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Review

Challenges and current limitations in using molecular methods for the analysis of food and foodborne pathogen isolates

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ABSTRACT

In the 'One Health' concept, animal health is linked to human health, through the matrix of food (including the food-processing environment). Application of molecular methods for pathogen analysis of food is, therefore, important in the 'One Health' approach. This analysis can involve direct analysis of food for pathogens, using a combination of traditional and molecular methods, or application of molecular methods to characterisation of pathogenic bacteria isolated. Molecular methods offer many advantages in terms of specificity, sensitivity (under certain conditions), time-to-result and in the characterisation of isolates, for example with whole genome sequencing. However, it is important to be aware of the current limitations of molecular methods used in food analysis. In this review, the advantages and challenges of molecular methods for the analysis of food and of pathogens isolated from food associated environments are discussed with particular focus on the opportunities and current limitations of such methods.

KEYWORDS: molecular methods, analysis, food pathogens, whole genome sequencing.

1. Introduction

Detection and confirmation of pathogens in food using bacterial agar plates, followed by biochemical tests are widely used for the microbiological analysis of food and isolates of foodborne microbial pathogens in the food industry. Such methods are specific to international standards and industrial specifications, and, therefore, results based on these traditional methods are usually required. There are advantages to such methods: they are widely used and accepted, there is a degree of standardisation and harmonisation so results can be compared, they are relatively cheap and expertise has been established.

Molecular methods for the microbiological analysis of food and of isolates from food offer great possibilities for more rapid analysis, with potential for timely and accurate results [1]. They offer additional advantages over traditional agar-based methods such as improved specificity. However, correct interpretation of the results is important, as the detection of a piece of DNA from a pathogen in a food sample does not necessarily imply that the food is unsafe.

Molecular methods include the polymerase chain reaction (PCR), that was first used in the 1980s [2], and since then has been used widely for many purposes, including pathogen detection, confirmation and characterisation [3-5]. More recently, other molecular methods such as pulsed field gel electrophoresis (PFGE), metagenomics, whole genome sequencing, and various adaptations of PCR, for example digital PCR, loop-mediated isothermal amplification (LAMP), etc. have been used [6]. Each has its own use in, for example, epidemiology, or source attribution, or tracking potential routes of contamination.

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The purpose of this review is to evaluate the use of molecular methods for the study of food and foodborne microbial pathogens, emphasising the advantages, current limitations, and challenges involved (Table 1). Food, being a link between animals and humans, is an integral part of the 'One Health' initiative (http://www.onehealthinitiative.com/index.php). This initiative merges animal health and veterinary medicine with food (including the processing environment) and human medicine, making application of molecular methods relevant to all sections of this multi-disciplinary concept. Other rapid methods for pathogen detection and characterisation, such as immunological or spectroscopic methods, will not be addressed.

2. Molecular methods for analysis of food

2.1. Metagenomics

Metagenomics is a molecular-based method that studies the total genetic material recovered from a sample, in order to profile the diversity of the microbial population in natural ecological communities such as complex foods and food processing environment samples. The majority of microbial populations that are found using this approach in food are not seen in culture-based approaches, as many microbial species do not culture. Metagenomics opens a broad range of opportunities for pathogen analysis: interactions of pathogens with the normal bacterial flora, prevalence of pathogens, etc. [7-9]. Recent studies have used either the shot-gun or PCR-based sequencing approaches to get a deeper understanding of the microbial populations, including pathogens, in milk and dairy samples [10-12].

Having the advantage of analysis of the total DNA in a sample, enrichment of pathogens may not be required; therefore, no handling of enriched pathogens is necessary, making the process safer. Additionally, enrichment media or the loss of non-culturable bacteria does not bias the results. As screening for all pathogens could theoretically be undertaken in one assay, this

Table 1. Summary of the advantages and challenges of various methods of food and foodborne pathogen analysis.

Method	Advantages	Challenges
Traditional culture	Sensitivity is good Cost - cheap Standardization	Time-to-result is slow Only analyses what is being looked for
PCR	Time-to-result can be fast Specificity is good	Sensitivity is generally poor in the absence of enrichment Inhibitors in food Live-dead cell differentiation is difficult
Metagenomics	Culture-independent method Community analysis Information on what you are not looking for	Live-dead differentiation is difficult Lack of standardization Variables such as DNA extraction, sequencing, and analysis programmes can influence the results No information on expression of genes
Transcriptomics	Information on expression of genes	Cost - expensive
Whole genome sequencing	The results of several tests obtained together Costs are decreasing	Lack of standardization Variables such as DNA extraction, sequencing, and analysis programmes can influence the results No information on expression of genes

technology is very attractive from a food safety perspective. However, the sensitivity is not good enough to substitute for traditional detection techniques for many foodborne pathogens, and metagenomic results can be biased by lack of consistency of the DNA extraction step, the sequencing approach, the instrumentation used or the subsequent bioinformatics analysis of the data [13-15]. Finally, bench top sequencers are still relatively expensive and not a common instrument in a food safety laboratory.

Screening of the food production chain for foodborne pathogenic bacteria using environmental swabs and raw materials, resulting in detection of their presence or absence, can give insight into possible contamination routes of food. The metagenomics of a beef production chain was analysed and the detection of foodborne pathogens conducted. Due to the basic algorithm used, the computational analysis introduced shifts in pathogen abundance. Hence, absolute quantification of individual species was not possible, but the abundance of individual species remained valid [8].

This example demonstrates the advantages, challenges, and current limitations of metagenomic approaches. Additionally, the use of different sequencing platforms and different software programmes makes comparing results difficult, since different sequencing platforms as well as different software will introduce different biases [13-15].

2.2. Direct analysis of food using PCR

Without an initial concentration step, molecular analysis on its own is not currently feasible for direct analysis of pathogens in food as the numbers present are generally too low for the sensitivity of PCR tests. Additionally, food has many inhibitors that may interfere with PCR tests. Therefore, increasing cell numbers, generally carried out using traditional enrichment or immunological methods, is required.

2.3. Enrichment of pathogens followed by molecular analysis

Enrichment has the advantage of minimizing the effect of inhibitors in PCR, allows differentiation of viable from non-viable cells and allows for repair of cells that were stressed or injured during food processing [16, 17]. The limitation of time means that the results of enrichment testing may not be suitable in a hazard analysis and critical control points (HACCP) system where the results are needed in order to make appropriate changes so as to manage food safety risks.

If the sample is enriched as recommended by the standard method, PCR can be used for detection of the pathogen in the enriched culture, while at the same time plating the enriched culture to get a valid result from the standard method. In this way, the time-to-result can be reduced by several days using PCR (for in-house use), while a valid result can be obtained simultaneously using the standard method. The risk that dead cells will be detected by PCR can be reduced by using Ethidium or Propidium MonoAzide (EMA or PMA) [18, 19], or other DNA-binding dyes that inhibit detection of DNA from dead cells. DNA binding dyes selectively penetrate the compromised membrane of dead cells, but not the intact membrane of viable cells, and thus intercalate into the DNA once inside the cell membrane. Upon exposure to intense visible light, the photoreactive azide group on the dye is converted to a highly reactive nitrene radical that cross-links with DNA from dead cells, making them unavailable for subsequent PCR amplification [20]. As these systems don't always work [21], validation using live-dead cell differentiation methods is essential in each food matrix to avoid false positives with dead cell detection and/or false negatives with PCR inhibitors present in the food matrix. It is important to be aware that combinations of enrichment and PCR are not yet incorporated into most standard methods, unless validation has be completed using ISO 16140 [21].

The standard method for detection of *L. monocytogenes* is the ISO 11290-1 method [22]. This involves a primary enrichment in half Frazer broth (Frazer broth with half the concentration of antibiotics) for 24 h, followed by a secondary enrichment in full Frazer broth (Frazer broth with the full concentration of antibiotics) for 24 h. An aliquot from each enrichment is spread onto agar plates, including chromogenic agar plates that are incubated for 24-48 h. In total, the process takes 4 days. After the 4 days, any suspect colonies (blue/green with a white precipitate on an ALOA chromogenic agar plate) must be confirmed as

L. monocytogenes, adding a further 2 days to the process, resulting in a 6-day period to isolate-confirmed positive colonies. By combining a real-time PCR assay (RTi-PCR) with the enrichment protocol from the ISO 11290-1 method, it was possible to reduce the time-to-result from 7 days to 3 days [23]. In that study, Dalmasso showed that the detection of *L. monocytogenes* could be achieved in 3 days, and that the results are even more reliable than the traditional plating method. Eight hundred and seventy-two samples from different matrices (food contact surfaces, non-food contact surfaces, food samples) from 13 different food chains in 6 European countries were analysed. The results from the ISO 11290-1 method were compared with the results obtained from RTi-PCR of the second enrichment of the same samples that were used for plating. The results showed that the number of positive samples from the RTi-PCR analysis of the second enrichment was greater than from the traditional plating. In a similar study, *L. monocytogenes* detection by RTi-PCR was less affected by the presence of *Listeria innocua* in the contaminated samples, demonstrating greater reliability than the standard method [24].

As a result of the *E. coli* O104 outbreak in Germany in 2011 due to contaminated beansprouts, ISO 13136:2012 was developed [25, 26]. The method describes the application of an RTi-PCR-based method for the detection of pathogenic shiga toxin producing *E. coli* (STEC) and the determination of *E. coli* O157, O111, O26, O103, and O145 serogroups in food. The method involves detection of the relevant genes from an enriched culture and from isolates from that enriched culture.

Studies on samples artificially inoculated with STEC-contaminated spinach have shown that the detection limit without enrichment was 1 x 10^6 CFU/g. Post a 23-h STEC-specific enrichment, a detection limit of 10 CFU/g was obtained. The combination of STEC specific enrichment and subsequent shot-gun sequencing could shorten the standard protocol by two days, not including the time for computational analysis [9].

Using the International Standardization Organization method for validation of alternative methods (ISO 16140), molecular methods have been incorporated into standard ISO methods [27]. Combining RTi-PCR with traditional agar-based methods for detection of *L. monocytogenes* has been applied to detection of other foodborne pathogens such as *Salmonella* [28] and *Cronobacter* [29].

As foodborne pathogens cause a major public health threat, molecular methods should be used for their detection in food. However, it is important that there is agreed interpretation of the results and awareness of the limitations.

2.4. Quantification of bacterial pathogens in food

Quantitative microbial risk assessment (QMRA) is an important approach to food safety assessment and management strategies. To conduct QMRA, there is a need for methods that generate quantitative data. Traditional methods using enrichment obtain a qualitative (positive/negative) result, and therefore, the results cannot be used for quantification purposes. However, by combining specific enrichment conditions with quantitative PCR (qPCR), an estimation of pathogen numbers is possible in some cases.

A short enrichment time has been successful for quantification of *Salmonella* in samples from pig carcasses [30]. The assay consisted of 8 h enrichment in buffered peptone water, so that most of the cells would be in the log phase, followed by DNA extraction and a qPCR assay. The limit of quantification was 1.4 CFU/20 cm^2 (approximately 10 g) of artificially contaminated sample, with the precision similar to the standard reference most probable number (MPN) method. Potentially naturally contaminated cork borer samples were screened for *Salmonella*-positive samples using the method of short enrichment followed by qPCR. A higher sensitivity was obtained with a low contamination level in naturally contaminated samples (<6.7 CFU/sample) using qPCR, where 15 of the samples were negative with the MPN method. A higher contamination level (6.7-310 CFU/sample) showed good agreement between the results obtained with the qPCR and MPN methods obtained [30].

A similar approach was used for enumeration of *E. coli* O157 and O26 in faeces and milk samples [31, 32], respectively. In faeces, 5 h enrichment in modified tryptone soya broth with novobiocin (20 mg/l) (mTSBn) was optimal to obtain a linear correlation between inoculum

of log 1 to 6 CFU/ml. In milk, an enrichment of 8 h in buffered peptone water was found to be optimal for a linear relationship between threshold cycles (CT values) and STEC log numbers over a five-log concentration range for six STEC strains (of serogroups O157 and O26). The bacterial numbers had increased by three log cycles.

For *L. monocytogenes*, it was demonstrated that short enrichment may not be suitable as during the first few hours competing bacteria may grow faster, interfering with the subsequent PCR reaction [33].

In using methods involving short enrichment times, it is important to be aware of the limitations of such methods. The methods are only applicable to the matrices and pathogens that are tested - verification is necessary for other pathogens and matrices. The short enrichment is dependent on an equal growth rate for all strains of the pathogen, an absence of stressed cells, or inhibition of the PCR reaction. In addition, the DNA extraction process is extremely important and must be consistent.

2.5. Comparative studies between molecular methods and traditional methods - validation and verification

In recent years, the development of PCR methods has improved with the use of fluorescent probes to target genes specific to the pathogens of interest. These probes can be detected at low numbers as the PCR method amplifies the genes. The introduction of ISO methods (Table 2) 16140:2003, ISO 22174:2005, ISO 20837:2006, ISO 20838:2006, ISO 22118:2011 and ISO 22119:2010 [21, 34-38] into standard methods has resulted in more widespread use of these methods. For their use as standard methods, the following are required:

1. Comparative studies to demonstrate that the 'alternative method' is equal to or better than the standard method, incorporating positive and negative controls as well as an internal amplification control for real-time PCR assays.
2. Demonstrated sensitivity, specificity, accuracy, reproducibility, ruggedness, and precision to ensure that the results are meaningful and appropriate to make a decision.
3. Reliability for its intended purpose, which includes emergency/contingency situations, rapid screening, high throughput testing and confirmatory analyses.
4. Validation for each food matrix analysed.

Commercial kits are now available, most of which are validated for use with dairy foods, although further validation of kits is required in new different matrices. Validation by AOAC, AFNOR, MicroVal and NordVAl has helped with significant harmonization in the area of detection, especially of *Listeria* and *Salmonella* species [27]. Commercially available diagnostic kits should be validated according to ISO guidelines as a minimum requirement. This demonstrates adequate confidence in the results obtained by these alternative methods,

Table 2. International Standardisation Organisation (ISO) standards that must be followed in PCR method development.

Microbiology of food and animal feeding stuffs - Polymerase chain reaction (PCR) for the detection of foodborne pathogens	
Title	
ISO standard	Title of standard
22174:2005	General requirements and definitions
20837:2006	Requirements for sample preparation for qualitative detection
20838:2006	Requirements for amplification and detection for qualitative methods
22118:2011	Performance characteristics of molecular detection methods
22119:2010	Real-Time PCR - general requirements and definitions

comparable to, or exceeding those obtained using the reference method. Care should be taken when using commercial kits that are not fully validated.

3. Characterisation of isolates

One of the major disadvantages of using RTi-PCR alone for direct analysis of food is that there is no isolate obtained for characterisation. Using traditional methods, isolates of the pathogen can be obtained and extraction of DNA from isolates allows further characterisation.

3.1. Extraction of DNA

Reliable analysis of food samples with RTi-PCR requires controlled sample preparation, and good quality DNA. The processed samples must be free of contaminants, especially inhibitors. There are many different DNA extraction kits for different purposes that require manual or automotive input, resulting in variability of the DNA content and quality depending on the operator. The current development of a broad range of fast, safe and easy-to-use automatic DNA and RNA extraction processes for any food, beverage or environmental sample have shown improved extraction in clinical diagnosis [39]. Bench-top automated processing systems can have a sample throughput in the range of 8 to 96 samples per hour.

3.2. Confirmation of isolates

While standard methods still rely, for the most part, on biochemical tests, such biochemical tests can be unreliable and can take several days to obtain a result [40]. Using molecular methods for confirmation of suspect isolates from agar plates offers the advantage of a faster time-to-result, and greater reliability and specificity. However, care in DNA extraction, PCR inhibitors, and false positives/negatives (for example because of a mutation in the target gene) must be considered. There are very many different assays targeting different genes for different pathogens, such as, PCR, multiplex PCR, LAMP-PCR, microarrays etc. For example, a multiplex PCR can be used for genus and species confirmation of suspect *Listeria* spp. isolates [41].

3.3. Sub-typing and comparison of isolates

Sub-typing of isolates using molecular methods can be used in epidemiology to determine strain relationships [42] that contribute to identifying the source of a contaminant and the degree of persistence in the food and food processing environment [43-45]. There are many different molecular-based subtyping methods.

Pulsed-Field Gel Electrophoresis (PFGE) is the 'gold standard' method for assessing strain interrelatedness. PFGE was first developed by Schwartz and Cantor [46] and has made possible the separation of large DNA fragments over 1,000 kbp. Bacteria are lysed in order to release DNA, then digested and cleaved into large fragments by rare-cutting restriction enzymes and embedded in agarose plugs. The DNA is separated in a horizontal agarose gel using pulsed-field migration. This results in DNA fragment patterns or pulsotypes which can be compared using analysis of the gel image with specific software. The PulseNet International network (www.pulsenetinternational.org) has proposed several standardized PFGE protocols for the study of foodborne pathogenic bacteria, allowing the creation of databases for the comparison of strains worldwide [47]. While the discriminatory power of PFGE is very good, it has the disadvantage of being difficult to perform and harmonise between laboratories.

Although Ribotyping does not have the discriminatory power of PFGE, it is a rapid and specific DNA-based sub-typing method that is widely used in clinical diagnostics and analysis of microbial communities in food, water, and beverages. Bacterial DNA is cut with restriction enzymes into smaller fragments (more than 300-500 fragments, 1-30 kb in size) than with PFGE. The fragments are separated according to their size by agarose gel electrophoresis, which is followed by hybridisation with a labelled 16S or 23S rRNA probe. Thus, only the fragments coding for such rRNA are visualised and then analysed. The resulting pattern is digitised and used to identify the origin of the DNA by comparison with reference organisms in a computer database [48, 49].

Multilocus sequence typing (MLST) characterises isolates of microbial species using the DNA sequences of internal fragments of multiple housekeeping genes. Approximately 450-500 bp internal fragments of each gene are used. For each

housekeeping gene, the different sequences in a bacterial species are assigned as distinct alleles and, for each isolate, the alleles for each housekeeping gene define the allelic profile or sequence type (ST). Due to the sequence conservation in housekeeping genes, MLST lacks discriminatory power to differentiate bacterial strains, which limits its use in epidemiological investigations.

MLST databases (http://bigsdb.web.pasteur.fr/; pubmlst.org; mlst.net) contain the reference allele sequences and sequence types for different organisms and for isolate epidemiological data. The websites contain interrogation and analysis software which facilitates a query of the allele sequences and STs in the database. A limitation of MLST is that it only uses approximately 0.1% of the genomic sequence to assign a ST. Multi-virulence-locus sequence typing (MVLST) is a similar principle focusing on virulence genes [50] and with the development of whole genome sequencing, whole genome MLST (wgMLST) and core genome MLST (cgMLST) are being established to utilise a greater percentage of the genome than MLST [51, 52].

Other DNA-based sub-typing methods include amplified fragment length polymorphism (AFLP) [53], randomly amplified polymorphic DNA (RAPD) [54] and clustered regular interspaced short palindromic repeats (CRISPRs) analysis [55].

4. Whole genome sequencing

Advancing whole genome sequencing (WGS) technologies and rapidly declining costs of this technology make the routine applications of WGS analysis of isolates from disease outbreaks and in food safety management possible. This has great potential to contribute to greater public health protection.

4.1. Methods for DNA sequencing

New DNA sequencing techniques are constantly being developed which offer reliable rapid sequencing. The first generation of DNA sequencing, namely the Sanger sequencing method, was developed in 1977. Although this method represented a major step forward in methodology, Sanger sequencing was very labour-intensive, time-consuming and expensive, making widespread use impossible [56]. Thus, sequencing techniques have been continuously advancing and moving towards methods which allow for higher throughput. Second-generation sequencing, e.g. Roche 454 sequencing which emerged in 2005, resulted in a gain in speed and reduction in cost due in part to parallel sequencing of multiple DNA strands. However, this gain in speed and cost reduction introduced a decrease in accuracy due to imprecise PCR amplification and shorter read lengths [57]. In more recent years, advances in sequencing techniques have been developed which help to address many of the problems posed previously [58]. In this review, some of the more commonly used DNA sequencing platforms for laboratory analysis is outlined.

In Illumina sequencing, fluorescently labelled nucleotides are used in a DNA sequencing-by-synthesis approach which allows the information to be recorded instantly. Illumina offers several different platforms for sequencing which vary in price, coverage and read length (between 50-600 bp). The Illumina MiSeq platform is now commonly used in laboratories and offers 1-25 million reads per run and a run time of approximately 27 h. The HiSeq platform offers significantly more reads per run, up to 3 billion, but has a greater instrument cost and longer run time and hence is not used in smaller laboratories [59].

The Ion Torrent Personal Genome Machine (PGM) offers a different method of sequencing in using pH measurement to detect base incorporations. Ion Torrent sequencing offers similar read lengths and requires similar DNA input to Illumina technology and the instrument costs are slightly less than an Illumina MiSeq. Although lower accuracy has been reported, a score of Q20 as opposed to Q30 for Illumina sequencing, the Ion Torrent PGM still offers a suitable sequencing method for laboratory analysis [60].

PacBio is a single strand sequencing-by-synthesis DNA sequencing technique. DNA replication is performed in picowells with fluorescently labelled bases. In each picowell one DNA polymerase is fixed and during base incorporation a light signal is detected at the bottom of the well. Picowells are so small that the light of bases that are not been incorporated does not reach the bottom of the picowell, making single strand sequencing possible. PacBio sequencing offers

a distinct advantage in terms of read length (on average 1500 bp) which allows for the closure of draft genomes, the sequencing of repetitive regions and mutations. However, PacBio sequencing, in part due to the longer read length, has a lower rate of throughput than other currently available technologies and so a comparatively higher price per base [61].

4.2. Whole genome sequencing data analysis

Due to its great detail, modern sequencing techniques generate enormous amounts of data, creating a requirement for storage, data processing capability, and bioinformatic skills. Analysis of WGS data can be conducted in various ways. Linux-based systems typically involve strategies that are performed with software that requires knowledge of Linux or Unix command-line language and/or requires the user to learn complex programmes. More recently, Windows-based systems, many of which are freely available on the internet, have been developed. Currently, the choice of platform and programmes is a personal choice, e.g. http://genometools.org/; http://bioinfo.wisc.edu/knowledge_base/next-gen-seq_software.php, and there is little uniformity in data analysis, partly due to the rapid development and advances in software.

Different sequencing platforms introduce different biases [13, 59], and therefore, the processing of raw output data is somewhat dependent on the sequencing platform. In addition, different sequence conditions, for example, extreme base compositions (high and low GC content) influence the sequencing results [62]. Although an increase in coverage can help to reduce errors, this introduces an even greater amount of data, requiring increased storage capacity, processing time and a higher cost.

Various programmes and algorithms perform sequence data processing and genome analysis differently and may therefore produce varying results [14, 15]. Certain approaches might function well for one type of genome or sequencing platform, but not for different genomes or sequencing platforms. Every assembled genome remains a hypothesis, and therefore, quality assessment and validation remains difficult, because of the lack of a proper reference. Threshold and parameter settings, different reference databases, and matrixes used for genome and gene function analysis can highly influence the results, leading to high variations in quality. Comparison between strains can similarly be affected not only by differences in sequencing but also differences in data analysis. This has to be taken into account when analysing and comparing genome sequences and whole bacterial genomes, especially between laboratories. Nonetheless, whole genome analysis is a powerful tool for understanding pathogenesis of foodborne pathogenic bacteria.

4.3. WGS for strain comparisons in disease outbreak situations

WGS offers a number of advantages over current strain comparison methods due to the much higher specificity offered in comparison to previous typing methods. Comparison of strains in outbreak scenarios makes case definitions and source tracking easier resulting in rapid responses and prevention of further cases. WGS is organism-independent as several pathogens can be sequenced in the same sequencing run, although analysis has to be organism-specific. Cost and speed have decreased dramatically in recent years making WGS a viable alternative to standard sub-typing or analysis methods in the management of food safety issues. Data can also be re-analyzed retrospectively as new information becomes available. WGS results can be used for more than one purpose simultaneously such as identification, subtyping, virulence marker detection, antimicrobial resistance (AMR) predictions, and genome-wide association studies. In 2014-2015, the US, UK and Denmark have initiated the use of WGS to analyse pathogen isolates from food and processing environment samples, and/or in outbreak scenarios. In such cases, the regulatory response was faster due to sharing of clinical, food and environmental WGS data, apparently sporadic cases could be epidemiologically linked, preventing further cases, and in one case investigation identified the root cause of a *Salmonella* outbreak, preventing future outbreaks [63].

Despite these advantages, there are a number of disadvantages to WGS application in disease outbreak situations. While the cost of an individual sequence has decreased, the capital investment is still significant, and requires

appropriate infrastructure and internet connectivity. There is a need for global harmonization and validation of protocols for DNA extraction, sequencing equipment, data analysis, data interpretation, and for data transfer across national borders. Data storage (physical and virtual space), and data handling (trained bioinformaticians) are major issues that need to be considered. Finally, WGS should be a tool for all and implementation in developing countries is a consideration [63].

4.4. Transcriptome sequencing

While sequencing the genome of a strain is important in determining the gene content, transcriptome sequencing is also important as it indicates the genes that are actually expressed. Among other things, understanding the transcriptome facilitates quantification of the gene expression levels of each transcript under different conditions, which is necessary to interpret the functional elements of the genome [64]. Transcriptome sequencing, followed by analysis using RNA-Seq was used by Fox *et al*. [47] in an attempt to understand gene expression in the presence of sub-lethal concentrations of a sanitiser. Using RNA-Seq, it is necessary to map the dataset against a reference genome. One of the advantages of an RNA-Seq dataset is that the data can be reanalysed as new information becomes available. Casey *et al*. [64] re-analysed the dataset of Fox *et al*. and mapped the dataset against the genome of the strain used for the transcritome sequencing. In doing that, additional mechanisms of sanitiser resistance were observed and a genetic basis of persistence of that particular strain was speculated on.

5. Requirements for molecular methods to be incorporated as regulatory methods

There is a current transition from standardized traditional agar-based methods based on phenotype and descriptive microbiology, to molecular-based methods. There is now a need to sustain the development of validated, standard protocols that are accredited by international standardization organisations. ISO standard method 16140 can be used to show equality between methods i.e. similarity between traditional and PCR methods. Standard protocols that are open access and feasible to implement in any laboratory worldwide, with reagents and equipment from any company are also required. Systems for providing reference material and proficiency testing are also needed. Finally, the same strict approach in terms of replication and controls that are applied to traditional methods is required for molecular methods.

6. Future perspectives

Undoubtedly, molecular methods have advantages in analysis of food and foodborne pathogens. The limitations are currently being addressed and it is increasingly difficult to keep up with developing technologies and analytical methods, be they for analysis of pathogens or data analysis. Sensitivity and specificity of detection methods continue to be improved, innovations in combinations of traditional and molecular methods continue to be developed, and validation of molecular methods will facilitate their incorporation into standard methods.

Advances in WGS will ensure its routine use in food safety management. Harmonisation and standardisation of methodologies for data generation and analysis is needed, although these issues are being addressed. The EU-funded project COMPARE (http://www.compare-europe.eu/), which aims to improve the response to disease outbreaks among humans and animals worldwide through the use of new genome technology, will address these issues. Additionally, the Global Microbial Identifier initiative (GMI; http://www.globalmicrobialidentifier.org/) envisions a global system of DNA genome databases for microbial and infectious disease identification and diagnostics. Such a system will benefit those tackling individual problems at the frontline, such as clinicians, veterinarians, etc., as well as policy-makers, regulators, and industry. By enabling access to this global resource, a professional response to health threats will be available to all countries with basic laboratory infrastructure.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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