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## ARTICLE

# A review of methods for the detection of pathogenic microorganisms

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The testing and rapid detection of pathogenic organisms is a crucial stage in the prevention and identification of problems to health, safety and wellbeing. In the United States and Europe, testing of faecal indicator bacteria where humans interact is routine. With the prospect of inevitable population growth and influx of tourism to certain water bodies testing will become a requirement to control and prevent possible outbreaks of potentially fatal illnesses. Legislation is already particularly rigorous in the food industry, where failure to detect pathogenic material represents a catastrophic event particularly for the elderly, very young or immune-compromised. In spite of the need and requirement for rapid analytical testing, conventional and standard bacterial detection assays may take up to seven days to yield a result. Given the advent of new technologies, biosensors, chemical knowledge and miniaturisation of instrumentation this timescale is not acceptable. This review represents an opportunity to fill a knowledge gap for an extremely important research area; discussing the main techniques, biology, chemistry, miniaturisation, sensing and the emerging state-of-the-art research and developments.

## 1. INTRODUCTION

The field of pathogenic microorganism detection is by no means a new one and microorganisms since time immemorial have been an integral part to life on Earth<sup>1</sup>. In fact, a recent renaissance on microbial impact has given researchers a new insight into the functional mechanisms into the symbiosis of microbes with human life<sup>2,3</sup>. However, some microorganisms are known to cause diseases and can have calamitous effects on humans and can cause detrimental health issues<sup>4</sup>. The ability of most microorganisms to evolve rapidly allows them to adapt and grow under various conditions like high or low temperatures, acidic or basic pH, various pressures and range of salinity<sup>5,6</sup>. These abilities allow some of these microbes to cause substantial damage to life<sup>7</sup>. Under this scenario, the commonly used control measures and sampling techniques are not robust enough maintain infection control. Moreover, current protocols do not provide us with adequate reactive mechanisms for such circumstances. The early detection of pathogens are very crucial and key for diagnosing and preventing the diseases<sup>4,8</sup>.

Currently, conventional methods are used to detect pathogens owing to their high selectivity and high sensitivity<sup>4</sup>. However, these methods are prone to lengthy experimental turnaround for results and miss the correct sampling period – leading to misinterpretation of growth of the organisms in question<sup>9,10</sup>. Some emerging methods such as biosensors are being developed, but their future is in reaching the same level of selectivity for a fraction of the cost<sup>11</sup>. Furthermore, the issue of ease of use, maintenance programme and in-situ, real-time approaches are requiring due consideration. This review aims to give a focussed overview of the field of pathogenic microorganism detection from basic conventional laboratory-based assays through to the emerging state-of-the-art particularly emerging from the material sciences.

### 1.1 Early Detection

Monitoring is the primary control point in the prevention of diseases caused by pathogenic microorganisms<sup>12</sup>. Effective detection methods are necessary to control this effect and have been realised through the ages via the use of conventional microbiological methods<sup>13,14</sup>. These standard methods have

been a common practice for nearly a century and continue to be a readily resourced practice for this detection type<sup>15</sup>. These conventional methods rely almost exclusively on the use of specific agar media lines to isolate, culture and enumerate viable cells in samples<sup>16</sup>. The standard sampling procedure typically includes the culturing and isolation of the pathogen, followed by subsequent confirmation by biochemical and serological tests sometimes taking up to five to seven days to get a confirmed result for a particular pathogenic organism<sup>17</sup>. The microbial quality of water is currently monitored through enumeration of faecal indicator bacteria: thermotolerant coliforms (ThC), *Escherichia coli* (*E. coli*), enterococci, and the spores of sulphate reducing anaerobes<sup>18</sup>. These organisms are used as markers for the level of faecal inputs and thus the probability of pathogens being present. Furthermore, enteric pathogens such as; viruses, protozoa and some bacteria have various survivability than faecal bacterial indicators in water environments owing to grazing protozoa and loss of culturability as they undergo different environmental stresses<sup>19,20</sup>.

## 1.2 Faecal Indicator Bacteria

Owing to the limitations for the direct monitoring of pathogens, it is standard practice to monitor faecal indicator bacteria (FIB)<sup>21</sup>. Epidemiology has already established a set of human health standards based on exposure limits to FIB in drinking, recreational and aquaculture waters<sup>22</sup>. The most serious threat to human health is thought to be of human nature, not animal, and thus most emphasis and research output has been driven from human sewage research<sup>23</sup>. It should be noted that indicator bacteria do not identify sources of contamination and thus the difficulty to identify point source markers becomes apparent. Therefore an adequate faecal indicator should be one that does not reproduce externally to the animal host, where both *E. coli* and enterococci can survive, grow and establish populations in natural environments: soils and sediments,<sup>24</sup> freshwater water bodies,<sup>25</sup> and within plant matrices<sup>26</sup>. An ideal indicator should be correlated with the presence of a pathogen and should ideally have the survival profile similar to the pathogen it is trying to indicate. For instance, *E. coli* and enterococci are not well correlated with *Salmonella spp.* For instance, the poor correlation of bacterial indicators with viruses is of a growing concern owing to the typical attributes of a virus: low infectious dose, linked to acute and chronic disease and involved in recreational water associated illness<sup>27</sup>.

## 1.3 Microbial Source Tracking (MST)

Establishing the sources of faecal contamination of water bodies has been termed MST or bacterial source tracking (BST). However, microbes that are naturally occurring are ubiquitous to surface waters with typical numbers at an average density of 10<sup>6</sup> cells per mL<sup>28</sup> and viruses at an even higher density<sup>29</sup>. The methods associated with MST include: culture-based and culture-independent based methods<sup>30</sup>. Some of these described methods require a library or host database: which have been predetermined through culture-based results<sup>30</sup>. The identification of the source is determined by a comparison between test-

organism fingerprints in the library or database<sup>31</sup>. Furthermore, the library and database methods typically include a range of genotypic and phenotypic tests<sup>32</sup>.

## 2. PATHOGEN DETECTION METHODS

Some of the most popular methods to confirm the presence of pathogens are typically based on culture and colony counting methods and also the polymerase chain reaction (PCR)<sup>33</sup>. These methods have typically been analytically concrete for both selectivity and reliability<sup>34</sup>. However, the culture and colony counting methods are more time intensive than PCR methods<sup>35</sup>. Both techniques provide conclusive, robust and unambiguous results<sup>8</sup>. The recent advances in PCR, in the form of real-time PCR now enable fast results to be realised<sup>36</sup>.

### 2.1 PCR

PCR, is a nucleic acid amplification technique developed in the 1980s and is widely used to identify bacteria<sup>37</sup>. In principle, the method is constructed on the isolation, amplification and quantification of a short DNA sequence, inclusive of the target bacteria's genetic material<sup>38</sup>, Figure 1.

Some examples of developed PCR methods for bacterial quantification are: real-time PCR, multiplex PCR and reverse

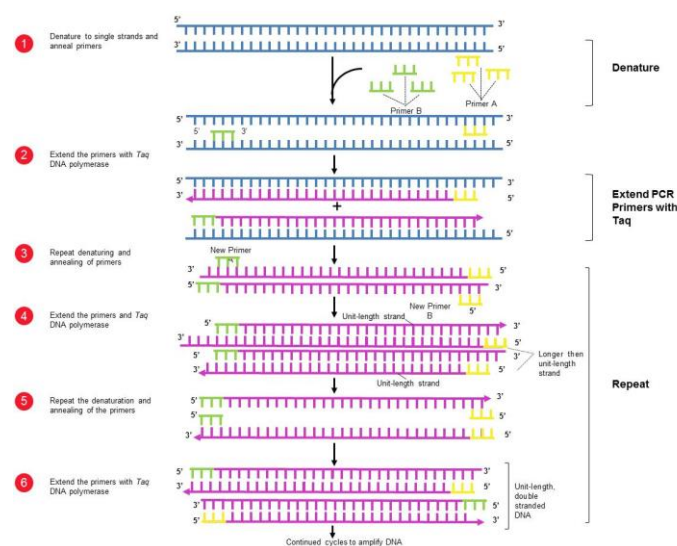


Figure 1: Schematic representation of a PCR cycle

transcriptase PCR. PCR is much less time consuming than other conventional techniques utilising culture and plating. PCR usually takes anywhere between 5 to 24 hours to produce a result but this is dependent on the specific PCR variation and excludes enrichment stages<sup>39</sup>. PCR technique is used for amplifying a specific DNA fragment from a complex pool of DNA<sup>38</sup>. PCR assay requires template DNA, primers, nucleotides and Thermostable DNA polymerase (Taq polymerase) enzyme. Nucleotides are referred to adenine, thymine, cytosine, and guanine (A, T, C, G)<sup>38,40</sup>.

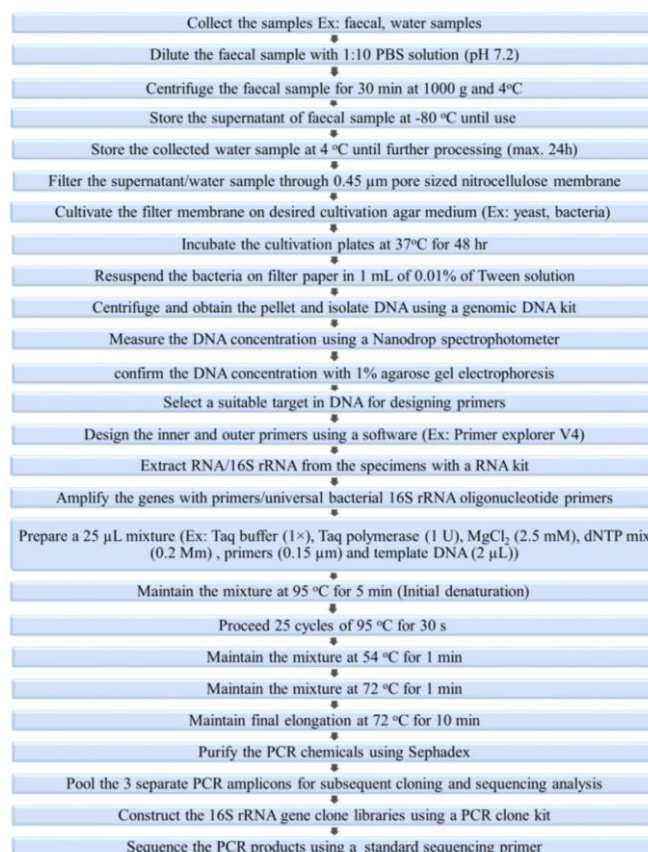
Figure 1 illustrates the basics of PCR whereby proceeds through 3 main phases; denaturation, primer annealing and primer extension<sup>40</sup>. Different synthetic cycles are performed and heat beyond melting point of DNA is applied to denature the targeted double stranded DNA. The DNA is then purified followed by an extension phase using primers and a thermostable polymerisation enzyme. Subsequently, each new double stranded DNA acts as a target for a subsequent cycle and exponential amplification is therefore attained. The primers are extended from 5' to 3' direction by polymerase enzyme to overlap the copies of original template<sup>40</sup>. Subsequent detection of the amplified sequence is detected through gel electrophoresis<sup>41</sup>. Of the methods listed multiplex-PCR is of most use<sup>42</sup>. Multiplex PCR allows the simultaneous detection of several organisms through the introduction of different primers to amplify DNA regions coding for specific genes of each bacterial strain targeted<sup>43</sup>. In real time PCR faster results can be obtained without the need for manipulation of the recognition stages<sup>44</sup>. Real-time PCR results are obtained through the detection of fluorescent emission by a specific dye that attaches to the targeted amplicon<sup>45</sup>. Fluorescence intensity is directly proportional to the amount of amplified product<sup>46</sup> and therefore it is possible to follow this response in real-time eliminating laborious post-amplification processes such as the already mentioned, gel electrophoresis<sup>47</sup>.

Faecal samples were obtained from cattle and other animals for analysis and quantification of O157 *E. coli* with the development of a relatively sensitive method using multiplex real time PCR (qPCR). This method essentially targets the O157 somatic antigen gene, along with Shiga toxin genes produced from several pathogenic strains of *E. coli*. The LOD for this method was detected to be 103 of genome copies; this method proved to be an effective rapid and sensitive technique while altogether reduces cost and time of screening<sup>48</sup>.

Kou, X. *et al.* employed a different aspect of PCR, loop-mediated isothermal amplification assay, for the detection of rotavirus in faecal samples and artificially seeded oysters. A specific region, the VP7 region of the rotavirus genome was selected for use of detection, where four primers were designed for identification of six distinctive sections located in that area. Results of amplified products were executed by different processes; gel electrophoresis, turbidity and colour changes, providing easy detected observations to the naked eye. The LOD of the technique was 0.5 pg of rotavirus RNA; this practice can be executed in laboratory and field work as a simple, sensitive and cost-effective screening tool<sup>49</sup>.

However, a current research lead by Yang, et al. 2014<sup>49</sup> has demonstrated a more effective technique analysing samples with more specificity and sensitivity, called the Droplet digital PCR (ddPCR). The scientists used two loci from a range of *Cryptosporidium* DNA templates to evaluate the specificity and linearity comparing conventional qPCR and the emerging ddPCR. The later acts as an autonomous nano-PCR, where

targeted nucleic acids are randomly assorted and contained in each droplet containing from zero to one (or more) copies of that particular nucleic acid. Each droplet fluoresces and is therefore measured individually – this also provides more readily detected intensity as small changes are more easily observed individually by the instrument as compared to an amount required by the qPCR. The major advantage of this method over conventional qPCR includes the non-requirement of a calibration curve; absolute quantitation is obtained without difficulty. Precision of droplet digital is also significantly higher than qPCR but decreases as DNA concentration decreases. Due to its nano-litre



volume, the ration between target DNA molecules to PCR reagents is substantially higher. Overall cost was also significantly lower than conventional qPCR<sup>50</sup>.

Considering the recorded studies a summarised example protocol for PCR analysis is given in Figure 2.

**Figure 2: A summarised protocol of a PCR analysis**

### 2.1.1 Limitations of PCR

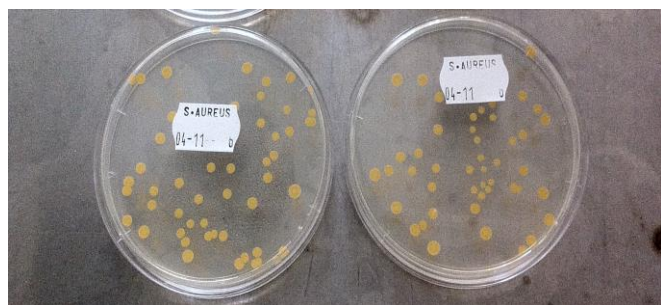
One of the most apparent limitations of PCR techniques comes from the lack of differentiation between viable and non-viable cells<sup>51</sup>. This is because DNA is always present whether the cell is alive or dead. Reverse transcriptase PCR (RT-PCR) was developed to counteract this lack of discrimination, and is capable of differentiating viable cells<sup>52</sup>. Reverse transcriptase is

an enzyme able to synthesis single-stranded DNA from RNA in the 5' – 3' direction<sup>53</sup>. Several other genes, specifically present during the growth phase of bacteria, can also be detected<sup>54</sup>.

## 2.2 Colony Counting and Culturing

Until recently, efforts to detect and identify organisms have depended largely on in-vitro methodologies. This is the most established method for bacterial detection and relies on directly culturing and plating the organisms, Figure 3.

Microbial cultivation has opened up a world of microscopic life detection?, whereby the rules were first conveyed by Robert Koch, known as Koch's postulates<sup>55</sup>. Koch's postulates are a set of ground rules that determine whether a given organism can cause disease. One of the rules states that a bacterium must be shown to be able to grow outside of the human body to demonstrate it is disease ready<sup>56</sup>. However, more than often culturing based assay are inconvenient for industrial applications that require immediate results i.e. food, water industries<sup>57</sup>. Researchers already know that traditional methods of culturing bacteria are effective at



**Figure 3: Photograph of culturing and plating technique for *S. aureus***

identifying only a fraction of bacteria in a given sample. In 1932, Razumov observed large discrepancies in viable cell counts and total microscopic counts of bacteria from aquatic environments<sup>58</sup>. A final observation was recorded where Razumov found an order of magnitude of higher number of cells in direct microscopic counting rather than in plate counting illustrating the limitation in reliability. This was fully validated by Stanley and Konopka in 1985 who showed that Razumov's discrepancies were indeed correct, validated through thousands of water samples from Lake Washington performed using two methods of counting; direct microscope counting using acridine orange and a count of bacteria in a sample of water validating the presumptions of Razumov some 50 years later<sup>59</sup>.

In a project involving the confirmation of health-beneficial administered probiotic strains *Bifidobacterium Lactis* (*B. lactis*) HNO19 in the gastrointestinal tracts of children, Prasad *et al.*<sup>58</sup> utilised standard colony counting techniques for quantitatively enumerate bacterial populations from collected faecal samples. Here the, sample size of the experimental procedure was limited

as well as lack of baseline data. Comparable to normal bacterial culture, PCR provided with significantly higher and consistent counts.<sup>60</sup> In another experimental study comparing conventional culturing method to slowly emerging techniques such as PCR and biosensors, results showed that colony culturing and counting failed to account for particular enteric pathogens otherwise detected in other comparable molecular methods.<sup>61</sup>

Faecal coliforms have the ability to survive extremely harsh environments inclusive of predation by other grazing organisms. In a study by Davies *et al.*<sup>61</sup> faecal coliforms, faecal streptococci and *Clostridium perfringens* spores were analysed in fresh and marine water sediments near sewage outlets. Following a study for 85 days, the authors demonstrated that the cells were still present even through die-off periods recognising the inherent ability for these cells to survive. The protocol employed by the authors was simple: collection of sediments, predation studies, in-situ microcosm survival assays followed by enumeration. In this work, enumeration was carried out by placing a membrane filter on a 15 mL layer of m-Enterococcus agar. Results demonstrated that the red and dark colonies were presumptive of faecal streptococci.<sup>62</sup>

Microbial pathogens are the major cause of worldwide waterborne diseases and *E. coli* has been accepted as a universal water health indicator and surrogate microorganism among them. In a recent study, the authors Rajapaksha *et al.*<sup>63</sup> recorded antibacterial efficiency of graphene oxide and metal nanoparticles doped graphene oxide materials against *E. coli* microorganisms. The protocol of the recorded study was *E. coli* bacteria cultures were plated on LB-agar medium with positive and negative treatments. The antimicrobial assays were then followed by enumerating the number of colonies on agar plates after the incubation period at 37 °C. Results demonstrated white colonies of *E. coli* bacteria on agar plates and recorded 100% antibacterial efficiency with metal doped graphene oxide materials.

A range of other staining methods exist, in particular, *BacLight* staining<sup>64</sup>. *BacLight* staining has several advantages over standard cell counting methods. Namely, it is a reliable, rapid and easy to use assay, which can yield total count and viable cells in one-step<sup>65</sup>. Preparations of the assay components are simple along with an easy to read (obvious differences in contrast) between the green colour of the viable bacteria and the red colour of the dead cells<sup>66</sup>. The method relies on the principle of epifluorescence to gain a result, and will be discussed accordingly in this review.

### 2.2.1 Advantages and Limitations of classical culturing methods

Culture-based methods constitute the majority of conventional testing for bacteria detection. They are the basic tools used for detection around the world, due to their reliability in efficiency, sensitivity and range of application bases. Conventional culturing methods are still regarded as the gold standard for

confirmation of the obtained results in emergency scenarios. Beyond these advantages, the standard culture techniques require considerable amounts of laboratory equipment, consumables and time to detect pathogens. Unfortunately, more than often laboratory personnel need to be trained to prepare and interpret results, which makes wide-spread, non-specialist utilisation difficult. It is therefore apparent, that although culturing techniques are still a method of choice, more rapid, reliable and user friendly systems need to be developed.

Considering the mentioned studies, a summarised example protocol for colony counting method is given in Figure 4. Sample selection should be carried out in aseptically.

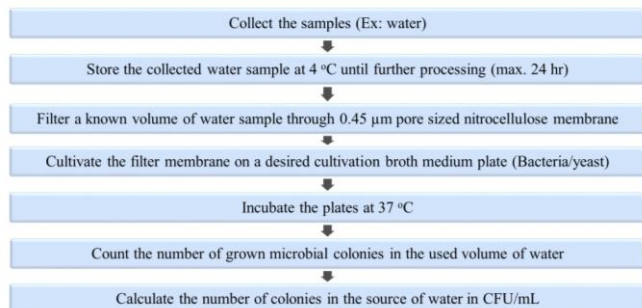


Figure 4: A summarised protocol for colony counting method

## 2.3 Flow Cytometry

Flow-cytometry is an optical detection based method for analysing individual cells in complex matrixes<sup>67</sup>. The method can be considered as a form of automated fluorescence microscopy in which a sample is able to be injected into a liquid which passes through the flow sensing area of the instrument<sup>68</sup>, Figure 5.

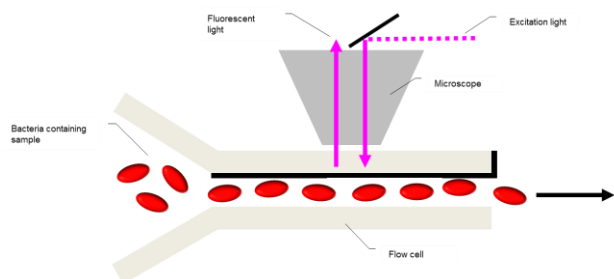


Figure 5: Flow cytometry method – single cell detection using optics and fluorescent markers i.e. propidium iodide.

The basic principle for microorganism detection include: (1) microorganisms are suspended in a liquid matrix (2) then pass through a beam of laser focused light. (3) When this occurs, the light is both scattered and absorbed by the target microorganisms. (4) The extent and the nature of this scattering process, which is an intrinsic property of the microorganisms with the light source, may be analysed by collecting the scattered

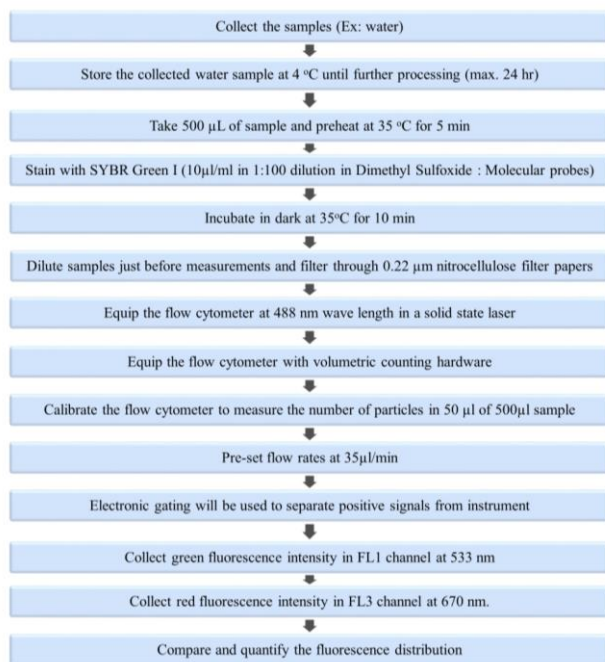
light with a system of lenses and photocells and can be used to determine the number, size, and shape of microorganisms<sup>69</sup>. More importantly, high sensitivity measurements can be achieved in very short times. Owing to the method's high sensitivity, flow cytometry is suitable for detecting low numbers of specific organisms in fluids, scrapes, rinses or whole liquid aliquots. Flow cytometry can not only examine bacteria but viruses. In particular, viruses in seawater<sup>70</sup> have been successfully counted after staining with a nucleic acid specific dye SYBR Green-I. In a typical experiment, samples were initially fixed with glutaraldehyde and then deep frozen prior to analysis using the flow cytometer instrument

Although the method preferably used in detecting bacterial numbers are direct immunofluorescence assay (DFA) and enzyme immunoassay techniques, Valdez *et al.*<sup>71</sup> proved flow cytometry can be modified and improved to deliver 15 times more sensitivity than these methods; appropriate fluorescent tags and a monoclonal antibody have been used and adapted as per study performed by Moss and Arrowood<sup>72</sup>. This experiment employed the use of faecal human samples in successfully detecting *Cryptosporidium* oocysts<sup>70</sup>.

Since 2011, flow cytometry techniques have been preferentially used alongside other methods such as qPCR and fluorescent biosensors to achieve more specific results. The reason flow cytometry is not used as a standalone technique is due to the numerous dilution steps needed to be performed prior to analysis – this is required to reduce the concentration of debris making it easier for antibodies to bind to their target and each individual cell analysed as they passed through the point of detection. Unfortunately, this decreases the potential sensitivity of the flow cytometer. Harba *et al.* have demonstrated the comparable differences between conventional staining methods utilising flow cytometry and IFAT<sup>73</sup>.

Figure 6: A summarised protocol for flow cytometer method

Another study conducted by El-Nahas *et al.*, comparing the direct immunofluorescence assay, direct microscopic examination and flow cytometry, showed that although flow cytometry is a rapid and sensitive method in the screening of large numbers of faecal samples for the presence of protozoan



cysts at exceptionally high speed; it did not show the expected sensitivity in their study<sup>70</sup>. A summarised example protocol for flow cytometer analysis is given in Figure 6.

### 2.3.1 Limitations of Flow Cytometry

Some of the inherent limitations of flow cytometry include; (1) the need for cells to be in a suspension affords a lack of tissue and cell architecture to be examined<sup>74</sup>, (2) Cell subpopulations with similar marker expressions are difficult to differentiate, where a method requiring more fluorophores are subjected to signal spill over<sup>75</sup>, and (3) Flow cytometer can generate large volumes of data, which makes the excess analyses cumbersome<sup>76</sup>.

### 2.4 Optical Biosensors

Optical transducers of an optical nature are an attractive technology for the direct detection of bacteria<sup>77</sup>. Optical transducers can detect the smallest of changes in refractive index, meaning that measurement can be sensitive to thickness changes when cells bind to the receptors on the transducer surface<sup>78</sup>. Several techniques have already been investigated: surface plasmon resonance, ellipsometry, and interferometry. Ellipsometry has a thickness resolution of 0.01 nm for studying layers of molecular dimensions on solid substrates<sup>79</sup>. Ellipsometry uses the principle of photons-in and photons-out, not requiring a vacuum, which makes it an attractive technique<sup>79</sup>. The method can be conveniently used directly at the solid and liquid interface making it practical for bacterial detection purposes. Recent advances in combined spectroscopic

ellipsometry has allowed analysis to extend beyond layer thickness and surface phenomena measurements to more qualitative and quantitative data such measurements include, surface dynamics, layer density effects, molecular orientation, clustering, and multilayering effects<sup>80</sup>.

Homola *et al.*<sup>81</sup> have extensively discussed the role of surface plasmon resonance (SPR) sensors for the detection of chemical and biological species<sup>81</sup>. Particular reference<sup>81</sup> has been made to pathogens; bacteria, protozoa, fungi and parasites. Fratamico and co-workers<sup>82</sup> first used SPR to detect *E. coli*. Since then, commercial SPR sensors have become available utilising monoclonal antibodies immobilised on a protein G-coated surface of the sensor<sup>82</sup>.

Bunyakul *et al.*<sup>83</sup> applied microfluidic biosensor techniques to detect cholera toxin found in stool samples, utilizing fluorescence and electrochemical detection by means of liposome amplification for the detection of Cholera toxin subunit B (CTB)-specific antibodies in real, spiked, faecal samples. Sample preparation was simple and non-laborious – centrifugation followed by filtration and magnetic capture practices. Stool samples were required to be free of simple debris for improved fluorescence detection, thus reaching a limit of detection of 9.0 mg/mL<sup>83</sup>.

Optical interferometry is a technique based on the detection of the interference pattern when polarised light is split into a reference and an analysis beam<sup>84</sup>. Optical interferometry is able to be coupled with real-time, liquid-phase, flow through and minimal processing. The technique has been used to characterise bacteria in food such as *Salmonella*, short time frames and low detection limits can be realised<sup>85,86</sup>. One of the biggest advantages to this technique is the short detection times, however, lack of sensitivity does not make this a method of choice.

### 2.5 Bioluminescent Sensors

Recent advances in the bioanalytical domains have led to the use of enzyme technology to emit photons as a by-product of their reaction mechanisms. This method has long been known as bioluminescence. The use of bioluminescence was initiated back in the 1980s by Ulitzur and Kuhn.<sup>87</sup> The team introduced genes encoding for luciferase of a bacteriophage. The virus infects the host bacteria, a bioluminescent phenotype is produced and thus bioluminescence is produced. Contag *et al.*<sup>88</sup> had developed a method for the detection of bacterial pathogens in a living host to evaluate disease processes for strains of *Salmonella typhirium* in a mouse model. In this work 3 strains of *Salmonella* were marked with bioluminescence through the transformation of a plasmid conferring constitutive expression of bacterial luciferase<sup>88</sup>. The authors observed patterns of bioluminescence that suggested the caecum might play a role in *Salmonella* pathogenesis.

Further assays have consequently been developed. In particular, bioluminescence assays that detect bacterial adenosine

triphosphate (ATP)<sup>89</sup>. Bacterial ATP is an excellent indicator for the presence of microbial contamination<sup>89</sup>. An important point to note that since ATP is present within all actively metabolising cells. This means that, in order for this method to solely detect microorganisms assays need to include steps to segregate microbial from somatic ATP – this is usually in the form of basic filtration. Turner *et al.*<sup>90</sup> demonstrated the efficacy and capability of an ATP-based system in detecting contaminants and harmful microorganisms from samples of faeces, rodent blood, urine, *E.coli*, *S. aureus*, *Toxocara canis* eggs, *Toxoplasma gondii* tachyzoites and epithelial cells. Gram-negative bacteria had a weak detection limit of 104 while being a 102 for Gram positive. Sonication of pure Gram-negative proved to be efficient in increasing limit of detection by applying a buffer agent, lysing the cell wall of the microorganism, releasing its ATP and providing a detection signal. Rodent faeces were successfully detected of up to a 1:105 dilution; faeces showed a notably improved detection limit as a community containing a mixed of various contaminants and bacteria.<sup>90</sup>

## 2.6 Molecular Detection Methods

Some emerging pathogens are not detectable by conventional microbiological techniques<sup>91</sup>. This has resulted in a move to use nucleic acid (RNA and DNA) based assays for the differentiation of pathogens in a matrix<sup>91</sup>. These nucleic acid based methods also include polymerase chain reaction (PCR), pulsed-gel electrophoresis, ribotyping, plasmid typing and randomly amplifies polymorphic DNA<sup>91</sup>.

### 2.6.1 Nucleic Acid Based Faecal Detection Assay

Over the past ten to fifteen years a significant increase in the development of genetic based methods for the detection and characterisation of pathogens has occurred<sup>92,93,94</sup>. The principle of genetic detection relies on the hybridisation of target DNA combined with a specific DNA-probe<sup>95</sup>. Dependant on the specificity of the detection albeit genus, species, strain, different regions of the genome can be used as targets<sup>96</sup>. Three types of nucleic acid based detection methods currently exist. (1) Nucleic acid hybridisation; is typically the interaction between DNA and RNA molecules present in the target organisms and a probe DNA which possesses a sequence complementary to the target sequence<sup>97</sup>. (2) Molecular subtyping methods are also used to identify different strains within a species. (3) Molecular subtyping has the ability to generate data with respect to taxonomic and epidemiologic purpose<sup>98</sup>. A nucleic acid based technique has the advantage over phenotypic identification as it is not influenced by environmental parameters<sup>99</sup>.

For the successful detection of *Clostridium difficile* *tcdA* and *tcdB* in fecal samples, Carroll, K. C. *et al.*<sup>100</sup> employed the use of a Verigene CDF test, a Nucleic Acid test, which operates by a multiplex qualitative PCR assay coupled with a nanoparticle-based array hybridization technique. The method has proven to increase significantly specificity, sensitivity and positive and negative predictive values as compared to either direct culture or a combination of direct and enriched culture techniques. It is

currently the only method known capable in detecting both toxins A and B of *C. difficile*, along with the ability to identify ribotype 027, where some studies have shown it may directly be associated to increased disease and mortality<sup>100</sup>.

Real-time PCR (rtPCR) differs from conventional PCR in a number of ways: (1) rtPCR does not require gel-based detection, (2) results are quicker since the amplification cycles are much shorter (3) rtPCR has different heating components, light sources and detectors<sup>101</sup>.

### 2.6.2 Advantages and Limitations of Molecular Detection Assays

Molecular methods provide a sensitive and rapid detection for many pathogens. Results can be achieved very quickly given the range of methods that are currently on the market<sup>102</sup>. The sensitivity of a molecular method can be improved if new primers are designed, thus these methods can be expected to evolve given advances in chemistry, biochemistry and pathogen types<sup>103</sup>.

PCR does have some drawbacks. PCR can be affected by the complex structure of certain matrices i.e. food. Also, some matrices may contain PCR inhibitors that influence amplification efficiency or primer binding, i.e. lipid content<sup>104</sup>.

## 2.7 Emerging Pathogenic Detection Assays

The discussion throughout this review has been centred on the tools available to analyse pathogens to date. The review will now focus on where some common research gaps are, and thus give insight into where emerging developments need to be considered. The general requirements of a recent detection method include:

- (1) Increased specificity;
- (2) Increased reliability;
- (3) Availability of use;
- (4) Rapid turnaround;
- (5) Low cost;
- (6) Having a standard method approach and;
- (7) High throughput.

### 2.7.1 Beyond State of the Art Considerations

There are several factors that need to be considered before adapting a new alternative or rapid method for pathogen and even microbial detection these are now described herein:

(1) **Accuracy Issues:** the detection of false-positive and false-negative results must be minimised or ideally close to zero. The designed method must be as sensitive as possible and limits of detection as low as possible. Analytical assays surrounding these agents need to only be qualitative. For more rapid screening assays, a higher false-positive ratio may be acceptable, where positive screening tests are followed by confirmation assays. The

**Figure 7: General costing of conventional, current and beyond state of the art techniques for pathogen detection**

overall sensitivity of an assay is the proportion of target organisms that can be detected; it can be calculated by the following equation:

$$\text{Sensitivity (\%)} = \frac{(p)}{(p) + (f)} \times 100$$

Where (p) is the number of true positives, and (f) is the number of false negatives.

If a target organism is not detected when the target is present, this is described as a false-negative result and will lower the overall sensitivity of an assay. The specificity of a method is described as the ability to discriminate between the target organism and other organisms or substances contained within the matrix:

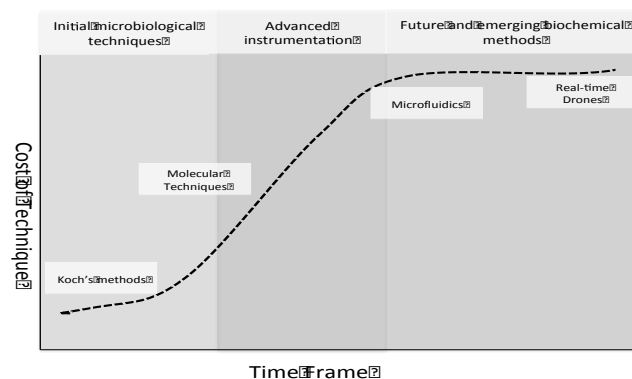
$$\text{Specificity} = \frac{(tn)}{(tn) + (fp)}$$

Where (tn) is the total number of true negatives, and (fp) is the total number of false positives. A positive result in the absence of the target is deemed a false-positive result and thus will lower the specificity of an assay

(2) **Turnaround of results** — for a rapid assay for the detection of pathogenic material should produce accurate results within hours, or at least within one day. Subsequently, many detection systems need an overnight enrichment stage for the resuscitation and further amplification of the target pathogens. This is owing to the reliance on the presence of at least  $10^4$ – $10^5$  organism/mL for results to be consistent. To advance the result turnaround real-time autonomous methods are required to be advanced. Microfluidic fabrication is one way of achieving this, where reagents are scaled down along with power supplies and footprint. This enables more options to produce smaller systems that can be left in the field or as a probe with telemetry so teams can analyse in real-time rather than waiting for extended lab based methods.

(3) **Sample matrix effects** — newly developed pathogenic detection methods should aim to give a good performance of the matrix that is to be tested within. A series of baseline extinction values may depend on the type of pathogen being tested. Considerations of the background flora, natural compounds and biochemical, or erroneous debris, can interfere with the assay and therefore invalidate the results produced. Sample matrix effects have long been known and have long plagued the analytical process. In order to produce improved detection of the target pathogen, filtration or pre-processing can be implemented.

(4) **Validation** — if an alternative method is to be developed, the assay should be validated against standard tests and be further evaluated by collaborative tandem studies. Outcomes obtained low pathogenic contamination levels should be stressed since there is adequate evidence that in most cases elevated numbers of target cells in samples will lead to positive test results.<sup>105</sup> To



deal with these problems, the European project MICROVAL was initiated by the European Community.<sup>106</sup>

(5) **Automation**— automation is the ability to test many samples at the same time, whereby improving replicates and statistical analysis of the given pathogen detection issue. Many systems use the microtitre plate format that can analyse up to 96 samples at one time, Figure 8.

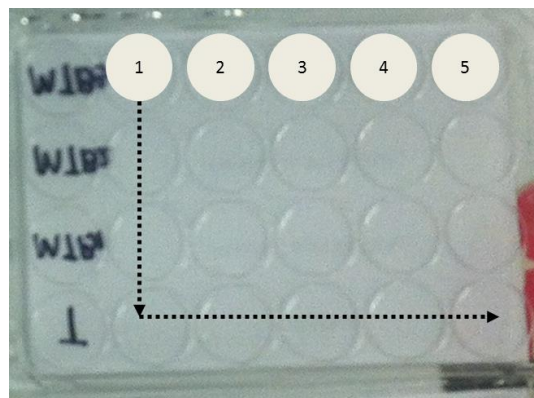


Figure 8: Photograph showing microtitre plate, illustrating the multiple sample assay approach.

(6) **Costs** — the purchasing of reagents and consumables, operational costs, personnel and instrumentation upkeep all contribute to the rising cost of basic validated, reliable and standard methods for pathogen detection. Laboratory costs if sending samples to accredited laboratories often exceed into the thousands. In conducting, some of the techniques discussed within this paper initial financial investment for rapid methods may be high, because many systems require expensive instrumentation and specialised reagents (molecular probes etc.). A summary of the cost related drivers have been highlighted in Figure 5 per technique per sample.

(7) **Others** — (a) Simplicity in the method; should be user-friendly, which means easy to operate and manipulate. (b) training where technical ability should be moderated and streamlined in order to obtain quick and cheap results, ideally suited to non-expert users. (c) Space, where small footprint instrumentation is a must. Large scale instrumentation makes space and laboratory rental space increase dramatically. The

knock on effects is to pass the cost on to the consumer. The development of microfluidic devices will enable this problem to be overcome dramatically.

### 2.7.1 Limitations to Beyond State of the Art

One of the major disadvantages of new and emerging, rapid assays over standard culturing assays is that most rapid methods include irreversibly, damaging the cells. Hence, viable cells for confirmation and further characterisation can only be attained through repeat experimentation using standard culturing procedures. Another limitation is that with rapid assays, the user is only able to detect one specific pathogen, while culturing assays offer a simultaneous detection result. In this discussion cost also forms a worthwhile discussion. Multipathogenic detection can be realised, but costs are unfavourable at present, highlighting a potential avenue for further research exploration.

### 2.8 Conclusions

Conventional methods have demonstrated a reliable, reproducible and resourceful tool for over a century in determining contamination with pathogens. Current state of the art techniques demonstrates reproducible sensitivity and are generally much quicker than conventional techniques. However, since no single approach satisfies all or even most of the emerging criteria for quick, effective, reproducible and sensitive results – there is still an obviously knowledge gap in research in this field. A summary table, Table 1 demonstrates the advantages and limitations of each technique reviewed in this paper.

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## ARTICLE

Table 1: A review of faecal detection techniques: limit of detection, result turnaround, specificity, advantages and limitations.

Technique	Detection Limit	Time before Result	Specificity	Advantages	Limitations	References
<b>Polymerase Chain Reaction</b>				Simple to understand and use Produces results fairly rapidly Sensitive and able to produce millions to billions of copies of a specific product, sequence, cloning and analysis	Owing to its high sensitivity, any form of contamination of the sample including trace amounts of DNA can induce misleading results Primers need to be produced Some prior sequence data is also required Primers can also anneal non-specifically to sequences that are similar but not identical to target DNA	<sup>107, 108, 109</sup>
<b>Plating Techniques</b>	1	1 – 3 days	Good	Robust and reliable method. The method has been well validated and well tested since Robert Koch first presented the technique	Time constraints, intense media preparatory steps	<sup>110, 111</sup>
<b>Bioluminescence</b>	10 <sup>4</sup>	0.5 h	No	Enhanced microbial support assay, rapid results. Powerful tool when coupled with photon counting and CCD imaging	Quenching can occur of emitted light adversely affecting measurements. Some matrix effect detection can also occur.	<sup>112</sup>
<b>Flow Cytometry</b>	10 <sup>2</sup> – 10 <sup>3</sup>	0.5 h	Good			
<b>Optical Biosensors</b>	Close to femtometre	0.5 h	No	Performing local, high resolution and label free molecular measurements. Are now being miniaturised for transport	Need for calibration, possible variability when coating surfaces are measured	<sup>113</sup>