. epidemiologist will need to understand the biological relevance of specific genetic markers while risk managers need comprehensive high-level summaries to inform their decision making. They are likely to struggle to extend their knowledge and training to all the different sub-discipline required for a successful molecular surveillance programme. With the increasing complexity of available data and analytical techniques the gaps in comprehension are widening. Without skilled guidance from experts, decision-makers will not be able to draw the right conclusions form the results presented.

To increase the quality of publications based on molecular data, standards have been defined regarding the minimum information that should be provided for correct interpretation (e.g. STROME-ID, STROBE-AMR). While most medical journals are currently endorsing these statements, the list of veterinary journals is more limited (Strobe Vet Statement, 2016). It is hoped that more journals, including journals relevant to aquaculture, will apply these standards in the future. A further challenge in the assurance of high-quality publications and

successful knowledge transfer is the competency of reviewers to recognize and assess the weaknesses of manuscripts using molecular and genomic data. Often, reviewers are either competent in molecular diagnostics or in surveillance design, but rarely in both. Inter-disciplinary collaboration and sound editorial decision-making (e.g. supported through experienced Editorial Board Members) will be required to assure quality of published articles and the appropriate interpretation of surveillance results based on molecular and genomic data.

Discussion

While molecular and genomic typing tools can clearly super-charge surveillance systems and create exiting new opportunities to prevent and control infectious diseases, they need to be integrated in the wider context of surveillance and should not be considered stand-alone approaches. This is true for animal health surveillance in general, but also for surveillance applied in aquaculture. While diagnostic advantages offered by such methods could be attractive for a specific human patient, or a high-value animal patient, the use of genome-based diagnosis is more complex in the context of population-based decision making (Ellington et al., 2017). There are additional, specific needs when operating in aquatic animals where the diversity as well as the interactions of aquatic systems presents a series of unique challenges and complexities due to the limited segregation between populations (Peeler & Taylor, 2011).

Regardless of surveillance design, the sound foundation on epidemiological concepts is of paramount importance. Before engaging in molecular surveillance activities, we need to reflect on basic principles of diagnostic validity, misclassification risk and surveillance design, including risk-based surveillance. At the moment, many such gaps persist in the

context of molecular or genome-based diagnostics. More efforts are needed to work towards standardised operating procedures that assure the comparability between results. This is comparable to the development of standardised diagnostic procedures as used in the context of other trade-relevant biological hazards. Efforts have started for the validation for molecular tests (e.g. Saunders & Sharp, 2015; Cunha & Inàcio, 2015; Gioia et al., 2016). It is the responsibility of the standard setting organisations to consider novel diagnostic approaches and how they can (or cannot) contribute to decision making that is relevant to early detection of outbreaks, prevention of transmission and to trade.

In animal disease surveillance, speed is not generally of a similar importance as if a human patient's life were on the line. The additional costs of rapid diagnosis might therefore be hard to justify for routine surveillance where lower cost alternatives exist. One exception is surveillance in the wake of an outbreak, particularly if a listed (exotic) disease incursion is suspected, and when fast confirmation as well as rapid comparison to similar samples and high resolution typing will be essential to establish the sequence of transmission (Köser et al., 2012). Particularly in aquaculture, where testing water may be attractive, a higher sensitivity will be an advantage for surveillance.

Aquaculture is among the fastest growing sectors world-wide and a substantial proportion of products is traded internationally (Asche et al., 2016). While this offers opportunities to the sector, there are also risks. Animal health emergencies lead to trade disruption, and surveillance is one of the key tools for providing assurance to trading partner and therefore for building resilience towards unjustified rejection of products (Peeler et al., 2014). The lack of validated and accepted diagnostic tools provides a substantial hurdle for the establishment of the health status of a farm, region or country. The aquaculture sector may be differently

organised from other sectors due to different hazards causing disease and disease control measures that differ substantially from terrestrial animals. Therefore, alternative approaches may be required for surveillance in general, such as farmer involvement (Brugere et al., 2017) or the sector may adopt advanced techniques and follow a different developmental pathway than terrestrial animal surveillance, which has long relied on comparative typing approaches. Finally, also the role of people in surveillance needs to be better understood and how they can best contribute and integrate with methods and tools to provide effective and efficient surveillance information to the sector's advantage.

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Figure 1: Strengths, weaknesses, opportunities and threats of surveillance based on molecular and genomic data