

Rapid Detection of Food-borne Pathogens

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Background

The main food-borne pathogens causing food-related illnesses in humans include *salmonella*, *E. Coli 0157*, and *Campylobacter*. These pathogens can enter the food chain in many different ways, and it is for this reason that their control is both difficult and inefficient (Buncic, 2006). Table A.1 summarizes the infective dose and incubation period for several common types of food-borne pathogens.

Food analysis has several key challenges that impede the proper detection of pathogens. These challenges include: uneven distribution of bacteria in food; presence of indigenous microbes that affect the identification of pathogens; and, the heterogeneous nature of food matrices (Mandal et al., 2010). Other important factors to consider in food analysis are time and sensitivity of the analyses, and to minimize human errors and labor costs.

The development of rapid and reliable detection methods for food-borne pathogens is ongoing. Examples of these methods include biosensors (e.g., bioluminescence biosensor, impedimetry, piezoelectric biosensors, etc), immunological methods, and nucleic acid based assays (Mandal et al., 2010).

Table 1.1: Common Pathogenic Microorganisms

Microorganism	Associated Foods	Infective Dose (no. of organisms)	Incubaton Period
<i>Campylobacter jejuni</i>	Raw milk, and raw or under-cooked meat, poultry & shellfish	400-500	2 to 5 days
<i>Salmonella spp.</i>	Raw/undercooked eggs, poultry, and meat; raw milk and dairy products; seafood; chocolate; salad and spices	15–20	12 to 24 h
<i>E. coli</i>	Raw/undercooked eggs, poultry, and meat; raw milk and dairy products;	<10	2 to 4 days
<i>L. monocytogenes</i>	Soft cheese, raw milk, improperly processed ice cream, raw leafy vegetables; raw meat and poultry	< 1000	2 days to 3 weeks
<i>Clostridium botulinum</i>	Improperly canned foods and vacuum packaged and tightly wrapped food	< nano grams	12-36 h
<i>Hepatitis A virus</i>	Sandwiches, fruits and fruit juices, milk and milk products, vegetables, salads, shellfish, and iced drinks	10-100	Unknown
<i>Norwalk virus/Norovirus</i>	Raw oysters/shellfish, water and ice, salads, and frosting	Presumed to be low	1-2 days

2

Methods

In order to be deemed an effective detection method for food-borne pathogens, methods should meet the following requirements (Mandal et al., 2010):

1. Detection method must be rapid
2. The method should detect the desired specific pathogens
3. Method must be sensitive to detect small numbers of pathogens
4. The detection method should produce a quantitative analysis to help determine the severity of the hazard
5. The method should be multiplex (i.e., capable of detecting more than one contaminant simultaneously)

Rapid detection methods are generally classified as either modified and automated conventional methods, biosensors, immunological methods, or nucleic acid based assays.

Table 2.1 provides some key characteristics of the above-mentioned methods.

Table 2.1: Characteristics of rapid methods (Adapted from Mandal et al. (2010))

Method	Detection Limit [cfu mL ⁻¹ or g]	Time Before Result [h]	Specificity
Bioluminescence	10 ⁴	0.5	No
Impedimetry	1	6-24	Moderate/Good
Immunological Methods	10 ⁴	1-2	Moderate/Good
Nucleic Acid Based Assays	10 ³	6-12	Excellent

2.1 Biosensors

Biosensors in their simplest form are analytical devices that convert a biological response to a measurable electrical signal proportional to the concentration of the analytes. A biosensor consists of a bioreceptor or biorecognition element and a transducer. A bioreceptor can either be a tissue, microorganism, organelle, enzyme, antibody, etc, while the transducer may be optical, electrochemical, thermometric, etc (Su et al., 2010).

Figure 2.1 shows a graphical representation of a biosensor. As the bioreceptor (*blue box*) recognizes the target analyte (represented by *yellow triangles*), a biological response occurs and is converted into an equivalent electrical signal by the transducer (*green box*). The electrical signal is passed to an amplifier (*red-brown box*) where it is converted to an essential waveform, and passed onto a signal processor. Figure 2.2 provides a summary of classifications for bioreceptors and transducers (Velusamy et al., 2010).

Biosensors are very useful in food-borne pathogen detection. For instance, they have a sensitivity in the range of ng/ml for microbial toxins; provide fast or real-time detection; and, the miniaturization of biosensors allow for integration in food production equipment and machinery (Rasooly and Herold, 2006).

The main disadvantage of biosensors is the instability of the biological sensing component, which tends to degrade and lose its effectiveness over a short-period of time. This is due to the various stresses encountered within an environment,

including pH, temperature or ionic strength (Cháfer-Pericás et al., 2010).

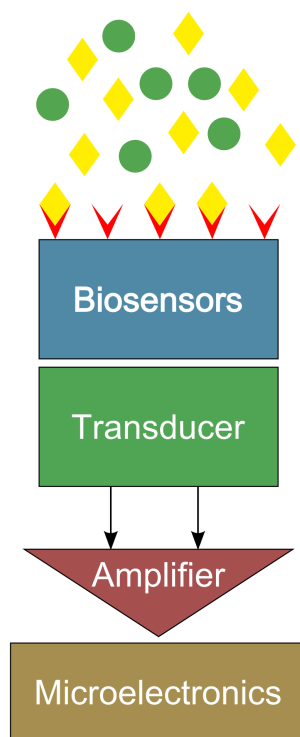


Figure 2.1: Principle of Biosensors

Sections 2.3, 2.1.2 and 2.1.3 provide a detailed summary of three types of biosensors.

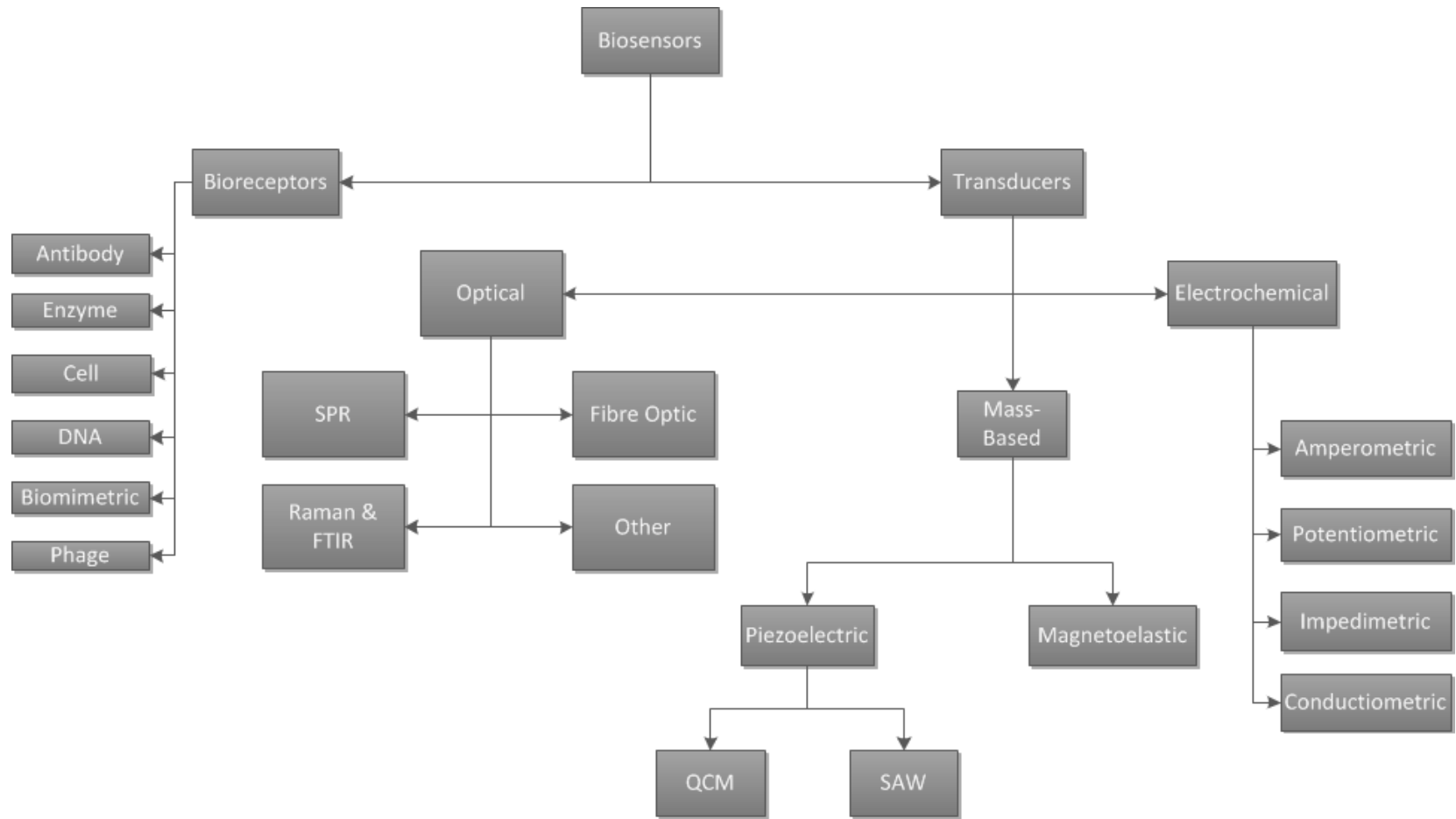


Figure 2.2: Biosensor Classification Flowchart

2.1.1 Bioluminescence Biosensor

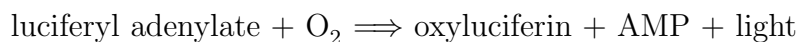
A bioluminescence biosensor measures the change in luminescence emitted by living microorganisms. There are two general types of bioluminescence used in the food industry: (1) ATP bioluminescence; and (2) Bacterial bioluminescence (Mandal et al., 2010).

ATP Bioluminescence

ATP bioluminescence techniques are commonly used to measure the effectiveness of cleaning surfaces and utensils. The general procedure takes a swab sample and combines it with a mixture of luciferase/luciferin (enzyme/substrate). As summarized in the following two reactions, the reaction of luciferin with luciferase requires the presence of ATP.



Light is produced once luciferyl adenylate reacts with Oxygen. Other products of this reaction include oxyluciferin and AMP.



The main advantages of using a bioluminescence biosensor to detect food-borne pathogens include extreme sensitivity and speed in detection, ease of implementation, and their ability to be used in portable field devices (Guisan, 2006).

One of the disadvantages of this method include the non-specificity of ATP assays since ATP is present in all living cells. For this reason, bioluminescence sensors alone do not provide any information regarding the origin of the ATP source. This problem has been addressed by coupling the sensors with other detection and identification techniques (Fratamico et al., 2005).

Bacterial Bioluminescence

The gene responsible for bacterial bioluminescence is known as the lux gene. DNA carrying this specific gene can be introduced into host-specific phages as shown

in Figure 2.3. Once the lux gene is transferred to a host bacterium during infection, bioluminescence occurs. Bioluminescence will only occur once the lux gene is transferred since the host-specific phage does not have the necessary intracellular components to express the gene (Mandal et al., 2010). Luminometers are used to accurately detect the light emission.

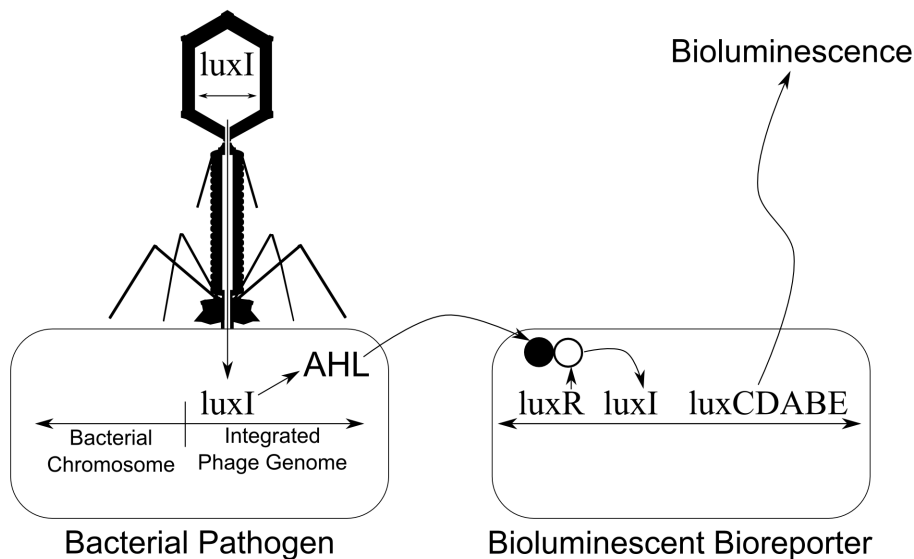


Figure 2.3: Bacterial Bioluminescence

Bacterial bioluminescence is capable of detecting 100 cells/hr with a specificity defined by the host-specific phage.

2.1.2 Impedimetry (Electrical Impedance)

During microbial growth in culture medium, small changes in impedance¹ can be continuously observed using electrochemical impedance spectroscopy (EIS) and passing known amounts of electrical current through the medium (Yang et al., 2008).

Advantages of impedimetry methods include the capability to monitor large numbers of samples simultaneously, good agreement between standard viable counts and detection times, and a relatively short detection time (between 6 to 24 hrs) (Hayes, 1992).

¹A material's opposition to the flow of electric current (measured in ohm's).

Disadvantages or drawbacks to this method include the limitation of this technique to large numbers of microorganisms and the influence of the food matrix on the analysis which requires a calibration curve for each particular food matrix (de Boer and Beumer, 1999). Also, the size, shape, materials, and biological receptors of the electrode greatly affect the overall performance of the biosensor. Recently, the use of nano-particles has improved the impedimetric biosensor by reducing the size of the electrode and for signal enhancement purposes (Yang et al., 2008).

2.1.3 Piezoelectric Biosensors

The piezoelectric principle usually describes the properties of crystals and their capability to generate electrical potential in response to a mechanical force. This makes piezoelectric biosensors suitable for direct, label-free detection of specific nucleic acid targets, making them useful for food-borne pathogen detection (Zourob et al., 2010).

A popular type of piezoelectric biosensor is the quartz crystal microbalance, as shown in Figure 2.4 (Zourob et al., 2010). Specific oligonucleotide patterns are immobilized on the surface of the quartz crystal and placed in a solution containing that contains potential target nucleic acids. Once the target nucleic acids start to bind to their complementary oligonucleotides the mass of the piezoelectric biosensor increases with a proportional decrease in the resonance frequency of the quartz oscillation (Zourob et al., 2010).

Piezoelectric biosensors offer a number of advantages including real time output, cost effectiveness and an inherently easy use. However, these biosensors are generally difficult to regenerate after hybridization. It is postulated that due to a decrease in manufacturing costs, these detectors may become a cost effective one-time use tool. Other disadvantages include lack of specificity and sensitivity, and interference at the sensor surface (Zourob et al., 2010).

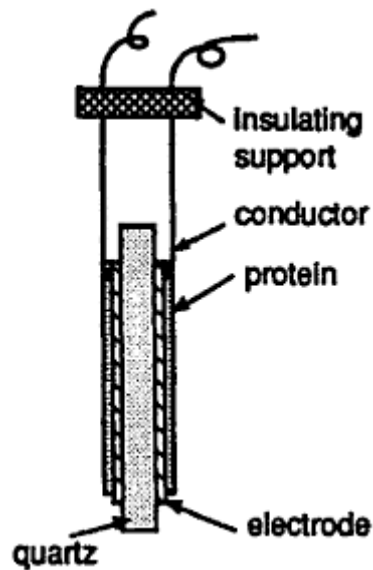


Figure 2.4: Piezoelectric Biosensor (Adapted from Tran (1993))

2.2 Immunological Methods

Immunological methods rely on the binding of antibodies to antigens. The specific binding of antibodies/antigens is determined qualitatively and quantitatively by immunological reaction by immunoassays. This technology is touted as being the only technology that has successfully detected bacterial cells, spores, viruses and toxins. This particular method has been used to detect *Escherichia coli*, *Salmonella*, *L. monocytogenes*, *Staphylococcal enterotoxins* and *Campylobacter spp.* in various studies.

There has been an increased use of immunoassays for the rapid food-borne detection of pathogens because of the:

- Availability of new and highly sensitive assays
- Mechanical devices that automate tedious steps
- Hybridoma Technology²

²A technique that produces monospecific antibodies from a single antibody-producing cell (Walsh, 2002).

Hybridoma technology has made the immunological detection methods more sensitive, specific and reproducible.

2.3 Nucleic Acid Based Assays

There are three main types of Nucleic Acid Based Assay techniques, including DNA hybridization, polymerase chain reaction, and DNA microarray (Gene chip technology).

These three types are explained in detail in Sections 2.3.1, 2.3.2 and 2.3.3.

2.3.1 DNA Hybridization

DNA hybridization (also known as gene probe assays) refers to the detection of DNA or RNA targets using complementary nucleic acid probes. This method identifies bacteria based on the presence or absence of particular genes (Mandal et al., 2010).

This procedure usually requires the denaturation of double-stranded DNA (ds-DNA) probes, whereas, single stranded probes do not require this step. The probes are labeled with a radioactive substance in one of two methods: (1) Nick translation or (2) Random priming technique (Mandal et al., 2010).

The target nucleic acids are also denatured at extreme conditions ($> 95^{\circ}\text{C}$ or $\text{pH} > 12$). The labeled gene probe is added and if the sample contains the same sequence as the gene probe, it will form a bond. Autoradiography is then used to detect probe target complexes.

DNA hybridization toolkits are commercially available. One particular example is Gene Trak, which is suitable for detection of *Salmonella typhimurium*, *Listeria monocytogenes* and *Escherichia coli*.

2.3.2 Polymerase Chain Reaction

General Information

PCR is an *in vitro* method that selectively duplicates a specific section of DNA. The section of DNA to be amplified is known as the target DNA and is defined by a pair of oligonucleotide primers (Patel, 1994).

There are three main processing steps in PCR:

1. Denaturation
2. Annealing
3. Extension

In the first step of the PCR method, DNA is heated to 95-98°C, at which point double stranded DNA separates into two single strands. In the annealing step of the method, synthetic oligonucleotide primers are added and bind to the single strands at a temperature of 55°C. Finally, in the last step of the process the primers are extended by DNA polymerase in the presence of adenine, guanine, cytosine, and thymine. The extension by polymerase occurs at a temperature near 72°C. These three steps result in new DNA strands that are complementary to the original strand that was separated in step 1 (Smith, 2004). Figure 2.1 shows a graphical representation of the PCR process.

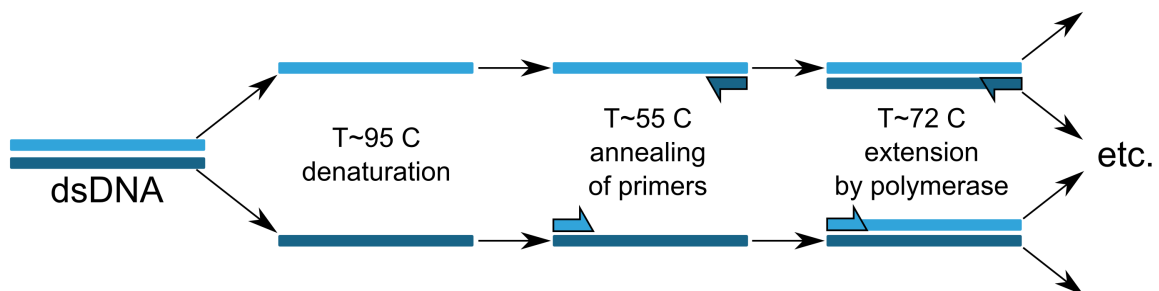


Figure 2.5: Polymerase Chain Reaction

The above cycle can be repeated many times. The amount of replication can be represented by Equation 2.1, where n represents the number of cycles.

$$\text{Number of replications} = 2^n \quad (2.1)$$

PCR techniques for detecting food-borne pathogens have many advantages. In particular, PCR has a high specificity and rapidity for tests when compared to traditional cultural techniques.

Real-Time PCR

Real-Time PCR is a technique used for the continuous collection of fluorescent signals from one or more PCRs. It is also known as quantitative real time polymerase chain reaction or kinetic polymerase chain reaction.

There are two general techniques used to obtain a fluorescent signal from the amplification of product in PCR. The first technique uses the inherent properties of fluorescent dyes such as SYBR Green I, as shown in Figure 2.6. As the dyes bind to dsDNA and undergo a change in shape, it increases their fluorescence (Logan et al., 2009).

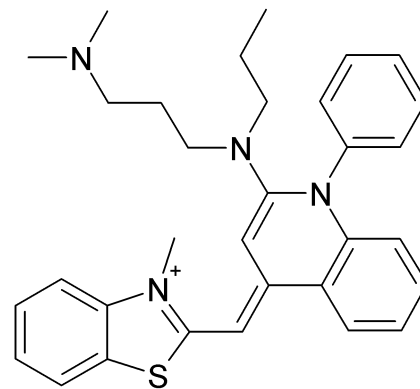


Figure 2.6: SYBR Green I

The second approach uses fluorescent resonance energy transfer (FRET). FRET relies on the presence of two molecules that interact with one another, where at least one of the molecules must have fluorescent properties. The fluorescent molecule is known as the donor, while the non-fluorescent molecule is known as the acceptor. During fluorescent resonance energy transfer, the donor molecule is excited by an external source. It emits light at a shifted, longer wavelength, which is then used to excite the acceptor molecule. It is not necessary for the acceptor molecule to emit light. The signal emanating from the acceptor molecule will be detected using the real-time instrument (Dorak, 2006).

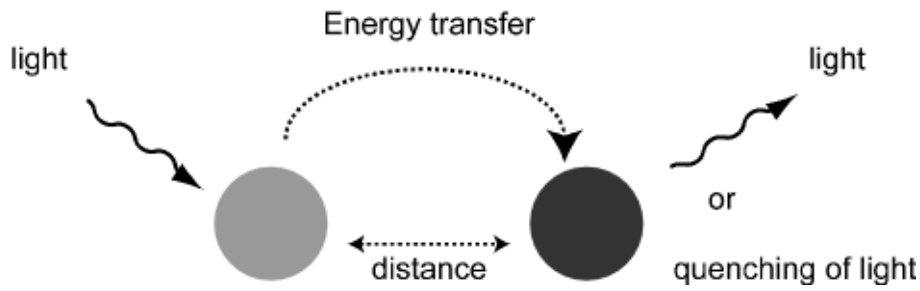


Figure 2.7: Fluorescent Resonance Energy Transfer

There are many advantages of using Real-Time PCR. One of the main advantages over traditional PCR is that real-time PCR collects data in the exponential growth phase as opposed to the plateau phase. Typical growth stages associated with the PCR method are indicated in Figure 2.8. Other advantages include an increase in dynamic range of detection, and no-post PCR processing (Padilla et al., 2009).

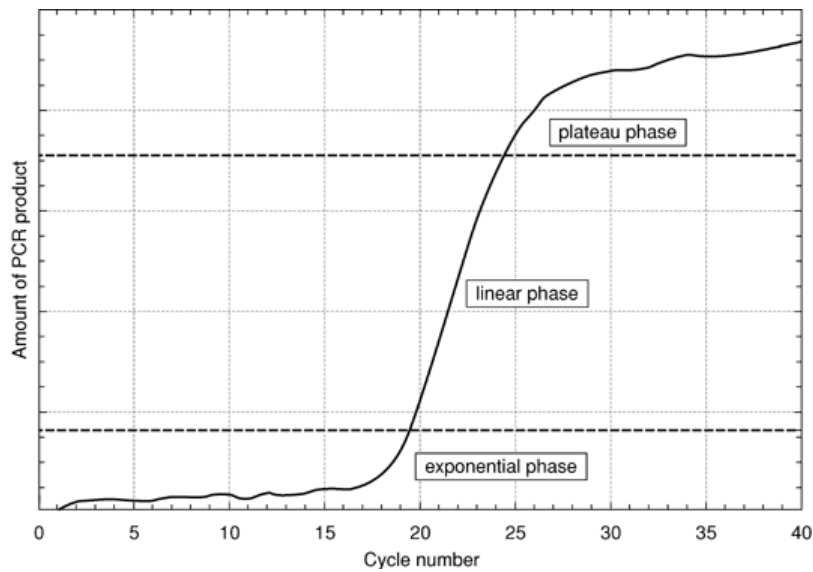


Figure 2.8: PCR Growth Stages (Adapted from Padilla et al. (2009))

Real-Time PCR has many different applications. It is currently being used in the fields of viral quantitation, drug therapy efficacy, pathogen detection, and genotyping, among others.

PCR Product Detection

The amplified DNA levels are typically detected by various methods. Common examples include gel electrophoresis, calorimetric, or chemiluminescent assays (Marriott and Gravani, 2006).

2.3.3 DNA Microarray (Gene Chip Technology)

The DNA Microarray technique uses photolithography, a popular method used for fabricating semiconductors in the computer chip industry. DNA arrays that are used for pathogen detection usually consists of glass slides or nylon membranes where PCR products are bound (Fratamico et al., 2005). Microarrays are a series of DNA molecules fixed on a substrate at predetermined locations. The sequence of the DNA molecules is know, and are referred to as features.

Figure 2.9 shows an example of a DNA array used for studying the difference in gene expression using two fluorescent dyes. Common dyes are Cy3 and Cy5. mRNA is extracted from the control and experimental cells, and labeled with a fluorescent dye. They are then subsequently hybridized to a platform containing DNA probes specific for a particular sequence (Fratamico et al., 2005).

DNA microarrays offer a rapid and efficient method of detecting simultaneously thousands of specific DNA sequences. They are also capable of revealing the level of expression. Presently, the technology is limited by the need for specially trained operators and the expensive equipment required (Kostrzynska and Bachand, 2006).

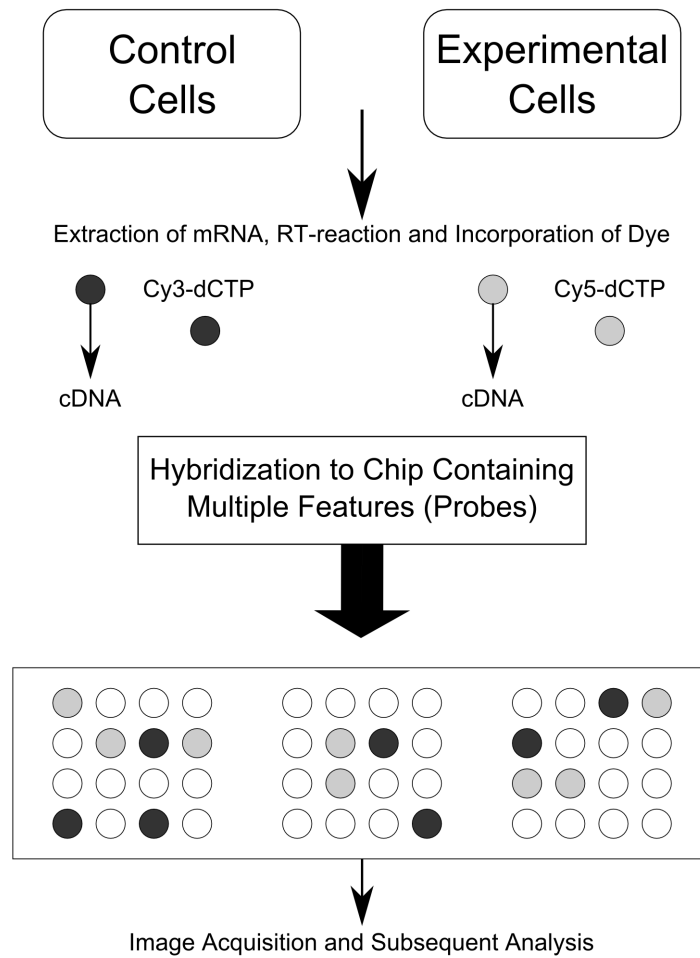


Figure 2.9: Microarray (Adapted from Fratamico et al. (2005))

3

Recent Developments

Biosensors based on micro- and nanofabrication techniques are emerging as rapid-detection methods for food-borne pathogens. For instance, the use of quantum dots as potential fluorescent probes for detection is being studied (Vinayaka and Thakur, 2010). Quantum dots are semiconductor nanocrystals that are generally considered dimensionless due to their small size. Their sizes range between 2 and 10 nanometers in diameter (Webb, 2010). They have narrow, highly specific, stable emission spectra, which make them very useful for pathogen detection as shown in Figure 3.1.

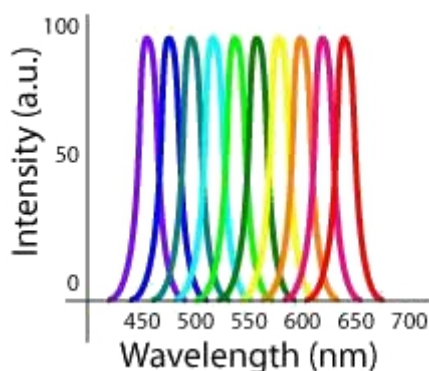


Figure 3.1: Photoluminescence Spectra of the Quantum Dots at Wavelength of Emission

Quantum dots offer many advantages to the rapid detection of food-borne pathogens. For instance, they have a high specificity, offer rapid detection, ease of mass fabrication and field applicability due to its mobility (Vinayaka and Thakur, 2010). When compared to traditional organic dye molecules used in fluorescent labeling, they show narrower emission peaks, higher emission intensity, and longer life time than dye molecules (Heo and Hua, 2009).

In a recent study by Sungkanak (2010), a microcantilever-based biosensor for detection of *V. cholerae O1*¹ was presented. The commercial gold-coated atomic force microscopy microcantilevers are immobilized with a monoclonal antibody and used to detect *V. cholerae O1* in concentrations ranging from 1×10^3 to 1×10^7 CFU/ml. This new technology will likely address the limitations of limited assay life-time, complicated detection steps, and inadequate sensitivity for detection of *V. cholerae O1*.

Another area where research is being focused is in the development of phage-based assays. Phages are highly host-specific, as they only infect specific species or strains². The specificity of phages is partly due to tail-associated proteins that can recognize surface molecules of specific bacteria.

Phages can be used for both detection and prevention of food-borne pathogen. As a detection tool, phages can carry reporter genes that are expressed only after infection. Such a tool would provide a detection method that is both rapid and also capable of differentiating between living and dead cells. Phages can also be used for specific killing of unwanted bacteria, such as those known to cause illnesses in humans (Ripp, 2010).

¹An important food-borne pathogen.

²There are a few exceptions. For example, *Listeria* phage A511

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Conclusions

Illnesses caused by food-borne pathogens have demonstrated the need for improved and reduced detection time. For example, in a recent Canadian case, a listeriosis outbreak in 2009 caused the death of 22 people. Other mentionable outbreaks have been summarized in Appendix B.

Since food-borne pathogens are generally present in very low numbers, mixed in with millions of other bacteria, it is imperative to develop detection methods that have high specificity but can also return results in a timely fashion. The techniques outlined in this report highlight some of the available tools, but as research continues the methods improve as well.

Recent developments have shown that there are promising tools on the horizon in the form of quantum dots, phage-based assays, and microcantilever biosensors. These new tools will reduce the likelihood of severe future contaminations.

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Appendices

Appendix A

Recent Outbreaks

Table A.1: Common Pathogenic Microorganisms

Pathogen	No. of Illnesses	Food Consumed	Place/Year
<i>Campylobacter jejuni</i>	33	Cooked Chicken	High school in Japan, 2005
<i>Salmonella spp.</i>	72	Egg, Squash & Seafood	Funeral Service in South Korea, 2007
<i>Salmonella spp.</i>	1435	Hard Pastry with vanilla cream	Spain, 2007
<i>Hepatitis A.</i>	601	Green Onions	USA, 2003
<i>Listeria monocytogenes</i>	22 deaths	Deli Meat	Canada, 2009
<i>Norwalk-like viruses</i>	130	Salid Sandwiches	India, 2002

Appendix B

Proposal

The main food-borne pathogens causing food-related illnesses in humans include *salmonella*, *E. Coli 0157*, and *Campylobacter*. These pathogen can enter the food chain in many different ways, and it is for this reason that their control is both difficult and inefficient (Buncic, 2006).

Food analysis has several key challenges that impede the proper detection of pathogens. These challenges include: uneven distribution of bacteria in food; presence of indigenous microbes that affect the identification of pathogens; and, the heterogeneous nature of food matrices (Mandal et al., 2010). Other important factors to consider in food analysis are time and sensitivity of the analyses, and to minimize human errors and labor costs.

The development of rapid and reliable detection methods for food-borne pathogens is ongoing. Examples of these methods include biosensors (e.g., bioluminescence biosensor, impedimetry, piezoelectric biosensors, etc), immunological methods, and nucleic acid based assays (Mandal et al., 2010).

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