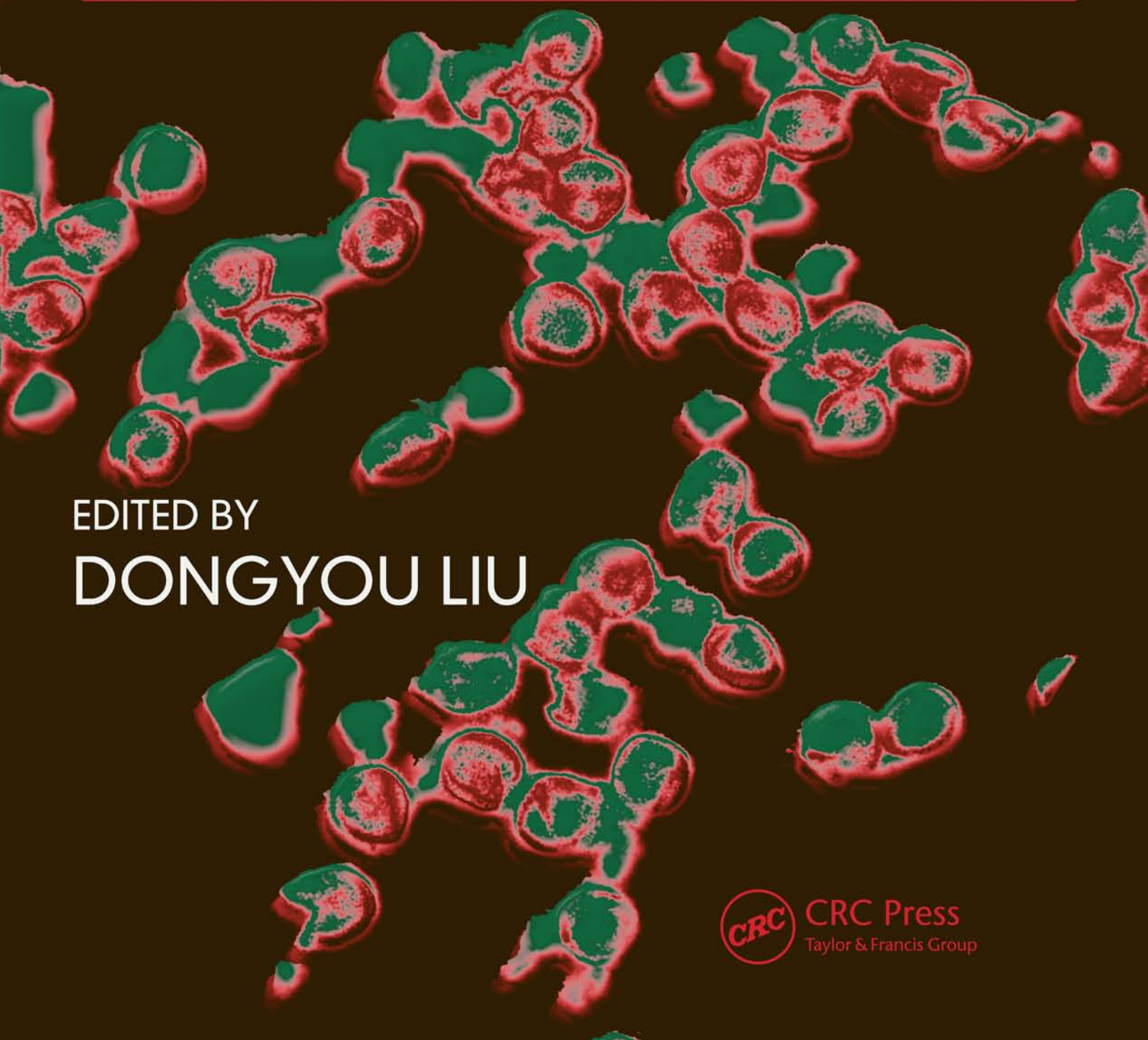
MOLECULAR DETECTION OF FOODBORNE PATHOGENS



EDITED BY

DONGYOU LIU

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This book is dedicated to my parents, Jiaye Liu and Yunlian Li, whose unselfish sacrifice and unrelenting love have been a constant source of inspiration in my pursuit of knowledge and betterment.

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Preface

Foodborne pathogens are microorganisms (e.g., bacteria, viruses, fungi, and parasites) that are capable of infecting humans via contaminated food and/or water. In recent years, diseases caused by foodborne pathogens have become an important public health problem worldwide, resulting in significant morbidity and mortality. Currently, there are over 250 known foodborne diseases. Due to the introduction of pathogens to other geographic regions through population movement and globalization of the food supply, new foodborne infections are continuously emerging. Furthermore, pathogen evolution, changes in human immune status and life-style as well as food manufacturing practices also contribute to increased incidences of foodborne illnesses. As a consequence, large outbreaks of foodborne diseases have been reported with alarming frequencies.

It is well known that one of the most effective ways to control and prevent human foodborne infections is to implement a surveillance system that includes a capability to rapidly and precisely detect, identify, and monitor foodborne pathogens at the nucleic acid level. The purpose of this book is to bring out an all-encompassing volume on the detection and identification of major foodborne bacterial, fungal, viral, and parasitic pathogens using state-of-art molecular techniques. Each chapter includes a concise review of the pathogen concerned with respect to its biology, epidemiology, and pathogenesis; a summary of the molecular detection methods available; a description of clinical/food sample collection and preparation procedures; a selection of robust, effective, step-wise molecular detection protocols for each pathogen; and a discussion on the challenges and continuing research needs to further extend the utility and performance of molecular diagnostic methods for foodborne diseases.

With each chapter written by scientists with expertise in their respective foodborne pathogen research, this book provides comprehensive coverage of the molecular methodologies for the detection and identification of major foodborne pathogens. It is an indispensable tool for clinical, food, and industrial laboratory scientists involved in the diagnosis of foodborne diseases; a convenient textbook for prospective undergraduate and graduate students intending to pursue a career in food microbiology and medical technology; and a reliable reference for upcoming and experienced laboratory scientists wishing to develop and polish their skills in the molecular detection of major foodborne pathogens.

Given the number of foodborne pathogens covered, and the breadth and depth of the topics discussed, an inclusive book like this is undoubtedly beyond the capacity of an individual's effort. It is my fortune and honor to have a large panel of international scientists as chapter contributors, whose willingness to share their technical insights on foodborne pathogen detection has made this book possible. Moreover, the professionalism and dedication of senior editor, Steve Zollo, and other editorial staff at CRC Press have contributed to its enhanced presentation. I hope the readers will find it as stimulating and rewarding as I do through reading this book, which by presenting relevant background information and ready-to-run molecular detection protocols will serve to save readers' time and patients' lives.

Dongyou Liu, PhD

Editor

Dongyou Liu, PhD, is currently a member of the research faculty in the Department of Basic Sciences, College of Veterinary Medicine at Mississippi State University in Starkville. In 1982, he graduated with a veterinary science degree from Hunan Agricultural University in China. After one year of postgraduate training under the supervision of Professor Kong Fangyao at Beijing Agricultural University (presently China Agricultural University) in China, he completed his PhD study on the immunological diagnosis of human hydatid disease due to the parasitic tapeworm *Echinococcus granulosus* in the laboratory of Drs. Michael D. Rickard and Marshall W. Lightowlers at the University of Melbourne School of Veterinary Science in Australia in 1989. During the past two decades, he has worked in several research and clinical laboratories in Australia and the United States, with an emphasis on molecular microbiology, especially in the development of nucleic acid-based assays for species- and virulence-specific determination of microbial pathogens such as ovine footrot bacterium (*Dichelobacter nodosus*), dermatophyte fungi (*Trichophyton, Microsporum*, and *Epidermophyton*), and listeriae (*Listeria* species). He is the editor of the *Handbook of Listeria monocytogenes* and the *Handbook of Nucleic Acid Purification*, both of which have been published recently by Taylor & Francis/CRC Press.

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1 Molecular Detection: Principles and Methods

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1.1 INTRODUCTION

While the vast majority of our food supplies are nutritious and safe, illness due to foodborne pathogens still affects millions if not billions of people each year. It is estimated that up to 30% of the population in industrialized nations suffer from foodborne illness each year.[1] In the U.S. there are an estimated 76 million cases each year that result in 325,000 hospitalizations, and 5000 deaths.[2] Estimates of the number of cases in developing countries are difficult to obtain due to differences in reporting of cases in different countries; however, the rates of illness are expected to be higher.[1,3,4]

Diarrheal diseases, a high number of which result from foodborne contamination, kill an estimated 1.8 million children worldwide.[3]

Table 1.1 summarizes the statistics of U.S. foodborne illness outbreaks for the year 2006 broken down by etiology. An outbreak is constituted by more than one person becoming ill by the same strain of an organism. The list displays only outbreaks from known etiologies of bacterial, viral, parasitic, and helminthic origin, and does not take into account outbreaks where an etiology could not be assigned. Nor does it take into account sporadic cases of illness, which far outnumber outbreak cases. Most of these sporadic cases are not reported to any official health tracking agency because they are not severe, or cultures are never obtained.[1] An even greater number of people with sporadic cases of foodborne illness do not seek medical attention.

Whether an illness is mild or severe, the underlying message from the statistics is that millions or billions of servings of food are contaminated with a pathogen or a toxin each year. Table 1.1 illustrates that the types of foods implicated is broad and comprises meats, dairy, produce, grains, processed foods, and water. While many cases of foodborne illness result from human cross-contamination in restaurants or in the home, a large amount results from foods that arrive into the kitchen already contaminated. These organisms can contaminate the foods directly by association with feed animals or plants prior to or during processing, through contaminated water used for watering or washing, and through handling by infected people.

One of the most difficult and fundamental issues in food safety is the detection of foodborne pathogens. The problem is terribly complex with a multitude of factors and variables with which to contend. With the infectious dose of some of the pathogens as low as <100 cells or particles, sensitivity is essential. In some instances, an enrichment step is necessary to amplify the number of pathogens in the sample simply so that they can be detected. However, enrichment does not work with viruses or toxins, and some organisms with long generation times can take weeks to enrich. Additionally nonprocessed or minimally processed foods are not sterile and native microflora can sometimes mask the presence of the pathogen. Finally the food matrix itself sometimes inhibits detection by affecting the chemistries used in detection methods. While an all-encompassing test that would detect every possible pathogen or toxin would be desirable, the technology does not yet exist. Ideally, the detection of pathogens should be fast and economical. Ultimately a balance between the financial burden of testing and the risk of selling of untested foods must

TABLE 1.1
Number of Foodborne Outbreaks with Confirmed Etiologies in the U.S. for the Year 2006

	Agent	No. of Outbreaks	No. of Cases	Suspected Vehicles
Bacterial	*Bacillus*	3	35	Produce, rice, meat
	Brucella	1	5	Cheese
	Campylobacter	22	283	Milk, cheese, seafood, produce, meat
	Clostridium	20	745	Produce, seafood, canned food, meat
	Escherichia	29	520	Milk, produce, meat
	Listeria	3	7	Cheese, salad
	Salmonella	116	2751	Meat, dairy, produce, peanut butter
	Shigella	9	183	Salad, produce, meat
	Staphylococcus	12	380	Meat, dairy, seafood
	Vibrio	8	427	Seafood
	Subtotal	223	5336	
Viral	Hepatitis A	3	34	Spring water, unspecified
	Norovirus	333	10,970	Salads, seafood, meat, produce
	Subtotal	336	11,004	
Parasite	*Cryptosporidium*	2	16	Unspecified
	Cyclospora	3	19	Fruit salad
	Giardia	2	11	Unspecified
	Subtotal	7	46	
Helminth	*Trichinella*	1	2	Bear meat
	Subtotal	1	2	
	Total	567	16,388	

Source: Compiled from Centers for Disease Control http://www.cdc.gov.

be met to ensure the safety of consumers and simultaneous profitability for food producers. The following chapters give detailed reviews of the latest methods and targets for detection of specific organisms. However, when reviewing the subject of molecular detection methods, common themes arise. These themes relate to the choices of detection methods and the molecular targets for detection.

1.2 DETECTION METHODS

A wide range of foodborne pathogen detection techniques have been developed including culturing methods, nucleic acid methods, immunological methods, microscopy, spectroscopy, and bioluminescence, with varying degrees of cost, specificity, sensitivity, and ease of use. The major considerations of a detection system include the cost of the process, the target for detection, and the specificity and sensitivity of the procedure selected for detection. In recent years painstaking methods of cell culture and microscopic observation have yielded faster, more efficient molecular methods of detection. While traditional microbial detection methods may yield adequate target specificity and sensitivity, the time to results is on the order of days, often relying upon pathogen growth. Numerous molecular techniques have emerged that offer the advantage of speed along with specific and sensitive detection. Molecular methods have also proven advantageous in cases where it is difficult to culture the target of interest, as can be the case with viruses. These methods require a solid understanding of the physiology of the target organism, its close relatives, and those with which it may coexist on a food surface.

1.2.1 Pathogen Detection in Complex Matrices—Sample Preparation

Simultaneous advances in detection methods and in sample preparation prior to analysis are needed to ensure a safe food supply.[5] Foodborne pathogens have been associated with a wide variety of foods including poultry, beef, shellfish, fruits, vegetables, and drinking water. Without appropriate preparation of a test sample prior to detection, a common potential problem to many detection methods is that the sample background material may drastically decrease the sensitivity of the detection step or even lead to false negative test results. Food derived polymerase chain reaction (PCR) inhibitors include Ca^{2+}, fats, glycogen, and phenolic compounds.[6] The presence of proteinases in cheese[7] and milk[8] may also inhibit PCR. Different approaches have been used to counteract poor PCR performance in difficult backgrounds. Bovine serum albumin has shown success in relieving PCR inhibition in certain cases,[9] and the type of DNA polymerase used can greatly affect the outcome of a reaction in the presence of biological samples.[10]

Additional potential challenges of detecting foodborne pathogens include their nonuniform dispersal and very low concentrations within foods. Therefore, considerable effort is often required to prepare a sample such that it is suitable for testing with a nucleic acid detection procedure. Methods that have been used for the removal of PCR inhibitors include physical separation techniques such as filtration, DNA extraction, and adsorptive methods such as immunomagnetic separation.[11] Lampel et al.[12] used filters capable of trapping and lysing microorganisms, then used these filters directly in PCR reactions. The detection limits of *Shigella flexneri* in artificially contaminated foods using the filter system were greatly improved as compared to unfiltered tests.

1.2.2 Nucleic Acid Based Detection

Advances in nucleic acid testing have included rapid amplification techniques and associated automated instrumentation, microarray based technology, and lab-on-a-chip platforms. The relatively low cost and speed of oligonucleotide synthesis, the wide range of 3′ and 5′ oligonucleotide modifications readily available, and powerful software to aid in molecular assay design and data analysis have facilitated the growth of a wide range of nucleic acid based techniques applied to the detection of foodborne pathogens.

1.2.2.1 PCR

Nucleic acid amplification techniques have an enormous range of applications and have become an indispensible tool in molecular biology and powerful rapid screening method in the detection of foodborne pathogens. By targeting and amplifying (or making copies of) DNA sequences *in vitro*, it has been possible to detect the presence of specific DNA sequences with sensitivities down to a single target copy per reaction, and in many cases quantify the results.

PCR is a method for the amplification of double or single stranded (ss) DNA sequences *in vitro*. The reaction proceeds in response to temperature driven steps of double stranded (ds) DNA denaturation, primer or ss oligonucleotide annealing to complementary ss target DNA sequences, and DNA polymerase extension. These steps are repeated, and under appropriate conditions will generate a doubling of the initial number of target copy sequences with each cycle. The primers define the 5′ ends of the discrete products that are subsequently formed. Three step PCRs use three individual temperature steps for denaturation, annealing, and extension, while two step PCRs use a combined annealing and extension step. Reaction reagents typically include a thermostable DNA polymerase, deoxyribonucleoside triphosphates (dNTPs), user selected primers for targeting specific sequences, magnesium chloride, and template or target DNA. The process is rapid, requiring between minutes and hours to generate enough discrete sized target sequences for detection; a single thermal cycle may require as little as a few seconds to complete. The length of time required for a reaction is typically a function of variables such as the length of the target sequence and the heating and cooling rates of the thermal cycler used. However, it is now possible to find PCR systems capable of thermal cycling speeds so fast that decreasing cycle time further would not be worthwhile without first finding a DNA polymerase capable of working faster than those currently

in use.[13] While it is possible for PCR to routinely detect low copy numbers in a reaction, many reactions use between 5 and 10 µl sample volumes, yielding a lower detection limit of close to 10^3 CFU/ml.[14]

Among the expanding array of nucleic acid amplification techniques, PCR remains the most popular method, presumably as a result of its cost and ease of use,[15] and has been used extensively for the detection of foodborne pathogens. By the early 1990s numerous primer sets had been developed for the detection of pathogens and the food industry had gained interest in this powerful method.[16]

The technique was initially reported in 1985,[17] explained in full detail in 1986,[18] and has since undergone several significant modifications including the use of a thermostable polymerase[19] preventing enzyme destruction at denaturation temperatures, and "hot start" enzymes[20] for temperature induced activation control, reducing the possibility of nonspecific product formation. Other major advances have included amplicon formation monitoring without opening the reaction tube,[21,22] yielding facile quantification of initial target copy numbers, and the use of melting curve analysis to evaluate product specificity, which in some cases, allows extension of the quantifiable range beyond what is possible with threshold cycle analysis alone.[23]

In addition to evaluating a reaction's specificity and detection limit, PCR reaction efficiency is often used to assess performance. The number of target copies generated after n cycles, x_n, is a function of the initial target copy number x_o and the amplification efficiency ε:

$$x_n = x_o(1+\varepsilon)^n, \tag{1.1}$$

with the amplification efficiency ranging from 0 to 1. Assay parameters that may influence reaction efficiency include primers, annealing temperature, and type of polymerase used. Annealing temperature optimization may be used to balance reaction efficiency and specificity.

Approaches to the quantification of real-time PCR products have been described,[22,24,25] and techniques typically involve the monitoring of fluorescence accumulation as a function of cycle number through specific or nonspecific dsDNA binding dyes. The threshold cycle, C_T, is the fractional cycle at which enough fluorescence has accumulated to rise above the background signal and may be used for quantification. Absolute quantification is possible with unknown samples by running reactions of known template copy numbers to obtain a relationship between the threshold cycle number and the amount of initial template in the reaction. A mathematical model for relative quantification purposes has also been described.[26]

1.2.2.1.1 *Practical Considerations for PCR-based Detection*

The strength of PCR is its weakness; the assay is incredibly sensitive to the detection of nucleic acids. Since PCR products serve as substrates for subsequent reactions, extremely large numbers of target copies may be generated. As a result care must be taken in reaction setup and amplicon handling following a reaction to prevent carry-over contamination. Kwok and Higuchi[27] list important steps to avoid the occurrence of false positive results, including physically separating the preparation of PCR reagents and the handling of PCR products, as well as frequently changing disposable gloves. While technique is paramount in the ability to generate reproducible results, a brilliant enzymatic approach has also been used to avoid false positive results. Longo et al.[28] used a strategy that involved using dUTP in place of dTTP for PCR. All subsequent reactions were treated with uracil DNA glycosylase (UDG), followed by thermal inactivation of this enzyme prior to starting thermal cycling. As a result, any carry-over contaminating DNA would contain uracil and ultimately be rendered unamplifiable through the action of UDG, while simultaneously leaving target DNA intact.

Nonspecific amplification arises from primers that bind to unintended targets such as themselves (primer dimers) or other unintended sequences present in the reaction mixture (e.g., DNA sequences from the natural microbiota present in foods). Methods to minimize nonspecific amplification include proper primer design, optimization of assay conditions, and the use of a hotstart DNA polymerase. Wittwer et al.[29] studied the influence of annealing time on product specificity. Tests indicated that as annealing time increased, so did the tendency of primer sets to form nonspecific products. Although specificity was generally improved with short annealing times, in some cases there was a tradeoff in the amount of product formed and the specificity of the products formed. Other techniques for optimizing PCR conditions include varying the concentrations of primers and $MgCl_2$, and evaluating two and three step thermal cycling formats. The ultimate test of a primer set's specificity is in evaluating the performance with target and nontarget DNA sequences.

Although it is possible to generate millions of amplicon copies in an hour or less, one complicating factor with PCR testing of food samples is that the level of inhibition is a function of the type of food tested.[30] PCR inhibitors may hamper cell lysis, making it difficult to extract DNA, degrade or sequester nucleic acids, or they may act on DNA polymerase.[6] In an effort to increase the likelihood of detection when pathogens are present in a sample, separation methods, enrichment procedures, and the extraction of DNA have been used.

A negative PCR result could indicate that the target sequence was not present in the reaction or that the reaction itself failed. In order to avoid the uncertainty of such a result in diagnostic PCR, it has been proposed that PCRs contain an internal amplification control.[31] Internal amplification controls are nontarget DNA sequences that will be amplified regardless of whether or not the target sequence was present in the reaction. If the internal amplification control is not amplified, then the reaction failed, and it is not possible to know if the target sequence was present in the failed reaction, so the detection step must be repeated.

The amplification of nucleic acids for detection purposes is usually just one step of a procedure that involves assay design and sample preparation prior to amplification, followed by specificity and sensitivity analysis. Some steps prior to and after amplification are shown in Figure 1.1.

1.2.2.1.2 Traditional PCR

Traditional PCR techniques involve amplification of a target sequence of interest followed by product size verification using a technique such as agarose gel electrophoresis to compare the mobility of standard DNA ladder to the mobility of the amplified DNA (Figure 1.2). Comparison of the known standards to the PCR products can be used to estimate the size of the products formed. This step may be followed by performing a Southern blot to evaluate sequence specificity. Two potential drawbacks to traditional PCR are that (i) endpoint quantification is challenging and (ii) it is necessary to open the reaction tube to verify reaction product specificity,

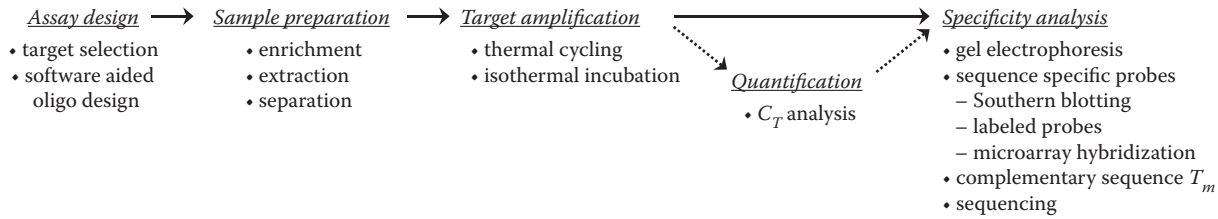

FIGURE 1.1 Steps used for the detection of nucleic acids by amplification. Quantification is not essential to verify specificity.

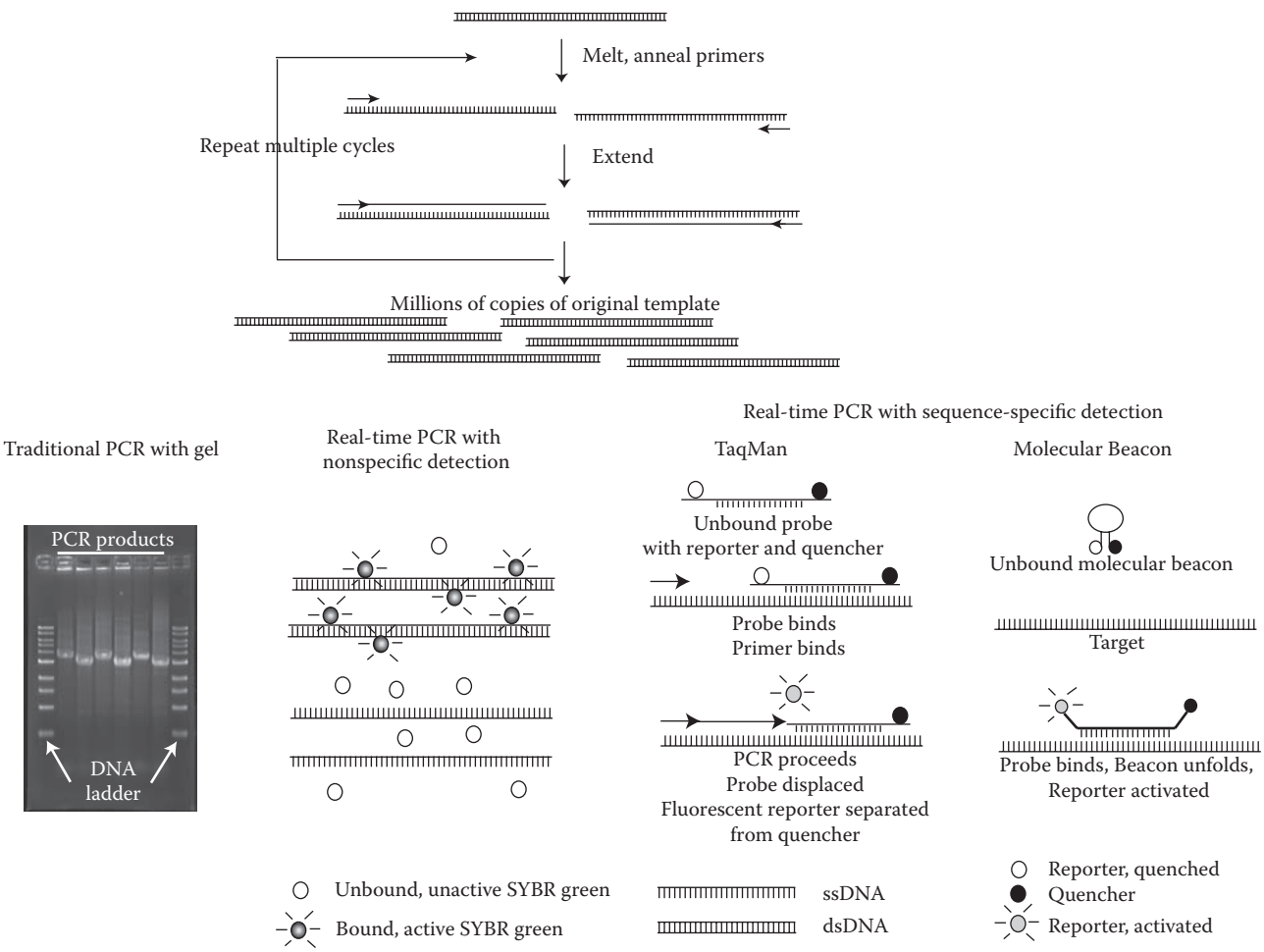

FIGURE 1.2 Representation of PCR and detection protocols. The principle of PCR is illustrated in the top part of the figure. On the bottom are detection techniques. These include gel electrophoresis after traditional PCR. Real-time PCR with nonspecific fluorescent dye is shown where the dye only fluoresces when associated with double stranded DNA. Real-time PCR with probe-based detection with TaqMan and Molecular Beacon technologies is illustrated where the fluorescent reporter must be physically separated from the quencher in order to fluoresce.

increasing the opportunity for carry-over contamination. Traditional PCR therefore requires separate instrumentation for the amplification and evaluation of dsDNA products.

However, provided that PCR products are handled carefully and that real-time quantification is not necessary, traditional PCR techniques can be used with great success for the detection of food pathogens. A single enrichment, thermal cycling protocol, set of PCR reagent components and concentrations were used for the detection of 13 foodborne pathogens by Wang et al.[32] Agarose gel electrophoresis on 2% agarose gels stained with ethidium bromide was used for separation of PCR products. The PCR detection limits reported ranged from two cells to 5×10^4 cells for *E. coli* O157:H7 and *Shigella* spp., respectively.

1.2.2.1.3 Real-time PCR

The ability to monitor amplicon accumulation as a reaction proceeds has drastically improved the field of nucleic acid detection. In addition to facilitating the quantification of initial target copy numbers, real-time PCR allows an operator to evaluate product specificity without opening the reaction chamber, saving time, and reducing carry-over contamination risk. Real-time PCR systems offer a wide range of capabilities. These include the ability to handle thousands of samples per day, perform 35 thermal cycles in under 40 minutes, and detect initial target copy numbers over a range from 10 to 10^{10}.[33]

The design of real-time PCR assays has been aided by commercially available software packages that can determine optimal primer, probe, and reaction conditions, given a specific sequence of interest. Real-time PCR assays are typically designed to target short DNA fragments using primers specifically selected to avoid the formation of primer dimers. The increase in fluorescence in response to amplicon formation is generally accomplished in one of two ways: through the use of a nonspecific dsDNA binding, or by sequence specific probes that generate a signal only in the presence of the target DNA sequence. Real-time PCR techniques and applications have been reviewed extensively,[14,34–37] and experimental comparisons among instrumentation and assay formats have been performed to compare sensitivities.[38]

1.2.2.1.4 Real-time PCR—Nonspecific Detection

Nonspecific dsDNA binding dyes have been used for real-time PCR fluorescence based detection systems for target quantification and specificity evaluation. Over the course of thermal cycling, an increase in the amount of fluorescence generated is recorded. The earlier this increase in fluorescence occurs, the larger the initial target copy number present in the reaction. Following thermal cycling, product specificity is verified by slowly raising the reaction temperature through a broad temperature range that includes the expected product melting temperature, while simultaneously recording fluorescence in order to determine the melting temperature (T_m) of any dsDNA products that have formed (Figure 1.2). The melting temperature of a dsDNA product is the temperature at which half of the product has become ss. This melting temperature is a function of the dsDNA length, GC content, solution salt concentration, and dye concentration. Advantages of using nonspecific ds binding dyes as compared to probe based systems include cost and ease of assay design. As a result, this approach may be used as a less expensive alternative for initial testing with a primer set. However, this technique does not provide information regarding the length or sequence of the amplified product, and GC rich regions within a single amplicon may create complex melting profiles with multiple peaks.[39]

SYBR Green is a nonspecific dsDNA binding dye that is frequently used in real-time PCR assays. This dye binds to the minor grove of dsDNA and does not give strong fluorescence when free in solution. SYBR Green real-time PCR assays have been successfully used for the detection of foodborne pathogens, with specificity verification performed by melting curve analysis.[40,41]

1.2.2.1.5 Real-time PCR—Sequence Specific Detection

A large number of real-time PCR strategies that are based on fluorescene increases in response to sequence specific detection have also been developed. Probe based real-time PCR techniques are advantageous over the use of nonspecific dsDNA binding dyes in that they may not require analysis of PCR amplicon melting temperatures for product specificity—fluorescence generation is a function of the probe binding to a specific sequence of DNA. In the case of real-time PCR development with probe based systems, excitation and emission wavelengths of the fluorophores selected must be kept in consideration.[34]

Sequence specific chemistries that have been incorporated into real-time PCR assays include those based on a sequence specific probe and DNA polymerase exonuclease activity, molecular beacons, and self-quenched hairpin primers. One real-time PCR chemistry (TaqMan®) that has been used extensively for the detection of foodborne pathogens relies upon the 5′ exonuclease activity of *Taq* polymerase. A probe containing a reporter and quencher in close proximity to one another binds to a target region between the two primers which define the ends of the discrete fragment ultimately formed. This probe is cleaved by the 5′ exonuclease activity of a DNA polymerase, separating the fluorophore and quencher, generating increases in fluorescence as a direct result of specific probe binding and target fragment extension (Figure 1.2). Numerous assays have been developed with this chemistry.[42–45]

Molecular beacons are stem and loop oligonucleotide structures used for sequence specific detection. The loop portion contains a sequence that is complementary to a chosen target, while the stem portion contains a short sequence of bases at the 3′ and 5′ ends that are complementary to one another but not the target.[46] Fluorescence and quenching moieties are attached to the ends of the beacon. The beacons are designed such that with no loop complementary sequence present the stem structure is stable, but in the presence of a

complementary target sequence the arms of the stem separate. This separation changes the conformation of the beacon to a more stable structure, allowing simultaneous separation of the fluorophore and quencher, leading to fluorescence generation (Figure 1.2).[46] Molecular beacons have been used in numerous applications,[47] outside of monitoring specific amplicon formation in real-time PCR. Molecular beacons have been used in multiplex PCR applications for the simultaneous detection of four pathogenic retroviruses[48] and four *V. cholerae* genes.[49]

Hairpin primers have also been used to monitor product formation as a function of cycle number. Blunt end hairpin primers using fluorophores with no quencher molecules were used with great success in a real-time PCR assay.[50] Nazarenko et al.[50] also demonstrated that these blunt end hairpin primers reduced the formation of primer dimers without PCR template present, thereby showing the outstanding specificity of the system. Nordgren et al.[51] used this type of chemistry to detect norovirus (NV) genogroups I and II. Using hairpin primers it was possible to distinguish between genogroups in a duplex PCR through melting curve analysis.

1.2.2.1.6 Reverse Transcriptase PCR

Enrichment procedures have successfully been used for the sensitive detection of viable foodborne pathogens, but this technique is time consuming, as it is a function of the target organisms growth. While PCR is capable of detecting low levels of target DNA, DNA detection does not provide information regarding the viability of a cell; food processing may destroy bacteria while leaving behind DNA and this DNA may be present even if its host cell is no longer alive.[52] On the other hand, RNA is easily destroyed, which makes it suitable for determining organism viability.[30] Reverse transcriptase PCR of mRNA targets has demonstrated that these molecules are indicators of cell viability.[53,54] Following RNA purification and degradation of contaminating DNA from a sample of interest, RNA is reverse transcribed and the synthesized complementary DNA or cDNA may be amplified as is typically done for any DNA target. Reverse transcriptase PCR has been used successfully for the detection of foodborne bacterial pathogens[55] and viruses.[56] A real-time reverse transcription PCR assay using a TaqMan minor grove binding probe was implemented for the quantitative detection of H5 avian influenza down to 100 target copies.[57]

1.2.2.1.7 Multiplex PCR

The amplification of several target sequences in a single reaction tube can be accomplished by optimized multiplex PCR assays. The motivation for such an approach includes cost efficiency[58] and a reduction in laboratory effort and time.[59] Conditions such as annealing temperature and reagent concentrations must be adjusted to allow for the simultaneous amplification of more than one target. Multiplex PCR optimization may be complicated, resulting in preferential amplification, poor sensitivity, and poor specificity[59] if satisfactory conditions for all primer and template combinations cannot be met. In comparison with single PCR reactions, multiplex PCR assay design considerations include designing long primers with higher melting temperatures and using elevated $MgCl_2$ concentrations.[60] Additionally, design considerations should include a method to distinguish between amplicons following thermal cycling. Methods may include designing target sequences of different sizes or melting temperatures for discrimination using gel electrophoresis or dissociation analysis with nonspecific dsDNA binding dyes, respectively. Using real-time PCR probes with different excitation and emission wavelengths may also be used to accomplish this goal.

Mutliplex PCR has been used to detect multiple gene targets for speciation and virulence determination in *Listeria monocytogenes*.[61] Other multiplex assays have been aimed at detecting food or waterborne pathogens of differing genera.[58,62–65] Lee et al.[64] simultaneously amplified sequences from *Salmonella enterica, Salmonella typhimurium*, *Vibrio vulnificus*, *Vibrio cholerae*, and *Vibrio parahaemolyticus* with multiplex PCR from seeded oyster homogenates. Following enrichment and DNA purification, it was possible to detect each pathogen at a level of 10^2 cells/g of oyster homogenate. Kong et al.[63] were able to simultaneously detect *Aeromonas hydrophila*, *Shigella flexneri*, *Yersinia enterocolitca*, *Salmonella typhimurium*, *Vibrio cholerae*, and *Vibrio parahaemolyticus* in marine water with detection limits ranging from 10^0 to 10^2 CFU in a total assay time of less than 12 hours.

Molecular beacons were used for the simultaneous detection of four retroviral target molecules in the same reaction tube.[48] Using different colored fluorophores with emission maxima separated over the visible range and target sequences less than 130 bp, Vet et al.[48] detected as few as ten retroviral genomes.

1.2.2.2 Isothermal Amplification

Within the last 20 years, many techniques have been developed that allow for amplification of nucleic acids under isothermal conditions. These techniques include loop mediated amplification (LAMP),[66] nucleic acid sequence based amplification (NASBA),[67] rolling circle amplification (RCA),[68] and strand displacement amplification (SDA).[69] Isothermal amplification simplifies hardware requirements as compared to PCR in that they do not require a system for thermal cycling, and may even work with a simple water bath setup. These techniques may use several sets of primers or more than one enzyme to carry out amplification of the target product without thermal cycling.

NASBA is an isothermal amplification process developed shortly after PCR began gaining widespread attention.[67] NASBA is a sensitive detection method for the detection of RNA or DNA. The reaction typically consists of three enzymes including T7 RNA polymerase, deoxyribonucleoside triphosphates, two specific primers, and buffering reagents and takes place at approximately 40°C. One major advantage of the procedure is that contaminating genomic DNA does not create problems with the assay as it will not be

amplified due to the fact that there is no thermal denaturation step involved with the process.[67]

NASBA has been used for the detection of Hepatitis A virus (HAV) using primers targeting major capsid proteins.[70] Jean et al. used Northern blotting and dot blot hybridization to verify the specificity of their reaction and found a detection limit of 0.4 ng RNA/ml as compared to a reverse transcriptase PCR assay used that yielded a detection limit of 4 ng RNA/ml. Other NASBA published methods for the detection of pathogens in foods have been listed by Rodríguez-Lázaro et al.[71] Real-time NASBA has also been used to show product formation as a function of time. Molecular beacons were used to generate fluorescence signals with NASBA assays for the detection of *Vibrio cholerae*[72] and HAV.[73]

LAMP is a procedure using four primers that have a total of six binding sites on the target DNA sequence. The isothermal reaction allows for the generation of 10^9 target sequences in less than one hour.[66] A LAMP assay targeting the *invA* gene of *Salmonella* was developed by Wang et al.[74] using an amplification time of approximately 60 minutes and run at 65°C. The detection limit of the LAMP assay was 100 fg of DNA per reaction, whereas a PCR approach gave a detection limit of 1 pg of DNA per reaction tube.

1.2.2.3 Microarray Detection

In addition to Southern blots, gel electrophoresis, melting temperature analysis with nonspecific dsDNA binding dyes, and probe based amplification detection, microarrays have been used to analyze the specificity of PCR products. DNA microarray technology (aka DNA chips or gene chips) involves the placement of user defined oligonucleotide probes in specific locations on a solid substrate such as glass. Following hybridization of target DNA sequences to probes anchored on a chip's surface, fluorescence detection can be used to monitor binding events. Depending on the sensitivity required, microarrays can be used with or without upstream amplification steps. Software analysis of large data sets that are generated greatly facilitates the process of data analysis. The advantages and limitations of several microarray software packages have been reviewed.[75]

Microarrays may be an effective way of distinguishing between nonspecific and target product formation and therefore this detection strategy may allow the use of more primers in a multiplex PCR assay than would normally be possible.[76] Amplification methods have been used in combination with microarray technology for the detection of *E. coli* O157:H7.[77] Wilson et al.[78] were able to specifically detect 18 pathogenic microorganisms including, prokaryotes, eukaryotes, and viruses using PCR in combination with a microarray containing over 50,000 probes and with a detection limit as low as 10 fg of DNA.

1.2.3 Fluorescence *in situ* Hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) is a technique for the probe-based identification of nucleic acids without amplification. The technique can be used to specifically identify microbial cells in environmental samples and rRNA molecules are frequently targeted.[79] Fluorescently labeled probes can be used to generate signals in the presence of specific target sequences, seen with fluorescence microscopy. Typical steps include sample preparation by fixation and permeabilisation, probe binding, removal of unhybridized probes by washing, and flow cytometry or microscopy detection.[79] A FISH technique for the detection of *Listeria monocytogenes* showed specific detection of the target microorganism and detection was possible in sheep milk samples.[80]

1.2.4 Immunological Detection Methods

At the core of all immunological assays is an antibody and antigen interaction. Numerous formats have been used to detect these binding events and immunological assays have been widely used for the detection of foodborne pathogens. Assay specificity and sensitivity is a function of the quality and type of antibodies used in binding to specific antigen epitopes.

Many immunoassay formats are based on the enzyme linked immunosorbent assay (ELISA).[81] ELISAs are commercially available for the detection of foodborne pathogens, and the method can be used for the detection of antibodies or antigens. The technique involves coating an antibody to a solid support surface, adding a sample of interest and incubating, and washing to remove nonspecific interactions. This step is followed by the addition of a second antibody to create a sandwich structure between the primary bound antibody, the target of interest, and this secondary antibody. The secondary antibody may be conjugated with an enzyme or fluorophore for detection and quantification with a plate reader. In this assay format, the target antigen must have at least two antibody binding sites.[82] Muhammad-Tahir and Alocilja[83] used a sandwich immunoassay with lateral flow disposable membranes and polyaniline-conjugated antibodies, and conductance measurements yielded detection limits of less that 100 CFU/ml.

Other methods for evaluating immunological binding events include fluorescence microscopy and surface plasmon resonance (SPR). Fluorescence microscopy has been used to evaluate antibodies against protozoan parasites *Giardia* and *Cryptosporidium*.[84] SPR sensors measure refractive index changes that result from surface plasmon excitation at the interface between a thin metal film and a dielectric material.[85] SPR is attractive because it is a label-free technique, but has sensitivity limitations in terms of the size range of molecules that can be detected. An SPR system was used to detect *Salmonella enteritidis* and *Listeria monocytogenes* using antibodies against the pathogens on a gold sensor surface.[86] The lower limit of detection was 10^6 CFU/ml for the pathogens, and it was noted that this sensitivity was comparable to an ELISA using the same antibodies.

Immunoassay sensitivity and potential cross reactivity should be carefully considered in comparing detection methods. Another consideration in using immunoassay based systems is that antibodies must be raised against antigens. As a result, immunological methods typically must be used

with microorganisms that have been sufficiently characterized.[87] The long development times associated with monoclonal antibodies and requirement of *in vivo* generation makes the widespread application of this technology complicated.[88] Also, in some cases it may be difficult to confirm the identity of a microorganism through immunological testing alone. On occasion, reference laboratories found that serotyping could not be used to verify the identity of strains that were initially identified as *Salmonella* sp.[89] Additionally, another nucleic acid based technology may be a suitable alternative for a range of molecular targets traditionally detected by antibodies. Aptamers, single stranded ss DNA or RNA that fold into conformations allowing specific binding to targets, have been proposed as alternative recognition molecules to antibodies.[88]

1.2.5 Combined Detection Methods

Due to limitations of individual detection methods, the combination of two or more techniques has been used for verification purposes, ensuring adequate specificity and sensitivity of results. In a study examining 244 stool samples from an outbreak of gastroenteritis, transmission electron microscopy (TEM), PCR, and ELISA formats were used for the detection of NV.[90] The results indicated that at least two of the methods should be used in order to increase the level of confidence in the diagnosis.

Combining methods has also been used to enhance the performance of individual assays. Immuno PCR (IPCR) was introduced in 1992[91] and is a method that can dramatically increase the sensitivity of immunoassays such as the commonly used ELISA. IPCR involves the use of an antibody-DNA conjugate to bind specifically to a target antigen. The antibody is bound to DNA that can then be amplified by PCR. The system is designed such that the presence of PCR product in a reaction means that the target antigen has been detected. One advantage of this technique over other types of PCR methods is that the sequence of DNA to be amplified can be entirely selected by the user.[92] An overview of IPCR applications, including pathogen protein detection assays, along with detection limits and sensitivity increases compared to ELISA results, is given by Niemeyer et al.[92]

A real-time IPCR assay to detect NV capsid proteins in food and fecal samples was developed by Tian and Mandrell.[93] They found that PCR inhibitors had a minimal impact on the antigen capture and were removed by wash steps. The real-time IPCR system was the first report to detect NV in contaminated foods without virus purification or concentration. Using a tri-antibody system, the results showed a greater than 1000 fold improvement in sensitivity in comparison to an ELISA assay alone.

1.2.6 Foodborne Pathogen Typing

Molecular typing can be used to determine variability within a population of closely related microorganisms and has been valuable in epidemiological investigations. It is especially important when distinguishing between multiple isolates of the same species. Frequently used methods for studying molecular genetics of bacterial pathogens include pulsed-field gel electrophoresis (PFGE), PCR, and PCR-RFLP (restriction fragment length polymorphism).[94] Schwartz and Cantor[95] developed PFGE for the separation of large DNA fragments on a 1.5% agarose gel. By alternating the direction of the electrical field across a gel in a perpendicular fashion and varying the pulse length of the different field orientations in a nonuniform fashion from 1 to 90 seconds, it was possible to separate fragments as large as 2000 kb. By changing the direction of the electric field across a gel over short time intervals, it was possible to separate much larger fragments of DNA than was originally possible with standard gel electrophoresis. Whole bacterial chromosomes may be cut by rare digestion enzymes, generating a moderate number of DNA fragments suitable for gel analysis, essentially creating a genetic fingerprint of banding patterns for comparison between strains of the same species.[94]

PFGE is a technique often used for typing of many bacterial foodborne pathogens and the technique has applicability in studying strain population variability. A typing scheme was created by Wong et al.[96] using over 500 strains of *Vibrio parahaemolyticus* collected from 15 countries and 115 PFGE patterns were identified. It was also found that the restriction enzyme *Sfi*I resulted in clearly separated bands, as opposed to the use of other restriction enzymes.

1.2.7 Microfabrication and Microfluidics

Advances in microfluidics along with development of integrated lab-on-a-chip or micro total analysis systems (μTAS) have generated platforms capable of small scale sample preparation, fluid transport, and biological detection.[97] Advantages of these microsystems over amplifications on larger scales are that reduced reagent volumes are required, and it may be possible to reduce the amount of time required for the reaction to take place.[98] Disadvantages of some microsystems include increased nonspecific binding and the reduction of signal intensity.[98]

Microchip PCR systems offer advantages of low power consumption as well as rapid heating and cooling. Belgrader et al.[99] developed the Advanced Nucleic Acid Analyzer using ten silicon reaction chambers, and detection limit ranges between 10^2 and 10^4 organisms/ml were achieved. Neuzil et al.[100] obtained heating and cooling rates in excess of 100°C/s using a 100 nl PCR volume with a silicon micromachined chip in a system was able to complete 40 cycles in 5 minutes and 40 seconds.

1.2.8 Other Molecular Detection Approaches

Manipulation of nanomaterial properties for targeting biomolecules has created the potential for new techniques that are competitive with ELISA and PCR methods.[101] The applications of nanostructures in biodiagnostics has been reviewed.[101]

Many silver and gold nanoparticle based methods have been used to detect DNA. A label-free platform using silver nanoparticles and smooth silver films was used for the detection of ssDNA by surface-enhanced Raman scattering (SERS).[102] In another system, gold nanoparticles were functionalized with thiolated oligonucleotides to detect DNA hybridization by transmission SPR spectroscopy.[103]

Aptamers are ss nucleic acids that can be generated by a process known as systematic evolution of ligands by exponential enrichment (SELEX), by using libraries of synthetic nucleic acids.[88] After folding into a particular conformation, the resulting nucleic acid ligands are capable of specifically binding to a wide range of targets including proteins, making these molecules a potential alternative to antibody based detection. Aptamers are beginning to emerge as molecules that can contend with antibodies in the fields of diagnostics and therapeutics.[104] Specific advantages of aptamers over antibodies include their ability to reform their structure following denaturation, an *in vitro* as opposed to animal or cell based selection process, and chemical synthesis of the selected sequence, making it possible to produce the selected ligand in a very repeatable fashion.[104,105]

Electrochemical nucleic acid detection techniques have emerged that are label-free and therefore do not require fluorescent dyes and optical components. A disposable electrode system has been used for sensing fM quantities of specific ssDNA sequences, and it was possible to verify hybridization specificity down to a single base pair mismatch by using melting curves.[106] Other electrochemical DNA systems have shown promising results in detecting DNA in blood serum[107] and PCR products amplified from the *gyrB* gene of *Salmonella typhimurium*.[108]

1.2.9 Assay Design and Data Analysis Software

Nucleic acid based detection techniques have grown at a staggering rate due to the availability of target sequence data, powerful methods for nucleic acid amplification, and the ability to easily design suitable nucleic acid sequences for a particular assay. The design of a sensitive, specific PCR assay includes many considerations, and some of the most important are selecting appropriate primers and target DNA sequences. Computer aided PCR assay design systems began appearing not long after the amplification technique was introduced. One such program provided the ability to evaluate DNA duplex stability, oligonucleotide specificity, and oligonucleotide self-complementarity.[109] Significant empirical optimization with poorly designed primers can be costly, time consuming, and may not yield adequate results. PCR assay design software can aid in finding primers that have minimal tendency to form secondary structures, closely match primer melting temperatures, find a suitable amplicon size, and predict its melting temperature, all in less time than it takes to select parameter constraints. Additionally, user defined criteria allow primer sets to be rated in terms of their ability to match desired characteristics. Many packages can be found online at no charge by using keywords phrases such as "PCR design software" and range in available features from displaying oligonucleotide secondary structure formation to design aides for multiplex real-time PCR assays.

1.3 DETECTION TARGETS

Just as important as the selection of a suitable method for detection is the selection of an appropriate target to detect. Targets for detection must be unique to the organism of interest. The ideal target would be a gene or a noncoding region with a unique sequence present only in the organism of interest. While unique genes exist, most detection systems take advantage of sequence variations in genes that are shared by many different organisms. Only by studying these organisms, comparing sequence data, and determining specificity have researchers elucidated targets suitable for detection systems in foods. While specificity is an issue, the target should not be so specific that it fails to detect most strains of a species. The detection sequence should be relatively stable within the species. Genes that undergo high rates of recombination, such as some surface antigens that change often to evade immune systems, are not desirable targets. The most common detection sequences are in genetic regions that share some common traits. These loci are common to most if not all the isolates of a species, and they have a high level of sequence conservation, but enough variability in sequence and/or length to distinguish them from similar loci in other genera and sometimes species within genera. Good candidates are genetic loci that are somewhat constrained in sequence because the gene products encode products of essential function but still display some amount of variability (e.g., ribosomal RNA or cytoskeletal proteins).

Technical considerations play a role in the choice of detection targets as well. Some high G+C regions may not have high PCR efficiency. This is something to keep in mind if universal primers are being used in a food with a large amount of natural microflora (such as produce or raw meat) when testing for a pathogen that may be present in low numbers. If the template for detection is present in very low numbers, it could be missed if PCR amplifies competing targets with higher efficiency. This is why targets should be tested in laboratory situations with food samples contaminated with pathogens. This allows for assessment not only of the target but the potential inhibition of PCR by components of the food matrix.

1.3.1 Viral Targets

The limited genetic information in viruses in relation to the rest of the organisms discussed in this book necessitates a separate discussion of viral detection targets. Viruses consist of genetic material within a proteinaceous capsid and sometimes a surrounding lipoglycoprotein envelope. They are completely dependent on host cells for the expression of their genetic material, their reproduction, and their assembly. Since they have neither organelle structures nor ribosomes,

protocols using those targets for detection are useless for viruses. They have very small genomes in comparison with other classes of foodborne pathogens, meaning that there is not a lot of variety in genes to choose for detection purposes. While some concepts of viral detection are shared by other pathogens, some detection targets are unique to viruses.

1.3.1.1 RNA Targets

Some of the viruses involved in foodborne outbreaks carry their genetic information as RNA, so reverse transcriptase-PCR is used for detection of them. Some RNA viruses contain a gene for an RNA dependent RNA polymerase, which is used for duplication of the viral genetic information. The mutation rate in RNA viruses is much higher than in DNA viruses because of the lack of proofreading ability in RNA dependent RNA polymerase.[110,111] This makes it difficult to find stable regions of the genome to use as sequence markers. The polymerase gene itself is one of the few regions of the RNA genome that is relatively conserved. It is an example of a gene that serves an essential function to the virus, so broad changes in sequence that may affect its function are not tolerated. It is used as a detection target for some RNA viruses including hepatitis A, Norwalk virus, and others.[112–115] DNA viruses lack such an accommodating gene to use for detection.

1.3.1.2 Viral Structural Genes

Capsid proteins are the viral components on display to the environment. They are the major antigenic determinants in viruses, and as such are unique to each virus. These sequence differences among structural proteins present in all viruses is probably the most exploited target for viral detection.[113] Capsid genes are used for detection of both DNA and RNA viruses. The V2 and V3 capsid genes in hepatitis A[116] have been used for detection of the virus in spiked food samples.[117,118] Similarly, primers to rotavirus conserved genome region 9, which contains genes for capsid structure have also been used as targets for detection.[118] Strain variability among the different strains of the same virus group results in divergence of capsid gene sequences, and in some viruses the capsid gene sequences are highly mutable. As a result some isolates are not detected by some capsid-directed primer targets. This has been reported for different varieties of Norwalk virus and others.[119] Therefore, capsid-designed primers work for detection as long as they target conserved regions in the capsid sequence.[120,121]

1.3.1.3 Other Viral Targets

Noncoding regions in viral genomes, as long as they are conserved and therefore usable between different isolates, have also been used as detection targets for several viruses.[122–124] This is the case with the 5′ noncoding region of HAV.[125]

In other cases unique genes, such as hemagglutinin in avian influenza virus,[126] are detection targets. Additionally, since viruses have small genomes multiple isolates of the same virus type can be sequenced to find unique regions shared among them. Whether or not they are coding regions, these unique sequences can then be used for detection targets for that virus. This procedure was used to find detection targets for Astroviruses.[127]

1.3.2 Nonviral Targets

1.3.2.1 Ribosomal RNA Genes

The most common target for molecular detection is the DNA encoding ribosomal RNA (rRNA). All organisms except for viruses contain these loci. These genetic loci are uniquely suited for diagnostic purposes because they have regions that are very highly conserved in sequence, as well as regions that are divergent. Depending on which regions of the rRNA are targeted they can give different levels of identification from kingdom through genus and species, as well as sometimes differentiating strains within a species.[128,129] These regions are also desirable for identification purposes because they share similar physical chromosomal structures (Figure 1.3). Ribosomal RNA in both prokaryotes and eukaryotes is synthesized as one precursor molecule which is then processed to make ribosomes. In prokaryotes the 16S, 23S, and 5S rRNAs are transcribed as one unit also containing a tRNA. In eukaryotes the 18S, 5.8S, and 28S rRNAs are also transcribed as a single unit. In both cases mature rRNAs are made by processing of the primary transcript. Because ribosomes perform an exact function in all living cells the sequence diversity among functional areas of rRNA is highly constrained, but some variation is tolerable. On the other hand, the nonfunctional regions of rRNA loci are under minimal selective pressure, and their sequences and lengths can vary greatly. These differences in rRNA sequence have been used to determine evolutionary relationships between organisms.[128] Another benefit of rRNA loci is that they are often present in multiple copies since many ribosomes are necessary for the functioning of growing cells. This means multiple copies of the template sequence for amplification.

Beyond the sequence variability in the nonfunctional regions of the rRNA, variable regions are contained within each of the rRNA subunits that provide targets for detection

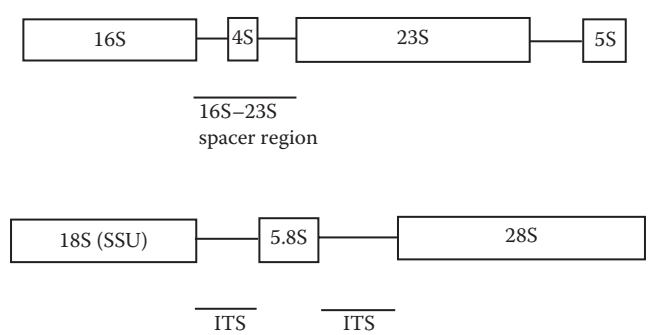

FIGURE 1.3 Basic physical map of ribosomal DNA loci in prokaryotes (top) and eukaryotes (bottom). ITS, internal transcribed spacer region, SSU, small subunit RNA.

of many foodborne pathogens. The 16S, 23S, 18S and 28S subunits have been utilized for detection in most cases. The 16S and 18S RNAs, sometimes referred to as the small subunit RNAs (SSU RNA) in protozoa, have more sequence diversity than the larger subunits (23S and 28S), and as a result the smaller RNAs are used more often for detection.[130,131] SSU RNA is a popular target among the protozoa and helminths.[132–134] The 16S rRNA sequences have defined bacterial phylogenetic relationships;[128] however, for differentiation between species in the same genus the 16S rRNA region is often not discriminating enough because of its low rate of mutation.

Even greater diversity in sequence can be obtained by using the spacer regions between the structural subunits. These regions get transcribed as part of the preribosomal RNA, but are cut away later. Since they are not functional RNAs, the spacer regions are not under selective pressure to retain their sequence, but closely related species share similarities in these regions. Because these spacer regions are bound on either side by conserved regions (Figure 1.3), universal primers exist that will bind to the conserved regions and allow their amplification. Specific probes are then used to detect pathogens. Among prokaryotes this spacer region is called the 16S–23S intergenic spacer region (ISR). In eukaryotes, the analogous region is called the internal transcribed spacer region (ITS). ITSs are present in high copy number, and display phylogenetic divergence such that they can show species differentiation.[135] The 16S–23S spacer region is widely used to probe for foodborne pathogenic bacteria,[131,136] and ITSs are used quite often for detection of fungi and for some helminths.[135,137–139]

While very useful, sometimes rRNA is not a preferred target. Since all prokaryotes and eukaryotes contain rRNA, primers will amplify regions from many different organisms. If a pathogen is present in low number among normal microflora in foods, then the pathogen target must compete with other templates present in a sample. If the target of interest has a lower PCR efficiency than others present, then the organism of interest may be missed. rRNA has met with mixed results in amplifications from *Giardia*, for example, because of a high G+C ratio in its 18S rRNA sequence.[140] Also, for differentiation of a pathogenic species from nonpathogenic relatives in the same genus, rRNA may not be discriminating enough.

1.3.2.2 Cytoskeleton Proteins

Similar to ribosomal DNA, gene sequences for cytoskeletal proteins have been conserved in eukaryotes. These proteins control vital functions such as growth and division of cells, motility, endocytosis, exocytosis, and maintenance of the cell structure. Because these genes arose early in eukaryotic evolution and their sequences have a slow rate of change, they are useful for phylogenetic comparison of species.[141,142] These same traits make them useful detection targets, especially if amplification of ITS regions is problematic. Actin and β-tubulin have been used as targets to distinguish between different species in genres of various fungi.[143] Giardin, a protein associated with the cytoskeleton in *Giardia* has been used as a detection target.[144,145] These proteins, as such, do not exist in prokaryotes, so cytoskeletal targets are limited to detection of eukaryotic pathogens.

1.3.2.3 Virulence and Toxin Genes

Among bacteria and fungi there are many cases where a genus consists of pathogenic and nonpathogenic species. Examples are *Listeria, Aeromonas, Aspergillus,* and *Penicillium*, to name a few. Often rRNA is not suitable to distinguish between the pathogen and their closely related nonpathogen in the same genus. One way to distinguish them is by assaying for virulence or toxin genes, which are unique to the pathogen genomes. In bacteria, virulence genes are often grouped together on the genome at discrete loci called pathogenicity islands. The altered G+C content of the DNA in many of the pathogenicity islands in relation to the rest of the genome, and repeated sequences on their edges hint that they arrived in these organisms by horizontal transfer.[146] Often the pathogens are genetically similar to their sister species except for the pathogenicity islands and other virulence genes. In order to ensure detection of the pathogen in the food sample, and not the innocuous species in the genus, it makes sense to screen directly for the virulence genes or toxins. Virulence gene sequences can be used as specific targets if the gene is unique enough, and the sequence does not vary much between different isolates of a pathogenic species. Hemolysins are a popular target, and they have been used for detection in foods of *Shigella, Vibrio, Listeria, Yersinia, Aeromonas,* and others.[55,147–154]

In addition to virulence genes which are involved in the infection process, some organisms produce toxins which are released from the cells. Sometimes these toxins are released as a part of the disease process; however, many organisms release toxins while growing in a food product. Food poisoning is actually caused by reactions to toxins present in food that are made by organisms that grew there. Toxin genes are usually of unique sequence, and so they are used as detection targets. Examples of using toxin genes as detection targets in foods are cereulide, the emetic toxin of *Bacillus cereus* in rice, botulinum toxin made by *Clostridium botulinum* in meat and canned corn, enterotoxin made by *Staphylococcus aureus* in dairy products, and an array of mycotoxins made by fungi such as *Alternaria, Aspergillus, Pennicillium,* and *Fusarium* in apples and grains.[137,155–162] Other organisms make toxins as part of the disease process once the organism has already grown in the individual, and these types of toxins are also used as detection targets in foods. Examples of these toxins are cytolethal distending toxin in *Campylobacter* sp. in poultry, and the shiga toxins in *Shigella* some *E. coli* strains in meat and dairy products.[163–165]

1.3.2.4 Unique Genes and Sequences

The best detection targets are genes that are absolutely unique to the organism of interest. Failing that, a gene that has unique sequences is desirable. In this section are several examples of unique gene sequences that are neither

rRNA nor related to virulence and toxicity, but have been found by studies of the physiology of the organisms in question.

In *Staphylococcus aureus*, the *nuc* gene is a thermostable nuclease.[166] While it is not unique to *S. aureus*, it has sequences in it that will distinguish it from other similar genes. Therefore, it has been used as a detection target for *S. aureus*.[167] The *per* gene, which encodes perosamine synthetase, has a sequence that is highly conserved among *Brucella* species, and primers were designed to take advantage of that specificity for detection.[168] A unique region in an open reading frame encoding part of the Type III secretion system was utilized as a target to differentiate *Burkholderia pseudomallei* strains from other bacteria as well as other *Burkholderia* spp.[169] The genus *Pseudomonas* encompasses a large number of species, some of which are very closely related, so rRNA can be problematic in distinguishing the pathogens from the nonpathogens. The *carA* gene which encodes carbamoyl phosphate synthase in *Pseudomonas* sp. was used to distinguish between different species in the genus in meats.[170] In order to differentiate between different strains of *E. coli* sequences in *gadA* and *gadB*, which encode glutamate decarboxylase, have been used in artificially contaminated wheat grain.[171,172] For the detection of *Salmonella* in poultry houses the *iroB* gene, which is absent in the closely related *E. coli*, was used.[173,174] The *cpn60* gene (also known as *groEL* or *hsp60*), which encodes a heat shock protein in bacteria, contains within it a fragment that has been useful for determining phylogenetic relationships among bacteria. A database of sequences exists to identify organisms found by using this gene as a detection target.[175]

The *rpsU- dnaG- rpoD* region is another locus has been used to differentiate between different bacteria. This region encodes proteins involved in the initiations of protein, DNA, and RNA synthesis, and is another example of a locus that has regions that are highly conserved and others that are variable. It has been found to vary between bacteria genera, but to be relatively conserved between species within a genus.[176,177] This region was used to distinguish the foodborne pathogen *Enterobacter sakazakii* from other *Enterobacter* sp. in infant formula.[178]

The *Toxoplasma* B1 gene, which is highly repetitive (35 copies) and highly conserved among various *Toxoplasma* spp. has been used as a detection target.[179,180] The cytoskeletal protein giardin is a major antigenic determinant that is unique *Giardia*.[181] It is also conserved between the different species of *Giardia*, and as such is a useful detection target. Among the helminths complete genome sequences are not available for many of the genera. However, sequences have been identified by researchers that are unique to some, and these have been used as detection probes. Organisms that have been detected in this fashion include *Clonorchis*, *Opisthorchis*, *Paragonimus*, *Taenia,* and *Fasciola*.[182–186]

1.3.2.5 Insertion Elements

In many cases rRNA gene is a fine target for differentiating between genera. However, it is sometimes not discriminating enough for the differentiation of species or subspecies. In the case of *Mycobacterium avium* subsp. *paratuberculosis*,[187,188] IS*900* used. This insertion sequence in the genomes of *M. avium* subsp. *paratuberculosis* strains allows discretion down to the subspecies level between different *M. avium* subspecies.[189] If simple detection of genus and species is required for the *Mycobacterium* genus then 16S rRNA is used.

Another insertion sequence, IS*711* is used as a species specific detection target for *Brucella abortus*.[190] Primers specific for IS*407A* have been used to differentiate *Burkholderia mallei* from *B. pseudomallei*.

1.3.2.6 Mitochondrial Genes

In cases where a whole genome sequence is not available for a eukaryotic organism, mitochondrial genome sequences often are available. These organelles, which are present in almost all eukaryotes, contain genomes on the order of 12–20 kb in length that can provide useful targets for detection. For the most part mitochondrial genomes contain the same complement of genes including those coding for proteins needed for oxidative phosphorylation, rRNA, tRNA, as well as noncoding spacer regions. The mitochondrial genome replicates on its own separate from the nucleus, and the coding regions differ at the rate at which they acquire mutations. The noncoding regions have the most variable sequences. Comparisons of mitochondrial genomes have been used to determine phylogenetic relationships between organisms.[191,192] Mitochondrial genes are used often for the molecular detection of helminths and some fungi in foods. The mitochondrial gene COX2, which encodes one of the subunits of cytochrome oxidaise, was used as a target for detection of *Saccharomyces cerevisiae*.[193] Cytochrome oxidase and NADH dehydrogenase genes from the mitochondrial DNA is also a popular target for the detection of helminths such as *Clonorchis, Opisthorchis, Fasciola,* and *Diphyllobothrium*.[194–196] Mitochondrial sequences can be used to differentiate between closely related species in a genus based on size differences in noncoding regions.[195]

1.3.2.7 Genes for Surface Expressed Markers

Similar to the genes that encode capsid proteins in viruses, genes that encode surface markers in other organisms have been used as detection targets. These surface markers are often antigenic determinants, meaning that their coding regions are sufficiently unique for use in detection. However, a problem with surface markers lies in strain variation. In organisms that change surface markers to evade the immune system, detection based on those markers is not useful. Yet bacterial capsule genes are sometimes used to detect *Streptococcus* species.[197] *Streptococcus suis* contains an extracellular protein factor encoded by the *epf* gene that is a specific marker for these strains.[198] Among Gram-negative bacteria genes encoding lipopolysaccharide markers were used for detection of *E. coli*, *Salmonella*, and *Vibrio*.[199] Flagellar genes in bacteria also fall into this category. Many of the flagellar components are expressed on the surface of the cell. They demonstrate unique signatures, so they are also antigenic. In

some instances flagella serve as virulence factors for bacteria when they are involved in attachment to animal host cells. Several bacterial detection target strategies utilize flagellar genes to detect and differentiate species of *Campylobacter,*[200] *E. coli*.[201] The *eap* gene in *Staphylococcus aureus* encodes a cell-surface associated protein that allows adherence of the bacterium to host cells. This gene was described as a potential detection target.[202] In *Brucella* the BCSP-31 gene encodes a cell surface antigen specific to *Brucella* spp. BCSP-31, and other outer membrane protein encoding genes have been used as *Brucella*-specific detection targets.[203,204] The gene for the oncosphere-specific protein *tso31* has been used as a detection target for the helminth *Taenia*.[205] The oocyst wall protein gene CpR1 from *Cryptosporidium* is also a target for food detection systems.[134,206,207]

1.3.3 Using Multiple Targets

The best way to screen for a particular pathogen in foods is to use multiple targets for detection. If a combination of rRNA, virulence, and other relevant target genes are used, then there can be a fair amount of confidence that most or all strains of any pathogen can be detected. The most efficient ways of detecting these multiple targets at the same time would be either with a multiplex procedure or with microarray analysis.[60,76,199] Panicker et al.[149] used both microarrays and multiplex PCR to detect *Vibrio* spp. in shellfish. Beyond multiple targets and methods, the chances of detection are enhanced with a pre-enrichment step before molecular analysis. This can take the form of a microbiological enrichment, a capture and concentration with immunomagnetic beads that bind to specific pathogens, or both. Just as multiple targets are used for detection of one pathogen, they are also used to detect several pathogens in one test. Screening for several potential pathogens with one protocol saves both time and money. *E. coli*, *L. monocytogenes*, and *Salmonella typhimurium* were each detected in contaminated wheat in one protocol utilizing a microbiological enrichment step followed by multiplex PCR using primers specific to all three bacteria.[171] Multiplex PCR was also used for simultaneous detections of *Salmonella* and *Vibrio* in shellfish,[62] and *Yersinia, Staphylococcus*, and *Shigella* in lettuce.[65] A microarray was used to detect the mycotoxin biosynthetic genes of *Fusarium, Penicillium,* and *Aspergillus*.[137] Wilson et al.[78] developed a microarray to detect eighteen prokaryotic, eukaryotic, and viral pathogens at once including *Brucella, Clostridium, Staphylococcus, Vibrio, Yersinia,* and *Fusarium*.

1.4 VALIDATION

Before widespread use of detection methods, they should be validated to make sure that they will detect most or all isolates of a species. False negatives can lead to disastrous consequences, and false positives can lead to costly, unnecessary recalls by manufacturers. A detection target is not very useful if a subset of strains of that organism do not possess it. The best methods have been validated by use in multiple laboratories to make sure they work with different sets of workers. This type of interlaboratory validation is routinely done. For example, 12 laboratories recently validated a *Campylobacter* detection protocol in spiked chicken carcass rinses,[208] and several labs were involved in detection of hepatitis A from spiked food samples.[117] Experiments within the food matrix are very important as they will not only assess the detection method within in the presence of potential inhibitors, but also determine how well the target organism competes with natural microbiota in the food. The competition aspect is important to test if an enrichment is involved prior to actual detection.

Targets should be screened to make sure they do not react with unwanted organisms. Potential primers and probes should be tested against multiple, closely related species for cross-reactivity. Also during these validations, sensitivity of the method can be determined with known levels of organisms added to foods.

1.5 CONCLUSIONS

Molecular biology has rapidly revolutionized food diagnostics, driven by biotechnology advances fueled by basic science.[209] Numerous molecular detection techniques have emerged that are rapid, sensitive, and specific in detecting nucleic acid sequences of foodborne pathogens. Several factors have led to an explosive growth in methods available for the detection and quantification of foodborne pathogens including the ready availability of synthetic oligonucleotide sequences of approximately 100 bp or less, the ability to modify these sequences at their 3′ or 5′ ends with fluorescence labels or conjugation chemistries, the development of extremely sensitive DNA amplification techniques such as PCR, software tools for molecular assay design and data evaluation, and DNA sequence databases to allow for the search for efficient target sequences. Rapid and simple sample preparation techniques, simplification of data analysis, standardization of molecular testing procedures, and identification of suitable detection targets will lead to the more widespread acceptance of molecular techniques. Additionally, cost effective solutions to pathogen detection and systems for on-site analysis that require minimal operator interface would benefit many industries. Microfabrication, microfluidics, and nanoparticle conjugation chemistries are likely to play significant roles in future systems. The extent to which foodborne pathogen detection solutions will converge upon widely accessible integrated instrumentation solutions that merge preparation, detection, and data interpretation capabilities in a seamless platform remains to be seen. However, molecular detection techniques will likely continue to simplify and increase the speed of detection procedures while simultaneously improving the sensitivity and specificity required for tracking pathogens in environmental, clinical, and food matrices.

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Section I

Foodborne Viruses

2 Adenoviruses

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2.1 INTRODUCTION

The potential for the transmission of adenoviruses by foods has only recently received attention and has been almost entirely focused on shellfish. Transmission of adenovirus eye and respiratory infections by recreational waters, however, has been well documented. Recent outbreaks have suggested that adenoviruses can also be transmitted by drinking water. Association of adenoviruses with water and food outbreaks is difficult because of the wide variety of illnesses that the viruses can cause, and of large number of asymptomatic cases. This is exacerbated by the fact that being a nonreportable disease, adenovirus infection is often associated with illnesses not considered foodborne (respiratory infections). Still occurrence of these viruses in food and water should be taken as an indication of their potential to be transmitted by these routes. The application of molecular methods has been key in our understanding of exposure by food and water.

2.1.1 Classification and Morphology

The human adenoviruses belong to the genus *Mastadenovirus* in the family Adenoviridae and consist of at least 51 serotypes. These serotypes are divided into six subgenera labeled A through F. Each serotype is distinguished by its resistance to neutralization by antisera to other known adenovirus serotypes.[1] Table 2.1 outlines the current classification scheme for human adenovirus serotypes.

Adenoviruses have a nonenveloped, icosahedral virion that consists of a core containing linear double-stranded DNA (26–45 kb) enclosed by a capsid.[2] The capsid is composed of 252 capsomers, 240 of which are hexons and 12 of which

are pentons. Each penton projects a single fiber that varies in length for each serotype, an exception being the pentons of the enteric adenoviruses (serotypes 40 and 41) that project two fibers.[1] Adenoviruses are approximately 70–100 nm in diameter.

2.1.2 Biology, Pathogenesis, and Medical Importance

Due to their physical, chemical, and structural properties, adenoviruses may survive extended periods of time outside host cells. They are stable in the presence of many physical and chemical agents, as well as adverse pH conditions. For example, adenoviruses are resistant to lipid solvents due to the lack of lipids within their structure.[4] Infectivity is optimal between pH 6.5 and 7.4; however, the viruses can withstand pH ranges between 5.0 and 9.0. Adenoviruses are heat resistant (particularly type 4) and may remain infectious after freezing.[5]

TABLE 2.1
Human Adenovirus Serotype Classification

Subegenera	Serotypes
A	12, 18, 31
B	3, 7, 11, 14, 16, 21, 34, 35, 50
C	1, 2, 5, 6
D	8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49, 51
E	4
F	40, 41

Source: Adapted from Shenk, T., Adenoviridae: The viruses and their replication. In *Fields Virology*, 4th edn. Knipe, D.M. et al. (Eds.), Lippincott Williams and Wilkins, Philadelphia, PA, 2001, and van Regenmortel, M.H.V. et al., *Virus Taxonomy: Seventh Report of the International Committee on Taxonomy of Viruses*, Academic Press, San Diego, CA, 2000.

Routes of infection include the mouth, nasopharynx and the ocular conjunctiva. Less frequently, the virus can become systemic and affect the bladder, liver, pancreas, myocardium or central nervous system.[6] Of the 51 currently recognized human serotypes (a serotype 52 has been proposed), only one-third are associated with a specific human disease (Table 2.2). Other infections remain largely asymptomatic.

Adenoviruses are associated with a variety of types of clinical illnesses involving almost every human organ system. Illnesses include upper (pharyngitis and tonsillitis) and lower (bronchitis, bronchiolitis, and pneumonia) respiratory illnesses, conjunctivitis, cystitis, and gastroenteritis. Several studies have found that the enteric adenoviruses are second only to rotaviruses as the causative agents of acute gastroenteritis in infants and young children.[7,8] Most illnesses caused by adenoviruses are acute and self-limiting. Although the symptomatic phase may be short, all adenoviruses can remain in the gastrointestinal tract and continue to be excreted for an extended period of time. Species within subgenera C may continue to be excreted for months or even years after disease symptoms have resolved. Adenoviruses can remain latent in the body (in tonsils, lymphocytes and adenoidal tissues) for years and be reactivated under certain conditions, such as a change in immune status. The long-term effect of such a latent infection is unknown.[5]

Adenovirus infections may be accompanied by diarrhea, though the virus can be excreted even if diarrhea is not present.[7] A large proportion of infections caused by subgenera A and D tend to be asymptomatic, whereas the species within subgenera B and E tend to result in a higher rate of symptomatic respiratory illnesses. Immunity is species-specific. The presence of pre-existing antibodies resulting from a previous infection is usually protective.

It is difficult to confidently link all adenoviruses to specific illnesses because many infections may be asymptomatic, healthy people can shed viruses.[5] Occurrence studies comparing infection in healthy and ill people have found

TABLE 2.2
Common Illnesses Associated with Human Adenoviruses

Disease	Individual at Risk	Serotypes
Acute febrile pharyngitis	Infants, young children	1–3, 5–7
Pharyngoconjunctival fever	School-aged children	3, 7, 14
Acute respiratory disease	Military recruits	3, 4, 7, 14, 16, 21
Pneumonia	Infants, young children, military recruits	1–3, 4, 6, 7, 14, 16
Epidemic keratoconjunctivitis	Any	8–11,13, 15, 17, 19, 20, 22–29, 37
Follicular conjunctivitis	Infants, young children	3, 7
Gastroenteritis/diarrhea	Infants, young children	18, 31, 40, 41
Urinary tract	Bone marrow, liver or kidney	34,35
Colon	transplant recipients, AIDS	42–49
Hepatitis	victims or immunosuppressed	1, 2, 5

Source: Adapted from Horwitz, M.S., Adenoviruses. In *Fields Virology*, 4th edn. Knipe, D.M. et al. (Eds.), Lippincott, Williams and Wilkins, Philadelphia, PA, 2001; and Enriquez, C.E., Adenoviruses. In *Encyclopedia of Environmental Microbiology*, Bitton, G. (Ed.), John Wiley and Sons, New York, 2002.

between 0 and 20% of asymptomatic people can shed adenovirus.

2.1.2.1 Gastroenteritis

Estimates of the incidence of adenovirus gastroenteritis in the world have ranged from 1.5 to 12%. Enteric adenoviruses are second only to rotaviruses as the leading causes of childhood gastroenteritis.[7,9] Diarrhea is usually associated with fever and can last for up to two weeks. Though diarrhea can occur during infection by any type of adenovirus, Ad40 and Ad41 of subgenus F specifically cause gastroenteritis and diarrhea. Adenovirus type 31 (Ad31) is also suspected of causing infantile gastroenteritis. Some estimate that Ad40/41 contribute from 5 to 20% of hospitalizations for diarrhea in developed countries.[10]

2.1.2.2 Respiratory Infections

Over 5% of respiratory illnesses in children younger than 5 years of age are due to adenovirus infections.[11] The initial transmission of adenoviruses is through the nasopharynx. Secondary transmission in households can be as high as 50% due to fecal-oral transmission from children shedding virus in the feces. Adenoviruses can be recovered from the throat or stool of an infected child for up to three weeks.[6] Adenovirus respiratory infections are also well documented in adults.

2.1.2.3 Pharyngoconjunctival Fever (PCF)

Pharyngoconjunctival fever (PCF) refers to a syndrome of pharyngitis, conjunctivitis, and spiking fever.[5] Symptoms of this syndrome include unilateral or bilateral conjunctivitis, mild throat tenderness, and fever. The illness usually lasts from 5 to 7 days, with no permanent eye damage.[4] The most commonly isolated adenovirus serotype is 3, although 7 and 14 have also been associated.[6] The disease is best known for centering on summer camps, pools, and small lakes.[12,13] Transmission of the agent appears to require direct contact with the water, allowing the virus direct contact with the eyes or upper respiratory tract. Secondary spread is common, although adults contracting the disease tend to have milder symptoms, usually only conjunctivitis.

2.1.2.4 Eye Infections

Epidemic keratoconjunctivitis (EKC) is a syndrome which causes inflammation of the conjunctiva and cornea. EKC was once referred to as "Shipyard Eye," as it was first described in shipyard workers.[6] EKC is considered highly contagious and begins with edema of the eyelids, pain, shedding tears, and photophobia. Serotypes 8, 11, 19, and 37 can cause EKC. Transmission occurs through direct contact with eye secretions from an infected person as well as through contact with contaminated surfaces, eye instruments, ophthalmic solutions, towels, or hands of medical personnel. Outbreaks have involved mostly adults.

Follicular conjunctivitis is often contracted by swimming in inadequately chlorinated swimming pools or in lakes during the summer.[6] Most cases result in only mild illness and complete recovery. Ad3 and Ad7 are the most commonly isolated species.[6]

2.1.2.5 Obesity

There is accumulating evidence that several viruses may be involved in obesity in animals and humans.[14] Studies in chickens, mice and nonhuman primates indicate that Ad36 can cause obesity.[15] Obese humans have a higher prevalence of serum antibodies to Ad36 than lean humans.[16] Other adenoviruses are capable of causing obesity in animals, but no correlation with antibodies has been demonstrated.[16] The metabolic and molecular mechanisms of how adenovirus infections cause obesity are not precisely understood; however, increases in food intake alone cannot explain the observed increases in adiposity (tendency to store fat), suggesting that Ad36 induces metabolic changes.[17] One mechanism appears to be that Ad36 influences the differentiation of preadipocyte.[17]

2.1.2.6 Morbidity and Mortality

Since adenovirus is not a reportable disease agent, there are no national or population-based morbidity and mortality figures available; most of the epidemiological data come from the study of select populations who appear to be most affected by adenovirus exposure. These include children in institutions such as hospitals and daycare centers, military recruits, immunocompromised individuals, and groups of families.

Enteric infection in children results in disease 50% of the time. This percentage is greater when the infection is centered in the respiratory tract.[5] Attack rates for waterborne outbreaks have been as high as 67% in children, with secondary attack rates (person-to-person transmission) of 19% for adults and 63% for children.[5]

2.1.2.7 Impact on the Immunocompromised

Although adenovirus infection may result in mild or asymptomatic infections in the immuno competent in the immunocompromised, the virus can disseminate into any body system and cause pneumonitis, meningoencephalitis, hepatitis (especially in liver and bone marrow transplant patients), and hemorrhagic cystitis (especially in kidney transplant patients).[5] According to Hierholzer[18] over 11% of transplant recipients become infected with adenoviruses, with an 18% case fatality rate. The enteric adenoviruses are rarely isolated from immunocompromised patients with gastroenteritis or diarrhea and are generally not associated with serious illness in the immunocompromised.

2.1.2.8 Water- and Foodborne Outbreaks

Although adenoviruses have been detected in shellfish no foodborne outbreaks have been documented to date. This may be a simple reflection that the virus is never considered a cause and thus no testing is perform to assess if adenoviruses could be involved. However, contact with recreational water has been associated with numerous adenovirus outbreaks over the years. Adenoviruses are the most reported cause of swimming pool outbreaks associated with viruses. Many outbreaks of PCF from nonenteric adenoviruses have come from people

swimming in pools and lakes. Ad3, Ad4, Ad7, and Ad14 have been associated with outbreaks in swimming pools[19,20] and the adenoviruses have been detected in pool waters after outbreaks.[12] It is clear that nondisinfected or inadequately disinfected recreational water is a source of adenovirus infection in swimmers. A routine monitoring of chlorinated swimming pools in South Africa demonstrated the presence of adenovirus by polymerase chain reaction (PCR) in 26 of 93 (15.4%) samples.[20] While the detection method did not assess virus viability, it did demonstrate the widespread occurrence of adenoviruses in swimming pools.

There have been three drinking water outbreaks reported in Europe in which enteric adenoviruses may have been a cause of gastroenteritis.[21–23] Multiple viral agents were involved and the water had not been adequately disinfected.

2.1.2.9 Occurrence in Water

Limited data has been available on the occurrences of adenoviruses in water. Only since the development of molecular methods for the direct detection of adenoviruses in water with confirmation tests performed in cell culture has data become available. Adenoviruses have been isolated from wastewater and river water, often more frequently and at higher concentrations than the enteroviruses.[24–27] Adenoviruses have also been detected in sewage, rivers, oceans, swimming pools.

Adenoviruses are commonly detected in raw and nondisinfected secondary sewage discharges, although little published data is available for the U.S. In Spain, monthly samples of raw sewage, effluent, river water, and seawater were tested using nested PCR amplification. Adenovirus was detected in 14 of 15 sewage, two of three effluent, 15 of 23 river water, and seven of nine seawater samples. Samples that were positive for enterovirus or hepatitis A were also positive for adenovirus, but there was no correlation between the fecal coliform level and adenovirus occurrence.[26] In Greece, 36 samples of sewage effluent were tested over a 15-month period using cell culture. Adenovirus was detected in all samples with concentrations ranging from 70 to 3200 cytopathic units (CPU)/l. In Australia,[24] raw sewage, primary effluent, and secondary effluent were sampled over a year using cell culture; 25 of 26 raw sewage, 23 of 26 primary effluent and 23 of 26 secondary effluent samples were positive for adenovirus.[28] The mean concentrations in sewage, primary effluent, and secondary effluent were 1950, 1350, and 250 infectious units (IU)/l, respectively. Enteroviruses were removed to a greater extent than adenoviruses by activated sludge treatment.[24]

Both respiratory and enteric adenoviruses have been isolated from surface waters worldwide. Nevertheless, survey data is limited in the U.S. An evaluation of 29 surface water samples in the U.S. yielded 38% positive for infectious Ad40 and Ad41.[29] The concentration of Ad40/41 ranged from 1.03 to 3.23 per 100 l. In this study, adenoviruses were more common in surface waters than enteroviruses and astroviruses. Likewise, when comparative studies have been conducted, adenoviruses usually outnumber enteroviruses in surface waters.

Infectious adenoviruses have been detected in conventionally treated and disinfected drinking water in Africa and Asia using genome detection with PCR in cell culture.[30,31] In both of these studies, adenoviruses were commonly detected in the raw, untreated surface water. In one study, adenoviruses were found in 4.4% of the finished drinking water samples that met the current acceptable bacteriological standards. In the other study, adenoviruses were detected at concentrations ranging from 0 to 0.9 most probable number (MPN)/100 l. In the van Heerden et al. study it was noted that none of the adenoviruses growing in cell culture produced cytopathic effects (CPE).

2.1.2.10 Occurrence in Shellfish

Interest in the occurrence of adenoviruses in shellfish largely stems from their potential as indicators of other enteric viruses and fecal pollution.[26] As with sewage and surface waters adenoviruses appear to be in greater numbers or at least isolated more commonly in shellfish than other enteric viruses, which may be a reflection of their greater stability in the environment.[32] In addition, being a DNA virus eliminates the added steps for detection enteric RNA viruses needed for reverse transcription-PCR (RT-PCR). Although cell culture has been used to a limited degree PCR has been the method of choice, because of the long incubation times required for production of cytopathogenic effects in cell culture required for adenoviruses.

Adenoviruses have been reported from every continent where shellfish are harvested. In Spain reported that of the mussels and oysters tested 47% contained adenoviruses, 19% enteroviruses, and 24% hepatitis A virus.[33] In a multilaboratory study of virus contamination of shellfish in Europe human adenoviruses detected by PCR where found to correlate with the presence of other human enteric viruses and suggested they could be useful as a molecular index of viral contamination of shellfish.[34] In a study in Korea adenoviruses was detected in 89% of the oysters collected from several locations.[35] In India adenoviruses were detected in 17% of the oysters and 27% of clam samples, but noroviruses and hepatitis A virus were detected.[36] However, enteroviruses were isolated with a greater frequency, 37% for oysters and 46% for clams.

2.1.2.11 Survival in the Environment and Shellfish

Limited data suggests that adenoviruses survive longer in water than enteroviruses and hepatitis A virus.[37] Adenoviruses also exhibit greater thermal stability than enteroviruses. This may explain their longer survival in water. They are capable of surviving for months in water, especially at low temperatures. The double-stranded DNA that comprises the genome of the virus may provide more stability in the environment. In addition, adenoviruses may use host cell repair enzymes to repair damaged DNA. This may also prolong their survival in the environment and enhance their resistance to inactivation by ultraviolet light.

Qualitative PCR analyses of adenovirus DNA in oysters and mussels demonstrated that Ad35 could be detected for 6–8 weeks.[38] In contrast the virus was detected in cell culture for 4–6 weeks.

2.1.3 Identification and Diagnosis

2.1.3.1 Culture-Based Techniques

Adenovirus subgenera A through E can be cultured in human cell lines, albeit slowly,[39,40] and thus may be overgrown by other faster growing viruses. They also require more than one passage in cell culture for expression of CPE. For this reason usually $TCID_{50}$ or MPN methods are used for their quantification in environmental samples[27] Guanidine can be added to cultures to selectively suppress enteroviruses while allowing adenoviruses to grow.[25] A variety of cell lines have been used to grow and/or detect adenovirus such as HeLa cells,[24,41] HEp-2 cells,[28,41] 293 cells,[42] Chang conjunctival cells,[43] CaCo-2 cells,[44] and PLC/PRF/5 cells.[43]

Hurst et al.[25] found that the number of infectious adenoviruses obtained by observing CPE in the 293 cell line was five-fold greater than the number detected via CPE in HEp-2 cells with sewage samples. Based on these findings and those of Takiff et al.[45] they suggested that HEp-2 cells might not be as appropriate for detecting Ad40 and Ad41 as 293 cells. The use of HEp-2 cells might miss the enteric adenoviruses that may constitute up to 80% of the adenoviruses found in raw sewage. This might explain the findings of Tani et al.[41] who, unlike other researchers, detected adenoviruses at much *lower* numbers than the enteroviruses in sewage, but relied on the use of the HeLa and HEp-2 cell lines.

Grabow et al.[43] determined that the PLC/PRF/5 liver cell line was more sensitive for detecting Ad41 and also exhibited CPE earlier than 293 cells and Chang conjunctival cells; however, while Ad40 may be grown using the PLC/PRF/5 cell line, CPE is not observed.[43] This cell line has been used to study the survival and recovery, respectively, of Ad40 and Ad41 in water.[19,37] also reported that the PLC/PRF/5 cell line was at least as sensitive as the HEp-2 cell line for isolating the lower-numbered serotypes (i.e., Ad1, Ad2, Ad3, Ad5, Ad6, and Ad7).

Although BGM is the most common cell line used for isolation of enteric viruses from environmental samples adenoviruses will not produce CPE in this cell line although they can be detected by ICC-PCR.[27,35]

2.1.3.2 Antibody-Based Methods

Antibody-based techniques have been developed for detecting and identifying adenoviruses in clinical samples, but have rarely been used with environmental samples. Both group-specific techniques (e.g., detecting all human or primate adenoviruses only) and species-specific techniques (e.g., detecting Ad40 or Ad41 only) have been developed. A group-specific indirect immunofluorescence technique has been used to observe nongrowing (do not replicate or produce CPE) adenoviruses obtained from stool samples in tissue cultures.[46] Only two studies have used antibody techniques for adenovirus detection in environmental samples. One used a group-specific immunofluorescence assay to detect adenoviruses in primary sludge from wastewater treatment plants.[47] The viruses were visualized in HEp-2 cell cultures in which primary sludge concentrate had been added. The second study compared cell culture, immunofluorescence, and *in situ* DNA hybridization methods for the detection of the enteric adenoviruses in raw sewage.[25] While one of the cell-culture methods (using HEp-2 cells) and the immunofluorescence method (using group-specific antibodies) yielded nearly equivalent results, the average levels detected using the *in situ* DNA hybridization technique were approximately 40% greater.

2.1.3.3 Nucleic Acid Probes

Gene probes have been developed to detect enteric adenoviruses in clinical and environmental samples, but have thus far seen limited use because they are not as sensitive or as easy to use as PCR methods. Genthe et al.[48] used Ad40 and Ad41 specific digoxigenin (DIG)-labeled DNA probes for enteric adenovirus detection in both raw and treated water. Nevertheless, the viability of the adenoviruses detected using this method was questionable since they were still detectable after exposure to 20 mg/l chlorine.

2.1.3.4 PCR-Based Techniques

The advent of PCR techniques has provided faster, more sensitive and more specific methods to detect adenoviruses in both clinical and environmental samples. These techniques do not demonstrate infectivity, however. Allard et al.[49,50] used PCR to detect adenoviruses in untreated domestic sewage via nested PCR. Puig et al.[39] compared cell culture, one-step PCR and nested PCR using sewage and river water samples. Nested PCR was found to be the most sensitive technique, allowing for the detection of < 10 particles. This is 100–1000 times more sensitive than traditional cell culture-based detection methods. Using similar techniques, Pina et al.[26] were able to detect human adenoviruses in sewage, river water, seawater, and shellfish. They suggested that the detection of human adenoviruses by PCR be used as an indicator of human viral contamination of the environment.

A nested multiplex PCR for detection of human enteric adenoviruses, hepatitis A virus and enteroviruses in sewage and shellfish was reported by Formiga-Cruz et al.[51] The limit of detection was approximately one genome copy for adenovirus and ten copies for enterovirus and hepatitis A virus per PCR reaction using cell cultured viruses. The lower detection of enteroviruses may reflect the addition steps to perform RT-PCR for the detection of the RNA viruses.

2.1.3.5 Integrated Techniques

A combination of cell culture and PCR has been used as a method to assess the viability of viruses and to increase the speed of identification (i.e., reduce the need for another passage in cell culture). In such methods, PCR is used to detect the presence of viruses growing in cell culture.[51] Chapron et al.[29] employed this method to detect Ad40 and Ad41 in surface water samples in BGM cells. The viruses did not produce CPE, yet could be detected by PCR. Ko et al.[52] developed an RT-PCR method for the detection of Ad2 and Ad41 mRNA in cell culture. Only infectious adenoviruses are detected using this method because only viable viruses are able to produce mRNA during replication in cell culture.

Choo and Kim[35] compared the detection of adenoviruses in oysters by ICC-PCR in BGM and human lung epithelial cells (A549) along with direct detection in the oyster samples by PCR. They found 23.6%, 50.9%, and 89.1% of all oysters positive by cell culture, ICC-PCR, and direct PCR, respectively. This suggests that not all of the adenoviruses in the oysters were viable. Rigotto et al.[53] also reported the greater sensitivity of nested PCR over IC-PCR. Nested PCR was capable of detecting 1.2 plaque forming units (PFU) of Ad5 per gram of tissue vs. 120 CFU/g by ICC-PCR.

2.2 METHODS

Virus detection in foods usually involves an extraction step followed by a concentration step. To date the only published methods for adenovirus detection in foods have been aimed at its isolation from shellfish. Shellfish extracts and concentrates can be toxic to cell cultures used in virus detection and may have more substances than other foods which may interfere with detection by molecular methods (PCR). No method for the extract/concentration or molecular detection is 100% efficient and should not be expected to be so because of variability in the individual genomes resistance to reagents used in extraction and processing. Another issue is that the volume of the extract may be large relative to the volumes used in the molecular assay limiting the sensitivity of the assay. All of these factors should be taken into consideration when reporting results.

2.2.1 Sample Preparation

Due to the low number of viruses found in most surface and ground water samples, viruses are first concentrated from volumes ranging from 10 to 1000 l. Methods commonly used are adsorption to positively or negatively charged microporous filters in a pleated cartridge format, adsorption to positively charged glass wool and ultrafiltration. Viruses are then desorbed or eluted in small volumes of liquid. Fields and Metcalf[54] first reported the concentration of adenoviruses using negatively charged filters. Enriquez and Gerba[19] demonstrated the use of positively and negatively charged filters for the concentration of Ad40 from tap, sea and waste waters. Jiang and Chu[55] used ultrafiltration to concentrate adenoviruses from surface waters and Van Heerden et al.[30] used positively charged glass wool to isolate adenoviruses from conventionally treated drinking water.

Methods have only been developed for the detection of adenoviruses from shellfish. This usually involves an extraction step from the shellfish, a concentration step and then preparation for PCR. These methods are identical to what is used for other enteric viruses. Methods for used isolating enteroviruses from other foods would likely be useful for adenoviruses.[56] The only consideration is that adenoviruses tend to be more sensitive to inactivation at pH levels above 9 than enteroviruses. After shucking usually several shellfish are pooled for processing. Sometimes certain organs such as the gills, digestive gland, or versa are extracted and then pooled. Since viruses may occur in the shellfish meat usually the shellfish are processed whole after removal from the shell. The shellfish meat is them homogenized in a blender or stomacher. This is followed by centrifugation to pellet the solids and virus. The supernatant is then discarded. Ultracentifugation can be used to ensure pelleting of the virus[26] or the pH lowered to 5.0 and conductivity adjusted to ensure adsorption of the virus to the tissue.[57] The virus is then eluted from the homogenized tissues by addition of glycine buffer at pH 9.5–10.0 or with glycine buffer at pH 7.5 and the salt concentration increased to 8000 mg/l and the solids discarded. The eluate is then concentrated further by ultracentrifugation, ultrafiltration, or acid precipitation at pH 4.5. The method reported by Pina et al.[26] involving glycine buffer elution and ultracentrifugation or minor modifications appears to be the most commonly used method at present for adenoviruses.[33,34,51]

The following methods described the processing of shellfish sample prior the analysis by PCR for the detection of adenovirus. This method consists of the use of an alkaline glycine solution (0.25 M pH 10.00) to promote the detachment of viruses from meat. Then the sample is clarified by centrifugation and the viruses are concentrated from the elution solution by polyethylene glycol (PEG) precipitation (8% PEG and 0.3 M of NaCl). The elution of the viruses from shell fish meet was described by Formiga-Cruz et al.[34] and Pina et al.[26] The conditions for PEG precipitation were described by Lewis and Metcalf.[58]

Materials:

(1) Blender
(2) 500 ml Erlenmeyer flask
(3) 0.25 M glycine solution: for 1 l distilled water, add 18.75 g of glycine and adjust the pH to 10.00 with 1 N NaOH
(4) 250 ml conical centrifuge bottle
(5) PEG 6000
(6) Sodium chloride
(7) Floor swing bucked refrigerated centrifuge
(8) Horizontal shaker
(9) 1 N HCl solution
(10) 1 N NaOH solution

Virus extraction procedure:

(1) Homogenize 33 g of shell fish meat with 167 ml of 0.25 M glycine solution in a blender at maximum speed for 30 sec
(2) Decant the homogenize solution in a 500 ml Erlenmeyer flask and stir for 15 min
(3) Adjust the pH of the solution to 7.3 with 1 N HCl solution
(4) Centrifuge at 2500×*g* for 30 min
(5) Keep the supernatant. Measure the volume of the solution

Concentration of virus by PEG precipitation:

(1) For 200 ml of eluted sample, add 16 g of polyethylene glycol, and 3.5 g of NaCl
(2) Decant the solution in a 250 ml conical bottle

(3) Put the bottle horizontally in the orbital shaker, shake at 100 revolutions/min overnight at 4°C
(4) Centrifuge at 4200 rpm for 1 h
(5) Add 5 ml of phosphate buffer, using the pipette, break the pellet and agitate the bottle with a voltex for 1 min
(6) Measure the volume on the reconstituted concentrated sample
(7) Store the sample at –20°C until further analysis

2.2.2 Detection Procedures

For the nucleic acids extraction, we describe a modification of the guanidine thiocianated extraction method described by Boom et al.[59] However, any commercial kit for the DNA extraction from plasma or stool sample would work. The described nested-PCR is specific for the hexon gene for all human adenovirus which includes the groups A through F. These set of primers were previously described by Avellon et al.[67] Nested PCR consists of two rounds of amplification: the first amplifies the target region from the viral genome and the second round amplifies a smaller region inside the product of the first round amplification. The PCR products are analyzed by agarose gel electrophoresis.

Materials:

(1) Guanidine lysis buffer: 120 g guanidine thiocyanate in 100 ml TE buffer, 11 ml of 5 M NaCl, 11 ml 3 M sodium acetate (NaOAc) pH 5.5, and 3.5 ml of poplyadenylic acid 5′ potassium salt (1 mg/ml)
(2) 100% proof ethanol
(3) 70% ethanol solution
(4) Sterile nuclease free water
(5) Silica spin minicolumns (high-bind RNA minicolumns, Promega Biotek, others manufacturers columns such as Qiagen will work)
(6) 2 ml collection tubes
(7) 1.5 ml microcentrifuge tubes
(8) 5 U/µl hot start taq polymerase (qiagen hotstart or Applied Biosystems gold Taq)
(9) 10×PCR buffer (provided with Taq polymerase)
(10) 25 mM $MgCl_2$ (provided with the Taq polymerase)
(11) 25 pmoles/µl stock primers
(12) 2.5 mM each dNTPs solution (10 mM total)
(13) Sterile nuclease free water
(14) Micropipets (0.5–10 µl, 10–100 µl, 100–1000 µl sizes)
(15) Barrier sterile tips
(16) 10% bleach solution (50 ml commercial bleach solution, 450 ml of water)
(17) First round PCR primers are:
ADHEX1F 5′-AACACCTAYGASTACATGAAC-3′
ADHEX2R 5′-KATGGGGTARAGCATGTT-3′
Fragment size of 473 bp
(18) Second round PCR primers are:
ADHEX2F 5′-CCCMTTYAACCACCACCG-3′
ADHEX1R 5′-ACATCCTTBCKGAAGTTCCA-3′
Fragment size of 168 bp

Nucleic acid extraction:

(1) Add 100 µl of lysis buffer to a 1.5 ml microcentrifuge tubes
(2) Add 100 µl of sample to the tubes and mix then with a voltex for 15 sec
(3) Incubate at room temperature for 10 min
(4) Remove the drops from the lip by a brief centrifugation
(5) Add 200 µl of 100% proof ethanol
(6) Mix with a voltex for 15 sec
(7) Load the 400 µl of sample mixture into the silica minicolumns
(8) Centrifuge for 1 min at 16000×*g*
(9) Place the column in a new collection tube
(10) Add 500 µl of 70% ethanol and centrifuge at max speed for 1 min
(11) Repeat the wash steps 9 and 10 one more time
(12) Place the column in a collection tube. Dry the column by centrifugation at 16000×*g* for 1 min
(13) Put the column in a sterile 1.5-ml tube
(14) Add 50 µl of sterile nuclease free water in the center of the column without touching the walls
(15) Let incubate for 1 min and then centrifuge 16000×*g* for 1 min
(16) Keep the flow through store at –20°C for future PCR analysis

Nested PCR procedure:

(1) Prepare first round PCR mixture (50 µl) consisting of 1×PCR buffer, 2.0 mM of $MgCl_2$, 200 µM of each dNTP, 25 pmoles of each primers (ADHEX1F and ADHEX2R) for the first PCR, 1.5 U of Taq polymerase, 2 µl extracted virus DNA, and nuclease free water for a final volume of 50 µl. Prepare a master mixture for multiple reactions and adjust by increasing the volume 5% (to consider the lost of mixture during handling). Specifically, the following calculation is for a master mixture for ten reactions:
   (i) In a sterile 1.5 ml Eppendolf tube add:
   52.5 µl of 10× PCR buffer
   42 µl of 25 mM $MgCl_2$ solution
   10.5 µl of Primer F (ADHEX1F)
   10.5 µl of Primer R (ADHEX2R)
   2.1 µl of Taq polymerase
   260 µl of water
   (ii) Aliquot 40 µl of PCR mixture into each PCR tube and add 10 µl of sample
(2) Conduct PCR amplification in a thermal cycler using the following program: one cycle of 94°C for 10 min; 35 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec; one cycle of 72°C for 10 min. Use the heated lip option. (Note: If this option is not available then add a drop of mineral oil to the reaction to avoid the vaporization.)
(3) Prepare second round PCR mixture (50 µl) consisting of 1× PCR buffer, 2.0 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM each primers (ADHEX2F

and ADHEX1R) for the second round of PCR, 1.5 U of Taq polymerase, 2 µl of first round PCR, and nuclease free water for a final volume of 50 µl. Specifically, the following calculation is for a master mixture for ten reactions:

(i) In a sterile 1.5 ml eppendolf tube add:
52.5 µl of 10× PCR buffer
42 µl of 25 mM $MgCl_2$ solution
10.5 µl of nested Primer F (ADHEX2F)
10.5 µl of nested Primer R (ADHEX1R)
2.1 µl of Taq polymerase
328 µl of sterile nuclease free water

(ii) Aliquot 48 µl of PCR mixture into each PCR tube and add 2 µl of first round PCR product. The cycling conditions of the second round PCR are the same used for first round PCR.

(4) Agarose gel electrophoresis

(i) Prepare a 2% agarose gel in 0.5×TBE in a 250-ml Erlenmeyer flask adding 2 g agarose and 100 ml 0.5×TBE buffer

(ii) Heat the gel in a microwave oven until the agarose dissolves. Add 5 µl of 10 mg/ml ethidium bromide stock solution (50 µg/ml working solution) after the agarose cools down (~45°C) and before pouring.

(iii) Pour the gel slowly, trying to reduce the formation of bubbles. Put the well comb in its correct position. Let the gel stand for at least 30 min.

(iv) Locate the gel wells in the negative side of the electrophoresis chamber. Add 0.5×TBE buffer until the lever reaches more than 2 mm above the gel.

(v) Mix 10 µl of the sample with 2 µl of 6×loading buffer.

(vi) Using a pipette with a fine tip, load the sample into the well. Load at step DNA ladder every six samples (amount recommended by manufacturer).

(vii) Check that the polarity is in the correct orientation (well in the negative side, running to positive side).

(viii) Applied as follows: 5 V per cm gel length.

(ix) Stop the electrophoresis when it reached 70% of the gel length. Use as reference the faster dye of the loading buffer.

(x) Visualize the PCR products using a UV transilluminator.

(xi) The PCR product size is estimated by comparing it with the step ladder and with the positive control. The 100 bp step ladder has step increases from 100 bp to 1000 bp. A positive sample for adenovirus should show a band between 100 bp and 200 bp with 168 bp. Also, a positive sample should have the same running distance as the positive control.

Comment on quality control:

(1) All the areas for the analysis should be separate in different rooms: one room exclusive for mixing PCR reagents, one room for handling the samples, one for the nested PCR, and another for gel electrophoresis.

(2) We recommend using a different PCR workstation/hood with UV lamp for preparing the master mix and another for the addition of the first round PCR product to the second round PCR mixture. Use a biological hood type 2 for handling the samples. Before and after uses, the hoods should be cleaned with 10% bleach solution and turn on the UV light for 30 min. The bleach can be inactivated with 2% sodium thiosulfate solution and washed with water.

(3) Open the reagents only inside the workstation, and the samples and PCR products are only opened in their respective workstation.

(4) Keep the equipment in each respective room and not used then in other areas (i.e., pipets, tips, and different lab coats are exclusively used in each room).

(5) The PCR product is only opened in the workstation for samples and in the electrophoresis room (negative pressure from the main laboratory).

2.3 CONCLUSIONS AND FUTURE PERSPECTIVES

Adenoviruses, like the enteroviruses, cause a wide range of illness many of which are not thought of as being food or waterborne. This, combined with the long time needed to produce cytopathogenic effects in cell culture previously resulted in few studies on their occurrence in the environment and role in food and waterborne diseases. While waterborne transmission is well documented, the role of food in their transmission is unknown. Certainly documenting their presence in food, especially shellfish suggests foodborne transmission is possible.

Human adenoviruses are more common in sewage contaminated waters and it has been suggested by several groups of investigators that they may be useful indicators of other enteric viruses in water and shellfish. Adenoviruses also have the added advantage of unlike most human enteric viruses, they have a DNA genome, eliminating the need for RT-PCR. However, recent research on the occurrence of adenoviruses in areas remote from human sewage contamination suggests that other sources besides humans or exceptionally long survival times in the environment.[61] Clearly additional research is needed to assess the usefulness of human adenoviruses as indicators of human viral contamination.

The application of PCR makes possible for the first time low cost and simple methods for the detection of adenoviruses in food and water. This will help us better understand the role of these vehicles in their transmission. Currently all molecular methods suffer from inability to determine viability without the use of cell culture, small assay volumes, quantification at low numbers of genome copies, interference with substances in concentrates, and the loss of virus during sample processing. These are major challenges that need to

be overcome to take full advance of molecular approaches for virus detection in foods.

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3 Astroviruses

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3.1 INTRODUCTION

Astroviruses are the members of *Astroviridae* family, which include both human and animal nonenveloped viruses possessing a plus-sense, ssRNA genome. In humans, astroviruses mainly produce gastroenteritis together with a broad spectrum of symptoms such as malaise, vomiting, diarrhea, fever and abdominal pain. Besides being one of the most common causes of viral gastroenteritis in young children in developed countries, astrovirus is the culprit for viral diarrhea in young children in some other parts of the world.[1] Although foodborne illness resulting from viral infections is a large and growing public health problem, most countries do not have good reporting systems; therefore realistic estimation of the true burden of foodborne diseases is difficult. Based on a recent study, astroviruses account for less than 1% of foodborne illnesses in the United States.[2]

3.1.1 History, Virion Structure and Classification

3.1.1.1 History

Astroviruses were first identified by Madeley and Cosgrove in 1975 in the feces of hospitalized infants with diarrhea.[3] Based on direct electron microscopy (EM) studies of fecal samples, astroviruses were observed as 28–30 nm particles in diameter with a distinctive five-six pointed star-like surface (Figure 3.1). This morphology distinguished astroviruses from other small, round viruses with similar size, such as picornaviruses and caliciviruses. In some preparations, bridging structures, which may be surface extensions of the virus, have been observed between adjacent astrovirus particles.[4] The term "astrovirus" was named after the star morphology (astron = star; Greek), although this surface structure can only be identified in approximately 10% of the particles by EM. Interestingly, particles isolated from infected rhesus

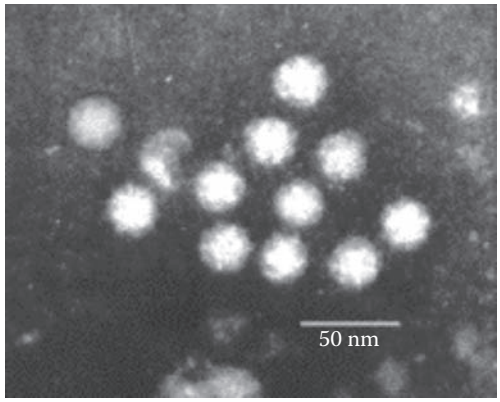

FIGURE 3.1 Image of human astroviruses by electron microscopy. http://www.virology.net/Big_Virology/BVRNAastro.html

monkey kidney epithelial (LLCMK2) cells lack the star-like morphology, but it can be induced by a brief exposure to pH = 10 environment. Later, based on high-resolution EM[5] and electron cryomicroscopy studies astroviruses seemed to be icosahedral particles with spikes or knob-like projections, and 41–43 nm in total diameter. Studies on purified cell culture-adopted HAstV-1 particles evaluated by cryo-electron microscopy and image analysis revealed a rippled solid capsid shell structure (330 Å in diameter) decorated with 30 dimeric spikes extending 50 Å from the virion surface.[6] These particles, however, have not been characterized by protein composition, which is a key factor for the virus-specific infectivity and may have an effect on virion structure.[7,8]

A few months before naming astroviruses, in 1975 Appleton and Higgins[9] reported an outbreak of mild diarrhea and vomiting among infants in a maternity ward. In their study, astroviruses particles were 29–30 nm in diameter, and did not display the special surface features, however by EM these viruses were distinct in size and morphology from the previously identified Norwalk viruses and rotaviruses. One year later specific immunologic reagents proved that these viruses were really astroviruses.[10]

Subsequently, viral particles, that were similar size and had the star-like surface features, were observed in gastroenteritis cases in several young mammals and birds, including mice,[11] kittens,[12] dogs,[13,14] lambs,[15] calves,[16] deer,[17] piglets,[18] minks[19] as well as turkeys.[20] Gough et al.[21] observed fatal hepatitis in ducklings due to astrovirus infection, and virus particles were found in liver of these animals in addition to feces. Astrovirus appears to cause species-specific infections.[22] In 1981, Lee and Kurtz[23] published the successful isolation of human astroviruses in the presence of trypsin in human embryonic kidney (HEK) cells and passage in LLCMK2 cells, which definitely distinguished them from noncultivatable small, round viruses, such as caliciviruses. In the late 1980s, Herrmann et al.[24,25] developed an enzyme immunoassay (EIA) that detects viral antigen. Although, the virus was well known since 1975, the real medical importance was only recognized in 1991.[26] Reverse-transcription polymerase chain reaction (RT-PCR) assays were first use in 1995 to detect human astroviruses. These techniques contributed to more detailed characterization of astrovirus strains by the analysis of nucleotide sequence information.[27–29] In the past few years spreading of RT-PCR technique could be observed in studies of the prevalence of astrovirus among children with diarrhea to detect and genotype strains.

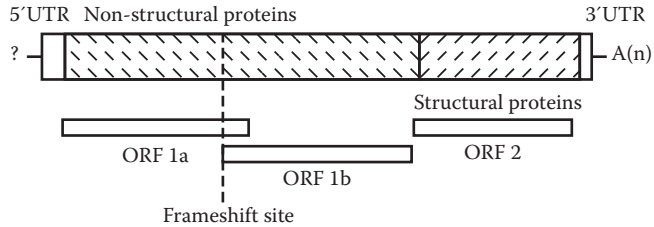

FIGURE 3.2 Genome arrangement of astroviruses. www.tulane.edu/~dmsander/WWW/335/Diarrhoea1.gif

3.1.1.2 Genome Structure

Astroviruses have a plus-sense, single-stranded RNA genome, which is approximately 6800 nucleotides (nt) (varies from 6.4 to 7.3 kb) in length. It is polyadenylated at the 3′ end, and surrounded by an isocahedral capsid. The genomial RNA includes 5′ and 3′ nontranslated region (UTR), and three open reading frames (ORFs), each encoding polyprotein that is proteolytically processed to yield smaller proteins (Figure 3.2). The two ORF located toward the 5′ end of the genome, designated ORF 1a (~2700 nt) and ORF 1b (~1550 nt), encode nonstructural proteins, such as an RNA-dependent RNA polymerase and a 3C-like serine protease that are involved in RNA transcription and replication. ORF 1a also encodes overlapping immunogenic epitopes that are recognized by antibodies produced to intact astroviruses.[30] An overlap of 60–70 nt is found between ORF 1a and ORF 1b of mammalian astroviruses (this region is only 12–45 nt long in avian viruses). This overlapping region contains signals which are essential for translation of the viral RNA polymerase through a frameshift mechanism.[31,32] This region is completely conserved among human and animal astroviruses in two characteristics: a heptameric AAAAAAC sequence, and the potential to form a downstream stem-loop structure. These features are critical for the ribosomal-1 frameshifting event during the translation of the genome.[31,32] The third ORF is located at the 3′ one-third of the genome, designated ORF 2, which has the greatest sequence variability in the astrovirus genome, and encodes the 90 kDa protein that is the precursor of the three capsid proteins that have been described for human astroviruses.[33,34] Structural proteins encoded by ORF 2 are translated from the so-called subgenomic (sg) RNA.[33,34] The more conserved amino-terminal region of the astrovirus capsid protein has an important function in assembly of the capsid core, while the hypervariable carboxy-terminal form the spikes of the virion and participate in the early interactions between the virus and the host cells.[35] ORF 1b and ORF 2 overlap in eight nt. The 3′ UTR of HAstV genome, which is located between ORF 2 and the polyA tail is 80–85 nt long (this sequence can be longer [130–305 nt] in avian viruses).[36]

The final 19 nt of ORF 2 and the 3′ UTR are thought to be important for interacting with the viral RNA replicase and cellular proteins. These regions are highly conserved among all known HAstV serotypes.[37]

During infection of susceptible cells, two RNA species have been observed: the full-length genomic RNA (gRNA), and an sgRNA (~2.4 kb in length).[38,39] Both RNA species are initially observed at 12 hours postinfection in LLCMK2 cells.[39] Synthesis of the negative-sense RNA of astrovirus has not been well studied. By all means, the gRNA is probably a template to synthesize the full-length negative-sense RNA, which is a template to produce both the full-length gRNA and the sgRNA. The synthesis of sgRNA probably requires an internal sequence in the full-length negative sense RNA to serve as promoter for the virus transcriptase. However, the identity of this promoter in astrovirus has not been defined; it is thought that about 120 nt of the ORF 2 region might be an important sequence of the promoter.[40] Part of this region includes the sequence AUUUGGAGNGGNACCNAAN$_{5\text{-}8}$ AUGNC (the ORF 2 start codon is underlined; N can be any of the four nucleotides), which is highly conserved among all members of *Astroviridae*.[41]

After astrovirus entry, the gRNA is used as a template to synthesize the virus nonstructural proteins. The primary protein product coded by ORF 2 is observed abundantly 12 hours after infection[39] (nonstructural proteins are initially detected 6 hours postinfection[42]).

3.1.1.3 Classification

Genome arrangement of astroviruses is similar to the genome of *Picornaviridae* and *Caliciviridae,*[43] but the size, number, and processing of polyproteins, the lack of an RNA-helicase domain in astroviruses, and the use of a ribosomal frameshifting mechanism distinguish them from similar viruses. Therefore astroviruses are classified into a separate family, the *Astroviridae*.[44]

Astroviruses have been isolated from both humans and several animal species. According to the origin of the virus and the genome structure, two genera have been distinguished within the family: *mamastrovirus* (infect mammals) and *avastrovirus* (including viruses from avian species). Viruses in the genus mamastrovirus are more closely related to each other than those viruses within the avastrovirus genus.[36,45] Serologic relatedness between viruses isolated from different species, even within the same genus has not been identified.[46,47]

Human astroviruses have been grouped into eight serotypes (HAstV-1 to HAstV-8) according to immunfluorescence and neutralization assays, as well as immunelectron microscopy that use hyperimmune sera to raise different culture adapted strains.[23,48–52] Common epitopes of the capsid protein in cases of all known HAstV serotypes were identified. These epitopes are widely used in many different diagnostic assays.[24,25] Recently, more sensitive molecular methods are used, that enabled the classification of human astroviruses on the basis of the sequence similarity of specific genome regions. Nowadays, different regions of the genome, obtained by RT-PCR, have been widely used to group HAstVs into different genotypes; though depending on the specific region of the analyzed genome (ORF 1a, 1b or ORF 2), strains can be grouped differently.[53]

Several animal serotypes of astroviruses have been identified. At least two, but probably three serotypes are found among bovine astroviruses (BAstVs) based on the crossreactivity to specific sera. Two serotypes have been identified among turkey (TAstVs)[36] and chicken astroviruses (CAstVs).[46]

Many serotypes of astroviruses have been identified in consequence of different serological studies, suggesting that additional types of astrovirus might exist.[47,54]

3.1.2 Pathogenesis and Pathology

3.1.2.1 Physical Features

Astroviruses are one of the most resistant viruses. They show resistance against different physical and chemical agents, such as chloroform, a variety of nonionic, anionic as well as zwitterionic detergents, even lipid solvents; they are able to maintain their infectivity at 60°C for 5 min (HAstV)[5,22] or 10 min (TAstV), and at ultralow temperature (–70 to –85°) for 6–10 years, but repeated freezing and thawing is detrimental[55] to them, particles are resistant to treatment from pH 3 to 10.[5,22]

The extreme stability of astroviruses against environmental factors suggests that traditional pasteurization procedures cannot completely inactivate them. Furthermore, astroviruses are able to persist under severe environmental conditions, they endure on inanimate surfaces, on human hands, in dried human and animal fecal materials, in water, on kitchen surfaces, food preparation areas, hospital as well as cruise ship cafeterias, on carpets and hospital lockers.[56]

There is a lack of information on the survival of astrovirus on foods. Information is also lacking on the efficiency of current washing and decontamination procedures for the removal of astrovirus. It was already demonstrated that traditional disinfection procedures do not eliminate astroviruses by water treatment.

3.1.2.2 Propagation of Human Astroviruses

Serial passage of astrovirus in HEK cells requires incorporation of 10 μg/ml trypsin in the serum-free growth media.[23] Higher levels of typsin do not improve the viral yield, while lower levels (for example the 0.5 μg/ml necessary for rotavirus growth) are not sufficient to maintain astrovirus in cell culture. After several passages of HEK-293 cells, viruses were able to grow in primary baboon kidney (PBK) cells and in a continuous cell line of LLCMK2 cells. Attempts to establish serial passage by direct inoculation of PBK or LLCMK2 cells with fecally derived astrovirus were unsuccessful. Willcocks et al.[57] could propagate the virus in a continuous cell line of human colon adenocarcinoma (CaCo-2) cells using 5 μg/ml trypsin in the culture medium. With this method, the first cytopathic effect (CPE) appeared after 2 days of infection. Nowadays, this cell line is commonly used to grow wild-type astrovirus strains.

However, adenocarcinoma cell lines (CaCo-2, T-84, HT-29, SK-CO-1 cells) are the most efficient cells to isolate HAstV, the virus are also able to grow in human liver hepatoma cells (PLC/PRF/5) and in monkey kidney-derived (MA104, Cos-1, vero) cells as well.[52,58–60] CaCo-2, T-84 and PLC/PRF/5 cell lines are the most efficient to directly isolate HAstV strains from fecal suspensions.[52,58–60]

Adaptation of field HAstV strains to grow in cell culture has low efficiency because different astrovirus strains have different susceptibility to trypsin. Although, 10 μg/ml of trypsin has been regularly used to activate virus infectivity; recent isolation of HAstV type-8 in CaCo-2 cells required much higher (200–400 μg/ml) trypsin concentration.[8] In consequence, the optimal concentration of trypsin to obtain completely activated virus can depend on the HAstV strain.

3.1.2.3 Propagation of Animal Astroviruses

To date bovine,[61] porcine[62] and avian astroviruses have been propagated in cell culture. Bovine astrovirus was isolated by Aroonprasert et al. in primary neonatal bovine kidney (NBK) cells in the presence of 50 μg/ml trypsin. This amount of trypsin in the culture media was necessary both for direct isolation from fecal samples and for serial passage of the virus in NBK cells. Primary bovine embryo kidney (BEK) cells were also efficient in growing bovine astroviruses using feces and cell culture adopted viruses as the starting material.[61]

A porcine astrovirus was isolated in embryonic swine kidney cells using 50 μg/ml trypsin in the culture medium. Fecal samples from infected pigs were filtrated and used directly for infection. However, maximal CPE was evident 4–5 days after inoculation of cell monolayers, the CPE was not formed if trypsin was removed from the medium or if the virus inoculum was mixed with convalescent phase serum.[62]

Chicken astroviruses were successfully adapted to both chicken embryo liver (CEL) and chicken hepatocellular carcinoma (LMH) cells. Tracheal swabs, lymphocytes, and intestine homogenates from infected chickens were particularly used as a source of the virus. Duck astroviruses were also adapted to these cells (CEL and LMH) without the addition of trypsin to the medium.[46] Adaptation of turkey astrovirus to avian (as turkey and chicken embryo-derived cells) and mammalian (e.g. CaCo-2, vero) cell lines was unsuccessful, although they could be replicated in turkey embrionated eggs.[63–65]

Astroviruses from other vertebrates, like lambs,[15] red deer,[17] cats[26] and dogs[13,14] have also been isolated in cell cultures, but serial passage of the virus has not been performed.

3.1.2.4 Transmission

Astrovirus can be transmitted through the fecal-oral route, by person-to-person contact, from fecally contaminated fingers to foods or to work surfaces and door handles. There is a significant risk of contamination from field workers who do not have adequate on-site toilet and hand-washing facilities.[56] Astroviruses can be disseminated over a wide area in aerosol droplets (produced by vomiting), which is a particular hazard for exposed food or surfaces with subsequent transfer to foods.

Astroviruses can be transferred with contaminated food and water from different origins.[52,66–70] Sequence analysis of HAstV strains detected from both clinical samples and water supplies verified that water could be an important source for HAstV contamination, because virus strains from both origins were identical, at least in the specific genome region analyzed.[69]

It was shown, that poliovirus can infiltrate into the roots and body of plants from the soil,[71] and consequently it is probable, that astroviruses also have the same feature.

3.1.2.5 Pathogenesis

Pathogenesis of human astrovirus infections has been extensively studied. Recent histopathologic examinations show that astrovirus infects the mature epithelial cells of the small intestine, especially in the jejunum and in the duodenum.[72] Severe diarrhea caused by villus atrophy in the intestine suggests that the inflammatory response does not play an important role in the pathogenesis of astrovirus.[72]

Other mammalian astroviruses can infect epithelial cells (OAstV, BAstV), subepithelial macrophages (OAstV), as well as M cells (BAstV) of the small intestine.[73,74] OAstV particles were also observed in vacuoles of the enterocytes.[73] OAstV infection was characterized by transient villus atrophy and crypt hypertrophy, which resulted in severe diarrhea after 2–4 days of infection. BAstV was unable to induce diarrhea in gnotobiotic animals, nevertheless, inflammatory mononuclear cells above the dome villi were observed on infection with this virus.[74] In addition, the lamina propria was infiltrated with neutrophils and cells with degenerate nuclei were present. Lymphoid cell depletion was noted in the central region of germinal centers beneath the infected dome villi. In the case of turkey astrovirus 2 (TAstV-2) infection, mild crypt hyperplasia was observed after 1 day of infection in the proximal jejunum, while after 3–5 days of infection the same manifestation was observed in the distal jejunum and ileum, as well as in the duodenum.[75] Electron microscopy studies revealed intracytoplasmic astrovirus aggregates in enterocytes on the sides and base of villi in the ileum and distal jejunum on day 3 postinfection.

Astrovirus infection do not cause inflammation in humans[72] and turkeys,[65] but induce apoptosis in cultured cells,[76] which suggest that this form of programmed cell death, could contribute to diarrhea in some species. Several other mechanisms could also contribute to the gastroenteritis due to astroviruses.

3.1.3 Clinical Features

3.1.3.1 Characteristics of Human Illness

HAstV are the causative agents of viral gastroenteritis worldwide mainly in children (under the age of 5). Within the four childhood gastroenteritis virus (rotavirus, enteric adenovirus, astrovirus and calicivirus) HAstV is the second most common viral agent that causes diarrhea in young children evaluated in outpatient settings.[26] Astrovirus infections have also been recognized in elderly, institutionalized patients[77,78] and

immunocompromised individuals. HAstV type 1 has been detected as the predominant strain in most countries.[22,51]

However, the incubation period in most HAstV infections is 3–4 days; a shorter incubation time of 24–36 hours was also documented during an outbreak of gastroenteritis in a Japanese kindergarten.[79–81] Generally, human astroviruses induce a mild, watery diarrhea that typically lasts for 2–3 days, associated primarily with vomiting, fever, anorexia, abdominal pain and a variety of constitutional symptoms lasting no more than 4 days.[22,82] Dehydration also can occur in patients with underlying gastrointestinal disease, poor nutritional status, or mixed infections[83] (Table 3.1). Prolonged lactose intolerance and sensitivity to cow's milk have been described.[84,85] Persistent gastroenteritis due to astrovirus has been associated with serotype 3.[86] Deaths related to astrovirus infection are extremely rare, although have been reported.[87] Severe intussusception caused by HAstV infection was also documented in a child hospitalized with gastroenteritiss.[88]

In an Argentinean outpatient study with children under 36 months of age, astrovirus was associated with 12.4% of the diarrhea episodes; fever was present in 41.6%, and 16.7% of the patients required hospitalization.[89] In Egypt among children under the age of 3 years, the total incidence of diarrhea due to astrovirus was equal to rotavirus; and severe dehydration arose out of 17% of astrovirus infected patients.[90]

Astroviruses cause infection at relatively low doses in humans, and appear in food which are usually obtainable in easy-to-use form, and therefore are not subjected to cooking conditions that kill them. Infected food workers may shed virus for longer periods of time, and for that reason may remain infectious even after full recovery.[92]

In children it may be difficult to distinguish diarrhea caused by astrovirus from that caused by rotavirus on clinical grounds alone.[22,26] However, in general astrovirus diarrhea is less severe when compared to symptomatic rotavirus infection, as it does not cause significant dehydration and patients are less likely to require hospitalization.[26,45,85]

3.1.3.2 Role in Immunocompromised Hosts

HAstVs cause chronic diarrhea among immunosuppressed patients in all age groups. HAstVs cause infection more frequently in patients with several immune diseases, such as chronic lymphocytic leukemia, congenital T-cell immunodeficiency, human immunodeficiency, combined immunodeficiency, Waldenstrom's macroglobulinaemia and immunodeficiency polyendocrinopathy.[93–96] Depletion of $CD4^+$ T-cells by disease or iatrogenic means (for example chemotherapy) develops prolonged astrovirus diarrhea.[93,97,98]

Among HIV-infected patients, several viruses (for example astroviruses, adenoviruses, picobirnaviruses) were found more often in the stools of those with diarrhea (n = 65) than those without diarrhea (n = 65).[95] HAstVs have been associated with outbreaks in bone marrow transplant patients.[99] Chronic astrovirus diarrhea has been published in a child, who received a bone marrow transplant for combined immunodeficiency.[22] The infection persisted until the child's death, but no antibodies to astroviruses were detected in the serum.

TABLE 3.1
Clinical Symptoms Associated with Human Astrovirus Infection

Diarrhea	Incidence	72–100%
	Duration	2–3 days (average)
	Maximum number of stools	4/24 hours
	Incidence of bloody diarrhea	0%
Abdominal pain	Incidence	50%
Vomiting	Incidence	20–70%
	Duration	1 day (average)
	Maximum number of vomiting	1/24 hours
Fever	Incidence	20–25%
	Maximum	37.9°C
Dehydration	Incidence to a degree	24–30%
	Incidence of severe dehydration	0–5%
Hospitalization	Incidence	6%
	Duration	6 days (average)
Bronchiolitis	Incidence	33%
Otitis	Incidence	13%
Severity score (1–20)*		5 (average)
Admission diagnosis of gastroenteritis		18.7–48%

Source: Adopted from Walter, J.E., Mitchell, D.K., *Curr. Opin. Infect. Dis.*, 16, 247, 2003.

*20 points scoring system according to Ruuska and Vesikari.[91]

3.1.3.3 Immunity

At present the determining factors of immunity to astrovirus are not well understood. Astroviruses primarily infect two age groups (young children and the elderly) and institutionalized patients. The age distribution of symptomatic infection suggests that antibodies to astrovirus acquired in childhood provide a certain protection from illness through adult life and that immunity decreases late in life. Studies revealed that in volunteers with detectable serum astrovirus antibody, diarrhea did not manifest clinically after virus challenge.[79] Indirect evidence suggests that astrovirus-specific antibodies play a role in limiting infection in the host.

Gamma globulin pools in the USA and Japan contain antibodies to human astroviruses, suggesting that astrovirus infection is common.[81,100] Studies in the UK have shown that antibodies to astrovirus are acquired in early childhood; 70% have antibody by school age, 75% by 10 years of age, and 77% by early adulthood.[101]

The normal mucosal immune system is important in the protection of individuals from repeated human astrovirus infections.[102] $CD4^+$ T-cells that recognize human astrovirus antigens in a human leukocyte antigen (HLA)-restricted manner have been found in the lamina propria of intestinal tissue of healthy adults.[102] Upon activation, these human astrovirus $CD4^+$ T-cells may play a role in preventing repeated astrovirus infections by production of helper T-cell subtype 1-type cytokines, interferon gamma and tumor necrosis factor, providing a defense barrier at the portal of entry.

The role of the humoral immune response in animals to restrict astrovirus infection is not clear. It was demonstrated, that virus replication in small turkeys infected with TAstV was limited, however the infection did not induce significant adaptive immune response. No protection was observed against TAstV on secondary challenge; and the restricted virus replication was attributed to an inherent response, cured by production of nitric oxide.[103]

3.1.4 Epidemiology

Human astrovirus infections have been detected worldwide, principally in young children suffering from diarrhea.[9,77,95,104,105] Human astroviruses cause disease in (i) infants and young children, (ii) elderly institutionalized patients, (iii) immunocompromised hosts, and (iv) otherwise healthy individuals that come into contact with astrovirus-contaminated food or water. Studies in Australia,[106] Thailand[26] and Guatemala[83] have revealed HAstV as the second most common cause of gastroenteritis in children, after rotavirus, with incidences varying from 4.2% to 8.6%.

Large outbreaks caused by astroviruses through contaminated food, which affect thousands of persons in Japan, have been reported among otherwise normal school-age children and adults.[107,108] Age distribution of HAstV can vary. In a Spanish study, 80% of children under 3 years of age were infected with astroviruses.[109] In Egypt age-specific HAstV and rotavirus incidences were similar (0.38 for infants under 6 months, 0.40 for infants between 6 and 11 months, and 0.16 for children 12–23 months).[90] Astrovirus infections occur primarily in the winter months in temperate regions and in the rainy seasons in more tropical climates.[45,83]

HAstV type 1 has been detected as the predominant strain in most countries,[48,69,106,110] and the circulation of other types in a given period is probably less frequent throughout the year in any single geographic area, but the most common serotype can vary with time and location. Hence, in the UK 72% of the community-acquired astroviruses, detected between 1975 and 1987 were serotype 1,[22] while in Australia serotypes 1, 3, and 4 were most frequently found in an 18-year period.[111] In Mexico, astrovirus type 2 was the predominant strain (35%).[112] Although in a study in 2004, where samples were analyzed from different regions of Mexico, types 1, 2, 3, 4, 6, 7, and 8 were found, and in a region, frequency of serotype 8 and type 1 was the same.[113] Both community-acquired[26,85] and nosocomial[9,84] infections have been described.

3.1.4.1 Role in Disease

Until recently it was complicated to determine the real incidence of astrovirus infection and its role in disease, partly due to the frequency of coinfections with other pathogens.[45] Development of more sensitive detection methods has clarified their role in gastroenteritis in different populations.

Astroviruses frequently cause outbreaks of diarrhea in child care centers, and children less than 36 months of age attending child care settings are at the greatest risk of developing diarrhea.[114] In several parts of the world astroviruses are known to be the main cause of viral diarrhea in young children.[1]

Astroviruses were identified as a foodborne pathogen only within the last 30 years, though they have probably been causing foodborne infections for centuries. Astroviruses are estimated to participate in less than 1% of all foodborne illness, similar to rotaviruses,[2] which mean 39000 cases per year out of 3.9 million total cases due to viruses.[2] Although, weaknesses and variations in foodborne disease surveillance systems (where such programmes exist at all), make the global estimation of foodborne diseases difficult. Symptoms of astroviral foodborne infection range from mild gastroenteritis to severe life-threatening syndromes. The seriousness of foodborne illness caused by astroviruses is dependant on the number of infective virus particles, moreover the age, genetic background, and general immune, health and nutrition status of the infected person, as well as the efficiency of sanitation systems. Thus foodborne diseases most seriously affect children, pregnant women, the elderly, and people already suffering from other diseases causing severe illness.

Any food that has been handled manually and not at all or insufficiently heated is a possible source of astrovirus infection.[115] Contamination of fresh produce by the astrovirus-containing fecal material frequently causes foodborne illnesses. The source of this contamination can vary from an infected person that has contact with food, to an entire harvesting area subjected to inefficiently or even untreated sewage or sludge (which is commonly used as fertilizer), and polluted water for irrigation.[56] Bivalve molluscan shellfish can concentrate astroviruses from large volumes of water, allowing accumulation of virus from fecally contaminated water.

3.1.4.2 Antibody Acquisition and Prevalence

Antibodies to astrovirus are generally acquired in early childhood. In 1978, an examination of 87 children under 10 years of age in the Oxford region of the UK demonstrated a rapid increase in antibody prevalence from 7% in 6–12-month-old babies, to 70% by school age.[101] Astrovirus antibodies could be detected in 75% of the 10-year-old children. Young adults were also examined, and 77% had antibodies to astrovirus. In this study HAstV antibodies were detected by immunofluorescence of astrovirus infected cells; consequently, the prevalence of astrovirus in the population was likely underestimated. In a seroprevalence study among hospitalized children in the UK rates of 86% for serotype 1, 1% for type 2, 8% for serotype 3, and 6% for serotype 4 were reported.[116]

3.1.5 Laboratory Diagnosis

Table 3.2 summarizes the current diagnostic methods for astroviruses. Most studies of astrovirus detection in food have focused on shellfish. Different protocols have been developed for the detection of astroviruses from foodstuffs; and comparative studies are needed to determine which assays should be recommended.[117] The detection of astroviruses from any type of food sample without an effective concentration

TABLE 3.2
The Current Diagnostic Techniques for Astroviruses

Method	Specimen	Sensitivity	Reagents	Limitations/Requirements
Electron microscopy (EM) Immunoelectron microscopy (IEM)	Negative stained fecal specimens	10^6 to 10^7 virus particles per gram of feces[119] 10^5 to 10^6 particles per gram of stool	• Organic solvents, resin, • Aqueous (or alcoholic) solutions of heavy metal stains	• A lack of specific antibodies to every serotype[119] • Experienced microscopist • Only 10% of the astroviral particles in a given specimen display the distinctive surface star-like structure[54] • Cannot be used for looking for the lower concentration of virus particles present in contaminated food or water
Enzyme immunoassay (EIA)	Clinical samples[25]	Comparable sensitivity (91%) and specificity (98%) to IEM (10^5 to 10^6 particles per gram of stool)	• A group reactive monoclonal antibody (8E7) • Viral antigen • A polyclonal antiserum as the detector antibody • Commercial kit is available (DAKO Corporation,Carpenteria, CA)	
Molecular techniques (molecular probes, reverse transcription-polymerase chain reaction/RT-PCR/)	Both clinical and environmental samples[69,118,120–125]	• Molecular probes: 10^5 to 10^6 particles per gram of stool[119] • RT-PCR: ten to 100 particles per gram of feces[119]	• Probe (fragment of astrovirus genome) in the case of molecular probes • Extracted astrovirus nucleic acid • Primers (oligonucleitodes from different regions of the genome)[29,53,126,127] • Deoxinucleotides • Reverse transcriptase • Polymerase	• Knowledge of human astrovirus genomes • The high variability in the genomic sequence does not allow the use of universal primers for all members of the family, *Astroviridae*[36] • Fail to distinguish between infectious and noninfectious virus particles • Diagnostic methods for the detection of astroviruses or viral RNA in food and water have not been adopted to routine laboratories in most parts of the world • Naturally occurring inhibitors can hamper the PCR reaction. Therefore, the incorporation of an internal nucleic acid standard into each RT-PCR tube is important to identify inhibitors and eliminate false negatives • Requires the extraction of the viral RNA from the sample (e.g. with borate buffer, glycine solution, saline beef, proteinase K digestion, washing of food samples with guanidinium thiocyanate, adding PBS to the food sample, then extract with Freon) • More sensitive real-time RT-PCR has been developed • Have also been used to confirm the presence of astrovirus in EIA-positive samples[28,95,104]. Good correlation between EIA and RT-PCR has been observed, although it was demonstrated that in some cases RT-PCR has a higher sensitivity than EIA in detecting astrovirus[28] • Have also been used to detect astrovirus genome in animals, such as minks[128] and turkeys[129]
Cell culturing	Both clinical and environmental samples		Media, trypsin	• Time-consuming, unreliable, expensive • Requires trypsin for isolation from primary specimens • Polluted water and shellfish samples are usually toxic for cell cultures

procedure and appropriate virus detection method is difficult and frequently unsuccessful. Several concentration methods have been developed in recent years. Concentration and purification of virions from shellfish rely on physicochemical procedures,[118] such as polyethylene glycol (PEG) 6000 or 8000 precipitation, organic flocculation, or application of positively-charged virosorb filters. In addition, many different viral detection techniques have been used, ranging from tissue culture methods to nucleic acid hybridization. It is important to apply a method, which is flexible, inexpensive and can be used without extensive pH treatment or use of multiple reagents. Furthermore, it can be used in a minimally equipped laboratory and by staff with a minimal level of training.

The traditional technique for detection of astroviruses is to isolate them in tissue culture. The isolation of astroviruses from food samples is not an easy task. Bacterial contaminations from shellfish also create difficulties in virus propagation in cell cultures. Treating samples with large amounts of antibiotics or chloroform might be effective, but toxic effects cannot be excluded. Therefore, at present, molecular techniques (such RT-PCR, real-time PCR, or immunomagnetic beads-PCR) offer the best alternative to develop sensitive and specific methods for the detection of astroviruses from environmental samples.[130]

Unfortunately, molecular techniques fail to distinguish between infectious and noninfectious virus particles, which may be a critical point in environmental virology and infection control.[131] Although, the RNA genome does not remain intact without the protective capsid in an RNase-rich environment; therefore, if the genomic RNA could be detected by amplification, there is a good reason to suppose that viruses are infective.

Virus detection in other foods, such as lettuce, strawberry, deli meats (ham, turkey, and roast beef), and green onion is not general.[132]

3.1.6 Treatment and Prevention

Astrovirus gastroenteritis is generally characterized as a mild, self-limiting diarrhea with or without nausea and vomiting. The infection can strike down the individual for a few days, but does not require specific therapy. Although, more severe disease can develop in patients who have other medical problems, such as underlying gastrointestinal disease, malnutrition, immunodeficiency, coinfection with other pathogens(s) or prolonged illness. In addition, dehydration in young children may demand oral or intravenous fluid resuscitation. Intravenous immunoglobulin may be a beneficial adjunct in patients with severe immunodeficiency who have no response to conservative therapies,[93] however, subsequent studies are required to determine effectiveness and to ascertain indications.

Determinant of prevention of astrovirus infection is to break transmission, particularly in hospitals and other institutions, day-care centers, and families where person-to-person transmission is probable. Immunization against astroviruses is the ideal solution to prevent outbreaks, considering that astrovirus is a medically important pathogen.[26,83] Vaccine development is not current, because the actual importance of astroviruses, as well as key factors of immunity are not well understood. Nevertheless, a prophylactic vaccine can play a remarkable role in reducing the incidence of food- and waterborne infections caused by astrovirus. Until then, general hygienic procedures, such as hand washing, disinfection of probably contaminated areas, surfaces (associated with feces or vomits containing astrovirus) with chlorine-based detergents, wearing of gloves for all points in the food chain where foodstuffs are handled manually, and appropriate treatment of potable water can prevent foodborne outbreaks and diseases due to astroviruses. It is also essential to not grow or wash foodstuffs in fecally contaminated water. Moreover, pasteurization procedures, disease control in animals, better regulation of shellfish beads, improvement in the safety of animals own feed and water supply, and protection from manure can prevent outbreaks; primarily because general hygienic procedures are not always sufficient to reduce astroviral infections and contamination.

3.2 METHODS

3.2.1 Sample Preparation

Frequently used virus concentration methods are summarized in Table 3.3, and general sample preparation and detection procedures of astroviruses from food samples are shown in Table 3.4. Food samples are usually obtained from cafeterias, coastal waters, as well as shellfish growing areas, and stored at 4°C, for a maximum of 24 hours until further processing. Generally, cold storage temperatures (2–8°C) delay senescence, product browning and growth of microorganisms in minimally processed fruits as well as vegetables, however promote the survival of astroviruses. Although, most studies of astrovirus detection in food have focused on shellfish. Shellfish are generally shucked, and the stomach and digestive verticula are removed by dissection, and frozen (–20°C) until analysis. For analysis, 20–25 g tissues need to be thawed on ice, then homogenized (e.g. in tryptose phosphate broth (TPB)-glycine buffer) and—in some cases—sonicated before virus concentration and nucleic acid extraction. In order to prevent cross-contamination, it is recommended to sterilize the homogenizing part of the blender with ethanol (70%) and to heat for 1 min after each sample. Some detailed sample preparation procedures are introduced in Section 3.2.3. Here we provide three step-wise protocols (Figures 3.3 through 3.5) for preparation of mussel, food and shellfish for RT-PCR detection of astroviruses.

3.2.3 Detection Procedures

(i) **Extraction of viral RNA:** Viral RNA is prepared with one of the methods outlined in Figures 3.3 through 3.5.

(ii) **RT-PCR:** The type-common primer pair Mon2/PRBEG (Mon2, 5′ GCT TCT GAT TAA ATC AAT

TABLE 3.3
Frequently used Virus Concentration Techniques from Foods

Method	Reagents and Equipment	Underlying Priniciples	Advantages/Disadvantages
Hydroextraction	Homogenized sample in dialysis bag surrounded by polyethylene glycol	Viruses remain inside the bag, at 4°C, complete within 18–24 hours	–Low recovery –Viruses are sometimes adsorbed to the dialysis membrane, –Toxic substances present in food may inactivate viruses during the 24-hour incubation period
Ultracentrifugation	Homogenized sample	Centrifuge at speeds above 50000 rpm	–Ultracentrifuge is very expensive
Ultrafiltration	Homogenized sample, membrane	Sample is driven through a membrane by applying pressure, viruses and macromolecules are retained	–Good recovery, –Clogging of the filter
Adsorption to membrane filters	Homogenized sample, positive/ negative filter	Membrane filters retain viruses	–Clogging, –Membrane coating components
Precipitation	Homogenized sample, PEG	Add 8% PEG	–Simple, cheap, –Good recovery

TABLE 3.4
General Sample Preparation and Detection Procedures of Astroviruses from Food Samples

Concentration	Viral extraction or elution	Viral nucleic acid extraction	Detection	Reference
Homogenization, centrifugation	PEG 6000 centrifugation	Proteinase K, phenol-chloroform extraction, ethanol precipitation	RT-PCR, hybridization	124
Homogenization, sonification, centrifugation	Freon TF centrifugation	Mix of glass powder matrix and guanidine isothiocyanate	Nested RT-PCR	133
Homogenization in 1:7 (wt/vol) 10% TPB-0.05 M glycine (pH 9), centrifugation	Chloroform extraction, PEG 6000 precipitation	Proteinase K digestion , phenol-chloroform extraction, ethanol precipitation	RT-PCR, cell culture	33
Homogenization, ultracentrifugation	Glycine buffer, PBS, centrifugation,	Guanidinium thiocyanate-silica particles	(nested) RT-PCR	134

TTT 3′; PRBEG, 5′ ACC GTG TAA CCC TCC TCT C 3′) targeting the hypervariable 3′ end of ORF2 region of astrovirus types 1–3 to 5–8, as well as Mon2/JWT4 (JWT4, 5′ GCA GAG AGC TTG TTA TTA AC 3′) for HAstV-4 are used for detection of HAstV by RT-PCR. These primers yield amplicons from 296 to 332 bp depending on the different HAstV types.[136]

(1) Prepare reverse transcription mixture (25 µl per tube) containing 0.1% bovine serum albumin, 0.4 mM dNTP mix (Promega, Madison, WI), 2 U AMV-RT (avian myeloblastosis virus RT, Promega, Madison, WI), 4 U RNasine (RNase inhibitor, Promega, Madison, WI), 0.05 µM dithiothreitol (Promega, Madison, WI), 2 µM negative-strand primer (Mon2) and 5 µl RNA suspension in the reaction buffer (10 mM Tris, 50 mM KCl, 3 mM $MgCl_2$, pH 8.3).
(2) Incubate the tubes for 1 hour at 42°C.
(3) Prepare PCR mixture (25 µl per tube) containing 10% dimethyl sulfoxide (Sigma, St. Louis, MO), *Taq* polymerase and 1 µM positive-strand primer (PRBEG, JWT4 or DM4 [DM4, 5′ CTA CAG TTC ACT CAA ATG AA 3′]) in the reaction buffer (same as the RT).
(4) Transfer the total volume (25 µl) of cDNA from step 2 into each tube containing 25 µl PCR mixture. Perform PCR amplification with an initial denaturation at 94°C for 3 min, followed by 40 cycles of amplification; denaturation at 94°C for

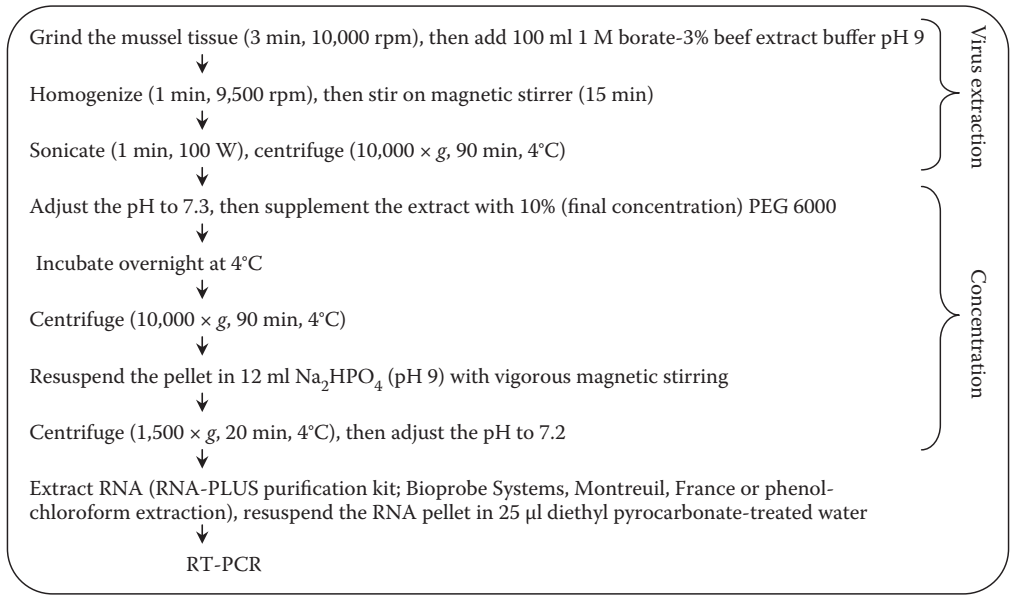

FIGURE 3.3 Protocol I: preparation of mussel sample for RT-PCR detection of astroviruses (based on Traore et al., *Appl. Environ. Microbiol*., 64, 3118, 1998.)

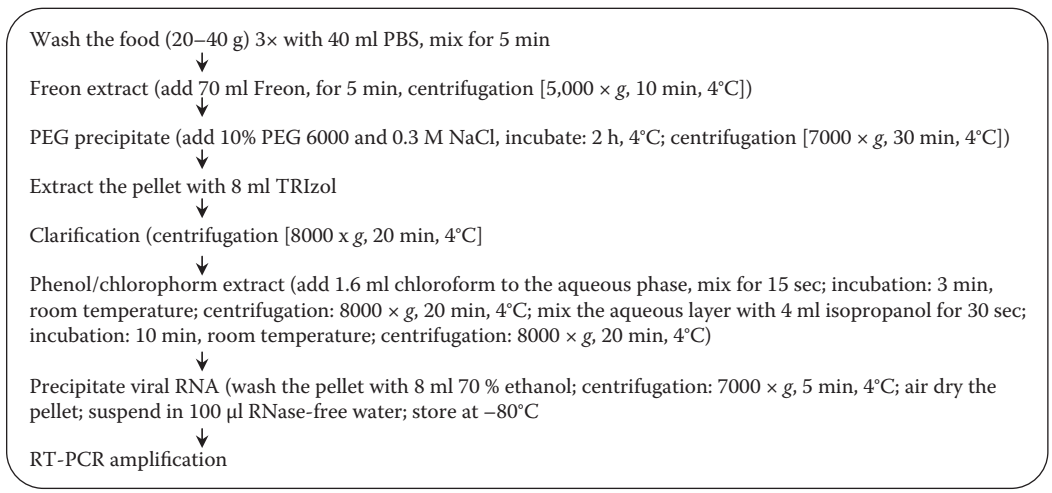

FIGURE 3.4 Protocol II: preparation of food sample for RT-PCR detection of astroviruses (according to Schwab et al., *Appl. Environ. Microbiol*., 66, 213, 2000.)

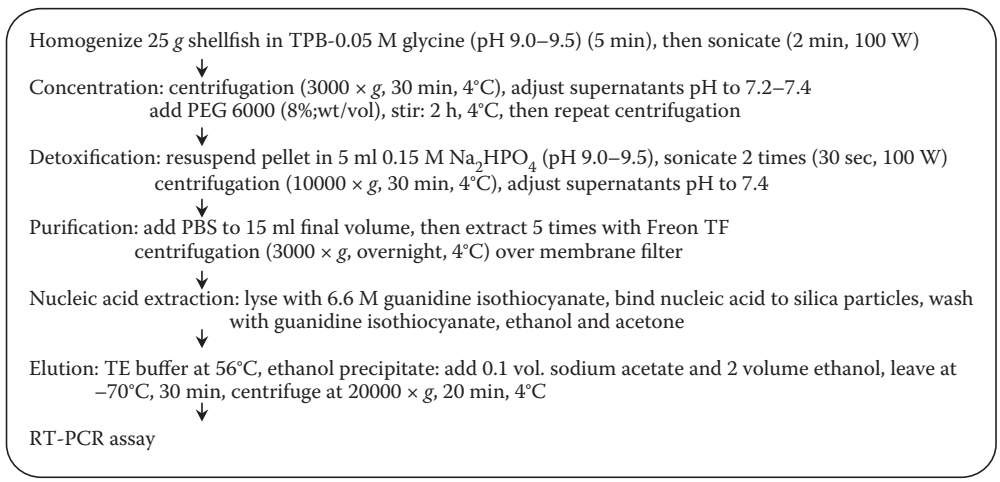

FIGURE 3.5 Protocol III: preparation of shellfish for RT-PCR detection of astroviruses (based on Lees et al., *Appl. Environ. Microbiol*., 60, 2999, 1994.)

1 min, annealing at 50°C for 1 min, and elongation at 72°C for 1 min. The final extension is at 72°C for 10 min.

(5) Separate PCR products by agarose gel (3%) electrophoresis in Tris–boric acid EDTA buffer, pH 8.0, containing ethidium bromide (0.5 μg/ml), and visualize amplicons by UV-transillumination at 320 nm.

(iii) **Sequencing and phylogenetic analysis:** The amplicons from the 3′ end of ORF2 obtained above are cloned into pGEM-T vector (Promega, Madison, WI) following the manufacturer's instructions. Two different clones of the same RT-PCR amplicon are sequenced using fluorescein-labeled primers and commercial sequencing kit (SequiTerm EXCEL II Long-Read DNA Sequencing Kit-ALF, Epicentre Technologies, Madison, WI) on an automated sequencer (ABI 310, Applied Biosystems, Foster City, CA) following the manufacturer's recommendations. Basic sequence manipulation and verification are performed using OMIGA software (v.2.0 Accelrys Co., San Diego, CA).

Nucleotide sequences of the Hungarian strains are compared to available reference strains and a genotype is assigned based upon similarity scores. Viruses with 97–100% nucleotide identity in the 3' end of ORF2 region of the genome are considered the same strain. In order to obtain a more accurate and reliable comparison, phylogenetic analysis is performed on representative strains using a longer, approximately 1.2-kb region (Mon2/DM4) of the 3' end of ORF2. ClustalW v1.7 (http://evolution.genetics.washington.edu) is used to create multiple alignments of the amino acid sequences of the selected partial capsid sequences. The nucleotide sequences are added and aligned by GeneDoc v2.3 (http://www.psc.edu/biomed/genedoc) using the corresponding amino acid sequences as template, resulting in a consensus length of 1183 nucleotides terminating at the 3' end of ORF2. A phylogenetic tree is constructed from the nucleotide sequence alignment using the maximum-likelihood algorithm in the program DNAML of PHYLIP v3.52c (http://evolution.genetics.washington.edu/phylip) running in a UNIX environment. The global rearrangement option is invoked and the order of the sequence input is randomized 50 times. The analysis is performed unrooted.

3.3 CONCLUSIONS AND FUTURE PERSPECTIVES

Since astrovirus is one of the main four viruses that cause foodborne infections, studies are needed to estimate the burden and cost of illness caused by foodborne astroviral infections, especially among susceptible individuals. Therefore, better surveillance systems are required. Molecular investigation of astroviruses throughout the whole food chain and through populations is also essential. The burden of foodborne illnesses due to astrovirus is highest in the elderly, and as a result of aging populations it will probably increase in the following years.[137] The extreme stability of astroviruses in the environment and their highly infectious nature contribute to the ease of foodborne transmission. Infected foodhandlers play a well known role in transmission of astrovirus. Nevertheless, virus contamination may occur anywhere in the food chain, consequently the role of the infected agricultural laborer should be taken into consideration. The use of sludge as fertilizer and wastewater for irrigation increase the risk for viral contamination.

There is a need to develop quick and simple molecular methods for detection of astroviruses from foodstuffs, and to urge rapid exchange of typing information between food laboratories and countries. New methods should be comparable and need to be standardized. Further studies are needed to evaluate the applicability of these methods in food microbiology.[137] Molecular methods may play an important role to better understand foodborne astroviral outbreaks. Further studies are needed both to evaluate the mechanism of emergence of epidemic strains and the plausible link with animal astrovirus infections. There is a need for international cooperation as well as a well-organized public health system with the involvement of researchers to find solutions for foodborne astroviral outbreaks. Development of rapid detection methods to combine epidemiological and virological information is also necessary in the limitation of foodborne outbreaks.

Contaminated products usually have a normal look, smell and taste, which present difficulties in the identification of astrovirus. Also, appropriate astrovirus detection methods are in general, not routinely available in food microbiology laboratories. While routine cell culture methods isolate astroviruses easily from blood or other sterile sites, specific methods are necessary to identify them from foodstuffs. A primary obstacle of linking epidemic outbreaks with foods is to detect the small amount of viruses that may be present in foodstuffs. In addition, by the time the infection is clinically manifest, the food under discussion has been consumed or discarded. In most countries the surveillance infrastructure for foodborne diseases of microbiological (or chemical) etiology, is weak or nonexistent. Moreover, in many countries, principally in the developing world, laboratory resources and skills to identify astroviruses are inadequate, and etiology-specific surveillance is often not possible. Even in the developed countries, laboratory-based surveillance is not well advanced. Absence of a simple and reliable diagnostic test to detect astroviruses in food makes surveillance difficult.

Currently available routine monitoring systems focus on bacterial pathogens. Nowadays, directives establish no specific microbiological criteria concerning the presence of enteric viruses in food or water; nonetheless it has clearly been shown, that no correlation between the presence of HAstVs and indicators of fecal pollution was found. Further investigations are needed to establish possible correlations between fecal contamination and HAstVs as viral

contaminants.[138] Nevertheless, the shortcomings of standard indicator organisms: fecal coliform bacteria, *Escherichia coli*, and *Salmonellae* as generally accepted indicators of fecal contamination of food, have been highlighted in several studies,[139,140] and this has led to calls for a reassessment of quality guidelines based on these indicator organisms. The food industry and the scientific community should work together to develop an integrated plan of action to address foodborne astroviral infections.

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4 Avian Influenza Virus

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4.1 INTRODUCTION

Recent outbreaks of avian influenza (AI) in birds occurring in Europe, in the Americas, Asia, and Africa, have provided field evidence of how challenging could it be to control this infection, particularly in densely populated poultry areas or in areas where free-range rural village poultry and backyard flocks are present.[1–3] The early detection of AI in domestic and wild bird populations as well as in poultry commodities has been recognized as crucial to the implementation of timely and adequate prevention and control strategies.

In the last decade, an increasing number of novel molecular technologies have become available to aid diagnosis of infectious diseases of animals and many of these have been applied to improve and accelerate the diagnosis of AI. In addition, more traditional diagnostic protocols, mainly based on classical viral culture methods and immuno-enzymatic assays, have been revisited or improved in the recent years.

The increased availability of diagnostic tests in conjunction with improved knowledge on the epidemiology and the pathogenesis of AI, is resulting in a modified approach to surveillance and diagnosis of this infection, with a wider application of the molecular-based technologies.[4]

4.1.1 Classification of AI Virus

Influenza viruses have segmented, negative sense, single strand RNA genomes. The viruses are classified in the family *Orthomyxoviridae*. At present the *Orthomyxoviridae* family consists of five genera, only viruses of the *Influenzavirus* A genus are known to infect birds. Influenza A viruses are subdivided into subtypes based on the antigenic relationships in the surface glycoproteins, hemagglutinin (HA), and neuraminidase (NA). At present, 16 HA subtypes have been recognized

(H1–H16) and nine NA subtypes (N1–N9).[5] Each virus has one type of HA and one type of NA antigen, apparently in any combination.

4.1.2 Biology and Pathogenesis

Type A AI viruses are very widespread in nature; up to now they have been detected in more than 105 different species of wild birds from 26 families.[6] Wild aquatic birds are natural reservoirs of these viruses and they can become infected by viruses of all HA and NA subtypes without showing signs of disease.

In domesticated poultry AI viruses can be grouped in two pathotypes based on the clinical signs they may cause mainly in gallinaceous species: low pathogenic AI (LPAI) viruses and highly pathogenic AI (HPAI) viruses. LPAI viruses mostly cause infections of the respiratory and the enteric tract and the infection is subclinical in most avian species. Infections may be characterized by mild respiratory signs, some depression and reduced egg production. HPAI viruses not only replicate in the respiratory and enteric tract but also in endothelial cells throughout the body with spillover to adjacent parenchymal cells. Disease signs involve the respiratory and enteric tracts and many other organ systems, such as the central nervous system. Lesions are characterized by multiple haemorrhages in visceral organs and the skin, and mortality rates approach 100%. Infection with HPAI viruses thus leads to wide dissemination in the body and virus presence in many organs and edible tissues.[7]

To date, only viruses of H5 and H7 subtype have been shown to cause HPAI in susceptible species, but not all H5 and H7 viruses can be classified as HPAI. It appears that H5 and H7 HPAI viruses arise by mutation after an AI virus precursor of low pathogenicity (LPAI) has been introduced into poultry. It follows that all HPAI viruses should have a LPAI progenitor, although the latter have only been identified in a limited number of cases.[8]

For all influenza A viruses the HA glycoprotein is produced as a precursor, HA0, which requires post translational cleavage by host proteases before it is functional and virus particles are infectious.[9] The HA0 precursor proteins of AI viruses of low virulence for poultry (LPAI viruses) have a single arginine at the cleavage site and another basic amino acid at position −3 or −4 from the cleavage site. These viruses are limited to cleavage by extracellular host proteases such as trypsin-like enzymes and thus restricted to replication at sites in the host where such enzymes are found, i.e., the respiratory and intestinal tracts. HPAI viruses possess multiple basic amino acids (arginine and lysine) at their HA0 cleavage sites, either as a result of apparent insertion or apparent substitution, and appear to be cleavable by an intracellular ubiquitous proteases. HPAI viruses are able to replicate throughout the bird, damaging vital organs and tissues, which results in disease and death.[10,11]

The factors that bring about mutation from LPAI to HPAI are not known. In some instances mutation seems to have taken place rapidly (at the primary site) after introduction in poultry possibly through the wild birds,[12,13] in others the LPAI virus has circulated in poultry for months before mutating.[14] Therefore, it is impossible to predict if and when this mutation will occur. However, it can be reasonably assumed that the wider the circulation of LPAI in poultry, the higher the chance that mutation to HPAI will occur.

HPAI viruses are not necessarily virulent for all species of birds and the severity of the clinical signs may vary with bird species, age of the host and virus strain.[15,16] In particular, ducks rarely show clinical signs as a result of HPAI infections although there are reports that some of the Asian H5N1 viruses have caused disease and the HPAI viruses A/duck/Italy/2000 H7N1 and A/chicken/Germany/34 (H7N1) have been reported to cause disease and death in naturally and experimentally infected waterfowl.[15,17]

Bird to bird transmission of AI viruses is complex and it largely depends on the virus strain, host species and environmental factors.[18] LPAI viruses are mainly excreted with feces, through the cloaca. Viral shedding through the respiratory tract is also considered important, at least for some species or some strains, as the Asian HPAI H5N1.

For this virus, and similarly for other HPAI viruses, transmission from poultry to humans is supposed to occur primarily through direct contact with secretions of the upper respiratory tract, infected feces, feathers, organs, and blood of infected animals. Inhalation of contaminated dust or droplets can be an alternative transmission route.[19]

4.1.3 Medical Importance and Zoonotic Implication of AI Viruses

Until recently, direct infection of humans with AI viruses had not been considered significant. Human cases were sporadically reported between 1959 and 1996 with only three documented cases, two in the USA (HPAI and LPAI H7N7) and one, likely to be of laboratory exposure-origin, in Australia (HPAI H7N7). In all cases the patients recovered and the main clinical sign was characterized by self-limiting conjunctivitis.[3,8] However, starting from 1996, a series of events has raised the concerns on the zoonotic potential of AI infections.

With some exceptions, since 1996 almost all the reported cases of AI virus infection in humans have been caused by HPAI viruses belonging to the H5 or H7 subtypes directly transmitted from infected birds to humans. Among the LPAI viruses, the first documented case of avian to human transmission was described in 1996 and caused by a LPAI H7N7 virus. The virus was isolated in England from the eye of a woman suffering from conjunctivitis. This person had ducks and the virus was shown to be genetically 100% of avian origin.[20,21] In 1999, LPAI viruses belonging to the H9N2 subtype were isolated from two young girls in Hong Kong.[22] They were suffering from an influenza-like syndrome and fortunately they recovered with no serious consequences. Subsequently, isolation of H9N2 viruses in human beings was reported in the Peoples Republic of China on five occasions during 1998.[23] In 2003, LPAI H7N2 was isolated from a patient with respiratory symptoms in the US. Also in this case, the patient recovered.[3]

In two other circumstances, evidence of contacts between LPAI viruses and humans were only detected serologically. A serological survey in human beings potentially at risk of exposure during the 2002–2003 LPAI H7N3 epidemic in Italy revealed the presence of specific antibodies in 3.8% of serum samples collected in poultry workers.[24] Serological prevalence with regard to H9N2 was also revealed by the hemagglutination inhibition test in the human population at risk of exposure in Iran.[25] However, this latter finding needs confirmation by means of other serological tests.

Certainly, more significant and severe are the human infections caused by HPAI viruses. The first documented evidence on how serious could be the consequences of avian to human transmission of HPAI viruses occurred in 1997 in Hong Kong. In that year, the HPAI H5N1 circulating in the domestic poultry was capable of infecting 18 people, causing the death of six of them.[26] Viruses belonging to the same antigenic subtype, H5N1, and genetically related to the 1997 viruses re-emerged in Hong Kong in 2003.[27] In that year, this HPAI virus circulating in poultry in South East China began to spread westward among wild and domestic birds throughout Asia, reaching Europe and Africa in 2005 and 2006. Since then, the continued infections of humans and other mammals, such as felines, caused by this virus have caused great concern over the capabilities of H5N1 to cross the species barrier and to potentially become easily transmissible among humans. To date (2nd February 2009), a total of 404 confirmed cases of HPAI H5N1 infections in humans and 254 human deaths have been reported to the World Health Organization (WHO) (available at http://www.who.int/csr/disease/avian_influenza/country/cases_table_2008_04_17/en/index.html).

Other HPAI viruses belonging to the H7 subtype were reported as etiological agents of severe human infections to a minor extent compared to HPAI H5N1. During the 2003 outbreak caused by the H7N7 HPAI virus in poultry in The Netherlands, 82 cases were reported in humans. Generally, symptoms were described as influenza-like illness and/or conjunctivitis, but one fatality also occurred.[28] In Canada, persons involved in outbreaks management suffered from conjunctivitis, headache, and flu-like syndrome. The H7N3 HPAI virus, responsible for the outbreak in poultry, was confirmed to be the causative agent of the disease. Fortunately, no fatal cases occurred.[29]

While such human infections generally result from direct and intensive contact with infected or diseased poultry, other routes of infection such as consumption of edible tissues from infected avians or contact with contaminated water have been suggested as possible sources of infection.

4.1.4 Diagnosis of AI Viruses

Surveillance and monitoring programmes have been implemented in many countries around the world as a result of the global spread of AI viruses and of the subsequent implications on public perception and animal health issues. These programmes are mainly targeting the wild birds—considered as the main reservoirs—and the poultry population. The main aims of these programmes are to detect and control AI viruses in the poultry compartment, thus preventing their spread to human beings.

Therefore, nowadays laboratory testing is mainly applied to trace viral circulation in a given area or in a susceptible population in order to implement an early warning system, in addition to diagnose the presence of the virus in a diseased flock or animal or in poultry derived products. This implies the use of rapid, sensitive and, possibly, cost-effective laboratory tests adaptable to very high throughputs.[4]

4.1.4.1 Virus and Antigen Detection

Traditionally, laboratory protocols for the detection and the identification of AI viruses were based on virus isolation (VI) in SPF eggs or in cell cultures. The application of these methods of laboratory investigation is mainly limited by the fact that they are not flexible to a sudden increase in demand, are not cost-effective and often require a long processing time. In fact, these methods are time-consuming and require a minimum of 12 days before a negative result may be issued. Office International des Epizooties (OIE) and EU official methods require two blind passages in eggs of 6 days, before the sample is considered negative.[30,31] At present, there seems to be only limited space for improving the time-efficiency of these methods.

What appears to be a major bottleneck is the obtainment of suitable substrates for VI. The primary cell cultures and the continuous cell lines tested so far provide variable results, mainly strain to strain dependant and, in general, they are less sensitive than SPF eggs. These are expensive and not always easily available. The use of eggs derived from AI and Newcastle disease virus (NDV) specific antibody negative (SAN) parent stocks are considered as an alternative method in the OIE manual[30] and, more recently, in the EU manual.[31]

VI implies the replication in laboratory of viable viral particles to a significant concentration thus, biosafety, and biocontainment should be regarded as a priority for laboratories in which AI VI is performed. Despite these major difficulties, VI in fowl's eggs still remains the gold standard for AI virus detection. Its sensitivity is equal or often superior to many alternative tests. In addition, genetic or antigenic variation of the viruses, as well as the presence of contaminants or PCR inhibitors in the samples, can impair the efficiency of molecular and immunoassays, but they have minor impact on VI.

Under certain circumstances, it might be desirable to test a certain number of samples in a short period of time. In this case, antigen capture immunoassays can be considered a very useful diagnostic tool. They are very easy to use, do not require sophisticated or expensive equipment and, in many instances, they can be applied on-site, thus avoiding the time-consuming and delicate phase of sample preparation and shipment. In many cases, test results can be available within minutes. To date, most of the antigen capture tests available on the market target the type A influenza virus nucleoprotein (NP), thus detecting any type A influenza virus. With the exception of one H5 subtype specific test for veterinary use, this kind of

assay does not provide any indication on the subtype involved. Furthermore, no indication on the pathotype (i.e., HPAI vs LPAI) can be obtained.

Their main limitations consist in the unsatisfactory sensitivity compared to VI and molecular tests, and their unit cost.[32,33] Due to their low sensitivity, sampling numbers should be increased before the presence of viral circulation in a given population can be ruled out. For the same reason, excessive dilution of the samples by pooling of swabs should be avoided. These features make this kind of assay unsuitable for monitoring and early detection programmes, where large number of samples must be cost-effectively screened with sensitive testing procedures.

4.1.4.2 Serology for AI Diagnosis

Serology represents a relatively inexpensive and practical methodology to assess the circulation and the prevalence of influenza viruses in poultry. It does not provide direct evidence on the presence of the virus but, in the framework of a surveillance effort in domesticated birds, serological diagnosis is considered a suitable approach to monitor the AI-free status of a given region or farm. On the contrary, the application of the serological methods available today in wild bird surveillance may generate information of very limited use, particularly for HPAI surveillance.[34] In fact, serology does not provide information concerning the pathotype and wild birds, specifically wild waterfowl, which are commonly infected by LPAI viruses, therefore serologically positive.

During monitoring or surveillance programmes, large numbers of animals have to be tested in order to guarantee acceptable statistical significance. In recent times the agar gel immuno-diffusion (AGID) test has been superseded by commercial ELISA tests. Both systems detect antibodies to the group antigen of influenza A viruses, and therefore are unable to give any indication on the virus subtype causing infection. These tests find their primary application in monitoring poultry flocks, although most of them lack validation data for minor species (ducks, geese, quails etc). In addition, AGID is not suitable for testing sera of waterfowl as the latter do not produce precipitating antibodies.[35]

The hemagglutination inhibition test is a simple, robust, and fully validated test that generates qualitative and quantitative information on antibodies that are a result of vaccination or of infection in most avian species. It is more labor-intensive than ELISA or AGID tests, but it yields information that under certain circumstances is more valuable and useful for managing field situations.

4.1.4.3 Molecular Tests

In implementation of surveillance and monitoring programmes for AI, cost-effective testing procedures capable of facing high and constant workflows are necessary. During AI outbreaks the main problems encountered by a diagnostic laboratory are represented by a sudden increase in sample testing and an increased pressure for faster turn-around-time (TAT), both combined with high quality test performances and cost effectiveness.[36]

In this respect, the possibility of diagnosing AI by using molecular methods offers important advantages compared to other protocols, such as VI and ELISA. For this reason, in the recent past there has been a significant increase in the development and application of testing procedures for the detection of AI viral RNA. Several RT-PCR and real time PCR protocols have been published in scientific journals (see Table 4.1 for references) and the most recent methodologies such as NASBA,[37,38] LAMP-PCR[39,40] and pyrosequencing[41,42]

TABLE 4.1
Main Representative PCR-Based Protocols for the Detection of AI Viruses Published Since 2000 in International Scientific Journals

Target	Assay	Notes	Reference
End point RT-PCR			
Type A influenza virus	One step RT-PCR	Positive samples confirmed by dot-blot hybridisation.	82
Type A influenza virus, H5 and H7 subtypes	Two step RT-PCR	Emi-nested PCR for H7 subtype. Laboratory evaluation.	83
Type A influenza virus, H5 and H7 subtypes	One step RT-PCR-ELISA	Partially validated on Eurasian lineage.	44
Type A influenza virus and Newcastle Disease virus	Two step duplex RT-PCR	Validated assay.	84
Type A influenza virus, avian pneumovirus and Newcastle Disease virus	One step multiplex RT-PCR	Laboratory evaluation.	85
Type A influenza virus, H5, H7, H9 subtypes	One step type A RT-PCR and one step multiplex RT-PCR (H5, H7, H9)	Laboratory evaluation.	86
Type A influenza virus, H5, H7 and H9	Two step, multiplex RT-PCR	Laboratory evaluation.	87
H5 AI subtypes	One step RT-PCR	Ring test evaluation on Eurasian strains.	56
H7 AI subtypes	Two step RT-PCR	Ring test evaluation on Eurasian strains.	56
H5 and H7 subtypes	One step multiplex RT-PCR	Limited clinical validation.	88

(*Continued*)

TABLE 4.1 (Continued)

Target	Assay	Notes	Reference
H5 subtype of the H5N1 HPAI virus	One step RT-PCR	Limited clinical validation.	89
H1-H16 AI subtypes	RT-PCR and sequencing	Limited validation.	61
H1-H16 AI subtypes	RT-PCR and sequencing	Laboratory evaluation.	60
H1-H15	RT-PCR and sequencing	Laboratory evaluation.	59
Real time RT-PCR (rRT-PCR)			
Type A influenza virus	One step rRT-PCR	Hydrolysis probe. Validated assay.	45, 56
Type A influenza virus	One step rRT-PCR	MGB-hydrolysis probe. Laboratory evaluation.	90
Type A influenza virus	One step rRT-PCR	Light upon extension fluorogenic primers. Laboratory evaluation.	91
Type A influenza virus	Two step rRT-PCR	SybrGreen chemistry. Limited field evaluation.	92
Type A influenza virus, H5 and N1	One step multiplex rRT-PCR	MGB-hydrolysis probes. Laboratory evaluation.	93
Type A influenza virus, H5 and H9	One step multiplex rRT-PCR	Hydrolysis probe. Laboratory evaluation.	94
Type A influenza virus, H9 and N2	One step multiplex rRT-PCR	SybrGreen 1 dye. Validated assay	95
Type A and B influenza virus, H5	One step multiplex rRT-PCR	Discrimination between type A and B not possible. Laboratory evaluation.	96
Type A and B influenza virus, H5 and N1	One step multiplex rRT-PCR	Hydrolysis probes. Limited clinical validation on human specimens.	97
H5, H7, H9 AI subtypes	One step rRT-PCR	Hydrolysis probes. Validated assay for Eurasian lineage.	46
H5 and H7 subtypes	One step multiplex rRT-PCR	Hydrolysis probe. Validated assay for American lineage.	45
H5 AI subtypes	One step rRT-PCR	Hydrolysis probes. Validated assay for Eurasian lineage.	54
H5N1 HPAI virus of the Qinghai lineage	One step rRT-PCR	Hydrolysis probe. Validated assay.	57
H5 subtype of the H5N1 HPAI virus	Two step rRT-PCR	MGB-hydrolysis probes. Laboratory evaluation on one human specimen.	98
H5 subtype of the H5N1 HPAI virus	Two step multiplex rRT-PCR	Hydrolysis probes targeting two distinct regions of the HA molecule. Validated on human specimens of Hong Kong and Vietnam origin.	99
H5 subtype of the H5N1 HPAI virus	One step rRT-PCR	Hydrolysis probe. Limited clinical validation on human specimens.	42
N1 subtype	One step rRT-PCR	MGB-hydrolysis probes. Validated assay on N1 subtypes pf the Eurasian lineage	100

have been applied, in many cases successfully, for the detection and typing of AI viruses. However, the use of these latter techniques is limited to research purposes at the moment.

With reference to the application of nucleic acid amplification protocols, sample processing appears less cold-chain dependant, as the preservation of cellular integrity and virus viability is not essential for these assays. The possibility to detect AI viral RNA in samples containing inactivated viral particles due to prolonged storage or shipment, or in samples treated to eliminate viral infectivity increases the chances to diagnose the disease in specimens collected in remote area of the world and addresses the biosafety issues. Reagents are also available to better preserve the integrity of a fragile molecule as the RNA at environmental temperatures.[43] Thus, unlike VI, molecular techniques can also be applied in small laboratories, providing that the basic equipment is available. This can contribute to the extension of a diagnostic laboratory network in the affected area and to the reduction of the TAT by avoiding the submission of the samples to a distant central and fully equipped laboratory of virology.

In terms of analytical and diagnostic sensitivity and specificity, the data available concerning the molecular tests for AI can be considered as optimal for their application in large scale diagnosis during an outbreak, showing values of diagnostic sensitivity, and specificity close to or above 90%.[32,44,45] An excellent agreement with the VI was reported during a LPAI outbreak investigation[32] and in clinical validation of real time protocols.[46] Data derived from experimental study[32]

indicate that RT-PCR based methods are capable of revealing viral RNA in tracheal swabs of experimentally infected birds for 10–15 days after challenge, similarly to VI. This period of time covers the first 7–10 days post-infection, when serology is not of diagnostic aid due to an undetectable immune response. Similarly, other studies provided experimental evidence of the usefulness of PCR-based techniques as an alternative to quali-quantitative VI methodologies.[47,48] Molecular techniques can provide a variety of data useful for surveillance, monitoring, and outbreak investigation. Once the RNA is extracted, it is possible to gain information not only concerning the presence of virus in the clinical specimen, but also about the HA and NA gene segments, the pathotype (LPAI vs HPAI virus) and other genomic sequencing data that can be used for molecular epidemiology. Most importantly, expensive, and time consuming *in vivo* tests for pathogenicity can be avoided, preserving animal welfare.

However, the recent and extensive applications of these kinds of molecular assays in the field of AI diagnosis have highlighted some drawbacks. The costs related to the equipment and reagents needed for PCR and real time PCR testing are still significant, although they decreased in recent times, mainly as a result of the widespread use of these technologies and the subsequent marketing competition.

Nucleic acids amplification methodologies are generally extremely sensitive assays, making them prone to easily reveal cross contamination of samples, leading to false positive results. Mishandling of the extracted RNA, improper use of reagents or use of nonsterile, non RNAse-free disposables, or inadequate reference controls, may result in false negative test response.

AI viruses exhibit a significant degree of genetic variability, particularly in certain important regions of the genome, as for segment 4 (HA) and 6 (NA). This might lead to diagnostic failures of some molecular tests based on primers and probes targeting these hyper variable regions when applied on mutated or new emerging viruses. A recent example for this is the PCR detection failure of viruses belonging to the H7 subtypes occurred during wild bird surveillance program in the US. In this case, a few nucleotides mismatches in the PCR probe targeting the HA molecule were the likely cause of failure.[49] Consequently, the recent initiatives concerning AI sequence data sharing[50] are crucial in the understanding of viral evolution and in updating probes and other molecular diagnostic tools as long as the viruses mutate.

Considering the extreme sensitivity of many nucleic acid amplification assays, some samples tested positive by a given molecular test might not be confirmed by any other test applied on the same sample, including VI.[51] Therefore, the adoption of fully validated protocols and harmonized test is mandatory. Examples of unexpected false positive results may indeed occur after extensive field application.[52]

Since AI is now threatening almost every continent, these considerations should seriously be taken into account when planning official guidelines and diagnostic protocols. Public institutions and private biotechnology companies are currently investing resources in research and development of more stabilized PCR reagents, robust molecular protocols and easy-to use, flock-side molecular tests.[53]

Currently, the most common types of molecular tests used for AI detection are RT-PCR or real time RT-PCR (rRT-PCR)-based protocols (Table 4.1). Schematically, these can be further subdivided in protocols for the so-called "generic" detection of type A influenza viruses and protocols for the detection and identification of specific type A influenza virus subtypes.

The common genomic targets of the first type of molecular tests are well conserved segments of the viral genome located in the genes encoding for the matrix proteins (M1 and 2) or the NP. Since these proteins are antigenically and genetically conserved regardless of the virus subtype, these types of tests are virtually capable of detecting type A influenza viruses belonging to subtypes H1–H16. Based on the available literature, these protocols exhibit high sensitivity and specificity, with higher performances of the rRT-PCR tests compared to the RT-PCR tests. For these reasons, these type A influenza tests are used as screening tests to evaluate the infectious status of an animal, flock or area (see Table 4.1 for references).

Infections caused by AI viruses belonging to the H5 and H7 subtype are of major concern for public health and economic impact. Regardless of their pathogenicity, which may vary, they are notifiable diseases of animals, according to the current international legislation for animal health.[30] Therefore, their occurrence must be detected promptly and immediately reported to the national and international veterinary authorities. As a consequence, all the samples tested positive in the screening for type A influenza virus should be PCR tested for the identification of these two subtypes and/or immediately submitted for VI attempts.[31,36] Several protocols have been described for the specific PCR detection of H5 and H7 directly in clinical specimens (Table 4.1) and some of them properly validated.[45,46,54]

Phylogenetic studies[55] demonstrated that H5 and H7 sequences could be divided into two major groups, related to the geographical origin of the viruses. Thus, so called "American" and "Eurasian" lineages were described among avian H5 and H7 viruses. These groups reflect the genetic variation observed in the targeted genes and this has also influence in the development and application of specific diagnostic assays. In fact, molecular tests designed on viruses belonging to the American lineage generally exhibit poor performances, in term of sensitivity, when applied on the Eurasian strains and vice versa.[45,56]

The major public health and veterinary concern raised by the spread of the Asian H5N1 HPAI also contributed to the development of several molecular tests specifically targeting this dangerous virus (Table 4.1). In many instances, these protocols are duplex RT-PCR or rRT-PCR tests targeting the H5 and the N1 gene segments of this specific virus, but sometimes they result in poor performances when applied on different H5 strains. One protocol has been described targeting one specific H5N1 HPAI genetic sublineage.[57]

LPAI viruses belonging to the H9 subtype are also of major interest for the economic losses they can cause to the

poultry industries[58] in addition to their sporadic capabilities to be transmitted to humans, fortunately without serious consequences.[22] These reasons have also led to the development of rapid molecular tests for its detection in clinical specimens (see Table 4.1 for references).

In some instances, there is the need to determine the specific subtype of type A PCR positive samples, once the involvement of the H5, H7, and H9 subtypes has been ruled out. RT-PCR protocols designed on conserved regions of the HA molecule were developed and subsequent sequence analysis of the PCR product should enable the identification of the H1–H16 subtype.[59,60,61] These protocols are useful for rapid subtyping of viral isolates, but the lower sensitivity of these methods may represent a limit for their direct application on clinical specimens. Currently, none of the published molecular tests have been specifically validated for AI detection in poultry-derived products such as meat, feathers, etc. Recently, a protocol describing the RNA extraction procedure and the subsequent RT-PCR protocol for the detection of AI RNA from manure has been described.[62]

4.2 METHODS

4.2.1 Sample Collection and Handling

4.2.1.1 Selection of Samples to be Collected

Protocols concerning sample collection for AI are mainly focused on the detection of the virus in holdings suspected to be infected, thus, in living, moribund or recently dead birds. As a reference, the EU diagnostic manual for AI, adopted in 2006 by the EU[31] or the *OIE Manual for Diagnostic Tests and Vaccines for Terrestrial Animals*[30] provide concise but practical guidelines concerning sample collection and transportation.

For molecular testing, viable viruses are not requested. However, the target RNA molecule is extremely fragile and its degradation due to improper sample handling and storage may result in false negative results.

The targeted replication sites of LPAI viruses are the respiratory (mainly trachea and lungs) and the digestive (intestines) tract. HPAI viruses are disseminated throughout the whole body tissues. Both, LPAI and HPAI viruses, are shed via the upper respiratory tract and feces but the quantity of shed viral particles and the duration of shedding may vary according to host species, host immune status, and virus strains.[63,64] Thus, tissue specimens from trachea, lungs, and intestines or oropharyngeal, tracheal, or cloacal swabs are suitable samples for AI detection in carcasses and/or living birds. In addition, for HPAI viruses, specimens from brain and other internal organs, such as spleen, heart and kidney, can be collected.

Intestinal contents of fresh feces also may contain high viral loads.[65–67] However, presence of potential PCR inhibitors, bacterial or fungal contaminations and proteases impose particular attention when dealing with this type of material as false PCR results (positives or negatives) may occur.

In case of HPAI infections, the virus causes viremia and therefore can be present also in the blood of recently infected birds.[68–70] Data on the duration and intensity of the viremic period are scarce and these parameters can vary greatly according to the virus strain, its pathotype or the host involved. For example, virus titres in experimentally infected chickens varied from $10^{1.4}$ EID_{50}/ml for H5N2 HPAI[69] to 10^{8} EID_{50}/ml for H5N1 HPAI.[68] In another study, ducks experimentally inoculated with A/H5N1 HPAI revealed virus titers in blood ranging from $10^{0.7}$ EID_{50}/ml to $10^{2.3}$ EID_{50}/ml.[71] For LPAI viruses, replication occurs at the epithelial surfaces of the respiratory and intestinal tracts; therefore viremia is not expected for this pathotype. However, in a few occasions H9 or H7 LPAI virus genomes have been detected in blood and viable viruses were isolated.[72,73] Based on the above mentioned data, blood does not represent an ideal sample for AI detection but it can be considered as a potential source of environmental contamination during handling and processing of blood samples collected from suspected HPAI infected animals or during slaughtering of poultry in the pre-clinical phase of the disease.

Viremia and viral replication in endothelial cells occurring in HPAI infections contribute to the colonization of muscles and other edible tissues (i.e., liver) in infected birds. Muscles and meat of domestic birds were found to be HPAI infected during field investigations on imported poultry products[74] as well as experimental infections.[69,75] Incomplete evisceration or contamination from lungs and intestine during slaughtering procedures may lead to LPAI or HPAI contaminated meat or carcasses, as recently demonstrated.[76] Therefore, muscles (particularly breast and tight muscles) or respiratory and intestinal tissues still present in poultry carcasses represent suitable specimens for the detection of both HPAI and LPAI viruses.

4.2.1.2 Transportation and Storage of Specimens

Swabs, tissue specimens, or feces should be immediately submitted to the laboratory for testing. Soon after their collection, samples should be refrigerated on ice or with frozen gel packs. In case a submission delay (>24 h) to the laboratory is expected, samples should be frozen in dry ice, liquid nitrogen or −80°C. Repeated cycles of freezing and thawing must be avoided to prevent reduction of the viral load, cell lysis, and consequent RNA degradation.

Storage of allantoic fluids of eggs inoculated with H5 and H7 HPAI, and LPAI viruses in the guanidine-based lysis buffer included in two commercial RNA extraction kits preserved the suitability of the original RNA template for real time PCR amplification up to 7 days at + 4°C; ambient temperature and at + 37°C.[77] Importantly, the same lysis buffers were able to inactivate both the HPAI and LPAI viruses tested after 4 h, thus increasing the biosafety of the handled specimens.

In one other investigation,[78] end point RT-PCR amplification was still possible after 2 weeks storage at ambient temperature of bird fecal samples spiked with influenza A viruses using guanidine buffer, commercial preservative or alcohols (ethanol or isopropanol) as preservatives. PCR products up to 521 bp were obtained in samples preserved with guanidine or commercial buffers and up to 206 bp in samples

preserved with alcohols. Obviously, all these procedures will make parallel VI attempts impossible.

In case VI is necessary to confirm results of molecular testing or to assess viral infectivity, viral transport medium (VTM) should be used. VTM are generally based on phosphate saline buffered solutions (PBS, pH 7.0–7.4) or protein-based media, such as brain heart infusion (BHI) or tris-buffered tryptose bacteriological media supplemented with antibiotics and/or antifungals.[31] VTM supplementation with glycerol (10–20%) contributes to better preserve sample stability and integrity, particularly during prolonged storage at low temperatures.

4.2.1.3 Handling of Specimens

Probably only one documented case of human infection caused by an AI virus following laboratory exposure has been reported so far in the scientific literature.[79] The case occurred in Australia and consisted of conjunctivitis caused by an H7N7 HPAI virus. In contrast, several cases of conjunctivitis, influenza-like illness and one fatal case of acute lung infection were reported during the H7N7 HPAI poultry outbreaks in 2003 in The Netherlands.[28] People confirmed to be infected were somehow involved in the outbreak management. Adequate protective measures should therefore be adopted during collection and handling of samples suspected to contain AI viruses, at the farm level, at the slaughterhouse and within the laboratory. Personal protective items (PPI), such as lab-coats, goggles, disposable gloves, should be properly worn during necropsies and collection of samples from animals suspected to be AI-infected.

Orthomyxoviruses are identified as biological agents of biohazard class 2.[80,81] According to the WHO recommendations,[81] they should be manipulated in a BSL2 laboratory, adopting BSL3 work practices. Good laboratory practices should be applied during the whole process of sample testing at laboratory level. Useful and practical guidelines and comprehensive information on biosafety-related issues in the laboratory can be found in the EU manual for AI and on the WHO website.[31,80,81]

4.2.2 Samples Preparation for PCR Testing

The following procedures for sample preparation can be followed prior to submitting the specimen for RNA extraction and subsequent RT-PCR amplification.

Organs and tissues (brain, trachea, lungs, intestine): extract the RNA from tissue homogenate. Using sterile scissors or surgical blades, cut small blocks (approximately 2–5 mm × 2–5 mm) of tissues. If possible, tissues blocks should be frozen in liquid nitrogen to better preserve RNA integrity and to facilitate tissue disruption. Animal tissues can then be disrupted simply by sterile pestle and mortar. Fiber-rich tissues (e.g., lung and trachea) may require the addition of sterile quartz powder or sand to better disrupt tissue cells. To facilitate the disruption and the homogenization process, 300–500 µl of sterile PBS can be added. This will make also possible the preparation of aliquots for other types of test (i.e., VI), starting exactly from the same homogenate. Homogenization is carried out simply using a syringe and needle. Alternatively, a commercially available, automatic homogenizer can be used. Add the requested amount of this suspension to the lysis buffer of the RNA extraction kit according to the manufacturer's instructions.

Cloacal swabs, tracheal, or oro-pharyngeal swabs: dilute swabs in PBS (max 1 ml) and extract the RNA from this suspension. It is possible to pool the samples (up to ten tracheal swabs/pool or five cloacal swabs/pool). Vortex briefly. Add the requested amount of this suspension to the lysis buffer of the RNA extraction kit according to the manufacturer's instructions.

Feces: a suspension is prepared by adding one volume of feces to four volumes of sterile PBS. Add the requested amount of this suspension to the lysis buffer of the RNA extraction kit according to the manufacturer's instructions.

Allantoic fluid: simply add the requested amount of sample (allantoic fluid) to the lysis buffer of the RNA extraction kit according to the manufacturer's instructions.

RNA extraction: several methods for the manual or robotic extraction of the RNA exist. Many commercial kits are available, some of them developed and optimized for the extraction of the nucleic acids on specific matrixes, such as tissues, blood, and stool. However, to facilitate the organization of sample processing within the laboratory and make it more cost-effective and practical, only some kits are presented in this chapter, which can be used on different matrixes with satisfactory results. The kits listed should be considered as examples, representatives of the most common types used in different laboratories or described in several scientific journals. They have been evaluated in many laboratories working on AI. The use of other kits not included in the list is possible, providing their performances are methodically evaluated.

Kits widely used for RNA extraction: NucleoSpin® RNA II (Macherey-Nagel GmbH & Co., Germany); RNeasy® MiniKit (Qiagen Gmbh, Germany); High pure RNA isolation kit (Roche Applied Science, Germany), not recommended for feces; MagMax (Ambion/Applied Biosystems), for swabs or other liquid matrix. Not recommended for tissues and organs. Useful for robotic extractions. Extraction protocol follows the manufacturer's instructions.

4.2.3 Detection Procedures

4.2.3.1 One Step RT-PCR for the Detection of Type A Influenza Viruses

This protocol is a modification of the method developed and described by Fouchier et al.[82] for the detection of type A influenza viruses in samples of human and animal origin, including birds. According to previous field investigation on swabs of avian origin, the relative sensitivity was 95.6% (CI_{95} = 93.1–98.0) and the relative specificity was 96.3% (CI_{95} = 94.4–98.1) when compared to VI.[32] In one other study on experimentally infected ducks[48] the protocol provided 100% of relative sensitivity and 94% of

relative specificity. In some cases, unspecific bands can be visualized on gel.[52] Careful examination of the gel and use of proper controls and size-markers are therefore extremely important.

Target: M gene

Sample: RNA 5 µl in 25 µl of total reaction volume

Reagents: One step RT-PCR (Applied Biosystems GeneAmp® Gold RNA PCR Core Kit Part No 4308207)

Primers:[82]

Forward M52 C: 5′-CTT CTA ACC GAG GTC GAA ACG-3′

Reverse M253 R: 5′-AGG GCA TTT TGG ACA AAG/T CGT CTA-3′

Reagent (Conc. Stock Solution)	Final Concentration	Volume Required for one Reaction
RNase-free water	/	4.7 µl
PCR buffer 5×	1×	5 µl
$MgCl_2$ 25 mM	2.5 mM	2.5 µl
dNTPs mix 10 mM	1 mM	2.5 µl
DTT 100 mM	10 mM	2.5 µl
Primer M52 C 10 µM	0.3 µM	0.75 µl
Primer M253 R 10 µM	0.3 µM	0.75µl
RNase inhibitor 20 U/µl	10 U	0.5 µl
Reverse transcriptase 50 U/µl	15 U	0.3 µl
Ampli Taq GOLD 5 U/µl	2.5 U	0.5 µl
Total volume Vortex the mix for few seconds. Aliquote 20 µl in 0.2-ml PCR tubes.		20 µl
RNA		5 µl
Final reaction volume		25 µl

Cycling conditions: One cycle of 42°C for 20 min; one cycle of 95°C for 5 min; 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min; one cycle of 72°C for 10 min.

Detection: Agarose gel 2 % or silver stained SDS-PAGE 7%

Expected amplified fragment: 244 bp.

4.2.3.2 One Step RT-PCR for the Detection of AI Viruses Belonging to the H5 Subtype

This one step RT-PCR protocol has been partially validated in two consecutive ring trials performed in the EU and the results were published.[56] According to the ring trials results, the protocol appears to be sensitive and useful for H5 AI virus detection in clinical specimens. Since the targeted region encompass the cleavage site segment of the HA gene segment, the subsequent sequence of the resulted amplified products will allow the determination of the pathotype. However, some results have revealed possible specificity problems. These include false positives with non-H5 AI specimens and/or multiple bands of similar size to the predicted amplicon. This may relate to the precise cycling conditions which are employed on a given thermocycler. The protocol has been tested mainly on H5 AI viruses belonging to the "Eurasian" lineage.

Target: HA gene

Sample: RNA 5 µl in 50 µl of total reaction volume

Reagents: One step RT-PCR (Qiagen OneStep RT-PCR Kit cat # 210212)

Primers:[56]

H5-kha-1: CCT CCA GA**R** TAT GC**M** TA**Y** AAA ATT GTC

H5-kha-3: TAC CAA CCG TCT ACC AT**K** CC**Y** TG

Note the inclusion of degenerate nucleotides indicated above in bold.

Reagent	Final Concentration	Volume Required for one Reaction (50 µl)
RNase-free water	/	28.8 µl
PCR buffer 5×from Qiagen OneStep RT-PCR Kit	1×	10 µl
dNTPs mix 10 mM each (from Qiagen Kit)	0.4 mM each	2 µl
Primer H5-kha-1: 50 pmol/µl (50 µM)	1 µM	1 µl
Primer H5-kha-3: 50 pmol/µl (50 µM)	1 µM	1 µl
RNase inhibitor 40 U/µl (Promega)	8 U	0.2 µl
One Step RT-PCR Enzyme Mix (Qiagen Kit)		2 µl
Volume minus target		45 µl
Volume extracted RNA		5 µl
Final reaction volume		50 µl

Cycling conditions: One cycle of 50°C for 30 min; one cycle of 94°C for 15 min; 40 cycles of 94°C for 30 sec, 58°C for 1 min, and 68°C for 2 min; one cycle of 68°C for 7 min.

Detection: Agarose gel 2 % or silver stained SDS-PAGE 7%

Expected amplified fragment: 300–320 bp.

4.2.3.3 Two Step RT-PCR for the Subtype Specific Detection of H7 AI Virus

This protocol consists in a two step RT-PCR first developed in The Netherlands and then selected for its good results among different tests during the AVIFLU European project.[56] By this protocol, amplicon is detected conventionally by agarose gel electrophoresis with ethidium bromide staining or by SDS-PAGE with silver staining. The amplicon span the HA cleavage site so sequencing can provide pathotyping information, i.e., LPAI or HPAI.

Target: HA gene

Sample: RNA 5 µl in 50 µl of total reaction volume

Primers:[56]

GK 7.3 5′-ATG TCC GAG ATA TGT TAA GCA-3′

GK 7.4 5′-TTT GTA ATC TGC AGC AGT TC-3′

Step 1. Preparation of cDNA

Reagent	Final Concentration	Volume Required for one Reaction
RNA	/	10 µl
Primer GK 7.3 50 µM	2.5 µM	1 µl
Heat to 95°C for 2 min and put the mix immediately on ice; then add the following reagents:		
RNase-free water	/	3 µl
M-MLV RT Buffer 5×	1×	4 µl
dNTPs mix 10 mM	0.5 mM each	1 µl
RNase inhibitor 40 U/µl	20 U	0.5 µl
MMLV-RT 200 U/µl	100 U	0.5 µl
Final reaction volume		20 µl

Reverse transcriptase thermal parameters: 15 min at 37°C; 45 min at 42°C.

Step 2. cDNA PCR amplification

This protocol has been evaluated using the reagents contained in the AB Gene Kit cat#AB-0575/DC/LD/A:

Reagent	Final Concentration	Volume Required for one Reaction
RNase-free water	/	18 µl
2×Reddy Mix PCR Master Mix with dNTPs	1×	25 µl
Primer GK 7.3 50 µM	1 µM	1 µl
Primer GK 7.4 50 µM	1 µM	1 µl
Total volume		45 µl
cDNA		5 µl
Final reaction volume		50 µl

Cycling conditions: one cycle of 94°C for 30 min; 35 cycles of 94°C for 30 sec, 52°C for 30 sec, 72°C for 45 sec; one cycle of 72°C for 4.15 min.

Detection: agarose gel 2% or silver stained SDS-PAGE 7%

Expected amplified fragment: 200–220 bp.

4.2.3.4 Detection of Type A Influenza Virus by Qualitative Real Time PCR (M gene)

The protocol uses the probe-primer set previously evaluated by Spackman et al.[45] The basic procedure has been previously evaluated by different authors.[45,56] In the one step rRT-PCR protocol described below the QuantiTect Multiplex RT-PCR Kit (Qiagen Cod. 204643) has been adopted.

Reagents: QuantiTect Multiplex RT-PCR Kit (Qiagen Cod. 204643)

Primers:[45]

Forward **M+25**: AGA TGA GTC TTC TAA CCG AGG TCG

Reverse **M-124**: TGC AAA AAC ATC TTC AAG TCT CTG

Probe:[45]

FAM **M+64** : FAM-5′-TCA GGC CCC CTC AAA GCC GA-3′-TAMRA

Reagent	Final Concentration	Volume Required for one Reaction
Probe FAM M+64 1 µM	100 nM	2.5 µl
2×QuantiTect Multiplex RT-PCR Master Mix	1×	12.5 µl
Primer M+25F 5 µM	300 nM	1.5 µl
Primer M-124R 5 µM	300 nM	1.5 µl
QuantiTect Multiplex RT Mix	/	0.2 µl
RNase-free water	/	1.8 µl
Total volume Vortex the mix for few seconds. Aliquote 20 µl per tube		20 µl
RNA		5 µl
Final reaction volume		25 µl

Cycling conditions: This protocol was developed on AB7300 (Applied Biosystems) and Rotorgene 6000 (Corbett) real time platforms. Other laboratories using different instrumentation platforms should first critically and carefully examine these cycling conditions as they may *not* perform optimally on other instruments. Hence cycling temperatures, times and ramp speeds may all need to be modified. Thermal parameters: one cycle of 50°C for 20 min; one cycle of 95°C for15 min; 40 cycles of 94°C for 45 sec, 60°C for 45 sec.

4.2.3.5 Detection of Type A Influenza Viruses of H5 HA Subtype by Qualitative One Step Real Time RT-PCR

Similarly to the H7 real time PCR protocol (Section 4.2.3.6), this protocol has been developed and properly validated recently.[46] The procedure can be coupled with H7 detection within the same real time PCR run, providing a fast and sensitive method for the detection of notifiable AI subtypes in poultry. The protocol has been validated on a wide variety of H5/H7 isolates of the Eurasian lineage, including the recent H5/H7 LPAI viruses circulating in poultry and wild birds as well as the H5N1 HPAI viruses circulating in Eastern and Central Asia, Middle East, Europe and Africa. The protocol was developed using the QuantiTect Multiplex RT-PCR Kit (Qiagen Cod. 204643).

Reagents: QuantiTect Multiplex RT-PCR Kit (Qiagen Cod. 204643).

Primers:[46]

Forward **H5 F** : TTA TTC AAC AGT GGC GAG

Reverse **H5 R**: CCA **K**AA AGA TAG ACC AGC

Note degenerate nucleotides indicated in bold.

Probe:[46]

FAM **H5**: FAM-5′-CCC TAG CAC TGG CAA TCA TG-3′-TAMRA

Reagent (Conc. Stock Solution)	Final Concentration	Volume Required for one Reaction
Probe FAM **H5** 1 µM	150 nM	3.75 µl
2×QuantiTect Multiplex RT-PCR Master Mix	1×	12.5 µl
Primer H5F 5 µM	300 nM	1.5 µl
Primer H5Rnew 5 µM	300 nM	1.5 µl
QuantiTect Multiplex RT Mix		0.2 µl
RNase-free water	/	0.55 µl
Total volume Vortex the mix for few seconds. Aliquote 20 µl per tube		20 µl
RNA		5 µl
Final reaction volume		25 µl

Cycling conditions: This protocol was evaluated on AB7300 (Applied Biosystems) and Rotorgene 6000 (Corbett) real time platforms. Other laboratories using different instrumentation platforms should first critically and carefully examine these cycling conditions as they may *not* perform optimally on other instruments. Hence cycling temperatures, times and ramp speeds may all need to be modified. Thermal parameters: one cycle of 50°C for 20 min; one cycle of 95°C for 15 min; 40 cycles of 94°C for 45 sec, 54°C for 45 sec.

4.2.3.6 Detection of Type A Influenza Viruses of H7 HA Subtype by Qualitative Real Time PCR

Primers:[46]

Forward **H7 F**: TTT GGT TTA GCT TCG GG

Reverse **H7 R**: GAA GA**M** AAG GC**Y** CAT TG

Note degenerate nucleotides indicated in bold.

Probe:[46]

VIC **H7**: VIC-5′-CAT CAT GTT TCA TAC TTC TGG CCA T-3 ′-TAMRA

Reagent	Final Concentration	Volume Required for one Reaction
Probe VIC **H7** 1 µM	150 nM	3.75 µl
2×QuantiTect Multiplex RT-PCR Master Mix	1×	12.5 µl
Primer H7F 10 µM	300 nM	0.75 µl
Primer H7R 10 µM	900 nM	2.25 µl
QuantiTect Multiplex RT Mix		0.2 µl
RNase-free water	/	0.55 µl
Total volume Vortex the mix for few seconds. Aliquote 20 µl per tube		20 µl
RNA		5 µl
Final reaction volume		25 µl

Cycling conditions: This protocol was evaluated on AB7300 (Applied Biosystems) and Rotorgene 6000 (Corbett) real time platforms. Other laboratories using different instrumentation platforms should first critically and carefully examine these cycling conditions as they may *not* perform optimally on other instruments. Hence cycling temperatures, times and ramp speeds may all need to be modified. Thermal parameters: one cycle of 50°C for 20 min; one cycle of 95°C for 15 min; 40 cycles of 94°C for 45 sec, 54°C for 45 sec.

4.3 CONCLUSIONS AND FUTURE PERSPECTIVES

The increasing importance of AI viruses for the veterinary and medical sciences in the last decade has contributed to a tremendous increase in actions related to this infection with the aim to better understand the pathogenicity and virulence mechanisms of these viruses and to develop better diagnostic tools for their detection. Taking advantages of the new technologies available nowadays for the diagnosis of infectious diseases, an impressive number of scientific papers has been published describing the application of technologies, such as NASBA, LAMP-PCR, or microarrays, to the detection of the viruses. Further, well established technologies, such as RT-PCR or real time PCR have been improved and applied for this infection. As for many other infectious and contagious diseases, it has been realized that rapidity in the detection and characterization of the responsible microorganism is essential for the disease control.

The key issues related to the major changes in the field of AI diagnosis in the last 10–15 years, can be summarized into two main points: improved rapidity and flexibility. If compared to the classical VI and typing methods, molecular technologies have probably not improved significantly the diagnostic sensitivity or specificity. Rather, they have allowed the detection of the causative agent, in association with its typing, subtyping, and with the characterization of its molecular determinant of pathogenicity in a more time-effective and flexible manner. Importantly, the molecular tests have made the screening of large susceptible populations, such as poultry or wild birds, sustainable and cost-effective for many countries.

Many molecular protocols are currently available for AI testing, but only few of these have been fully and properly validated at present. Provision and harmonization of molecular test validation procedures is therefore necessary to expand the number of validated protocols, getting to more standardized laboratory results. With very few exceptions, the vast majority of the available protocols have been developed and tested for the detection of AI viruses in clinical specimens, from alive or dead animals. To better perform epidemiological investigations, as well as to better address public health and food safety issues, it would be desirable to expand the application of the molecular tests to environmental specimens, such as water, soil, surfaces, etc., and to poultry-derived products such as meat, feathers, and eggs. Thus, the development of new protocols or the validation of the existing testing procedures on these materials appears to be necessary.

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5 Hepatitis A and E Viruses

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5.1 INTRODUCTION

5.1.1 Hepatitis A Virus (HAV)

5.1.1.1 Overview of Hepatitis A Virus Infection and Pathogenesis

Viral hepatitis is a major health concern worldwide with higher incidence in developing countries than in the developed countries.[1,2] Hepatitis A virus (HAV) is one of the etiologic agents of acute viral hepatitis. Most infection occur in children and generally are self-limiting.[1] Transmission of HAV is primarily via the fecal-oral route, either by contact with an infected person or by ingestion of contaminated food and water.[1] In the industrialized countries, due to improvements of public health and socioeconomic conditions, there has been a shift of HAV infection toward a higher age with an increase of hospitalized and severe cases associated with outbreaks.[3] The illness severity is age-dependent. Generally, HAV infection in children is asymptomatic and rarely develops jaundice, whereas in older children and adults are symptomatic infection with a wide range of clinical manifestations from mild and anicteric infection to severe and fulminant hepatic failure (FHF).[1] Infected people can excrete HAV in feces for about 3 months or longer, and viremia is detectable by reverse transcription-polymerase chain reaction (RT-PCR) in the majority of patients at the onset of symptoms and can persist for several weeks after aminotransferase peak.[4] Viral replication occurs primarily within hepatocytes, and then the viruses are secreted into bile through the bile ducts which results in large amount of virus being shed in the feces.[5]

5.1.1.2 Hepatitis A Virion, Genome Organization, and Proteins

HAV is classified as the only member of the genus *Hepatovirus* within the *Picornaviridae* family.[6,7] It is a

spherical, icosahedral symmetry, nonenveloped RNA virus. The virus particle is 27–32 nm in diameter with a positive single-stranded RNA genome of 7.5 kb.[1] The viral genome composed of 5′ nontranslated region (NTR), structural protein regions, nonstructural protein regions, 3′ NTR, and followed by a short poly(A) tail.[8] A single large open reading frame (ORF) of HAV genome can be divided into three distinct functional protein-encoded regions termed P1, P2, and P3. The P1 region encodes the capsid polypeptides VP1–VP4. The P2 and P3 regions encode the nonstructural polypeptides which are necessary for virus replication.[8]

5.1.1.3 HAV Classification and Genetic Diversity

The HAV strains isolated from various parts of the world constitute a single serotype. However, genetic variability between strains allows the classification of HAV into six different genotypes based on phylogenetic analysis of nucleotide sequences in the VP1/P2A region.[9,10] Based on this region, the HAV strains that differ from each other at least 15% of nucleotide sequences are considered to be different genotypes, while the strains that their nucleotide sequence differ over 7–7.5% belong to different HAV subgenotypes.[9] Of six HAV genotypes, genotype I, II, and III are associated with human infections, and are further divided into subgenotype IA, IB, IIA, IIB, IIIA, and IIIB, respectively. The remaining three genotypes IV, V, and VI are represented by strains of simian HAV.[9,10] From the epidemiological data, HAV genotypes I and III are the vast majority of human strains which comprise more than 80% of strains circulating worldwide.[1,9,11]

5.1.1.4 Foodborne and Waterborne HAV

Foodborne and waterborne viral infections are increasingly recognized as the causes of illness in humans. HAV is one of the leading causes of foodborne and waterborne viral infections. It can be transmitted from person-to-person, or indirectly via food and water contaminated with virus-containing feces. HAV has previously been isolated directly from food or environmental sources. Salad vegetables, soft fruits, green onion, strawberry, lettuce, clam, shellfish, and oyster samples have been reported to be the sources of HAV infections.[12–17] A large epidemic outbreak of HAV, reported in 1988, was attributed to the ingestion of raw clams and caused illness in 300,000 persons in Shanghai, China.[18] Moreover, waterborne outbreaks of HAV associated with HAV contamination in water supply have also been reported from several countries. HAV has also been detected in river, canal, ground/tap water and sewage.[12,16,19–21]

5.1.1.5 Diagnosis of HAV

For the detection of HAV genomic RNA, several genome segments have been amplified by different sets of primer pairs. The genomic regions which have been used widely to detect and to define HAV genotypes, included the junction of VP3/VP1 region.[17,22–25] the junction of VP1/P2A region,[4,24–28] the entire VP1 region,[29] and the junction of 3C/3D region.[30] The primers used for the detection of HAV are summarized in Table 5.1. Currently, the genomic region that most commonly used for the detection and identification of HAV genotypes is the VP1/P2A junction region. To facilitate the molecular analysis of HAV, the amplification of their genomic RNA and sequencing of DNA amplicon should be performed and HAV genotypes are identified based on sequence analysis. Sequence variation within the VP1/P2A junction has defined six HAV genotypes and two subtypes within genotypes I, II, and III.[9–11]

In contrast to most picornaviruses, HAV of human origin replicates poorly in cell cultures with a relatively low concentration of viruses and viral antigen being produced into the cultured supernatant. For these reasons, development of a number of RT-PCR-based assays that enable the rapid and specific detection of small amount of viral nucleic acid in environmental sources, food samples, and clinical specimens have been developed recently. Several kinds of clinical samples have been used for the detection of HAV genome, including stool, serum, saliva, and liver suspension.[9,26,31] In addition, HAV has also been detected by other sensitive molecular techniques such as restriction fragment length polymorphism (RFLP),[32] Southern blotting,[33] real-time RT-PCR,[34] and reverse transcription loop-mediated isothermal amplification assay (RT-LAMP).[35] Currently, the amplification of viral RNA by RT-PCR is the most sensitive and widely used method for the detection of foodborne HAV RNA.[15,16,21,25,36]

The efficiency of the extraction methods for HAV RNA from clinical specimens is of great important for molecular diagnosis. Therefore, choosing appropriate RNA extraction methods is a critical step for a successful and valid use of PCR amplification of viral genome in clinical samples. Traditionally, proteinase K digestion and guanidinium isothiocyanate (GTC)-phenol-chloroform extraction method followed by ethanol precipitation has been widely used to extract RNA from serum or stool samples.[37] Recently, numerous protocols for RNA extraction, i.e., GTC-silica method, antigen-capture method, and magnetic beads coated with anti-HAV have been used for the isolation of HAV RNA and to separate viral genome from the potential inhibitors of RT-PCR reaction that might exist in the clinical samples.[9,22,31,37–41] The total genomic RNA of HAV can also be easily isolated from clinical samples with high sensitivity and reproducibility by using commercially available RNA extraction kits, i.e., Trizol LS® Reagent (Invitrogen Life Technology, Carlsbad, CA)[30,41] or QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany).[27,31,35,42] Recently, the use of the QIAamp Viral RNA Mini Kit (Qiagen) has been reported to be an efficient method for extraction of HAV genomic RNA with a detection limit of 6×10^3 copies/ml in clinical samples.[31]