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New approaches in microbial pathogen detection

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Abstract: Viruses are common causes of foodborne outbreaks. Viral diseases have low fatality rates but transmission to humans via food is important due to the high probability of consuming fecally contaminated food or water because of poor food handling. Because of the low infectious doses of some foodborne viruses, there is a need for standardization and the development of new sensitive methods for detecting viruses. The focus is on molecular and non-molecular approaches, and emerging methods for the detection of foodborne viruses. The detection of noroviruses, hepatitis A and E viruses, rotaviruses and adenoviruses will be discussed. The chapter will conclude with insights into future research directions.

Key words: foodborne outbreak, virus, detection, food safety, nanotechnology.

11.1 Introduction

An estimated 40 million illnesses each year are caused by foodborne pathogens, including 5.2 million (13%) due to bacteria, 2.5 million (7%) due to parasites and 30.9 million (80%) due to viruses (Mead *et al.*, 1999). In the last decade, the occurrence of gastroenteritis in humans as a result of consumption of foods contaminated by viruses has increased (WHO, 2000). The transmission of viruses has been predominantly associated with the consumption of shellfish, mainly, raw oysters (Koopmans and Duizer, 2004; Widdowson *et al.*, 2005), which have been contaminated by polluted water or virus-infected food handlers (Bosch *et al.*, 2011). Common symptoms of viral gastroenteritis include vomiting and diarrhea (FAO/WHO, 2008). Foodborne viruses can be divided into three categories based on disease symptoms: those that cause gastroenteritis (noroviruses, rotaviruses and adenoviruses), those that cause fecal-orally transmitted hepatitis (hepatitis A and E viruses), and those that cause other illnesses after they migrate to other

organs, such as the central nervous system or the liver (enteroviruses). Of these, noroviruses and the hepatitis A virus have been recognized as the most important human foodborne pathogens in terms of the number of outbreaks reported and the number of people affected in the world (Koopmans and Duizer, 2004; Cook and Rzezutka, 2006; Verhoef *et al.*, 2008). Even though these viruses are the major cause of foodborne outbreaks, there is still an urgent need for standardization and the validation of detection methods at the national and international levels (FAO/WHO, 2008). In this chapter, an overview of existing detection methods for foodborne viruses are presented including non-molecular and molecular approaches as well as promising emerging methods for virus detection. The detection of noroviruses, hepatitis A and E viruses, rotaviruses and adenoviruses will be discussed as these viral agents have a high potential for foodborne outbreaks. The rest of this section briefly gives the essential characteristic features of these viruses.

Noroviruses (NVs) comprise a genus in the family Caliciviridae. They are mostly linked to non-bacterial gastroenteritis in humans and are estimated to cause 93% of the food-related outbreaks of gastroenteritis in the United States (Widdowson *et al.*, 2005; Fankhauser *et al.*, 2002). An NV is a small round virus, 27 to 35 nm in diameter, with a single-stranded, positive-sense, polyadenylated RNA genome of 7400 to 7500 nucleotides (Atmar and Estes, 2001). These small viruses show high genomic diversity and antigenic variation within five genogroups (GI, GII, GIII, GIV and GV) based on the genome sequence of the RNA-dependent RNA polymerase (RdRp) and the capsid regions (Vinjé *et al.*, 2004).

Hepatoviruses (or hepatitis A viruses or HAVs) comprise a genus in the family Picornaviridae with a diameter in the range between 27 and 32 nm. They were first identified by electron microscopy in 1973 (Lemon and Robertson, 1993; Koopmans *et al.*, 2002). They are small, non-enveloped, spherical viruses with a single (positive-) stranded RNA genome of approximately 7.5 kb in length (Koopmans *et al.*, 2002). Each year, approximately 1.4 million people become ill with HAV costing $1.5–3.0 billion worldwide (WHO, 2000).

The hepatitis E virus (HEV), one of the major causes of viral hepatitis other than HAV, causes infection with a high rate of mortality particularly in pregnant women (Widen *et al.*, 2011; Ahn *et al.*, 2005). A HEV, similar to a HAV, is a small, non-enveloped, positive sense, single-stranded RNA virus, reclassified in the genus *Hepevirus* of the family Hepeviridae although previously classified as a member of Caliciviridae family (Berke and Matson, 2000; Gyarmati *et al.*, 2007). The primary source of infection is the consumption of fecally contaminated drinking water in developing countries (Koopmans *et al.*, 2002). Based on geographical origin, all isolated HEV strains can be identified into four genotypes as Asian/African, Chinese, Mexican or US/European (Emerson and Purcell, 2001). Among these genotypes, genotypes 1 and 2 only infect humans, while genotypes 3 and 4 appear to infect other hosts, particularly pigs, and are associated with zoonotic transmission (Gyarmati *et al.*, 2007).

Rotaviruses comprise a genus in the family *Reoviridae* and are one of the major causes of acute diarrhea in infants and young children with high morbidity

and mortality, especially in developing countries (Kapikian *et al.*, 1996). These round, non-enveloped viruses are estimated to cause the death of more than 600 000 children worldwide every year and infect almost all children under five years of age (Pineda *et al.*, 2009; Gutierrez-Aguirre *et al.*, 2008).

Adenoviruses are non-enveloped viruses with double-stranded DNA (Vasickova *et al.*, 2005). Human adenoviruses are the only human enteric DNA viruses and are often detected in association with other human enteroviruses or hepatitis A viruses (Rigotto *et al.*, 2005). Outbreaks have caused gastroenteritis in children (Vasickova *et al.*, 2005). Adenovirus types 40 and 41 cause gastroenteritis when transmitted through a fecal–oral cycle (Koopmans *et al.*, 2002).

11.2 Detection methods

HAVs and other enteric viruses may be found in large numbers in clinical samples ($\geq 10^6$ virus particles per gram of stool); however, they are usually found in much lower numbers in food, e.g. 0.2–224 particles per 100 g shellfish meat (Sanchez *et al.*, 2007). The infectious dose of HAVs and NVs is estimated to be as low as 10–100 infectious viral particles even though the ingestion of thousands of cells is required for bacterial infection to occur with the same probability (Sair *et al.*, 2002; Gerba, 2006; Guevremont *et al.*, 2006). Unlike bacterial pathogens, viruses cannot multiply in foods, making the traditional food microbiological techniques of cultural enrichment and selective plating inapplicable (D'Souza and Jaykus, 2006). Therefore, methods with high reliability and sensitivity are required for viral detection. In the sections below we discuss some of the currents methods.

11.2.1 General approaches

Conventional assay systems to detect enteric viruses in clinical specimens cannot be directly used for food (Rodriguez-Lazaro *et al.*, 2007). In general, electron microscopy, tissue cultures and immunological and molecular methods are used to detect viruses in food. Viruses were diagnosed historically by scanning a stool suspension under an electron microscope (EM) (Koopmans and Duizer, 2004). Many of the small round viruses, including HAVs, astroviruses, noroviruses, sapovirus and parvoviruses, were first discovered through the use of EM (Greening, 2006). EM is fairly insensitive, labor intensive and requires a minimum of 10^6 virus particles per milliliter of sample for detection in patient fecal samples, thus, using this method, detecting viruses at low levels in contaminated food, water and environmental samples is not possible (Koopmans and Duizer, 2004; Seymour and Appleton, 2001).

Detection by cell culture depends on cytopathic effects, and virus quantification is performed by plaque assay, the most probable number or 50% tissue culture infectious dose ($TCID_{50}$) (Bosch *et al.*, 2011). Cell-culture-based assay can differentiate between infectious and non-infectious viruses; nevertheless it is limited and not practical, mainly due to the lack of sensitivity, the long analysis

time and the lack of susceptible cell lines for many epidemiologically important enteric viruses (Casas and Sunen, 2001; Verhoef *et al.*, 2008). Even though these assays are commonly used to enumerate levels of viable polioviruses and adenoviruses, they are inadequate for the detection of the two most important foodborne viruses, HAVs and NVs, since neither of these replicate or express themselves efficiently in cell cultures (Goyal, 2006; Jiang *et al.*, 2004; Koopmans and Duizer, 2004). Thus, HAVs and NVs have been detected conventionally using EM and enzyme-linked immunosorbent assay (ELISA) but even these methods are insensitive, lengthy and expensive (Morales-Rayas *et al.*, 2010).

Non-culture-based detection methods, such as immunoassays, have been developed to detect viruses over the years (Lees, 2000). Although immunoassays, such as ELISA, have been used to detect viruses in water and HAVs in shellfish, reports are very limited and not always successful (Lees, 2000). The limited success of this approach is probably due to the lack of sensitivity of the immunoassay and like EM requires a thousand or more virus particles for a positive result (Kogawa *et al.*, 1996). Therefore, new approaches have focused on molecular methods as these techniques for detecting enteric viruses are faster and more sensitive compared to infectivity tests performed with *in vitro* cell cultures or with immunological methods, even though molecular methods cannot discriminate between infectious and non-infectious particles (Green and Lewis, 1999; Morales-Rayas *et al.*, 2010).

11.2.2 Molecular approaches

Several molecular methods using nucleic acid amplification have been developed for virus detection in food (Jean *et al.*, 2003). In recent years, polymerase chain reaction (PCR)-based methods in particular, have become the gold standard for virus detection in food due to their high sensitivity, specificity and potential to detect even a single virus particle (Bosch *et al.*, 2011; Martinez-Martinez *et al.*, 2011; Richards *et al.*, 2003; Cook and Rzezutka, 2006). Selected examples with detection limits are listed in Table 11.1.

Reverse transcription PCR (RT-PCR)

Reverse transcription PCR (RT-PCR), a modified form of PCR that allows the amplification of viral RNA, is currently the most sensitive and widely used method for foodborne virus detection (Casas and Sunen, 2001; Morales-Rayas *et al.*, 2010). However, the application of this technique for routine analysis of food matrices is elaborate due to the need for sample concentration and the presence of residual food-related PCR inhibitors (Sair *et al.*, 2002). Since only low numbers of viruses are present in food, inhibition is a more serious issue (Morales-Rayas *et al.*, 2010). Therefore, several methods have been developed to concentrate and purify viruses and remove inhibitors from food samples before RT-PCR (Dubois *et al.*, 2002; Croci *et al.*, 2008).

The sample preparation procedures for detecting viruses in food typically involve one or more of the following: (i) elution of the virus particles from the

Table 11.1 Selected examples of molecular approaches for detection of foodborne viruses

Type of virus	Method of detection	Detection limit	Samples tested	References
Norovirus	TaqMan qRT-PCR	0.01 PDU	Clinical	Lamhoujeb *et al.*, 2009
	Real-time NASBA	0.01 PDU		
	RT-PCR	1 RT PCRU/25 g	Green onion	Guevremont *et al.*, 2006
	RT-PCR	1–10 PCRU/mL	Ham	Kim *et al.*, 2008b
	Nested PCR			
Hepatitis A	Duplex qRT-PCR	10 PFU/1.5 L	Bottled water	Blaise-Boisseau *et al.*, 2010
		100 PFU/1.5 L	Tap water	
		50 PFU/25 g	Fresh raspberries	
		100 PFU/25 g	Frozen raspberries	
	TaqMan RT-PCR	14 PFU/g	Tomato sauces	Love *et al.*, 2008
		33 PFU/g	Blended strawberries	
	Nested RT-PCR	1 $TCID_{50}$/10 g	Mollusks	Croci *et al.*, 1999
Hepatitis E	TaqMan qRT-PCR	1.2 PID_{50}	Water	Jothikumar *et al.*, 2006
Rotavirus	qRT-PCR	125 PFU/g	Oyster	Kittigul *et al.*, 2008
	NASBA-ELISA	0.2 PFU	Water	Jean *et al*., 2002a, 2002b
		15 PFU	Sewage effluent	
Adenovirus	Nested PCR	1.2 PFU/g	Oysters	Rigotto *et al*., 2005
	Conventional PCR	1.2×10^2 PFU/g		
	ICC-PCR	1.2×10^2 PFU/g		
	Nested mPCR	1 copy of adenovirus DNA/ PCR reaction	Sewage Shellfish	Formiga-Cruz *et al*., 2005

Note: PFU: plaque-forming unit; PCRU: RT-PCR amplifiable unit; $TCID_{50}$: 50% tissue culture infective dose; PID: pig infectious dose.

food using a variety of buffers and solutions including solutions of glycine and sodium chloride, borate and beef extract, saline and beef extract, and beef extract alone; (ii) extraction with an organic solvent, most commonly with Freon to remove insoluble or poorly soluble organic compounds in the water; (iii) concentration of the viruses using sedimentation by antibody or ligand capture, flocculation, ultra-centrifugation or precipitation (commonly polyethylene glycol precipitation); and (iv) extraction of viral nucleic acids (there are two main approaches using phenol: chloroform extraction and guanidinium isothiocyanate extraction) (Cook and Rzezutka, 2006; Goyal, 2006; Rodriguez-Lazaro *et al.*, 2007). Various strategies have been proposed to improve the performance of each step over the years.

There are several commercial kits for nucleic acid purification, which are reliable, produce reproducible results and are easy to use. Most of these kits are based on guanidinium lysis and the capture of nucleic acids on a column or bead of silica (Bosch *et al.*, 2011). However, sample preparation methods still require improvement to isolate viral particles from diverse food matrices without decreasing the sensitivity of the molecular method used for detection (Morales-Rayas *et al.*, 2010).

The sensitivity and specificity of RT-PCR assays depends mainly on primer selection (Atmar and Estes, 2001). The major obstacle in NV detection with PCR arises from the very high genomic diversity of NV since new variants continue to evolve constantly (Widen *et al.*, 2011). Therefore, it is difficult to select a single or even a small number of probes that can detect all possible NV variants (Atmar and Estes, 2001). Although ORF1 of the RdRp gene has been targeted in most of the assays (Nakayama *et al.*, 1996; Jiang *et al.*, 1999), the ORF1-ORF2 region has also been shown to be well conserved and is used in several assays (Katayama *et al.*, 2002; Hohne and Schreier, 2004; Jothikumar *et al.*, 2005b). One of the first enteric viruses detected by RT-PCR was HAV (Jansen *et al.*, 1990). The VP1 capsid region was previously commonly targeted by primers in HAV detection; however, nowadays the 5′ non-coding region is highly preferred for targeting. It has similar performance as VP1, approximately 1 RNA copy per reaction (Sanchez *et al.*, 2007). For HEV detection, various specific sets of primers have been developed to amplify conserved regions within ORF1, ORF2 and ORF3 (Enouf *et al.*, 2006). Most of the RT-PCR assays developed for rotaviruses target the structural genes VP4, VP6 and VP7 (Atmar, 2006). The hexon gene in adenoviruses is most commonly used as the target in PCR assays; it has been shown to be reactive in all adenovirus species (Jothikumar *et al.*, 2005a; Atmar, 2006). More recently, a FRET-based real-time assay, which amplifies the adenovirus fiber gene, was described. It showed slightly better performance in terms of detection limits of AdV40 and AdV41 compared to TaqMan assays (Jothikumar *et al.*, 2005a).

The major limitation of RT-PCR is its inability to distinguish between infectious and non-infectious viruses (Richards, 1999). Integrated cell culture PCR (ICC-PCR) and ICC/strand-specific RT-PCR have been proposed to compensate for this problem (Atmar, 2006; Jiang *et al.*, 2004). ICC/strand-specific RT-PCR is a

combination of cell culture and molecular biology-based methods, which requires initial propagation of infectious virus particles in a cell culture and the detection of a negative-strand RNA replicative intermediate as an indicator of viral replication (Jiang *et al.*, 2004). The limitations of RT-PCR were eliminated in environmental samples by increasing the equivalent sample volume and thereby reducing the effects of inhibitory compounds (Reynolds *et al.*, 1996). ICC-PCR and ICC/strand-specific RT-PCR assays targeting the VP3 genes, which code for a major HAV capsid protein, have been developed to detect viruses in water (Jiang *et al.*, 2004). The ICC/strand-specific RT-PCR used in this study was demonstrated to be a novel, rapid, sensitive and reliable method, since it can detect infectious HAVs at inoculation level of 100 $TCID_{50}$ per flask within four days in water samples.

Even though RT-PCR is a rapid and sensitive method and can detect viruses that are difficult or impossible to culture (Casas and Sunen, 2001), several different types of RT-PCR have been developed to improve the specificity and sensitivity of the standard method for foodborne virus detection such as nested RT-PCR (Love *et al.*, 2008; Croci *et al.*, 1999) and multiplex RT-PCR (Rosenfield and Jaykus, 1999; Formiga-Cruz *et al.*, 2005; Coelho *et al.*, 2003).

Nested PCR

In nested PCR, two different primer pairs are used successively to amplify a target sequence (Haqqi *et al.*, 1988). Nested PCR was developed to ensure detection specificity, to minimize false-positive results and to enhance the amplification signal (Rigotto *et al.*, 2005). It has been widely used in the performance evaluation and verification of different PCR-based methods as well as viral extraction, concentration and purification (Kim *et al.*, 2008a, 2008b; Di Pinto *et al.*, 2003; Jothikumar *et al.*, 2005b).

The superior sensitivity of nested PCR over other methods has been demonstrated in several studies (Croci *et al.*, 1999; Rigotto *et al.*, 2005; Love *et al.*, 2008). Nested PCR gives a more sensitive and specific identification of HAV at concentrations as low as 1 $TCID_{50}$/10 g of mollusk compared to 10^3–10^4 TCID/10 g of mollusk after one round of PCR (Croci *et al.*, 1999). It had a higher level of sensitivity in shellfish compared to conventional PCR and ICC-PCR when detecting adenoviruses (Rigotto *et al.*, 2005) (Table 11.1). Recently, TaqMan RT-PCR has been used to detect HAV RNA from artificially inoculated tomato sauce and blended strawberries (Love *et al.*, 2008). The lower limits of HAV detection were reported as 14 PFU/g (plaque-forming units per gram) of tomato sauce and 33 PFU/g of blended strawberries at initial seeding levels. Moreover, the nested RT-PCR was not inhibited by undiluted final RNA extracts of tomato sauce or blended strawberries unlike TaqMan RT-PCR.

The sensitivity of standard RT-PCR was further increased when combined with semi-nested or nested PCR by using an aliquot of the product from the primary RT-PCR as a template for the second round of amplification (O'Connell, 2002; Abad *et al.*, 1997). Nested multiplex real-time PCR (mRT-PCR) has also been developed to provide a highly sensitive, rapid and cost-efficient approach for HAV,

adenovirus and enterovirus detection in urban sewage and shellfish (Formiga-Cruz *et al.*, 2005). This method was able to detect as little as one copy of adenovirus DNA, and ten copies of both enterovirus and HAV RNA, which was shown to be similar to the previously determined sensitivities of monoplex PCR with 1–10 viral particles for adenoviruses, and 5–10 viral particles for enteroviruses both in sewage and shellfish samples (Formiga-Cruz *et al.*, 2005). Most recently, RT nested PCR targeting the VP7 gene of rotaviruses in naturally contaminated oyster samples was shown to give the highest sensitivity and the lowest detection limit of 125 PFU/g of oyster with acid adsorption–alkaline elution (Kittigul *et al.*, 2008).

Multiplex PCR

In multiplex PCR, two or more primer sets are used simultaneously in the amplification of different target sequences in a single tube (Chamberlain *et al.*, 1988). Thus, this method could be used for the detection of more than one virus in a single reaction tube (Rosenfield and Jaykus, 1999; Coelho *et al.*, 2003; Beuret, 2004). A multiplex reverse transcription polymerase chain reaction (mRT-PCR) method has been described for the simultaneous detection of the human enteroviruses, HAV and NV (Rosenfield and Jaykus, 1999). Detection limits lower than 1 infectious unit (poliovirus type 1 (PV1) and HAV) or RT-PCR-amplifiable unit (NV) for all viruses were obtained by the multiplex method. In a similar vein, mRT-PCR has been developed to concentrate and purify HAV, PV1 and simian rotaviruses (RV-SA11) simultaneously from experimentally seeded oysters (Coelho *et al.*, 2003). However, this method could not detect the three viruses simultaneously when tested on experimentally contaminated raw oysters. This was attributed to the low concentration of viral RNA present in the oyster extract as a result of an ineffective extraction method.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) is used to amplify and quantify simultaneously a targeted DNA molecule by using DNA-binding fluorophores or, commonly, by specific fluorescently labeled oligoprobes (Atmar, 2006). In recent years, qRT-PCR has been widely used in food virology as the most promising nucleic acid detection method, since it offers several advantages over conventional RT-PCR, including high sensitivity, the possibility of simultaneous amplification, detection and quantification of the target nucleic acids in a single step, and with minimum risk of carry-over contamination through the use of a closed system (Mackay *et al.*, 2002; Bosch *et al.*, 2011; Houde *et al.*, 2007). Sensitive and specific detection with real-time PCR is achieved using novel fluorescent technology probes (Espy *et al.*, 2006). In qPCR assays, three types of fluorescently labeled target-specific probes have been used most often: TaqMan probes, molecular beacons and fluorescence resonance energy transfer (FRET) hybridization probes (Sanchez *et al.*, 2007). These detection methods all depend on the transfer of light energy between two adjacent dye molecules, a process known as fluorescence resonance energy transfer (Espy *et al.*, 2006).

TaqMan-based assays have been widely used to detect HAVs using qPCR in recent years (Sanchez *et al.*, 2007). These assays combine a specific linear dual-labeled oligoprobe in the TaqMan master mix to eliminate the need for post amplification steps and also offer the opportunity of multiplexing amplification reactions (Houde *et al.*, 2007). Several studies targeting the 5′ non-coding region (NCR) have been performed with TaqMan qRT-PCR to detect HAVs (Costa-Mattioli *et al.*, 2002; El Galil *et al.*, 2005; Jothikumar *et al.*, 2005b, Costafreda *et al.*, 2006). The detection limits ranged from one to five copies per reaction. In NV detection, real-time TaqMan-PCR targeting in the well conserved ORF1-ORF2 region has been developed (Hohne and Schreier, 2004; Jothikumar *et al.*, 2005b). These TaqMan RT-PCR assays were able to detect as few as 100 genomic equivalents of different NV strains, including subtypes of GI and GII, rapidly, sensitively and reliably. A TaqMan RT-PCR assay targeting a conserved region in ORF3 has also been developed to detect HEVs in clinical and environmental samples (Jothikumar *et al.*, 2006). This assay was shown to be sensitive and specific for detecting HEV genotypes 1–4 with the detection limit as few as four genome equivalent copies of HEV plasmid DNA and as low as 0.12 50% pig infectious dose (PID_{50}) of swine HEV. Moreover, the detection of different concentrations of swine HEVs (120–1.2 PID_{50}) in a surface water concentrate was performed successfully.

Molecular beacons (MBs) are single-stranded fluorescent probes and have a stem-loop structure that is labeled both with a fluorescent dye and a universal quencher at the 5′ and 3′ ends, respectively (El Galil *et al.*, 2005). MBs undergo a fluorogenic conformational change upon binding to their target, which allows the progress of the reaction to be followed in real-time PCR (El Galil *et al.*, 2004; Valdivia-Granda *et al.*, 2005). A qRT-PCR based on the amplification of 5′-NCR was used to detect genome copies of HAVs using TaqMan and MB probes in clinical and shellfish samples (Costafreda *et al.*, 2006). MB had a lower sensitivity and reproducibility compared to TaqMan probes, which was able to detect as little as 0.05 infectious unit and 10 copies of a single-stranded RNA (ssRNA) synthetic transcript.

Two FRET hybridization probes, made from DNA, are used: one with a fluorescent dye on the 3′ end and the other with an acceptor dye on the 5′ end. They are intended to anneal next to each other in a head-to-tail configuration on the PCR product (Espy *et al.*, 2006). These probes are also referred to as LightCycler probes and are commercially available (Espy *et al.*, 2006; Sanchez *et al.*, 2006). A commercial qRT-PCR assay, the LightCycler HAV quantification kit (Roche Diagnostics), coupled with immunomagnetic separation (IMS) pretreatment, has been shown to be sensitive and specific in the detection of HAVs in fresh produce (Shan *et al.*, 2005). IMS is based on the isolation of an antigen from the sample with a monoclonal antibody against HAV (anti-HAV 1009) combined with streptavidin-coated magnetic beads to recover low levels of viruses and to remove PCR inhibitors. In this assay, 5′ NCR was chosen as the highly conserved target region and a detection limit as low as 1 PFU was obtained. In a similar study, two commercial qRT-PCR HAV assays, the LightCycler HAV

quantification kit (Roche Diagnostics) and the RealArt HAV LC RT PCR kit (artus GmbH), were compared in terms of precision, accuracy, linearity and detection limits (Sanchez *et al.*, 2006). The results showed that both kits were suitable for detecting and quantifying HAVs; however, the Roche kit had a slightly better detection limit with the capability of differentiating between different HAV strains and it was also able to detect HAVs in spiked water and food samples.

Several commercial kits for detecting and quantifying NVs have been developed due to the high incidence of NV outbreaks (Butot *et al.*, 2010). The NV qRT-PCR Kit (AnDiaTec GmbH and Co. KG, Kornwestheim, Germany) and the NV Type I and Type II kits (Generon S.r.l., Castelnuovo, Italy) were evaluated and compared with the assay designed by the CEN/TC/WG6/TAG4 research group in the specific detection and quantification of 59 NV samples, including different subtypes of NV genogroups I and II (Butot *et al.*, 2010). The commercial kits failed to detect the vast majority of NV strains, showing poor performance.

The challenges associated with the detection of foodborne viruses, such as PCR inhibitors and low virus concentrations in foods, affect the efficiency of real-time assay adversely, therefore, for process control (PC) an internal amplification control (IAC), which is extracted and amplified with the target sequence, is crucial in the evaluation of PCR and to prevent false negatives (Di Pasquale *et al.*, 2010). A real-time PCR IAC has been developed recently for the simultaneous detection of GI and GII NVs, which may also reduce the cost of the assay (Stals *et al.*, 2009). Likewise, the use of non-pathogenic viruses, such as the mutant mengovirus MC_0 strain, the MS2 bacteriophage and feline calicivirus (FCV), as sample process controls has been proposed in detecting HAVs in different food matrices (e.g. shellfish, raspberries and strawberries) (Costafreda *et al.*, 2006; Blaise-Boisseau *et al.*, 2010; Di Pasquale *et al.*, 2010). In these studies, no loss of HAV detection sensitivity was observed after the addition of controls.

Nucleic acid sequence-based amplification (NASBA)

NASBA is an alternative approach to PCR-based molecular methods. In this method, an RNA template is amplified under isothermal conditions using three enzymes (avian myeloblastosis virus reverse transcriptase, RNase H and T7 RNA polymerase) in the reaction tube (Compton, 1991). NASBA is particularly suitable for detecting RNA viruses since the direct amplification of RNA targets is possible without a separate reverse transcription step (Jean *et al.*, 2001, 2004). It has also been shown to be less susceptible to environmental PCR inhibitors (Rutjes *et al.*, 2006). Even though the amplification power and the sensitivity of NASBA assays are comparable or even better than that of RT-PCR (Jean *et al.*, 2001), NASBA assays have been used in relatively few studies for detecting enteric viruses compared to RT-PCR.

NASBA assays have been multiplexed or coupled to RT-PCR and ELISA assays to achieve lower detection limits, high sensitivity and specificity in virus detection (Jean *et al.*, 2002a, 2002b, 2003, 2004). Amplification of viral RNA from HAVs and human rotaviruses with selected primers in the multiplex NASBA mixture had detection limits of 40 and 400 PFU/ml for rotaviruses and HAVs,

respectively (Jean *et al.*, 2002b). In this study, highly conserved regions in rotavirus gene 9 and in the HAV VP2 gene encoding a major capsid protein were targeted for amplification. Accordingly, multiplex NASBA has been used to detect HAVs, GI and GII noroviruses from representative ready-to-eat foods (Jean *et al.*, 2004). All three viruses were detected in the food matrix simultaneously through targeting relatively conserved genomic regions for each of these, with detection limits ranging from 2×10^2 to 2×10^3 PFU/9 cm^2. These results show that NASBA is a promising alternative to RT-PCR as it offers rapid and simultaneous detection in a single reaction tube.

A semi-quantitative form of real-time NASBA estimated a viral load in less than half an hour (Patterson *et al.*, 2006). Molecular beacons can be used with NASBA coupled with RNA amplification to produce a specific fluorescent signal, which can be monitored in real time. The measurable fluorescence is directly proportional to the concentration of the target sequence (Leone *et al.*, 1998). More recently, real-time NASBA using a MB probe has been demonstrated to be a sensitive and specific assay for NV detection in clinical and environmental samples (Lamhoujeb *et al.*, 2009). Molecular methods, despite being sensitive and specific, cannot differentiate between infectious and non-infectious viruses. Hence, an enzymatic treatment followed by molecular beacon NASBA targeting of the highly conserved ORF1-ORF2 junction has been developed to distinguish infectious from non-infectious NVs in ready-to-eat food (Lamhoujeb *et al.*, 2008). The proposed enzymatic pretreatment utilized proteinase K and RNase at the same time to digest non-infectious virus particles (Nuanualsuwan and Cliver, 2002).

11.3 Emerging methods

In general, current detection methods have poor sensitivity and selectivity at low virus concentrations. In the main, PCR-based methods have been used to overcome the challenges associated with virus detection; however, these methods also have limitations in terms of complexity in sample preparation and amplification. Thus, the following section is an overview of emerging detection methods.

11.3.1 Spectroscopic approaches

Spectroscopic techniques to detect and identify viral infections are promising owing to their sensitivity, speed, cost and simplicity (Erukhimovitch *et al.*, 2011).

Surface enhanced Raman spectroscopy

Surface enhanced Raman spectroscopy (SERS) and electrochemical impedance spectroscopy are the most commonly used spectroscopic approaches in virus detection.

Even though Raman spectroscopy has been used previously to characterize virus structures, it lacks sensitivity due to the extremely small cross section of

Raman scattering, which is about 12–14 orders of magnitude less than fluorescence cross sections (Porter *et al.*, 2008; Shanmukh *et al.*, 2006; Kneipp *et al.*, 2002). With the help of metallic nanostructures, SERS amplifies low-level Raman signals within highly localized optical fields on metallic surfaces. It overcomes the limitations of conventional Raman spectroscopy because of the electromagnetic field or chemical enhancement (Kneipp *et al.*, 2002). SERS spectral fingerprints have been used to discriminate between different types of viruses (Fan *et al.*, 2010; Shanmukh *et al.*, 2006). Recently, several food and waterborne viruses, namely noroviruses, adenoviruses, parvoviruses, rotaviruses, coronaviruses, paramyxoviruses and herpesviruses, were detected and identified using a gold substrate (Fan *et al.*, 2010). Viruses with or without an envelope were differentiated using multivariate statistical analyses (SIMCA) with more than 95% classification accuracy. For SERS, the detection limit was a titer of 10^2, demonstrating promise for the rapid detection and identification of viruses in food and water samples.

In addition to discriminating between different virus types, SERS has also been used to detect different strains of a single virus type (Tripp *et al.*, 2008). Using silver nanorod arrays fabricated by oblique angle deposition (OAD), SERS was able to detect trace levels of DNA viruses (adenoviruses) and RNA viruses (rhinoviruses and human immunodeficiency viruses (HIVs)) in real time. Moreover, it was able to discriminate between respiratory viruses, virus strains and viruses with gene deletions in biological media (Shanmukh *et al.*, 2006). Further studies indicated that SERS spectra could be used to differentiate between respiratory syncytial virus (RSV) strains and detect viruses with gene deletions using partial least squares (Shanmukh *et al.*, 2008). In a similar study, SERS-active silver nanorod arrays prepared by OAD detected and differentiated between the molecular fingerprints of several important human pathogens, including RSV, HIV and rotavirus (Driskell *et al.*, 2008). SERS also showed high sensitivity and specificity in the identification and classification of rotavirus strains (Driskell *et al.*, 2010). Even though the spectra were similar for each strain, the relative intensities were different (Fig. 11.1). Besides being determined as rotavirus positive or rotavirus negative, samples could be classified by the difference in spectral shapes.

Recently, tip-enhanced Raman spectroscopy (TERS), a combination of optical spectroscopy with SERS, was used to obtain a representative virus spectrum in the identification of different virus strains of avipoxvirus and adeno-associated virus (Hermann *et al.*, 2011). In recent years, SERS substrates and probes have been developed to detect viral genes from HIVs, West Nile viruses and RSVs (Liang *et al.*, 2007; Malvadkar *et al.*, 2010; Zhang *et al.*, 2011a). These studies indicate that the specificity, speed and sensitivity may make SERS-based virus detection a competitive alternative to current detection methods used for food matrices.

Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) monitors the electrical response of a system when a periodic, small amplitude AC signal is applied (Hassen *et al.*,

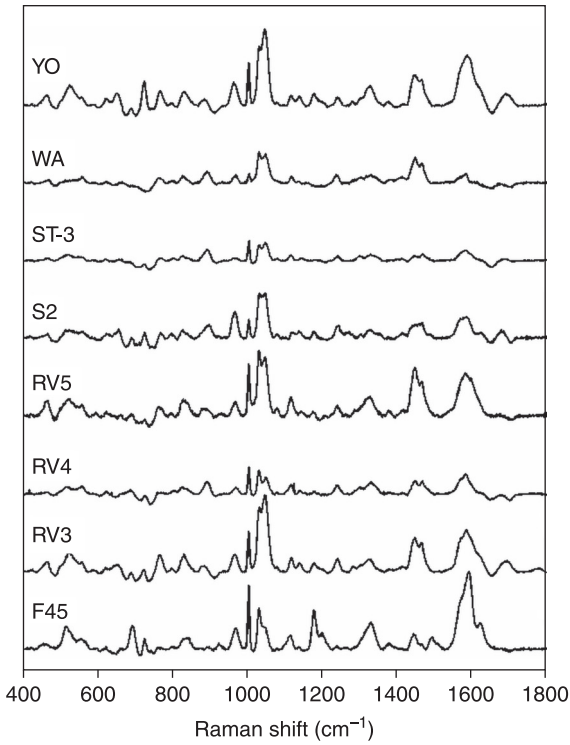

Fig. 11.1 Average SERS spectra of eight strains of rotavirus and the negative control (MA104 cell lysate). Taken from Driskell *et al.* (2010) with permission.

2008). EIS has been used to detect several viruses including the influenza virus, the rabies virus, the hepatitis B virus and HIV (Kukol *et al.*, 2008; Hassen *et al.*, 2008, 2011; Hnaien *et al.*, 2008). Many types of biosensor are based on EIS (Hassen *et al.*, 2008). Recently, the influenza A virus was detected using EIS with an antibody-neutravidin-thiol structure immobilized on the surface of an Au electrode in solutions of phosphate buffer saline with large amounts of non-target protein, which showed the detection sensitivity and selectivity (Hassen *et al.*, 2011). The detection limit was as low as 8 ng/ml, which shows the efficiency of this approach for virus detection. A biosensor based on EIS has been used to detect the label-free viral DNA hybridization of avian influenza virus (Kukol *et al.*, 2008). Even though EIS has not been used for foodborne virus detection, it is a promising approach in terms of sensitivity, selectivity and response.

11.3.2 Immunoassays

Immunoassays are analytical methods that produce a sensitive, selective and measurable response based on highly specific antibody and antigen interactions

(Li *et al.*, 2011a; Lee *et al.*, 2011). Until recently, ELISA and enzyme immunoassays were widely used in foodborne virus detection. Even though these methods are reliable, they are time-consuming and labor intensive. An immunoassay using microsphere technology can overcome the limitations associated with traditional ELISA (Go *et al.*, 2008). The well-known microsphere assay system, the xMap system (Luminex Corp., Austin, TX), combines three well-established technologies: bioassays, solution phase microspheres and flow cytometry (Go *et al.*, 2008). A liquid suspension array consisting of unique color-coded microsphere polystyrene beads is coupled to antigens and antibody reactions, and the emissions are then measured by a flow-based detector (Deregt *et al.*, 2006). Microsphere immunoassays offer several advantages, including accuracy, high sensitivity, specificity, reproducibility, high-throughput sample analysis and multiplexing capability, over traditional ELISAs (Go *et al.*, 2008). In particular, the multiplexing capability enables the detection of a multiplex analyte in a single reaction tube based on individually identifiable, fluorescently coded sets of polystyrene microbeads (Binnicker *et al.*, 2011; Khan *et al.*, 2006). In the last decade, a number of microsphere-based immunoassays have been described for the antigen and antibody detection of several viruses including HIV (Bellisario *et al.*, 2001), non-human primate viruses (Khan *et al.*, 2006), avian influenza virus (Deregt *et al.*, 2006), West Nile virus (Johnson *et al.*, 2007), Epstein–Barr virus (Binnicker *et al.*, 2008) and hepatitis C virus (Fonseca *et al.*, 2011).

Immuno-PCR (IPCR) is a method similar to ELISA. Reporter DNA is used instead of an enzyme in IPCR, which may have a 10^2 to 10^5 increase in sensitivity as a result of the amplification of the reporter DNA (Deng *et al.*, 2011b). More recently, this method has been used in rapid screening for trace levels of avian influenza viruses (Deng *et al.*, 2011b), Newcastle disease viruses (Deng *et al.*, 2011a), RSVs (Perez *et al.*, 2011) and foot and mouth disease viruses (Ding *et al.*, 2011). IPCR had an approximately 1000-fold improvement over conventional ELISA, and a 100-fold enhancement over RT-PCR. The detection limit was as low as 10^{-4} EID_{50} (50% egg infective dose) for the H5 subtype avian influenza virus (Deng *et al.*, 2011b).

11.3.3 Microelectromechanical systems and microfluidics

Microelectromechanical systems (MEMSs) can act as transducers for sensing and actuation in various engineering applications. They can be used to integrate micron-sized mechanical parts with electronics and they can be batch fabricated in large quantities (Gau *et al.*, 2001). MEMS-based and microfluidic-based biosensing approaches have received considerable interest in recent years owing to their advantages over conventional methods including low cost and sample volume, portability, disposability, parallel processing and automation (Wang *et al.*, 2011). More recently, a MEMS biosensor has been developed to detect hepatitis A and hepatitis C viruses (HCVs) in serum using dynamic-mode microcantilevers without any labels or preamplification (Timurdogan *et al.*, 2011). Electroplated nickel MEMS cantilevers functionalized with HAV or HCV

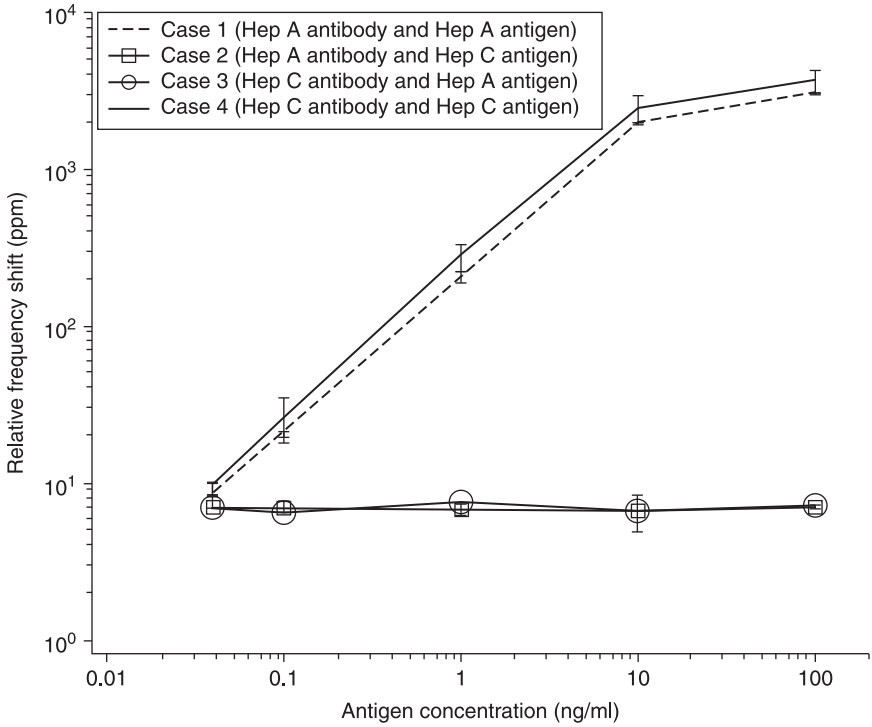

Fig. 11.2 Hepatitis detection measurement results using two biosensor chips. Measurements were taken at different concentrations ranging from 0.04 to 100 ng/ml for negative and positive controls. Different HAV and HCV concentrations were introduced into fetal bovine serum. Taken from Timurdogan *et al.* (2011) with permission.

antibodies were exposed to either HAV antigens (Case 1 and Case 3) or HCV antigens (Case 2 and Case 4), in increasing concentrations in an undiluted serum (Fig. 11.2). The minimum detection limit concentration was 0.1 ng/ml for both HAVs and HCVs, which is comparable with labeled sensing detection methods such as ELISA. Moreover, it was shown that the dynamic range of this biosensor was in excess of 1000:1 for the specific type of hepatitis antibody used.

MEMS technology enables PCR using microfluidics and consequently the synthesis of complementary DNA (cDNA) on microfluidic devices (Li *et al.*, 2011c). This microfluidic-based PCR method has several advantages including lower thermal capacitance giving rapid thermal cycling, reduced analysis times, low consumption of sample and reagent, portability and the potential for high automation and integration of various analytical procedures (Li *et al.*, 2011b). Microfluidic-based RT-PCR has been developed to detect foodborne viruses (Li *et al.*, 2011b, 2011c). An integrated microfluidic system for continuous-flow RT-PCR reactions with online fluorescence detection has been developed for the rapid identification of NVs and rotaviruses; the limit of detection (LOD) is

6.4×10^4 copies per μl using a one-step RT-PCR process (Li *et al.*, 2011b). This restricted LOD was mainly attributed to the inhibition effect of the channel surface. Detection of the amplified products was carried out online using fluorescence microscopy with SybrGreen I. This method did not require the time-consuming and labor-intensive agarose gel electrophoresis and ethidium bromide staining and had much faster reaction times compared to conventional RT-PCR.

11.3.4 Nanostructures

Spherical (quantum dots) and linear particles (nanowires, nanotubes or nanorods) with specific optical, electrical, mechanical, thermal and magnetic properties can be fabricated by combining different metals, semiconductors and carbon (Valdivia-Granda *et al.*, 2005). Nanoparticles (NPs) can be used to provide additional functional properties, including signal enhancement or purification, in virus detection (Fournier-Wirth and Coste, 2010).

Quantum dots (QDs), clusters of a few hundred to a few thousand atoms, are synthesized from metallic materials such as gold, silver or cobalt and semiconductor materials such as cadmium sulfite, cadmium selenide and cadmium telluride (Valdivia-Granda *et al.*, 2005). QDs have often been used to label biomolecules owing to their outstanding properties such as negligible photobleaching, fairly high quantum yield, stability, narrow emission spectrum and broad excitation spectrum (Zhang *et al.*, 2011b). These particles have been conjugated to antibodies and nucleic acids and used as a label in the detection of several viruses including RSV (Agrawal *et al.*, 2005), porcine reproductive virus (Stringer *et al.*, 2008), cauliflower mosaic virus (Huang *et al.*, 2009), Newcastle disease virus and avian virus arthritis virus (Wang *et al.*, 2010) and the Epstein–Barr virus (Chen *et al.*, 2010); however, QDs have never been used to detect foodborne viruses.

Carbon nanotubes (CNTs) are widely used in novel nanostructures and devices due to their large surface area per unit mass and excellent mechanical and electrical properties (Bhattacharya *et al.*, 2011). Moreover, the functionalization of CNTs through the alteration of the surface chemistry increases their potential for use as biosensing markers (Valdivia-Granda *et al.*, 2005). Using the surface functionalization feature, CNTs can be used to immobilize antibodies or nucleic acid that target a type of virus. The process can be monitored using a change in the mechanical or electrical property of the CNTs (Bhattacharya *et al.*, 2011). This concept has been recently used to detect hepatitis C viruses (Dastagir *et al.*, 2007), avian influenza viruses (Zhu *et al.*, 2009; Tam *et al.*, 2009) and swine influenza viruses (Lee *et al.*, 2011) other than foodborne viruses. As CNT-based biosensors are easy to produce, have reproducible results and are inexpensive, and since they have better sensitivity and time responses than current techniques, they are very promising for detecting viruses.

One other promising approach for detecting biomolecules is the use of a semiconducting nanowire where the conductance is proportional to the viral load. The change in conductance is in response to binding between the target and the probe, which is attached to the nanowire (Patolsky *et al.*, 2004; Valdivia-Granda

et al., 2005). Nanowires act as a capture agent on the sensor surface and selectively bind target biomolecules much like CNTs (Ishikawa *et al.*, 2009). Nanowires have several attractive features for the real-time detection of a single virus with high selectivity (Valdivia-Granda *et al.*, 2005). Silicon nanowires have been used in label-free field effect transistor (FET)-based biosensors to detect influenza A viruses (Zheng *et al.*, 2005) and dengue viruses (Zhang *et al.*, 2010). The results showed that silicon nanowire-based sensors are more sensitive and have a more rapid response compared to traditional methods. Recently, an alternative nanomaterial to silicon nanowire, a metal oxide nanowire, has been used to detect a protein related to severe acute respiratory syndrome (SARS) at a subnanomolar concentration in a background of 44 μM bovine serum albumin (Ishikawa *et al.*, 2009).

11.4 Future trends

Research into the detection of foodborne viruses has grown in recent years due to the high incidence of outbreaks. Currently, immunological and PCR-based methods are commonly used to detect viruses in food samples. Despite their reliability, most of these methods have limitations in terms of speed and sensitivity owing to low viral concentrations and inhibitory substances present in food. Even though methods for concentrating and purifying viruses in food samples have been widely investigated and developed, the inhibitory substances can remain and cause false-negative results. Therefore, new detection methods that are rapid and sensitive are necessary for direct detection in food samples.

Some of the approaches described for detecting viruses are relatively new and some are still in their infancy. It is expected that electrochemical-based detection techniques will become more prominent, while spectroscopic and microfluidic assays will be developed in parallel. It is anticipated that research using microfluidics will focus on combining the pretreatment of a viral sample and multiplex detection into a biochip. Thus, the microfluidic approach could be a promising platform for rapid detection of viruses. Additionally, the conjugation of antibodies, antigens and nucleic acids with quantum dots, nanowires and carbon nanotubes offers several advantages over current detection methods in terms of sensitivity and speed. These systems may also be used for the label-free detection of very low concentration of viral particles, or even for detecting a single virus without amplification. These approaches can be further improved with the advent of novel nanostructures. Even though most of these approaches have not been used to detect foodborne viruses, all of them are promising and can complement the existing methods.

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