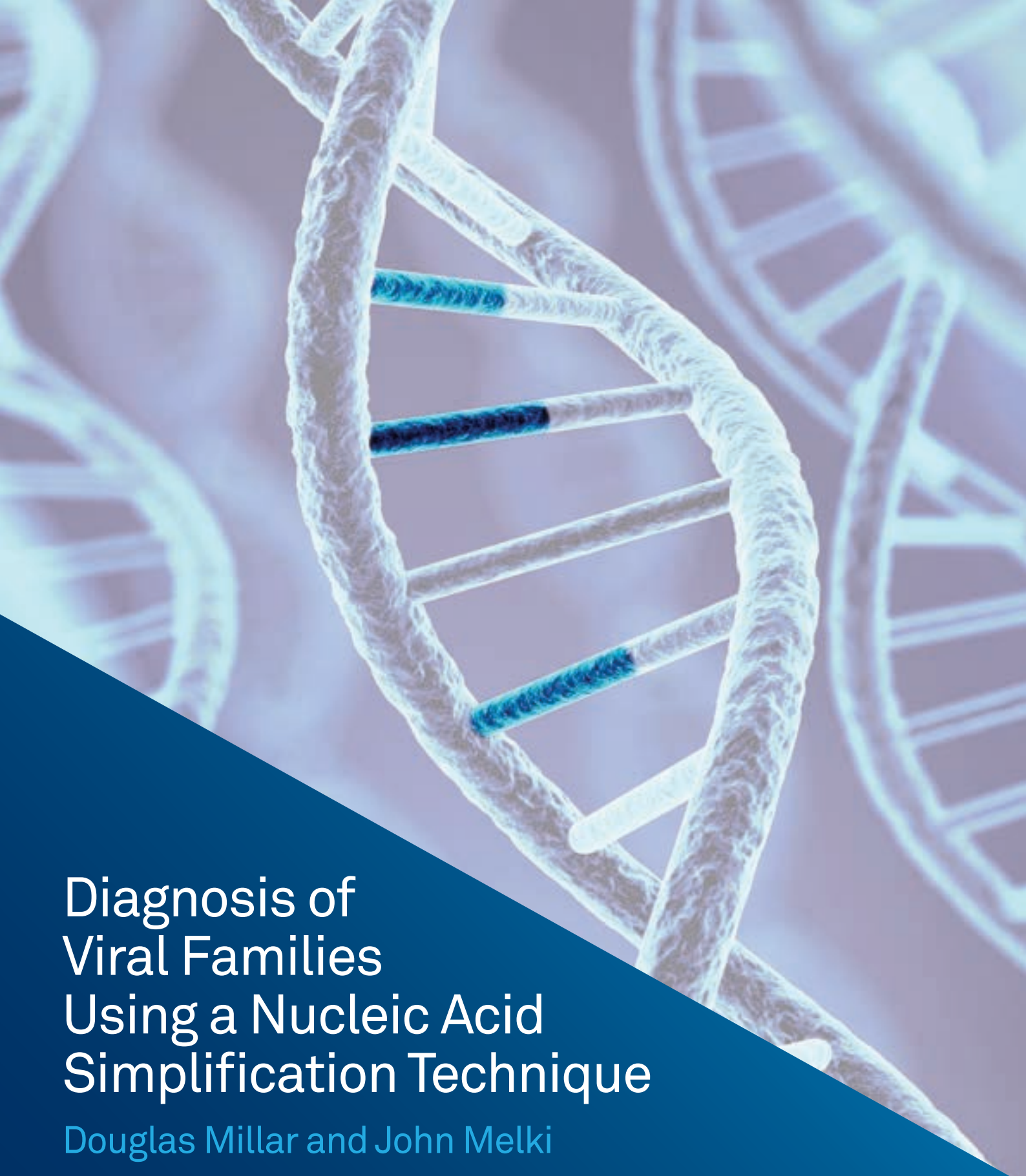

Diagnosis of Viral Families Using a Nucleic Acid Simplification Technique

Douglas Millar and John Melki

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Chapter

Diagnosis of Viral Families Using a Nucleic Acid Simplification Technique

Douglas Millar and John Melki

Abstract

We have developed a novel strategy to simplify microbial nucleic acids termed 3base™. This technology uses the chemical sodium bisulphite to reduce the genome from adenine, cytosine, guanine, and thymine or uracil, in the case of RNA containing viruses, to adenine, guanine and thymine thus reducing genome complexity. The method has been applied to the detection of high-risk human papilloma virus (HPV), gastrointestinal pathogens, alphaviruses, flaviviruses, dengue and more recently coronaviruses. Currently, there are very few real-time RT-PCR based assays that can detect the presence of all members of these viral families using conventional approaches. This strategy allows the design of assays that are capable of pan-family detection. The pan-viral assays provide a sensitive and specific method to screen and thereafter speciate viral families in clinical samples. The assays have proven to perform well using clinical samples and additionally during an outbreak of dengue fever that occurred in 2016/17 on the islands of Vanuatu. The 3base™ assays can be used to detect positive clinical samples containing any viral family generally in less than 3 hours making them ideally suited to viral surveillance and perhaps the discovery of emerging viruses in families without prior sequence knowledge of the pathogen.

Keywords: human papilloma virus, gastrointestinal pathogens, flavivirus, alphavirus, dengue, coronavirus, simplification, RT-PCR

1. Introduction

Many viruses are members of large families in which the individual viruses can be diverse at the molecular level. For example, SARS-CoV-2 belongs to the family *Coronaviridae* that contains 4 distinct genera namely the Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus. Many other viruses such as *Flaviviridae* and *Togaviridae* again contain many individual viruses in their designated family (see **Table 1** for examples).

Due to the genomic heterogenicity of viral families virtually all molecular diagnostic tests target individual viruses for disease diagnosis. However, even this can be challenging as viruses such as Influenza A contains many different strains based on the composition of their haemagglutinin and neuramidase genes. This chapter

Family	Genera	Notable pathogens	Species
Coronaviridae	Alphacoronavirus, Betacoronavirus, Deltacoronavirus and Gammacoronavirus	SARS-CoV-2, SARS, MERS	46
Filoviridae	Cuevavirus, Dianlovirus, Ebolavirus and Marburgvirus	Ebola, Marburg	12
Flaviviridae	Flavivirus, Hepacivirus, Pegivirus and Pestivirus	Zika, dengue, West Nile virus, Japanese encephalitis virus, yellow fever virus, Kyasanur forest disease, Alkhurma disease, Omsk hemorrhagic fever	54
Orthomyxoviridae	Alphainfluenzavirus, Betainfluenzavirus, Gammainfluenzavirus, Deltainfluenzavirus, Isavirus, Quaranjavirus and Thogotovirus	Influenza A, Influenza B	8
Papillomaviridae	53 members	HPV16, HPV18	>100
Picornaviridae	68 members	Enterovirus, Poliovirus, Hapatovirus	158
Poxviridae	Chordopoxvirinae and Entomopoxvirinae	Smallpox, Monkeypox, Cowpox	23
Rhabdoviridae	Alpharhabdovirinae, Betarhabdovirinae, Gammarhabdovirinae (plus 6 unassigned)	Lyssavirus	33
Togaviridae	Alphavirus	Chikungunya, Western Equine Encephalitis virus	32

Table 1.
Examples of the diversity contained within a number of different viral families.

describes a novel genomic simplification technique that enables the use of pan-family primers and probes to detect the presence of viral pathogens such as high-risk HPV, gastrointestinal pathogens, flavivirus, alphavirus, dengue and coronaviruses in clinical samples.

2. 3base™ a novel RNA simplification method

In order to simplify and improve the detection of viral families in clinical samples, we have developed an assay that is able to detect the presence of any high-risk HPV, gastrointestinal pathogen, flavivirus, alphavirus, dengue or coronavirus virus using a single primer and probe set for each type. These assays are based on the use of the chemical sodium bisulphite to reduce the complexity of genomes from 4 to 3 bases by deaminating cytosine to an uracil intermediate. The deamination reaction of cytosine to uracil was first described in 1970 by Hayatsu [1, 2] and has been studied in detail since. The first step of the reaction involves the sulphonation of cytosine to cytosine sulphonate followed by deamination to an uracil sulphonate intermediate and subsequently the removal of the sulphate adduct to uracil, traditionally by the use of strong alkali (**Figure 1**).

Sulphonated uracils are unable to be copied by DNA polymerases [3] due to steric hindrance as a result of the presence of the sulphate group at the C6 position. This causes distortions of DNA geometry and reduced stacking interactions [4]. Therefore, this adduct has to be removed if the resulting template is to be copied by a polymerase or reverse transcriptase enzyme. The bisulphite reaction was further refined in 1992 by Frommer and her colleagues [5] and used to differentiate cytosine from 5-methyl-cytosine in mammalian DNA as 5-methyl-cytosine is resistant to the deamination reaction. Since the publication of the genomic sequencing method, it has become the gold standard for studying the presence of methylated cytosine residues in the human genome (**Figure 2**).

However, this method resulted in up to 96% degradation of the DNA template [6] and would completely destroy RNA due to the need to desulphonate the uracil adduct with strong alkali. We have subsequently refined the method so that the degradation of DNA and RNA has been eliminated allowing "simplification" of both microbial DNA and RNA.

The 3base™ protocol deaminates all cytosine residues in nucleic acid to uracil, which are subsequently copied as thymine by a polymerase (**Figure 3**) or reverse transcriptase enzyme [5]. After simplification individual species become more similar in base composition resulting in reduced complexity of primer and probe sets for pan-family identification. The resulting primer and probe sets have fewer mismatches to the original sequences thus allowing binding of these to regions of nucleic acid that were previously heterogeneous in nature. The use of the simplification method does not result in a loss of specificity as it is still possible to design individual primer sets that can detect the viral species responsible for disease.

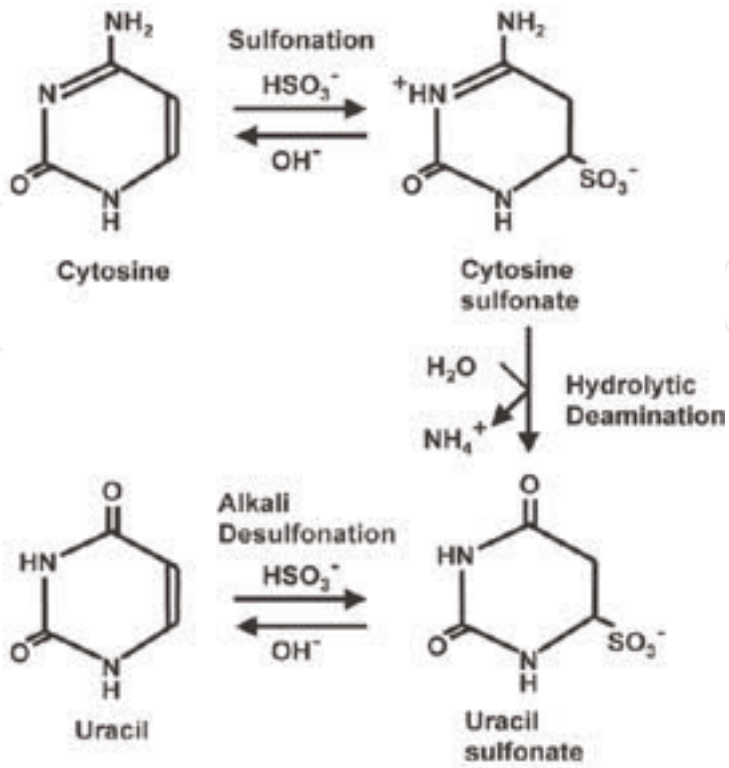

Figure 1.
Shows the reaction of cytosine with sodium bisulphite.

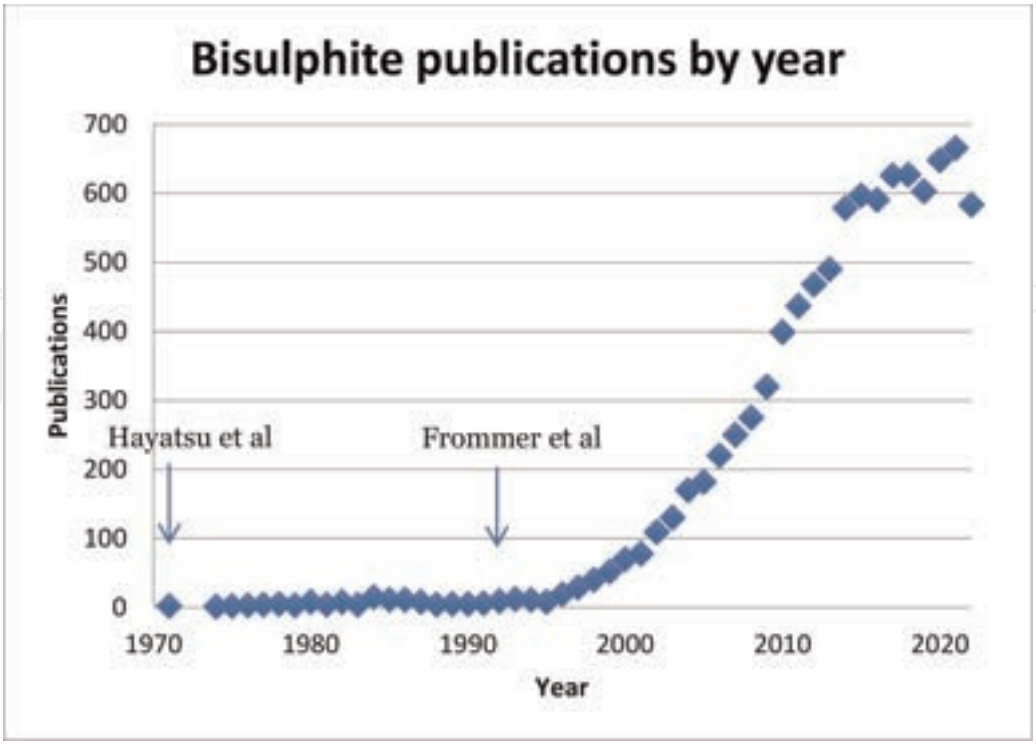

Figure 2.
Schematic representation of published papers using the bisulphite method with arrows representing the publication of the description of the cytosine deamination method and the bisulphite sequencing protocol.

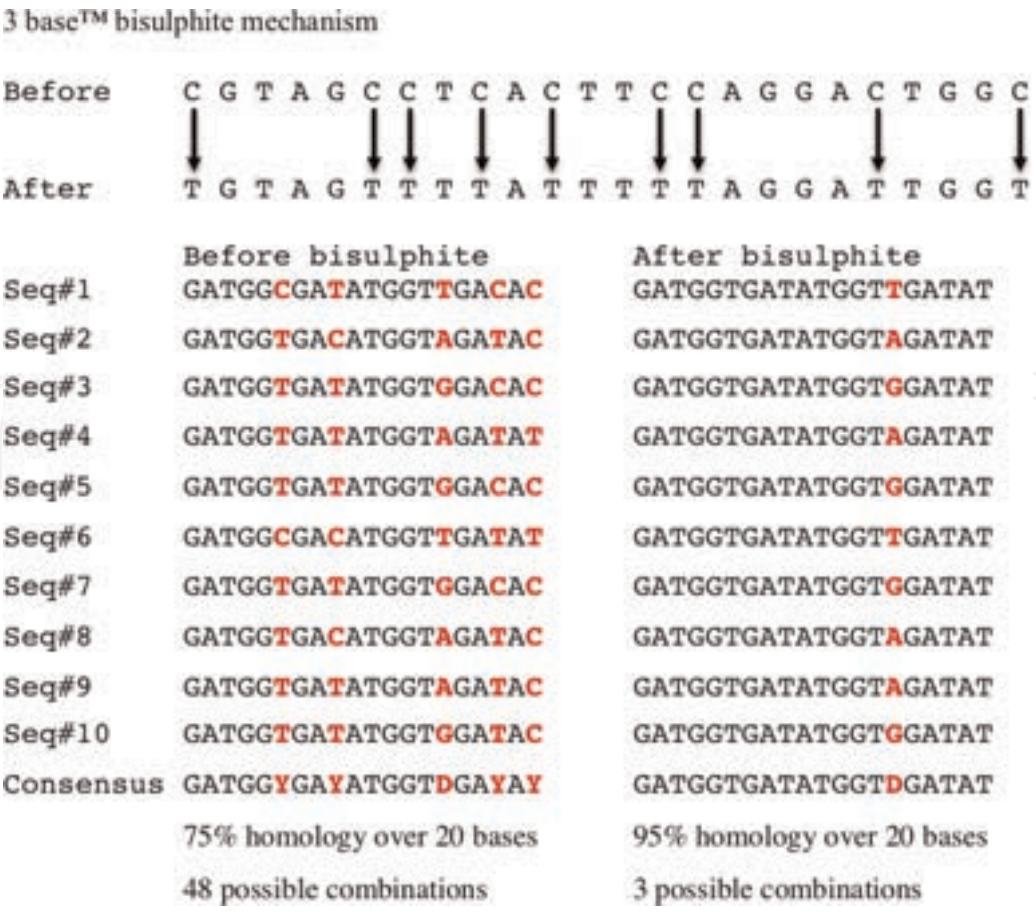

Figure 3.
Shows the simplification process where cytosine residues are converted to uracil.

3. Viral surveillance

New viruses will continue to appear due to evolutionary pressure, climate change and the demise of natural habitats as a result of human intervention. The Zika virus epidemic that began in 2016 demonstrates that a flavivirus originally thought to be relatively benign can emerge as a significant public health threat within a relatively short space of time [7]. SARS-CoV-2 emerged in 2019 and subsequently gave rise to a global pandemic which to date has resulted in over 620,000,000 confirmed cases and over 6,500,000 deaths [8]. SARS-CoV-2 more than likely emerged from an animal reservoir, and it is highly likely that other coronavirus threats will emerge in the future likely by the same route. While it is impossible to predict the rise of a particular virus in the human population it is almost certain that in the future new viral threats will emerge which will more than likely result in widespread morbidity and mortality.

As a result of the recent SARS-CoV-2 pandemic it is likely that governments will in the future invest in a more extensive network of testing equipment, stockpile reagents and enable easier regulatory protocols. While this could reduce the time required for testing, a critical phase exists of when a new pathogen becomes infectious to the general population, and when reliable diagnostic tests are generally available. A strategy that may allow for less-restricted screening for novel pathogens during this period is the use of pan-family assays: molecular diagnostic tests which target a family of viruses rather than a single species [9].

The use of species-specific PCR is unlikely to pick up new strains of a virus and this was demonstrated in the case of SARS-CoV-2, which was only detected on Next Generation Sequencing (NGS) and not by conventional PCR using species-specific primers and probes [10]. Interestingly, we had already developed a pan-coronavirus 3base™ assay that on publication of the complete genome of SARS-CoV-2 [11] would have picked up this variant without prior knowledge of the viral genomic sequence. The pan-family PCR approach is thus perhaps a simpler and more cost-effective alternative to NGS for viral surveillance?

It has been postulated that one of the more obscure viruses in the *Flaviviridae* family such as Spondweni virus (SPOV), Usutu virus (USUV), Ilheus virus (ILHV), Rocio virus (ROCV), Wesselsbron virus (WSLV) or tick-borne flaviviruses may be the next pathogen to emerge into the human population [7]. The use of the pan-flavivirus 3base™ assay would be the ideal tool to screen for emerging flaviviruses entering the population without the expense and labour costs of screening each and every sample for all of the individual flavivirus species that are currently known.

4. Pan viral diagnosis

There are several methods available for molecular pan-viral diagnosis (see **Table 2**). Perhaps the first was the use of arrays fabricated with large numbers of oligonucleotides probes specific for individual pathogens. Hybridisation of a clinical sample to such arrays was then be used to detect the presence of viral genomes in infected individuals [39]. However, using this approach prior sequence knowledge of the pathogens are required to design the specific oligonucleotides to be arrayed, and as stated previously emerging pathogens are highly likely to contain divergent nucleic acid sequences. Another approach for the detection of novel pathogens is the use of Next Generation Sequencing (NGS). Unlike the array approach no prior knowledge of an emerging viral sequence is required as all nucleic acids in the sample can be

Pan-virus target	Method	Reference
Adenovirus	RT-PCR	Kosulin et al. [12]
Bluetongue virus	RT-PCR	Mulholland et al. [13]
Coronavirus	RT-PCR	Erlichster [9], Holbrook [14]
Dengue	RT-PCR	Hu et al. [15], Waggoner et al. [16], Simmons et al. [17], Waggoner et al. [18]
Filovirus	RT-PCR	Jääskeläinen et al. [19]
Flavivirus	RT-PCR	Khongwichit et al. [20]
Foot and mouth disease	RT-PCR	Bachanek-Bankowska et al. [21]
Hepatitis B	Numerous (review article)	Wose Kinge [22]
Hepatitis C	RT-PCR	Walker et al. [23]
lyssavirus	RT-PCR	Marsten et al. [24], Condori et al. [25], Fischer et al. [26]
Orthopox	PCR	Grant et al. [27]
Phlebovirus	RT-PCR	Klimentov et al. [28]
Pegivirus	Microarray	Fridholm et al. [29]
Poxvirus	RT-PCR	Li et al. [30]
HPV	PCR	Chang et al. [31], Chouhy et al. [32]
Paramyxovirus	RT-PCR	Schatzberg et al. [33]
Simbu virus	RT-PCR	Fischer et al. [34]
Viral meningitis	NGS	Guan et al. [35]
Viral pathogens	Microarray	Chen et al. [36]
Viral pathogens	Microarray (genus specific oligonucleotides)	Kang et al. [37]
Viral pathogens	Microarray	Tang et al. [38]

Table 2.
Examples of the pan-family approach applied to molecular diagnostics.

sequenced then assembled by alignment with established genomes to produce a best match. Recently the costs associated with NGS have reduced dramatically from when the technology was in its infancy thus it is now possible to apply this technique to viral discovery [40]. However, the use of viral arrays and NGS is still more costly, labour intensive and less sensitive compared to the more routine technique of RT-PCR which can generate clinically meaningful data in around 1 h.

Many viruses that infect humans cause non-specific symptoms such as headache, fever, arthralgia, myalgia, and lethargy making initial diagnosis based on clinical symptoms challenging. This is especially true of respiratory viruses thus pan-family diagnosis can reduce the number of primer and probe sets that are require for molecular syndromic testing. **Table 3** shows that if the pan-family approach was used for respiratory viruses screening the number of individual reactions that would be required to identify the infectious agent is reduced from 20 to 7 reducing costs and the labour involved. After identifying the family responsible for infection individual typing primers could then be used to detect the exact species if required. Likewise,

Species-specific approach	Pan-family approach
Influenza A virus (Flu A)	Pan-orthomyxoviridae
Influenza A-H1 (Flu A-H1)	
Influenza A-H1pdm09 (Flu A-H1pdm09)	
Influenza A-H3 (Flu A-H3)	
Influenza B virus (Flu B)	
Respiratory syncytial virus A (RSV A)	Pan-orthopneumovirus
Respiratory syncytial virus B (RSV B)	
Parainfluenza virus 1 (PIV 1)	Pan-parainfluenza
Parainfluenza virus 2 (PIV 2)	
Parainfluenza virus 3 (PIV 3)	
Parainfluenza virus 4 (PIV 4)	
Coronavirus 229E (229E)	Pan-coronavirus
Coronavirus NL63 (NL63)	
Coronavirus OC43 (OC43)	
Coronavirus HKU-1 (HKU-1)	
SARS-CoV-2	
Human rhinovirus (HRV)	Pan-picornavirus
Enterovirus (HEV)	
Adenovirus (AdV)	Pan-Adenovirus
Metapneumovirus (MPV)	Metapneumovirus

Table 3.
Shows that using the pan-family screening approach the number of individual reactions required for a comprehensive respiratory screen is reduced from 20 to just 7.

infection with arboviruses manifest in similar symptoms thus the use of the pan-family screen can provide a rapid diagnosis of the family involved without the need to perform multiple individual PCR reaction to determine the cause of infection. After determination of the species responsible for infection again samples can then be typed using species-specific PCR if required.

5. Human papilloma virus (HPV)

The family *Papillomaviridae* contains a group of double stranded DNA viruses containing a circular genome of approximately 8000 base pairs [41, 42] that were first described to be associated with skin warts in 1907 [43]. The family papillomavirus contains over 100 individual members many of which cause no symptoms with the vast majority (90%) resolving after 2 years [44]. HPV can infect many different sites in the body including the skin, throat, tonsils, mouth, cervix, vulva, vagina, penis, and anus.

It was first postulated in 1976 that HPV could be associated with the development of cervical cancer [45]. Genital HPV infection can be caused by at least 50 individual

	Low risk	Probable high risk	High risk	Highest risk
HPV type	6, 11, 42 and 44	26, 53, 66, 68, 73 and 82.	33, 35, 39, 51, 52, 56, 58 and 59	16, 18, 31 and 45

Table 4.
HPV viruses classified according to the risk of cervical cancer development.

viruses that can be split into four classes as shown in **Table 4** [46]. The high-risk types of HPV have been shown to be associated with the development of cervical cancers [47–49].

Traditional methods for the diagnosis of cervical cancer have relied heavily on cytology in which cells of the cervix are observed under the microscope for the presence of cancerous or precancerous lesions. This test, known as the Papanicolaou (Pap) test was invented in the 1920s by Georgios Papanikolaou and Aurel Babeș and subsequently named after Papanikolaou. A simpler version of the test was discovered by Anna Marion Hilliard in 1957. The use of the Pap test when used in combination with molecular methods has been shown to increase the sensitivity in which pre-cancerous lesions can be detected in the cervix [50].

5.1 Molecular detection of HPV

There are a number of molecular methods that can be used to detect the presence of HPV in clinical samples [51–55]. One common primer pair, the MY set, was first described in 1989 [56] detects a common region of the viral L1 gene that is found in all HPV types. Improvements on these primers generated the GP set that are able to detect more strains of the virus [57]. However, these primer sets are unable to differentiate the presence of high-risk HPV from low risk therefore amplicons must be sequenced or hybridised to oligonucleotide arrays to determine the strain of the virus responsible for infection.

One of the earliest FDA approved molecular tests for HPV was the hcII HPV test (Digene Corporation, USA). This test used oligonucleotide probes that were specific for each of the high and low risk viruses. The method was based on capture of specific HPV sequences present in the clinical sample coupled with a chemiluminescent read-out. However, it has been demonstrated that this assay could generate both false positive and negative results [58].

5.1.1 3base™ detection of high-risk HPV types

To produce an assay capable of detecting specifically the high-risk viruses we aligned the sequences of the complete genomes of the high-risk HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 along with the low-risk types 6, 11, 43 and 44 to serve as reference for non-target HPV strains. Using this approach, we were able to design a nested PCR assay that was specific for high-risk HPV types only. The primers were tested extensively on a large number of previously typed ThinPrep® liquid-based cytology samples to ensure that the assay was specific for the high-risk-types.

5.1.2 3base™ high-risk HPV clinical trial

A total of 834 ThinPrep® samples were tested using the 3base™ simplification method and compared to the reference hcII method. Discordant samples were the

amplified using a reference method (MY09/MY11 and the GP5+/GP6+ primer sets) and the amplicons subsequently sequenced to identify the strain of virus present. As can be seen from **Table 5** both methods demonstrated a similar sensitivity which was not statistically significant (p = 0.398). However, the specificity of the 3base™ was significantly higher than the hcII method (p = 0.001) and as would be expected the PPV for the 3base™ test was also significantly higher [59].

	Reference method (PCR)					
	Positive	**Negative**	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**
3base™ method						
Positive	197	49	63.1	90.6	80.1	80.4
Negative	115	473				
Digene hcII method						
Positive	202	80	64.7	84.6	71.6	80.1
Negative	110	442				

Table 5.
The results generated in an independent clinical trial comparing the 3base™ to a commercially available assay for the detection of HPV in cervical samples.

6. Gastrointestinal disease

Gastrointestinal (GI) diseases occur globally and are a major cause of morbidity and mortality. In developed countries the mortality due to GI disease is relatively low compared to that of developing countries [60]. It has been estimated that in developing countries up to 2 million children under the age of five die from GI infections annually [61].

There are many viral, bacterial, and protozoan agents that are responsible for gastroenteritis in humans. Perhaps the most notable of these are the viral agents that include Norovirus, Rotavirus, Sapovirus, Astrovirus and the Adenoviral group. One of the most common causes of gastroenteritis is Norovirus which is responsible for outbreaks of disease especially in children with an estimated 685 million cases and around 200,000 deaths occurring annually worldwide [62].

The viruses that cause gastroenteritis are a diverse group of pathogens with many genotypes and genogroups responsible for disease (see **Table 6** below).

There are also a wide range of bacterial species that are responsible for gastrointestinal illness with the most common agents that of *Campylobacter* spp. and *Salmonella*. The CDC estimated that in the USA that 43% of bacterial gastrointestinal cases are caused by *Salmonella* spp. followed by *Campylobacter* spp. representing a further 33% [66]. Other notable causes of GI disease are *Shigella* spp., *Yersinia enterocolitica*, *Clostridium difficile* and pathogenic strains of *Escherichia coli*. Different bacterial agents also show distinct geographical distributions with species such as *Vibrio cholera* and *Shigella* spp. more common in developing countries [67].

Protozoan species also contribute to the burden of GI diseases with notable agents such as *Giardia* spp., *Cryptosporidium* spp. and *Enteramoeba histolytica* the most

Virus	Genome	Genotypes	Genogroups	Ref
Norovirus	Positive sense RNA	10	49	Chhabra et al.[63]
Rotavirus	Double stranded RNA	7	36G and 51P	Wahyuni et al. [64]
Sapovirus	Positive sense RNA	19		Tang et al. [65]
Astrovirus	Positive sense RNA	8 (+2 novel)		Tang et al. [65]
Adenovirus	Double stranded DNA	7		Tang et al. [65]

Table 6.
The diversity of viral agents responsible for GI disease.

common causes. Other agents such as *Dientamoeba fragilis* and *Blastocystis hominis* have also been implicated in the aetiology of gastroenteritis [68–70].

The symptoms of gastrointestinal disease can include diarrhoea, vomiting, abdominal pain, fever, general lack of energy, and dehydration. These symptoms are shared between the many organisms that cause symptoms thus traditionally disease was diagnosed by a combination of culture, microscopy and EIA for bacteria, protozoan and viral disease respectively. These techniques are laborious and in some cases such as conventional culture can take up to 4–5 days to yield positive results [71].

To simplify the detection of gastrointestinal pathogens and streamline the process we sought to use 3base™ technology to not only detect the complex viral causes of gastroenteritis but also the individual bacterial and protozoan agents responsible for gastroenteritis [72].

6.1 Viral pathogens

Sequences for all genotypes of Norovirus, Rotavirus, Astrovirus, Sapovirus and Adenovirus were downloaded from the NCBI nucleotide database and aligned using the free web-based alignment tool Dialign (https://dialign.gobics.de). Regions were then chosen to produce primer and probe sets to amplify each viral group. After initial optimisations the best sets were used to screen a bank of archived clinical samples with the results are shown in **Table 7**.

As can be seen from **Table 7** the assay was able to detect the presence of all viral targets. In addition, the assay was validated independently yielding similar results.

6.2 Bacterial and protozoan pathogens

Although bacterial and protozoan causes of gastroenteritis are not as complex as the viral targets it was important that the assay was able to detect these pathogens as gastroenteritis is a syndromic disease. **Tables 8** and **9** demonstrate the ability of the 3base™ method to detect organisms at the species level.

As can be seen from the data the 3base™ assay does not suffer from a loss of specificity when primer and probe sets are designed to detect organisms at the species level. The syndromic multiplex PCR assay is thus a useful tool for the detection of viral, bacterial, and protozoan causes of gastroenteritis without the need for time consuming and labourious conventional methods. In addition, testing can be centralised with a turnaround time of less than 4 h.

Viral specimens (n = 109)			3rd party evaluation	
Species	**EIA**	**3base™ assay**	**Conventional**	**3base™ assay**
Norovirus	81	81	15	16
Rotavirus	21	21	15	15
Astrovirus	5	5	1	3
Adenovirus	2	2	2	2
Sapovirus	0	0	0	0

Table 7.
Results generated using the 3base™ on stool samples with viral gastroenteritis.

Bacterial specimens (n = 80)			3rd party evaluation	
Species	**culture**	**3base™ assay**	**Conventional**	**3base™ assay**
Campylobacter spp.	40	41	13	13
Salmonella spp.	32	31	5	5
C. difficile	4	4	17	18
Shigella spp.	1	1	2	2
Y. entercolitica	0	0	2	2
Listeria monocytogenes	0	0	0	0
Negative	3	3		

Table 8.
Detection of bacterial causes of gastroenteritis.

Parasite specimens (n = 81)			3rd party evaluation	
Species	**Microscopy**	**3base™ assay**	**Conventional**	**3base™ assay**
G. intestinalis	33	37	2	4
Cryptosporidium spp.	15	15	0	0
D. fragilis	12	13	4	5
Entamoeba complex	N/A	7	0	0
E. histolytica	0	0	0	0
B. hominis	15	20	2	3
Negative	11	6		

Table 9.
Detection of protozoan causes of gastroenteritis.

7. Coronaviridae

The Coronavirus family members are sub classified as alpha, beta, gamma and deltacoronaviruses [73, 74]. Alphaconoronaviruses contain least 10 known species including human coronavirus (hCoV) 229E that causes the common cold, many bat, feline, canine coronaviruses, and the porcine transmissible gastroenteritis coronavirus. The Betaconoronaviruses contain members such as SARS-CoV-1,

MERS-CoV, human coronavirus 0C43 and now SARS-CoV-2. The Gammacoronaviruses genera contain avian, duck coronavirus and the infectious bronchitis virus. Finally, the deltacoronaviruses members include HKU11, HKU12, HK13 that cause the common cold. **Figure 4** illustrates a phylogenetic tree showing the relatedness of various coronavirus strains. Most of the members of the coronavirus family exhibit a zoonotic lifecycle, that in rare occasions result in a spill over event to the human population.

A number of notable human coronaviruses have emerged in the last two decades which can result in severe respiratory disease. The severe acute respiratory syndrome (SARS) originated as a mystery illness in Guangdong, China in 2002 and resulted in an epidemic that killed 10% of the 8000 people it infected [75]. The etiological agent was subsequently identified as the severe acute respiratory syndrome coronavirus (SARS, now renamed SARS-CoV-1). This was the fifth hCoV to be identified and is thought to have originated as an animal virus from an unknown animal reservoir. The disease was characterised by flu-like symptoms, high fevers exceeding 38°C, myalgia, dry non-productive cough, difficult breathing, and an infiltrate seen on chest radiography.

Ten years later in 2012, a sixth hCoV was isolated from a patient presenting with severe respiratory illness in Jeddah, South Arabia [76]. The etiological agent was later designated Middle East respiratory syndrome coronavirus (MERS-CoV). MERS-CoV has been detected in more than 27 countries across the Middle East, Europe, North Africa, and Asia. There has been a total of 2040 MERS-CoV laboratory confirmed cases, with 712 deaths (34%) making this the most lethal coronavirus to date.

Another novel coronavirus (SARS-CoV-2) emerged into the human population in December 2019 in Wuhan, China, and has subsequently become the deadliest coronavirus to emerge in the human population in the past two decades [77], bringing the number of hCoV to seven. The disease (Covid-19) is believed to have been contracted from an animal virus that crossed over into the human population, more than likely from bats. The virus has spread globally and infected over 620,000,000 people resulting in over 6,500,000 deaths [8] which although far more than the MERS-CoV epidemic represents only a 1% case fatality rate compared to 34% for MERS-CoV. Such a large-scale spread is a result of efficient human-human transmission as the virus evolves to improve its ability to infect its human host.

The severity of Covid-19 and the rapid spread of the virus is a wakeup call to rethink diagnostic approaches, especially for the coronavirus family that has many members maintained by a variety of animal reservoirs such as bats, birds, pangolins, and snakes [78–80]. Covid-19 is an example of what can happen if a spill over event involves a virus well attuned to human-human transmission. The severity of coronavirus disease and the potential for new emerging viruses calls for rapid diagnostic tests which can quickly and accurately detect these viruses in clinical samples and animal hosts. The pan-family molecular approach could be an ideal method to screen for coronaviruses in general and detect novel strains as they emerge.

7.1 Design of the pan-coronavirus assay

Whole genomic sequences of SARS-CoV-1, MERS, HKU-1, NL63, 229E and OC43 were downloaded from the data base and aligned using the Geneious Prime™ software to generate optimal regions for the design of 3base™ primers and probes. These were them tested using synthetic constructs to determine assay sensitivity and specificity (see **Table 10**).

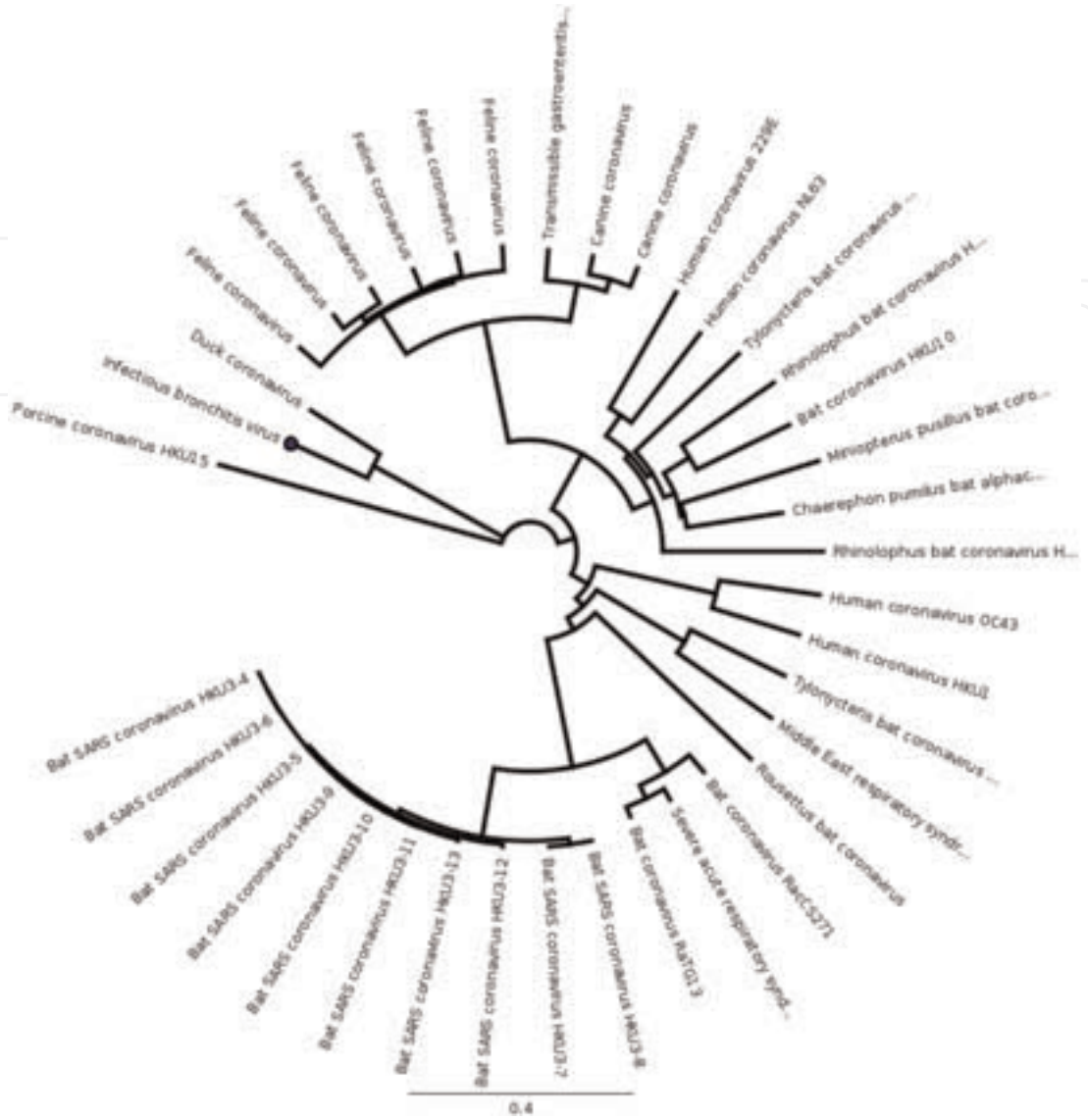

Figure 4.
Shows a phylogenetic tree generated using whole genomes of various coronaviruses using the Geneious prime™ tree building software.

After initial assessment and assay validation including cross-reactivity studies the pan-coronavirus component was then multiplexed with the SARS-CoV-2 E and N genes for clinical studies using cultured SARS-CoV-2 virus. **Table 11** shows that the triplex assay could detect low levels of SARS-CoV-2 virus.

The clinical performance of the assay was established using 1, 662 patient samples sourced from a local hospital in 2020 when the virus was still relatively rare in Australia. Twenty-five samples were found to be positive for SARS-CoV-2 by both the pan-coronavirus and gene specific assays. In addition, a further 45 samples were positive using the pan-coronavirus assay and negative with the SARS-CoV-2 specific primer and probe sets. These samples were then tested with a confirmatory assay that detected the presence of seasonal coronaviruses. This assay detected 37 samples as either NL63, 229E, OC43 or HKU-1. Of the eight samples that were negative by the confirmatory assay, five were available for sequencing using the pan-coronavirus amplicons. On sequencing the results showed that these samples contained a novel HKU-1 variant not targeted in the confirmatory test.

	Pan-Coronavirus (replicates positive)						
Copies/PCR	**MERS**	**SARS-CoV-2**	**SARS-CoV-1**	**NL63**	**229E**	**HKU1**	**OC43**
1E4	5/5	5/5	5/5	5/5	5/5	5/5	5/5
1E3	5/5	5/5	5/5	5/5	5/5	5/5	5/5
1E2	5/5	5/5	5/5	5/5	5/5	5/5	5/5
50	5/5	5/5	5/5	5/5	5/5	5/5	5/5
25	5/5	5/5	4/5	5/5	5/5	5/5	5/5
12.5	5/5	5/5	3/5	5/5	5/5	5/5	4/5
6.25	4/5	3/5	2/5	5/5	4/5	4/5	4/5
3.125	0/5	2/5	3/5	1/5	2/5	4/5	1/5

Table 10.
Sensitivity of the pan-coronavirus assay tested on synthetic construct.

SARS-CoV-2	**Pan-Coronavirus**	**E-gene**	**N-gene**
Dilution	Positive	Positive	Positive
1\10	2/2	2/2	2/2
1\100	2/2	2/2	2/2
1\1000	2/2	2/2	2/2
1\10,000	2/2	2/2	2/2
1\20,000	2/2	2/2	2/2
1\40,000	2/2	2/2	2/2
1\80,000	2/2	2/2	2/2
1\160,000	1/2	2/2	2/2

Table 11.
Shows the results using the pan-coronavirus triplex assay on cultured viral samples.

8. Current pan-flavivirus/alphavirus assays

A PubMed.*gov* search was performed using the keywords pan-flavivirus real time PCR (RT-PCR), pan-alphavirus RT-PCR and pan-dengue RT-PCR to determine the number of assays that employed a pan-family approach. Although this is not a definitive search the results give an idea of what is possible at present using conventional real-time PCR. From 1996 to 2022 a total of 1, 182 paper were found that used real-time PCR to detect the presence of flaviviruses in general. Of these only 2 papers used the pan-flavivirus detection approach. Similarly, from 2004 to 2022 a total of 294 papers mentioned RT-PCR for the detection of alphavirus with only 1 using a pan-species approach with this assay using multiple primers due to target sequence degeneracy. With dengue virus from 2001 to 2022 a total of 782 papers were published that mentioned dengue virus and real-time PCR with 32 using pan-dengue RT-PCR primers and probes.

As the dengue virus family contains only 4 members it makes sense that this was the target to which most pan-family assays were designed. The flavivirus and

alphavirus virus families are much more complex and contain 54 and 32 members respectively and are much underrepresented with pan-family tests compared to dengue. The reduction in the number of assays able to detect pan-flavivirus and pan-alphavirus is presumably due to sequence divergency of the individual members making the selection of suitable primers and probes for pan-family identification using conventional RNA more challenging.

This is where the use of the chemical simplification step can make the selection of regions to design primers and probes easier (see **Table 12**). As can be seen before the genomic simplification process the consensus sequence for a pan-alphavirus primer would contain a total of 576 individual primers to produce sequences that were a perfect match for all targets. However, after the simplification process the primer pool would be reduced to just 27 representing a major reduction in genomic complexity.

8.1 Flavivirus/alphavirus and dengue

The *Flaviviridae* family of viruses contain many individual members that result in a heavy toll in terms of morbidity and mortality globally on an annual basis. Notable members include dengue which has been estimated to cause over 400 million

	Sequence	
Alphavirus species	**Before conversion**	**After conversion**
Barmah Forest Virus	CCUUACUUCUGUGGAGGAUUU	TTTTATTTTTGTGGAGGATTT
Ndumu virus	CCGUAUUUCUGCGGCGGGUUC	TTGTATTTTTGTGGTGGGTTT
Chikungunya virus	CCUUACUUUUGUGGAGGGUUU	TTTTATTTTTGTGGAGGGTTT
O'nyong-nyong virus	CCAUACUUCUGUGGGGGAUUU	TTATATTTTTGTGGGGGATTT
Middelburg virus	CCCUACUUCUGCGGAGGGUUU	TTTTATTTTTGTGGAGGGTTT
Mayaro virus	CCCUACUUUUGUGGAGGUUUC	TTTTATTTTTGTGGAGGTTTT
Ross River virus	CCAUACUUCUGCGGCGGGUUU	TTATATTTTTGTGGTGGGTTT
Semliki forest virus	CCAUAUUUUUGUGGGGGAUUC	TTATATTTTTGTGGGGGATTT
Una virus	CCUUACUUCUGCGGAGGAUUC	TTTTATTTTTGTGGAGGATTT
Aura virus	CCUUACUUUUGCGGCGGAUUU	TTTTATTTTTGTGGTGGATTT
Rio Negro virus	CCAUACUUUUGUGGAGGGUUU	TTATATTTTTGTGGAGGGTTT
Mucambo virus	CCGUACUUUUGCGGCGGGUUU	TTGTATTTTTGTGGTGGGTTT
Everglages virus	CCCUAUUUUUGUGGAGGGUUU	TTTTATTTTTGTGGAGGGTTT
Venezuelan equine encephalitis virus	CCCUAUUUUUGUGGAGGGUUU	TTTTATTTTTGTGGAGGGTTT
Eastern equine encephalitis virus	CCGUACUUUUGCGGAGGGUUC	TTGTATTTTTGTGGAGGGTTT
Western equine encephalitis virus	CCCUACUUCUGUGGGGGAUUU	TTTTATTTTTGTGGGGGATTT
Consensus sequence	CCNUAYUUYUGYGGDGGDUUY	TTDTATTTTTGTGGDGGDTTT
Number of variants	**576**	**27**

The red colouring indicates bases that are degenerate in the sequences before and after the simplification process.

Table 12.
Genomic simplification of alphavirus sequences reduces the number of primer variations from 576 to just 27.

infections yearly with 100 million cases in 2010 [81]. Other species include Zika which caused epidemics between 2014 and 2017, yellow fever virus which is endemic in Africa and South America, Japanese encephalitis virus and West Nile virus which has been associated with sporadic outbreaks in the USA. Flaviviruses infections range from asymptomatic to life threatening conditions such as hemorrhagic fevers. Flaviviruses are characterised by a positive sense single stranded RNA genome that ranges in size from 10 to 11 Kb. The genome consists of 8 non-structural and 3 structural proteins [82].

Alphaviruses are members of the *Togaviridae* group of viruses with genomes of around 11–12 Kb that like flaviviruses contain a single stranded positive sense genome [83]. Alphaviruses infect a wide range of birds, fish and mammals including humans. Probably the best-known alphaviruses are chikungunya, Barmah Forest virus and O'nyong'nyong virus. Both flavi- and alphaviruses are arboviruses and are most commonly transmitted to the human population via a bite from an infected mosquito or tick.

The global distribution of flaviviruses and alphaviruses can be overlapping or unique with some viruses specific for certain geographical locations (**Figure 5**). Epidemics of flavivirus and alphavirus occur on an annual basis with different degrees of severity thus rapid and specific molecular diagnostic approaches are required to aid in patient management.

8.2 Design of 3base™ primers and probes

To determine if the pan-flavivirus, pan-alphavirus and pan-dengue simplification method could be used in screening and outbreak management we designed 3base™ assays for each family of pathogens.

8.2.1 3base™ pan-flavivirus/pan-dengue assays

The complete genomes of the following flaviviruses were analysed using Geneious software to determine the optimal regions for 3base™ primers and probes; Karshi virus (AY863002), Powassan virus (EU670438), Kyasanur forest disease virus

Figure 5.
Shows the global distribution of a number of important arboviruses (this map was prepared using information in Socha et al. [84] using the free web based MapChart software).

(AY323490), Langat virus (NC_003690), Omsk hemorrhagic fever virus (AB507800), Tick-borne encephalitis virus (KU761572), Yellow fever virus (MF423374), Sepik virus (DQ859063), Wesselsbron virus (JN226796), Dengue 4 (EU854296), Dengue 2 (AF038402), Dengue 3 (AB189125), Dengue 1 (AB189120), Zika (KU820899), Saint Louis encephalitis virus (MN233312), West Nile virus (KT57320), Kunjin virus (KX394405), Japanese encephalitis virus (AF080251), Usutu virus (AY453411) and Murray Valley encephalitis virus (AF161266).

8.2.2 3base™ pan-alphavirus assay

The complete genomes of the following alphaviruses were analysed using Geneious software to determine the optimal regions for 3base™ primers and probes; Barmah Forest virus (NC_001786), Ndumu virus (NC-01659), Chikungunya virus (NC_004162), O'nyong-nyong virus (NC_001512), Middelburg virus (NC_024887), Mayaro virus (NC_003417), Ross River virus (NC_001544), Semliki forest virus (NC_003215), Una virus (NC_043403), Aura virus (NC_003900), Rio Negro virus (NC_038674), Mucambo virus (NC_038672), Everglades virus (NC_038671), Venezuelan equine encephalitis virus (NC_001449), Eastern equine encephalitis virus (NC_003899) and Western equine encephalitis virus (NC_003908).

8.3 Assay performance

Numerous primer/probe sets were designed for the pan-flavivirus, pan-dengue and pan alphavirus assays and sets then wet tested to determine optimal sensitivity and specificity. After initial screening the best performing sets were tested using individual synthetic oligonucleotoides specific for each virus. The pan-flavivirus assay was able to detect the presence of DENV-1, DENV-2, DENV-3, DENV-4, TBEV, WNV, YZV and Zika virus with a lower limit of detection (LLOD) of 12.5 copies/PCR for all species tested.

Likewise, the pan-alphavirus assay was able to detect the presence BFV, CHIKV, EEEV, MVE, NV, RRV, VEEV and WEEV with a sensitivity of 10 copies/PCR for VEEV, RRV, NV, BFV and MV, 25 copies/PCR for CHIKV and EEEV and 50 copies/PCR for WEEV.

To assess potential cross reactivity with other viruses after the 3base™ simplification process a large number of RNA and DNA samples were obtained from a number of human viruses. No cross reactivity was observed with any component of the assays using a wide range of both DNA and RNA containing human pathogens.

Molecular quality assurance panels obtained from QCMD for dengue, Zika virus and chikungunya from 2016 to 2018 demonstrated that the pan-flavivirus/pan-alphavirus/pan-dengue assays were in 100% concordance with the expected results. These results indicate that the simplification assays are performing well, if not better than other molecular assays used worldwide.

8.4 Vanuatu 2016/2017 dengue outbreak

T0 date traditional methods such as Enzyme Immuno Assays (EIAs) have been the method used for the detection of both flavi- and alphaviruses. It has been shows that dengue EIAs show and high degree of cross reactivity with Zika virus and likewise

Zika EIAs cross react with dengue [85, 86]. Unlike molecular approaches conventional EIAs are unable to differentiate the individual dengue serotypes and in addition are generally less sensitive than molecular assays. However, unlike the RNA simplification approach there are very few RT-PCR assays can target all members of complex groups such as flavivirus or alphavirus using a single primer and probe set.

There have been numerous outbreaks of arboviruses in the South pacific regions. From 2012 to 2014 it was estimated that at least 28 outbreaks of disease have occurred which were attributed mainly to dengue virus but notable outbreaks as a result of chikungunya and Zika virus were also recorded [87]. These outbreaks cause severe stress on both the public health system and on the islands economy which for the most part are tourist driven.

During 2016/2017 an outbreak of dengue fever occurred on the islands of Vanuatu [88]. Vanuatu consists of a group of over 80 islands that are located in the South Pacific region the largest of which is Efate home to over 86, 000 residents. The population on the rest of the islands range from as many as 46,000 to as low as a few hundred. From the 12th to 24th March 2017, we tested both archived and fresh samples obtained from Port Villa central hospital, Efate, to determine if the 3base™ pan-flavivirus, pan alphavirus and pan-dengue assays were useful in an outbreak situation. We included a dengue 2 specific primer and probe set since this was the genotype responsible for the outbreak. Samples were extracted using a small footprint automated extraction platform along with a small portable PCR machine weighing less than 2 kg.

Over the study period we tested 187 serum sample for the presence of dengue (see **Table 13**). One hundred and sixteen samples tested positive for the presence of pan-flavivirus, pan-dengue and the specific dengue 2 assay representing a positivity rate of 62%. Seven samples were inconclusive as only signals were obtained with the pan-dengue component of the assay which could be explained by a very low viral load in these particular samples.

When we plotted the dengue positivity from December to March (see **Figure 6**) we found that the number of positive dengue cases peaked in the month of January followed by a marked decline in positivity in February. Routine testing of patients with dengue like symptoms using the pan-family assays commenced in the middle of March and we found that the number of cases began to increase again at this time. As molecular methods are more sensitive than the conventional EIA assays the rise could be attributed to increased sensitivity of the pan-family assays [80].

	Number	**% Positive**
pan-flavivirus	116	62
pan-alphavirus	0	0
pan-dengue	123	66
DENV-2	116	62
dengue not typed	7*	3.2
Negative	64	34

**Weak samples with a Ct value >40.*

Table 13.
Results of clinical sample obtained during the Vanuatu outbreak.

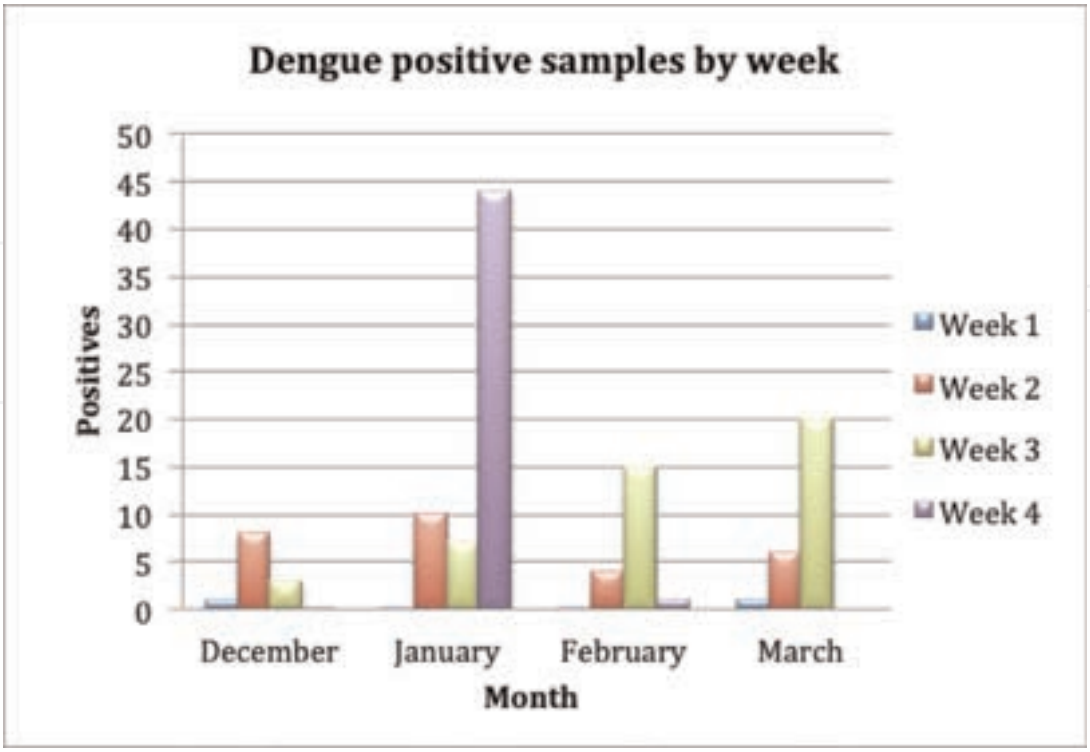

Figure 6.
Shows the weekly positive results from December to the 24th March 2017.

In addition, when we looked at the distribution of dengue cases across the islands, we found a statistically significant concentration of infection on the islands of Emae ($p < 0.00001$), Tongoa ($p < 0.00001$) and Ambae ($p < 0.00001$) compared to the regional average, which was calculated to be 0.416 per 1000 people, suggesting that these islands may possibly harbour animal reservoirs infected with the dengue virus.

9. Conclusion

In summary it has been shown that the pan-family screening approach is a sensitive and specific method for the detection of viral families that contain a large number of diverse pathogens. Viruses will continue to emerge from animal and avian hosts in the future and at present there are very few assays that can detect complex viral families. Coronaviruses are a good example of a family of viruses that have adapted well to human-to-human transmission. In just 20 years three significant pathogens, SARS-CoV-1, MERS-CoV and SARS-CoV-2, have emerged from zoonotic hosts and resulted in two epidemics and one global pandemic which has infected more than 620,000,000 people. It is likely that in the near future a new coronavirus variant will emerge and spill over into the human population resulting in significant morbidity and mortality.

Individual flavivirus and alphaviruses have to date shown different global distribution patterns. Yellow fever is predominately found in African and South America with JEV mainly confined to Asia. It has been suggested that new flaviviruses will continue to emerge or re-emerge into the human population which may cause more serious infections than previously realised as was the case with the recent Zika virus epidemics. Climate change [88] will challenge the current distribution of these

viruses globally as was demonstrated recently with JEV which for the first time was found in Victoria and New South Wales, Australia [90]. The pan-family assays have been tested using insect vectors to screen for flavi- and alphaviruses and preliminary results look promising (John Waitumbi, personal communication) opening the potential of these assays to be used to screen arbovirus vectors for the presence of novel or re-emerging pathogens. One advantage of the current pan-flavivirus/pan-alphavirus/pan-dengue screening test is that the assays can be used in any region worldwide to quickly detect the presence of an unknown arboviral infection and with the boundaries to infection expanding their use is even more urgent.

It would be possible to design unique primer and probe sets that covered the major families of viruses that are pathogenic to the human population. These assays could be multiplexed to produce screening panels that could be used in front line hospitals or sentinel laboratories to screen animal, bats, birds, or vectors such as mosquitoes at regular intervals for emerging viruses. If a sample is positive using the pan-family assay but negative using species specific primers the sample could then be quickly screened by NGS to determine if a novel virus is present. In this way we would be forewarned to the presence of an emerging viral threat.

This simplifies and reduces the costs of broad screening approaches in disease outbreaks or during pathogen surveillance in humans, animal or vectors and importantly has the possibility to identify emerging pathogens without prior sequence knowledge.

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Conflict of interest

DM and JR are paid employees of Genetic Signature the inventors of 3base™ technology.

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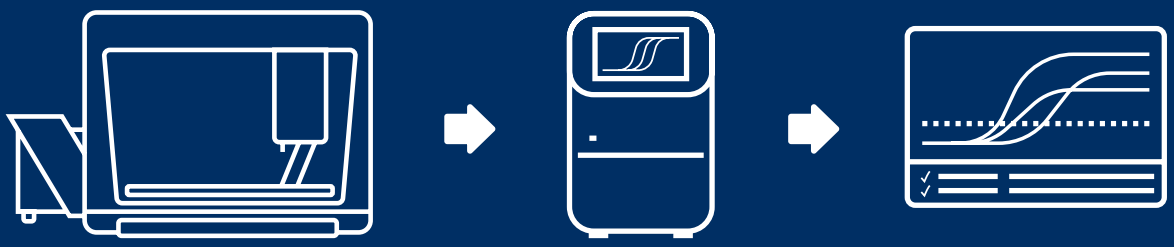

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