Towards Routine Diagnosis of Gastrointestinal Infections by Molecular Technology in Australian Laboratories

Shane Byrne

Departments of Microbiology and Molecular Pathology,

Sullivan Nicolaides Pathology, Taringa, Australia

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Shane Byrne, Departments of Microbiology and Molecular Pathology, Sullivan Nicolaides Pathology, 134 Whitmore Street, Taringa, Queensland, 4068, Australia.

Shane_Byrne@snp.com.au
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Abstract

Routine diagnosis of gastrointestinal tract infectious disease remains a significant component of the diagnostic pathology laboratory and the core techniques employed have remained relatively unchanged for many years. The provision of traditional gastrointestinal disease diagnosis remains labour intensive and final results for conventional bacterial culture techniques have a time to result of 2-5 days. Microscopic diagnosis of parasitological disease generally has inferior sensitivity when compared to immunoassay and molecular based testing. In addition, the high specificity of molecular techniques has also proven superior for organisms that resemble each other closely or are indistinguishable by visual examination such as certain Entamoeba.

The combination of reduced time to result and improvements in sensitivity and specificity suggest that diagnosis of gastrointestinal disease is arguably the next most ready component for progressing to routine molecular based testing. The organisms currently known to cause gastrointestinal disease are relatively well characterised and this enables the collation of a definitive number of organisms for specific molecular detection. This number of organisms is not overly large in comparison to recent advances in the diagnostic density formats of molecular techniques. Three systems that provide cost effective PCR testing formats that can cover sufficient organisms are discussed. These systems realise the potential of becoming genuine replacements for conventional techniques. As therapy is often empiric or not recommended, antimicrobial resistance testing is not as linked to the diagnostic protocol to the extent it is when characterising organisms from extra-intestinal disease. This allows the application of molecular methods focused on detection of organisms without the absolute need to elucidate antimicrobial resistance.

Several commercial multi analyte enteropathogen kits have also recently become available and the coverage of these kits is compared to enteropathogens recovered in our laboratory from 2007-2011. Available performance evaluations and specifications are also presented. The funding model for Australian laboratories is discussed. Current remuneration for molecular testing is currently insufficient to cover the costs required to utilise commercial enteropathogen molecular testing covering a large range of enteropathogen targets.

Processing of stool for nucleic acid recovery is a key component of using molecular techniques. The use of currently available methods and instrumentation supporting the processing of this complex sample type are discussed. The multiple layers of process control used in molecular techniques are presented and these are compared to the level of control of conventional techniques.

A workflow describing the use of molecular screening using cost effective multi analyte technology in parallel with a minimised bacterial culture technique is presented as a possible solution. This protocol combines the benefits of routine molecular testing with the ability to maintain provision of isolates for public health outbreak tracing and antimicrobial resistance surveillance/testing as required.
Introduction

Routine diagnosis of lower gastrointestinal tract infectious disease remains a significant component of the diagnostic pathology laboratory, accounting for 15% of non-urine samples received for processing in our laboratory. The core techniques employed have remained relatively unchanged for many years. These techniques are also labour intensive when compared to processing of other microbiology sample types. Dependent on the specific testing request from the patients' clinician, conventional diagnostic workup of a stool specimen typically involves some or all of the following techniques. Recording a macroscopic description, performing culture for bacterial pathogens on a variety of solid media with or without prior broth based selective enrichment. Preparing wet mounts and faecal parasite concentration slides to examine stool microscopically. Use of ELISA or lateral flow immunoassays and/or specific stains for amoebic, flagellate, coccidial or microsporidial parasites. And often the use of ELISA, lateral flow or latex agglutination assays for viruses and bacterial toxins. Antibody testing of whole blood/serum/plasma for host response to enteropathogens is also utilised where appropriate.

Diagnosis and identification of bacterial infections using culture, biochemical and serological assays is considered the gold standard but the process is both costly and time consuming. Often final diagnosis of a bacterial aetiology is not available until several days post collection of a stool sample and often after the infection has run its course. Reducing the time to diagnosis assists patient management and allows timely action to be taken to limit further transmission in the community. Typical bacterial culture techniques require 2-5 days dependent on the protocols used. Culture also requires significant labour for screening culture plates and in follow up identification techniques. Although treatment for bacterial infections is often not recommended, performing antimicrobial sensitivity testing may be valuable for monitoring the development and spread of antimicrobial resistance. This is particularly important if the isolate is recovered from a patient who may have acquired the symptoms during or shortly after international travel. Such isolates could potentially harbour emerging transmissible antimicrobial resistance determinants that have implications for the patient and for the community. Isolates are also often required to be sent to reference laboratories for epidemiological tracing in the interests of public health and protecting the food supply.

Dependent on volumes of samples received for testing in the laboratory the microscopy component of the diagnostic workup can be onerous. This may be reflected in repetitive strain injury or more commonly general fatigue from the efforts required in scanning large numbers of slides by medical technologists. Microscopic diagnosis of parasitological disease is documented to have inferior sensitivity compared to molecular based testing. The sensitivity of microscopy for detection of parasites has been shown to be approximately 60% at best (1). The high specificity of molecular techniques is also superior for organisms which resemble each other closely or are indistinguishable by visual examination but have quite different relevance clinically e.g. *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii* (2).

The typical viral workup for gastrointestinal disease includes rotavirus and group F adenovirus. When outbreaks are identified in a community or health care setting,
testing for other viral agents such as Norovirus, Astrovirus or specific enteroviruses
supplement the virological workup as required.

It has been reported that in approximately 40% of investigations for symptomatic
diarrhoeal illness no aetiological agent is identified (3). Evidence suggests that
expanding the range of pathogens tested is likely to increase the rate of diagnosis of
an aetiological agent.

Molecular techniques have proved very useful for expanding the diagnostic
repertoire and have become a routine component of infectious disease diagnostics
since the mid 1990’s. From the late 1990s diagnostic virology underwent a shift from
what was primarily cell culture based diagnostics to molecular virology, most
commonly employing Polymerase Chain Reaction (PCR) as the nucleic acid
amplification technique (NAAT) utilised. As sequence based information is generally
required to develop NAAT based assays, reference laboratories have continued to
maintain cell cultures for emerging viruses. However recent advances using direct
application of nucleic acid detection techniques have been used as a culture
independent methodology for viral discovery to good effect (3-7).

The clinical microbiology laboratory has seen a gradual shift from the conventional
methods of culture and microscopy towards molecular testing. The pathogens
involved have been those that generally fall within one or more of the following
contexts: difficult or impossible to recover in culture, poorly diagnosed by
conventional means, recovered by conventional means after lengthy incubation, or
those with tedious and/or expensive methodology required for diagnosis. The
transition from conventional methods to molecular testing is most evident in clinical
diagnostic virology, closely followed by diagnosis of Chlamydia trachomatis and
Neisseria gonorrhoeae. As an example C. trachomatis, N. gonorrhoeae and Herpes
simplex virus testing together now account for 51% of total molecular testing
volumes in our laboratory. Organisms that have been difficult to culture including
Mycoplasma pneumoniae, Bordetella pertussis, Bartonella henselae and
Tropheryma whipplei are also now commonly diagnosed by molecular technology.
Slow growing organisms such as Mycobacteria continue to see expansion in the
utilisation of molecular technology. Recently the WHO endorsed the use of a
molecular cartridge based integrated assay called the ‘Xpert MTB/RIF’ developed by
Cepheid as the diagnostic test of choice in developing countries. Rapid detection of
disease states that are only caused by a specific subset of strains e.g. Human
papillomavirus infections of the cervix, has also been possible utilising type specific
nucleic acid based diagnostic techniques. In recent years, the lack of sensitivity of
microscopy techniques has also led to adoption of molecular assays for the routine
diagnosis of Trichomonas vaginalis. Pneumocystis jirovecii diagnosis has also
transitioned away from direct fluorescent antibody testing of samples to the use of
quantitative analysis available by real-time PCR.

When looking at the remaining conventional activities of a clinical microbiology
laboratory, diagnosis of gastrointestinal disease is potentially the next most ready
component for progressing to routine molecular based testing. The organisms
currently known to cause gastrointestinal disease are relatively well characterised
and this enables the collation of a definitive number of organisms for specific
molecular detection. This number of organisms is not overly large in comparison to
recent advances in the diagnostic density formats of molecular techniques. As
therapy is often empiric or not recommended, antimicrobial resistance testing is not as linked to the diagnostic protocol to the extent it is when characterising organisms from extra-intestinal disease. This allows the application of molecular methods focused on detection of organisms without the absolute need to elucidate antimicrobial resistance. The use of bacterial culture for provision of isolates for public health outbreak tracing however remains an important consideration. Using molecular technology as an enteropathogen screening technique with a strategy of follow up 'reflex' conventional culture based on molecular detection of certain bacterial pathogens appears a plausible strategy to preserve this important public health requirement (8, 9).
Current Techniques for Diagnosis of Gastrointestinal Infections

Diagnostic techniques currently utilised in clinical microbiology laboratories for stool examination can be broadly classified into three components: Microscopy, Culture and Immunodiagnostics. Often all three are combined within the workflow of a laboratory to provide the final results to clinicians. Antibody testing from serum, plasma or whole blood for certain enteropathogens is also of use.

**Microscopy**

Conventional processing of stool typically involves a macroscopic description being recorded. Stool is added to normal saline and a drop placed on a microscope slide. Use of a small amount of Lugol’s iodine mixed with the stool suspension assists observation and identification of parasites. A cover slip is applied to produce a wet preparation for microscopic analysis. This initial microscopic examination provides diagnostic information on the presence of white cells, red cells, Charcot-Leyden crystals, fat globules, observable pathogenic and commensal parasites and yeast. Further processing of stool is then undertaken by combining chemical clarification and centrifugal concentration to prepare what is known as a ‘conc’ or faecal parasite concentration microscopy preparation. This concentrated deposit is examined microscopically for the presence of parasites and improves diagnostic yield. Examination for certain parasites such as *Schistosoma* requires that all of the concentrate be examined, which can number several slides. Smears of faecal concentrate and/or stool supplied in parasite preservation fixatives can also be made on slides. Smears are fixed and subsequently stained with stains such as the Wheatley modified Gomori Trichrome, Iron Haematoxylin or the Modified Kinyoun Acid Fast which allow targeted observations for parasites amongst stool debris. Some staining methods also highlight morphological characteristics important in the identification of the parasite. Use of microscope eyepieces with calibrated measuring scales printed within allows accurate measurement of ova, cysts and parasites which can also be required to differentiate and correctly identify them.

**Culture**

Culture on/in bacteriological media is the primary diagnostic method for bacterial enteropathogens. As one third of the solid matter in stool is comprised of bacteria, recovering pathogenic bacteria typically involves the combination of selective agars and the use of selective enrichment broths to reduce normal flora. Plates are incubated at specific temperatures and appropriate atmospheric conditions. Media for recovering *Campylobacter* is incubated in a modified microaerophilic atmosphere and media for *Clostridium difficile* is incubated anaerobically. Incubation periods from two to five days are commonly used before plates are discarded and final results issued. The typical media applied for routine investigation of bacterial
enteropathogens generally covers *Salmonella*, *Shigella*, *Campylobacter*,
Aeromonads, Vibrionaceae and *Yesinia*. Additional non-routine media may be
added if clinical information indicates it may be appropriate e.g. recovery of *Listeria*
monocytogenes or O157 sorbitol non fermenting shiga toxigenic *Escherichia coli*.
Some laboratories also routinely diagnose toxigenic *Clostridium difficile* infection by
recovery of the organism on solid media in anaerobic incubation conditions. Use of
chromogenic agar media (e.g. bioMerieux ChromID Salmonella) which assists in
improving recognition of pathogenic bacteria amongst normal faecal flora has also
become commonplace (9, 14).

**Immunodiagnostic Assays for Enteropathogen Antigens**

Two distinct immunoserological formats are utilised for enteropathogen antigen
detection.

In settings of limited resources including at point of care (PoC) and/or where
expediency is required within the laboratory rapid easy to use lateral flow
immunochromatographic assays including those packaged in cartridge form have
been of value. These lateral flow assays are rapid and often provide a result in a
time ranging from 10 to 30 minutes with minimal hands on time. However, the
techniques are generally not amenable to routine processing of large numbers of
samples. Costs range from $5 to $16 AUD per single test dependent on
manufacturer, antigen target and quantity ordered.

The second formats are the classical serological assays of ELISA (enzyme-linked
immunosorbent assay), CFT (complement fixation test) and IHA (indirect
hemagglutination assay) which are typically utilised in microplate format. These
classical techniques generally involve the use of equipment and reagents within the
laboratory setting and generate results in timeframes between and hour and two
hours. Many of these microplate format assays are also adaptable to high
throughput automated instrumentation where a larger number of samples need to be
tested. There is a large range of commercially produced assays available for
enteropathogen antigen detection. Costs range from $4 to $9.50 AUD per test well,
again dependent on manufacturer, antigen target and quantity ordered.

Use of “combi” type formats where more than one antigen are detected on/in the
same cassette, strip or microwell reduces the overall costs for the common
applications of these tests. Some of the common products available in the
Australian market are presented below.
Parasite Enteropathogen Antigen Detection

The performance of several antigen detection assays for *E. histolytica* are presented in the reviews of Fotedar et al. (2) and Tanyuskel et al. (15) When compared to microscopy the antigen detection assays were mostly in the range of >90% sensitive and generally 99-100% specific.

Rapid Methods

Cassette or immunochromatographic strip products have mainly been developed with a focus on the larger market of *Cryptosporidium* and *Giardia*, often in a combined format. Examples of these assays include the TechLab Giardia/Cryptosporidium QUIK CHEK; R-Biopharm RIDA®QUICK (cassettes or strips) for Cryptosporidium, Giardia, and in combination; Savyon CoproStrip™ for Cryptosporidium, Giardia, and in combination; Remel Xpect® for Cryptosporidium, Giardia, and in combination; and the Meridian Bioscience ImmunoCard STAT!®Crypto/Giardia.

The Alere Triage® Micro Parasite Panel and R-Biopharm RIDA®QUICK Cryptosporidium/Giardia/Entamoeba Combi products provide for detection of *Giardia lamblia*, *Entamoeba histolytica/dispar*, and *Cryptosporidium parvum* in the one product.

ELISA Microplate Methods

ELISA antigen detection kits have been produced that cover a slightly wider range of enteric parasites than the rapid methods. *Cryptosporidium* and *Giardia* ELISA tests are available both as single assays and in combination assays. Examples include; Techlab GIARDIA II, CRYPTOSPORIDIUM II, GIARDIA/CRYPTOSPORIDIUM CHEK®; R-Biopharm RIDASCREEN®Cryptosporidium, Giardia; Savyon CoproELISA™ Giardia, CoproELISA™ Cryptosporidium and CoproELISA™ Giardia-Cryptosporidium; Remel ProSpecT™ Giardia, ProSpecT™ Giardia/Cryptosporidium, ProSpecT™ Giardia EZ, ProSpecT™ Cryptosporidium; Cortez Diagnostics Crypto/Giardia Ag Combo, Cryptosporidium, Giardia; Novagnost Giardia lamblia Antigen in stool and Cellab Giardia CELISA.

Comparative or improved sensitivity compared to microscopy and the ability to automate the testing have seen the adoption of combo microplate ELISA assays for *Cryptosporidium* and *Giardia* utilised as screening methodology within the workflow of our laboratory.
Several *E. histolytica* ELISA assays are available such as the; Techlab E. HISTOLYTICA II, R-Biopharm RIDASCREEN®Entamoeba; Savyon CoproELISA™ Entamoeba; Remel ProSpecT™ Entamoeba histolytica; Cortez Diagnostics E. histolytica/dispar ELISA kit; Novagnost Entamoeba histolytica and Cellab Entamoeba CELISA Path.

Savyon also produce an ELISA product for *Blastocystis* (CoproELISA™ Blastocystis), and have a *Dientamoeba* ELISA (CoproELISA™ Dientamoeba) under development.

**Indirect immuno-fluorescent antibody assays (IFA):**

Slide based IFA assays are also utilised to detect parasitic antigens from stool. Bordier Affinity produces an IFA to detect antigens of the microsporidia *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* from stool.

### Bacterial Enteropathogen Antigen Detection

#### Rapid Methods

A wide range of cassette and immunochromatographic strip tests for bacterial enteropathogens is also available. The largest product range are assays that detect *Clostridium difficile* toxins and/or cellular antigens. Examples include; Techlab C. DIFF QUIK CHEK®, C. DIFF QUIK CHEK COMPLETE®, TOX A/B QUIK CHEK; R-Biopharm RIDA®QUICK Clostridium difficile Toxin A/B, RIDA®QUICK Clostridium difficile GDH, Remel Xpect® C. difficile Toxin A/B; and Meridian Biosciences ImmunoCard®Toxins A&B, ImmunoCard®C. difficile GDH.

Rapid format assays for *Campylobacter* have been developed including; R-Biopharm RIDA®QUICK Campylobacter and Meridian Biosciences ImmunoCard STAT!®CAMPY;

Shiga toxins of STEC and O157 cell wall antigen specific rapid tests also exist for direct testing from stool. These include the R-Biopharm RIDA®QUICK Verotoxin/O157 Combi and the Meridian Biosciences ImmunoCard STAT!® E. coli O157 Plus and ImmunoCard STAT!®EHEC.

SD Bioline (Alere) produce a rapid test for *Vibrio cholerae*, the SD BIOLINE Cholera Ag 01/0139 which is specific the Ogawa serotype 01 and Inaba serotype 0139.

In addition to rapid direct testing of stool, rapid format antigen detection kits are also available for testing of blood for diagnosis of typhoid fever including the Cortez
Diagnostics Salmonella Typhi Antigen Rapid Test RapiCard™ Insta Test. The
Biocan TELL ME FAST™ Salmonella typhi/paratyphi A Test Strip is also available
which can be used to test stool, serum or plasma. LumiQuick produce both the S.
typhi/paratyphi Antigen Duo Test and Salmonella typhi Antigen Test for which stool,
serum, plasma or whole blood can be utilised as the input sample. DIALAB
manufacture the Salmonella typhi/paratyphi Ag Cassette for which stool/whole
blood/serum and plasma can all be tested.

ELISA Microplate and Chemiluminescent Immunoassay (CLIA) Methods

*C. difficile* toxin(s) and antigen assays include; Techlab *C. difficile* Toxin/Antitoxin
Kit, C. DIFFICILE TOX-B TEST, C. DIFFICILE TOX A/B II™ and C. DIFF CHEK™ -
60. The R-Biopharm RIDASCREEN®Clostridium difficile GDH and RIDASCREEN®
Clostridium difficile Toxin A/B. The Savyon CoproELISA™ C.difficile GDH and
CoproELISA™ C.difficile Toxin A/B. The Remel ProSpecT™ C. difficile Toxin A/B,
and the Meridian Biosciences Premier® Toxins A&B and Premier® C. difficile GDH.

Two kits utilising Chemiluminescent Immunoassay methodology are available for *C.
difficile* on the Diasorin Liaison instrument platform LIAISON® C. difficile Toxins A
and B and LIAISON® C. difficile GDH.

Campylobacter* antigen assays include the R-Biopharm
RIDASCREEN®Campylobacter; Remel ProSpecT™ Campylobacter; Cortez
Diagnostics Campylobacter ELISA kit and the Meridian Biosciences Premier®
CAMPY

Detection of STEC and/or serotype O157 is available using R-Biopharm
RIDASCREEN® Verotoxin; Remel ProSpecT™ STEC (Shiga Toxin E.coli); Cortez
Diagnostics E.Coli O157 (Fecal) ELISA kit and E.Coli Verotoxin (Fecal) ELISA kit;
Meridian Biosciences Premier® EHEC

Viral Enteropathogen Antigen Detection

Rapid Methods

R-Biopharm RIDA®QUICK Norovirus, RIDA®QUICK Rotavirus, RIDA®QUICK
Rotavirus/Adenovirus Combi; Remel Xpect® Rotavirus; Cortez Diagnostics
Rotavirus Rapid Test (Cassette) and Adeno / Rota Antigen Rapid Test (Cassette)

RapiCard™ InstaTest; bioMerieux Vikia ROTA-ADENO; Meridian Biosciences

ImmunoCard STAT!®Rotavirus; Alere SD BIOLINE NOROVIRUS Ag Test and SD BIOLINE ROTA/ADENO Ag Rapid Kit; Famouze Diarlex MB (Rota and Adeno) strip and Actim NORO Cassette.

Sensitized latex particle agglutination: Famouze Diarlex Rota-Adeno and Rotalex (Rota).

ELISA Methods

R-Biopharm RIDASCREEN® Rotavirus, RIDASCREEN® Adenovirus, RIDASCREEN® Astrovirus, RIDASCREEN® Norovirus; OXOID IDEIA Amplified
IDEIA Astrovirus kit, IDEIA Adenovirus kit, IDEIA Norovirus kit and IDEIA Rotavirus kit; Remel ProSpecT™ Rotavirus, ProSpecT Adenovirus ProSpecT Astrovirus
Microplate Assay; Cortez Diagnostics Adenovirus (Fecal) ELISA kit and Rotavirus (Fecal) ELISA kit; Meridian Biosciences Premier® Rotaclone®, Premier® Adenoclone® and Premier® Adenoclone®– Type 40/41.

Immunodiagnostic Assays for Enteropathogen Antibody Production

Several antibody detection assays are available for enteropathogens. These assays have been developed in several formats including rapid tests and each of the routinely utilised serological techniques (ELISA, CFT, and IHA) in microplate formats. Slide and tube agglutination assays have also been manufactured.

Detection of host response to infection has generally proven most useful in patients from areas of low endemicity of the particular enteropathogen that is being tested for (2). A positive antibody result from a returning traveller with no previous risk of exposure to the enteropathogen is of high value, whereas positive IgG serology for enteropathogens that can be encountered locally may represent prior exposure and not active infection (16). IgM and IgG combination assays are useful in elucidating the currency of the infection, particularly in areas where the enteropathogen is endemic (17, 18).

Examples of available antibody assays and assay formats include the following.

Rapid lateral flow/immunochromatographic tests

Rapid format antibody detection kits are available for testing of blood for diagnosis of typhoid fever including the SD BIOLINE Salmonella typhi IgG/IgM Test. LumiQuick
produce the Quick Profile™ S. Typhi IgG/IgM Duo Test Card for use whole blood, serum or plasma.

**Rapid slide or tube agglutination kits**

Famouze manufacture an extraintestinal BLA-Bichrolatex Amibe Slide test for *Entamoeba histolytica*. IDL Biotech manufactures the TUBEX TF magnetic and latex particle agglutination assay in tube format that detects IgM antibodies to *Salmonella* Typhi O9 antigen. Both of these assays compare very favourably performance wise to other immunodiagnostic assays, can be completed in very rapid time (5-10 minutes) and both are useful in front line/PoC settings and within the laboratory (18, 19).

**ELISA format antibody kits**

R-Biopharm RIDASCREEN® Entamoeba histolytica IgG, RIDASCREEN® Taenia solium IgG; RIDASCREEN® Adenovirus IgA, IgG.

Cortez Diagnostics Fasciola IgG ELISA kit, Strongyloides IgG ELISA kit, Cysticercosis IgG (T. Solium) ELISA kit, E. histolytica IgG ELISA kit and Schistosoma IgG ELISA kit.

DIASORIN Adenovirus IgA, Adenovirus IgG, Adenovirus IgM, Ascaris lumbricoides IgG, Entamoeba histolytica IgG, Schistosoma mansoni IgG and Taenia solium IgG.

Novatec Adenovirus IgA/IgG/IgM, Ascaris lumbricoides IgG, Schistosoma mansoni IgG, Taenia solium IgG and Entamoeba histolytica IgG.

Virion-Serion Campylobacter jejuni IgA/IgG/IgM, Yersinia IgA/IgG/IgM and Campylobacter jejuni IgA/IgG/IgM.

Bordier Affinity Schistosoma mansoni ELISA, Strongyloides IgG ELISA and Microsporidial ELISA.

**CFT format kits**

Virion-Serion Adenovirus, Campylobacter jejuni, Listeria monocytogenes, Rotavirus, Yersinia enterocolitica O3 and Yersinia enterocolitica O9.

**IHA format kits**

Famouze Schistosome IHA, Amoebiasis IHA and Fasciola hepatica IHA; Cellognost Amoebiasis Combipack and Cellognost Schistosomiasis.
Immunodiagnostic Assays Specifically for Bacterial Toxins

An additional diagnostic modality for gastrointestinal disease is direct detection of the excreted toxins of certain bacterial enteropathogens in the stool sample. In addition to the commercial assays for the toxins of *C. difficile* and STEC, immunodiagnostic antigen tests also exist for other bacterial toxins involved in gastrointestinal disorders.

For some bacteria (e.g. *Bacillus cereus*, *Staphylococcus aureus* and *Clostridium perfringens*), clinical symptoms represent ingestion of large numbers of organisms and/or their toxins within foodstuffs rather than infection of the intestinal tract by the organism (20-22). Commercial assays that detect these toxins generally employ reverse passive latex agglutination (RPLA). Examples include the set of RPLA kits available from OXOID: *E. coli* heat-labile enterotoxin and *Vibrio cholerae* enterotoxin; shiga toxins of EHEC; *Bacillus cereus*; *Clostridium perfringens* enterotoxin and a kit for Staphylococcal enterotoxins. ELISA based assays for *C. perfringens* enterotoxin are also produced by Techlab (Techlab *C. perfringens* Enterotoxin Test) and R-Biopharm (RIDASCREEN®*Clostridium perfringens* Enterotoxin).

Comparison of Immunodiagnostic Assays to Molecular Assays

Rapid and conventional immunoassays for enteropathogens generally show similar specificities but are often reported to be less sensitive than molecular testing. The rapid lateral flow kits and agglutination methods offer superior time to result than molecular techniques, and require only basic technical skill and no specialist equipment to perform. Direct protein toxin detection is of course unable to be performed using nucleic acid based molecular testing. Detection of a specific nucleic acid sequence encoding the toxin is a useful proxy for some organisms such as toxigenic *C. difficile* and STEC which both carry specific genetic sequences responsible for toxin production and which differentiate these strains from non-toxigenic strains.

Imunoassays for *C. difficile* toxins A and B and glutamate dehydrogenase (GDH) antigen are used widely within clinical microbiology laboratories. The GDH assays compare favourably to molecular methods sensitivity wise but are not quite as specific as PCR or immunoassay based toxin detection(23, 24). Eastwood et al. compared nine ELISA toxin assays, a GDH assay and a Toxin B real time PCR against the gold standard assay of cytotoxin neutralisation on cell lines. Sensitivity and specificity of the GDH assay was reported as 90.1% and 92.9%, a PCR assay for TcdB was 92.2% and 94% and the mean of the toxin assays was 82.8% and
95.4% respectively (24). An international review of the immunoassays for detection of toxin A or B by Planche et al. found that despite reasonable sensitivities the low prevalence resulted in unacceptably low positive predictive values for all of the six common assays examined (Meridian Premier, TechLab Tox A/B II, TechLab Tox A/B Quik Chek, Remel Xpect, Meridian Immunocard and BioMérieux VIDAS (25)). To address these issues several screening and confirmation algorithms have been proposed incorporating combinations of the immunoassays and PCR testing of specific genes in the pathogenicity locus (PaLOC) (23, 26, 27). The suggested algorithm in Australia is that proposed by the Australasian Society for Infectious Diseases (ASID) which recommend the use of a lower cost sensitive screening assay such as GDH, followed by a specific confirmation assay such as Toxin A/B or PCR against genes in the PaLOC (26).

Two STEC immunoassays were compared to stx1/2 PCR and chromogenic CHROMagar STEC cultures by Hirvonen et al. (9). The R-Biopharm RIDA®QUICK Verotoxin/O157 Combi rapid assay detected 14 of the 16 positives, whereas both the Premier® EHEC ELISA assay and stx1/2 PCR assays detected all 16 positives.

There are several studies examining the use of ELISA based kits for detection of STEC (28-31). The Premier® EHEC, and R-Biopharm RIDASCREEN® Verotoxin assay have been reported to have similar sensitivity when compared to culture, with sensitivity of 89% (29, 31). The Remel ProSpecT™ STEC, was found to be ten fold less sensitive than those two assays in the study of Willford et al. (31).

Rovida et al. performed a comparison of the R-Biopharm RIDASCREEN® and RIDA®QUICK immunoassays against molecular assays for the four main viral enteropathogens (32). Results of the immunoassays were variable, specificity was high for all assays, but sensitivity was generally lower than PCR. The Astrovirus ELISA assay performed equivalently to PCR testing with a sensitivity and specificity of 100%. The Norovirus ELISA product had a poor sensitivity of 49.5%, an outcome that was inconsistent with other investigations that reported sensitivity between 60% and 90% (32-34). The Adenovirus component of the combi rapid product performed poorly with sensitivity of 28.6%, however the Rotavirus component had a sensitivity of 88.8% (32).

Three papers have been published by an Australian laboratory comparing two rapid Norovirus immunoassays and an ELISA assay against RT-PCR (35-37). Reported sensitivity of the immunoassay products was 62%, 83% and 66% for the Bioline SD Norovirus, RIDA®QUICK Norovirus and the DAKO IDEIA Norwalk like Virus kit respectively. Specificities of 100%, 98.6% and 85% were also reported respectively for those tests. These results are consistent with international reports that also
indicate high specificity but lower sensitivity of Norovirus immunoassays when compared to RT-PCR methods (38-40).

All of the R-Biopharm assays for Cryptosporidium, Giardia and E. histolytica, including the rapid tests and the ELISA kits were evaluated by Goni et al. (41). The immunoassays were compared to microscopy results and conventional PCR assays. Each assay provided similar sensitivity and specificity against the two reference methods. Sensitivity compared to PCR for Cryptosporidium was 65-70% with a specificity of 92.9-96.4%. Sensitivity compared to PCR for Giardia was 87.5-90.6% with a specificity of 99.2-96.9%. And sensitivity compared to PCR for E. histolytica was 62.5-75% with a specificity of 92.8-96.1% (41). A positive predictive value of just 56.5% for Cryptosporidium with the ELISA method was also reported (41). This low PPV is consistent with low prevalence of target and use of lower sensitivity assays. In our laboratory, positive results for Cryptosporidium in ELISA screening methodologies are confirmed using stained microscopy for this reason.

An IFA assay by Alfa Cisse et al. using monoclonal antibodies against Enterocytozoon bieneusi and Encephalitozoon intestinalis was shown to generate comparable results to conventional PCR with 100% sensitivity and specificity observed (42). Testing time was reported as three hours with hands on time of one hour and conventional PCR was listed as an eight-hour test including nucleic acid extraction.

Bessede et al. compared one rapid (ImmunoCard STAT!®CAMPY) and two ELISA based Campylobacter immunoassays (RIDASCREEN®Campylobacter and Premier® CAMPY) to two PCR assays and culture (43). The sensitivity of all three immunoassays was reported as being 5-10% higher than that of either PCR method. All five alternate assays were significantly more sensitive than culture, which was only 63% sensitive in the study.

Results for the immunoassays were consistent with those reported by Granato et al. who compared two ELISA methods Premier® CAMPY, and ProSpecT™ Campylobacter and the rapid ImmunoCard STAT!®CAMPY to culture utilising PCR analysis for discrepancy assessment (44). The two ELISA tests had identical sensitivity of 99.3% and specificity of 98%, and the rapid ImmunoCard STAT!®CAMPY had a sensitivity of 98.5% and specificity of 98.2% (44). Interestingly the study of Granato et al. reported a sensitivity of 94.1% for culture, which was significantly different to that of Bessede et al. at 63% sensitivity.

Irrespective of comparative analytical performance, in Australia there is an important consideration that often makes the choice of using immunodiagnostic assays or
molecular assays a “one or the other” decision. The funding of testing for enteropathogens by both of these methods is reimbursed from the same Medicare items 69494-69496. These items can only be claimed just once per patient sample. The text of the items are; 'Detection of a virus or microbial antigen or microbial nucleic acid (not elsewhere specified)’. Consequently combining both methods in the diagnostic workflow needs careful consideration of costs.
Human gastrointestinal infection is the second leading cause of deaths due to infectious disease worldwide, responsible for millions of deaths in children under age five each year, predominantly in developing countries (45-48). In Australia community and hospital acquired infectious diarrhoea is a significant cause of morbidity reportedly causing 17.2 million episodes of gastroenteritis a year (49). OzFoodNet estimated that there are approximately 3.7 million visits to general practitioners for gastroenteritis and that this resulted in more than 500,000 stool tests being performed in Australian diagnostic laboratories (49). The 80% discrepancy between actual doctor visits and estimated cases arises due to the self-limiting nature of the majority of gastrointestinal diseases. Australian health departments have estimated that, for every case of salmonellosis notified by a laboratory there may be up to 7 infections that remain unreported in the community, and that there are may be up to 8 cases unreported in the community for every notified case of Campylobacter or Shiga toxin producing Escherichia coli (STEC) (49, 50). Based on those estimates there is an 86% difference between doctor visits for gastrointestinal disease and stool samples actually submitted for testing. This may also reflect that the existing diagnostic tests are not viewed as offering results in a relevant time frame and that by the time results are available the patient is most likely well. In the majority of cases of uncomplicated gastroenteritis, only supportive therapies are recommended by clinicians. In an Australian national gastroenteritis survey it was found that for most patients symptoms lasted 24-48 hours with an average of five episodes of diarrhoea per 24 hours (51). The same survey indicated that the age groups with the highest disease burden were children under five years, and women between 20-40 years of age. Those aged over 60 often did not report gastroenteritis but were reported to have the longest duration of symptoms (51). It was also observed that most samples are submitted when people have symptoms that continue for three days or more. If symptoms were less than three days only one in 142 cases are submitted for testing. As the duration of symptoms increased to 3-4 days, one in 14 cases were submitted for testing. Patients with symptoms of 5 or more days had samples submitted for testing in 25% of cases.(51). This appears to correlate well with conventional detection methodologies generally not being complete before 3 days and therefore often providing only retrospective information. Transmission of enteric disease within the community and the healthcare setting causes a significant economic drain due to time losses. In Australia it was estimated at over six million days of lost paid work, nearly half due to a carer needing to look after the affected individual (51). Any improvement in diagnostics that can assist with greater coverage and earlier identification of enteric pathogens may help to reduce these burdens. Sinclair et al. examined pathogens causing community gastroenteritis in Australia and concluded that, “Many of the pathogens responsible
for cases of gastroenteritis in the Australian community are likely to go undetected by
current surveillance systems and routine clinical practice" (52). Incorporation of
molecular technologies into clinical practice due to the “unsophistication of
conventional diagnostic methodologies and strategies” has also been promoted by
Bennett & Tarr (53). The overwhelming challenge in diagnosing infectious
diarrhoea is the large number of causative enteropathogens, including viruses,
bacteria and protozoa. Current methodologies are inefficient, as the protocols require
multiple tests and testing modalities. This results in a workflow that is time
consuming and labour intensive. Standard viral enteropathogen diagnostics
available in the routine laboratory are also limited. Routine molecular diagnosis of
an extended panel of enteropathogens appears warranted. Amar et al. published
data on a retrospective analysis of samples from the English case-control Infectious
Intestinal Disease Study (1993-1996) where PCR techniques were utilised to
examine 4627 archived faecal samples for enteropathogens. The percentage of
archived samples from cases in which at least one agent was detected increased
from 53 to 75% after the application of PCR assays (54).

Addressing several needs not met by conventional methods, the high sensitivity and
rapid time to results provided by molecular diagnostic technologies have seen
increasing application of these techniques to the diagnosis of gastrointestinal
disease. Selected examples of the use of molecular amplification technology for
diagnosis of bacterial, viral and parasitic enteropathogens are presented below.

**Bacterial Pathogens**

The traditional diagnosis of bacterial enteropathogens involves growth of isolates on
solid media after direct inoculation of faeces to the plates, and enrichment/selection
in liquid broths followed by inoculation to solid media. The approach utilises several
types of media and incubation atmospheres to provide appropriate selectivity and
growth parameters for the target organisms and to reduce the recovery of normal
flora. Plates are examined by skilled laboratory staff and suspicious isolates are
confirmed via morphological, serological and biochemical methods, and more
recently by use of proteomic mass spectrometry. The culture methodology performs
well but is dependent on the skill of the individual observing the cultures and the
initial fitness of the organisms in the specimen. Unfortunately, observational
characteristics may not always allow appropriate recognition of pathogens. For
example it is not possible to distinguish all pathovars of *E. coli* from commensal
varieties based on the phenotype of the organism on culture media. The major
disadvantage of the culture techniques however is that time is necessarily required
for bacteria to grow into colonies that can be adequately observed by the human
eye. Bacterial culture from faeces can take from 2-5 days to complete dependent on
the protocol utilised and what bacterial pathogens are sought. Diagnostic yields
between 6% and 11% have been reported for the bacterial culture techniques (8, 55). In our laboratory for the period 2007-2011 a yield of 19080 bacterial enteropathogens recovered from a total of 254940 stool cultures (7.5%) was observed (56).

Many PCR assays have been developed for the detection of human bacterial enteropathogens (8, 57-77), including several multiplex PCR assays targeting more than one organism in the one assay (54, 78-85). Compared to conventional culture techniques the use of PCR approaches has reduced detection times from 2-5 days to a single day turn around (8, 85). Many molecular assays exist for Salmonella and Campylobacter, as these two genera are responsible for the majority of bacterial gastrointestinal disease. However there are at least 12 other genera which contain species that have been reported to cause human enteric infections. Table 1 provides a list of the majority of bacterial enteropathogens known to be responsible for cases of human gastroenteritis. Diagnostic PCR based assays have been developed for all of the bacteria listed in Table 1 and the use of real-time PCR is the most common approach. The use of real-time PCR offers two significant benefits over conventional amplification techniques. The first benefit is speed due to the combination of amplification and detection. The second is that the reaction is ‘closed tube’ and amplicon detection does not require open tube manipulation thereby reducing the risk of amplicon derived contamination of future assays. The cost of a ‘home-brew’ or ‘in-house’ developed real time PCR assay including extraction is between $10 and $20 per reaction dependent on the reagents and extraction methodology utilised. Multiplexing on modern real time PCR instruments generally allows up to five or six unique fluorescent labels to be reported. In our experience with the development of four or five target multiplex assays where more than two of those targets are reasonably expected to amplify concurrently is normally problematical. In circumstances where more than one target amplification process is occurring other assay components may not achieve optimal sensitivity. This is because of competitive inhibition deriving from amplification of the most abundant target sequences. Dual amplification methodologies employing a nested PCR approach with limited cycle preliminary target enrichment have been designed to overcome some of the multiplexing deficiencies that arise due to variation in multiple target abundance (86, 87).

Bacterial enteropathogens can be classified into those acquired in the community from sources such as food, water or fomites; and those acquired as a healthcare facility associated disease due to C. difficile, generally as sequelae to antibiotic treatment. Unfortunately the incidence of community acquired C. difficile gastroenteritis also appears to be rising (88). Discussion of the common molecular approaches to diagnose specific groups of bacterial enteropathogens follows.
Enterobacteriaceae (*Salmonella spp.*, *Shigella spp.*, *Yersinia enterocolitica*, *Plesiomonas shigelloides, Edwardsiella tarda*, pathogenic *Escherichia coli*, *Escherichia albertii*)

**Salmonella**

Salmonellosis is caused by *Salmonella enterica* subsp. *enterica* which is the second most common bacterial enteropathogen in Australia after *Campylobacter*. The disease is characterised by rapid development of symptoms including abdominal pain, fever, diarrhoea, muscle pain, nausea and/or vomiting. People can become infected via faecal-oral transmission, ingesting contaminated food, animal contact and from environmental exposures (89). There were 11,993 notified cases of salmonellosis in Australia in 2010; a rate of 53.7 per 100,000 (89). *Salmonella enterica* serotype Typhi is only isolated from humans where person-to-person transmission results in a serious bacteraemia called Typhoid fever. There were 96 notified cases of typhoid during 2010 (rate 0.4 per 100,000). Overseas travel was the primary risk factor for notified cases in 2010, with 76% of notified cases known to have been acquired overseas (89). Routine diagnostic procedures require selective culture methods preceded by enrichment broth, and subsequent biochemical or proteomic identification, as such it can take 3-4 days before a final result is available (8). A number of authors have published molecular assays for use on human faecal samples which have included *Salmonella* detection in multiplex with other bacterial enteropathogens (8, 80-82, 84, 85). The genetic targets utilised have primarily been the invasion gene *invA*, although the *ttr*BCA (tetrathionate reductase response regulator) locus has also been used. Results from multiplex enteropathogen assays for detection of *Salmonella* indicate either equivalent performance to culture (85) or indeed improvement in sensitivity for *Salmonella* detection of up to 13% (8).

**Shigella**

Shigellosis is characterised by acute abdominal pain and fever, small-volume loose stools, vomiting and tenesmus (89). Humans and apes are the only known reservoirs of *Shigella* and transmission is generally person-to-person via the faecal-oral route, but has also been caused by ingestion of contaminated food or water (90). In Australia in 2010, there were 552 notified cases of shigellosis; a rate of 2.5 per 100,000 (89). Successful molecular detection methods for *Shigella* targeting the *ipaH* invasion plasmid antigen H gene have been published including multiplex methods with other bacterial enteropathogens (80, 84, 85, 91-93). Two authors have reported that utilisation of a molecular detection methodology has shown improved sensitivity for detection of *Shigella* from human samples as compared to culture techniques (92, 94).
Pathogenic *Escherichia coli*

E. coli is generally the most abundant commensal in the human intestine. However, certain pathotypes of *E. coli* which have acquired distinct virulence determinants and are recognised bacterial enteropathogens. The use of conventional techniques offers minimal assistance in recognising the majority of these pathogenic *E. coli*. Recognition of sorbitol non fermenting shiga toxin producing *E. coli* (STEC) of particular serotype O157 is possible on specific variations of MacConkey media, and on chromogenic agars. There were 81 notifications of STEC in Australia in 2010; a rate of 0.4 per 100,000 population (89). Due to the lack of utility of conventional culture for detection of STEC or other enteropathogenic *E. coli* presenting in community samples, the reported rates of STEC infection in Australia are influenced by state policy. South Australia routinely tests all bloody stools by PCR for genes coding for shiga toxins and other virulence factors, and consequently has the highest notified rates at 2.0 per 100,000 (89). In our laboratory, any stool sample exhibiting 2+ or more red blood cells in a wet preparation during faeces microscopy is also screened for STEC. Screening is performed using an assay published by Paton and Paton, which is a five target PCR for associated virulence factors including *stx*1, *stx*2, *eae*A, *ehxA* and *saa* (95). The diagnosis of the entire range of pathovars of *E. coli* is generally achieved only via molecular detection of the associated virulence factors that confer the pathogenic phenotype. Vidal et al. describe a multiplex assay to identify the six categories of diarrhoeagenic *E. coli*. The multiplex assay was applied to testing 509 stool samples from children <9 years of age presenting with acute diarrhoea in Chile and 76 patients were found to have a diarrhoeagenic *E. coli* present (77). Pathovars and their typical virulence mechanisms enabling molecular identification are as follows. Enterotoxigenic *E. coli* (ETEC), LT and ST toxin genes. Enteropathogenic *E. coli* (EPEC), *eae* encoding intimin (without the presence of *stx*1/2), the EAF plasmid and the *bfp* pilus gene. Enterohaemorrhagic *E. coli* (STEC or EHEC), *stx*1, *stx*2 shiga toxin genes and *eae*. Enteroinvasive *E. coli* (EIEC), *ipaH* invasion plasmid though this is also present in *Shigella*. Enteroaggregative *E. coli* (EAggEC), AAF-II adhesion fimbriae. And Diffusely Adherent *E. coli* (DAEC) have the *daa* gene producing F1845 fimbriae.

A bacterium originally described as *Hafnia alvei* has also been implicated in gastrointestinal disease via the same attachment and effacement mechanism associated with STEC and EPEC (96). Hyma et al. reported that subsequent studies have identified similar *H. alvei*-like strains that were positive for an intimin gene (*eae*) probe. Based on DNA relatedness these were classified as a distinct *Escherichia* species, *Escherichia albertii* (96). These *E. albertii* strains were found to be closely related to strains of *Shigella boydii* serotype 13(96). Molecular detection of *eae* (although not species specific) could assist in recognising gastrointestinal disease potentially due to *E. albertii*. 
Yersinia

*Yersinia enterocolitica* can cause gastroenteritis via the ingestion of contaminated food (and in particular raw pig meat) or water (90). *Y. enterocolitica* is divided into a number of biogroups some of which are not pathogenic or only weakly pathogenic. Accordingly the significance of detection of this organism in stool also requires clinical input. Biogroup 1B is the most pathogenic and possesses a virulence plasmid as well as chromosomal genes that encode the heat stable toxin YST(90). The specific gene targets that have been utilised for molecular detection of *Y. enterocolitica* from stool include the *ail* outer membrane attachment, *ystA*, *ystB*, *virF* transcriptional activator, *yadA* adhesion and the *lysP* lysine transporter (67, 80, 82, 84, 85, 97, 98). *Yersinia pseudotuberculosis* has also been known to cause a self limiting diarrhoea in young children and adults and other species of *Yersinia* are of uncertain significance (90). The *Yersinia spp.* assay of Cunningham et al., targeting *lysP* was found to detect thirteen species of *Yersinia* excluding *Y. kristensenii* (85).

The use of such an assay may fulfil recommendations that detection of any *Yersinia* species from stool in symptomatic patients with no other diagnosis should be reported (90).

Plesiomonas shigelloides

*Plesiomonas shigelloides* generally causes a self limiting secretory watery diarrhoea but can also present as a dysenteric bloody diarrhoea (90). Gastroenteritis due to *P. shigelloides* is generally observed in travellers returning from tropical countries and is associated with ingestion of seafood (99). Molecular detection of the organism has been performed from human faeces utilising an assay targeting the 23S rRNA gene, and also from fish portions using the *hugA* haem receptor protein gene (64, 65).

Edwardsiella tarda

*Edwardsiella tarda* is typically associated with water and animals such as turtles or fish. The organism is recognised as a rare cause of gastroenteritis and possesses cell associated haemolysins and cell invasion mediators (90). In our laboratory, all of the cases of gastroenteritis from which we have recovered *E. tarda* have been associated with owners of pet turtles cleaning their tanks. The most recent case we have observed was in 2011. To date all of the published molecular detection assays for *E. tarda* have been directed towards diagnosis in marine animals (100, 101).
**Vibrionaceae** (*Vibrio cholerae, Vibrio parahaemolyticus, Vibrio fluvialis*)

*Vibrio* species inhabit marine and brackish water systems, all requiring some amount of Na⁺ for growth. *V. cholerae* causes endemic, epidemic and pandemic cholera disease. There are three distinct sub groups *V. cholerae* O1, non-O1 and O139. O1 and O139 disease is similar with copious watery diarrhoea where severe dehydration can lead to death, non-O1 results in a milder disease as cholera toxin is rarely produced (90). *Vibrio parahaemolyticus* can cause a watery diarrhoea and is usually acquired via ingestion of contaminated raw fish or shell fish (102). *Vibrio fluvialis* is also recognised as being responsible for sporadic gastroenteritis outbreaks and has been reported as the cause of severe gastroenteritis (103, 104).

A number of multiplex PCR assays for identification of several species of *Vibrio* including *V. cholerae, V. parahaemolyticus* and *V. fluvialis* have been published using target genes such as *dnaJ, rpoB, ftsZ, tdh, trh* and *toxR* (105-109). The performance of the multiplex assay of Nhun et al. (106) was evaluated specifically utilising human stool and a sensitivity of 10⁵ to 10⁶ cells/mL of stool was observed.

**Aeromonadaceae** (*Aeromonas hydrophila complex, caviae complex, veronii complex*)

Gastroenteritis caused by *Aeromonas* most commonly presents as an acute self limiting watery diarrhoea (90). Although cholera like presentations have been reported which have dysenteric presentations similar to that observed with *Shigella* (90). Complications can include HUS, and recently homologs to the *stx1* and *stx2* genes of STEC were identified in strains of *Aeromonas* (110). The organisms are inhabitants of aquatic environments and are recovered from food and water sources associated with human infection. The consensus is that not every isolation of *Aeromonas* from stool reflects involvement in intestinal disease, though some most certainly do. Both conventional and molecular detection requires careful interpretation in relation to the significance of detecting the organism (90, 111). An *Aeromonas hydrophila* complex specific Taqman molecular assay has been published with primers and probes that target specific sequences of the 16S rRNA gene as well as the cytolytic enterotoxin gene (*aerA*) in a duplex assay (74). *Aeromonas hydrophila* complex is more associated with severe infections and septicaemia than the other species groups (90).
**Curved and Spiral Shaped Gram Negative Rods (Campylobacter spp., Arcobacter butzleri, Brachyspira)**

*Campylobacter jejuni* and *Campylobacter coli* are the most common cause of bacterial gastroenteritis in Australia and globally (59, 89). Sporadic outbreaks are also caused by two other species *C. lari* and *C. upsaliensis* (90). The severity of campylobacteriosis varies and is characterised by diarrhoea often with blood, abdominal pain, fever, nausea and or vomiting. In 2010, there were 16,966 notified cases of campylobacteriosis in Australia, a rate of 112 per 100,000 (89).

Conventional culture requires selective agar incubated in a microaerophilic atmosphere for up to three days. In attempt to simplify identification and to improve the speed of diagnosis a number of authors have published assays specifically for *C. jejuni* and *C. coli* detection and/or differentiation targeting genes such as *hipO*, *glyA*, *mapA* and *ceuE* (60, 102, 112). Debruyne et al. published an assessment of seven different assays for detection of *C. jejuni* and *C. coli* reporting that two multiplex assays were highly reliable (113). Other assays have been published for detection of the four major species infecting humans including an assay targeting the *glyA* gene and using hybridisation techniques (114) and an assay using a genus specific 16SrDNA PCR assay followed by a second bi-probe melting curve PCR analysis to determine species identity (115). Real time molecular detection assays for *Campylobacter* in food products have also been published for *C. jejuni* and *C. coli* (68) as well as an assay that includes specific detection of *C. lari* (70). The results reported indicate that molecular techniques when used for detection of *Campylobacter* in complex matrices such as stool or food result in significant improvements of 15-20% or greater in sensitivity of detection compared to culture (8, 59, 70).

In our laboratory spiral and curved organisms are sometimes observed in faecal microscopy techniques but without *Campylobacter* being recovered in culture, a phenomenon also observed by Bessede et al. (59). Aside from the previously mentioned poor sensitivity of culture methods for *Campylobacter*, some of the observations of spiral or curved bacteria when followed up in our laboratory have in fact been *Arcobacter butzleri* and *Brachyspira pilosicoli*. *A. butzleri* can be recovered on certain *Campylobacter* culture media if the incubation temperature utilised is 37°C, and it has been reported as the fourth most common “*Campylobacter* like organism” isolated from patients with diarrhoea (116). A recent study in New Zealand found *Arcobacter* as the causative agent in 0.9% of diarrhoeal patients which is consistent with a report from France where *A. butzleri* was recovered in 1% of patients (117, 118). Molecular assays for detection of *Arcobacter* have targeted several genes including *rpoB/C*, 16S rDNA and 23S rDNA in both human stool and food matrices (57, 61, 63, 71, 117, 119, 120). *Brachyspira pilosicoli* and *Brachyspira aalborgi* are spirochaetes which colonize the intestines of a number of animals, *B. pilosicoli* causes disease in pigs and both species may be under...
reported as human pathogens though this is not yet proven (90, 121). Large
numbers of spirochaetes attached to the intestinal epithelium may form a ‘false
brush border’, and this condition is referred to as ‘intestinal spirochaetosis’ or IS (90).
Gad et al. suggest that “heavy infestation of the gut surface epithelium by
spirochaetes acts as a barrier for the normal absorptive processes and leads to
diarrhoea” (122). Brachyspira have been detected in human faeces utilising PCR
assays targeting the 16S rDNA gene (123, 124).

Clostridia (Clostridium perfringens, Clostridium difficile)

Diarrhoea due to Clostridium perfringens type A results from ingestion of $10^8$ or more
vegetative cells from contaminated food (generally meat). Release of an enterotoxin
(CPE) during sporulation causes mild self limiting diarrhoea within 7-30 hours of
digestion of the food. It has also been implicated as a cause of persistent diarrhoea
in the elderly in healthcare facilities (90). CPE is encoded by the cpe gene carried
on a large plasmid of enterotoxin producing strains and has been utilised as a
diagnostic PCR target (125).

C. difficile strains that possess certain toxin genes can cause a spectrum of enteric
disease from mild self limiting diarrhoea, a bloody-slimy diarrhoea often referred to
as C. difficile associated diarrhoea ‘CDAD’, or the more serious pseudomembranous
colitis (126). The organism causes a significant burden and is prevalent in
healthcare facilities due to a combination of factors including resistance of the spore
form to disinfectants, interference of the protective effect of normal flora via the use
of antibiotics (in particular clindamycin, expanded spectrum cephalosporins and
fluoroquinolones) and patient age (90).

Toxigenic strains of C. difficile that are capable of causing symptomatic intestinal
disease possess a pathogenicity locus termed the ‘PaLoc’(127, 128). Genes within
the PaLoc including tcdA (enterotoxin), tcdB (cytotoxin) and the accessory genes
tcdC, and tcdE have also been utilised as molecular diagnostic targets. The
existence of strains without tcdA or with variant tcdA has seen tcdB become the
most common molecular target even though tcdB itself contains variability (129,
130). In Australia, approximately 1.1% of toxigenic isolates have been reported as
tcdA-/tcdB+ (131). This is an important consideration as one of the more common
commercialised molecular technologies being implemented in Australian diagnostic
laboratories this year has been the Illumigene C. difficile LAMP assay (Meridian
Bioscience) which targets non-variant regions within the tcdA gene with isothermal
loop- mediated amplification. In part to address the question of performance of the
Illumigene assay for detecting tcdA-/tcdB+ isolates the manufacturers have
published data that has shown that the 5’ end of tcdA is not deleted in the common
tcdA-/tcdB+ toxinotypes VI and VII and these strains are still detected by the
technology (132). Of all of the bacterial enteropathogens, toxigenic *C. difficile* is rapidly becoming the most widely detected by molecular technologies in diagnostic laboratories and it has the greatest number of commercialised molecular testing products available.

Hyper virulent strains of *C. difficile* (PFGE type NAP1), which are more correctly known as PCR ribotype 027 strains have been observed in Australia and elsewhere and have been associated with increased morbidity and mortality (133). Molecular investigation of deletions within tcdC and/or the presence of the binary toxin genes cdtA and cdtB have been used as indicators of the possible presence of a hyper virulent strain requiring further investigation at a reference laboratory (134).

The presence of *C. difficile* in human faecal flora by PCR requires clinical correlation due to the possible presence of asymptomatic toxigenic *C. difficile* at high levels in children and neonates, and also in approximately 3-5% of healthy adults (90, 135).

Arguments have been made for *C. difficile* to be included in future commercial multi analyte assays for two reasons. The first is an observation that 30% of requests for single investigation of *C. difficile* associated diarrhoea based on clinical suspicion were negative for the organism but were in fact positive for other enteropathogens (136). The second is the increased observation of community acquired symptomatic *C. difficile* diarrhoea that supports the inclusion of this organism in any multi panel enteropathogen investigation screening technique.

**Listeria monocytogenes**

In the immunologically competent host gastroenteritis due to *Listeria monocytogenes* generally occurs 24 hours after ingestion of a food source containing large number ($10^5-10^9$ CFU/g or mL) of the organism and the illness lasts about 2 days (90). Gastroenteritis and invasive listeriosis following ingestion may affect the elderly or immunocompromised. Listeriosis in pregnancy can result in infections of the foetus. In 2010, there were 71 notified cases of invasive *L. monocytogenes* infection in Australia (89).

The majority of PCR assays targeting *L. monocytogenes* have been developed in the context of screening the food supply or for applications involving assessment of waste water treatment efficacy (73, 75, 83, 137). The hly (hemolysin listeriolysin O) gene target has been utilised most successfully and provides a *L. monocytogenes* species specific assay target with a detection limit between 3 and 30 template molecules per PCR reaction (72). One author incorporated three marker genes for *L. monocytogenes* (iap, hly and prfA) as targets for a PCR-Array based diagnostic assay for human stool samples but none of the 34 clinical samples tested contained the organism (84). *L. monocytogenes* has also recently been included in the
bacterial enteropathogen PCR panel developed by local diagnostic company Genetic Signatures (Easy-Screen Enteric Bacteria Detection Kit) (138).
Several viruses are known to cause gastroenteritis and together they are responsible for significant morbidity and mortality. On a global basis viral enteric disease accounts for 60% of illness in developing countries and 40% in developed countries (139). The most common viruses causing gastrointestinal disease are Rotaviruses, Noroviruses, Astrovirus and certain Adenoviruses. Other less recognised viruses include Sapovirus, certain Enteroviruses and human Parechovirus. Given many symptomatic patients are not diagnosed with a known enteropathogen, suspicion remains that undiscovered enteropathogens and in particular viruses are likely to exist. In fact recent work using new technologies to enable sequence independent viral discovery techniques has identified unique viral nucleic acids and variants of known enteropathogenic viruses in stools from symptomatic patients (3). Though treatment for viral gastroenteritis is supportive only, rapid diagnosis assists in isolation, outbreak tracing and cessation of antibiotic courses if they have been started due to suspicion of bacterial or parasitological disease (102).

Most of the enteropathogenic viruses are RNA in nature and require a reverse transcriptase component to convert RNA into cDNA prior to utilising any DNA based molecular amplification technique for diagnosis or characterisation. Discussion of the common molecular approaches to diagnose specific viral enteropathogens follows.

**Rotavirus**

Rotavirus is the most common viral cause of gastroenteritis. It has a segmented dsRNA genome and is divided into seven serogroups with serogroup A being the predominant cause of infections in humans (140). In Australia, the dominant genotype changes year to year, in 2010-2011 the genotype G2P[4] was predominant representing 51% of samples examined under the Australian Rotavirus Surveillance Program (141). The development of two live oral rotavirus vaccines Rotarix® (GlaxoSmithKline) and RotaTeq® (Merck) and their use in Australia since 2007 has shown an early impact with significant declines in hospitalisation and emergency room visits reported since vaccine introduction (141). Conventional testing techniques are generally rapid antigen tests either as RPLA, lateral flow immunoassays, or ELISA detection kits, and usually detecting the VP6 antigens. Molecular methodologies have not been widely adopted for initial diagnostic testing despite the fact that they have been shown to have superior analytical and clinical sensitivity compared to immunoassays (142). The potential benefits of using multiplex approaches for multi viral detection from stool has resulted in the development of a number of highly sensitive multiplex assays targeting viral enteropathogens (139, 140, 143). Aside from reducing the overall costs, multiplexing for viral enteropathogens is viewed as worthwhile because many are
clinically indistinguishable (102). Molecular diagnosis of Rotavirus has focused predominantly on the VP7 or VP6 outer capsid protein genes as the diagnostic targets. It has also been suggested that assay specific cycle threshold cut-offs be utilised for real-time PCR based rotavirus diagnostics, due to the observation that higher Ct value detection (e.g. Ct of >28) of Rotavirus was less associated with gastroenteritis and that 20-40% of rotavirus infections are subclinical (139).

**Adenovirus**

A subgroup of Adenoviruses known as Group F serotype 40 and 41 are associated with gastroenteritis (90). Adenoviruses have a dsDNA genome and conventional diagnosis is normally via the use of rapid immunoassays or ELISA. Variation in the fiber gene determines amino acid changes conferring serotype in the terminal portion of the fiber protein (144). Many of the other Adenovirus serotypes are shed in faeces so it is important that Group F specific molecular assays are utilised for diagnosis of enteropathogenic Adenovirus (102). At least two Adenovirus real-time PCR assays have been developed targeting specific regions of the fiber protein gene to deliver Group F specificity when detecting diarrhoea due to Adenovirus serotype 40 or 41. The fiber gene Group F specific assay of Jothikumar et al. reportedly has a sensitivity of 3-5 copies per reaction (145, 146). The authors also designed a second assay targeting a conserved region of the Hexon gene to report all Adenovirus groups A-F (145). As was found for rotavirus, adoption of RT-PCR methodology has been reported to increase the recovery of Adenovirus significantly, Logan et al. reported a 175% increase compared latex agglutination testing when using a Group F specific molecular assay (146). Two of the three multiplex/panel viral enteropathogen assays previously mentioned also include primers and probes specific for Adenovirus Group F (140, 143).

**Norovirus and Sapovirus**

Norovirus and Sapovirus are both members of the Caliciviridae possessing ssRNA genomes which exhibit a high level of genetic diversity and are divided into five genogroups GI to GV, GI and GII predominantly cause acute viral gastroenteritis, and GIV occasionally (140). Each genogroup can be further subdivided into multiple genotypes with GII/genotype 4 strains predominant globally (147). The burden of disease from these viruses is high, the CDC estimated that each year in industrialised countries these viral infections cause 64,000 episodes of diarrhoea that require hospitalisation as well as 900,000 clinic visits by children and up to 200,000 deaths of children under five years old in developing countries (147). As there is no effective method for culturing these viruses, molecular technology has been the dominant diagnostic modality and is already considered the gold standard. Consequently there are a significant number of commercialised molecular test kits
available, as well as a substantial number of published molecular assays. The most
successful molecular target utilised for detection of Norovirus is a region at the
ORF1-ORF2 capsid-polymerase junction and derives from the work of Kageyama et
al. (148). Singleplex or multiplex Taqman based adaptations containing specific
component assays for GI and GII genogroups designed in this region have become
widely used with clinical sensitivity reported as 91-97% (102, 149-151). Sapovirus
detection assays have evolved identically to Norovirus assays and the most common
approach is also real-time Taqman assays (152-155). Diagnostic targets utilised
are the capsid-polymerase junction or conserved regions of the polymerase. Two of
the three multiplex/panel viral enteropathogen assay publications include assays for
Norovirus GI and GII as well as Sapovirus (139, 140).

Astrovirus

Astroviruses are positive sense, ssRNA viruses and cause acute gastroenteritis in
children and sporadic outbreaks in adults (156, 157). A number of real time PCR
assays have been published for the detection of Astrovirus and the ORF1a, 3'NCR
and capsid sequence regions have been used (143, 158, 159). Logan et al.
compared RT-PCR to electron microscopy and observed a tenfold increase in the
rate of detection of Astrovirus (154).

Human Parechovirus (HPeV)/Enteroviruses

Human Parechoviruses (HPeVs) are associated with gastroenteritis however the
aetiology is not clear (139). Although the majority of HPeV infections occur in
children without specific symptoms, symptoms have been described ranging from
gastroenteritis and respiratory infections to neurological disease especially in the
very young (160). Braun et al. recently published a study following viral
gastrointestinal disease in a childcare centre and observed HPeV was present in
21% of symptomatic children, but also in 27% of samples from asymptomatic
children (161). Molecular detection techniques have been published and generally
target HPeV specific sequences in the 5'UTR giving specificity compared to other
Picornaviridae (139, 162, 163). Amongst the Enteroviruses a number have been
associated with diarrhoea, in particular certain Echovirus types (164, 165).
Molecular detection targets are also primarily designed at conserved specific regions
of the 5'UTR, however a recent publication by Harvala et al. discusses specificity
problems with 5'UTR assays for diagnosis of enteroviruses in symptomatic patients
presenting to hospital due to apparent gastrointestinal rhinoviruses (166).
An Australian study by Bowman et al. reported a detection rate of 2.0% (10/505) from submitted stools for all parasites by combination of routine and concentration microscopy (167). For the period 2007-2011 in our laboratory a diagnostic rate of 12.3% (31299/255018) for all parasites was observed from wet preparation, faecal parasite concentration and fixed stain microscopy techniques on 255018 stool samples (56). Ova, cysts and parasite examination by faecal concentration microscopy and/or wet prep, has been reported as having a diagnostic yield of 2.9% for Entamoeba complex in an Australian study by Fotedar et al. (168).

Dependent on volumes of samples received for testing, the microscopy component of the diagnostic workup poses a large burden to the workflow of a lab and indeed on the general health of the laboratory staff examining the slides. Repetitive strain injuries are becoming more common as volumes of samples requiring extended field examination by microscopy increase. The sensitivity of microscopy for detection of parasites has been shown to be approximately 60% at best (1). The high specificity of molecular techniques is also superior for organisms which resemble each other closely or are indistinguishable by visual examination but have quite different relevance clinically e.g. Entamoeba histolytica, Entamoeba dispar and Entamoeba moshkovskii (2).

There have been a significant number of publications for molecular parasitology in recent times. Amoebae, Flagellates, Coccidia, Blastocystis hominis, and Microsporidial assays predominate, but there are also an increasing number of publications of molecular assays for Helminths.

Specific conventional diagnostic techniques for soil transmitted helminths require several days of observation for results and can pose an infection risk to laboratory staff e.g. the Harada-Mori larval stage nematode culture (169) and the Strongyloides faecal culture (170). Both of these conventional techniques appear highly suited to replacement by molecular technology.

The utility of real-time multiplex PCR for concurrent testing of multiple parasites is viewed favourably due to decreased cost/target, an increased range of parasites examined and improved speed of analysis (171). Articles using at least triplex multiplex testing include the following combinations: E. histolytica/G. intestinalis/C. parvum (172, 173); E. histolytica/G. intestinalis/Cryptosporidium sp./Dientamoeba fragilis (1); Ancylostoma/Necator americanus/Ascaris lumbricoides/Strongyloides stercoralis (174); E. histolytica, E. dispar and E. moshkovskii (175); a Luminex implementation for E. histolytica/G. intestinalis/Cryptosporidium sp./Ancylostoma duodenale/ Necator americanus/Ascaris lumbricoides/Strongyloides stercoralis (176); Ancylostoma duodenale/Necator americanus/Oesophagostomum bifurcum (177); and Cyclospora/Cystoisospora/Microsporidia (178).
Amoebae present in the human intestine can be divided into three groups. The first is the pathogen *Entamoeba histolytica*, which is responsible for amoebic dysentery and invasive disease. The second is *Entamoeba moshkovskii* and *Entamoeba dispar* which are generally considered non pathogens, but both have various case reports postulating their involvement in intestinal disease and discomfort (2). And the third is a range of non-pathogens which include *Entamoeba coli*, *Entamoeba hartmanni*, *Entamoeba polecki*, *Endolimax nana* and *Iodamoeba buetschlii*.

Molecular techniques have been particularly useful for detection and differentiation of *E. histolytica* from *E. dispar* and *E. moshkovskii* as these organisms are morphologically identical by conventional microscopy techniques and clearly have very different clinical relevance. The target utilised is generally 18S rDNA which encodes the small subunit rRNA as it offers appropriate specificity for distinguishing between the three species and is also multi copy which enhances assay sensitivity. Many other molecular targets have been utilised and the review of Fotedar et al. provides a comprehensive overview (2). The multiplex assay of Verweij et al. for *E. histolytica/G. intestinalis/C.parvum* was shown to exhibit 100% sensitivity and specificity (173). A multiplex real time PCR assay for detection and differentiation of *E. histolytica*, *E. dispar* and *E. moshkovskii* has also been published which targets 18S rDNA and utilises hybridisation probes and melting temperatures for species specific identification (175).

Traditionally the presence of the non pathogens *E. coli*, *E. hartmanni*, *E. polecki*, *E. nana*, and *I. buetschlii* has been made via observing cysts and/or trophozoites in faecal specimens via concentrated wet mounts and/or permanent stained smears. One of the disadvantages of the current multiplexed molecular assays for parasites is that the non-pathogens are no longer identified. Arguments exist both for and against the need to incorporate detection of non-pathogenic protozoa. What is clear is that acquisition of the non-pathogenic protozoa is normally via the same faecal-or oral route as pathogenic protozoa and the detection of non-pathogens indicates possible exposure to faecal material warranting further examination in symptomatic patients (179). Santos et al. also promote the value of reporting non pathogenic amoebae when observed and have published an 18S rDNA based PCR-DNA sequencing approach for identifying the *Entamoeba* species present in samples (180). A sequencing based approach is not realistic for routine diagnostic detection of the non-pathogenic *Entamoeba* and the development of real-time PCR assays for non-pathogenic protozoa would be a better approach.

Flagellates
Flagellates in the human intestine include two pathogens; *Giardia intestinalis* and *Dientamoeba fragilis*, and three non-pathogens; *Chilomastix mesnili*, *Retortamonas intestinalis* and *Trichomonas hominis*.

*Giardia intestinalis* is the most common non viral enteropathogen causing gastroenteritis in developed countries (173). Prevalence of 2-5% in developed countries and 20-30% in developing countries has been reported (181). *G. intestinalis* accounted for 18% (5752/31299) of the total reported parasites from our laboratory between 2007 and 2011 as shown in Table 3. Diagnosis can be via microscopy and observation of cysts and trophozoites. However often the initial screening is performed using ELISA or other immunoassay methods which generally also detect *Cryptosporidia*. Molecular assays for *G. intestinalis* also primarily target specific regions of the multi copy ribosomal genes including the 18S rDNA and intergenic spacers (182, 183). Other targets utilised in real-time PCR assays include the beta-giardin gene (184). As *G. intestinalis* is the second most reported parasite after *Blastocystis hominis* it features prominently in all of the multiplex parasite methods published excluding those designed for helminths alone (1, 172, 173, 185-187). The performance of molecular testing for *G. intestinalis* has proven to be very good, several publications show PCR to have excellent sensitivity and specificity when compared to ELISA methodology and PCR exhibited at least a 10%-13% improvement in sensitivity compared to microscopy (182, 183). Bruijnestijn et al. reported PCR for *G. intestinalis* to be 34% more sensitive than microscopy (185).

*Dientamoeba fragilis* genetically is characterised as a flagellate, but in fact is ‘aflagellate’ and morphologically resembles amoebae (102). *D. fragilis* is particularly difficult to diagnose even when using preserved stools and fixed stain microscopy based techniques due to the fact the trophozoites degenerate rapidly and lose nuclear definition (188). The Australian prevalence of *D. fragilis* has been reported as being from 0.9 to 16.8% in patients symptomatic with gastroenteritis (189). In our laboratory despite the difficulties in diagnosing the organism, it comprised 2.5% (791/31299) of the total parasites that were reported between 2007 and 2011, Table 3. Molecular detection of *D. fragilis* has been shown to offer considerably better performance and has been reported to be 42%-65% more sensitive than microscopy (185, 188). Targets utilised for molecular detection of *D. fragilis* have been the 5.8S rRNA gene (185, 190) and the SSU 18S rDNA (191).

The relevance of detecting the non-pathogenic flagellates is similar to the detection of the non-pathogenic amoebae as previously discussed. The non-pathogenic flagellates have also not had molecular methods published other than the sequencing approach also previously described for amoebae.

**Coccidia**
Coccidial enteropathogens include *Cryptosporidium parvum*, *Cryptosporidium hominis*, *Cyclospora cayetanensis* and *Cystoisospora belli* (previously *Isospora belli*). All are obligate intracellular pathogens.

Cryptosporidiosis is a parasitic infection of the lower intestine; symptoms generally include abdominal cramping and usually large-volume watery diarrhoea. The ingestion of contaminated water is the major risk factor for infection (89). In 2010, there were 1,480 notified cases of cryptosporidiosis reported in Australia (89). The major burden of disease is generally in children <5 years of age (172). In our laboratory *Cryptosporidium* spp. comprised 7.4% (2313/31299) of the total parasites we reported between 2007 and 2011, Table 3. Diagnosis is generally via modified acid-fast staining techniques and observation of the oocysts using microscopy. Often the initial screening is performed using ELISA or other immunoassay methods which generally also detect *G. intestinalis*. Consensus primers/probes for molecular detection of *Cryptosporidium* spp. are recommended, as there are a number of *Cryptosporidium* species known to infect humans for example; *C. parvum*, *C. hominis*, *C. felis* and *C. meleagridis* (1). Molecular assays have targeted a variety of genes including the SSU 18S rDNA (1, 192), a *C. parvum* specific ‘segment A’ sequence (172, 173, 185, 193), the COWP gene (194), actin gene (195), and the hsp70 gene (196). PCR has been shown to be specific and was reported as being 16% more sensitive than microscopy techniques (197).

*Cyclospora cayetanensis* is considered an emerging pathogen, generally associated with ingestion of contaminated food in travellers returning from SE Asia or South America (198). Symptoms reported are prolonged diarrhoea including nausea and abdominal cramps (199). Conventional diagnosis is similar to other coccidian parasites and relies on modified acid-fast staining techniques such as the modified Kinyoun acid-fast stain and observation of the oocysts via microscopy. In our laboratory 12 cases of *Cyclospora cayetanensis* were diagnosed between the years 2007 and 2011 from 255018 stool samples submitted for enteropathogen testing (Table 3). The molecular target utilised in published methods is the SSU 18S rDNA (178, 198, 199). Sensitivity has been reported as 1 oocyst per 5uL PCR reaction volume (199).

*Isospora belli* is primarily associated with immunologically compromised patients in which it can cause chronic and severe diarrhoea. However, *I. belli* has also been reported as causing infections in children and travellers to tropical regions (200, 201). For the other coccidian parasites conventional diagnosis again relies on modified acid-fast staining techniques such as the modified Kinyoun stain and observation via microscopy. At our laboratory, we did not detect *I. belli* in the four years between 2007 and 2011. Ten Hove et al. have developed a real time PCR assay for the detection of *I. belli* with very good performance characteristics, reporting 100% sensitivity and 100% specificity (201). The molecular targets utilised for *I. belli* PCR assays include the internal transcribed spacer 2 (ITS-2) region of the ribosomal genes and the SSU 18S rDNA (201, 202).
Ciliates

*Balantidium coli* is an opportunistic parasitic pathogen with pigs as the primary host, humans become infected via direct or indirect contact with pigs and develop balantidiasis. The infection may be subclinical (203), or can develop as a fulminant infection with bloody and mucus containing diarrhoea and possible perforation of the colon (203). To date no PCR detection assays have been published but ITS sequencing (204) and the SSU 18S rDNA (205, 206) have been utilised in taxonomical studies with other *Balantidium* species and would likely be suitable targets for the development of specific real-time PCR assays.

Blastocystis hominis

*Blastocystis hominis* is classified in its own Kingdom (Stramenopila) (90). The organism is by far the most common parasite observed in human stool samples in all countries (207). In our laboratory, *Blastocystis hominis* comprised 64.4% (20148/31299) of the total parasites we reported between 2007 and 2011 (Table 3). Some patients from which *B. hominis* is recovered report abdominal pain, diarrhoea, bloating, and flatulence. However detection of *B. hominis* is of uncertain clinical relevance, recent studies indicate that the parasite is actually a species complex with perhaps up to nine different types found in humans, all with variation in virulence (208). Subtype 3 is the most common in prevalence studies, followed by subtype 1 (208). Roberts et al. have published a very well designed assay targeting the SSU 18S rDNA with primer sets carefully designed to avoid the variation inherent in the wide range of genotypes (209). Sensitivity was reported as 94% (92/98) which was 51% better than the sensitivity of microscopy from a modified iron-haematoxylin permanent stain (209). An incidence of 19% in symptomatic Sydney population was much higher than that observed by our laboratory in Brisbane which was 6.3% (3576/56876) for 2011, Table 3.

Microsporidia

Microsporidia are small spore-forming obligate intracellular organisms and were originally considered protozoa but have since been recognised as being fungi (102). *Enterocytozoon bieneusi,* and *Encephalitozoon intestinalis* are the predominant microsporidia infecting humans and for the most part only those patients with immunosuppression are infected. The small size, intracellular nature and poor staining with histological stains results in under-reporting of microsporidial infections (200). The conventional diagnostic technique utilised is modified trichrome staining and examination under the microscope for the typical morphology. Several molecular assays have been published, and potentially the most useful is the TaqMan multiplex of Verweij et al. which detects both *E. bieneusi,* and
Encephalitozoon intestinalis via the ITS of the ribosomal genes (210). Another molecular approach uses a multiplex Luminex bead array assay including *E. bieneusi*, and *E. intestinalis* and this method yielded 87–100% sensitivity and 88–100% specificity (178).

Helminths

Soil-transmitted helminths (*Strongyloides stercoralis, Ascaris lumbricoides, Ancylostoma duodenale, Necator americanus* and *Trichuris trichiura*) infect an estimated one-sixth of the global population (211). The rate of infection by helminths is reportedly the highest for children living in sub-Saharan Africa, and this is followed by Asia, Latin America and the Caribbean (211). Molecular PCR amplification as a diagnostic modality has been compared against both the Harada-Mori larval stage nematode culture (169) and the *Strongyloides* faecal culture (170). Gotuzzo reported a sensitivity of 95% for *Strongyloides* diagnosis by PCR, equivalent to the performance of both the Harada-Mori which had a sensitivity between 70 and 100% (dependent on the study) and also *Strongyloides* Agar Plate Culture which had a sensitivity of 85-97% (also dependent on the study) (212). Several multiplex real-time molecular assays have been designed combining some of the soil transmitted helminths and are listed as follows; *Ancylostoma/Necator americanus/Ascaris lumbricoides/Strongyloides stercoralis* (174); a Luminex implementation for *Entamoeba histolytica/Giardia intestinalis/Cryptosporidium sp./* Ancylostoma duodenale/ Necator americanus/Ascaris lumbricoides/Strongyloides stercoralis* (176) and an assay for *Ancylostoma duodenale/Necator americanus/Oesophagostomum bifurcum* (177).

Molecular targets in the Luminex bead array assay reported by Taniuchi et al. for *Ascaris lumbricoides* and the hookworms were the ITS1 and ITS2 regions respectively. The SSU 18S rDNA target was utilised for *Strongyloides stercoralis*. Reported sensitivity of the multiplex compared to parent singleplex assays was between 83% and 100% (187). Basuni et al. utilised the same target genes for a pentaplex multiplex for *Ancylostoma/Necator americanus/Ascaris lumbricoides* and *Strongyloides stercoralis*, which also included a phocine herpesvirus internal control reaction (174). Basuni et al. compared the results of the multiplex to those of the conventional methods and reported that 48/77 samples were positive with the molecular test and just 6/77 were positive with the microscopy.

A limited number of real time PCR based molecular assays have been published for other helminths. Design and validation of real-time assays for many of the helminths listed in Table 1 would be required to complete any comprehensive molecular approach.

An assay was published for detecting the trematode *Clonorchis sinensis* in stool samples (213). The internal transcribed spacer 2 (ITS2) region was utilised to
design a specific TaqMan real time PCR assay. The authors reported a diagnostic
sensitivity of 95.9% and 100% sensitivity at 100 eggs/g, though egg counts as low as
1 egg/g were PCR positive.

A multiplex real-time PCR assay for detecting *Schistosoma mansoni* and *S.
haematobium* in stool samples was published by ten Hove et al. (214). This assay
utilised primers and probes targeting the cytochrome c oxidase gene. A detection
rate of 84.1% compared favourably to microscopy at 79.5%. The authors reported
100% sensitivity for the *S. mansoni* assay at 100 eggs/g (EPG).

Alasaad et al. published a real-time PCR assay for the identification of the trematode
*Fasciola* spp (215). The ITS-2 region was utilised to design genus specific primers
for *Fasciola* and to design species specific internal TaqMan probes to identify *F.
hepatica* and *F. gigantica*. The authors reported a sensitivity of 1 pg/µL of *Fasciola*
DNA. Other diagnostic targets have also been utilised and Le et al. have published
an assay targeting the mitochondrial DNA genes *cox1-trnT-trrnL* (216).

Intapan et al. designed a real-time FRET based PCR assay for detection of the
trematode *Opisthorchis viverrini* in human faecal samples (217). The primers and
probes were designed to bind to a specific region of the pOV-A6 specific DNA
sequence. Diagnostic sensitivity was reported as 97.5% and specificity as 100%.
There was 100% sensitivity at 100 EPG, 94.4% at 1-99 EPG and the lowest EPG
detected by the PCR was 17 EPG.

Conventional PCR has been utilised with a wide range of genetic targets for the
following helminths. ITS2 for the trematodes *Paragonimus westermani*, *Fascioloops
buski* and *Fasciola gigantica* (218). The non-coding HDP2 sequence and the *cox1*
sequence has been used for the tapeworms *Taenia solium* and *Taenia saginata*
(219, 220). The 28S D1 ribosomal DNA (rDNA) and mitochondrial cytochrome c
oxidase subunit I (mtCOI) fragments contain appropriate species specific regions for
designing a real time PCR assay for the small trematode *Metagonimus yokogawai*
(221). The repetitive element DL1 has been utilised for detection of the cestode
tapeworm *Diphyllobothrium latum* (222) as has the cytochrome c oxidase subunit 1
gene of mitochondrial DNA (223). ITS1 was utilised to detect the nematode round
worm *Trichostrongylus spp.* (224). SSU 18S rDNA was utilised to detect the
whipworm *Trichuris trichiura* (225). And the mitochondrial Cytochrome C Oxidase
Subunit 1 (*cox 1*) has been used for species specific detection of the cestodes
*Hymenolepis nana* and *H. diminuta* (226).
Current Commercially Produced Multi Analyte Molecular Products

A summary of commercial molecular assays and the enteropathogens detected is presented in Table 2. The enteropathogen coverage of the currently available multi analyte molecular assays is quite variable. Before adopting these products, the scope of enteric pathogens usually observed by the laboratory and the desired workflow would need to be considered. In general, bacterial pathogens are reasonably well represented. Coverage of enteric viral disease ranges from none (Savyon) to a reasonably comprehensive set of five viruses (Genetic Signatures and Film Array). The commonest parasites detected in developed countries are represented in the majority of assays although Seegene currently has no parasite detection component. The maximum number of different parasites detected by the commercial assays is six (Genetic Signatures). When compared to faecal parasite concentration microscopy the available molecular assays provide limited parasite coverage.

In our laboratory a review of the enteric pathogens reported from 255018 faeces samples submitted for testing between 2007 and 2011 indicated that 17 different parasites were reported and 11 different bacterial species, refer Tables 3 and 4 (56). Viral agents reported reflected the existing scope of testing which included Rotavirus, Adenovirus and Norovirus. Comparison to conventional methodology shows that the most comprehensive multi analyte option for parasites the Easy Screen Enteric Parasite Detection Kit (Genetic Signatures) which provides 6 parasitological assays would have covered 95.9% (30010/31299) of the total parasites reported by our laboratory. The NanoChip Gastrointestinal Panel (Savyon) and combinations of Easy-Plex kits (AusDiagnostics) offer four parasites (differing from Easy Screen by exclusion of Blastocystis hominis and Entamoeba complex) and would have resulted in coverage of 28.7% (8997/31299) of the parasites reported. The Luminex xTAG GPP parasite assay component (Cryptosporidium, Giardia intestinalis and Entamoeba histolytica) would have resulted in coverage of 26.2% (8206/31299) of the parasites reported. Based on types of parasites detected, using the molecular assay with the widest coverage would have resulted in 1299 patients between 2007 and 2011 (324/year) not having a parasite reported that would otherwise have been when using faecal parasite concentration microscopy.

Overall detection of bacterial enteropathogens at our laboratory from 2007-2011 was 19080 reported positives from 254940 cultures with a total of 10 different bacterial enteropathogens reported, refer Table 4 (56). In addition to the 10 bacterial species reported, toxigenic C. difficile has also been reported by PCR based testing alone in our laboratory since 1997. The coverage of gastrointestinal disease due to bacterial enteropathogens by the available molecular kits is also variable. The Luminex XTag GPP bacterial target range would have resulted in 79.4% coverage (15143/19080) of bacteria reported by our conventional culture. The ‘Diarrhea ACE’
product (Seegene) compares very well to the range of organisms we reported using conventional culture based methods and would have resulted in 99.8% coverage (19078/19080) of the bacteria reported. Thirty seven *Arcobacter*, one *Plesiomonas* and one *Edwardsiella* isolate would not have been reported by the Seegene molecular assay. The Savyon NanoChip Gastrointestinal Panel and the AusDiagnostics Easy-Plex Panel bacterial target range are identical and would have resulted in 77.3% coverage (14748/19080) of the bacteria reported by our conventional culture. The Genetic Signatures EasyScreen Enteric Bacteria Detection kit target range would have resulted in 79.1% coverage (15093/19080) of bacteria reported by our conventional culture methods.

For laboratories receiving moderate to high volumes of samples per day use of the available commercial molecular options might also present processing challenges. In our laboratory more than 240 samples/day are received six days per week for testing. The highest single instrument commercial kit ‘throughput/single test batch’ is provided by the NanoChip (Savyon), at up to 95 patient samples per batch in 4 hours, however this solution does not offer any diagnostic coverage of enteric viruses. The other methods are just 24, 32, 7 and 15 samples per batch (Luminex, Seegene, AusDiagnostics and Genetic Signatures respectively) in similar time frames on single instrumentation. One solution to throughput constraints is use of multiple instruments, but this adds cost and the need for additional laboratory space. Available laboratory space, particularly in controlled purpose built molecular laboratories, is an important consideration in modern pathology laboratory practice.

A number of evaluation studies examining analytical performance have now been completed for some of these commercial multi analyte enteropathogen detection kits, in particular the Luminex xTAG GPP test which was the first to carry approval as an IVD assay. Reported performance characteristics from various sources for the commercial products are as follows.

**Luminex xTAG GPP**

The xTAG GPP assay is based on the Luminex bead array hybridisation platform and offers up to 15 bacterial viral and parasitic pathogens in a single test as summarised in Table 2. The assay is based on a single one-step RT-PCR, a target specific primer extension, subsequent hybridisation to specific xTAG beads and then laser based detection on the Luminex Analyzer.

Chong et al. examined the performance of the GPP assay on 313 stools collected in a high prevalence paediatric population from Botswana for the comparative detection of viral targets including: Norovirus (GI/GII), Rotavirus A, and Adenovirus (serotypes 40 and 41) (227). The xTAG GPP assay results were compared to the authors in-house multiplex real-time PCR viral assay that detected Adenovirus, Norovirus GI and GII and Rotavirus A. Discordant results were subsequently tested with
singleplex PCR assays for the individual viruses (227). Six samples were shown to
be inhibitory by failure of the MS2 internal control assay, leaving 307 valid results in
the study. Overall 280/307 (91.2%) of the stools tested with the GPP assay were
positive and 108 (35.2%) of these were positive for more than one pathogen (227).
Positivity rates for various targets were as follows: Norovirus GI/GII 43/307 (14.0%),
Rotavirus A 176/307 (57.3%), Adenovirus 40/41 34/307 (11.1%), C. difficile
(4.2%), Campylobacter 49/307 (16.0%), ETEC LT/ST 28/307 (9.1%), Shigella 35/307
(11.4%), E. coli O157 4/307 (1.3%), Salmonella 15/307 (4.9%), STEC stx1/stx2
4/307 (1.3%), Yersinia enterocolitica 1/307 (0.3%), Cryptosporidium 19/307 (6.2%)
and Giardia 10/307 (3.3%) (227). When comparing the xTAG GPP with the authors’
in house viral multiplex real-time PCR assay, the Luminex platform showed a
sensitivity of 98.3% (176/179) for Rotavirus A, 97.7% (42/43) for Norovirus and
100% (34/34) for Adenovirus 40/41. Three rotavirus samples were negative by GPP
and positive by multiplex and singleplex rotavirus assays, subsequent examination of
the crossing points for the in-house assays were greater than 33 cycles, indicating
low viral loads in these samples (227).

Vocale et al. compared the xTAG GPP kit on 385 stool samples from hospitalized
symptomatic patients against culture for bacterial detection, EIA assays for virus
detection and microscopy for parasite identification (228). xTAG GPP produced
positive results in 27% of the samples tested (104/385) compared to a 13%
combined detection rate of the conventional methodologies. 10% of the positive
patients had two enteropathogens detected by the xTAG GPP system, with one
patient having three enteropathogens detected (228).

Marimón et al. assessed the performance of the xTAG GPP against a panel of 387
stool samples including 233 retrospective frozen samples and 154 prospective
samples (229). Conventional techniques were positive for 225 of the stools (173/233
retrospective and 52/154 prospective), and there was an 84% agreement with the
xTAG GPP. The xTAG GPP detected most of the Campylobacters (61/63),
Rotaviruses (63/65) and Noroviruses (25/26)(229). The performance for detecting
Salmonella was less reliable with 7/42 Salmonella not detected. Use of another
commercial molecular assay for discrepant analysis of these seven Salmonella
results also resulted in a not detected result (229).

Ciardo et al. assessed the performance of both the xTAG GPP and the Seegene
‘Diarrhea ACE’ kits on 126 stool samples from symptomatic patients (230, 231).
Results were compared to culture on selective media, EIA testing, and Norovirus RT-
PCR. Pathogens were found in 42 of the stool samples. In six samples only one of
three methods was positive, in a further 6 samples 2 of three methods were positive.
The two multiplex assays gave identical results in 115/126 samples. No pathogen
was found in 64 specimens(231).

Mansuy et al. utilized the xTAG GPP assay on 365 diarrhoeal stools samples
collected from children and adults in the hospital context and compared results to
bacterial cultures, detection of *E. coli* shiga toxin like genes and two immunoassays, the Exacto Combo Adeno Rota All Diag and the ImmunoCard STAT Norovirus (232). Utilising all testing modes there were 156 enteropathogens detected. This included 98 viruses, 42 bacterial targets, and 16 parasites. There were also 29 co-infections which all included a viral pathogen (232). Contrasting markedly with the findings of Marimón et al. the xTAG GPP assay was found to be more sensitive than *Salmonella* culture. Results also indicated the GPP assay was more sensitive than the alternate assays used for *C. difficile* Toxin and the three viral enteropathogens (232). The xTAG GPP produced similar results to conventional methodologies for *Campylobacter* detection (232). The xTAG GPP assay was reported as being less sensitive than PCR detection for STEC (232).

Wessels et al. repeated a previous analysis of 300 samples using a more recent version of the Luminex xTAG GPP assay. The newer assay included improved *Campylobacter* species specific targets replacing a 16S rDNA target that was used in a preliminary version of the xTAG GPP kit in the prior study (233). 62 out of 200 prospectively collected samples were found positive in the GPP assay, and 80 pathogens were detected in total. In 55 cases *Campylobacter* was detected in the preliminary version of the kit. Other targets were *C. difficile* (n=7), *Giardia* (n=4), Norovirus GII (n=4), *Shigella* (n=4) and the ETEC ST toxin (n=4)(233). 74 of the 100 selected samples were positive by GPP and 101 pathogens were detected. *Campylobacter* was the most prevalent (n=39), followed by *Giardia* and Norovirus (n=10), rotavirus (n=8), *C. difficile* (n=7), *Cryptosporidium* (n=6). In total there were 94 *Campylobacter* GPP positive results from the preliminary version of the kit, compared to only 10 recovered isolates. When the improved IVD kit was utilised, just the 16 specific *C. jejuni*, *C. lari* and *C. coli* positives were detected, which matched what was previously confirmed by real-time PCR (233). The high rate of detection of *Campylobacter* by the preliminary 16S target was attributed by the authors as representing ‘apathogenic *Campylobacter*’(233). It must be noted that using bacterial 16S rDNA as a diagnostic molecular target has resulted in unexpected specificity issues in other assays, and therefore this should also be considered as an explanation for the findings (234). Nucleic acid testing on a microbial biosphere as diverse as the human faecal flora necessitates careful evaluation of primers and/or probes in respect of the expected specificity.
Seegene ‘Seeplex Diarrhea ACE’ Detection Kits

The Seeplex Diarrhea ACE assays involve the use of three PCR amplification reactions containing dual priming oligonucleotides. Amplicons are then detected and specifically identified by size using auto-capillary electrophoresis (ACE) (235). When used together the kits cover four viral enteropathogens, nine bacterial pathogens and the toxin gene of toxigenic C. difficile. Seegene does not currently have a parasite kit available.

Higgins et al. utilised the Seegene Diarrhea-V ACE viral component to perform a retrospective analysis of 200 clinical specimens. From these samples 177 had also been previously examined for viral enteropathogens by electron microscopy and/or real-time RT-PCR (236). Discordant analysis was performed with other commercial kits or in-house assays. The diagnostic sensitivities of the kit were 100% for Adenovirus, Rotavirus, and Norovirus GI and 97% for Norovirus GII with specificities of 100% for each of the first three viruses and 99.4% for Norovirus GII (236). The limits of detection were reported as 31, 10, 2, and 1 genome equivalent per reaction for Adenovirus, Rotavirus, and Norovirus GI and GII respectively (236). Viral co-infections were observed for 12 (6.8%) stool specimens (236).

Bessède et al. examined the performance of the Seegene Seeplex Diarrhea-B1 bacterial component for detection of Campylobacter on 242 samples (59). Conventional culture, three immunoassay methods (ProSpecT Campylobacter, RidaScreen Campylobacter and ImmunocardStat!Campy) plus an in-house PCR were also performed on the samples. In total there were 23 positive samples, which included 16 positive by culture. Seven other samples were culture negative but positive for Campylobacter using both the immunoassays and PCR (59). A total of 37 specimens were positive by at least one method (59). Of the 16 positive culture specimens, three were not detected by the Seegene PCR and four were not detected by the in-house PCR. Two positive cultures were not detected by the ImmunoCard, and one was not detected by the ELISA methods (59). For 6 of 7 samples that fulfilled the positivity criteria when the culture was negative, all of the non culture methods were positive (59). Of 14 cases where only one technique was positive, 9 were positive using immunoassay methods only, and 5 were positive using molecular methods only (59).

Results for Salmonella enterica detection were also presented. The Seeplex Diarrhea-B1 multiplex kit detects Salmonella spp. (Salmonella enterica and Salmonella bongori), Shigella spp. (Shigella flexneri, Shigella boydii, Shigella sonnei, and Shigella dysenteriae), Vibrio spp. (Vibrio cholerae, Vibrio parahaemolyticus, and Vibrio vulnificus), and C. difficile toxin B (235). The assay detected 14 samples of S. enterica that were also positive by culture. There were another eight samples which were positive by the multiplex PCR only, and five samples that were culture positive and PCR negative (59).
Ciardo et al. also assessed the performance of both the xTAG GPP and the Seegene ‘Diarrhea ACE’ kits on 126 stool samples from symptomatic patients and found the two multiplex assays gave identical results in 115/126 samples (230, 231). Results were compared to conventional bacterial detection techniques of culture on selective media, EIA testing, and Norovirus RT-PCR and pathogens were found in 42 of the stool samples. In six samples only one of three methods was positive, in a further 6 samples 2 of three methods were positive.

Savyon NanoCHIP Gastrointestinal Panel

The NanoCHIP Gastrointestinal Panel from Savyon Diagnostics is a microarray panel that can detect four bacterial and four parasitic enteropathogens. Detection of the array occurs on the NC400 NanoChip molecular electronic microarray system. The bacterial detected include *Salmonella*, *Shigella*, *Campylobacter*, and *C. difficile*. The parasites detected include *Entamoeba histolytica*, *Giardia intestinalis*, *Dientamoeba fragilis* and *Cryptosporidium spp.* No viral enteropathogens are detected by the assay.

Savyon have recently presented data on the NanoCHIP microarray based Gastrointestinal Panel (237), however no third party evaluation has yet been published. The NC400 NanoCHIP system itself has been successfully applied to detection of Influenza A and B, Respiratory Syncytial and Parainfluenza viruses (238). According to Greenberg et al. from Savyon Diagnostics the performance of the NanoCHIP Gastrointestinal Panel was compared against culture, microscopy, EIA and RT-PCR for 142 known positive samples and the NanoCHIP detected all as positive except for two of the 39 samples positive for Giardia and two of the 38 samples positive for Dientamoeba fragilis (237).

AusDiagnostics EasyPlex FaecalProfile-10

The analysis uses the principle of Multiplexed Tandem PCR employing two sequential PCR steps (239). Firstly, a short (15 cycles) multiplexed pre-amplification reaction using primers homologous to all targets in the panel and including a reverse-transcriptase component is performed. This is then followed by amplicon dilution into individual wells for real time PCR reactions using primers ‘nested inside’ those used in the initial short amplification step (86). This process is automated by the Easy-Plex liquid handling robotic system. The secondary PCR reaction is performed in the Rotor-Gene instrument and positives are determined by observing increasing fluorescence of the intercalating dye Eva-Green associated with the accumulation of amplification products. Specificity of the amplicons generated for
the diagnostic targets and result calling is determined by post amplification melting curve analysis. The ten targets and controls are amplified together in the initial amplification and then real-time PCR is performed in individual wells of a 72 position Easy-Plex ring (86). The specific targets included in the kit are *Salmonella spp.* (tetrathionate reductase structural gene), *Shigella spp.* (invasion plasmid antigen H gene), *Campylobacter* (16s rRNA gene), *C. difficile* (toxin B gene), Adenovirus subgenus F (hexon gene), Norovirus GII/1 and II/4 (capsid protein gene), Norovirus GII/2+3 (capsid protein gene), *Giardia spp.* (small subunit Ribosomal RNA), *Cryptosporidium spp.* (18S Ribosomal RNA). There is also an artificial sequence detected for the assay control which is termed ‘SPIKE’ (86).

Data has been published for sub component assays of the AusDiagnostics Faecal Profile-10 Assay, but none yet for the complete assay. As the initial amplification is a large combined outer primer multiplex assay, the sensitivities quoted for the subcomponent assays may or may not necessarily reflect the sensitivities observed in the Faecal Profile-10 assay. High level multiplexing requires appropriately designed compatible primer sets, preferably with little or no cross homology at the 3’ ends of any two primers in the multiplex mix. Any 3’ homology will be conducive to selective amplification of primer dimers formed when two primers anneal to each other via their 3’ ends and result in short length amplification products. Dependent on the stringency of the reaction, cross homology in as little as three consecutive bases from the 3’ end is sufficient for primer dimer formation due to the excess of primer compared to target in PCR reactions. The selective amplification of short length amplicons drains PCR reaction resources sufficiently to reduce optimal sensitivity of the intended PCR reactions. The problem may not be as acute with MT-PCR due to the ‘pre-amplification’ function of the initial multiplex, prior to the second stage PCR testing, and of course would be eliminated if the manufacturer has designed the 10 outer primer pairs to be free of any cross homology leading to dimerisation.

Stark et al. published an evaluation of the MT-PCR ‘Gastrointestinal Parasites – 5’ kit comparing the MT-PCR to conventional microscopy techniques for 472 faecal samples (1). The authors compared the assay to iron haematoxylin microscopy and an existing set of real-time PCR assays (1). Enteropathogenic parasites detected by the MT-PCR were as follows: 28 *G. intestinalis*, 26 *D. fragilis*, 11 *E. histolytica*, and 9 *Cryptosporidium spp.* (1). The results indicated 100% correlation to the RT-PCR assays. In comparison the microscopy results were reported as having sensitivities and specificities of: 56% and 100% for *Cryptosporidium spp.*, 38% and 99% for *D. fragilis*, 47% and 97% for *E. histolytica*, and 50% and 100% for *G. intestinalis* (1).

Thomas et al. presented performance data on the MT-PCR ‘Faecal Profile – 6’ kit comparing the MT-PCR to conventional bacterial culture techniques and immunoassay (TECHLAB C diff Quick Chek Complete antigen/Toxins A and B) for 147 selected stool samples (240). The samples contained the following enteropathogens: 41 *Salmonella*, 4 *Shigella*, 73 *Campylobacter spp.*, 18 *C. difficile*.
toxinB positive stools and 11 negative stools (240). Of the 73 Campylobacter
culture-positive stools tested; 72 were MT-PCR positive (98.6% sensitivity 100%
specificity)(240). Interestingly the authors state that the only negative sample was a
mixed infection of C. difficile (toxinB) and Campylobacter which raises questions
regarding competitive inhibition for samples containing multiple enteropathogens.
No data is provided for the relative abundance of these two targets in that sample.
Two positive Shigella culture results were identified and both were MT-PCR positive
(100% sensitivity) (240). There were 41 Salmonella culture-positive stools of which
40 were MT-PCR positive (97.8% sensitivity)(240). There were also 9 Salmonella
culture-negative but MT-PCR positive stools, of which one stool was positive on re-
culture, two more were confirmed as positive by alternative methods, five were
determined as negative by other methods, and one that was determined to be an
incorrect melt temperature (240). The resultant specificity for Salmonella was
therefore 96%. All 18 of the TECHLAB C. difficile toxinB positive stools were
identified correctly by the MT-PCR (240).

Genetic Signatures 'EasyScreen Enteric' Kits (Parasite, Bacteria, Viral)

The most recent commercial product in Australia is the Genetic Signatures set of
EasyScreen kits.

The three kits utilise target nucleic acids that have undergone a bisulfite conversion
of unmethylated cytosine bases into uracil bases under the trade name of ‘3base’
testing (241). This conversion reduces sequence complexity from four possible
bases to three possible bases (uracil and thymine bases being able to hydrogen
bond to adenine). Two distinct advantages are promoted for utilising the ‘3base’
technology (241). Firstly it allows unique design opportunities for assays without
requiring degenerate designs in regions of known variation, and subsequently offers
some ‘future proofing’ of assay designs potentially reducing the impact of unforeseen
future sequence variation at the location of primes and/or probes. Secondly, as the
target sequence is effectively converted to a synthetic nucleic acid, patents
applicable to the originating target regions no longer apply (241).

The kits ship complete with a choice of specific extraction protocols both manual and
automated which can be adapted to most common automated nucleic acid extraction
instruments. The extraction of the stool is a rapid process with swab of stool,
placement of the swab in a buffer, heating for 15 minutes and then purification by
standard Boom (242) based extraction methodology on silica columns or magnetic
silica beads (243). As the nucleic acids are bisulfite converted in the extraction
process they may not be suitable for other downstream molecular assays that have
not been designed to utilise the converted sequences.
The EasyScreen Bacterial Detection Kit is a real-time PCR kit for the detection of *Salmonella, Campylobacter, Shigella, Listeria, Yersinia* and *C. difficile* (243). The EasyScreen Viral Detection Kit is a real-time PCR kit for the detection of Norovirus I/II, Astrovirus, Adenovirus, Sapovirus and Rotavirus (243). The EasyScreen Parasite Detection Kit is a real-time PCR kit for the detection of *E. histolytica, D. fragilis, Cryptosporidium, Giardia* and *B. hominis* (243). Microsporidial detection is listed as ‘coming soon’. All of the three kits are each multiplexed over two reaction wells per patient per kit and include an Internal Positive Control and an Extraction Control.

Melki et al. from Genetic Signatures presented assay performance data indicating that all of the component assays in the kits were linear from $10^1$ to $10^6$ target copies and that there was no specificity issues when the assay was validated against a collection of bacteria and fungi (138). Data was presented for more than 400 clinical samples that had been compared to culture, EIA and microscopy and results indicated equivalent performance for bacteria and viruses, and improved recovery of parasites (138). Stark et al. have a manuscript currently accepted in press that presents an evaluation of the parasite component of the Easy Screen kits (244). Results indicated a 92%-100% sensitivity and 100% specificity. The authors also comment that this panel should not routinely replace microscopy as it does not cover *C. cayetanensis* or *C. belli* (244).

**Idaho Technology/BioFire – FilmArray GI Panel**

This product is yet to be made available to the Australian market, but has been utilised in selected laboratories. The manufacturer has very recently presented performance data on the use of the film array nanofluidic pouches containing the Gastrointestinal Panel at several conferences (136, 245-247). The data is derived from comparative testing at the Primary Childrens Medical Center Laboratory, a laboratory located in the manufacturers’ local area in Salt Lake City, Utah. The nanofluidic pouch technology employed in the FilmArray Gastrointestinal Panel is currently FDA and CE-IVD cleared for a 20 target respiratory panel for respiratory viruses and bacteria, and has been shown to perform very well compared to conventional viral and bacterial respiratory diagnostics and also a commercial xTAG Luminex respiratory panel (248). The pouch itself contains compartments for cell lysis, DNA/RNA purification, an initial large multiplex reaction chamber, and then a 102 chamber reaction module also referred to as a ‘chemical circuit board’ in which individual singleplex second-stage PCR reactions occur, result calling is performed by end point melting curve analysis (87). The two stage nested PCR amplification and melt curve detection strategy is very similar to that employed in the MT-PCR utilised in the AusDiagnostics kits, but in a totally closed system. The construction of the nanofluidic pouch is illustrated in Figure 1.
The FilmArray GI Panel allows rapid simultaneous identification of 26 enteropathogens as summarised in Table 2. Testing requires minimal pre-processing of specimens. The stool sample is diluted 1:10 in Cary Blair media, subsequently filtered by gravity-flow through a coarse strainer, and loaded into the FilmArray GI pouch using a novel filter-injection vial (245). An automated report listing all of the pathogens detected is ready approximately one hour later.

Vaughan et al. presented data from clinical testing results of 620 sequential patient stool samples submitted to the Primary Children’s Medical Center laboratory (247). From the 620 stool investigations by conventional methodologies 13.7% of patients were diagnosed with a causative pathogen and 86.3% remained undiagnosed (247). The FilmArray GI panel was then utilised on 118 samples out of the 620 submitted. The 118 samples comprised 43 known positive samples and 75 samples negative by the conventional methods in use (247). FilmArray GI panel testing detected 95 organisms compared to 45 detected by standard clinical methods and as ordered by the patients clinician (247). The authors attributed the increased rate of positives to two factors: improved detection sensitivity of the FilmArray GI Panel for enteropathogens, and the increased range of enteropathogens in the panel some of which were not requested or offered in the routine workup at that laboratory. Overall concordance of the FilmArray system and conventional methods was reported as 97% (247). In 30% of cases multiple pathogens were detected, and 20 of the 75 previously undiagnosed specimens were found positive with at least one pathogen using the FilmArray GI Panel, leading to a potential 26% increase in pathogen detection (247).

Harrel et al. presented data specific to the performance of the FilmArray GI panel for viral enteric disease using a set of previously characterised samples and also prospective clinical samples (245). Assessment of the FilmArray GI Panel was made in comparison to the conventional methods used in the clinical laboratory. Specimens previously determined to be positive for viruses were re-tested using the Film Array GI Panel. 66/69 (95.7%) RT-PCR confirmed Norovirus samples (36 Norovirus GI and 33 Norovirus GII) were identified as Norovirus by the FilmArray GI Panel (245). 19/22 (86.4%) Rotavirus EIA positive faecal specimens were identified as Rotavirus by the FilmArray GI Panel (245). The authors suggested that sample degradation during storage could be responsible for the false negatives observed. The second component of the evaluation utilised randomly selected clinical samples obtained from children with undiagnosed diarrhoea. All samples were tested with the available conventional methodology in the laboratory but these did not cover all of the viruses present in the FilmArray GI Panel (245). All five viruses were detected, Rotavirus 4 positives/134 tests, Adenovirus 6/150, Norovirus 26/210, Astrovirus 2/97 and Sapovirus 5/88 from the clinical samples (245). Compared to the routine testing performed in the laboratory the data suggested that the Film Array GI Panel would potentially improve viral enteropathogen detection by an additional ~25% in children who remained undiagnosed by existing common conventional methods (245).
multi-target testing available in the FilmArray GI panel also indicated that multiple infections were common and these were again found in about 30% of clinical specimens (245).

In a separate study, Harrel et al. reported the performance of the FilmArray GI Panel on samples submitted for assessment of *C. difficile* alone, or in combination with requests for other pathogens. In the 406 samples submitted for *C. difficile* testing alone, 74 were determined as positive by the Illumigene *C. difficile* LAMP assay (Meridian Diagnostics). 83 of the 406 specimens, comprising 35 positive and 48 negative specimens by the Illumigene method were retested with the FilmArray GI panel and showed 100% concordance. In this sample set of 83, the authors also reported the detection of 16 other enteropathogens in the 48 samples that were negative for *C. difficile* by using the Film Array GI panel. The pathogens detected in the *C. difficile* negative samples included *Aeromonas*, *Campylobacter*, EPEC (*eae*), *Giardia intestinalis*, Astroivirus, Norovirus, Rotavirus, Sapovirus and ETEC (*lt/st*).

Interestingly in 15 of the 35 *C. difficile* positive samples co-infections were also detected by the FilmArray GI Panel. Additional enteropathogens detected in the *C. difficile* positive subset included Adenovirus F 40/41, *Campylobacter*, EAggEC (pAA Plasmid), EPEC (*eae +/- bfpA*), Norovirus, *Plesiomonas*, *Salmonella* and Sapovirus. The authors argued that the inclusion of *C. difficile* into the FilmArray GI panel was clearly warranted given that another enteropathogen was detected in 19.4% of samples which were originally only requested for *C. difficile* testing (136).

Asymptomatic carriage rates of toxigenic *C. difficile* are contentious but have been reported from 4-20% in adults dependent on the population and technology utilised to determine the data (249). Interestingly in this data set, 18% (15/83) of the samples tested showed co-infection of toxigenic *C. difficile* with other recognised enteropathogens. It is worth noting that the laboratory performing the evaluations is the ‘Primary Childrens Medical Center Laboratory’. As 49.7% of the samples tested were reported to be from patients <6 years of age (136), a bias to samples collected from children may reflect the origin of the findings. Asymptomatic carriage of *C. difficile* has been reported at levels from 75% to 23.5% in infants and children at 1 year of age and 5 years of age respectively (135).

Whilst the FilmArray GI Panel product offers a very comprehensive suite of targets and a very simple workflow incorporating the nucleic acid extraction component, throughput is limited at just one pouch per instrument per hour. Due to this, it is unlikely that the product could be utilised in larger diagnostic laboratories as a primary screening methodology without acquiring significant numbers of instruments. The product would be very useful as a comprehensive ‘stat’ method for urgent samples, especially within larger laboratories using batched workflows when the sample might not be able to be tested immediately. Currently our laboratory utilises a variety of lateral flow immunoassay devices to provide urgent results for toxigenic *C. difficile* prior to subsequent PCR follow up, and also for Rotavirus and Adenovirus testing as required in-lieu of ELISA testing in normal batch process. At present the
FilmArray products are not yet available in Australia. Cost per test for the FilmArray system and test kits is expected to be $150 AUD per test and $50,000 AUD for the instrument platform. Pricing may be revised now that BioFire has been acquired by bioMerieux.
Existing commercial multi analyte assays for gastrointestinal pathogens range from eight to 26 targets, and cover up to 95% of the pathogens usually reported. There are a much greater possible number of known enteropathogens than 26. This effectively means that using the existing commercial test kit with the highest coverage would still leave a number of patients for whom conventional methodology potentially would have reported an enteropathogen being issued a result of no pathogens detected by the molecular kit. Depending on the multi analyte kit utilised this ‘missed’ enteropathogen may be bacterial, viral or parasitic.

Faecal parasite concentration microscopy is labour intensive and has comparatively poor sensitivity to molecular amplification technologies. Microscopy can however detect a vast range of parasites by operator skill and visual observation alone. The highly sensitive molecular assays are limited to reporting only those organisms that have specific primers/probes included in the assay. At the moment it can be argued that existing multi analyte nucleic acid amplification kits do not offer sufficient parasite coverage to supplant parasite microscopy. Arguments also exist for the need to incorporate detection of non pathogenic protozoa as well. Detection of non-pathogenic intestinal protozoa in microscopy and the reporting of these is common. Many laboratories attach report comments highlighting that the organisms are non-pathogenic or of unknown significance. Opinion is divided in regards reporting non-pathogenic protozoa. Some argue that these should be reported because the acquisition of the protozoa is generally via the same faecal-oral route as pathogens and detection in symptomatic patients indicates possible exposure to faecal material warranting further examination for known enteropathogens, and unknown viral enteropathogens (3, 179). Routine reporting of non-pathogens also ensures laboratory staff maintain expertise in differentiating pathogenic from non pathogenic protozoa (250). Others contend that reporting of non pathogenic protozoa actually complicates patient management and doing so incurs significant health costs in unnecessary patient follow up, be that clinician or patient driven (250, 251).

Molecular assays offer improved sensitivity, simplified workflow, and reduced time to results. However, in order to offer appropriately comprehensive enteropathogen diagnostics using molecular assays the use of alternate molecular technologies will be required. Many of these technologies have been developed for use in human genetics research but are adaptable to microbial diagnostics. Until recently, use of these products in pathology diagnostics was prohibitively expensive. This has recently changed and as summarised in Table 5, if a laboratory has sufficient scale the cost per patient for testing as many as 80 individual enteropathogen targets using nucleic acid amplification technology on these systems has reduced to <$25 AUD. The capital expenditure for the equipment required has also dropped commensurately.
A considerable amount of literature exists utilising microarray technology in clinical microbiology and the many and varied uses are summarised in the review of Miller and Tang (252), and the papers by Booth et al. (253) and Versalovic (254). The liquid array of the Luminex platform and its commercial application as the xTAG Gastrointestinal Pathogen Panel has already been discussed. A number of authors have published work on the detection of bacterial enteropathogens using universal primer sets in the bacterial 16S and 23S ribosomal RNA genes and intervening spacer sequences with post amplification hybridisation detection on solid microarrays tiled with organism specific probes (82, 255, 256). One author has utilised virulence associated gene targets with microarray detection to good effect (84). Although care is required as there is evidence that virulence determinants are not necessarily specific to defined enteropathogenic bacteria. Examples relating to virulence determinants associated with enteropathogenic strains of *E. coli* being detected in other enteric flora include the detection of the *eae* gene in *Escherichia albertii* and *Citrobacter rodentium*; the *stx2* gene in *Enterobacter cloacae* and *Citrobacter freundii*, *stx1* and *stx2* in Aeromonads, and O157 antigen expression in *Citrobacter freundii*, *Citrobacter sedlakii*, and *Escherichia hermanii* (96, 110, 257-260). Earlier publications with solid phase microarray approaches showed a detection sensitivity in the order of $10^3$ to $10^5$ CFU/mL and a resultant correlation of only 80% with culture techniques (255). A more recent publication testing 1700 stool samples has shown a similar detection sensitivity of $10^3$ CFU/mL but in this study all 440 culture positive samples were also detected by the microarray method, as well as an additional 51 positive results for which culture was negative (256). Of interest 29 of these 51 were identified by the microarray as *Shigella spp.*, and it is known that the differences between *E. coli* and *Shigella* in particular in the ribosomal gene sequences are minimal. Solid phase microarray platforms in current form are viewed as unlikely to be suitable for high volume routine use in a diagnostic laboratory as detection platforms for amplified nucleic acids. This is due to a number of potential problems. The current high cost of the microarrays themselves, the limits on depth of multiplexing possible in the amplification component whilst preserving appropriate compatibility of the resultant amplicons to stringency parameters for the hybridisation processes, and finally the ongoing contamination risk due to requirement for open tube manipulation of amplicons for hybridisation onto the arrays.

A large amount of work has been undertaken towards profiling the microbial biosphere and the human microbiome including the human intestinal tract using a variety of techniques (261-266). Several interesting clinical applications have emerged from this work, in particular culture independent approaches to identification of organisms (3, 6, 267). Metagenomic approaches based on sequence independent PCR amplification and sequencing of 384 clones per sample have been successful in identifying existing, highly divergent and also novel viruses from faecal samples of 12 symptomatic patients (3). The review article by Barnard et
al. summarises the applications evolving for detection of novel viruses directly from
clinical materials (4). A commercial system the Plex-ID (Abbott-IBIS) exists for
profiling samples for unknown organisms. The PLEX-ID Analyzer is a high
resolution electrospray ionization mass spectrometry (ESI-MS) instrument which
uses multi-locus nucleic acid base composition analysis based on combined mass
assessment of amplicons for microbial identification (5). At present the current cost
excludes the use of this platform for routine enteropathogen diagnostics.
Underpinning much of the future technology in profiling microbial content in clinical
samples is improvements in nucleic acid sequencing using next generation high
throughput sequencing technologies. Leveraging the depth of sequencing available
in next generation sequencing technologies allows determination of the bacterial
population either directly or via selective enrichment of pathogens by specific pre-
amplification (268-270). However, the direct application of these technologies to
routine enteropathogen diagnostics is at present too costly, has insufficient
throughput and requires significant computational analysis and associated
information technology resources. Delivering several hundred patient results per day
in clinically relevant time frames is not currently realistic, though it is worth noting
that the technology is rapidly advancing.

Amongst the currently available adaptable new technology platforms that can offer
appropriate numbers of target enteropathogens at an appropriate price point and
which are suited to current diagnostic laboratories, three genetic technologies
appear to meet the requirements. The three systems and relevant performance
parameters for this application are summarised in Table 5. As test scale increases
so does risk and the resultant cost of assay failure. Table 5 summarises the
potential lost testing as a percentage of the daily workload should an initial batch of
testing fail for some reason for each of the three systems on which it is currently
viable to perform 96 samples for 80 targets in a realistic processing time.

Life Technologies Open Array Plate and QuantStudio 12K Flex

OpenArray technology utilizes a microscope slide-sized plate with 3,072 through
holes as shown in Figure 2. Each plate contains 48 sub arrays with 64 through holes
and each through-hole is 300μm in diameter and 300μm in depth. Through holes are
coated with hydrophilic and hydrophobic coatings. Plates with user defined Taqman
assays are manufactured by Life Technologies and dried down into the appropriate
through holes. Liquid reagents, which are the mastermix base and the patient
nucleic acids are then added to complete the PCR reaction and are retained in the
through holes via surface tension. QuantStudio 12K Flex OpenArray plates come
encased with an alloy base to assist with handling of the array without touching the
through-holes prior to sealing and filling the case with immersion fluid and loading
onto the QuantStudio 12K Flex instrument for amplification and detection. Four
individual OpenArray plates can be run in a single run on the QuantStudio 12K Flex
system, which equates to 32 traditional 384-well qPCR plates (12,000 data points).
Assay time on the QuantStudio 12K Flex system is 2.5 hours. The use of a specialised liquid handler called the AccuFill System is required to dispense master mix and patient sample nucleic acid onto the appropriate number of target wells for the assays required for that sample. The final reaction volume is 33nL and the manufacturer claims this has been shown to enable 7 logs of dynamic detection range and a sensitivity to 1 copy (271). The nanolitre scale reaction volume enables low cost for multiple reaction targets whilst still using the reliable performance of Taqman probe based nucleic acid amplification and detection. The ‘128’ format array plate is utilised to obtain up to 128 singleplex Taqman q-PCR enteropathogen targets for 24 patients per slide, and with 4 slides able to be loaded on to the QuantStudio 12K Flex at the same time. This equates to a throughput of 96 patients tested for up to 128 targets by Taqman PCR chemistry every 2.5 hours. Costing of format 128 OpenArray plates and reagents for detecting 80 targets based on volumes of 250 patient samples a day, 6 days a week have been quoted as 20 cents per data point which equates to <$22 per patient. An example of 80 possible enteropathogen targets is provided in Table 1. Being able to expand the parasite coverage economically is a key feature of this technology and suggests that it is possible to realistically replace conventional microscopy of faeces concentrates or fixed smears.

The QuantStudio 12K Flex system also has the capability of interchangeable blocks allowing the use of 384-well TaqMan Array Cards, and TaqMan assays in 96 and 384 well plates. Life Technologies have recently presented data at the ECCMID conference on the use of a TaqMan array card developed by their company for the detection of 16 enteropathogens. Van Hannen et al. described the use of the microfluidic TaqMan Array molecular device to detect these 16 enteropathogens (272). The authors assessed the real-time PCR assays in the card against several hundred clinical stool samples and compared the results to bacterial culture and microscopy (272). The assay was also compared directly to a set of in-house PCR assays for 103 of the samples. The enteropathogen targets contained in the array were 3 viruses (Adenovirus, Norovirus and Rotavirus), 8 bacteria (STEC, *Shigella*, *Salmonella*, *C. coli*, *C. lari*, *C. jejuni*, *Y. enterocolitica*, *C. difficile*) and 5 parasites (*Giardia intestinalis*, *Entamoeba histolytica*, *Cryptosporidium parvum*, *Dientamoeba fragilis* and *Blastocystis hominis*) (272). Assays were designed with the assistance of Life Technologies who used specific assay design software (272). Internal controls for inhibition and/or inefficient nucleic acid extraction were included. Three co-extracted seeded internal control materials were used, the cyanobacterium *Synechococcus*, RNA Phocine Distemper Virus and the DNA Phocine Herpesvirus (272). The authors first tested the assay designs in 96 well plate formats for sensitivity and specificity against clinical isolates after which the assay designs with acceptable performance were spotted on 384 well, microfluidic cards (272). Results showed that the TaqMan Array Card was more sensitive than culture and
microscopy and correlated well to the in-house PCR assays (272). Life Technologies is currently working with our institution on a 32 target OpenArray plate format enteropathogen panel to be used as proof of principle for a subsequent 80 target enteric OpenArray 128 format solution.

Fluidigm Genetic Analysis System with 96.96 Dynamic Array IFC and BiomarkHD

A miniaturised microfluidic circuit in the form of a dynamic array suitable for nanolitre scale qPCR testing is available from Fluidigm. The Integrated Fluidic Circuits (IFC) are available in a variety of formats including a 96.96 IFC which allows combining up to 96 patient samples or controls with up to 96 individual 5' nuclease assays in singleplex or up to triplex formulation within the space of the array. Dynamic Array IFCs have an on-chip network of microfluidic channels, chambers and valves that automatically assemble individual PCR reactions (273). Figure 3 shows the construction of the IFC 96.96 product. The IFC requires filling in a specialised loading instrument called the IFC-HX IFC Controller which takes 80 minutes (273). Once the IFC is loaded the amplification and fluorescence based detection take place in a second instrument called the BioMark HD System in real-time or in end point modes which takes a further 60 minutes (273).

Reaction volume is lower again in this system at 6nL which offers 5.5logs of dynamic range (273). The drop of precision correlates with decreasing reaction volumes due to stochastic limitations as defined by the Poisson distribution, i.e. by the application of small volumes of patient nucleic acid these samples may simply by chance not contain the target of interest if it is also present at limited concentration in the nucleic acid extract (274). The principle just described underpins the use of ‘Digital PCR’ on these platforms as a quantitative methodology which does not require comparison to internal or external standards (275, 276).

The disadvantage of this system is the requirement to pre-prepare up to 96 master mixes and then load these to one side of the IFC chip, and then to load up to 96 patient samples to the other side of the IFC chip. Whilst the IFC-HX controller conducts the filling and mixing process this still equates to a significant amount of pipetting and construction of PCR mastermixes for use on the IFC plate. The additional pipetting required extends the time required from assay setup to result to around 5 hours. The costs provided by the supplier indicate a price point between $15 and $18 AUD per patient for up to 96 targets. Capital costs are in the order of $480,000 AUD per system including the IFC HX Controller and Biomark HD instrumentation.

Seqenom MassARRAY Genetic Analysis System in 384 well PCR Plate format
Sequenom have a high-resolution mass spectrometry solution for characterisation of nucleic acids called the MassARRAY System. Combined with software called iSEQ, an analysis of sets of highly multiplexed PCR amplicons is possible following extension of a target specific probe and measurement of the specific probe mass in the MALDI-TOF instrument. In genetics the application effectively reports SNP information at the 3’ end of the probe location. In this application a given PCR amplicon derived from the large homogenous multiplex reactions and to which a target specific probe needs to hybridize onto for single base extension will either be present (positive for the enteropathogen) or not present (negative for the enteropathogen). The compatibility of the amplification primers as well as the designs of the probes for the specific target amplicons is managed in the design phase by specific assay design software. This software is capable of creating compatible single reaction multiplex assay designs of up to 29plex. Even at such high multiplexing, the use of appropriate assay design preserves the ability of the MALDI-TOF system to distinguish each extension probe specifically by its possible full-length masses.

In order to achieve the specific aims of 80 enteric targets, three multiplex reactions at 29plex would provide up to 87 enteric PCR targets per patient. Using 384 scale microwell plates at three reaction wells per patient allows up to 128 patient samples or controls per 384 well microplate for 87 targets. Conventional thermocyclers with 384 compatible blocks are utilised. The amplification reaction volumes are 5µL and as there are no fluorescent dyes required by this technology the costs of the amplification component are comparatively low. The manufacturer has quoted a price of between $10 and $12 AUD per patient for up to 87 enteric microbial targets based on the volume of patient testing previously described.

To adapt the MassARRAY workflow to direct detection of microorganisms there are important additional considerations relating to the design of the amplification and probe extension components. Optimal microbial targets for detection are those which are specific to the intended target only and where possible those that are also multicopy to enhance sensitivity. The use of multicopy genetic targets can be problematic, as often these targets comprise part of mobile genetic elements such as insertion sequences or plasmids. An unintended consequence of using mobile genetic elements is that they may at some point in time transfer to other species, could already be present in other species that have not yet been recognised, and indeed could also potentially even be lost altogether from certain strains of the target organism. Examples of these events have been observed on a number of occasions where molecular techniques have been applied to microbial diagnostics. Examples include loss of the cryptic plasmid of *Neisseria gonorrhoeae* containing the cppB target resulting in false negative results (277). False positives being reported due to the use of the cytosine DNA Methyl transferase *cmt* gene target in *N. gonorrhoeae* that was found to also have homology to other *Neisseria* species that were transient.
colonisers of urogenital sites (234). False positives reported by using the IS481 multicopy insertion sequence for detecting *Bordetella pertussis* that was later found to be present in *B. holmesii* and certain strains of *B. bronchiseptica* (278, 279). Structural defects that emerge can also result in false negative results where sequence might be deleted in certain strains of the target organism. Such a deletion was observed in the *Chlamydia trachomatis* cryptic plasmid resulting in false negative results for many commercial assays (280, 281).

The specificity of the nucleic acid amplification technologies is underpinned by the base content of primers and probes and the ‘pair bonding’ of these bases specifically to the intended target sequence. In general, the combination of 20 or more base pairs from each of two primers and a probe interrogates upwards of 60 bases of information contributing to the pair-bonding responsible for amplification and detection to successfully occur. Assay stringency controlled via the temperature of annealing, cation concentrations and buffering work to ensure that in a well optimised PCR reaction this will reflect in the outcome of amplification of the intended target only. The reality is however that detection assays can generally tolerate minor single base mismatches of sequence information under the primers and/or under longer probes. This generally results in delayed cycle threshold values for real time PCR. Mismatches to target template at the 3’ ends of primers are not tolerated well and generally will prevent amplification.

In respect of the MassARRAY assay design, the amplification reaction and the probe could be expected to confer similar specificity and tolerance of mismatches to other nucleic acid amplification technologies, however the MassARRAY result interpretation relies heavily on the single base extension reaction. Since the identification of this single base is what determines the final mass of the analyte in the flight tube, it is in effect the arbiter of whether a result is scored as positive or negative for the microorganism. Temporal variation in the local and global epidemiology of the organism is also an important consideration when validating molecular assays. A reliance on a single extension base to determine a specific identification requires that additional assay validation steps be performed so that there is appropriate confidence in the reliable conservation of that base at that genetic location. The examination of sequence data from genomic databases should be performed as should sequencing of organisms sourced from divergent locales and from different chronological periods. Closely related organisms should also be thoroughly examined for cross homology at the target region for the assay. Performing these investigations in the design phase is important so that there is confidence that the sequence targeted in general and the location of the single base extension more specifically are both sufficiently robust as to provide reliable results.

Despite the extra rigour required in the design phase, the major problem with the MassARRAY approach is that the process also takes much longer than the other
technologies. The process is described in Figure 4 and it requires up to 570 minutes
(9.5 hours) from PCR setup to the first batch of 128 patient results despite the use of
liquid handling robotics (MassARRAY Liquid Handler) in the time consuming iterative
primer extension process. Nanodispensing to the MALDI-TOF SpectroCHIP (Figure
5) is performed by the MassARRAY RS1000 Nanodispenser. The MassARRAY
RS1000 Nanodispenser can hold up to two 384-well microplates, and can dispense
all of 384 samples to the SpectroCHIP in less than 10 minutes. The actual MALDI-
TOF mass spectrometry takes only 45 minutes. Whilst the total time to the first
batch of results is quite long, overlapping setup using staggered amplifications in
multiple thermalcyclers and the speed of the Nanodispenser and mass spectrometry
allow the full workload to be achieved in a ten hour time frame. This is similar to the
overall time taken for the daily workload on the other two platforms. There is
however a considerable risk that if the first batch fails for some reason then
troubleshooting and repeating is both costly and almost time prohibitive at 9.5 hours.
When using overlapping batch processing, if the same problem is evident in other
batches in progress, then the entire days workload might be lost by the time the
results of the first batch indicate a problem. The possibility of backlog might require
that two entire systems running from entirely separate reagent lots would be needed
for redundancy.
Funding of pathology testing is under increasing pressure, cost containment measures including fee schedule freezes, tendering of service provision and caps on testing or growth rate have all been implemented in response to pressures on healthcare budgets around the world (282). The great proportion of diagnosis of gastrointestinal disease in Australia occurs in the private sector based on community disease burden. Reimbursement of private pathology testing in the Australian context primarily occurs via recovery of payments for service from the federal government under a taxation funded national health insurance scheme called Medicare. The Medicare benefits paid per service from January 2000 to June 2008 grew by 6%, in the same time period average weekly earnings grew by 48% and the Consumer Price Index by 32% (283). In Australia the lack of reimbursement growth in real terms has contributed to the merger of many individual pathology companies into large market listed private entities where economies of scale can be achieved. Economies of scale include the heavy use of automation and access to supplier volume discounting that are justifiable only by access to sufficient test volumes. Public sector pathology services have also seen an increase in ‘centralisation’ of services in order to achieve appropriate efficiencies associated with combining test volume but are primarily funded via State Governments for public patient services.

Diagnostic technology that delivers more with the same (or less) funding will indeed become of further importance due to projections of significant retirement in the workforce and an increasingly ageing population placing increasing demands on the health care sector (284).

The funding of gastrointestinal disease diagnostics in Australia is facilitated within Medicare by using a number of benefit ‘items’. The 85% rate is the rate normally recovered by private pathology laboratories for testing performed and reimbursed under these items. Four items in particular are of relevance in regards conventional diagnostic techniques performed on stool for the purposes of detecting enteropathogens.

**Conventional Technology Items:**

- **Item 69300**
  Microscopy of wet film material other than blood, from 1 or more sites, obtained directly from a patient (not cultures) including:
  - (a) differential cell count (if performed); or
  - (b) examination for dermatophytes; or
  - (c) dark ground illumination; or
  - (d) stained preparation or preparations using any relevant stain or stains;
  1 or more tests
  Fee: $12.60 Benefit: 75% = $9.45 85% = $10.75
Item 69336
Microscopy of faeces for ova, cysts and parasites that must include a concentration technique, and the use of fixed stains or antigen detection for cryptosporidia and giardia - including (if performed) a service mentioned in item 69300 - 1 of this item in any 7 day period
Fee: $33.65 Benefit: 75% = $25.25 85% = $28.65

Item 69339
Microscopy of faeces for ova, cysts and parasites using concentration techniques examined subsequent to item 69336 on a separately collected and identified specimen collected within 7 days of the examination described in 69336 - 1 examination in any 7 day period
Fee: $19.25 Benefit: 75% = $14.45 85% = $16.40

Item 69345
Culture and (if performed) microscopy without concentration techniques of faeces for faecal pathogens, using at least 2 selective or enrichment media and culture in at least 2 different atmospheres including (if performed):
(a) pathogen identification and antibiotic susceptibility testing; and
(b) the detection of clostridial toxins; and
(c) a service described in item 69300;
- 1 examination in any 7 day period
Fee: $53.25 Benefit: 75% = $39.95 85% = $45.30

The reimbursement available for the standard workup of a stool submitted for analysis for Micro/Culture/Sensitivity and Ova/Cysts/Parasites amounts to $73.95 (Item 69336 + Item 69345).

Three items in particular are of relevance in regards utilising Molecular diagnostic techniques performed on stool for the purposes of detecting enteropathogens.

Molecular Technology Items

Item 69494
Detection of a virus or microbial antigen or microbial nucleic acid (not elsewhere specified) 1 test
Fee: $28.85 Benefit: 75% = $21.65 85% = $24.55

Item 69495
2 tests described in 69494
Fee: $36.10 Benefit: 75% = $27.10 85% = $30.70

Item 69496
3 or more tests described in 69494
The reimbursement available for three or more targets in a multi analyte molecular diagnostic assay for enteropathogens is defined by the item 69496 and is $36.85. This calculates as a difference of $37.10 in funding between the conventional techniques for enteropathogen detection of culture and parasite concentration, and the available funding for a multi analyte molecular technique.

At the moment diagnostic molecular testing in Australian laboratories as applied to gastrointestinal pathogens is primarily supplemental and provided by a limited set of in-house assays at a cost base of about $10-16 for a single reaction (singleplex or in multiplex up to a maximum of 4 or 5 targets). Reimbursement for the molecular testing is then obtained from items 69494 – 69496 dependent on the number of targets to a maximum of three. The use of in-house PCR testing even in multiplex format is therefore cost-prohibitive if attempting to examine a large number of enteropathogens. When more than three to four reaction tubes per patient are utilised the cost will exceed the reimbursement available under the MBS microbial nucleic acid detection items.

As described in Table 2, current commercially available multi analyte molecular based enteropathogen diagnostics range in cost from $25 to $51 with variable enteropathogen coverage. From the currently available kits arguably the Easy-Screen product at $51 per test matches most closely the scope of pathogens regularly detected by the conventional methodologies but at $51 the cost per test exceeds the remuneration available.

On that basis the use of multiplex PCR assays on existing common molecular equipment either has too restricted a scope of pathogens and/or is too costly compared to conventional techniques of microscopy/culture and antigen detection to be viewed as a suitable replacement technology.

There is also a reticence by patients to pay any premium ‘co-payment’ for pathology testing, and bulk billing to Medicare item fees without patient contribution occurs for 90% of pathology tests performed (283). Though there is precedent for patient contributions where referrers and patients can clearly perceive value. The take up of liquid based cytology (Thinprep, SurePath) for cervical screening which does not have a Medicare reimbursement item and is an additional cost compared to conventional Papanicolaou screening has proved that if a particular testing modality is seen to provide improved diagnostic value, referrers will recommend such testing. And importantly more than 25% of patients will accept additional out of pocket costs of up to $40 for this testing (285). To improve the value proposition for referrers and patients in enteropathogen diagnostics two improvements are required. Matching and extending the range of enteropathogen targets beyond what is currently routinely reported. And achieving significant improvement in the time to results. If those two challenges can be met it might be possible that such an assay
would become preferred by referring practitioners and be recommended to patients
as offering improved value perhaps worthy of an additional cost. Recent advances
in molecular technology look promising for delivering this ideal solution of an
improved range of enteropathogens detected in a significantly reduced time frame
and potentially within the available molecular funding available under Medicare Item
69496 ($36.85).

The price per patient sample based on 80 PCR targets performed in acceptable time
frames using the previously described adaptable high throughput, ‘high’ multi analyte
molecular technologies has been calculated based on volumes of 250 patient
tests per day, six days of the week. The cost/patient test using these
technologies is $25 or less, and under the $36.85 maximal funding available for
multiple microbial nucleic acid detection. In the context of the growing fiscal
pressures on healthcare budgets, delivering routine detection of both existing and
additional enteropathogens with higher sensitivity, in shorter time frames and at
lower cost appears to meet many aims.

Aside from standalone use of the molecular technology, a proposed model of parallel
multi analyte molecular testing with concurrent minimised bacterial culture is
proposed in a later section of this paper. The primary rationale for maintaining a low
cost parallelised bacterial culture system is recovery of isolates for epidemiological
use and sensitivity testing. In such a dual scenario the minimum criteria required of
the bacterial culture item 68345 - culture in two different atmospheres and wet prep
analysis could also be achieved, but with the primary diagnostic screen being a ‘high’
multi analyte molecular test. Funding for such an algorithm could also then be
recovered from the MBS Item 68435 at $45.30. The additional remuneration in this
model would offset the costs of labour and additional processing required for
performance of simple wet preparation and reflex culturing bacterial enteropathogen
PCR positive samples.

Government funding models eventually adapt to the emergence and use of new
technology, but usually take time. Governments would need to determine by cost-
benefit analysis if the additional costs of bacterial isolate recovery alongside
molecular screening justified this additional expenditure.
Nucleic Acid Extraction from Stool

Use of any nucleic acid based technology necessarily requires the availability of the target nucleic acids from sample material. Direct application of nucleic acid based reporting probes to cells for in-situ hybridisation techniques such as oligonucleotide or peptide nucleic acid FISH requires only physical preparation of cells on slides. Free nucleic acid may be present in any sample, but optimal diagnostic utility when using nucleic acid amplification technologies requires specifically recovering intra organism DNA and/or RNA. The nucleic acids must also be recovered in a sufficiently intact state for later enzymatic amplification processes. They should also be recovered free from inhibitors of amplification, and in a format that preserves them from degradation. The extraction may also serve as a process that concentrates the nucleic acids relative to the abundance in the original sample processed.

Composition of the Stool Matrix and Inhibition

Recovery of nucleic acids from stool poses some particularly unique challenges. The stool matrix itself is comprised of 75% water and 25% solid matter in normal stool. The solid matter is comprised of 30% as bacteria, 30% as food matter (10-20% as fats), 10-20% inorganics, 2-3% protein, and the remainder derives from the patient (cells, bile, secretions, leucocytes, bilirubin) (286). PCR can be inhibited by known inhibitors in stool specimens. Bile acids, bilirubins, haem, pectins, phenolic compounds, glycogen and complex carbohydrates have been reported as potential inhibitors (286, 287). Clearly many inhibitors of PCR amplification are prevalent in stool. Macro sized particulates that remain in solutions prepared from stool can also block liquid handling pipettes and columns that are utilised in the nucleic acid extraction processes. Blocked pipettor tips and columns can result in failure of extractions or reduced yields of nucleic acids. Diluting the nucleic acid extracts prior to testing has also been described as a method for removing inhibition(286). The dilutions performed reduce the concentration of inhibitory substances in the extract to a level where they are no longer inhibitory to the PCR process. However diluting out inhibitors also carries the risk of diluting out the intended diagnostic target nucleic acid below the limit of detection of the assay resulting in a false negative result.

Processing of Stool

Processing of faeces in a pathology lab is normally performed within the Microbiology laboratory. Molecular testing for toxigenic C. difficile has contributed to processing of stool in molecular laboratories also becoming common in recent years. Stool extraction protocols that simplify and/or limit the hands-on component offer improved workflow in the molecular laboratory. One of the techniques that assists
handling is the use of ‘flocked’ nylon swabs as a tool for sub sampling from faeces collection jars. This provides a sample format (i.e. a swab) that molecular technicians are already very comfortable processing. Diatherix Laboratories who offer a ‘Fed-Ex’ in sample service in Huntsville AL utilise the provision of E-Swab (Copan, Spain) collection in transport media either from rectal swab or from a swab collected from a stool sample, subsequently testing 11 enteric pathogens via a TEM (Target Enriched Multiplex) PCR (288). Genetic Signatures provides a flocked swab for sampling from the stool collection into proprietary extraction buffer utilised to produce nucleic acid for 3base molecular testing of stool samples (138). Despite increasing use of stool swabs, most of the commonly available stool specific extraction methods utilise an input sample of 200mg of stool or 200uL liquid stool into a buffer.

It is important to recognise that stool does not necessarily represent the actual infection site in the intestine but is an excretion vehicle that delivers a convenient sample for labs to test. As such a stool sample may not carry consistent numbers of causative enteropathogens dependent on the diarrhoea frequency and volume. Additionally the enteric pathogens within the stool matrix from which we wish to extract nucleic acids are diverse in structure and form. The nucleic acid extraction process utilised must first be able to disrupt several different varieties of ‘packaging’ i.e. cell and cyst walls and capsids in order to release the nucleic acids. Parasites can be free living trophozoites, cysts/oocysts or helminth worms and eggs; viruses can be RNA or DNA and intracellular and/or packaged in capsid protein coats, bacteria can possess gram negative or gram positive bacterial cell walls with different amounts of glycopeptide.

Nucleic Acid Extraction Chemistry

The protocols for the extraction of nucleic acids from stool can be divided into two distinct modalities. The first category are those relying on variants of the protocol first described by Boom et.al. (242). Such protocols utilise buffered chaotropic agents such as guanidium thiocyanate to facilitate sample lysis, nuclease deactivation and nucleic acid binding to silica. This is then followed by several washing steps with ethanol to remove non nucleic acid components from the silica/nucleic acid and drying to remove residual ethanol. Release (or elution) of the nucleic acids from the silica is then performed by washing with a low salt buffer or water. In the available extraction kits, the silica component is either in a membrane column format in tubes or in microwells, or as liquid paramagnetic silica beads. Dependent on the format of the silica, one of either centrifugation, application of vacuum or magnetic attraction are subsequently utilised to achieve manipulation of the liquid solutions and silica to extract the nucleic acids. The particulate nature of some stools can make it difficult to utilise columns/microwell membranes and
pipettes for aspiration of the sample itself. Pre-clarification of a suspension of stool using centrifugation after the lysis has been performed has been used as a method for limiting the particulates carried forward to the binding component of the extraction (172, 227, 289).

The second category of extraction from stool is a relatively new methodology reliant on using direct preparation of a stool sample in a single vessel. This form of extraction is utilised by the Focus Diagnostics Clostridium difficile assay. The stool is prepared via swab into a buffer which is centrifuged and a heating step applied. Supernate is then directly added to the mastermix in the test well. The formulation of the PCR reaction mix is proprietary but clearly it is lytic, preserves nucleic acids and appears to be effective at preventing the action of PCR inhibitors present in stool samples.

**Commercially Available Kits and Modifications**

A non exhaustive list of currently available methods includes the following. Manual methods such as the QIAamp DNA Stool mini kit, Qiagen, Hilden. Semi automated methods such as the Qiagen Stool mini kit on the QIACube instrument. And automated applications of specific kits on magnetic bead instruments such as the Nuclisens Easymag (bioMérieux), Magnapure32, Magnapure96( Roche), KingfisherFlex (Thermo), QIASymphony (Qiagen), EZ-1 (Qiagen), Maxwell (Promega), kPCR (Siemens) and EpMotion 5075 (Eppendorf). Column/membrane based kits are used on vacuum manifold instruments such as the Xtractor Gene (Qiagen) and EpMotion 5075 (Eppendorf). Highest throughput extraction is available using the Magnapure96 or Kingfisher Flex instruments both of which can purify 96 samples by magnetic bead technology in less than 1 hour from the post-lysis silica binding step.

A number of modifications to commercial kits have been utilised that improve extraction of nucleic acids from stool. The Qiagen DNA Stool mini kit utilises InhibitEX a proprietary substance to remove impurities prior to the silica binding component of the extraction(289). Use of chelex resins or polyvinyl pyrrolidone (PVP) has also been shown to assist in removal of inhibitors of amplification technologies without loss of target DNA (140, 173, 187, 286).

Due to greater difficulty in releasing nucleic acids from enteropathogens with gram-positive bacterial cell walls, oocysts and ova some authors have also utilised a variety of physical disruption methods including freeze/thaw and/or mechanical disruptions prior to extraction. Zirconia, glass, ceramic or stainless steel particles and beads utilised in homogenising equipment such as the TissueLyser
(Qiagen, Hilden), MagNAlyser (Roche, Rotkreuz), FastPrep (MP Biomedicals, Sydney), Mini Bead Beater-8 (Bio-Spec, Bartlesville, Oklahoma) have been used (139, 187, 290-293). Similar processing has been applied to disruption of fungal hyphae for downstream nucleic acid testing (294). By selecting an appropriate specific gravity of particle and an appropriate oscillation frequency, the impact between the particles and the organism produces effective disruption without excessive shearing of nucleic acids.

A dual approach has been suggested by Amar (286) whereby a faecal sample is processed via mechanical disruption with zirconia beads in a MagNAlyser and concurrently via a parallel traditional lysis followed by recombination of the two methods after silica bound nucleic acids have been obtained, with washing and elution performed on the combined silica. This combined method reportedly caters for the different robustness of viruses and gram negative bacteria (traditional lysis) versus that of gram positive bacteria and protozoal oocysts (mechanical disruption) (286). Successfully utilising a mechanical disruption extraction protocol for viral enteropathogen detection without loss of target has also been reported in the literature (139).

Recovery of RNA from Stool Samples

Several important viral enteric infections are caused by RNA viruses e.g. Rotavirus, Norovirus, Coronavirus, Enterovirus, Astrovirus, Sapovirus and Human Parechovirus. Successful extraction of RNA from faecal samples requires both an appreciation for the functionality of the extraction methodology utilised as well as attention to laboratory equipment and practices. In general, RNA is recovered in sufficient quantity via co-purifying with DNA to enable appropriate detection of viral enteric illness. Either DNA or 'Total' Nucleic acid extraction kits have been utilised successfully for enteric RNA virus diagnostics (139, 140, 295). Nonetheless, endogenous RNases are released from cells in clinical samples, and inactivation of these should occur as soon as possible to prevent RNA degradation. The chaotropic guanidinium isothiocyanate found in the majority of extraction kits is also an effective inactivator of RNAses, and specific nuclease inhibitor solutions are also available. After elution of RNA or total nucleic acids, RNA degradation may still occur if attention to laboratory equipment and processes do not meet an appropriate level of rigour. It is essential that any item that could contact the purified RNA is RNase-free. Laboratory surfaces and molecular equipment such as pipettors should be decontaminated with a solution such as RNase Away (Molecular BioProducts). RNase-free certified pipette tips and other laboratory consumables should be utilised and staff should be encouraged to change gloves frequently. Conversion of RNA to cDNA by randomly primed reverse transcriptase protocols as soon as possible after extraction has also been suggested (286).
Control of Molecular Gastrointestinal Infection Testing

As described in the previous section, stool is a complex matrix containing inhibitory substances that may co-extract with nucleic acids. Modern extraction chemistries and instruments have however proven to be very reliable at removing inhibitors within our laboratory. A rate of <0.4% inhibition has been observed from stool extracted for *Clostridium difficile* testing in our laboratory. In total, there were 132 PCR inhibitory stools from 35210 total stools extracted for the period 2007-2011. These 132 stools remained inhibitory even after nucleic extraction was repeated indicating that in some patient samples the particular matrix might still rarely result in PCR inhibition and a failure of nucleic acid testing methods. Appropriate controls to detect inhibition and assay performance in general are required.

The practice of diagnostic laboratory nucleic acid amplification technology in Australia is controlled by application of standards from the National Pathology Accreditation Advisory Council (NPAAC) (296). Several distinct layers of controls are mandated or recommended and these are consistent with best practice internationally.

**Positive Controls**

Positive control material controls for acceptable performance of the testing reagents. When examined quantitatively via crossing points (which correspond to target abundance), positive control material may also control for acceptable sensitivity of the amplification reaction. The use of a positive control near the limit of detection of the assay is required by the standards applicable in Australia. Failure to amplify a positive control with a target abundance set near to the limit of detection would indicate that the current test batch is exhibiting lower than optimal sensitivity and should be repeated.

Positive controls can be obtained or prepared from a number of potential sources. Previously positive patient material can be utilised, purified nucleic acid from cultivated organisms produced within the laboratory or from commercial culture collections, synthetic nucleic acids such as plasmids, target matching oligonucleotide sequences and synthetic viral particles can also be produced or obtained commercially. When selecting or preparing positive control material it is prudent to incorporate a definable difference that will enable a positive reaction due to the positive control to be distinguished from a positive reaction from the target analyte. This is commonly achieved by the positive control material generating a longer amplicon than the true analyte. Our laboratory routinely includes several Uracil bases replacing thymine bases in the positive control sequence. Including uracil bases throughout the positive control material provides the option if the positive control is suspected to be contaminating other processes that it can be specifically
removed/destroyed from test wells pre-reaction with the enzyme Uracil-N-Glycosylase.

In event of a positive control testing negative in a test batch, the status of any positive patient test is not in doubt, but all negative test results need repeat testing and an investigation of the cause for failure of the positive control is required.

**Negative Controls**

Negative control materials are required to be extracted along side the test samples. These negative controls are utilised as a control measure that confirms that there is no unexpected positivity in the test batch. Unexpected positivity can have a number of causes which are all related to contamination events in any of the analytic process. Contamination between a positive sample and negative samples could occur in primary sample handling, contamination during sample loading into extraction methods, contamination during the extraction process, contamination whilst handling extracts, contamination during PCR assay setup, and contamination between reaction wells during amplification.

In addition, contamination of the testing process deriving from positive control material (as opposed to positive patients in the test batch) is also a consideration if the negative control is unexpectedly positive.

It is also possible for the Negative control material itself to become contaminated and be positive before it is introduced to the current test batch and this needs to be considered in investigations of unexpected positivity.

In event a Negative control returns a positive reaction in a test batch, the reliability of negative results on the test batch are not in question, but all positive results are in doubt and require investigation of potential causes and repeat testing.

Negative control material should ideally be of the same matrix as the samples themselves such that they are handled identically through the full test process. In most cases the negative material is derived from a pool of patient samples known to be negative for the analytic target.

**No Template Controls**

A ‘No Template Control’ (NTC) is a specific type of negative control that serves primarily as a sentinel for contamination introduced upon preparation of the PCR mastermix. The NTC also detects inadvertent cross-contamination during loading of sample materials during the PCR setup, and also contamination occurring during the
PCR run, NTCs in most laboratories represent a test well containing PCR mastermix with molecular grade water substituting for the usual patient sample. If all samples are positive on a test run including the NTC this almost certainly represents contamination of the PCR mastermix or contamination of the entire batch during amplification.

If all patient samples and the negative control are positive on a test batch, but the NTC remains negative this indicates severe contamination of the pre-PCR processes including sample preparation and extraction.

NTC reaction wells are normally placed between patient test samples and positive control wells to assist with detection of contamination during PCR loading.

Co-Extracted Seeded Internal Controls

To prove unequivocally for each patient that a target was truly “Not detected” by an amplification assay another specific type of control is required. This control is defined as a Co-Extracted Internal Control, and optimal utility is obtained if it is also calibrated and seeded at a specific target copy number. The specific target copy number will produce expected cycle threshold results in the amplification component that provide valuable information regarding extraction and amplification assay performance.

The use of the control involves a calibrated seeded internal control target being placed into the patients sample before the extraction process begins. A specific amount of ‘seeded co-extracted’ internal control in which the quantity of seeded material reflects the minimal amount that can routinely be extracted and recovered as a detectable result with an amplification assay. Co-recovering this nucleic acid and detecting it in the same well as the patient sample provides the only reliable confirmation of a patients ‘not detected’ result for a diagnostic target. Using an internal control in duplex reactions (or greater plex) provides ‘in well’ control over several performance characteristics of a molecular assay. The control can detect the presence of total or partial PCR inhibition by either failure to be detected or being detected with delayed cycle threshold respectively. The co-extracted internal control can also detect if there has been unacceptably low yield or failed nucleic acid extraction performance for each patient sample. Total failure of extraction would be observed as a negative result, and reduced performance of the extraction methodology would reflect as a delay to the controls cycle threshold. It is not possible to immediately determine if total or partial failure of the internal control is due to PCR inhibition or problems with the extraction (or both). Importantly the co-extracted internal control is the only control that monitors and controls for events in the amplification environment in the individual reaction vessel for each patient.
An important design consideration is that the seeding level of the control and the robustness of the molecular assay designed to detect the internal control both require careful development. The assay that detects the internal control should be designed and optimised such that if the diagnostic target is also present then the sensitivity of the diagnostic assay(s) should not be compromised by competitive inhibition from the internal control reaction. In properly designed internal control/assay combinations, this commonly results in what is a perfectly acceptable outcome of the Internal Control assay testing as negative due to competitive inhibition from the diagnostic target assay. In this scenario, a detectable result is validly reported for the specific diagnostic target even though the internal control has apparently “failed” albeit by design.

Control of Reverse Transcription for RNA Targets

Multi analyte testing of faeces including viral enteropathogens requires both RNA and DNA to be recovered and tested as some of the common enteric viruses have RNA genomes. Consequently, a seeded co-extracted control for both RNA and DNA should be utilised. It is important to control for recovery of sufficient amplifiable viral RNA from stool samples. It is also important to control for the acceptable performance of the enzymes responsible for conversion of RNA to cDNA in the PCR mastermix prior to DNA amplification. Controls such as MS2 phage(297), phocine or equine herpes virus(187, 272), phocine distemper virus(272), synthetic armoured RNA (Asuragen) and bacterial ‘BioBall’ (BTF) have been utilised. All of these products can be quantified appropriately to establish correct seeding levels. Importantly all contain nucleic acids that require functional extraction from within capsids or cell walls which makes them superior to naked DNA or RNA controls such as constructed plasmids, synthetic gene segments or amplified nucleic acids.

Comparative Control to Traditional Methodology

When incorporating molecular technologies as replacements or adjuncts to the current processes, comparison with the level of control currently practiced with traditional methods in microbiology is worth discussing. Culture media has pre-release QC performed on sub samples, but individual plates themselves cannot be confirmed as functional before use. When in use, there are no controls on each specific plate undergoing the daily incubation/removal for plate reading/ and return to indicate that during this process it was and indeed remained possible to cultivate a given organism on that plate.
Culture reading is reliant on the observational skill of the scientist, and this can be compromised when other flora (normal or pathogenic) is present due to visibility difficulties. Active inhibitory effector molecules released from other organisms can also delay or prevent the growth of pathogens. The sample itself may also contain bacteriostatic or bactericidal substances delaying or impeding possible growth. Importantly unlike other disciplines very little if any secondary assessment of an individuals result interpretation (in this case of what has grown) is performed.

Results of microscopy are also not checked by a second person and are dependent on operator skill and fatigue levels. Seeding of a control organism into every faecal parasite preparation or fixed stain is not performed to indicate the success of each individual preparation.

Attempts at repeat testing of the sample are likely to provide results that differ due to cellular degradation, overgrowth, death of labile organisms and so forth.

ELISA testing for antigenic epitopes of pathogens does not have in built controls of negative results in each well that prove equivocally the sample was applied correctly and/or did not contain substances that interfered with the performance of the assay. Control lines in lateral flow immunoassays do offer some control for correct sample application.

Conventional microbiology is highly reliant on viability/growth and morphology techniques which are applied diagnostically at an ecological interface between humans and microorganisms. This contrasts significantly to the comparatively well characterised human cells, extracellular proteins and other biochemical metabolites which are the relatively static analytes in other pathology disciplines. It is possible to argue that microscopy and culture for microorganisms are perhaps the least controlled diagnostic pathology techniques and these techniques are applied to perhaps the most dynamic analytes i.e. microorganisms.

Molecular technology though also reliant on the individual training, competency and performance of staff for optimal outcomes benefits from the many layers of process controls previously discussed. Due to the controls utilised it is likely the molecular techniques offer improved confidence in the reliability of results for enteropathogen testing.
Potential Workflow Replacing Conventional Testing

An example of a potential workflow using the three ‘high’ multi analyte molecular systems is described in Figure 6. A similar approach has been proposed by Schuurman et al. in relation to pre-culture screening of bacterial enteropathogens by PCR and reflex culture of positive PCR samples (8). The proposed protocol described in Figure 6 is likely to be more effective at preserving bacterial enteropathogens than that proposed by Schuurman et al. The suggested protocol incorporates molecular screening, a ‘mini’ culture bacterial enteropathogen maintenance/enrichment system, plus wet preparation faecal microscopy on patient samples. Viral and parasite PCR results (positive or negative) are issued directly to the referrer, as are the negative bacterial culture results. Positive PCR testing for a bacterial enteropathogen initiates a reflex culture from the mini culture tubes onto conventional agar plates to attempt recovery of the bacterial enteropathogens. The strategy suggested reduces labour costs, significantly reduces time to result for negative patient samples, and reduces time to result for positive viral and parasitological enteropathogens. If reporting of ‘presumptively’ positive bacterial PCR results is also included prior to culture results being available this would complete rapid result turnaround for all three pathogen types.

The potential multi analyte molecular enteropathogen detection components in this suggested workflow remove the need for routine faecal parasite concentration microscopy. Performing simple wet preparation is primarily for meeting the requirements of the culture funding item. Wet preparations require only minimal field assessment and reporting of the presence of leucocytes, erythrocytes, observable parasites, and miscellaneous other findings such as Charcot-Leyden crystals and fat globules. The ‘mini’ culture is based upon culture media and enrichment broths being prepared in smaller format tubes that are amenable to automated “cherry picking” of samples which are PCR positive for bacterial enteropathogens. Identification of PCR positives requiring subculture can be directed via laboratory information systems to facilitate efficient workflow and minimisation of human error in selecting the appropriate tubes. The format of the media is ideally in SBS format racks, and the use of 1.4mL tubes in a 96-cluster arrangement is suggested. The three selected media types are Cary Blair (298), Selenite Broth (299) and Campylobacter Broth (300). Using these three media types enables preservation and enrichment of all of the bacterial enteropathogens in the time period between sample receipt and the completion of the molecular testing. Cary Blair media has also been successfully utilised as the input sample for molecular testing (85) and therefore serves as a useful source of material if any repeat molecular testing is required. A semi-solid broth supporting the growth of Campylobacter has been added to the protocol because decreased sensitivity of culture for Campylobacter was observed where stool culture was delayed waiting for bacterial enteropathogen screening by PCR testing in the protocol of Schuurman et al. (8). By miniaturising
the volume of media the cost is also significantly reduced, as are the requirements for incubator space. The hands on component of initial stool processing in the microbiology laboratory would change to using flocked swabs as the inoculation device for three media tubes, creation of a wet preparation and inoculation of a tube containing PVP Buffer and micro particles for sample disruption and subsequent nucleic acid extraction.

As mentioned previously the benefits of recovering isolates reside with epidemiology and antimicrobial sensitivity testing and surveillance. Additionally a part of ongoing molecular assay validation requires that there is periodic evaluation of the suitability of the primer/probe sequences for detecting organisms which might have genomic drift over time (296). The ability to re-validate performance of the molecular assay by occasionally culturing all samples including those PCR negative for bacterial enteropathogens is also possible with the suggested workflow.

Cost-benefit analysis is required as there are implications in regards what Medicare item(s) can potentially be billed, with the suggested approach potentially reimbursed under one or both of Item 69345 $45.30 for the culture and wet preparation and Item 69496 $36.85 for the molecular testing.
Available molecular technology continues to progress in scale, speed and affordability each year. It is difficult to predict the form and scope to which the technology will be applied in routine diagnostic Microbiology or indeed just when various conventional techniques might be replaced, if at all. There is no doubt however, that molecular based technology will continue to increasingly supplement the clinical microbiology diagnostic repertoire in Australian laboratories. The evolution of high multi target analytical instruments coupled with reducing cost per data point has recently bridged a gap between the number of potential organism targets required for comprehensive molecular screening of certain clinical samples and what has been economically possible with existing multiplex real-time PCR testing. The utility of screening faecal samples with these systems has been discussed in this report and proof of principle is in progress in our laboratory.

Arguably traditional mycology especially of dermatophytes may be another component of the routine laboratory functionality that could potentially be adapted to such systems. Extension of the current multiplexed molecular testing panels for respiratory virus diagnostics appears to also be a logical use of the systems described.

There are compelling benefits for using high multi analyte molecular technologies for screening for enteropathogens. Healthcare costs can be reduced from $73.95 per patient test using conventional methods to $36.85 whilst also delivering extended scope of testing, enhanced sensitivity and improved time to result.

For bacterial enteropathogens when compared to culture the completion time is reduced from 2-5 days to a single day. Equivalent or improved detection has been observed, particularly for *Campylobacter* with 15-20% increases in sensitivity. *E. coli* pathovars and *E. albertii* can be detected molecularly via virulence genes where otherwise they are not obvious. As is currently the case for culture, interpretation of the significance of certain molecular results also requires clinical correlation. The detection of *Aeromonas* or *Brachyspira* may not always represent the causative agent, some species of *Yersinia* are of unknown significance but reporting is still suggested, and finding toxigenic *C. difficile* can represent asymptomatic carriage particularly in children but also in a percentage of adults. Due to emergence of increased rates of community *C. difficile* disease a case has also been made for inclusion of toxigenic *C. difficile* in any routine panel of bacterial enteropathogens.

For viral enteropathogens, adopting molecular techniques has always resulted in an immediate increase in the detection rate. This phenomenon arises for two reasons (a) increased detection of viruses previously tested for because of increased sensitivity, and (b) use of multiplex testing and inclusion of additional virus types. Molecular panels of enteric virus detection assays have also raised issues that require clinical input including high rates of co-infections, and a recognition that
asymptomatic and subclinical infections exist for a number of viruses including rotavirus and human parechovirus.

Utilisation of molecular technology for parasitic enteric disease has been shown to offer substantial benefits compared to microscopy preparation techniques and the requirements on laboratory staff to screen slides. Sensitivity of the microscopy techniques has been shown to be particularly poor for some organisms such as *D. fragilis*, and using molecular techniques results in sensitivity increases of at least 10% (and up to 60% for *D. fragilis*). Replacement of larval stage nematode culture methods by multiplex PCR assays has also exhibited at least equivalent sensitivity for those helminths and reduced time to result from several days to a single day.

Several commercial multi analyte enteropathogen systems/kits are either available or about to be released. These solutions were reviewed in comparison to the range of pathogens reported in our laboratory across 4 years of data. When considering using one of these commercial products as a comprehensive ‘replacement’ solution to the conventional methodologies there were some gaps in coverage amongst the kits that would have resulted in more than 300 patients a year failing to have an enteropathogen reported. The best coverage for bacterial enteropathogens was 99.8% (Seegene), and the worst 77.3% (AusDiagnostics). The best coverage for parasites was 95.9% (Genetic Signatures) and the worst 26.2% (Luminex) though Seegene was 0% having no parasite component. Issues with the ability to process a daily workload of 250 samples were identified and the throughput per batch/instrument in a 4-hour run time ranged from 95 to 7 patients. The Idaho Technology FilmArray was comprehensive and rapid with a one hour time to result but only one patient per run per instrument is possible. Diagnostic yields using these commercial multi analyte enteropathogen kits were in the order of 30% higher than conventional methods and co-infections were increasingly observed ranging from 6.8% to 35% dependent on kit and study.

The time to obtain negative results for all enteropathogens, positive results for viral and parasitological infection and also positive presumptive bacterial infection are all reduced significantly when using molecular assays compared to the existing conventional techniques found in Australian laboratories. Provision of results in a timely fashion before an illness has resolved is of use for limiting community transmission and supports endeavours for appropriate use of antimicrobials.

Using widely available standard real time PCR instrumentation to perform a large number of real-time multiplexed home brew PCR assays is cost prohibitive when four or more of these assays are required. As the existing commercial products currently also do not provide an appropriate range of organisms to be able to realistically replace existing methods, three systems have been described for which a high number of PCR targets can be tested. Each system is capable of turning over 250 samples with 80 PCR targets/sample within 10 hours. Based on a test volume
of 250 samples/day for six days a week the cost per patient is ~$25 or less
dependent on the system.

In an environment of increasing health costs and fiscal pressures on health budgets
for pathology in Australia the use of these high multi analyte systems potentially
drives the cost down from $73.95 (Item 69336 + Item 69345) for conventional M/C/S
and O/C/P to $36.85 (Item 69496) for microbial nucleic acid determination. The
potential has been shown for these systems to deliver improved testing for lower
cost when utilised as stand alone methods. If the utility and value of the results
increase due to improved enteropathogen coverage and decreased time to reporting,
potentially more stool samples would be submitted for testing than the current rate of
one stool from every seventh patient presenting to a clinician with gastroenteritis.
This would actually increase testing costs in the health budget in a direct sense but
may help reduce community enteric disease burden and the economic costs of lost
time by patients and carers

Conceptually these systems meet the requirements but there are of course a number
of challenges to overcome. Stool is a complex matrix containing many inhibitors of
PCR and the organism nucleic acids themselves are ’packaged’ in a variety of ways
from cell walls, cyst walls, capsids, teguments and cuticles. Rapid, effective
automated nucleic acid extraction technology and appropriate control material to
seed into extractions is required. Even so, a percentage of samples will remain
inhibitory and not be suitable for PCR testing, which will require reverting to
traditional diagnosis, or sample recollection for repeat molecular testing. Several of
the helminth targets are yet to have a published real-time PCR developed and
require target evaluation and assay design. Validation of the large number of target
assays many of which detect organisms that have very low prevalence will be very
challenging. Adjusting to the inevitable increases in pathogens identified, the
increased observation of co-infection, and delivering appropriate interpretation to
clinicians and patients for organisms of variable or unknown significance and/or
asymptomatic carriage will all pose a challenge. There will also be an invariable
deskilling of the scientific staff in the techniques of faecal parasite identification by
microscopy, and bacterial enteropathogen recognition. Skills in bacterial culture
reading would be maintained if the proposed workflow of concurrent reflex culture
was adopted.

There appears to be two divergent projections of the functionality of future diagnostic
microbiology laboratories. The traditional global commercial microbiology
companies (bioMerieux and Becton Dickinson) are promoting 1-5 million dollar total
lab automation systems which bring manufacturing style automation and digital
imaging to the traditional phenotypic modalities of agar plates and microscope slides.
The products such as Previ-Isola/Smart Incubator (bioMerieux) or Kiestra (BD) utilise
automated agar plate inoculation robotics, attached track based smart incubation
modules, automated plate handling with high-resolution image capture of plate
growth, analysis of growth remotely via image observation, and downstream colony
manipulations for identification techniques and sensitivity test setup initiated by software based colony picking. There is also rapid progression of molecular technology which is acting as a disruptive technology to the traditional methods and replacing 'agar plate amplification' with 'nucleic acid amplification', the latter being considerably faster than the former, with equivalent or much improved specificity and sensitivity, and decreasing cost. The challenges of antimicrobial sensitivity testing will likely see molecular technology restricted to components of the workflow that are reliant primarily on detection of the organism in the medium term. However, it appears likely that enteropathogen detection within Australian laboratories is likely to transition to routine molecular methods over the next few years.
Table 1: Example List of 80 Potential Enteropathogen Targets for High Multi-Analyte Systems. The expanded target list for parasites represents a level of coverage where consideration might be given to not performing conventional microscopy based parasite testing on faeces. Many additional bacterial targets are present and cover numerous pathogens not examined for in routine bacterial culture. The expanded viral coverage also adds several viruses not routinely examined for by existing common tests in diagnostic pathology laboratories.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Viruses</th>
<th>Parasites</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella enterica</em></td>
<td>Rotavirus A/B/C</td>
<td><em>Entamoeba histolytica</em></td>
</tr>
<tr>
<td><em>Salmonella spp.</em></td>
<td>Adenovirus F 40/41</td>
<td><em>Entamoeba dispar</em></td>
</tr>
<tr>
<td><em>Campylobacter spp.</em></td>
<td>Norovirus G1/GII/GIV</td>
<td><em>Entamoeba moshkovskii</em></td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>Norovirus G1</td>
<td><em>Entamoeba coli</em></td>
</tr>
<tr>
<td><em>Campylobacter coli</em></td>
<td>Norovirus GII</td>
<td><em>Entamoeba polecki</em></td>
</tr>
<tr>
<td><em>Arcobacter spp.</em></td>
<td>Sapovirus</td>
<td><em>Idamoeba baetschlii</em></td>
</tr>
<tr>
<td><em>Brachyspira spp.</em></td>
<td>Mamastrovirus/Astrovirus</td>
<td><em>Endolimax nana</em></td>
</tr>
<tr>
<td><em>Shigella spp.</em></td>
<td>Human Parechovirus</td>
<td><em>Entamoeba hartmanni</em></td>
</tr>
<tr>
<td><em>Shigella dysenteriae</em></td>
<td>Enterovirus</td>
<td><em>Giardia intestinalis</em></td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td>Picobirnavirus</td>
<td><em>Dientamoeba fragilis</em></td>
</tr>
<tr>
<td><em>Shigella sonnei</em></td>
<td>Aichivirus</td>
<td><em>Chilomastix mesnili</em></td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em></td>
<td>Coronavirus</td>
<td><em>Retortamonas spp.</em></td>
</tr>
<tr>
<td><em>Yersinia spp.</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vibrio cholera</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vibrio fluvialis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> EHEC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> EPEC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> ETEC</td>
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<tr>
<td><em>Escherichia coli</em> EIEC</td>
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</tr>
<tr>
<td><em>Escherichia coli</em> EAggEC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aeromonas spp.</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Plesiomonas shigelloides</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Edwardsiella tarda</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Clostridium difficile</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hymenolepis nana</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hymenolepis diminuta</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Dipylidium caninum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fasciolopsis buski</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Echinostoma ilocanum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Heterophyes heterophyes</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Metagonimus yokogawai</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Comparative Summary of Commercial Enteropathogen PCR Testing Kits.

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Luminex</th>
<th>Seegene</th>
<th>Idaho Technology</th>
<th>Savyon</th>
<th>AusDiagnostics</th>
<th>Genetic Signatures</th>
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</thead>
<tbody>
<tr>
<td>Product Name</td>
<td>xTAG-GPP</td>
<td>Diarrhea ACE Detection 3 kits</td>
<td>FilmArray GI Panel</td>
<td>NanoCHIP Gastro-intestinal Panel</td>
<td>Easy-Plex Faecal Profile 10</td>
<td>EasyScreen Enteric Detection 3 Kits</td>
</tr>
<tr>
<td>Technology</td>
<td>Multiplex RT-PCR, BeadHyb Tag</td>
<td>Multiplex RT-PCR, CE Detection</td>
<td>Multiplex RT-qPCR in Pouch Nanofluidics</td>
<td>PCR plus Microarray</td>
<td>Multiplexed Tandem RT-qPCR</td>
<td>3base Multiplex RT-qPCR</td>
</tr>
<tr>
<td>Cost/patient AUD</td>
<td>Unknown</td>
<td>$39</td>
<td>Not available</td>
<td>$40</td>
<td>$24.70</td>
<td>$51</td>
</tr>
<tr>
<td>Throughput/batch</td>
<td>24</td>
<td>Up to 32</td>
<td>1</td>
<td>Up to 95</td>
<td>Up to 7</td>
<td>Up to 15</td>
</tr>
<tr>
<td>Extraction to Result Time for maximum batch</td>
<td>&lt;5 hours</td>
<td>&lt;5 hours</td>
<td>1 hour</td>
<td>4 hours</td>
<td>&lt;4 hours</td>
<td>3 hours</td>
</tr>
</tbody>
</table>

TARGETS:
- Salmonella
- Campylobacter
- Shigella
- Yersinia
- Vibrio spp.
- Vibrio cholerae
- EHEC
- Other Pathogenic E.coli
- Aeromonas
- Listeria
- Clostridium difficile
- Clostridium perfringens
- Plesiomonas shigelloides
- Rotavirus
- Adenovirus
- Norovirus
- Astrovirus
- Sapovirus
- Cryptosporidium parvum
- Giardia intestinalis
- Entamoeba histolytica
- Dientamoeba fragilis
- Entamoeba complex
- Blastocystis hominis
- Cyclospora cayetanensis
Table 3: Analysis of Reported Parasites 2007-2011 Sullivan Nicolaides Pathology(56). The results show that whilst the majority of recovered parasites are Blastocystis, Giardia, Cryptosporidium, Dientamoeba and Entamoeba histolytica, in total up to 13 distinct parasites of clinical significance and 4 types of commensal parasites have been reported from patients over the time period examined.

<table>
<thead>
<tr>
<th></th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blastocystis</td>
<td>4057</td>
<td>4177</td>
<td>4636</td>
<td>3702</td>
<td>3576</td>
<td>20148</td>
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<tr>
<td>Giardia</td>
<td>1118</td>
<td>1047</td>
<td>1179</td>
<td>1122</td>
<td>1286</td>
<td>5752</td>
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<tr>
<td>Cryptosporidium</td>
<td>321</td>
<td>430</td>
<td>997</td>
<td>214</td>
<td>351</td>
<td>2313</td>
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<tr>
<td>Dientamoeba fragilis</td>
<td>301</td>
<td>122</td>
<td>78</td>
<td>118</td>
<td>172</td>
<td>791</td>
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<tr>
<td>Endolimax nana</td>
<td>146</td>
<td>213</td>
<td>242</td>
<td>206</td>
<td>163</td>
<td>970</td>
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<tr>
<td>Entamoeba coli</td>
<td>182</td>
<td>201</td>
<td>198</td>
<td>152</td>
<td>132</td>
<td>865</td>
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<tr>
<td>Entamoeba histolytica</td>
<td>20</td>
<td>22</td>
<td>34</td>
<td>31</td>
<td>34</td>
<td>141</td>
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<td>Iodamoeba butschlii</td>
<td>23</td>
<td>24</td>
<td>24</td>
<td>22</td>
<td>18</td>
<td>111</td>
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<tr>
<td>Enterobius vermicularis</td>
<td>16</td>
<td>13</td>
<td>15</td>
<td>8</td>
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<td>70</td>
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<tr>
<td>Hymenolepis nana</td>
<td>5</td>
<td>6</td>
<td>2</td>
<td>5</td>
<td>0</td>
<td>18</td>
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<tr>
<td>Hookworm ova</td>
<td>9</td>
<td>1</td>
<td>9</td>
<td>3</td>
<td>3</td>
<td>25</td>
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<tr>
<td>Chilomastix</td>
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<td>11</td>
<td>7</td>
<td>8</td>
<td>3</td>
<td>48</td>
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<tr>
<td>Cyclospora</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>1</td>
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<tr>
<td>Taenia sp ova</td>
<td>2</td>
<td>1</td>
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<td>0</td>
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<tr>
<td>Strongyloides larvae</td>
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<td>7</td>
<td>7</td>
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<td>1</td>
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<td>Ascaris spp</td>
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<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>4</td>
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<tr>
<td>Trichuris ova</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
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<tr>
<td>Total Parasites</td>
<td>6230</td>
<td>6276</td>
<td>7435</td>
<td>5596</td>
<td>5762</td>
<td>31299</td>
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<tr>
<td>Total Faeces</td>
<td>47781</td>
<td>48308</td>
<td>52927</td>
<td>49126</td>
<td>56876</td>
<td>255018</td>
</tr>
</tbody>
</table>
Table 4: Analysis of Reported Bacterial Enteropathogens 2007-2011 Sullivan Nicolaides Pathology (56). The results show that whilst the majority of recovered bacterial enteropathogens are *Campylobacter*, *Salmonella*, *Aeromonas*, *Clostridium difficile*, *Yersinia* and *Shigella*, in total up to 13 distinct pathogens have been reported from patients over the time period examined.

<table>
<thead>
<tr>
<th></th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Campylobacter</em></td>
<td>1927</td>
<td>2106</td>
<td>1985</td>
<td>1857</td>
<td>2205</td>
<td>10080</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>844</td>
<td>703</td>
<td>827</td>
<td>1025</td>
<td>1077</td>
<td>4476</td>
</tr>
<tr>
<td><em>Aeromonas</em></td>
<td>856</td>
<td>863</td>
<td>868</td>
<td>679</td>
<td>632</td>
<td>3898</td>
</tr>
<tr>
<td><em>Yersinia</em></td>
<td>52</td>
<td>113</td>
<td>87</td>
<td>82</td>
<td>11</td>
<td>345</td>
</tr>
<tr>
<td><em>Shigella</em></td>
<td>44</td>
<td>45</td>
<td>36</td>
<td>39</td>
<td>28</td>
<td>192</td>
</tr>
<tr>
<td><em>Vibrios</em></td>
<td>6</td>
<td>7</td>
<td>14</td>
<td>9</td>
<td>13</td>
<td>49</td>
</tr>
<tr>
<td><em>Arcobacter</em></td>
<td>18</td>
<td>12</td>
<td>1</td>
<td>6</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td><em>Edwardsiella</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>EHEC</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>Plesiomonas</em></td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>19080</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Positive by PCR Detection

<table>
<thead>
<tr>
<th></th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. difficile PCR</td>
<td>309</td>
<td>328</td>
<td>453</td>
<td>493</td>
<td>962</td>
<td></td>
</tr>
<tr>
<td><em>stx1/2</em> PCR</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>7</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><em>eaeA</em> PCR</td>
<td>8</td>
<td>4</td>
<td>6</td>
<td>15</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

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Table 5: Comparison of Systems Capable of Cost-Effective ‘Comprehensive’ Multi Analyte Testing and High Throughput – Based on volumes of 250 patients/day and 6 testing days per week

<table>
<thead>
<tr>
<th>Format and Density</th>
<th>Fluidigm 96.96 IFC Chip</th>
<th>Sequenom MassARRAY</th>
<th>Life Technologies Open-Array Plate QuantStudio 12K Flex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>96x96 IFC Chip</td>
<td>3 x 27-29plex multiplex in 384 well plate format and secondary primer extension per sample</td>
<td>Open Array Plate Format (up to) 112 Assays by 24 samples per Plate by 4 plates per run</td>
</tr>
<tr>
<td>Analytical Volume</td>
<td>6nL</td>
<td>5μL</td>
<td>33nL</td>
</tr>
<tr>
<td>Dynamic Range</td>
<td>5.5logs</td>
<td>7logs</td>
<td>7logs</td>
</tr>
<tr>
<td>Multiplex PCR</td>
<td>Multiplex capable but meets criteria as singleplex</td>
<td>Yes – requires Multiplexing to meet criteria</td>
<td>No - singleplex only</td>
</tr>
<tr>
<td>Optimisation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Considerations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR Assay Setup</td>
<td>PCR assays to be individually constructed and loaded to IFC chip ports</td>
<td>PCR assays to be individually constructed and loaded into PCR amplification plates</td>
<td>Specific PCR Assays pre-filled into defined wells at manufacture, sample loaded with mastermix base only</td>
</tr>
<tr>
<td>Considerations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assay to Result Time</td>
<td>&lt;5 hours per 96 batch dependent on liquid handling for filling IFC chip</td>
<td>9.5 hours to first 128, 45 minutes MALDI-TOF end point analysis allows overlapping batch processing</td>
<td>3 hours per 96 batch Requires Accufill liquid handler to apply samples and mastermix base</td>
</tr>
<tr>
<td>Completion Time 250 samples each for &gt;80 specific PCR targets</td>
<td>Single Biomark HD 10-12 hours (3 runs)</td>
<td>Single System 10.25 hours with overlapping batches in process simultaneously Double System 9.5 hours</td>
<td>Single QuantStudio 9 hours (3 runs) Double QuantStudio 7 hours</td>
</tr>
<tr>
<td>Possible daily workload loss in event of first batch fails</td>
<td>33%</td>
<td>100%</td>
<td>33%</td>
</tr>
<tr>
<td>Capital Cost (AUD)</td>
<td>$480,000 per system</td>
<td>$450,000 per system</td>
<td>$160,000 per system</td>
</tr>
<tr>
<td>Contamination</td>
<td>Closed Amplification</td>
<td>Open amplified reaction tubes for secondary primer extension reaction setup</td>
<td>Closed Amplification</td>
</tr>
<tr>
<td>Considerations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indicative Cost/Patient including reagent volume discounting (AUD) Excludes Capital Costs</td>
<td>$15-$18</td>
<td>$10-$12</td>
<td>$22</td>
</tr>
</tbody>
</table>
Figure 1: Construction of the FilmArray Nanofluidic pouch, adapted from Poritz et al. (301). The pouch contains fluidic components manipulated by the Film Array cycler to achieve integrated extraction of nucleic acids, construction of PCR mastermixes and PCR in a set of wells in an array format.

Figure 2: Open-Array plate for Life Technologies QuantStudio 12K Flex qPCR System(302). Each Open-Array plate has 3072 through holes arranged as 48 squares of 8x8 through holes. User defined assays are added to sets of defined through holes on manufacture of the Open Array. Custom array formats of 32, 64, 128 and 192 through holes per patient test can be defined and primers and probes are deposited in the through holes assigned. Patient nucleic acid and the remainder of the reagent required to complete the PCR mastermix are loaded to the appropriate through hole set by the Accufill liquid handler. A maximum of 4 Open-Array plates can be run at the same time on the QuantStudio 12K Flex real time PCR cycler.
Figure 3: High Precision 96.96 Genotyping IFC Fluidigm Chip for Biomark HD qPCR System. (303). The 96.96 Fluidigm IFC Chip is loaded with 96 PCR assays on one side and 96 patient extracts on the other. Each patient sample is combined with each reaction mix within the IFC in a specific individual chamber utilising integrated thermal and pneumatic processes.
Figure 4: Seqenom MassARRAY Workflow (304). The amplification and iterative primer extension steps account for 7 hours of processing before MALDI-TOF analysis is performed. Failure of a batch of testing would not be apparent until approximately 8 hours after the first batch had been processed. If the cause of the failure affected other batches currently part way through the pre-MALDI-TOF process, this may render an entire day’s testing unsuccessful. This would delay patient results and incur high costs for the laboratory.

Figure 5: Seqenom MassARRAY SpectroCHIP for MALDI-TOF Mass Spectrometer (305). Extension oligo products are dispensed by robotic nanodispenser onto the SpectroCHIP array which is loaded into the MALDI-TOF MassARRAY Analyzer. Up to 384 reactions containing extended oligo products can be loaded to one SpectroCHIP for analysis.
Figure 6: Example workflow for incorporation of a high multi analyte PCR and ‘mini’ preservation culture within the laboratory. Stool is processed via three concurrent modalities; nucleic acid extraction with high multi analyte PCR testing, inoculation of Cary Blair, Selenite and Campylobacter Broth with appropriate incubation, and performing wet preparation microscopy. Positive PCR results for viruses and parasites are reported directly. Positive bacterial PCR results initiate a presumptive report and reflex culture from the mini cultures onto full agar plates. Reflex culturing allows recovery of the bacterial pathogen for antimicrobial sensitivity testing and epidemiological use where indicated.


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