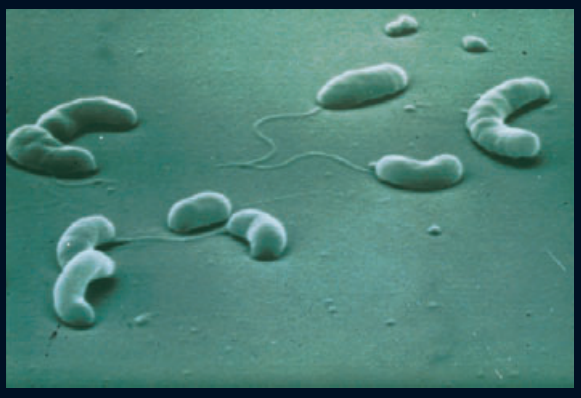
VOLUME 119, SUPPLEMENT 133, NOVEMBER 2011

APMIS

ACTA PATHOLOGICA,
MICROBIOLOGICA
ET IMMUNOLOGICA
SCANDINAVICA

Rapid Detection, Characterization and Enumeration of Foodborne Pathogens



Doctoral thesis

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Rapid Detection, Characterization and Enumeration of Foodborne Pathogens

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Preface

The current increase in public attention to food safety, especially foodborne microbes, has increased research into new, rapid methods of detection of foodborne pathogens. Faster detection and characterization of pathogens are the cornerstones of the fight against foodborne pathogens.

The food industry and control authorities are putting substantial efforts into testing to ensure safer foods, prevent product recalls, and limit economic losses. However, how are the current rapid methods validated, what is the cost-benefit of using rapid methods, and which rapid method is the appropriate one to choose? I have attempted to answer some of the questions based on what I have learned from 20 years or so of practical work, sometimes failed attempts.

My work has introduced the following novel developments into the field of rapid methods: (i) serological tests for large-scale screening, surveillance, or eradication programs using in particular non-invasive samples, (ii) same-day detection of *Salmonella* that otherwise was considered as difficult to achieve, (iii) enumeration following a short log-phase enrichment, (iv) detection of foodborne pathogens in air samples, and finally (v) biotracing of pathogens based on mathematical modeling, even in the absence of a positive test result.

In the present review, I have tried to discuss the aforementioned developments into a broader picture on why there is a need for rapid methods, the current developments, and where the field is moving. Whether the review has succeeded in drawing the big picture, it is up to peer colleagues to judge, but editing the ASM book, cited frequently in the present review, has certainly opened my eyes to the global perspective of this fast moving field.

I have been fortunate to work with the bright scientists, who carried out much of the work from my laboratory that is included in this dissertation. DTU-National Food Institute has given a strategic priority to method development and provided me with excellent working conditions and continuous support to expand the work on method development.

Last but not least, I thank my family for putting up with me sitting many nights in front of a computer to do this work.

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Conflicts of interest: The author has no potential conflicts to declare.

THE MAIN PART OF THE THESIS IS BASED ON THE FOLLOWING PAPERS:

(These are marked as **boldface** in the text).

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APMIS 119 (Suppl. 133): 1–24

DOI 10.1111/j.1600-0463.2011.02767.x

Rapid detection, characterization, and enumeration of foodborne pathogens

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J. Hoorfar. Rapid detection, characterization, and enumeration of foodborne pathogens. APMIS 2011; 119 (Suppl. 133): 1–24.

As food safety management further develops, microbiological testing will continue to play an important role in assessing whether Food Safety Objectives are achieved. However, traditional microbiological culture-based methods are limited, particularly in their ability to provide timely data. The present review discusses the reasons for the increasing interest in rapid methods, current developments in the field, the research needs, and the future trends. The advent of biotechnology has introduced new technologies that led to the emergence of rapid diagnostic methods and altered food testing practices. Rapid methods are comprised of many different detection technologies, including specialized enzyme substrates, antibodies and DNA, ranging from simple differential plating media to the use of sophisticated instruments. The use of non-invasive sampling techniques for live animals especially came into focus with the 1990s outbreak of bovine spongiform encephalopathy that was linked to the human outbreak of Creutzfeldt Jakob's Disease. Serology is still an important tool in preventing foodborne pathogens to enter the human food supply through meat and milk from animals. One of the primary uses of rapid methods is for fast screening of large number of samples, where most of them are expected to be test-negative, leading to faster product release for sale. This has been the main strength of rapid methods such as real-time Polymerase Chain Reaction (PCR). Enrichment PCR, where a primary culture broth is tested in PCR, is the most common approach in rapid testing. Recent reports show that it is possible both to enrich a sample and enumerate by pathogen-specific real-time PCR, if the enrichment time is short. This can be especially useful in situations where food producers ask for the level of pathogen in a contaminated product. Another key issue is automation, where the key drivers are miniaturization and multiple testing, which mean that not only one instrument is flexible enough to test for many pathogens but also many pathogens can be detected with one test. The review is mainly based on the author's scientific work that has contributed with the following new developments to this field: (i) serologic tests for large-scale screening, surveillance, or eradication programs, (ii) same-day detection of *Salmonella* that otherwise was considered as difficult to achieve, (iii) pathogen enumeration following a short log-phase enrichment, (iv) detection of foodborne pathogens in air samples, and finally (v) biotracing of pathogens based on mathematical modeling, even in the absence of isolate. Rapid methods are discussed in a broad global health perspective, international food supply, and for improvement of quantitative microbial risk assessments. The need for quantitative sample preparation techniques, culture-independent, metagenomic-based detection, online monitoring, a global validation infrastructure has been emphasized. The cost and ease of use of rapid assays remain challenging obstacles to surmount.

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1. INTRODUCTION

Microbiological testing of foods has always been an integral part of food production, but most often applied for end-product control, which we now recognize as an ineffective means of assessment due to logistical complexities in sampling and the heterogeneous distribution of contamination. The implementation of the hazard analysis and critical control points (HACCP) system has shifted the burden from testing to process control as a means of ensuring food safety (FAO/WHO, 2003).

However, microbiological testing remains a critical tool in process control monitoring, quality control, surveillance, and in providing inputs to risk assessment. In addition, environmental sampling and analysis, as part of the total-chain approach, have become routine in food production as well as in outbreak tracing and tracking (Scheule & Sneed, 2001).

As food safety management further develops, microbiological testing will continue to play an important role in assessing whether Food Safety Objectives (maximum levels of hazards at the point-of-consumption) are achieved (Cole & Tompkin, 2005). However, traditional microbiological culture-based methods are limited, particularly in their ability to provide timely data to meet current Food Safety Objectives. The present review discusses the reasons for the increasing interest in rapid methods, current developments in the field, the research needs, and the future trends. Rapid methods are herein defined as alternative microbiological testing methods that are able to provide reliable test results in a shorter time than those obtained by culture cultivation.

1.1. Reasons for testing

Microbiological testing is performed with two primary purposes: to establish the absence of pathogens or their toxins to ensure the safety of foods, and to enumerate total or indicator microbial load to monitor effectiveness of hygienic processing and to verify product quality and shelf-life stability. In addition, there are a number of secondary purposes for testing: (i) food safety emphasis on the farm-to-fork approach has increased field testing, (ii) increased number of tests for large-scale data collection for risk assessment, (iii) surveillance and monitoring at the primary production levels require increased screening methods, and (iv) the increasing need of food producers to quantify the level of pathogens in food samples.

These food testing programs have long been performed with culture-based methods, which, have evolved into laborious and time-consuming procedures. With a few exceptions, pathogen testing is usually done to conform to the zero-tolerance limits (ISO, 2003). Nevertheless, analysis for pathogens in foods is challenging due to the complexities of food matrices, the heterogeneous distribution of low levels of pathogens, the high levels of normal bacterial flora, especially in raw foods, and the presence of ingredients that can interfere with assays and bacterial stress-injury that may occur from food processing (Stevens & Jaykus, 2004; Dwivedi and Jaykus, 2011). As a result, food samples often had to be processed through a series of culture enrichments to compensate for these logistical problems prior to analysis. Although effective, culture enrichment is time-consuming and often extends the analysis to several days (Hoorfar & Baggesen, 1998; Hoorfar & Holmvig, 1999; Josefsen et al., 2002).

In contrast, an analysis for total or indicator bacteria in foods is done as enumeration assays, and so culture enrichment is usually not used (Goodridge & Bisha, 2011). Interestingly, recent reports show that it is possible both to enrich a sample and enumerate it by pathogen-specific real-time PCR, if the enrichment time is short (**Josefsen et al., 2004**; **Krämer et al., 2010**). This can be especially useful in situations where food producers ask for the level of pathogen in a contaminated product. An increasing number of routine food testing laboratories face customer demands for quantitative data.

In general, the levels of bacteria in foods varies markedly, ranging from low or absent in some processed foods to exceeding log 7 CFU/g or mL in some raw foods. As a result, a food homogenate has to be subjected to labor-intensive, multifold, serial dilutions followed by lengthy incubation periods to obtain an accurate count. Thus, conventional microbiological methods are inadequate to provide quick assessments on the microbiological safety of foods and inadequate to determine whether the

product meets established microbial specifications (Feng, 2001).

Moreover, traditional microbiological methods are not fast enough to keep up with the pace of today's food production and distribution networks. Distribution networks are able to move food (and contaminants) from the farm to virtually any table around the globe in a remarkably short period of time. Faster detection of foods contaminated with specific pathogen strains can prevent tainted food from being consumed, and thereby minimize the risk of an outbreak of foodborne illness.

Rapid detection is especially critical in situations where products have already been set in motion. For instance, once the food product has been shipped, the producer may not know whether it is still in transit on a truck, on a store shelf, or even in a consumer's refridgerator. Thus, when identifying and tracing the contaminated product, time is of the essence. This has become more complex, because some foods, such as black pepper, peanut butter, or hydrolyzed vegetable proteins, can be used as base ingredients in other food products. For example, *Salmonella* contamination of these base ingredients has led to thousands of product recalls as well as many cases of salmonellosis (CDC, 2009).

1.2. New platforms

The advent of biotechnology introduced new technologies that led to the emergence of rapid diagnostic methods and altered food testing practices (**Lübeck and Hoorfar, 2002**). Rapid methods are comprised of many different detection technologies, including specialized enzyme substrates, adenosine tri-phosphate, antibodies, and DNA, ranging from simple differential plating media to the use of sophisticated instruments, as in automated enzyme-linked immunosorbent assay (ELISA), real-time PCR, and microarrays (Goodridge et al., 2011).

Some of these assays take only minutes to perform, whereas others merely reduce the time of analysis in comparison with traditional methods, thus demonstrating how fast these assays are is subject to interpretation. However, rapid methods are indeed faster and require less hands-on manipulations than culture-based methods. There are several other characteristics to rapid methods that are less emphasized: They can be miniaturized, and thereby take less laboratory space, they are amenable to automation and large-scale performance on many samples, and can result in a higher diagnostic sensitivity for some pathogens (Abubakar et al., 2007).

Although convenient, the application of rapid methods to food testing is not without complications, and there are many logistical issues to consider, including validation and regulatory implications (**Hoorfar et al., 2004**; Qvist, 2011). Furthermore, there are no assays without limitations; hence, the user needs to be aware of the advantages and disadvantages of rapid methods to maximize the benefits of their application (Abubakar et al., 2007).

1.3. Serologic screening

With the introduction of farm–fork concept in 1990s, development of serologic methods in ELISA or other formats gained widespread popularity (Smith et al., 1995). ELISA formats that detected herd-based immune reaction of food-producing animals to major pathogens such *Salmonella enteric* (**Hoorfar et al., 1994**), *Yersinia enterocolitica* (Nielsen et al., 1996), and *Toxoplasma gondi* (Lind et al., 1997) were able to point out the infected herds with a high degree of certainty. A number of intervention measures were then developed to sanitize the infected herds or eradicate the pathogens (Wegener et al., 2003).

Later on, other and less invasive sample than serum were taken for use in ELISA, including tank milk samples from cattle herds (**Hoorfar et al., 1995; 1995a**) or meat exudates from slaughter carcass (**Hoorfar et al., 1997**; Nielsen et al., 1998).

Hoorfar et al. (1997) compared muscle fluid (meat exudate) with serum samples for detection of antibodies to *Salmonella* lipopolysaccharide, where muscle fluid and serum samples were taken from two cattle populations: one from the island of Bornholm with no history of salmonellosis (n = 39), and the other from the *Salmonella dublin*-enzootic areas of Jutland (n = 144). The samples were tested in three ELISA platforms: *S. dublin* (O:1,9,12), *Salmonella typhimurium* (O:1,4,5,12), and *Salmonella* O:9-blocking ELISA. In the *S. dublin* ELISA, all serum and muscle fluid samples from cattle

on the island of Bornholm had OD450 values well below the cutoff value (0.5). For samples obtained from cattle in the enzootic areas of Jutland, high correlation was found between serum and muscle fluid samples. The same samples had similar significant correlation in the *S. typhimurium* ELISA and the O:9-blocking ELISA. The results indicate that muscle fluid samples taken at slaughter can be a practical alternative to serum samples for surveillance of *Salmonella* infections in cattle.

In another study (**Hoorfar et al., 1995**), O-antigen ELISA was used for screening of milk samples for *S. dublin* infection in dairy herds. Levels of antibodies to the O antigens (O:1,9,12) of *S. dublin* were tested in 1355 serum, 1143 cow milk, and 160 bulk milk samples from dairy herds. To define the background reaction, milk samples from all lactating cows and serum samples from nine animals were collected in each of 20 salmonellosis-free herds located on the island of Bornholm, where cattle salmonellosis has not been reported. Similar samples were collected from all stalled animals in 10 herds with recent (<6 months) outbreaks of salmonellosis located in Jutland, where salmonella infection is enzootic. Using herd history of salmonellosis, herd location, and clinical status of the herds as criteria, the optimal cutoff in the milk ELISA was determined as being at least 5% of the samples having optical density >0.5, resulting in herd sensitivity of 1.0 and herd specificity of 0.95. Although none of the sera in the herds from Bornholm was ELISA positive, two herds had a few reactors in the milk ELISA. Using the same cutoff, all but one bulk milk sample from 150 herds on Bornholm was ELISA-negative, and all 10 salmonellosis-positive herds from Jutland were ELISA positive. A significant correlation was found between ELISA reactions in milk and in serum of cows. The results showed the potential of assay as a practical and cost-effective tool taking advantage of the existing tank milk sampling schemes. The assay is currently used a national eradication program in Denmark.

The use of non-invasive sampling techniques for live animals especially came into focus with the 1990s outbreak of bovine spongiform encephalopathy that was linked to the human outbreak of Creutzfeldt Jakob's Disease (Martucci et al., 2009). Serology is still an important tool in preventing foodborne pathogens to enter the human food supply through meat and milk from animals (Wegener, 2010). However, serology is not applicable to food commodities downstream the production chain. In addition, although serology can be a cost-effective indicator of herd infections, its ability to point to single animals, especially latent carriers, remains to be further refined (**Hoorfar et al. 1996**). This can be partly due to lack of specific humoral immune response from some animals, delayed response from others, or elevated response for a long period despite clearance of the infections.

1.4. Screening vs confirmation

One of the primary uses of rapid methods is for fast screening of large number of samples, where most of them are expected to be test-negative leading to faster product release for sale. This has been the main strength of rapid methods such as PCR (Abdulmawjood et al., 2004; **Krause et al., 2006**).

This practice is most cost-effective for food manufacturers, but from a food safety standpoint, the occurrence of false negative results are of great concern, as these will not be identified until the product has been implicated in infections or outbreaks. Moreover, in screening applications, positive results are only regarded as 'presumptive' and need to be confirmed, often using culture-based methods. In regulatory or outbreak settings, confirmation goes even further, to the point where an isolate is obtained, identified, and subtyped. Herein, PCR can be a faster alternative to culture (**Hoorfar et al., 2000**).

Although tedious, confirmation is a critical component of food testing, as unconfirmed false positives not only lead to destruction of otherwise safe products but can also mislead epidemiological outbreak investigations. The need for confirmation may not pose great inconveniences because the majority of the samples are expected to be negative. Even so, for large manufacturers, the need to confirm even 1% of 'presumptive' positives may add to significant time, labor, and cost expenditures. Hence, false positive and negative rates associated with an assay can have a severe economic and food safety impact.

Rapid methods use many different technologies and assay formats and expectedly, their detection efficiencies can be food dependant.

For example, a food may contain ingredients that inhibit PCR, but have no effect on antigen–antibody reactions and the converse may occur in another food. Furthermore, these methods have different detection sensitivities that can range from log 1–6 organisms. As the level of contamination in food varies, it is not surprising that an assay may be effective in some situations, but not in others. It is critical, therefore, that assays are comparatively evaluated or validated to ensure their effectiveness in the intended testing situation prior to implementation (**Malorny et al., 2003**; Qvist, 2011).

1.5. Improved detection sensitivity

Aside from speed, rapid methods also can have better detection sensitivities compared with cultivation methods. For example, the previous standard method used to detect staphylococcal enterotoxins was the micro-slide gel diffusion assay that had a sensitivity of 10 μg. This is poor, compared with 10-pg sensitivity that can be attained with ELISA (Feng, 2007).

However, as detection methods improve, diagnostic sensitivity also increases, and this can create logistical challenges (such as product recalls, export disputes) to the food industry and the regulatory agencies. Most countries have adopted the regulatory position of 'zero tolerance' or 'absence' for most pathogens in foods, but the determination of 'absence' is method-dependant, and the differences in sensitivities have given rise to situations where foods previously analyzed using traditional methods and found to conform to the requirement of 'absence', no longer meet specifications when more sensitive methods are used (D'Agostino et al., 2004). In other words, as assays become more sensitive, so does the stringency of 'absence', which forces the food industry to modify processing and quality control procedures to comply with increased test sensitivity (Feng, 2001). It also inevitably leads to discussions on which concentration levels in food still pose a threat to public health when considering the dose–response curves (**Hoorfar et al., 2011**).

1.6. Return on investment

The cost and ease of use of rapid assays remain challenging obstacles to surmount. Rapid assays need to be cost-effective to facilitate their employment within the food chain. Similarly, although such methods have been developed to facilitate their ease of use, the need for sample manipulation and enrichment protocols prior to detection often complicates their adoption for food testing (see below). This means the majority of rapid assays are still largely confined to the laboratory, where they are performed by skilled technicians. This again adds to the cost of setup and training (Hoorfar & Cook, 2002).

Some scientists and test kit manufacturers lament that the food industry or regulatory agencies are unwilling to adopt rapid detection technologies. To understand who would require rigorous pathogen monitoring and who would be forced or required to institute these monitoring programs, it is necessary to understand the inherent market forces at play as to who would be willing to invest in education and technology adoption and why. The value of a commodity increases significantly, as it moves through the supply chain from the farm to the retail level. For growers, therefore, the return on investment of a particular intervention strategy or program is quite small at the farm level when compared with the return on investment at the retail stage.

Thus, the increased value of the commodity at the retail level provides the necessary impetus for ensuring that the commodity meets the food safety expectations of the customer. The incentive therefore rests mainly with the distributors and the retail industry to invest in education and adoption of new technologies to protect their brand name, customer base, and avoid recalls and associated economic ramifications. However, from a regulatory and public health perspective, the best value for money is to prevent pathogens from entering the production chain from the primary production source.

However, the adoption and implementation of rapid methods by industries are not easy, and there are many other complex issues to consider in the worldwide implementation of rapid methods for food testing. These include factors such as local economic situations and assay costs, distribution and availability of assays worldwide, shelf-life stability, and ruggedness of the reagents to time and temperature abuses that may occur during shipping, import regulation, and tariffs.

2. GLOBAL HEALTH IMPACT OF RAPID METHODS

Global health is the health of populations in a global context and transcends the perspectives and concerns of individual nations (Brown et al., 2006). The term reflects the realization that the health of populations around the world is inter-connected. Though travel and trade are often still regarded in terms of monetary values, it is increasingly recognized that these are also main drivers of modern global health. Humans, animals, raw materials, and food products are being transported faster and in greater volumes than ever before. As such, national health issues of one country, e.g. food safety and zoonoses, can have global implications.

Despite efforts to understand the mechanisms that determine the occurrence and emergence of zoonotic pathogens in the food chain, it is still not possible to predict zoonotic events with any great certainty. Intervention and control strategies are mainly targeted toward known threats and hazardous practices. As zoonotic pathogens move rapidly through human, animal, and food vectors, the timely detection and identification of these pathogens is of great importance to prevent its spread locally, nationally, and internationally.

Fast testing results are critical to minimize infected humans from traveling or to prevent contaminated batches of food from further distribution. In addition, emerging zoonotic threats such as, Severe Acute Respiratory Syndrome and influenza type H1N1, or bioterrorism attacks on the food supply all serve to illustrate the need for rapid diagnostic methods in relation to safeguarding global health. Hence, rapid detection to identify causative agents at an early stage linked with fast communication of food contamination can minimize the impact of an 'attack', reduce further distribution, and prevent an international crisis. To protect human health globally from cross-border events related to international travel and trade, the International Health Regulations were developed (WHO, 2005). The aim of International Health Regulations is to prevent, protect against, control, and respond to the international spread of disease while avoiding unnecessary interference with international traffic and trade. These regulations, which entered into force in 2007, require countries to report disease outbreaks and public health events that may be of international concern to the World Health Organization (WHO). To meet the requirements of WHO, many countries will have to strengthen disease prevention programs, increase surveillance reporting, and become more vigilant in their disease control and outbreak response systems, including those caused by foodborne pathogens.

2.1. Significance of rapid methods for international trade

The ability to trade food among countries is subject to a strong legal framework that is consistent with the rules outlined in the World Trade Organization (WTO) Agreement on the Application of Sanitary and Phytosanitary Measures (WTO, 1995) aimed at protecting human, animal, and plant life, or health.

Benchmark standards are developed at the international level by the Codex Alimentarius Commission, the International Plant Protection Convention, and the World Organization for Animal Health. However, each exporting and importing country is responsible for putting systems and measures in place which aim at ensuring that the food that is traded nationally or internationally is safe for consumers. Given that the value of exported agriculture commodities was approximately USD 876 billion in 2007, about 70% of which was food (FAO/WHO, 2009), this is an enormous task.

Even though modern approaches to food control place increased emphasis on process control, testing remains an important component of any system which aims to produce safe food. The food sector is in fact the largest market for microbiological tests. Testing is also a valuable tool for import control as part of programs to check and assess compliance with import requirements. For example, in the year March 2009 to March 2010, the European Rapid Alert System for Food and Feed (RASFF) issued 325 notifications related to *Salmonella* alone in food and feed based on testing within the EU import control program (RASFF, 2010). Monthly import refusal reports from the United States Food and Drug Administration (US-FDA) reflect a similar importance of testing in their import control program (US-FDA, 2010).

Another important factor is the increase in international trade of perishable products such as fresh fruit and vegetables (FAO/WHO, 2009). With conventional test methods, irrespective of the outcome, the lengthy delay in getting a result can mean that by the time a shipment is released, it is no longer marketable. The ability to test and subsequently transport food quickly is a critical element of international trade today; hence, the availability of rapid test methods offers a major advantage.

However, there is also the potential that as we begin to use rapid methods, which are often more sensitive than conventional methods, pathogens and other contaminants will be detected at lower levels leading to the rejection of larger amounts of imported food. The application of rapid methods needs to be linked to policy considerations, and the potential need to address the implications of detection of, and the risk associated with, very low levels of pathogens. This adjustment has already occurred in the area of testing for veterinary drug residues, where the detection of very low levels of residues using new methods led to a major trade disruption before the risk management and policy aspects were addressed to reflect the change in detection levels (FAO, 2004).

The availability of a rapid method alone does not mean that regulators are immediately going to take it on board as a standard method. The performance of the method compared with conventional cultivation methods is an important consideration for global regulators, not only in relation to their own methods but also to those used by regulators in export markets. There are numerous bodies that can certify the performance of such methods or provide guidance on their validation compared with currently accepted methods that can facilitate equivalence, but this can be time-consuming.

In addition, the users should be cognizant to the possibility that a method validated by one country for the analysis of a particular product may not be applicable to the same product in another country. Different countries can have different production and processing practices that may result in foods containing different ingredients, pathogens, or levels and types of normal bacterial flora. Thus, the acceptance and use of such methods by official authorities is, usually for valid reasons, slow, and tends to lag behind the use of such methods by the private sector.

2.2. Rapid methods in developing countries

The contribution of developing countries to international food trade is increasing. In 2007, almost 40% of agricultural commodities in international trade came from developing countries, 87% of which were food items (FAO/-WHO, 2009). For many of these countries, increased access to international markets is an important element of their development strategies. This can neither be achieved nor sustained without addressing food safety, and thus there is a strong interest and demand for testing of foods from these countries, particularly given the challenges posed to food safety management by highly fragmented production systems, contaminated water supplies, unreliable electricity supplies, weak storage facilities, and poor road infrastructure.

In the African region, some of the food safety challenges include weak foodborne disease surveillance, inability of small- and medium-scale producers to provide safe food, outdated food regulations, weak law enforcement, inadequate capacity for safe food storage, and inadequate cooperation among stakeholders (WHO, 2007).

Over the last decade, many developing countries have made significant improvements to their national systems of food control. However, in many cases, considerable work is still required for these systems to afford the level of public health protection from foodborne hazards achieved in many industrialized countries. Lack of reliable and efficient laboratory services and trained laboratory personnel are major constraints facing both regulators and food chain operators in these countries. High capital costs for investment in testing facilities and high running costs, due to inadequately trained human resources, distant technical services for the repair and maintenance of equipment and for provision of supplies and consumables, contribute to the reluctance of authorities to invest in the development of laboratory infrastructure. Even when such infrastructure exists within a region or country, getting samples to a laboratory in good condition may be problematic.

Capacity building that also includes improved analytical skills for monitoring raw material going into food production and control of foods on the market is critical. Herein, cost-effective, non-complex rapid methods, such as simple, disposable assays that do not require instrumentation can play a key role to improve the public health and food safety in developing countries.

Furthermore, the use of quick and easy-to-use tests, particularly for screening purposes, can increase the number of samples to be tested, thus improving the overall statistical reliability of testing programs. The potential to reliably and accurately test product on site, be it a border inspection post, market or primary producer, can provide many benefits.

If rapid field tests are to offer value, then they need to be robust and reliable under the storage conditions of test kits and use in developing countries, taking into consideration the often high ambient temperatures and lack of refrigeration. In addition, they must be affordable and readily accessible. Many field tests or diagnostic kits are manufactured by a few selected countries, but have limited technical support, service, and distribution abroad. The customs and importation requirements of various countries can also affect the accessibility of diagnostic kits.

3. RAPID METHODS FOR RISK ASSESSMENTS

The primary objective of risk assessment and the process of modeling contamination in the food chain are improved decision-making and risk management actions, such as identifying intervention points to reduce risk effectively. This is achieved by developing a model of a food production system that can span the entire 'farm to fork' chain.

Models use mathematical equations to describe the system and rely on data to inform it. The reliability of the data is a key factor in ensuring that the model reflects reality and consequently that decision-making advice can go beyond vague risk management guidelines to more specific interventions and their expected risk reduction. Typically, however, the information and data that have been available for modeling the food chain has often been insufficient, uncertain, and too often qualitative in nature (FAO/WHO, 2002; FAO/-WHO, 2009).

3.1. Increased data and information

Rapid methods offer the promise of improving the risk assessment and modeling process by increasing the amount of data and information available for use in developing risk assessments, as well as reducing the uncertainty associated with the data that is used.

The process of developing a risk assessment model can improve our understanding of the food system under investigation by closely examining how different parts of the system interact with each other and by evaluating what data exists or what information we have about the system. A key part of developing a model is to use pathogen contamination data at the start of the process and simulate in the model how this changes as the food product moves through the food production system. Some models have included the use of expert opinion, data from surrogates, data from other countries, or other systems as proxies (FAO/WHO, 2003; FAO/WHO 2008a; Boone et al., 2009; Ross and Sumner, 2002), although without good, reliable quantitative data on pathogen presence and levels, these models describe contamination with a high degree of uncertainty.

The absence of quantitative data for use in risk assessment models has been a concern in those models developed to date. The use of rapid methods, by reducing the time and cost associated with collecting quantitative data, offer the potential to dramatically increase both the quantity and quality of the quantitative data available for risk modeling activities.

The potential of rapid methods to be more sensitive can skew risk assessments. Although the quantification of a pathogen in a food product is an important part of the risk modeling process, an equally important part of the process is the characterization of the prevalence of contamination. A risk model needs to determine the probability of exposure as well as the amount of exposure to a pathogen. Historically, there has been recognition that the probability of exposure that was typically used was a

function of the method, and risk assessments have taken this data at face value. However, a problem that has often been ignored is diagnostic method sensitivity, which most of the times has resulted in underestimations (Lindqvist et al., 2002; Oscar, 2004).

The problem is exacerbated when the product is assumed to be uncontaminated in the risk model and when it subsequently experiences time and temperature abuse that allows for growth, or even more significantly, is mixed with uncontaminated product that subsequently allows for growth. In the former situation, a product that was assumed to have no risk can in fact, expose an individual to a dangerous infectious dose, and in the latter case, multiple people that would have been estimated to have no-risk could be at significant risk of illness.

3.2. Outbreak data

To translate exposure to pathogens to a health-risk outcome, risk assessments require dose–response models that predict the probability of infection or illness upon exposure to a dose of pathogen. Although human feeding trial data from the 1960s does exist, it is unlikely now, due to ethical considerations, that any new dose-response data will be collected using human feeding trials. As a result, risk assessments are faced with the intractable problem of either a complete lack of information on the probability of infection for some pathogens or a large uncertainty associated with other pathogens and no way of addressing this information gap.

One source of information that can help overcome this problem is to make use of outbreak data. However, the ability to obtain samples of food, process them rapidly and with sufficient sensitivity to estimate a dose that was likely to have caused illness during an outbreak has been historically difficult to achieve. The development and use of rapid methods can clearly contribute to the later two issues (rapid processing and sensitivity) and should increase the likelihood that outbreak data can contribute to the overall understanding of dose-response and improve the ability of risk models to estimate the probability of illness.

4. CURRENT DEVELOPMENTS

4.1. The crude beginning

The greatest part of the work done on new and rapid methods is dealing with fancy detection step. However, the crude sample preparation step right at the (forgotten) beginning of any protocol is a precondition for advanced laboratory diagnostics. The newer methods that claim less time to reach a result have a major limitation with how much volume they can take prior to the rapid analysis for the target molecule. For example, PCR reactions, which require tens to hundreds of microliters, represent a good example of the problems facing the modern food microbiologist. The technical challenge is how to obtain an accurate representation of 25 g of cheese into the 100 μL sample that will go into a real-time, reverse transcriptase PCR reaction; this issue should be the center of focus. Herein, using some of the technologies described may be useful, although nucleic acid-based methods currently require 10^3–10^4 in the primary enrichment broth, which includes food particles, etc. (Jensen & Hoorfar, 2002; **Hoorfar et al., 2004**; Hoorfar et al., 2004a; **Josefsen et al., 2004**; Josefsen et al., 2004a).

Although many commercial PCR kits include easy-to-use sample treatments protocols that (apparently) work, independent studies show a remarkably low diagnostic sensitivity when testing naturally contaminated samples (D'Aoust et al., 2007).

Irrespective of whether the target is DNA, RNA, or proteins, it is not currently possible to overcome the problem posed by sample size without some sort of enrichment step, which may not be replaceable in the foreseeable future. This is especially important as the scientific community is now entering the so-called 'nanotechnology' phase. Pathogens that are known to enter the viable, but non-culturable state or that are stressed pose further challenges when shortening or eliminating the enrichment period, normally required for bacterial pathogens to reach levels amenable to detection by various platforms.

Clearly, foodborne viruses and parasites (cysts, oocysts) pose perhaps the greatest challenges as they cannot be enriched at all (Schultz et al., 2007; Schultz et al., 2010; Croci et al., 2008; Dixon et al., 2011). For these organisms,

sample preparation is the key step in being able to apply any downstream detection strategy. Unless the sample preparation method can isolate the 10 oocysts or 100 norovirus particles, for example, no amount of advanced downstream technology will help with the analyses required. This detection limit may be reached by combining simple filtration or floatation with RT-PCR (Wolffs et al., 2005; Morales-Rayas et al., 2009).

Although enrichment is a limitation in terms of assay speed, it is necessary for several reasons beyond increasing bacterial numbers, including diluting the effects of inhibitors, allowing the differentiation of viable from non-viable cells, and allowing for resuscitation of injured cells. The aforementioned reasons have been carefully reviewed, and the current sample treatment methods have been recently reviewed by Dwivedi & Jaykus (2011) and Rossmanith et al. (2011).

Recently, alternatives to enrichment may become more common in the future; Tolba et al. (2010) and Cademartiri et al. (2010) have described methods to immobilize bacteriophages on paramagnetic beads and surfaces, so that they retain infectivity. These immobilized bacteriophages can be used to capture host bacterial cells and, following propagation, bacteriophage nucleic acid can be detected using real-time PCR. This provides two cycles of amplification of the target; the first due to replication of the phage in the host and the second by PCR amplification of unique phage nucleic acid sequences. Using this technique it might be possible to detect one cell of the host bacterium within 4 h. As the bacteriophage requires metabolically active cells to propagate, the assay should, at least in theory, only detect live cells.

4.2. Quantitative sample preparation

One of the most promising target extraction techniques is the use of non-specific DNA-binding paramagnetic beads, especially in combination with real-time PCR to ensure the target specificity (Nogva et al., 2003; **Josefsen et al., 2004**; **Josefsen et al., 2007**). It was shown that the crucial parameter when using magnetic beads is the spherical shape, uniformity of the beads and correct binding and dissociation buffer conditions (Strömberg et al., 2008; Peeters et al., 2010). Magnetic beads can have the advantage of consistency and reproducibility due to controlled amount of beads and buffer volumes used in a protocol, provided choice of robust and sensitive probe chemistry (**Josefsen et al., 2009**).

Using paramagnetic beads, **Josefsen et al. (2004)** have developed a semi-quantitative-strategy to quantify low numbers of *Campylobacter* from chicken rinse samples. It was shown that even after a short pre-enrichment phase under standardized conditions, the initial numbers of *Salmonella* in carcass rinse was reversely correlated to the Ct values (**Krämer et al., 2011**). However, the precision of the method needs to be elucidated in more detail. Generally, this strategy was also applicable to *Salmonella* using adapted enrichment media and time-course followed by real-time PCR quantification (**Krämer et al., 2011**). Thus, careful consideration should be given to the enrichment strategies for *Salmonella* cells in combination with subsequent quantitative real-time PCR analysis.

For direct PCR-based quantification of pathogens from food samples, Aprodu et al. (2011) evaluated three different pre-PCR sample treatments, i.e. immuno-magnetic separation based on phage-derived cell wall binding molecules, matrix solubilization and flotation to establish their suitability for quantifying low numbers of *Staphylococcus aureus* in milk. All three procedures succeeded to remove *S. aureus* from the milk matrix, either raw or pasteurized, and, as a result of the concentration of the target cells, minimized the effect of milk associated PCR inhibitors. However, immuno-magnetic separation albeit being user friendly, specific, and rapid failed to allow quantification of low and medium numbers ($< 10^4$ CFU) of *S. aureus*.

Löfström et al. (2010) developed a quantitative flotation protocol based on traditional buoyant density centrifugation, for culture-independent enumeration of intact *Salmonella* in pig carcass gauze swabs (100 cm^2) prior to quantitative PCR. This novel approach excluded the homogenization step prior to flotation to improve the detection limit, and speed up the quantification procedure. The buoyant density of two *Salmonella* strains in different growth conditions was determined to be 1.065–1.092 g/mL. Based on these data, an optimal discontinuous flotation with three different density layers was designed,

which allowed quantification from 4.4×10^2 to at least 2.2×10^7 CFU *Salmonella* per swab sample using real-time PCR (without preceding DNA extraction) or selective plating. Samples with 50 CFU could be detected occasionally, but fell, however, outside the linear range of the standard curve, which indicate the need to improve the detection limit of the method. In addition, the swab samples showed a broad biologic diversity; for seven samples not inoculated with *Salmonella*, the microbial background flora was determined to 5.0 ± 2.2 log CFU/mL sample withdrawn after flotation. Interestingly, it was found that the proceeding PCR step was inhibited by background flora concentrations of $\geq 6.1 \times 10^8$ CFU/swab sample, but not by concentrations $\leq 6.1 \times 10^6$ CFU/swab sample.

The authors showed that using the gauze swabs directly in the flotation procedure, the homogenization step normally used for preparation of food-related samples could be excluded, which simplified the culture-independent quantification method considerably. The use of aforementioned protocol (having in mind its limited detection sensitivity) can facilitate quantitative risk assessment in the meat production chain.

A number of intervention strategies against *Campylobacter* contaminated poultry focus on post-slaughter reduction of the number of contaminating cells, emphasizing the need for rapid and reliable quantitative detection of only viable *Campylobacter* to document the effect. A new and fast quantitative real-time PCR approach has been reported based on the use of a simple propidium monoazide sample treatment (**Josefsen et al., 2010**). The method generated a signal from viable, but non-culturable *Campylobacter* with an intact membrane in < 3 h (total assay time). The method performance was evaluated for variability of the individual chicken carcass rinse matrices, species variation, and the efficiency of DNA extraction at different cell contents. The method showed reproducible results when compared with culture-based enumeration on 50 presumably naturally contaminated chickens from a known *Campylobacter*-positive flock.

4.3. Same-day screening of negative samples

The very first efforts to reduce the pre-enrichment time for *Salmonella* to be able to provide the testing results within the same day of receiving the samples failed (Myint et al., 2006). Recently, with the improved sensitivity of real-time PCR and improved pre-analytical sample preparations, it was possible to develop and ring-trial validate a 12-h assay, which fits within the working schedule of large slaughterhouses (**Josefsen et al., 2007**). Herein, the preliminary studies found that limited pre-enrichment of 8-h in buffered peptone water (BPW) was unable to produce sufficient number of *Salmonella* consistently. The addition of novobiocin or other substances to inhibit the background microflora did not improve the detection reproducibility. The solution was a combination of DNA purification by paramagnetic nano-particles, a sensitive, but still robust probe technology, and finally increased volume to purified DNA sample to the PCR tube (**Josefsen et al., 2009**).

A similar same-day approach, but without any enrichment, was used for detection of *Campylobacter* in poultry slaughterhouses (**Krause et al., 2006**). The strategy should be expanded to other pathogen protocols that require overnight resuscitation of the samples in some sort of enrichment media. In general, this protocol is useful in situations, where the number of contaminated samples in slaughterhouse is expected to be low; PCR positive results should still be confirmed, and the isolates submitted to a reference laboratory for serotyping should be used for safety management and as part of surveillance or source-attribution studies. In countries with a very low contamination level, same-day screening of large number of (non-contaminated) products can speed up the product dispatch, whereas in countries with expectedly higher number of contaminated carcass the product release should still await the culture-confirmation step. Another advantage of this protocol is faster release of fresh ground meat or poultry that has obviously a very short shelf-life. In addition, faster release means less storage space and thereby improved productivity for the food producer.

4.4. Untapped potential of PCR for industry

Quantification using real-time PCR is an established technique. Despite this, PCR is still mostly used only to declare food products free from pathogens. For the food producers, the more advanced use of PCR would be to directly

link patients who have eaten such foods to the pathogen, but such investigations are difficult to conduct at the current industry settings.

One of the unexploited potentials of real-time PCR for the food industry is to gain information on the number of pathogens present in the food or food production environment. This could help food producers to assess the impact of hygiene measures or the implementation of an improved quality assurance program. When personnel involved in quality assurances observe a reduction of the contamination they have been fighting against, they would be encouraged to continue the efforts. Fortunately, the new generation of real-time PCR technologies takes us closer to achieving this aim (**Malorny et al., 2008**), especially when using a log-phase enrichment medium (**Josefsen et al., 2004**; **Krämer et al., 2011**).

Another new application of PCR is its use by routine analysis laboratories to determine the source of contamination; whether due to raw material, personnel, equipment, or the food processing environment. By simultaneous detection and sub-typing of pathogens, it will be possible for food producers to accumulate in-house data on persistent strains. Adding DNA cutting-enzymes to post-PCR tubes could be easily done, but at present, time requires highly trained personnel. There is no question that this will be automated in the future, where different versions of the same PCR kit will be commercially available.

An example from clinical settings to follow is high-resolution melting curve analysis that takes advantage of differences in the melting curve of multiple DNA products, all in the same PCR tube (Tajiri-Utagawa et al., 2009). The software included in the PCR instrument produces several distinct graphs, each representing one subtype of target pathogen. This is all done without any additional laboratory work (Rudi et al., 2007a).

4.5. Automation and multi-pathogen detection

Another key issue is automation, where the key drivers are miniaturization and multiple testing, which mean one instrument is flexible enough to test for many pathogens, but also many pathogens can be detected with one test. Almost all existing rapid methods are designed to detect a single target, which makes them ideal for use in quality control programs to quickly screen large numbers of (mostly negative) food samples for the presence of a particular pathogen or toxin. The bottleneck in developing multi-pathogen PCR methods is the DNA probe chemistries. Instrument platforms can detect four probes in the same analysis, some probes showing higher fluorescence yield than others (**Reynisson et al., 2006**; **Josefsen et al., 2009**).

Another challenge is the use of different software and logarithmic calculations among instrument makers to transform the PCR fluorescence readings to measurable values. Unless some kind of standardization is done in this area, it would not be possible to develop universal multi-pathogen testing that can be used across real-time PCR platforms. Until then, laboratories may be forced to acquire new (and costly) instruments, every time a new multi-pathogen method is implemented.

Fortunately, the downstream detection, whether for nucleic acid, protein, or specific analyte, has seen an increase in methods used normally in other fields such as chemistry, biochemistry, and physics. These include Fourier Transform Infrared spectroscopy (FTIR), aptamers, biosensors, etc. (reviewed by Goodridge et al., 2011).

Strategies to detect multiple pathogens or to analyze microbial diversity in food or a clinical specimen with a single assay platform are highly desirable for food safety and food bio-security applications. It would not only cut costs for pathogen testing but also aid in providing total microbiological food safety.

Such screening capabilities would also allow researchers to study microbial diversity in a sample, food processors to release products in a timely manner, and regulatory agencies to make prompt decisions about whether the food is safe for consumption. In the event of an outbreak due to known or unknown agents, it is necessary to first provide a general assessment of the nature of the pathogens/toxins present in a product to initiate corrective or preventive actions such as treatment regimen, further processing, disposal of product, etc. At a later point in time, more rigorous laboratory-based protocols can be initiated to determine the identity of the agent using e.g. DNA array-based methods.

As such, it is likely that molecular-based amplification assays including PCR will dominate future diagnostic developments, when multiplex detection is a requirement. However, multi-pathogen detection requires that different classes of microorganisms (bacteria, viruses, parasites) be detected at the same time. Such requirements will pose challenges to the current rapid methods, due to issues associated with the fact that some microorganisms (bacteria) can be enriched, whereas others (viruses and parasites) are difficult, if not impossible, to culture. Recently, researchers have developed a confirmation, which contains 388 000 probes that together may screen for about 2000 viruses and 900 bacteria (Jiang et al., 2008).

An improved understanding of strain diversity from whole-genome microarray analysis and other genetic comparisons, plus the availability of an increasing number of whole-genome sequences, has led to a second generation of DNA microarrays that focus on smaller number of more informative genetic targets (Bruant et al., 2006; Huehn et al., 2009a). Low density microarrays consisting of a few to several hundred probes that detect specific genes or loci have been developed for several foodborne pathogens. Several different arrays employing oligonucleotides 50–70 residues in length and targeting genes related to virulence, prophages, plasmids, antimicrobial resistance determinants, and other variable elements have been described. Total genomic DNA from the strain of interest is labeled with a fluorescent dye and hybridized to the array; the analysis of total genomic DNA allows the characterization of strains without preconceived knowledge of the gene content of the strain.

Huehn & Malorny (2009) developed a microarray that represented 282 genetic loci from *Salmonella*. Probes were 40–60-mers and detected genes associated with serotype, virulence, prophages, fimbriae production, and antimicrobial resistance. The microarrays have been successfully used to understand the relationships between and virulence of specific *Salmonella* serotypes (Huehn et al., 2009a; 2009b), and identified diversity among *Salmonella* serovar Typhimurium strains of possessing the same PFGE pattern (Litrup et al., 2010). The next issue to be addressed here is the critical parameters for diagnostic robustness and reproducibility (**Grønlund et al., 2011**).

With regard to other pathogens, Bruant et al. (2006) developed a microarray to detect 189 genes that have been associated with virulence in *Escherichia coli* and 30 antimicrobial resistance genes, represented by 315 70-mer oligonucleotides. The array was used to characterize *E. coli* isolates from surface waters, showing that 24% of the isolates possessed a virulence gene profile suggestive of specific *E. coli* patho-types (Hamelin et al., 2007).

5. FUTURE TRENDS

5.1. Real-time testing

To be truly useful, microbiological testing must provide results in 'real-time'. Although this is difficult to achieve in food systems where the prevalence and incidence of the target are invariably low and it is not homogeneously distributed throughout the food, there are model approaches that may in the future allow detection without the need for enrichment or crude sample pre-treatments (Rider et al., 2003).

Among these approaches, the use of signal amplification techniques has received attention (Tolba et al., 2010). If it is possible to generate results in near real-time, then applications of microbiological testing could be extended to on-line monitoring, on-farm monitoring, as well as devices that consumers could use to verify the safety and quality of foods.

An increasing number of monitoring devices are becoming more compact to facilitate on-site detection of pathogens. In general, diagnostic technologies are becoming mobile, digital, virtual (software-based), and personal. This development can facilitate their use at the source of infection (field, farm, animal, slaughterhouse, etc.). In clinical and veterinary settings this is point-of-care diagnostics, but in food testing this development will result in on-site, on-line, or in-line monitoring systems that obviate the need to ship samples to testing laboratories.

An example of this system is the possibility of semi-continuous monitoring of *Campylobacter* in the air of chicken houses (**Olsen et al., 2009**). The development of improved methods to monitor for *Campylobacter* infection is important

for production of *Campylobacter*-free chickens. **Olsen et al. (2009)** for the first time detected *Campylobacter* in air samples and compared with the detection using real-time PCR in feces and dust. The study was conducted during the lifetime of chickens in broiler houses and concluded that detection of *Campylobacter* in air is comparable in sensitivity to detection in other sample materials. *Campylobacter* could not be cultivated from precipitates on Modified cefoperazone charcoal deoxycholate agar (mCCDA) plates, suggesting that the airborne particles including *Campylobacter* DNA may not be viable, consistent with the notion that *Campylobacter* is not an airborne infection.

Profiling of airborne particles revealed that the aerodynamic conditions were dependant on the age of the chickens and very comparable in broiler stables with low proportions of particles. *Campylobacter* could also be detected in air samples collected at the hanging stage during the slaughter process, but not at any other stage at the slaughter house. The exploitation of airborne dust in broiler houses as sample material for detection of *Campylobacter* and other pathogens provide an intriguing possibility in conjunction with new detection technologies allowing continuous or semi-continuous monitoring of infectious status.

The sampling of microorganisms in air and subsequent analysis by PCR can circumvent the PCR inhibition problem. PCR amplification of air samples has been reported to be less sensitive to inhibition than feces samples, and detection of as low as 1–10 CFU and spores pr air sample has been reported (Alvarez et al., 1995; Makino & Cheun, 2003). However, high amounts of non-specific DNA is known to inhibit PCR sensitivity in general, and may influence detection in air depending on the quality of air sampled (Alvarez et al., 1995; Stetzenbach et al., 2004).

In the ideal situation, a non-invasive, handheld scanning device (similar to barcode scanning at a local grocery store) would be used to quickly and accurately assess all aspects of a food commodity. This would include presence or absence of pathogens and their associated toxins down to a single-cell level even when they are not uniformly distributed in a food matrix. This may not be as far out of reach as one might think. Devices have been developed based on molecular fluorescence spectroscopy that can analyze microbial signature in wounds (DaCosta et al., 2007). As usual, the clinical diagnostics is much ahead of food testing, partly because of the higher number of pathogens in clinical samples, and partly because of the willingness to pay much more for a clinical testing.

With advances in genome sequencing and bioinformatics, single nucleotide polymorphisms and the various assay formats that have been designed to address them can allow for an assay to detect pathogenicity features unique to a foodborne pathogen (Goodridge et al., 2011).

Alternatively, assays can be designed to detect pathogenicity islands, virulence factors, etc., that might preclude the need to isolate the intact organism. In this light, the key feature will be the ability to generate a molecular fingerprint within the detection signal, so that it may be possible to help epidemiological investigations (Pagotto et al., 2008; **Grønlund et al., 2011**).

5.2. Culture-independent detection and typing

Diagnostic methods in general are being increasingly influenced by the 'omics' technologies, especially genomics, which have reached a stage where they are used on a routine basis in many reference laboratories. These technologies are expected to move towards culture-independent detection and characterization techniques based on the purification of total DNA from diagnostic samples and subsequent metagenomics analysis.

Nevertheless, the limitations of the information obtained when using genomics must be recognized. First, DNA-based assays detect genetic targets (such as virulence genes), which only indicate that bacteria with those gene sequences are present, whereas they do not indicate whether the gene is actually expressed or whether the bacterium is viable. Second, current legislations require the isolation of unintended microorganisms in situations such as food recalls, i.e. cultivation methods need to be used in addition to DNA-based methods during outbreak investigations. Third, DNA-based methods cannot detect proteins such as enterotoxins, which can indeed cause foodborne intoxications. Herein, proteomic techniques, including protein arrays or protein PCR (Gullberg et al., 2004), may be increasingly used for food safety. Despite those limitations, as the 'omics' technologies become increasingly accepted, obtaining

an isolate may not be a regulatory requirement in the future. One of the most promising developments is taking place in the areas of intestinal microbiology and geomicrobiology using metagenomics techniques, i.e. detection and identification of specific genes directly from the sample without any cultivation steps (Rudi et al., 2007a, 2007b; Morgan et al., 2010).

5.3. Metagenomics

The emphasis of future testing developments could be on the use of metagenomics (gene-based) rather than the phenotypic methods used today. One challenge will be to identify genotypes that are associated with infection. To accomplish this, a significant investment in bioinformatics will be required in order to translate large amounts of metagenomics data into a format that provides useful diagnostics, surveillance and epidemiological information.

The rationale behind metagenomics approach is that infectious diseases are caused by various pathogens, including yet-unidentified microorganisms. As procedures for detecting and identifying pathogens vary according to the target microorganism, diagnostic methods require a variety of media, reagents, and skills. As an alternative approach, DNA sequencers can determine more than 100 megabases of DNA sequences per run, and new sequencing technologies eliminate the bacterial cloning step used in traditional Sanger sequencing. Instead, they amplify single isolated DNA molecules and analyze them with computers capable of massive parallel processing (Reynisson et al., 2011).

Consequently, an increasing number of so-called metagenomics methods tap into the potential of novel sequences to promptly detect and identify various infectious pathogens (Nakamura et al., 2008). However, these sequencing-based methods are not without drawbacks, as it has been pointed out that care should be taken to avoid bias caused by different sample preparation protocols (Morgan et al., 2010).

Currently, the ability to subtype microorganisms is dependent on obtaining the isolate in pure culture. Culture-independent diagnostic tests are becoming available and increasingly used, particularly in clinical medicine (Bauer & Reinhart, 2010). In food production chains, where obtaining isolate can be challenging, new tracing approaches can combine metagenomic-based diagnostics with domain modeling to identify the source of contamination.

5.4. Future risk assessments based on metagenomics

The food safety risk assessment as first described by FAO/WHO (1995) states that it should be the role of the official bodies to use risk analysis to determine realistic and achievable risk levels of foodborne hazards. In recent years, this has been followed up by a discussion on setting risk-based metrics, i.e. Food Safety Objectives, Performance Objectives, and Microbiological Criteria (van Schothorst et al., 2009).

By moving toward real-time testing, it may also be appropriate to change the risk assessment concept toward on-line product risk assessments based on the microbiological testing. This requires the development of mathematical risk assessment tools, as part of the testing scheme, so that the microbiological test result is immediately modeled into an estimation of the public health outcome. An example here is a 30-times reduction of risk of campylobacteriosis following a 2-log CFU reduction of *Campylobacter jejuni* concentration in the chicken gut (Rosenquist et al., 2003). This is only possible, if the testing is done at slaughter, or as close to the slaughter time as possible.

As a result of this paradigm shift (from isolate-based to gene-based diagnostics), the new 'omics' technologies will further challenge the currently applied risk assessment methodologies, because bacterial detection and characterization will no longer be based on isolation of a specific bacterial strain, but rather on detection of genes or gene pools from a sample. Furthermore, these genes need not necessarily come from the same bacterial strain, but may represent several strains and even different bacterial species in the same sample.

Therefore, the development of metagenomics-based diagnostic tools should be linked to mathematical models that relate the information obtained from gene-based diagnostic assays to the risks posed to public health. One of the benefits of this approach would be improved source attribution i.e. models for quantifying the relative contribution of different food sources to a particular disease burden (Pires et al., 2010).

This may not be so far, as whole-genome sequencing, completed in less than a week, has been applied to outbreak investigations (Gilmour et al., 2010; Lewis et al., 2010). However, the major limitations in applying whole-genome sequencing to sub-typing and other biologic questions are the need for improved computational power to analyze the data, and the need for a means to store the massive datasets. Both these limitations will need to be addressed before whole-genome sequencing can be applied widely. Furthermore, as with any other sub-typing method, whole-genome sequencing must be validated in the context of the biologic questions it attempts to answer. It remains to be seen whether the straightforward concordance between whole-genome sequences and epidemiologic data, as in the report of a *Listeria* outbreak investigation (Gilmour et al., 2010), is the exception or the rule.

5.5. Biotracing

A rapid method by itself can be useful, but it will be more beneficial to food producers, regulators, and control authorities, if it is part of an integrated source-tracing system.

In outbreak situations, epidemiologists point to a certain food product as being suspected to have caused the outbreak. In most cases, this is indicated through case–control studies (Neimann et al., 2003), interviewing the affected patients, or re-testing suspicious food samples. However, in some cases, it is not possible to isolate the microorganism that caused the infection, especially in ingredient-driven outbreaks, where the same base product is part of many ready-to-eat foods (CDC, 2007a; CDC, 2007b).

In the future, the source identification may be modeled in Bayesian Belief Networks to point to the source. This new concept, referred to as biotracing (**Hoorfar et al., 2010**), can combine laboratory results obtained from detection of pathogen-specific genes, with information collected during the entire food process to make sound decisions about possible product recalls (food 'forensics') or determine the source of possible contamination (Barker et al., 2009). The concept is the foundation of the European Integrated Project BIOTRACER (http://www.biotracer.org; **Hoorfar et al., 2010**).

The advantages of improved biotraceability are faster intervention, limited recalls, and more targeted remedial action. Biotracing is not dealing with risk assessments, but developing tools that can be used in 'second-generation' risk assessments involving quantitative microbiology and genotyping methods.

The main advantage of biotracing is the high degree of integration of laboratory data into different steps of the production chain (Jordan et al., 2011). Although HACCP focuses on the critical production points, biotracing deals with the entire chain, from the primary production at farms, through transportation, storage, distribution, shelf-life issues, and consumption by consumers. An example here is the biological information on the significance of strains that are isolated during routine quality control checks: does the strain have virulence traits, can it confer pathogenicity, is it recurrent, can it survive the environment of the food production in question? (Champion et al., 2005; Wein & Liu, 2005).

Another aspect of biotracing, compared with HACCP or risk assessment, is virtual contamination scenarios; i.e. food producers work out for each food chain, a contamination scenario that may or may not happen sometime in the future. The contamination can be inadvertent (accidental) or caused on purpose as part of an attack on food supply chain.

5.6. Validation infrastructure

Despite the enormous supply of commercial kits and published protocols on rapid methods, surprisingly, many end-use laboratories still use culture-based methods. Is this because there is a lack of demand for faster methods? The answer is no. This is partly caused not only by the history of microbiology and laboratory traditions but also because of the lack of a universal validation infrastructure; it can be costly to develop a new method, but it also requires significant resources to thoroughly validate a new method against culture-based methods (Qvist, 2011).

New detection methods must perform equal to, or even better than, standard culture-based methods through extensive comparative and collaborative trials, and even then it may be difficult to obtain regulatory approval for their use.

Numerous studies have shown that rapid assays perform better in some foods than in others, mainly due to differences in the bacterial microflora and the sample matrix. Thus, all rapid assays should be carefully validated in the exact type of food matrix that is intended for routine testing.

The way ahead is the creation of a single validation body with universally accepted criteria. No organization is more suitable to take over this task than the United Nations. Although International Standards Organization (ISO) has published the validation guideline 16140 (ISO, 2003), ISO does not have the infrastructural capacity to carry out the actual validations.

One solution would be for the UN organizations dealing with food safety (World Health Organization or Food and Agriculture Organization) to delegate the validation performance to a series of regional private or public institutions, which could carry out the necessary comparative and collaborative trials. The benefit of this system for test procedures and industry is to have only one validation that is recognized globally.

Another factor that seems to be a major hindrance to the wide spread use of rapid methods is the lack of reference material (Trapmann et al., 2004; Madej et al., 2010) and harmonized standard methods and validation protocols (**Malorny et al., 2003**), which not only affects usage but also severely impacts international trade. This issue was one of the core activities of the European research project FOOD-PCR (Hoorfar, 1999).

5.7. PCR standardization

Several standardization issues must be dealt with, if real-time PCR is to obtain wider use. Despite the increasing number of probe chemistries and PCR instruments, the current applications are mostly limited to duplex detection, in rare cases triplex. This is far from the promised potential of real-time PCR for parallel testing of many targets in the same tube. Most scientists have alleviated this limitation by distributing the same sample in several parallel tubes, each containing different dyes. However, this is not what real-time was supposed to deliver. Instrument producers must develop true multiplex applications to support customers to take the full advantage of real-time PCR, but in most cases they are just competing on the procurement price of instruments that are basically doing the same.

Scientist or end-users should develop new applications to exploit the enumeration capacity of real-time PCR. Most publications report new tests that merely detect presence or absence of certain genes. More intelligent sample preparation designs are needed to obtain quantitative data that provide us with more meaningful indication of the extent of the initial contamination or the extent of interventions that are necessary to deal with a situation. Interpretation of PCR results, in particular fluorescence readings close to baseline values, is much dependent on the software algorithms that follow different instruments. The algorithms used to normalize output reading vary, in turn, among software producers. Some producers use sophisticated calculations to normalize PCR result for within sample controls (spontaneous fluorescence release, internal amplification control, etc.), sample-to-sample variations, coefficient of variations, baseline readings, and so on, whereas other instrument producers just subtract a fixed value of, e.g. the first five cycles, from the all fluorescence readings to produce threshold cycle values for test samples. Thus, there is an urgent need to agree upon a standard algorithm to facilitate data comparison among current and future PCR platforms.

To make matters worse, the DNA or RNA standard dilutions used among different research groups to produce quantification curves could vary considerably in genome and gene copy numbers, depending on the purity, nucleic acid integrity, and method of measurement. International standard organizations should agree upon a uniform guideline for the preparation and measurement of reference DNA dilutions.

There are certainly other issues that can be added to the challenges above. Nevertheless, new real-time PCR platforms and chemistries will continue to merge, in particular, with connection of low-density microarray platforms that can result in true multiplex detection of hundreds of targets. To achieve this goal, scientists involved in real-life diagnostics must collaborate more closely with instrument producers, and instrument producers must welcome new application needs that are put forward by scientists.

6. CONCLUSIONS

The traditional argument for developing and using rapid methods has been faster detection of pathogens, although it is becoming increasingly evident that rapid methods can contribute to other aspects of food safety, public health, and global trade. Another voice of support for the use of rapid methods has come from the recent concerns about bioterrorism attacks, which require much faster response and highly innovative technologies. This has moved rapid methods further up the agenda of regulators, health policy makers, and research organizations.

The present review has attempted to cover other important aspects of rapid methods than the mere test development. These aspects are often overlooked when new detection technologies are developed. Looking back at the many fancy molecular technologies that have been reported during the past 20 years or so, not so few has ended in 'technology cemetery'. A few techniques such as PCR or ELISA have proven sufficiently robust to be used in routine applications for the benefit of public health. That is why the works presented in this review have been mainly been built upon the proven technologies, but has also introduced four novel developments into the field of rapid methods: (i) same-day detection of *Salmonella* that otherwise was considered difficult to achieve, (ii) enumeration following a short log-phase enrichment, (iii) detection of foodborne pathogens in air samples, and finally (iv) biotracing of pathogens.

Nevertheless, substantial work is ahead to investigate the usefulness of these developments for other pathogens than *Salmonella and Campylobacter*, for other food matrices, for other sample types, and for new food chain models. For instance questions remain to be answered on how is it possible to further standardize the enrichment-enumeration techniques to minimize the impact of background microflora, when changing from one sample type to another.

The concept of same-day detection of *Salmonella* should be expanded to *Listeria monocytogenes*, *Yersinia enterocolitica*, *S. aureus*, and other important pathogens. New developments indicate the possibility to substantially shorten the 12-h *Salmonella* assay, if phage techniques or B-cell induced antibodies are used (Rider et al., 2003). Detecting pathogens in air samples can provide us with a short cut to onsite or online techniques, in particular, in production facilities that operate with zero tolerance of pathogens in air, e.g. in the case of *Cronobacters* in milk powder plants (FAO/WHO, 2008b). It cannot be ruled out that the number of pathogen to be tested is higher, the closer the sample is taken to the source of contamination, resulting in improved diagnostic sensitivity.

Dynamism in the emergence of new pathogens, and new pathogen/matrix combinations will continue. Thus, the need for developments in biotracing will remain. Future tracing work will include improved identification of pathogen markers, integration of detection and subtyping into a single test, metagenomic tools; incorporation of molecular data into modeling, the ability to study entire bacterial communities, and also the non-culturables. All of this will result in improved decision-making tools, better risk communication, and more targeted product recall.

Notwithstanding the limitations of rapid assays, the development of this class of detection methods continues to increase. Assays will need to be developed that focus on 'overlooked' pathogens including viruses and parasites. Biosensors, at the center of the marriage between biology and electronics, should be designed with the food matrix in mind, and should be able to test larger, rather than smaller, volumes of samples. Finally, research must commence to find a truly viable alternative to bacterial enrichment. Such an invention will revolutionize the food diagnostic field, leading to true real-time testing during the food production.

7. ACKNOWLEDGEMENTS

This work was supported in part by the European Union-funded Integrated Project BIOTRACER (Contract 036272) under the 6th RTD Framework.

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DANSK RESUMÉ

Så længe fødevaresikkerhed er i fokus, vil mikrobiologisk kontrol spille en vigtig rolle i vurderingen af, om målsætningerne for fødevaresikkerhed (Food Safety Objectives) er opnået. Dog er udbyttet af traditionelle mikrobiologiske dyrkningsbaserede metoder begrænset, især hvad angår deres evne til at levere rettidige data. Den foreliggende afhandling gennemgår årsagerne til den stigende interesse for hurtigmetoder, den aktuelle udvikling på området, forskningsbehovet og de fremtidige tendenser. Udviklingen i bioteknologi har ført til nye teknologier, der har banet vejen for hurtigere metoder og dermed ændret laboratoriediagnostik i praksis. Hurtige metoder består af mange forskellige påvisningsteknologier, baseret på bl.a. specialiserede enzymsubstrater, antistoffer og DNA, og spænder fra simple chromogene medier til brugen af avancerede instrumenter. Brugen af ikke-invasive metoder til prøveudtagning fra levende dyr kom især i fokus med 1990'ernes udbrud af kogalskab, der blev knyttet til den menneskelige Creutzfeldt-Jakob sygdom. Serologi er stadig et vigtigt redskab i forebyggelsen af zoonotiske fødevarebårne patogener, som kan smitte via kød og mælk fra dyr. En af de primære anvendelser af alternative metoder er hurtig screening af et stort antal prøver, hvoraf de fleste forventes at blive test-negative, og en dermed hurtigere frigivelse af fødevarer til salg. Dette har været styrken ved hurtigmetoder såsom realtids PCR. Enrichment-PCR, hvor en opformeret prøve testes i PCR, er den mest almindelige tilgang til hurtig test. Nylige resultater viser, at det er muligt både at opformere en prøve (dog i en kortere periode) og kvantificere antallet af patogener vha. realtids PCR. Dette kan især være nyttigt i situationer, hvor fødevareproducenter ønsker at kende niveauet af en patogen i et forurenet produkt. Et andet centralt spørgsmål er automatisering, hvor de vigtigste drivkræfter er mikrotest i et multipleksformat, hvilket betyder at et instrument er fleksibelt nok til at teste mange patogener, men også at mange patogener kan detekteres i samme test. Denne gennemgang er hovedsageligt baseret på forfatterens videnskabelige arbejde, der har bidraget med følgende nye udvikling på området: i) Serologiske test til omfattende screening, overvågnings- eller

udryddelsesprogrammer, ii) Samme-dags påvisning af *Salmonella*, iii) Kvantificering af patogener efter en kort log-fase præopformering, iv) Påvisning af fødevarebårne patogener i luftprøver, og endelig v) Eftersporing (Biotracing) af patogener baseret på matematisk modellering, selv i mangel af isolater. Hurtige metoder er diskuteret i et bredt globalt, sundhedsmæssigt perspektiv, i relation til international fødevareforsyning, og til forbedring af kvantitative mikrobielle risikovurderinger. Reviewet omfatter også en diskussion af behovet for kvantitative prøveforberedelsesteknikker, kultur-uafhængig, metagenom-baseret påvisning, online overvågning, og en global valideringsinfrastruktur. Omkostningerne og brugervenlighed af nogle af avancerede metoder forbliver en udfordring at overvinde.