INVESTIGATING MOLECULAR APPROACHES FOR DETECTION OF PATHOGENS IN HORSES

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Abstract

Infectious disease of equines has significant impacts on the New Zealand equine industry, causing avoidable morbidity and mortality. The correct identification of the aetiological agents involved in such infections allows for prompt and accurate administration of an appropriate therapeutic strategy, reducing the economic impact associated with equine wastage.

Traditional culturing methods are widely used for the diagnosis of infections in horses, however, conventional PCR offers a novel and rapid approach, through the targeting of unique genes for diagnosis of pathogens. PCR is an attractive alternative to microbial culturing, which can take anywhere between 24 hours to 1 week to determine the causative infectious agent, and in a number of cases the results are not as specific or as accurate as they need to be. It is the lack of sensitivity of the traditional culturing techniques that continues to contribute to the economic loss and welfare issues associated with equine infections.

Initially, it was found that an SDS lysis extraction method worked best for extraction of DNA which was used to extract microbial DNA from various types of clinical samples. These were subsequently used as DNA templates within PCR alongside primers associated with up to 28 genes associated with common equine infections. From a total 146 clinical samples received for testing 66.4% returned a PCR positive for one or more microbial genera, whereas traditional bacteriological culturing produced an identification rate of 46%. In addition, PCR was significantly faster than culturing methods (6 hours vs 48 hours) and allowed more specific identification of the microbes present, which provides a significant advantage for infection management and increasing animal welfare.

The results found from these experiments show rapid PCR identification holds promise as a diagnostic technique in the future, however, further development is required before this can be used as a primary diagnostic technique. Recommendations include further research into trends of

infection in NZ equines and respectively increasing the diversity of PCR primers to represent the larger scope of microbial species. PCR diagnosis would also benefit from the standardisation of the DNA extraction methods used to allow consistency and increase throughput of various samples. Lastly, the simplification of the individual PCRs for each infectious agent could be developed into multiplex reactions to decrease time processing time and reduce human pipetting error.

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Abbreviations

3’	Three prime DNA end
5’	Five prime DNA end
°C	Degrees Celsius
µl	Microlitre
µM	Micomolar
aspA	Aspartate ammonia-lyase
BLAST	Basic Local Alignment Search Tool
CHO	Cholesterol Oxidase
CTAB	Caty trimethylammonium bromide
DEICA	Diethyldithiocarbamic acid
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
et al.	And others
EtBr	Ethidium bromide
gpB	Glycoprotein B (gB) of Equine herpesvirus
GITC	Guanidinium thiocyanate
GuHCL	Guanidinium chloride
INCI	Sodium lauroyl sarcosinate
kb	Kilobase
KOH	Potassium hydroxide
LiCl	Lithium chloride
M	Molar
mg	Milligram(s)
ml	Millilitre(s)
mM	Millimolar
MQ water	Milli-Q water
N/A	Not available
Na	Sodium
$Na_3C_6H_5O_7$	Sodium citrate
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information

NGS	Next generation sequencing
netF	*Clostridia perfringens* netF toxin
ng	Nanogram(s)
NZ	New Zealand
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PVP-40	Polyvinylpyrrolidone 40
rcf	Relative centrifugal force
SDS	Sodium dodecyl sulfate
Strangles	*Streptococcus equi* subspecies *equi*
TcdA	*Clostrida difficile* toxin A
TcdB	*Clostrida difficile* toxin B
TE buffer	Tris-EDTA buffer
TD	Touchdown
TK	Thymadine kinase
TM	Primer melting/annealing temperature
V	Volt(s)
VAP	Virulence Associated Protein

Nucleotides:

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

Chapter One

INTRODUCTION

There are a number of bacterial and viral diseases New Zealand horses may encounter. Understanding the causes and susceptibilities associated with microbial disease is important for welfare, successful farming, and to allow the most effective treatment. Detecting the causative agent early in infection is key to this, and there are a number of methodologies available. However, more needs to be done to improve these to allow better detection of pathogens within clinical samples.

1.1 Effect of disease on the New Zealand equine industry

The equine sport horse industry contributes over $1 billion to the New Zealand economy annually, with the greatest component being the thoroughbred sector (IER Pty Ltd., 2010; Matheson & Akoorie, 2012). This economic contribution makes the loss of individuals from treatable disease and infection an important issue, putting strain on the New Zealand industry (Fennessy, 2010; IER Pty Ltd., 2010; Matheson & Akoorie, 2012; Tanner *et al.*, 2012). Infections affecting horses can be localised (gastrointestinal or respiratory) or become systemic (sepsis), and pose a significant welfare, and economic burden through foal crop decline, time wasted in training and failure to race, and reduced welfare for infected individuals (Dunowska *et al.*, 2002a; Furr, 2003; McKenzie III & Furr, 2001; Sanchez, 2005).

In New Zealand, localised and systemic equine infections contribute significantly to wastage in the equine industry. However, compared to gastrointestinal and systemic infections, respiratory infections have lower rates of mortality (Dunowska *et al.*, 2002a). Nationally and internationally, mortality caused by neonatal sepsis causes a great economic loss, through lost export. Up to 50% of foals in New Zealand fail to race, contributing

significantly to wastage within the industry. (Matheson & Akoorie, 2012; Tanner *et al.*, 2012).

1.2 Importance of a New Zealand diagnostic scheme

Illness bought on by infection impacts the performance of sport horses and reduces the welfare of all infected horses (Christley *et al.*, 2001; Perkins, 2005). Due to New Zealand's geographical isolation and strong biosecurity regulations, equine diseases and infectious sources are expected to vary from those seen on a global scale (Toombs-Ruane *et al.*, 2016). However very little is known about causes and strains of infection, as there is a distinct absence of epidemiological studies on New Zealand equine infections. Therefore, a reliable diagnostic scheme is needed to begin collecting information on the epidemiology of infectious disease, along with communication from local veterinarians.

The requirement for specific diagnostic techniques is also being driven by the need for New Zealand to be antibiotic free by 2030, where ideally antimicrobial treatment will only be applied to infections where the pathogenic cause has been identified. In order to achieve responsible antimicrobial use by veterinarians, increased detection and diagnostics are required to determine the causative organism of infection within a short time frame. This will allow the most appropriate therapeutic strategy to be determined before antimicrobial treatment is administered (New Zealand Veterinary Association, 2016). In addition, the increase in antimicrobial resistance is also a driving factor for more accurate diagnostics within a veterinary setting in New Zealand. Antimicrobial resistance is a known factor that has increased mortality associated with sepsis and other bacterial infections within the equine industry (Dunkel & Johns, 2015).

1.3 Welfare implications

Effective diagnosis of infections will help address welfare issues associated with sepsis and inadequate treatment. Early recognition of infection in

horses followed by the correct diagnosis allows for the application of the most effective treatments, as show in studies with septic neonatal foals (Fielding & Magdesian, 2015). Identification of the pathogenic species is essential for increasing survival rates in critically ill neonates, and it has been suggested that appropriate treatment targeting the infectious source needs to be administered within three hours of recognising sepsis (Fielding & Magdesian, 2015). This critical timeframe, and specificity of infectious organisms for treatment, makes the development of a rapid diagnosis system critical to reducing mortality caused by infections, such as sepsis, and improving the overall clinical prognoses. Rapid diagnosis would not only improve the diagnosis and treatment of horse diseases, but would also provide economic benefits, and improved welfare of the animals.

1.4 Groups of horses susceptible to infections

Infectious diseases in horses are diverse and can manifest in a variety of ways. The manifestation can be detrimental to the welfare of a horse, often ending a racing career, or in some cases become fatal, causing the death of an animal if left untreated. Factors such as age, immune status, and the environment an animal is kept in can leave an individual susceptible to certain pathogens and can be useful to know during diagnosis.

1.4.1 Neonates

There are multiple contributing factors that leave equine neonates susceptible to life threatening infections. Factors such as reduced immunity and multiple routes of invasion make this group of animals highly vulnerable to a variety of infections. Neonatal sepsis is a highly fatal disease found in newborn foals and is the leading cause of morbidity and mortality in foals under seven days old (Fielding & Magdesian, 2015; Furr, 2003). Despite increasing diagnostic and treatment technologies, sepsis remains a major cause of mortality in New Zealand horses, causing economic loss and reduced welfare for infected individuals (Furr, 2003).

Equine neonates are born with minimal immunity, therefore the acquisition of immunoglobulins to build immunity is dependent on the ingestion of colostrum from the mare during the first 24 hours of life. Failure of passive transfer results when a neonate fails to ingest the colostrum and antibodies contained within it, causing an increased risk of infection and therefore death (Clabough *et al.*, 1991). An experimental study compared the acquisition of bacterial infection between colostrum fed and colostrum deprived foals (Robinson *et al.*, 1993). With colostrum deficiency treatment, 62% of foals developed sepsis compared to 0% who were fed colostrum, demonstrating the severe impact of colostrum deficiency and its role in equine disease.

Another risk factor of infection in neonates relates to the health of the mare, as before parturition, blood is shared between the mare and foal. Bacteria associated with placental disease can enter the amniotic fluid, gaining access to either the respiratory of gastrointestinal tracts of the foetus. Additionally, the exposed umbilical stump can become contaminated with bacteria, or bacteria can be ingested or inhaled leaving newborns at greater risk of infection from the environment (McKenzie III & Furr, 2001).

1.4.2 Weanlings

The weaning stage in foal development occurs between 6-12 months and can cause significant stress to the animal. Changes around this time in development include the loss of immunoglobulins, microbiota, and immunity from the mother's milk, changes in the foals environment or housing, and increased freedom which may leave young foals at increased risk of infection or disease (Heleski *et al.*, 2002; Palmer, 1985). Due to the inquisitive nature of young animals, it is observed that there can be an increase of microbial infection in foals as a result of ingesting irritating or infectious substances (Palmer, 1985).

Weaning is recognised as a stressful event to young horses and as a result has been recorded as associated with susceptibility to certain infectious

microbes, such as *Lawsonia intracellularis*, a known gastrointestinal pathogen in young horses (Heleski *et al.*, 2002; Page, 2013). Additionally, the stress involved with weaning may induce latent Equine herpesvirus 1 (EHV-1), acquired before partition, or from the environment and other animals shedding the virus. This poses a significant risk to young horses, as the clinical impact of these infections leave them susceptible to increased risk of respiratory infection, particularly when there is no vaccination programme implemented (Ostlund, 1993; Pusterla *et al.*, 2009b).

1.4.3 Close quartered animals

There is high incidence of indirect transmission between individuals, particularly with highly virulent or contagious diseases, such as *Streptococcus equi* subspecies *equi* (Strangles). Indirect transition methods occur through fomites, which are objects which carry infection, and include; housing, water sources, feed, handlers, and equipment which facilitate the transmission of microbes causing disease (Sweeney *et al.*, 2005).

Hygienic housing is extremely important for health regulation in horses, specifically to prevent cross infections between different individuals (Mills & Clarke, 2007). Some housing, such as boxstalls, leave horses sharing a common airspace, which can promote the spread of disease as infected animals may shed infectious particles that can affect other horses (Mills & Clarke, 2007). *Rhodococcus equi*, an infectious agent that causes respiratory disease in horses, is known to be spread through the air, and results in direct transmission in stabled horses via shared air supply (Mills & Clarke, 2007).

Although stabling is not common practice in New Zealand thoroughbred horses, the housing of horses can also increase risk of the transmission of respiratory diseases, particularly EHV-1, Strangles, and *R. equi* which are spread through horse-to-horse contact, shared equipment, and are exacerbated by limited air and housing (Ministry for Primary Industries, 2014; Muscatello *et al.*, 2009). Stress caused by confinement has also been recognised to exacerbate and accelerate the spread of diseases, such as EHV-1 (Ministry for Primary Industries, 2014).

1.4.4 Environmental and on farm management

Bacterial species such as *R. equi* thrive and multiply within horse faeces, particularly in summer months (Giguère & Prescott, 1997). Therefore, the removal of faeces from paddocks and shared environments is recommended to reduce shedding of disease and infection of animals (Mills & Clarke, 2007; Prescott, 1991). Other infectious organisms, such as *Clostridia difficile* which is known to survive well in the environment, are often resistant to common disinfectants (Båverud, 2002). Because of this, care must be taken in hospitals and areas with infected animals to ensure the risk of transmission from fomites is reduced.

1.5 Systemic infections

Septicaemia (sepsis) is a prominent disease in the equine community and is one of the largest causes of foal crop loss and wastage in the equine breeding industry. Sepsis encompasses a broad range of disorders originating from a variety of infectious agents, including bacterial, fungal, viral or mixed infections, resulting in different clinical presentations and prognosis (McKenzie III & Furr, 2001; Wong & Wilkins, 2015). Early recognition of sepsis and its microbial cause, along with prompt, accurate, and aggressive antimicrobial administration is critical for successful clinical outcomes (Fielding & Magdesian, 2015; Taylor, 2015).

1.5.1 Detection

Sepsis is an exaggerated, systemic inflammatory response to infection and is identified by physiological symptoms within an animal. Septic horses will commonly present with an abnormal heart rate, blood pressure, and blood counts (McKenzie III & Furr, 2001). Current recognition and diagnostic techniques through the use of septic scores and bacterial cultures demonstrate poor sensitivity, low throughput and slow processing times. These diagnostic inadequacies are thought to contribute to an estimated 50% of mortality in septic neonates (Fielding & Magdesian, 2015). Overall, the

prognosis for a sepsis diagnosis has been highly variable leading to short term survival rates in septic neonatal foals, ranging from 10-70%. However, this outcome has improved significantly, likely due to early detection and treatment of vulnerable foals and it is now more appropriate to quote a prognosis for short-term survival of approximately 50–60% (Taylor, 2015). Survival rates in mature horses with sepsis are also shown to vary considerably, however it is difficult to prognosticate as no studies have been done to evaluate a large population of adult horses with sepsis.

1.5.2 Who is affected?

Sepsis is predominantly seen in neonates due to their immunocompromised status, however, it can affect any age animal if a localised infection progresses into the bloodstream (Taylor, 2015). As foals are born with limited immunity, the risk factors for the development of neonatal sepsis are significantly raised by a number of issues including the foaling environment, the health of the mare and the success of normal behaviours following parturition. The development of sepsis in neonates has been shown to correlate to the failure of passive transfer, which greatly increase the risk of sepsis development (McKenzie III & Furr, 2001; Robinson *et al.*, 1993; Taylor, 2015). Premature birth and significantly reduced colostrum intake increase the risk of infection in foals. Reduced colostrum, which is the primary cause of passive transfer failure, accounts for up to 40% of septic foals, as colostrum provides pathogen specific immunity and immunoglobulins needed for immunocompromised neonates to fight potential pathogens (Furr, 2003; Robinson *et al.*, 1993).

1.5.3 Causes of sepsis

Sepsis is caused by the introduction of pathogens into the bloodstream. Microbial infection can occur prenatally (via the mare) or postnatally, through infection of the umbilical stump, placenta, secondary wounds, ingestion, or inhalation with respiratory routes being the primary routes of infection (Furr, 2003). Bacterial infections are the predominant cause of sepsis in equine neonates (Taylor, 2015). Worldwide studies have identified both Gram negative and Gram positive bacterial isolates that can cause

sepsis. In the past, Gram positive organisms were seen as the most ubiquitous sepsis-causing organisms, however, the frequency of infections caused by Gram negative bacteria has been increasing (McKenzie III & Furr, 2001). Horses with Gram negative infections are considered to be at greater risk of mortality than those infected with Gram positive bacteria due to differences in the morphological, physiological, and virulence factors between species. Structural differences mean different antibiotic strategies are required for effective treatment (Taylor, 2015). An additional complicating factor is the presence of polymicrobial infections, which account for up to 50% of sepsis-causing infections, which can easily be missed in cultures (Furr, 2003). Lastly, geographical and temporal changes to common infectious pathogens also complicates sepsis diagnosis.

Limited information is available surrounding the causes of sepsis infections in New Zealand horses. However, in a study of foal sepsis, it was concluded in a test of 127 samples that Gram positive bacteria accounted for roughly 65% of cultured bacteria and Gram negative accounted for 35% (Toombs-Ruane *et al.*, 2016). The most common bacterial species associated with sepsis included *Streptococcus* spp., *Staphylococcus* spp., *Enterococcus* spp. and *Escherichia coli*, which were found in 79% of 127 samples. Globally, *Streptococcus* spp. have been recognised as the most common Gram positive bacterial species, and *E. coli* as the most common Gram negative species causing sepsis (Toombs-Ruane *et al.*, 2016). Other Gram positive bacteria recognised include; *Staphylococcus* spp., *Enterococcus* spp., *Bacillus* spp., *R. equi*, and *Clostridium* spp. Frequently identified Gram negative isolates include *Enterobacter* spp., *Acinetobacter* spp., *Klebsiella* spp., *Proteus* spp., *Citrobacter* spp., *Pseudomonas* spp., *Pasteurella* spp., *Salmonella* spp. and *Serratia* spp. (McKenzie III & Furr, 2001; Russell *et al.*, 2008; Toombs-Ruane *et al.*, 2016). Although bacteria have been recognised as the dominant source of sepsis-causing infections there is also evidence of EHV-1 and Equine herpesvirus-4 (EHV-4) causing septic infections. EHV-1 has been described as the most frequently occurring viral infectious species in neonatal sepsis (McKenzie III and Furr (2001) with EHV-4 predominantly causing respiratory infections (Ostlund, 1993). Fungal species can also cause infection and disease within horses, with

Candida recognised as noted pathogenic species, particularly infecting neonates (McKenzie III & Furr, 2001).

1.5.4 Diagnosis

Bacteriological blood culture and phenotypic analysis of culturing results currently provides the most definitive diagnosis of the infectious organism. Culturing methods have a substantial processing time, with each taking approximately 48-72 hours before a result is obtained (Pusterla *et al.*, 2009a; Taylor, 2015). These cultures exhibit low efficiency and poor sensitivity, typically giving a culture result 60% of the time, and in case studies efficiency has been shown to be as low as 25% (Russell *et al.*, 2008; Taylor, 2015). This poor sensitivity is exacerbated by a bacterial cultures inability to detect viral or fungal infections, and only growing bacteria supported by the culture medium. Additionally, false negatives with bacterial cultures are reported approximately 12-37% of the time (Furr, 2003; Rieger *et al.*, 2014). To overcome this, the use of the sepsis score also accompanies bacteriological culture diagnosis, and allows for the recommendation of treatment. However, the sepsis score does not provide information on the source of infection or the agent causing it. Rapid diagnosis is critical, and it has been suggested that a delay of more than 3 hours before administration of antimicrobials can increase mortality (Fielding & Magdesian, 2015). However, in order to achieve this more effectively, there needs to be a physical exam (early recognition of sepsis using the sepsis score) along with a more rapid lab diagnosis to confirm the infectious agent (Fielding & Magdesian, 2015).

1.5.5 Route of treatment

Bacterial sepsis is potentially able to be treated successfully through appropriate antimicrobial therapy, which requires the correct identification of the infection causing microbe. This allows the application of the appropriate antimicrobial against the infectious organisms and the correct administration method (Furr, 2003). Generalised treatment for septic foals currently includes prompt implementation of a combination of broad-spectrum penicillin based antimicrobials, hemodynamic support, and fluids

(Fielding & Magdesian, 2015; Furr, 2003). However, knowing the strain of the causative organism is important for determining treatment as infections with either Gram positive, Gram negative, or viral organisms make a significant difference to treatment and prognosis (Silhavy *et al.*, 2010). Additionally, information about the site of infection should also be considered in order to determine the best route of drug administration, pharmacokinetics and cost of treatment (Furr, 2003; Palmer, 2014).

Antimicrobial susceptibility testing is also becoming an important aspect of sepsis, diagnosis and clinical treatment (Fielding & Magdesian, 2015; New Zealand Veterinary Association, 2016). ß-lactams, aminoglycosides, cephalosporins, or a combination of penicillin and gentamicin are currently the first line of treatment in a majority of New Zealand sepsis cases as 81% of isolates are recorded as responding to either antimicrobial (Toombs-Ruane *et al.*, 2016). However, with the rise of antimicrobial resistance identified in bacterial isolates in New Zealand foals, care now needs to be taken when administering drugs to unknown infections (Toombs-Ruane *et al.*, 2016).

1.6 Respiratory infections

Respiratory infections are prevalent among thoroughbred horses and is a known contributor to airway infections and inflammation frequently observed in racehorses (Hughes, 2013). Respiratory disease is the largest cause of wastage in equine industries worldwide and is noted to interfere with training (McBrearty *et al.*, 2013). Equine respiratory infections are considered multifactorial, normally involving a combination of microbial infection, environmental interactions, and host interactions (McBrearty *et al.*, 2013). Unfortunately, respiratory diseases often go undiagnosed and failure of correct diagnosis leads to inappropriate treatment and the absence of subsequent preventative measures being undertaken to mitigate the spread of infection (Christley *et al.*, 2001).

1.6.1 Detection

Respiratory infections are suspected upon detection of characteristic symptoms, such as airway inflammation, coughing, and nasal discharge (Christley *et al.*, 2001). Unlike sepsis, clinical symptoms can be indicative of the pathogen causing infection. Coughing is consistently linked to bacterial infections, therefore is an avenue that should be investigated (Christley *et al.*, 2001). Viral infections often show serous or mucopurulent (mucous and pus) nasal discharge, cough, enlarged retropharyngeal or submandibular lymph nodes, and poor performance in training and racing (Dunowska *et al.*, 2002a).

1.6.2 Who is affected?

Lower airway inflammation in thoroughbred horses is seen to occur in 20%-50% of racehorses in training (Christley *et al.*, 2001). In New Zealand 56% of horses presenting with respiratory disease were under 3 years and 11% were under 1 year (McBrearty *et al.*, 2013).

1.6.3 Causes of respiratory infection

Fomites and shared airspace spread respiratory diseases, particularly *R. equi* pneumonia which can be transmitted by breathing, and therefore close proximity (Mills & Clarke, 2007; Muscatello *et al.*, 2009). Poor environmental management can also cause increased spread of infection as *R. equi* grows rapidly in faeces left within the environment, and oral-faecal ingestion is a primary route of *R. equi* infection (Mills & Clarke, 2007; Takai, 1997).

Respiratory diseases are very common among horses globally, and there are a number of pathogens known to cause respiratory diseases in horses (Dunowska *et al.*, 2002a). In the past, the cause of respiratory disease had been associated exclusively with viral infections, however, further epidemiological investigations have shown the importance of bacteria as a primary pathogen in horse respiratory disease (Christley *et al.*, 2001). Pathogenic microbes associated with respiratory disease include bacteria,

namely S*treptococcus* species and viruses, such as equine influenza virus and herpesvirus (Christley *et al.*, 2001; Dunowska *et al.*, 2002a). Similar pathogens have also been recognised within New Zealand, with *R. equi, S. equi* subspecies *equi, S. equi* subspecies z*ooepidemicus*, EHV-1 and EHV-4 recognised as the primary cause of respiratory infection and disease (Toombs-Ruane *et al.*, 2015).

i) Equine influenza

Equine influenza is the prominent respiratory disease in horses worldwide and causes significant damage to the equine industries (Cullinane & Newton, 2013). Caused by an orthomyxovirus of influenza A, equine influenza presents itself as the dominant microbe causing respiratory disease in horses of many countries (Daly *et al.*, 2011; Timoney, 1996). At present, there is no knowledge of equine influenza existing within New Zealand, likely due to strict biosecurity and quarantine laws, as well as geographical barriers (Cullinane & Newton, 2013; Rosanowski *et al.*, 2012). Equine influenza causes clinical symptoms very similar to other respiratory diseases, including pyrexia, nasal discharge, and dry coughs, therefore, vaccinations and quarantine are widely used to mitigate risk of transmission and identification schemes should be rapid in the case of transmission into New Zealand (Cullinane & Newton, 2013; Rosanowski *et al.*, 2012).

ii) Equine herpesvirus (EHV)

Equine herpesvirus are a well characterised group of viruses known to cause respiratory disease in horses and cause a significant loss within the horse industry on a worldwide scale (Harless & Pusterla, 2006; Varrasso *et al.*, 2001). The clinically significant EHV-1 and EHV-4 are dominant viral infections, affecting horses and are endemic in New Zealand (Ministry for Primary Industries, 2014). Currently, Equine herpesviruses are the only viruses shown to cause respiratory infection in New Zealand horses (McBrearty *et al.*, 2013).

a) EHV-1

EHV-1 is a common pathogen in horses distributed both worldwide and within New Zealand (Dunowska, 2014; Lunn *et al.*, 2009). There are

multiple forms of EHV-1, all of which can cause a myriad of symptoms ranging from mild to severe respiratory disease, abortion, reduced reproductive performance, and neurological disease (Dunowska, 2014; Lunn *et al.*, 2009; Ostlund, 1993). Initially EHV-1 infections occur within the respiratory tract, targeting the respiratory epithelium before becoming established in varying tissue types (Lunn *et al.*, 2009). EHV-1 causes sporadic mild respiratory disease in young horses under 2 years and in adult horses can cause abortion and outbreaks of equine myeloencephalopathy (Lunn *et al.*, 2009).

Figure 1.1: Equine abortion caused by suspected Equine herpesvirus infection (Photo source: Dr Lee Morris, Equibreed NZ).

Historically, methods for identification of EHV-1 and EHV-4 involved the use of virus isolation in cell culture with serological typing (Varrasso *et al.*, 2001). Due to the clinical importance of both Equine herpesviruses and their potential to severely damage the equine industry, there is a drive for rapid diagnostic methods. This has been successfully achieved using PCR which amplifies regions of the conserved thymidine kinase (TK) and glycoprotein B (gpB) genes of the herpesvirus genomes (Carvalho *et al.*, 2000; Varrasso *et al.*, 2001).

b) EHV-4

EHV-4 causes respiratory disease occasionally involved in equine abortion, and is indistinguishable from EHV-1 in a clinical setting (Carvalho *et al.*, 2000; Ostlund, 1993). Epidemiological studies have shown that EHV-4

causes significant respiratory disease outbreaks in young racehorses around the time of weaning (Ostlund, 1993). EHV-4 has been found to be present in 40% of all diseased horses presenting respiratory infection and in 0% of healthy horses (McBrearty *et al.*, 2013). This is more abundant than EHV-1 which is found in 6% of all diseased horses presenting respiratory infection (also 0% in healthy populations), supporting that EHV-4 is a more abundant and causative agent of respiratory infection compared to other herpesviruses.

iii) Streptococcus spp.

Lower respiratory infections in horses are commonly caused by aerobic *Streptococcus* species, including *S. equi* subspecies *equi*, *S. equi* subspecies *zooepidemicus*, *S. dysgalactiae* subspecies *equisimilis* and *S. pneumoniae* (Timoney, 2004; Wood *et al.*, 1993).

a) S. pneumoniae

S. pneumoniae is a known, yet somewhat uncommon pathogen in horses (Blunden *et al.*, 1994; Burrell *et al.*, 1986; Timoney, 2004). It is recognised as a cause of respiratory inflammation and infection when found in high numbers within the lower respiratory tract, however, it can be found in high numbers in horses not presenting any respiratory infection (Burrell *et al.*, 1986). When seen to be pathogenic, *S. pneumoniae* is predominantly observed in young training racehorses (Timoney, 2004). It is likely to be transmitted due to the shedding of bacteria from infected individuals or the transmission through handlers and therefore care should be taken during outbreaks of infection (Burrell *et al.*, 1986).

b) S. equi subspecies *equi*

S. equi subspecies *equi* is responsible for Strangles which is a highly contagious and a severe cause of respiratory disease in horses (Sweeney *et al.*, 2005; Timoney, 2004). Strangles affects young horses and is transmitted via inhalation or ingestion of the bacterium (Taylor & Wilson, 2006). Strangles infections are characterised by abscessation of head and submandibular lymph nodes and has a highly variable incubation period of 4-14 days. This means preventative measures such as isolation of sick animals should be undertaken to ensure infection is not spread, as Strangles

transmits very easily with stress and overcrowding (Laus *et al.*, 2007; Timoney, 2004). Strangles has a recorded morbidity rate of 100% and a mortality of 10%, which highlights the requirement for rapid and accurate detection (Laus *et al.*, 2007). Unfortunately, it can be difficult to identify as it shows low culture sensitivity when clinical samples are analysed using traditional microbial culturing. Recently, better identification has been achieved through the use of molecular methods, such as polymerase chain reaction (Patty & Cursons, 2014).

c) S. equi subspecies *zooepidemicus*

S. equi subspecies *zooepidemicus* has been strongly associated as a primary pathogen in lower airway disease in thoroughbred horses. It causes necrotizing legions and pneumonia in mature horses and is associated with the clinical symptom of coughing (Christley *et al.*, 2001; Timoney, 2004). Infection occurs through inhalation, and *S. equi* subspecies *zooepidemicus* is seen as a highly opportunistic pathogen, thought to often form concurrent infections with viruses, particularly equine influenza (Timoney, 2004).

d) S. dysgalactiae subspecies *equisimilis*

S. dysgalactiae subspecies *equisimilis* is an opportunistic *Streptococcus* species found on the skin and mucous membranes of various animals and known to cause infection in horses (Laus *et al.*, 2007; Timoney, 2004). It has been suspected to present symptoms similar to Strangles (Laus *et al.*, 2007), leading to cases being misidentified, however, is generally considered a facultative parasite (Laus *et al.*, 2007).

iv) Rhodococcus equi

*R. equi (*previously known as *Corynebacterium equi)* is a known respiratory pathogen derived from soil and faeces that causes pneumonia and severe infection in horses, particularly in foals (Takai *et al.*, 1991). Abundant within soil on horse and stud farms, affected farms are heavily contaminated with virulent forms of *R. equi* (containing VAP A plasmid). Animals are at increased risk of infection as *R. equi* is spread easily through the air in stalls, significantly exacerbated on dry and windy days. This weather dependent

spread determines why *R. equi* is also recognised to have a temporal prevalence as a respiratory pathogen (Takai, 1997).

Clinical isolates of *R. equi* are more pathogenic than those acquired from the environment due to virulence factors, such as cholesterol oxidase (CHO) and phospholipase C exoenzymes (Prescott, 1991). In addition, virulent strains contain a 85-90kb plasmid carrying genes for virulence associated proteins (VAP), such as VAP A, which specifically cause pneumonia in foals, and can be used to differentiate virulent *R. equi* from harmless environmental strains (Muscatello *et al.*, 2009). *R. equi* infections most commonly manifest as pneumonia with lung abcessation and lymphadenitis, alongside increased respiratory rate and fever (Giguère & Prescott, 1997; Prescott, 1991). Severely affected individuals, predominantly foals less than 6 months of age, can die as a result of severe and rapid onset infection despite treatment, due to early pneumatic pyogranulomas that occur prior to respiratory symptoms. However, mortality from *R. equi* is rare in adult horses (Giguère & Prescott, 1997; Prescott, 1991). Exhalation of virulent *R. equi* occurs 65% of the time and is noted to be a major cause of spread and a main cause of *R. equi* pneumonia on farms (Muscatello *et al.*, 2009).

As an intracellular pathogen, *R. equi* can be difficult to identify as retrieval can be difficult from the pyrogranulomas that it forms (Muscatello *et al.*, 2009). This same intracellular habitat also makes treatment more difficult as an *in vitro* approach is needed, however this is achieved using a combination drug approach of erythromycin and rifampin (Båverud, 2002).

v) Fungal infection

Fungal infections are rare in New Zealand equine disease literature, however, have the potential to cause damaging and life-threatening respiratory infections (Stewart & Cuming, 2015). Fungal species recognised as pathogens in horses include; *Cryptococcosis* spp., *Pseudoallescheriosis* spp., *Aspergillosis* spp., *Conidiobolomycosis* spp., *Blastomycosis* spp., *Histoplasmosis* spp., *Coccidiomycosis* spp., *Scopulariopsis* spp., *Adiospiromycosis* spp., *Canndidasis* spp., and *Epichloe coenophila*. Due to

their abundance, they need to be considered during diagnosis (Munday *et al.*, 2017; Pacynska, 2013; Secombe *et al.*, 2017; Stewart & Cuming, 2015).

Fungi are ubiquitous in farm environments, and may be found in soil, feed, damp hay, and bedding. They can cause infection as primary pathogens, or cause opportunistic infections in immunocompromised horses, such as those undergoing treatment using antimicrobials or steroids (Pacynska, 2013; Stewart & Cuming, 2015). Fungal infections are predominantly acquired through the inhalation of a high level of fungal spores, the inhalation of a highly virulent strain, or through fungal penetration into an open wound or compromised gastrointestinal tract (Secombe *et al.*, 2017; Stewart & Cuming, 2015). Some fungal infections can be directly traced to environmental causes such as the relationship between fescue grass infected with *E. coenophiala* causing equine fescue oedema (Munday *et al.*, 2017).

In respiratory infection, fungal pathogens manifest as upper or lower respiratory tract infections, often causing pneumonia, rhinitis, meningitis, granulomas, and in some cases abortion (Secombe *et al.*, 2017; Stewart & Cuming, 2015). Fungal infections often present only as a cough, or poor athletic performance, contributing to delayed diagnosis (Stewart & Cuming, 2015)

Diagnosis involves the use of patient history, clinical presentation, diagnostic imaging, histopathology, culture, and cytology of infection (Stewart & Cuming, 2015). Traditional diagnostic techniques are limited as cytology cannot differentiate the dead and live fungal cells, making it difficult to determine whether treatment has been effective or not (Secombe *et al.*, 2017). Treatment involves medical intervention with the application of pathogen specific drugs, and surgery. Early detection of fungal infections is rare, and this contributes significantly toward high mortality rates associated with fungal infections (Stewart & Cuming, 2015).

1.6.4 Diagnosis

The most significant factor to consider during diagnosis of respiratory disease is the bacteria found in the lower airways are frequently part of the normal and environmental flora found in the upper airways (Christley *et al.*, 2001). This shared bacterial load creates a need to carefully interpret any diagnostic result, particularly those obtained from cultures (Hughes, 2013).

1.6.5 Route of treatment

Upon confirmation of infection, appropriate measures must be taken to treat the affected animal and reduce the risk of transmission between other animals (Sweeney *et al.*, 2005). Treatment is multifaceted, firstly by segregation and quarantine of infected animals, then followed by appropriate treatment targeting the area of infection specific to cause of infection (Sweeney *et al.*, 2005).

Intermuscular administration of ceftiofur sodium and ampicillin sodium has been shown to be successful in the treatment of horses with unidentified respiratory infections (Folz *et al.*, 1992). The treatment of Strangles involves topical and systemic application of benzylpenicillin, and in the case of guttural pouch involvement, treatment may also involve the application of a topical penicillin gel mixture directly into the infected pouch (Sweeney *et al.*, 2005). Long term antimicrobial treatment is needed for long term/systemic respiratory infections.

Non-pneumonia respiratory infections caused by *Streptococcus* species are often treated with penicillin as the primary antimicrobial. *S. equi* isolates are also highly susceptible to trimethoprim-sulfadiazine (TMS) in the occurrence of penicillin allergy, however, *S. zooepidemicus* does not respond to this, highlighting the importance of the correct identification of pathogenic species before treatment (Sweeney *et al.*, 2005). In Strangles treatment, resistance is noted with aminoglycosides, particularly gentamicin (Sweeney *et al.*, 2005).

For the treatment of *R. equi* there are a wide variety of antimicrobial treatments, with gentamicin, and ciprofloxacin showing sensitivities over 95% and low resistance rates (Giguère & Prescott, 1997). The most common drug approach for treatment of *R. equi* infections involves a combination of erythromycin and rifampin, which demonstrated dramatically reduced mortality in foals since its introduction likely due to the combination of erythromycin which penetrates caesous material associated with *R. equi* pyrogranulomas (Giguère & Prescott, 1997).

Figure 1.2: Examples of clinical treatment applied to respiratory infection causing pleuropneumonia. Application of systemic antibiotics, IV fluids, non-steroidal anti-inflammatories, and antibiotics administered via nebuliser (Photo source: Dr Alex Leander, Equibreed).

1.7 Gastrointestinal infections

Gastrointestinal disease or enterocolitis is a significant section of equine disease and causes significant morbidity and mortality in mature horses and foals (Weese *et al.,* 2001). In horses, particularly foals, diarrhoea can occur for a number of reasons ranging from foal heat diarrhoea, nutritional diarrhoea, ulceration, or microbial infection (Palmer, 1985). This diverse distribution of disease causes and symptoms provides a requirement for a

thorough understanding of gastrointestinal infections to provide accurate diagnosis and treatment. However, diagnosis is often confounded by the diversity of the gut microbiota, meaning that the aetiological agent of any gastrointestinal infection is only identified in less than 10% of infections (Weese *et al.*, 2001).

1.7.1 Detection

The symptoms of gastrointestinal infection remain similar regardless of the aetiological agent of infection, therefore leaving the infectious pathogen indistinguishable based on clinical symptoms (Båverud, 2002; Feary & Hassel, 2006; Pusterla & Gebhart, 2013). For example, salmonellosis presents foul watery diarrhoea, but this factor alone is not significant enough to distinguish it from other causes of disease, as infections by *C. difficile*, and other pathogens present similar symptoms (Feary & Hassel, 2006; Palmer, 1985). Common symptoms of gastrointestinal infection include fever, depression, mild abdominal pain, increased heart rate, a 'toxic' mucous membrane and diarrhoea (Palmer, 1985).

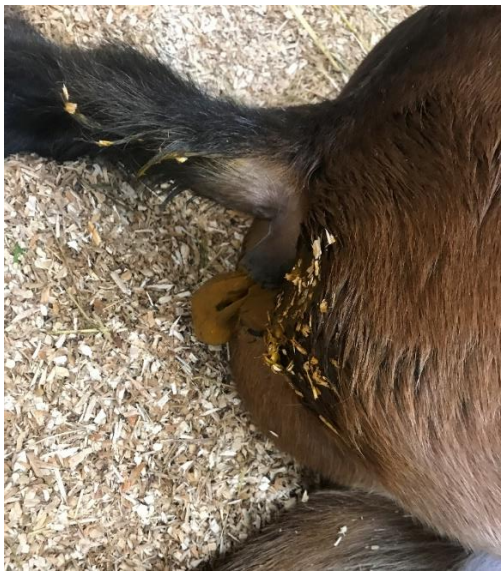


Figure 1.3: Foal with suspected gastrointestinal infection presenting with acute diarrhoea (Photo source: Dr Alex Leander, Equibreed).

1.7.2 Who is affected?

Intestinal microbiota is regarded as the first line of defence against gastrointestinal infection, as normal flora prevents colonisation of pathogenic bacteria (colonization resistance), but disruption to normal flora allows potentially pathogenic species to become established within the gut and produce toxins, causing disease (Båverud, 2002). Antibiotics in particular disrupt the normal bacteria in the gut, and affect the balance of protective flora, allowing overgrowth of opportunistic pathogenic species known to cause gastrointestinal infection (Diab *et al.*, 2013). Disruption can also occur due to changes of feed, which may occur seasonally, during weaning, or through the acquisition of microbes or spores from the environment, such as bacterial shedding from an infected horse (Palmer, 1985). Infections such as *C. difficile* and *R. equi* can be acquired through contaminated soil, faeces, animals, or fomites such as veterinary equipment used with sick animals (Diab *et al.*, 2013). Around 70-80% of foals under six months old are affected with some form of diarrhoea, which can be caused by a multitude of reasons, not just microbial infection (Palmer, 1985). In mature horses many infections manifest as a gastrointestinal infection, but these are often self-limitating (Palmer, 1985).

1.7.3 Causes of gastrointestinal infection

A significant cause of diarrhoea in foals is from bacterial infection by *Salmonella* spp., with equine salmonellosis being well documented (Palmer, 1985). Infection can also be associated with *Clostridium* spp. such as *Clostridia perfringens*, and *C. difficile*, however these occur normally in soil and the intestinal tract of animals, so are not always diagnostically relevant when found within a sample (Båverud, 2002; Palmer, 1985). Due to the variation of pathogens seen within gastrointestinal infection, common bacterial and viral isolates found associated with disease are discussed below.

i) Salmonella spp.

Salmonellosis infection from *Salmonella* spp. is a major and serious cause of bacterial gastrointestinal infection in horses (Palmer, 1985). Colitis

caused by *Salmonella* spp. causes enterocolitis, which is often associated with rapid onset diarrhoea (Feary & Hassel, 2006). Latent Salmonellosis can occur as a result of stressors including; transport, gastrointestinal tract disorders, changing feed, abdominal surgery, high temperatures and antimicrobial therapy (Feary & Hassel, 2006).

ii) Lawsonia intracellularis

The causative organism of equine proliferative enteropathy (EPE) is *L. intracellularis*, which is a major pathogen in equine gastrointestinal disease and causes symptoms similar to many other bacterial infections (Frazer, 2008; Pusterla & Gebhart, 2013). Infection occurs post weaning in foals aged 3 to 6 months and in young adults and is found as a dominant pathogen in horses worldwide (Feary & Hassel, 2006; Pusterla & Gebhart, 2013). *L. intracellularis* infections are becoming endemic on farms, increasing in frequency and there has been a rise in infections, particularly of foals, weanlings and horses under 12 months over recent years (Frazer, 2008; Pusterla & Gebhart, 2013).

L. intracellularis can infect multiple animal types including pigs, horses, and rabbits, and as a consequence exposure to the faeces of these animals is known to cause the spread of infection (Pusterla & Gebhart, 2013). Faecal-oral routes of infection are a dominant cause of the spread of infection and coincides with increased infection rates in weanlings as the naive inquisitive nature of foals leads them to obtain infectious material (Palmer, 1985; Pusterla & Gebhart, 2013). *L. intracellularis* prognosis is positive with accurate diagnosis and prompt treatment, but fatal if not (Feary & Hassel, 2006). Vaccines may offer the best prevention strategy to mitigate morbidity associated with *L. intracellularis* infection (Pusterla & Gebhart, 2013).

iii) Clostridia spp.

a) Clostridia difficile

C. difficile is noted as one of the dominant causes of diarrhoea in foals and mature horses and associated strongly with antibiotic-associated diarrhoea (Båverud, 2002; Diab *et al.*, 2013). The symptoms caused by *C. difficile* are

vague and indistinguishable from many other common gastrointestinal pathogens, including other *Clostridium* and *Salmonella* spp. (Diab *et al.*, 2013). *C. difficile* causes rapid onset diarrhoea, causing high mortality rates of up to 42%, particularly in foals (Diab *et al.*, 2013). *C. difficile* produces two toxins, enterotoxin A (*C. difficile* TcdA) and cytotoxin B (*C. difficile* TcdB), both of which are involved with virulence and can be used as targets for identification of infection (Båverud, 2002).

In normal cases, *C. difficile* is rarely found in faecal samples of adult horses (0-1%) and foals (0-3%) and therefore not considered part of the normal intestinal flora in adult horses (Feary & Hassel, 2006). However, *C. difficile* is found in abundance within the environment, particularly soils and waters (Båverud, 2002). *C. difficile* is transmitted through ingestion of faecal matter or through shared fomites or handlers. Spores survive for long periods in the environment and are often resistant to basic disinfectants, therefore, surviving months following infection (Båverud, 2002; Diab *et al.*, 2013). The spread of infection can be reduced by handlers using thorough aseptic technique with handlers and care taken with feeding. This is particularly important for animals undergoing antibiotic treatment, to minimise oral-faecal routes of spore ingestion (Båverud, 2002). Risk factors associated with the acquisition of *C. difficile* infection include hospitalisation, and treatment with erythromycin and rifampin, trimethoprim/sulfonamids, β-lactam antibiotics, clindamycin and gentamicin due to the development of antibiotic associated colitis (Båverud, 2002; Diab *et al.*, 2013). Foals under 2 weeks old are highly susceptible to *C. difficile* infection even without prior antibiotic application (Båverud, 2002) and it is suspected that colonisation early in life is what causes *C. difficile* to be a dominant cause of gastrointestinal infection in new born horses.

b) Clostridia perfringens

C. perfringens is associated with enterocolitis in both mature and young horses (Feary & Hassel, 2006) and along with *C. difficile*, are considered the major *Clostridia* species to cause infection in equines. *C. perfringens* is widely distributed within the environment in the form of spores or vegetative cells and can be isolated from the faeces of healthy horses (Feary

& Hassel, 2006). *C. perfringens* is often seen to cause enterocolitis in equine neonates, due to colonization of the gastrointestinal tract within the first 24-72 hours postpartum (Feary & Hassel, 2006).

C. perfringens is dividing into multiple strains, type A, B, C, D and E. Type C most commonly infects foals (Uzal *et al.*, 2012) and produces the virulence factors cytotoxic β-toxin and α-toxins, and is rarely found in the environment (Feary & Hassel, 2006). Infections in foals are characterised by abdominal pain, fever, and diarrhoea and may progress to sepsis, meaning infections can have a mortality rate of 54%-68% even with medical intervention (Feary & Hassel, 2006). In mature horses infection presents itself less commonly and less aggressively, often caused by Type A, commonly found in normal horses along with the β_2-cytotoxin (Feary & Hassel, 2006). Type A is also known to contain genes for production of *C. perfringens* enterotoxin (CPE) and although the mechanisms of how CPE causes symptoms associated with Type A infection is unknown, this gene can be exploited by molecular methods for diagnosis (Weese *et al.*, 2001).

It is important to note that there is also suspected interactions between *Clostridia* spp., specifically *C. perfringens* Type C and *C. difficile* which may further complicate diagnosis and create a need for more thorough diagnostic testing, where both can be identified if a polymicrobial infection is present (Uzal *et al.*, 2012).

iv) Rotavirus

Globally, Group A rotaviruses are recognised as a cause of gastrointestinal infection within equines, with particular effects seen in foals. Nearly all foals under the age of 3 months experience a bout of rotavirus prior to weaning and as rotavirus causes between 50%-90% of diarrhoea in foals under 3 months, rotavirus is considered the major cause of gastrointestinal infections in neonates (Bailey *et al.*, 2013; Papp *et al.*, 2013). Although the effects are mild, rotavirus has been known to cause death as a result of reduced absorption of liquids through the small intestines (Papp *et al.*, 2013).

Rotaviruses are prevalent worldwide, including New Zealand, and considered endemic to most horse populations (Bailey *et al.*, 2013). Transmission occurs through oral-faecal route and through contaminated animals and fomites (Bailey *et al.*, 2013). Vaccination and on farm management prevents the spread of rotavirus and significantly decreases the infection rates (Papp *et al.*, 2013). The self-limiting nature of rotavirus in horses, means it is an infection of least concern, however, it is important to correctly identify rotavirus to ensure a more pathogenic and potentially fatal microbe is not causing infection (Papp *et al.*, 2013).

v) *Rhodococcus equi*

As in respiratory disease, *R. equi* can be a common cause of gastrointestinal infection in horses. It has a temporal prevalence and is recorded to infect foals particularly within the summer months, coinciding with foals that are 0-6 months old (Prescott, 1991). *R. equi* infection in mature horses is less common and can manifest either as a respiratory or gastrointestinal infection, where it causes ulcerated lesions within the gut (Giguère & Prescott, 1997; Takai, 1997).

Although present worldwide in the soils, *R. equi* is shed through faeces and easily contaminates farms, making faecal shedding a predominant mechanism of infection transmission (Takai, 1997). Although normally present in soil, *R. equi* is significantly higher in soil in areas with horses (Giguère & Prescott, 1997). Bacterial growth is enhanced 1000x more in faeces left in the environment (Giguère & Prescott, 1997), making on farm management, such as removal of faeces, an important process in mitigating the spread of infection (Takai, 1997). Inhalation and ingestion of *R. equi* through oral-faecal transfer is the infectious route, and *R. equi* is highly opportunistic often affecting young foals with failure of passive transfer (Takai, 1997). Ingestion of *R. equi* infected sputum can also lead to ulcerative colitis in foals (Prescott, 1991).

It has been shown that 50% of foals with *R. equi* respiratory infections can also develop *R. equi* gastrointestinal infection, characterised by intestinal lesions. However, only 4% of foals have intestinal lesions without *R. equi*

respiratory infections (Giguère & Prescott, 1997). Diarrhoea may also present itself when *R. equi* respiratory infections are present, due to an increase in mucus build-up in the gut (Prescott, 1991). With combined treatment using erythromycin and rifampin, survival rates are as high as 88% (Giguère & Prescott, 1997). Early recognition, passive immunization (colostrum), and environmental management reduce the risk of spread and initial infection (Giguère & Prescott, 1997).

1.7.4 Diagnosis

Diagnosis of gastrointestinal infection is difficult due to the complexity of the normal gastrointestinal tract flora, and the fact that most infections present with clinically indistinguishable features (Båverud, 2002; Feary & Hassel, 2006; Pusterla & Gebhart, 2013). The similarity of presentations means symptom recognition is difficult and a differential diagnosis combining symptoms, serological results, and culturing are often used for a confident diagnosis (Pusterla & Gebhart, 2013). Salmonellosis is often identified through symptom recognition along with faecal cultures obtained from at least five faecal samples (Feary & Hassel, 2006; Palmer, 1985). Unfortunately this 'gold standard' diagnostic method takes a minimum of 2-5 days for diagnosis and produces results with less than desirable accuracy, only providing a diagnosis 55% of the time (Feary & Hassel, 2006). Diagnosis is further confounded by poorly standardised culturing methodology between diagnostic laboratories (Feary & Hassel, 2006).

Faecal cultures have in the past been the best diagnostic tool for identification of *C. difficile*. However, following a 48 hour incubation time cultures remained less sensitive than molecular tools, with the inability to differentiate or identify TcdA and TcdB (Båverud, 2002). Using faecal cultures for diagnosis is tedious, as these need to be collected in rectal sleeves or bags and maintained aerated during a 36-48 hour incubation period (Feary & Hassel, 2006). Additionally, *C. difficile* strains need to be identified rapidly, as within 72 hours of waiting time the sensitivity can decrease from 76% to 29% and culture techniques cannot differentiate between non-toxigenic and toxigenic *C. difficile* strains. However,

molecular methods such as PCR or enzyme-linked immunosorbent assay (ELISA) have the potential for toxin identification and therefore present a more accurate approach for diagnosis (Diab *et al.*, 2013). *L. intracellularis* can be identified using post-mortem tissues for histology, although ante mortem diagnosis is preferred and can be used with PCR detection of *L. intracellularis* in faeces or rectal swab and/or serology (Pusterla & Gebhart, 2013).

1.7.5 Routes of treatment

Treatment of gastrointestinal infections differ significantly dependant on the aetiological agent and is mainly supportive with the application of fluids and electrolytes. There is a strong requirement of correct diagnosis of the aetiological species, as narrow-spectrum antibiotics provide the best treatment in order to treat the pathogenic species while maintaining normal gut flora to avoid re-colonization by other opportunistic pathogenic species (Feary & Hassel, 2006).

1.8 Traditional diagnostic methods

Traditional diagnostic methods used to identify horse diseases are highly variable dependant on the nature of the organism and the site of infection. Optimal culturing methods are centred on setting up ideal growth conditions, which differ greatly between sample types and microbes. This means many different specific conditions are needed in order to identify pathogens within a clinical sample. These methods involve bacteriological and microbiological culturing followed by phenotypic identification. These methods are extremely time consuming and often insufficient, leading to misidentification and therefore misdiagnosis in cases such as Strangles and *S. dysgalactiae* subspecies *equisimilis* (Laus *et al.*, 2007). In addition, isolates that had been previously phenotypically identified as *R. equi*, have eventually been reclassified with the introduction of molecular methods. These molecular methods have allowed the taxonomic identification of distant species, such as *S. epidermidis*, (Stefańska *et al.*, 2016) suggesting

phenotypic identification and culturing may have been misleading in a number of cases previously.

1.8.1 Bacterial cultures

In humans and other animals, blood culture remains the gold standard for the diagnosis of septic infections, however these take a minimum of 24-48 hours to identify (Lehmann *et al.*, 2008). Additionally, culturing can be achieved using any serum from the sight of infection, allowing the culturing of bacteria found in infected joints, synovial fluid, or pus. Most often, blood cultures return negative, leaving the application of correct treatments being based only on observational symptoms (Lehmann *et al.*, 2008). At other times cultures may return false negatives, which are found to occur 12-37% of the time (Furr, 2003; Rieger *et al.*, 2014). Cultures demonstrate poor efficiency and low sensitivity, with results only being correctly diagnosed 60% of the time, with actual case studies showing efficiency to be lower than 25% (Russell *et al.*, 2008; Taylor, 2015). False-negatives often occur with culture diagnosis due to low levels of bacteria that circulate in collected samples (Pusterla *et al.*, 2009a). Good culturing results are very dependent on bacterial growth which can be affected and or inhibited by storage, preparation and transport conditions (Stefańska *et al.*, 2016). The use of antibiotics, before blood is taken also creates a huge hurdle for the identification of pathogens with cultures as they will not grow successfully, meaning detection of the aetiological agent may be impossible (Mencacci *et al.*, 2011).

1.8.2 Viral cultures

Traditional viral identification methods such as histology and ELISA have been found to be time consuming, expensive, and laborious (Carvalho *et al.*, 2000). Viral detection without molecular techniques has only been performed by specialists who compare information on the effect of the virus on the host, alongside bio-assays such as neutralisation, fluorescent antibody, negative staining, or electron microscopy (Boonham *et al.*, 2014). Similar to bacteria, the culturing of viruses also requires multiple cell culture types in order to provide optimal growth conditions. A large amount of specialist

knowledge is required for viral cultures and the consideration of the clinical symptoms provides significant insight for diagnosis, and is therefore used heavily to guide culturing conditions (Storch, 2000). During cell growth, morphological changes can be indicative of viral species, allowing diagnosis. Viral cells can also be tested with fluorescent antibodies or monoclonal antibodies specific for suspected viruses. These diagnoses can take 24-48 hours to culture, and an additional 1-3 weeks for inoculation (Storch, 2000). The complications associated with traditional viral diagnostics means they are often overlooked as equine pathogens, and can therefore cause otherwise avoidable morbidity and mortality.

1.8.3 Fungal cultures

Fungal cultures are the current gold standard diagnostic techniques for fungal infections in animals (Brilhante *et al.*, 2016). Species, such as *Coccidioides* spp. known to affect equines are slow growing and as a result may take three to five days to grow before diagnosis is possible (Brilhante *et al.*, 2016). The slow growing phase and infrequency of fungal infections means these are also often overlooked during diagnosis.

1.9 Molecular methods for diagnosis

Due to the requirement of an accurate diagnosis before the application of treatment, determining the aetiological agent of infection is of the utmost importance when dealing with sepsis and other infections in equines and other animal species (Mencacci *et al.*, 2011). Because of the time sensitivity of diagnosis there has been a requirement of a rapid diagnostic strategy for equines. Molecular methods are being recognised as more specific and sensitive, especially when detecting low levels of microbes within an infection (Stefańska *et al.*, 2016).

1.9.1 Polymerase chain reaction (PCR)

PCR is a widely used diagnostic tool, recognised to demonstrate high specificity and sensitivity. PCR uses enzyme replicating methods to

replicate small amounts of DNA exponentially within a short time period of 1-2 hours (Rahman *et al.*, 2013). Applications of PCR are broad and have been used in molecular and biomedical research for cloning, DNA sequencing and the analysis of genes (Rahman *et al.*, 2013). However, has also been applied diagnostically for the detection of infectious diseases and cancers. The rapid nature of the PCR protocol allows it to be a rapid method for early diagnosis and detection of disease and infection. Diagnostic PCR uses small reaction volumes containing DNA template, pathogen specific primers, buffers, free nucleotides, and selective enzymes which are then cycled between varying optimal temperatures by a thermal cycler (Rahman *et al.*, 2013). Desired PCR products can be viewed on agarose gels and analysed quickly to determine the success of amplification, indicative of the presence of pathogens (Settanni & Corsetti, 2007).

An important component of a PCR reaction is the template DNA which is formed by purified or high quality DNA obtained by performing a DNA extraction method on a serological sample or tissue. The next critical factor are the primers which determine the start and end of the desired region to be amplified. Primers are short DNA strands between 18-25bp that are designed to gene areas unique to the suspected organisms involved in infection. The primers adhere to the DNA template at complimentary regions, allowing the DNA to be copied (Rahman *et al.*, 2013). The enzyme involved in the amplification of the DNA is also very important and involves TAQ polymerases that anneal complementary nucleotides, allowing the formation of a new strand, which is initiated by primers binding. TAQ polymerase, derived from *Thermus aquaticus* is a thermostable polymerase which is available in different forms (Kralik & Ricchi, 2017). Hot-start is the most commonly used TAQ polymerase, which uses a thermally activated DNA polymerase enzyme for the annealing of nucleotides during PCR. This greatly increases the specificity of PCR products while reduces background noise, nonspecific binding, and primer dimers which commonly occur during a reaction set up with alternative polymerase reagents (Birch, 1996). Hotstart TAQ polymerase is activated by a 95°C pre-PCR hold, which can be easily incorporated into a regular thermocycling program. The use of this highly specific polymerase

in diagnostics is extremely important, as it mitigates non-specific binding that may be misinterpreted as a false positive results. Other factors important in PCR are the single bases of A, T, C and G deoxynucleotide triphosphates (dNTPs), which become incorporated and allow the synthesis of the new DNA strands and the PCR buffer which aid in protecting the template DNA and creates the optimal conditions for the enzymes activity.

Once reagents are combined, specific temperatures in thermal cycling cause PCR to occur, replicating specific gene areas from template DNA. A period of 95°C initiates Hotstart TAQ polymerase and denatures double stranded template DNA. A lowered temperature of between 50-70°C allows primers to adhere to the single stranded template DNA. Lastly, a temperature of 72°C provides the optimal temperature for the enzymatic activity of the TAQ polymerase, which then extends the primers, binding free dNTPs and forming amplicons. These temperatures cycle around and exponentially replicate the selected gene areas (**Figure 1.4**).

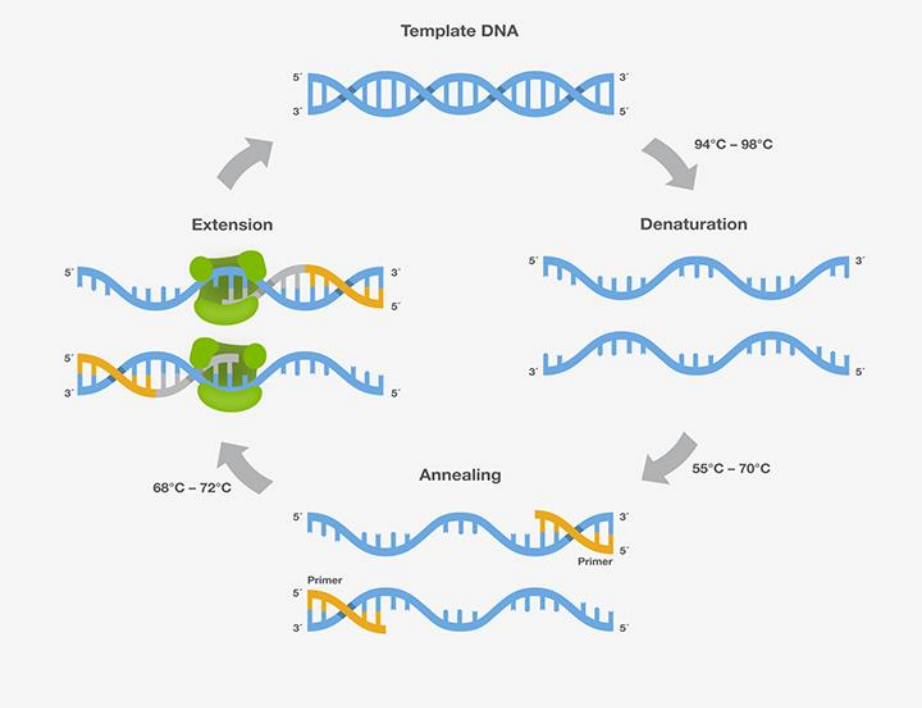


Figure 1.4: Primary steps of PCR; melting, annealing, and extension necessary for the successful amplification of target DNA sequence from DNA template (Thermo Fisher Scientific, 2017).

PCR protocols themselves can also be altered to improve amplification efficiency, such as incorporating touchdown PCR (TD-PCR), which can reduce nonspecific primer annealing through lowering annealing temperatures between cycles (Rahman *et al.*, 2013). This is recommended when using PCR for diagnostic purposes (Korbie & Mattick, 2008). The melting temperature of a primer is determined by the composition of oligonucleotides and helps to calculate an expected annealing temperature of the primers being used. However, the simplified method of calculating Tm is limited due to additional factors, including the arrangement of nucleotides and buffers used within a reaction. As a result, PCR will always require optimisation with each new set of primers in order to determine optimal Tm, which can be achieved in combination with TD-PCR (Korbie & Mattick, 2008). TD-PCR approach begins with a temperature above the calculated Tm and gradually lowers following each cycle. This helps to compensate for miscalculations in the annealing temperature, resulting in higher quality amplicons (Korbie & Mattick, 2008; Yamamoto, 2002).

Multiplex PCR is another modification that allows multiple primer sets to be used to identify multiple microbes in a single PCR reaction. It builds on traditional PCR for the detection of multiple microbial species by containing a pool of primers that allow the simultaneous amplification of multiple gene areas in a single reaction (Settanni & Corsetti, 2007). This offers a useful strategy to identify multiple infectious isolates rapidly, with ease and at a lower cost (Elnifro *et al.*, 2000). The simultaneous amplification of multiple target genes within a reaction is achieved through the addition of multiple primer sets and adjustments to buffers and other reaction components. To develop this, the expected primer size and Tm must be considered, and careful optimisation is critical for reliability of results (Elnifro *et al.*, 2000). Amplified products can then subsequently be analysed on a high density agarose gel using electrophoresis (Settanni & Corsetti, 2007).

1.9.2 Quantitative PCR (qPCR)

qPCR is another variation on traditional PCR, which has become an established diagnostic method within human and veterinary medicine. qPCR

monitors DNA amplification during a PCR reaction, by measuring fluorescence following each cycle. A fluorescent protein is included in the PCR reaction, which will intercalate between double stranded DNA that is formed at the end of each elongation cycle. Using a laser, the fluorescent protein is excited, and the fluorescent signal generated allows an accurate quantification of the DNA that has been amplified, providing a quantitative result when compared to a reference for control. This approach mitigates the need to completely wait for the PCR to finish and to run the products out on an agarose gel to visualise and determine successful amplification (Kralik & Ricchi, 2017). Florescent detection within a qPCR can be achieved using either non-specific fluorescent DNA dyes or fluorescently labelled oligonucleotides. Similar to traditional PCR, it is possible to design a qPCR protocol for every pathogenic microorganism, through the design of primers. Also, due to the high through-put, speed of amplification, and result analysis qPCR offers a unique method for pathogen detection with clinical samples. However, still requires optimisation, particularly when using a multiplex approach (Kralik & Ricchi, 2017).

A limitation with any PCR approach is the methodology cannot differentiate between live and dead cells, which may lead to false positives by amplifying genes for organisms not responsible for the current infection (Kralik & Ricchi, 2017).

1.9.3 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is an additional molecular technique that has applications in the diagnosis and detection of microbial infections. ELISA uses serological samples to measure antibodies associated with disease and infection, and has wide uses in human and veterinary diagnosis though its potential use as mass screening assays. Some microbial species with proteins present on the surface of the bacteria allow for the identification via ELISA. ELISA utilises immunoassays to detect the presence of an antigen or antibody in a sample, binding or attachment of the antibody or antigen to the assay causes an interaction that can be measured as a colour change for detection. ELISA have been applied to the detection

of select viruses and bacterial infections in equines and other animal species (Prescott *et al.*, 1996). Unfortunately these were recognised to have the potential to cross-react and produce false positives (Prescott *et al.*, 1996).

ELISA is considered alongside PCR to be a successfully established virus detection method, allowing for increased assay sensitivity with monoclonal antibodies, high specificity, and high versatility with testing sample types (Boonham *et al.*, 2014). ELISA is an extremely useful tool in diagnostic medicine due to its speed, ease of use, and sensitivity compared to traditional culture based identification methods (Kralik & Ricchi, 2017).

1.9.4 Efficacy of molecular methods

The efficacy of PCR diagnosis has been shown to differ depending on sample types, showing high accuracy with control samples and lowered accuracy with real clinical samples, but has been proven to be more sensitive than blood cultures, particularly when antibiotics have already been administered (Mencacci *et al.*, 2011). Although PCR is sensitive, qPCR has been shown to have higher sensitivity and a reduction in carryover of contamination which may result in false positives (Pusterla & Gebhart, 2013). ELISA has the potential to be a highly effective molecular technique for detecting certain pathogens, particularly in terms of rapid diagnosis and can mitigate the requirement of a wet lab, however, does face challenges associated with lowered sensitivity (Kralik & Ricchi, 2017).

1.10 Use of PCR in diagnostics

PCR is a molecular method that offers a unique approach for the detection of infectious organisms present in the blood stream, tissue, fluid or environment of an infected organism. All PCR requires is an extracted DNA sample and primers specifically designed to amplify a particular gene region unique to a particular pathogen to act as a marker for diagnosis. Currently there are a number of areas, where it has been employed for the detection of particular pathogens.

1.10.1 PCR for sepsis identification

In human clinical samples, qPCR has been used for the rapid diagnosis and identification of bacterial pathogens within the blood stream. PCR amplification of DNA extracted from whole blood has been tested within human samples and has been shown to have a greater accuracy compared to traditional culturing methods (Lehmann *et al.*, 2008). By using universal 16S and 23S ribosomal RNA (rRNA) sequences of bacteria and the 18S and 5.8S rRNA of fungi target sequences, 25 important human microbial pathogens were able to be amplified using PCR and visualised for diagnosis, even following serial dilutions of the pathogen (Lehmann *et al.*, 2008). PCR can also detect microbial pathogens in other samples, such as synovial fluids and purulent exudates, having a higher sensitivity compared to traditional culturing methods, even with multiple sample types (Mencacci *et al.*, 2011).

PCR has also been applied to the veterinary setting for the detection of bacteraemia in neonatal foals (Pusterla *et al.*, 2009a). With a small panel of potential pathogens tested, it was possible to detect pathogens relevant to the disease profile with no cross-reactivity and increased speed and accuracy (Pusterla *et al.*, 2009a).

1.10.2 PCR for respiratory diseases

In horses, EHV-1 and EHV-4 are closely related, making it difficult to distinguish between the two species using typical serological methods (Carvalho *et al.*, 2000). PCR has been used to increase the sensitivity of EHV diagnosis and enable the identification of EHV-1 and EHV-4 infections (Carvalho *et al.*, 2000). Further studies have confirmed the sensitivity of PCR for virus detection compared to cultures in a study that showed EHV-4 was only identified in 4/21 (19.05%) samples using viral isolation, whereas 14/21 (66.67%) was identified using PCR (McBrearty *et al.*, 2013).

PCR has also proved more sensitive than cultures for the identification of Strangles allowing for the rapid identification of infectious respiratory disease causing pathogens (Patty & Cursons, 2014). In addition, PCR

technology has been successfully applied to the identification of *R. equi* from various respiratory samples including; tracheal washes, nasal swabs, and tissue samples. All of which were amplified with higher sensitivity than those using traditional culturing methods (Stefańska *et al.*, 2016).

1.10.3 PCR for gastrointestinal diseases

A confounding factor in the diagnosis of gastrointestinal disease is related to the shedding of bacterial cells or spores from the infected animal. As bacterial shedding may not be constant, multiple faecal samples are required in order to obtain a sufficient amount of bacterial cells to culture and confirm a diagnosis (Feary & Hassel, 2006). PCR has been optimised for the detection of gastrointestinal infections, such as *Salmonella* spp. and shown to have higher sensitivity when detecting low numbers, which is common during early infection and with irregular bacterial shedding. Low numbers like these are often undetectable with traditional culturing methods (Feary & Hassel, 2006). Additional work has demonstrated PCR was a sensitive method for detecting the presence of *L. intracellularis* within a sample, however, caution was still required with diagnosis, as the presence of *L. intracellularis* did not imply it was the causative agent of the gastrointestinal infection (Frazer, 2008).

1.11 Aims

Molecular approaches, such as PCR offer a unique approach for the detection of pathogenic DNA in multiple clinical samples from equines with infections. PCR is highly sensitive, and therefore provides significant diagnostic value for the detection of microorganisms for samples that may be difficult to culture *in vitro* (Yamamoto, 2002). Techniques, such as multiplexing primers into a single PCR reaction also offer a more rapid and sophisticated method for the simultaneous identification of multiple organisms, allowing large scale diagnostics to be achieved, which is useful within a diagnostic laboratory (Yamazaki-Matsune *et al.*, 2007). PCR diagnostics has the potential to overcome limitations of traditional culturing methods, saving time and increasing sensitivity.

This investigation aims to utilise the molecular method of PCR, and exploit gene regions unique to major equine pathogens, recognised in previous literature to prove the efficacy of PCR as a standalone diagnostic method. This project will measure the efficiency of using PCR with clinical samples from multiple infection types and begin to provide valuable information on disease trends within the New Zealand equine industry.

Chapter Two

METHODS AND MATERIALS

All experimental work for this project was undertaken in the Molecular genetics laboratory, part of the Faculty of Science and Engineering, University of Waikato, Hamilton, NZ.

Glassware for media and solutions was vigorously washed using a laboratory glassware-grade dishwasher (Miele Professiona, G7893) prior to use. Routinely work spaces were disinfected, and were wiped down with 70% ethanol before work commenced to minimise microbial contamination, and cross contamination between samples. PCR reactions were prepared in a dedicated ESCO PCR cabinet to minimise the incorporation of DNases and cross contamination of samples.

Chemicals were obtained from Sigma-Aldrich (NZ) unless stated otherwise. Centrifugation steps were undertaken in a Biofuge pico Heraeus centrifuge and incubation steps in a 1.5ml Thermomixer comfort (Eppendorf). PCR reactions were carried out in a T100 thermocycler (BioRad). Molecular weight ladders used were provided by Genscript and Solis BioDyne. Agarose gel electrophoresis was carried out using a dedicated OWL electrophoresis tank (ThermoFisher Scientific) containing 1x Super Buffer (See Appendix 1) and a LighteningVolt 250P-OSL electrophoresis machine (Biolab scientific).

2.1 Obtaining ethical approval

Ethical approval was not required, as the collection of samples by veterinarians were part of normal animal husbandry process and a proportion of these were provided for research purposes.

2.2 Determining prevalent species

Communications with NZ equine veterinarian, Dr Angela Hawker (BVSc (Dist) MACVSc) and research into NZ veterinary literature was used to determine common isolates seen to cause diseases within NZ horses (**Table 2.1**). The estimated prevalence of pathogens determined helped to decide which microorganisms to focus on and guided primer design.

Table 2.1: An example showing the culture results of bacterial species found in blood samples of septic neonatal foals in New Zealand (Toombs-Ruane *et al.*, 2016), which helped direct primer design.

Species	Frequency (%)	Gram +/-
***Streptococcus* spp.**	25	+
***Staphylococcus* spp.**	24	+
***Enterococcus* spp.**	9	+
Escherichia coli	20	-
***Bacillus* spp.**	21	+
Rhodococcus equi		+
***Corynebacterium* spp.**		+
***Micrococcus* spp.**		+
***Klebsiella* spp.**		-
***Enterobacter* spp.**		-
***Acinetobacter* spp.**		-
***Morexella* spp.**		-
***Pseudomonas* spp.**		-

2.3 Specimens

Specimens were collected by veterinarians from around the Greater Waikato Region and around Auckland and sent through to the University of Waikato Molecular genetics laboratory by post. Specimen types provided included; blood sample (in an EDTA tube), blood cultures, abscess material, nasal swabs, nasopharyngeal swabs, tracheal wash, faecal swabs, and faecal samples. Samples received were categorised into three groups; sterile, respiratory, and gastrointestinal.

Samples which should be free from microbial cells in a non-infected animal were considered sterile, this included blood, blood cultures, pus, and synovial fluid. Sterile samples were retrieved aseptically, with veterinarians instructed to clip and scrub the site of infection wearing sterile gloves before inserting a sterile catheter and drawing the sample into a sterile syringe using a sterile needle. Once drawn, samples were placed into an EDTA tube and capped before being stored at room temperature. Some blood samples were directly injected into a BD BACTEC™ blood culture media bottle before delivery.

Respiratory samples included nasal swabs, nasopharyngeal swabs, and tracheal washes. Nasal swabs were retrieved by inserting a sterile swab as far as possible into the nasal cavity using a gloved hand, swabbing before removing the swab while trying to avoid the external nares, and placing the swab back into the sterile container for storage and transport. Nasopharyngeal swabs were collected by passing a 40cm cotton tipped swab at least 15cm into the nasopharynx and allowing secretions to absorb into the swab. Swabs were then cut and placed into a sterile pot containing sterile saline for transport and storage (University of Kentucky-Veterinary Diagnostic Laboratory, 2017). Tracheal wash samples were obtained using sterile saline, following common transtracheal wash procedure methodology, and stored in a sterile pot for transport and storage (Animal Health Diagnostic Center, 2016).

Gastrointestinal samples included faecal samples and faecal swabs, as well as DNA that had been extracted from previous faecal samples in a previous study. Faecal samples were retrieved by using a gloved hand to swab or scoop a small amount of fresh faeces from the affected animal into a sterile pot.

All clinical samples were assigned an identification number to maintain anonymity. Sample identification (Sample ID) was assigned based on sample type; Sterile (S), Respiratory (R), and Gastrointestinal (G), and numbers were assigned based on the chronological reception of samples. All

specimens were stored at room temperature (RT) until extraction, however any remaining blood was frozen at -20°C for future tests.

2.4 Bacteriological culturing and identification

2.4.1 Bacteriological culturing from blood samples

After inverting the EDTA blood sample, 250-1000µl was aseptically added to a 50ml culture flask containing 20ml Columbia broth (See Appendix 1) and placed into a culturing CO_2 incubator at 37°C (inCu safe, IR SENSOR) over night.

2.4.2 Bacteriological culturing streaking

Incubated cultures were streaked out onto a sheep blood agar plate (See Appendix 1) using a sterile 5µl inoculation loop. Single colony streaking was achieved (**Figure 2.1**) by starting at point A and using the inoculation loop to spread the culture solution back and forward across a $1/5^{th}$ section of the plate around five times before rotating the plate and repeating at point B and rotating again and repeating at point C. Following this, the loop was heated and cooled before rotating the plate and repeating the streaking procedure at point D. Again the inoculation loop was sterilised using a Bunsen burner flame and cooled before forming a single colony streak (point E) by moving the loop from the far end of the D streaking section, and bringing the colonies into the middle of the plate. The lid of the plate was replaced before incubating lid side down at 37°C overnight. Some culture samples were additionally streaked onto an amikacin treated blood agar plate (See Appendix 1).

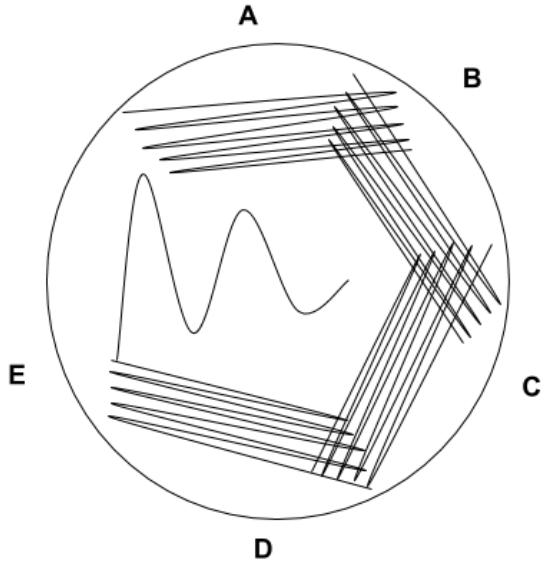


Figure 2.1: Single colony streaking directions.

2.4.3 Gram stain from culture media and single colony

A Gram stain pen (Securline®) was used to mark a 1cm diameter circle on a microscopic slide (Fronine). For Gram stain test being performed on a single colony, 20µl of sterile water was placed within the marked circle and a single colony was emulsified into the water. For Gram stain tests being performed on a culture, then 5µl of culture was transferred from the flask into the marked area. The culture was heat fixed onto the slide using a Bunsen burner and allowed to cool. Crystal violet was applied to the slide, ensuring all heat fixed material was immersed and allowed to sit for 1 minute, following which the crystal violet was rinsed off using tap water and a paper towel used to blot around the slide and remove excess water. Gram's iodine was then applied, fully immersing all culture material and left to sit for 1 minute. The Gram's iodine was then rinsed off with tap water and subsequently rinsed with 95% ethanol until the ethanol running off the side was clear. The slide was blotted with a clean paper towel before dilute carbol fuchsin was applied over the cultured material and allowed to sit for 1 minute. Slides were then rinsed under running water, blotted dry and allow to dry fully in a 37°C incubator (Precision, Economy Incubator). Gram staining was visualised using a CP-ACHROMAT oil immersion 100x lens (Zeiss axiostar) and analysed by observing microbial colony size, shape,

bunching, and Gram staining. Gram stain pictures were acquired and stored as .jpeg files by photography using either a COOLPIX 4500 camera (Nikon, Japan) or Olympus DP30 camera.

2.4.4 Identification of bacteria

Identification of bacteria was performed by observing morphological characteristics of cultured bacteria. Growth characteristics noted included; turbid appearance in culture, non/haemolytic growth on blood agar, and Gram positive, Gram negative, or polymicrobial appearance following Gram staining. Individual colonies were extracted from plates, DNA extracted, and used as DNA templates in PCR for confirmation of bacterial identification.

2.5 Specimen processing and DNA isolation

All samples received underwent genomic and bacterial DNA extraction and subsequent PCR diagnosis using selected primers designed to identify selected microorganisms. Before all DNA extraction commenced, work benches were thoroughly cleaned with 70% ethanol to prevent contamination between samples. A thermomixer was preheated to 95°C and 10% CTAB was place in an incubator at 80°C to liquefy.

2.5.1 Microbial DNA extraction from equine blood using SDS lysis

To ensure microbes were consistently found within samples the blood was mixed by inverting the EDTA tube by hand and 200µl of blood was aseptically removed from the EDTA tube using a 1.5ml sterile transfer pipette (Sarstedt). This was placed into a DNase/RNase free 2ml microcentrifuge tube (MULTIMax™) to which 800µl of 1% SDS lysis solution (See Appendix 1) was added to lyse the blood and bacterial cells, and mixed using a transfer pipette. This solution was placed into a thermomixer at 95°C for 10 minutes to agitate at 650rpm and heat the sample, promoting an even lysis of the cells. Following this the sample was

removed and thermomixer temperature was reduced to 65°C. The solution was left to cool for 5 minutes before adding 1ml of pH8 phenol (Sigma) and placing on a rotating wheel (Stuart SB2 rotator) to gently mix for 10 minutes. The solution was subsequently centrifuged at 16,060g for 10 minutes and the supernatant was carefully removed using a fine tipped 1.5ml sterile transfer pipette (Sarstedt) and dispensed in a DNase/RNase free 2ml microcentrifuge tube (MULTIMax™) before discarding the organic phenol layer. To the supernatant, 100µl of liquefied 10% CTAB was added and was placed in a 65°C thermomixer for 10 minutes and mixed at 650rpm. Following this incubation, 750µl of chloroform was added to the solution and shaken vigorously by hand for 20 seconds before being centrifuged for 10 minutes at 16,060g. Following centrifugation the supernatant was transferred into a DNase/RNase free 1.5ml microcentrifuge tube using a 1.5ml sterile fine tipped transfer pipette (Sarstedt) and precipitated using equal parts of isopropanol (approximately 500µl). The microcentrifuge tube was then placed into a spark free freezer (Lec Medical, Rollex) at -20°C for between 20 minutes or overnight to allow DNA precipitation. After sufficient precipitation the microcentrifuge tube was centrifuged at 16,060g for 10 minutes to form a pellet on the side of the tube. Isopropanol was gently poured off and discarded and 1ml of 70% ethanol was added to the tube to wash the pellet and remove excess salts. This ethanol solution was centrifuged at 16,060g for 5 minutes before being carefully discarded from the tube without disturbing the pellet. The microcentrifuge tube containing the DNA pellet was left open in a fume hood for 5 minutes to evaporate any excess ethanol for maximum purity. Once thoroughly dried 50µl of TE buffer (10mM Tris 1mM EDTA) was added and the tube was briefly vortexed to resuspend the pellet. Extracted DNA was then labelled and stored at RT for quantification and use as a template for PCR.

2.5.2 Microbial DNA extraction from equine blood using GITC

To ensure microbes were consistently found within samples the blood was mixed by inverting the EDTA tube by hand and 250µl of blood was aseptically removed from the EDTA using a 1.5ml sterile transfer pipette

(Sarstedt) into a DNase/RNase free 2ml microcentrifuge tube (MULTIMax™) to which 750µl of GITC viral extraction mix solution (See Appendix 1) was added and placed in a thermomixer at 80°C for 10 minutes at 650rpm. Following this 1ml of isopropanol was added and the tube was inverted 10 times and then centrifuged immediately at 16,060g for 10 minutes. Following centrifugation the supernatant was discarded and the sample briefly centrifuged again for 10 seconds to ensure removal of residual liquid. The precipitate was then dissolved in 350µl of 1% SDS lysis solution (See Appendix 1), 350µl 5M LiCl, and 85µL 10% CTAB and placed in a thermomixer at 65°C for 10 minutes at 650rpm. Following this incubation 750µl of chloroform was added to the solution and shaken vigorously by hand for 20 seconds before being centrifuged for 10 minutes at 16,060g. Following centrifugation the supernatant was transferred into a new DNase/RNase free microcentrifuge tube (MULTIMax™) using a 1.5ml sterile fine tipped transfer pipette (Sarstedt) and precipitated using equal parts of isopropanol (approximately 500µl). The microcentrifuge tube was then placed into a spark free freezer (Lec Medical, Rollex) at -20°C for between 20 minutes or overnight to allow DNA precipitation. After sufficient precipitation the microcentrifuge tube was centrifuged at 16,060g for 10 minutes to form a pellet on the side of the tube. Isopropanol was gently poured off and discarded and 1ml of 70% ethanol was added to the tube to wash the pellet and remove excess salts. This ethanol solution was centrifuged at 16,060g for 5 minutes before being carefully discarded from the tube without disturbing the pellet. The microcentrifuge tube containing the DNA pellet was left open in a fume hood for 5 minutes to evaporate any excess ethanol for maximum purity. Once thoroughly dried 50µl of TE buffer (10mM Tris 1mM EDTA) was added and the tube was briefly vortexed to resuspend the pellet. Extracted DNA was then labelled and stored at RT for quantification and use as a template for PCR.

2.5.3 Microbial DNA extraction from pus (abscess)

For pus, a 1.5ml sterile transfer pipette (Sarstedt) was used to combine 100µl of pus with 350µl of 1% SDS lysis solution (See Appendix 1) in a

DNase/RNase free 2ml microcentrifuge tube (MULTIMax™) and mixed thoroughly with a transfer pipette. The tube was placed into a thermomixer at 95°C for 10 minutes at 650rpm. Following this the sample was aliquoted into two new DNase/RNase free 2ml microcentrifuge tube (MULTIMax™) for balance and 350µl of 5M LiCl and 100µl of 10% CTAB added to each tube which were then placed into a thermomixer at 65°C and 650rpm for 10 minutes. For swabs, the cotton section was snapped into a DNase/RNase free 1.5ml microcentrifuge tube (MULTIMax™) containing 350µl of 1% SDS lysis solution (See Appendix 1) and placed in a thermomixer at 95°C for 10 minutes at 650rpm. Following incubation, the swab was removed and 350µl of 5M LiCl and 100µl of 10% CTAB was added to the tube which was then placed in a thermomixer at 65°C and 650rpm rotation for 10 minutes.

After lysis, 750µl chloroform was added to each tube and shaken for 20 seconds before centrifuging at 16,060g for 10 minutes. The supernatant was transferred into a new DNase/RNase free 1.5ml microcentrifuge tube (MULTIMax™) using a 1.5ml sterile fine tipped transfer pipette (Sarstedt) and precipitated using an equal volume of isopropanol (approximately 500µl). The microcentrifuge tube was then placed into a spark free freezer (Lec Medical, Rollex) at -20°C for between 20 minutes or overnight to allow DNA to precipitate. After sufficient precipitation the microcentrifuge tube was centrifuged at 16,060g for 10 minutes to form a pellet on the side of the tube. The isopropanol was gently poured off and discarded and 1ml of 70% ethanol was added to the tube to wash the pellet and remove excess salts. This ethanol solution was centrifuged at 16,060g for 5 minutes before being carefully discarded from the tube without disturbing the pellet. The microcentrifuge tube containing the DNA pellet was left open in a fume hood for 5 minutes to evaporate any excess ethanol for maximum purity. Once thoroughly dried 50µl of TE buffer (10mM Tris 1mM EDTA) was added and the tube was briefly vortexed to resuspend the pellet. Extracted DNA was then labelled and stored at RT for quantification and use as a template for PCR.

2.5.4 Microbial DNA extraction from gastrointestinal sample

For faecal samples arriving in a pot, a sterile swab was dipped into pot to collect faecal matter. The cotton part of the swab would then be snapped off into a DNase/RNase free 1.5ml microcentrifuge tube (MULTIMax™), 500µl of Plant CTAB (see Appendix 1) was added and vortexed for 10 seconds to thoroughly mix into solution. Plant CTAB (containing DIECA) was used at this step to inhibit phenoloxidase from plant matter that may have downstream inhibitory effects during PCR. The microcentrifuge tube was then placed into 95°C thermomixer at 650rpm for 10 minutes. Following incubation the swab was removed using sterile tweezers and discarded, and 500µl of phenol chloroform added to the solution before being shaken and left on a rotating wheel (Stuart SB2 rotator) for 10 minutes. This solution was then spun down in a microcentrifuge at 16,060g for 10 minutes, following this the supernatant was transferred to a new DNase/RNase free 1.5ml microcentrifuge tube (MULTIMax™) using a fine tipped 1.5ml sterile transfer pipette (Sarstedt) and precipitated using equal parts Na isopropanol to limit PCR inhibitors (see Appendix 1) and again, centrifuged at 16,060g for 10 minutes before supernatant and dregs were removed. To the pellet, 350µl of 1% SDS lysis solution (See Appendix 1), 350µl of 5M LiCl, and 100µL of 10% CTAB were added and vortexed for 10 seconds before placing in a thermomixer for 10 minutes at 65°C and 650rpm mixing speed. After mixing, 750µl chloroform was added to the solution and shaken for 20 seconds before centrifuging at 16,060g for 10 minutes. The supernatant was transferred into a new DNase/RNase free 1.5ml microcentrifuge tube (MULTIMax™) using a fine tipped 1.5ml sterile transfer pipette (Sarstedt) and precipitated using an equal volume of isopropanol. The microcentrifuge tube was then placed into a spark free freezer (Lec Medical, Rollex) at -20°C for between 20 minutes or overnight to allow DNA to flocculate. After sufficient precipitation the microcentrifuge tube was centrifuged at 16,060g for 10 minutes to form a pellet on the side of the tube. The isopropanol was gently poured off and discarded and 1ml of 70% ethanol was added to the tube to wash the pellet and remove excess salts. This ethanol solution was centrifuged at 16,060g for 5 minutes before being carefully discarded from the tube without disturbing the pellet. The microcentrifuge tube containing the DNA pellet

was left open in a fume hood for 5 minutes to evaporate any excess ethanol for maximum purity. Once thoroughly dried 50µl of TE buffer (10mM Tris 1mM EDTA) was added and the tube was briefly vortexed to resuspend the pellet. Extracted DNA was then labelled with sample name and stored at RT for use as a template for PCR.

2.5.5 Microbial DNA extraction from respiratory sample

For respiratory samples in the form of a nasal or nasopharyngeal swabs, the respiratory swab was removed from agar casing and the cotton part was snapped into a clean DNase/RNase free 1.5ml microcentrifuge tube (MULTIMax™) containing 400µl of sterile 0.85% saline and vortexed for 10 seconds. Using ethanol and flame sterilized tweezers the swab was flipped over in the tube so the cotton was above the level of saline and centrifuged at 16,060g for 2 minutes to allow any remaining fluid to leave the swab. Sterile tweezers were used to remove and discard the swab and the saline solution was vortexed for 10 seconds to resuspend solid material into solution. For respiratory samples in the form of a nasal wash or saline from around a large swab the tube or pot the solution and swab was in was vortexed for 20 seconds and 1.5ml of saline was transferred into a DNase/RNase free 1.5ml microcentrifuge tube (MULTIMax™).

Saline solution from either swab or wash samples were then centrifuged at 16,060g for 10 minutes and saline supernatant was removed leaving a pellet in the microcentrifuge tube. This pellet was then resuspended in 350µl of 1% SDS lysis solution (See Appendix 1), 350µl of 5M LiCl, and 100µl of 10% CTAB and vortexed for 10 seconds before placing in a thermomixer at 65°C for 10 minutes at 650rpm. To this solution, 750µl chloroform was added and shaken for 20 seconds before centrifuging at 16,060g for 10 minutes. The supernatant was then transferred into a new DNase/RNase free 1.5ml microcentrifuge tube (MULTIMax™) using a fine tipped 1.5ml sterile transfer pipette (Sarstedt) and precipitated using an equal volume of isopropanol (approximately 500µl). The microcentrifuge tube was then placed into a spark free freezer (Lec Medical, Rollex) at -20°C for between

20 minutes or overnight to allow DNA to precipitate. After sufficient precipitation the microcentrifuge tube was centrifuged at 16,060g for 10 minutes to form a pellet on the side of the tube. The isopropanol was gently poured off and discarded and 1ml of 70% ethanol was added to the tube to wash the pellet and remove excess salts. This ethanol solution was centrifuged at 16,060g for 5 minutes before being carefully discarded from the tube without disturbing the pellet. The microcentrifuge tube containing the DNA pellet was left open in a fume hood for 5 minutes to evaporate any excess ethanol for maximum purity. Once thoroughly dried 50µl of TE buffer (10mM Tris 1mM EDTA) was added and the tube was briefly vortexed to resuspend the pellet. Extracted DNA was then labelled and stored at RT for quantification and use as a template for PCR.

2.5.6 DNA extraction from cultured bacteria

For bacteria grown in media, culture flasks were gently swirled to resuspend microbes and a sterile transfer pipette or a syringe and needle were used to remove 1.5ml of culture broth and place solution into a DNase/RNase free 1.5ml microcentrifuge tube (MULTIMax™) which was then centrifuged at 16,060g for 10 minutes. The supernatant was removed and pellet resuspended in 350µL of 1% SDS lysis solution (See Appendix 1), the tube was then placed in a thermomixer at 95°C at 650rpm rotation for 10 minutes. For bacteria grown on plates, a single colony was selected from the agar growth medium using a sterile metal inoculation loop to ensure the extraction will only obtain DNA from one organism and placed in 350µl of 1% SDS lysis solution (See Appendix 1) in a DNase/RNase free 1.5ml microcentrifuge tube (MULTIMax™) and placed in a thermomixer at 95°C at 650rpm rotation for 10 minutes.

After incubation in lysis solution, 350µl of 5M LiCl and 100µl of 10% CTAB was added to each microcentrifuge tube which were then placed in a thermomixer at 65°C and 650rpm rotation for 10 minutes. After mixing, 750µl chloroform was added to the solution and shaken for 20 seconds before centrifuging at 16,060g for 10 minutes. The supernatant was

transferred into a new DNase/RNase free 1.5ml microcentrifuge tube (MULTIMax™) using a fine tipped 1.5ml sterile transfer pipette (Sarstedt) and precipitated using an equal volume of isopropanol (approximately 500µl). The microcentrifuge tube is then placed into a spark free freezer (Lec Medical, Rollex) at -20°C for between 20 minutes or overnight to allow DNA to precipitate. After sufficient precipitation the microcentrifuge tube was centrifuged at 16,060g for 10 minutes to form a pellet on the side of the tube. The isopropanol was gently poured off and discarded and 1ml of 70% ethanol was added to the tube to wash the pellet and remove excess salts. This ethanol solution was centrifuged at 16,060g for 5 minutes before being carefully discarded from the tube without disturbing the pellet. The microcentriduge containing the DNA pellet was left open in a fume hood for 5 minutes to evaporate any excess ethanol for maximum purity. Once thoroughly dried 50µl of TE buffer (10mM Tris 1mM EDTA) was added and the tube was briefly vortexed to resuspend the pellet. Extracted DNA was then labelled and stored at RT for quantification, and use as a template for PCR.

2.5.7 Analysis and quantification of genomic DNA

A Thermo Scientific NanoDrop™ 2000 spectrophotometer was used to measure the purity and quantity of extracted DNA. To blank the spectrophotometer, 2µl of TE buffer (10mM Tris 1mM EDTA) was placed on the clean NanoDrop pedestal. Next, 2µl of extracted DNA resuspended in TE buffer (10mM Tris 1mM EDTA) was placed onto the pedestal for quantification. DNA quantity was measured in ng/µl and absorbance ratios at 260nm/280nm and 260nm/230nm were determined, which were indicative of purity. Samples with 260nm/280nm ratios between 1.8 and 2.0 and samples with 260nm/230nm rations between 2.0 and 2.2 were accepted as of sufficient quality indicating little to no contamination from proteins, or solvents used in the DNA extraction process.

2.6 PCR setup

2.6.1 PCR primers

Primers used within this project were determined from previous papers, purchased from Sigma Alrich or IDT, and made up to manufacturer's instructions (See Appendix 2). A primer catalogue for each sample group has been generated including forward (F) and reverse (R) sequences, approximate PCR product size, and where the primers have been published (**Table 2.2**, **Table 2.3**, **Table 2.4**). Primers were chosen to target unique genus specific, species specific, or pathogenic genes present in microbial species recognised to cause infection in equines.

Table 2.2: PCR primers used for identifying microorganisms within sterile grouped samples. PCR primers highlighted were used against all samples.

Microbe	Gene target	Primer name	Primer sequence (5' to 3')	Expected size (bp)	Reference
Gram positive	16S rRNA	F (DG74) R (p143)	AGGAGGTGATCCAACCGCA GAYGACGTCAARTCMTCATGC	370	(Klausegger *et al.*, 1999)
Gram negative	16S rRNA	F (785F) R (68d)	AGGAGGTGATCCAACCGCA AYGACGTCAAGTCMTCATGG	370	(Klausegger *et al.*, 1999)
Staphylococcus (Genus)	tuf	F (TStaG422) R (Tstag765)	GGCCGTGTTGAACGTGGTCAAATCA TIACCATTTCAGTACCTTCTGGTAA	370	(Martineau *et al.*, 1996)
Staphylococcus aureus	SPA	F (SPA F) R (SPA R)	CAGCAAACCATGCAGATGCTA CGCTAATGATAATCCACCAAATACA	101	(Nakagawa *et al.*, 2005)
Streptococcus (Genus)	tuf	F (Str1) R (Str2)	GTACAGTTGCTTCAGGACGTATC ACGTTCGATTTCATCACGTTG	761	(Picard *et al.*, 2004)
Escherichia coli	uidA	F (UidA F) R (UidA R)	TGGTAATTACCGACGAAAACGGC ACGCGTGGTTACAGTCTTGCG	162	(Bej *et al.*, 1991)
Coliform (Species)	lacZ	F (lacZ3 F) R (lacZ3 R)	TGGAAAATGGTCTGCTGCTG TATTGGCTTCATCCACCACA	234	(Molina *et al.*, 2015)
Pseudomonas (Genus)	PA-GS 16S RNA	F (PA-GS F) R (PA-GS-R)	GACGGGTGAGTAATGCCTA CACTGGTGTTCCTTCCTATA	618	(Spilker *et al.*, 2004)
Bacillus (species)	16S rRNA	F (463 F) R (263 R)	CTAAAACTCAAAGGAATTGACG AATACGTTCCCGGGCCTT	463	(Kadyan *et al.*, 2013)
Enterococcus (Genus)	*Enterococcus* specific	F (Ent1) R (Ent2)	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	112	(Ke *et al.*, 1999)
Panfungal species	ITS	F (ITS1d) R (ITS4d)	TCCGTAGGTGAACCTGCGG CCTCCGCTTATTGATATGC	848	(Clara Gutierrez Galhardo *et al.*, 2008)
Equine	Glycoprotein B	F (EHV-1.F)	GGGAAAGCCGGCATCATC	326	(Hu *et al.*, 2014)

Microbe	Gene target	Primer name	Primer sequence (5' to 3')	Expected size (bp)	Reference
herpesvirus-1		R (EHV-1.R)	CCGTGAAGTTTCTCCCAAGG		
	Thymidine Kinase	F (TkF4)	CCTTGGTTCCTTTGGCGAC GCAC	226-268	(Carvalho *et al.*, 2000)
		R (TkR2)	CTGGCGAG AACGCT ACCC		
Equine herpesvirus-4	Glycoprotein B	F (EHV-4.F)	TTACAAAACTCTATGCGCTCCTC	269	(Hu *et al.*, 2014)
		R (EHV-4.R)	TAGGTGCTGATCACTTCGATTTCA		
	Thymidine Kinase	F (TkF5)	TTGGGCCGTGGCCGAAAAC	333	(Carvalho *et al.*, 2000)
		R (tkR6)	CTAGCCAAAACCTTGCCT		
Rhodococcus equi	Cholesterol Oxidase	F (CHO-F)	GCTCGCTTCCAGTTCAATTC	191	(Cursons, 2017)
		R (CHO-R)	AGCGGGTGGTATGTGAAGTC		
VAP	Virulence plasmid A	F (VapAF)	GACTCTTCACAAGACGGT	200	(Ocampo-Sosa *et al.*, 2007)
		R (VapAR)	TAGGCGTTGTGCCAGCTA		
PvapA	Virulence associated protein A	F (PvapAF)	GAGCAAGCGATACCGCCGG	286	(Ocampo-Sosa *et al.*, 2007)
		R (PABR)	CTGGATATGGCCGAGGAAGC		
PvapB	Virulence associated protein B	F (PvapBF)	TGCGATAGCCACAGCCGCT	477	(Ocampo-Sosa *et al.*, 2007)
		R (PABR)	CTGGATATGGCCGAGGAAGC		
TraA	Conjugal plasmid transfer gene TraA	F (TraA-F1)	AGAGTTCATGCGTGACAACG	959	(Ocampo-Sosa *et al.*, 2007)
		R (TraA-R1)	GTCCACAGGTCACCGTTCTT		

Table 2.3: PCR primers used for identifying microorganisms within respiratory grouped samples. PCR primers highlighted were used against all samples.

Microbe	Gene target	Primer name	Primer sequence (5' to 3')	Expected size (bp)	Reference
16S	16S rRna	F (16SF) R (16SR)	GGATTAGATACCCBBGTAGTCC GACGTCRTCCNCDCCTTCCTC	410	(Boye *et al.*, 1999)
S. zooepidemicus	sorD	F (F3) R (B3)	ATGGCCTCTGAGGCAGG TCTGGTCAACGGTTTTTCCT	220	(Kinoshita *et al.*, 2014)
S. equisimilis	Streptokinase precursor gene	F (*eqsim*) R (*eqsim*)	TCAAATCGGTTGGCACAGAC CGTCCTTAGCATAGAAGGATTGG	279	(Preziuso *et al.*, 2010)
S. equi	Superantigenic toxin	F (*seel*F) R (*seel*R)	GAAGGTCCGCCATTTTCAGGTAGTTTG GCATACTCTCTCTGTCACCATGTCCTG	520	(Alber *et al.*, 2004)
S. zooepidemicus	Putative sortase	F (*srtz*) R (*srtz*)	GCTGACTGATCAAGCCACAA AACTGATCGTGTTCCCAAGC	217	(Patty, 2012)
S. equi equi	eqbE	F (*eqbE* F) R (*eqbE* R)	GATGCTTTCAGGGGATTGGA CACCTCATCCCATCTTGTTCG	342	(Patty, 2017)
Equine herpesvirus-1	Glycoprotein B	F (EHV-1.F) R (EHV-1.R)	GGGAAAGCCGGCATCATC CCGTGAAGTTTCTCCCAAGG	326	(Hu *et al.*, 2014)
	Thymidine Kinase	F (TkF4) R (TkR2)	CCTTGGTTCCTTTGGCGAC GCAC CTGGCGAG AACGCT ACCC	226-268	(Carvalho *et al.*, 2000)
Equine herpesvirus-4	Glycoprotein B	F (EHV-4.F) R (EHV-4.R)	TTACAAAACTCTATGCGCTCCTC TAGGTGCTGATCACTTCGATTTCA	269	(Hu *et al.*, 2014)
	Thymidine Kinase	F (TkF5) R (tkR6)	TTGGGCCGTGGCCGAAAAC CTAGCCAAAACCTTGCCT	333	(Carvalho *et al.*, 2000)
Rhodococcus equi	Cholesterol Oxidase	F (CHO-F) R (CHO-R)	GCTCGCTTCCAGTTCAATTC AGCGGGTGGTATGTGAAGTC	191	(Cursons, 2017)

Microbe	Gene target	Primer name	Primer sequence (5' to 3')	Expected size (bp)	Reference
VAP	Virulence plasmid A	F (VapAF) R (VapAR)	GACTCTTCACAAGACGGT TAGGCGTTGTGCCAGCTA	200	(Ocampo-Sosa *et al.*, 2007)
PvapA	Virulence associated protein A	F (PvapAF) R (PABR)	GAGCAAGCGATACCGCCGG CTGGATATGGCCGAGGAAGC	286	(Ocampo-Sosa *et al.*, 2007)
PvapB	Virulence associated protein B	F (PvapBF) R (PABR)	TGCGATAGCCACAGCCGCT CTGGATATGGCCGAGGAAGC	477	(Ocampo-Sosa *et al.*, 2007)
TraA	Conjugal plasmid transfer gene TraA	F (TraA-F1) R (TraA-R1)	AGAGTTCATGCGTGACAACG GTCCACAGGTCACCGTTCTT	959	(Ocampo-Sosa *et al.*, 2007)

Table 2.4: PCR primers used for identifying microorganisms within gastrointestinal grouped samples. PCR primers highlighted were used against all samples.

Microbe	Gene target	Primer name	Primer sequence (5' to 3')	Expected size (bp)	Reference
16S	16S rRna	F (p201) R (DG74)	GAGGAAGGIGIGGAIGACGT AGGAGGTGATCCAACCGCA	201	(Tseng *et al.*, 2003)
Salmonella (Genus)	Invasion protein (InvA)	F (*invA*-156F) R (*invA*-288R)	CATTTCTATGTTCGTCATTCCATTACC AGGAAACGTTGAAAAACTGAGGATTCT	132	(Pusterla *et al.*, 2010)
L. intracellularis	aspA	F (aaL-F) R (aaL-R)	TCTGCTGCCATTGTGACTCT CTCTCAAGTGGTCCTCGCTG	195-329	(Wattanaphansak *et al.*, 2010)
TcdA	Enterotoxin A	F (tcdA F) R (tcdA R)	GGTAATAATTCAAAAGCGGCT AGCATCCGTATTAGCAGGTG	201	(Luna *et al.*, 2011)
TcdB	Cytotoxin B	F (398CLDs) R (399CLDas)	GAAAGTCCAAGTTTACGCTCAAT GCTGCACCTAAACTTACACCA	177	(van den Berg *et al.*, 2005)
C. sordellii	Phospolipase C of *C. sordellii*	F (CSP-09F) R (CSP-09R)	TGGGATGATTGGGATTATTCAG TCAGTTCCTGCATATTCATTGT	176	(Bhatnagar *et al.*, 2012)
C. perfringens	Phospolipase C of *C. perfringens*	F (PL3-F) R (PL7-BR)	AAGTTACCTTTGCTGCATAATCCC ATAGATACTCCATATCATCCTGCT	283	(Bhatnagar *et al.*, 2012)
C. perfringens	CPE	F (CPE-F) R (CPE-R)	GGTTCATTAATTCAAACTGGTG AACGCCAATCATATAAATTACAGC	154	(Cursons, 2017)
C. perfringens	netF	F (net-F) R (net-R)	AACAATATGTACAGGTATAACT TTGATAGGTATAATATGGTTCT	862	(Prescott *et al.*, 2016)
Rhodococcus equi	Cholesterol Oxidase	F (CHO-F) R (CHO-R)	GCTCGCTTCCAGTTCAATTC AGCGGGTGGTATGTGAAGTC	191	(Cursons, 2017)
VAP	Virulence plasmid A	F (VapAF)	GACTCTTCACAAGACGGT	200	(Ocampo-Sosa *et al.*,

Microbe	Gene target	Primer name	Primer sequence (5' to 3')	Expected size (bp)	Reference
		R (VapAR)	TAGGCGTTGTGCCAGCTA		2007)
PvapA	Virulence associated protein A	F (PvapAF) R (PABR)	GAGCAAGCGATACCGCCGG CTGGATATGGCCGAGGAAGC	286	(Ocampo-Sosa *et al.*, 2007)
PvapB	Virulence associated protein B	F (PvapBF) R (PABR)	TGCGATAGCCACAGCCGCT CTGGATATGGCCGAGGAAGC	477	(Ocampo-Sosa *et al.*, 2007)
TraA	Conjugal plasmid transfer gene TraA	F (TraA-F1) R (TraA-R1)	AGAGTTCATGCGTGACAACG GTCCACAGGTCACCGTTCTT	959	(Ocampo-Sosa *et al.*, 2007)

2.6.2 PCR reaction set up

Either a PCR containing one set of primers (uniplex) or multiple sets of primers (multiplex) was used for identifying microorganisms in the clinical samples received. All PCRs were made up in a dedicated DNAse free hood (ESCO). For most uniplex PCRs, in a 300µl volume PCR tube (Axygen® unless otherwise stated), 200µl of 2mM master mix (**Table A2.1**), 1µl of Solis BioDyne Hot FIREPol® DNA polymerase, and 4µl of a mixture of forward and reverse primer (10µl of forward, 10µl of reverse, and 80µl TE stored at 4°C) was combined (**Table 2.5**). Primers were at 20mM concentration unless otherwise stated. The reagents were gently mixed using a sterile pipette and 25µl aliquoted into eight separate PCR tubes. PCR mixes were stored at -20°C until required for diagnosis. To each tube 1µl of template (100ng/µl concentration) was added. This uniplex PCR setup was used for identification of *Staphylococcus* genus, *S. aureus*, *E. coli*, Coliforms, *Bacillus* genus, *Pseudomonas* genus, *Enterococcus* genus, Panfungal, EHV-1, EHV-4, *R. equi* (CHO, VAP, VapA, VapB, TraA), *L. intracellularis*, *C. sordellii*, and *C. perfringens* (PLC, CPE).

Table 2.5: PCR set up for all uniplex reactions.

PCR set up for uniplex reactions	
200µl	2mM master mix
4µl	F & R primer mix (each at 20µM)
1µl	Hot FIREPol® Taq polymerase

For the uniplex PCR carried out for the identification of Gram positive and Gram negative microbes, the Taq polymerase required sterilization to reduce unspecialized amplification. The same setup was used (**Table 2.5**), except 2µl of ethidium monoazide bromide was also added before aliquotting 25µl into clear PCR tubes (MULTIMAX™) with the UV hood lights off. These clear tubes were subsequently placed in a dark cabinet for 10 minutes to bind any endogenous DNA and prevent amplification, and following this left on a white light box near a window for 15 minutes to

inactivate the ethidium monoazide bromide. PCR mixes were stored at -20°C until required for diagnosis.

For the multiplex PCRs carried out to identify multiple microorganisms in each reaction, each PCR was made up in a dedicated DNAse free hood (ESCO). Depending on the multiplex being used, various amounts of 2mM master mix (**Table A2.1**) or 4mM master mix (**Table A2.2**) were used, along with 1µl of Solis BioDyne Hot FIREPol® DNA polymerase and different combinations of primers (**Table 2.6** to **Table 2.10**).

Table 2.6: PCR set up for respiratory multiplex A.

Respiratory Multiplex A		
184µl	4Mm master mix	Expected amplicon size
6µl	seel (equi) F & R (10µM each)	520bp
3µl	soda (equi/zoo) F & R (10µM each)	235bp
3µl	Equisim (equisimilis) F & R (10µM each)	279bp
2µl	16S F & R (20uM each)	410bp
2µl	Hot FIREPol® Taq polymerase	

Table 2.7: PCR set up for respiratory multiplex B.

Respiratory Multiplex B		
187µl	2mM master mix	Expected amplicon size
6µl	EqbE (equi) F & R (10µM each)	324bp
6µl	sorD (zoo) F & R (10µM each)	220bp
1µl	Hot FIREPol® Taq polymerase	

Table 2.8: PCR set up for respiratory multiplex C.

Respiratory Multiplex C		
194µl	2mM master mix	Expected amplicon size
4µl	EHV-1 F & R (20µM each)	326bp
1µl	EHV-4 F & R (20µM each)	269bp
1µl	Hot FIREPol® Taq polymerase	

Table 2.9: PCR set up for *Salmonella* spp. multiplex.

16S *Salmonella* Multiplex		
200µl	2mM master mix	Expected amplicon size
4µl	16S Faecal F & R (20µM each)	400bp
4µl	SiiA (Salmonella) F & R (20µM each)	132bp
1µl	Hot FIREPol® Taq polymerase	

Table 2.10: PCR set up for *C. difficile* multiplex.

***C. difficile* Multiplex**		
200µl	2mM master mix	Expected amplicon size
4µl	*C. difficile* Tox A F & R (20µM each)	201bp
4µl	*C. difficile* Tox B F & R (20µM each)	177bp
1µl	Hot FIREPol® Taq polymerase	

Table 2.11: PCR cycling conditions for sterile and gastrointestinal samples (Excluding EHV-1 and EHV-4).

Lid at 105°C		
1.	95°C	15 minutes
2.	95°C	15 seconds
3.	60°C	15 seconds (-1°C per cycle)
4.	72°C	45 seconds
5.	Go to step 2 (10x)	
6.	95°C	15 seconds
7.	50°C	15 seconds
8.	72°C	45 seconds
9.	Go to step 6 (35x)	
10.	72°C	5 minutes

Table 2.12: PCR cycling conditions for respiratory samples (Including EHV-1 and EHV-4)

Lid at 105°C		
1.	95°C	15 minutes
2.	95°C	15 seconds
3.	65°C	15 seconds (-1°C per cycle)
4.	72°C	20 seconds
5.	Go to step 2 (10x)	
6.	95°C	15 seconds
7.	55°C	15 seconds
8.	72°C	20 seconds
9.	Go to step 6 (35x)	
10.	72°C	5 minutes

2.6.3 Visualising results

2.6.3.1 Gel electrophoresis

Following PCR, 10µl of each PCR product were mixed via pipette with 2µl 6x loading dye (See Appendix 1) and loaded into a 2% agarose gel (HydraGene LE Agarose) made with 1x Super Buffer (See Appendix 1) with a final concentration of 0.00075mg/mL EtBr. Samples were run together with 2.5µl of a Solis BioDyne 100bp molecular weight ladder (See Appendix 1) or 10µl of a Genscript 100bp molecular weight ladder (See Appendix 1). The gel was run in a dedicated OWL electrophoresis tank (ThermoFisher Scientific) containing 1x Super Buffer (See Appendix) with a LighteningVolt OSP250-L power supply at 200V and 500mA for 15 minutes at room temperature. The gel was viewed using a TFX-35M GIBC UV transilluminator (ThermoFisher Scientific) and imaged as a .TIFF file using Omega Lum™ G imager (Aplegen®, USA).

2.6.3.2 Restriction digest

For the confirmation of EHV-1 and EHV-4 using the specific gpB primers, a restriction digest was required, where HincII (New England Biolabs) was used to cut EHV-1 and HindIII (Boehringer Mannheim, Germany) used to cut EHV-4. To achieve this, 10µl of PCR products were transferred into a new PCR tube and 1µl of the required restriction enzyme added. This was then incubated at 37°C for 4-12 hours with a DNA engine peltier thermal cycler (BioRad). Confirmation of successful enzyme cutting was measured using gel electrophoresis with a 2% Super Buffer gel at 200V for 15 minutes and visualised using a UV transilluminator, as mentioned above.

2.6.3.3 Quantification of amplified DNA

DNA quantification was calculated by estimating the band intensity in the gel by eye. The brightness of each PCR product band was compared to the known DNA concentrations of the Genscript or Solis BioDyne ladder (**Figure A1.1** and **Figure A1.2**).

2.6.4 Preparation of samples for DNA sequencing and sequencing analysis

2.6.4.1 PCR purification for optimal sequencing results

To 15µl of PCR product, 1µl of Fast AP shrimp alkaline phosphatase (Roche, Germany) and 0.5µL of exonuclease (Roche, Germany) were added. Each tube was then placed into a BioRad T100 thermocycler and incubated at 37°C for 15 minutes, followed by 85°C for 15 minutes. Once complete, samples were prepared for sending to either the Waikato DNA Sequencing Facility (Hamilton, NZ) or the Massey University Genome services (Palmerston North, NZ) for sequencing.

2.6.4.2 Waikato DNA Sequencing Facility

Following ExoSap samples were sent to Waikato DNA Sequencing Facility (Hamilton, NZ). Forward and reverse primers were diluted to 5pmol/µl in TE, the template concentration was reported in ng/µl using gel estimate quantification method, and PCR product size was reported from known and expected bp length. These details were critical for successful sequencing reactions in order to get consensus sequences that could be used to determine the accuracy of primers. Waikato DNA Sequencing Facility uses dye chain terminator sequencing through Applied Biosystems 3130xl Genetic Analyser and BigDye Terminator 3.1 reaction mix (ThermoFisher Scientific).

2.6.4.3 Massey University Genome Service (Palmerston North, NZ)

Following purification, PCR product were diluted to 2.5ng for every 100bp, following the table from Massey University Genome Service (**Table 2.11**). After dilution, 1µl of each sample was placed into two PCR tubes (labelled forward and reverse). Primers were diluted to 4pmol by diluting 1µl of 200pmol in 50µl of TE buffer (10mM Tris 1mM EDTA). To the respective labelled tube, 1µl of diluted forward or reverse primer was added and each were topped up with 18µl of double distilled water, making the final volume the 20µl. Samples were analysed using ABI3730 DNA with BigDye Terminator 3.1 reaction mix (ThermoFisher Scientific).

Table 2.11: Dilution table for Massey University Genome Service PCR products.

PCR product template	**Template Total Quantity (in final volume)**	**Primer Total Quantity (in final volume)**	**Final volume required**
100-200bp	1.25-5ng	4pmol	20µl when using own primer
200-500bp	5-12.5ng		
500-1000bp	12.5-25ng		

2.6.4.4 Analysis of PCR product sequencing

ABI files received from either Waikato DNA Sequencing Facility or Massey Genome Services were opened in Geneious (Geneious® 7.1.8, Biomatters Limited). Sequences were trimmed to remove areas of low quality from each the forward and the reverse sequence. The forward and reverse sequences were then aligned using a pairwise global alignment with free end gaps (gap open penalty of 12 and gap extension penalty of 3). These aligned sequences were then used to search nucleotide databases using basic local alignment search tool (BLAST) found at the National Center for Biotechnology Information (NCBI) or the DNA Databank Japan (DDBJ). BLAST engines were used to carry out blastn through NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and DDBJ (http://blast.ddbj.nig.ac.jp/). Results allowed the accuracy of primers to be determined, if the search returned a high quality match to the species that was primed for then it was considered to be of sufficient quality to be used as a primer for future PCRs. Additionally, BLAST allowed the pathogenic species at a species level to be determined, that would otherwise be indistinguishable using genus specific primers.

Chapter Three

RESULTS

Results presented are from molecular and traditional methodologies used to identify the cause of infection in horses, and measure their efficacy. In addition, sequencing was carried out to assess and to confirm the sensitivity and reliability of primers used in the identification of infectious microbes.

Clinical samples (n=146) were obtained for this study and DNA was isolated from them for the detection of pathogens. Results from these experiments have been divided into three major groups for analysis. Sterile samples, which include blood, pus, and synovial fluids retrieved aseptically from sites that would normally have no microbes present in healthy animals. Respiratory samples, which includes nasal swabs, washes and saline from around stored nasal swabs. Gastrointestinal samples, which includes faecal samples, faecal swabs and 50 randomised DNA samples that had been submitted for previous gastrointestinal tests were revisited. Lastly, culture samples which included samples received as a culture or samples cultured in Columbia broth overnight. These were streaked on a blood agar plate, and observed for growth and morphological characteristics of the microbes.

Age groups were divided based on stages of development and estimated susceptibility to infection (**Table 3.1**).

Table 3.1: Age classifications of equine samples. Used to analyse infection trends associated with age or developmental stage.

Age Group	Age
Newborn	Birth – 1 week
Neonate	1 - 4 weeks
Foal	1 - 3 months
Weanling	3 - 12 months
Yearling	1 -2 years
2+	2+ years
N/A	No age data available

The success of the PCR positive results obtained were determined by comparing amplicon size to a 100bp ladder and ensuring concurrence between expected PCR amplicon sizes determined and primer information and design. Selected amplicons of the correct size were also submitted for sequencing to confirm the accuracy of the primers designed.

3.1 Culture results

Selected sterile samples were cultured on blood agar and underwent more traditional diagnostic approaches to determine the identity of the microorganisms within the clinical samples (**Table 3.2**).

Table 3.2: Culture results from Columbia Broth growth, Gram staining, and morphological growth characteristics on blood agar.

Sample ID	Culture details	Gram stain		Growth on blood agar		Identification
		Gram positive	Gram negative	Haemolytic	Non haemolytic	
S001	No growth	N/A	N/A	N/A	N/A	N/A
S002	Grown and streaked on blood agar. Colonies appear slightly haemolytic suggesting *Staphylococcus*. DNA extracted from single colony and PCR performed.	N/A	N/A	+	-	Coagulase negative *Staphylococcus* spp.
S004	Blood culture grew coagulase negative *Staphylococcus* species. Extracted single colony proved difficult to lyse for DNA extraction.	+	-	N/A	N/A	Coagulase negative *Staphylococcus* spp.
S005	Did not grow - possibly anaerobic.	N/A	N/A	N/A	N/A	N/A
S008	Culture grew readily on blood agar with teardrop colonies (suggest Gram negative species). Gram stain showed cocci and potential rods, colonies were successfully extracted and amplified. Culture was streaked on an amikacin plate and grew *Staphylococcus*.	+	+	N/A	N/A	*Staphylococcus* spp.
S009	Gram stain showed small Gram positive cocci.	+	-	N/A	N/A	Unidentified Gram positive cocci
S011	Gram stain showed single small Gram positive cocci. Unable to grow on plates.	+	-	N/A	N/A	Unidentified Gram positive cocci
S012	Small Gram positive cocci present in culture and Gram stain	+	-	N/A	N/A	Unidentified Gram positive cocci

Sample ID	Culture details	Gram stain		Growth on blood agar		Identification
		Gram positive	Gram negative	Haemolytic	Non haemolytic	
S013	Culture grew readily on blood agar and amikacin plates. Culture Gram stained and showed Gram positive cocci. Following this DNA was extracted from each plate and underwent PCR.	+	+	N/A	N/A	*Staphylococcus* spp.
S014	Culture grown from infected knee joint and coffin showed small, sparse Gram positive cocci.	+	-	N/A	N/A	Unidentified Gram positive cocci
S015	Didn't properly grow. Gram stain was unclear (large pink blobs, some very small Gram positive cocci).	+	-	N/A	N/A	Unidentified Gram positive cocci
S016	A 24 hour culture failed to change turbidity to indicate growth, and subsequent Gram stain was negative. Following a longer incubation culture flask had scummy growth around top which when Gram stained showed Gram positive cocci. DNA was extracted from scum and PCR results showed positive bands for *R. equi*, *Staphylococcus spp*, and *S. aureus*. The material was re-streaked onto a blood agar, and the following day was observed to be growing a pure haemolytic species, a single colony was Gram stained and showed Gram positive cocci.	+	-	+	-	*Staphylococcus* spp.
S022	Culture initially failed to grow in BacTech culture media (Provided by veterinarian) due to antibiotic application. Culture material was removed from BacTech and placed into Columbia broth overnight where it readily grew a significant colony of Gram positive rods.	+	-	N/A	N/A	*Bacillus* spp.

3.2 Gram stain results

Example of gram stain results from culture material that help to illustrate the morphological characteristics of bacteria and stain colour that were used to identify the types of microbes present at 100x oil immersion (**Figure 3.1** – **Figure 3.4**).

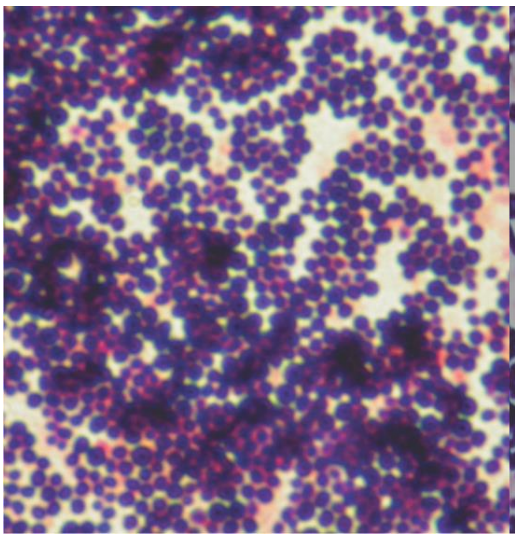


Figure 3.1: S016 Gram positive cocci.

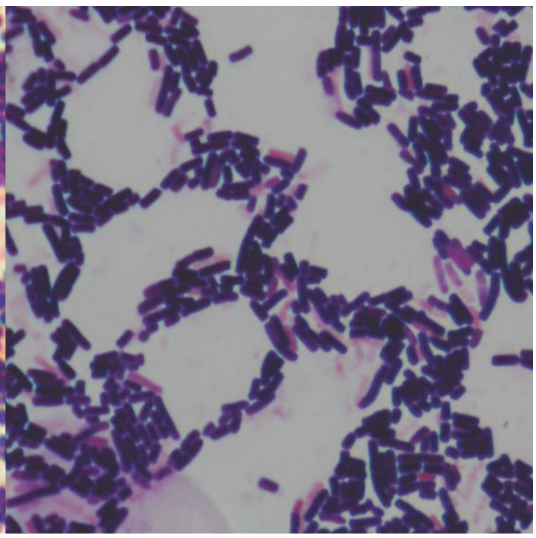


Figure 3.2: S023 Gram positive.

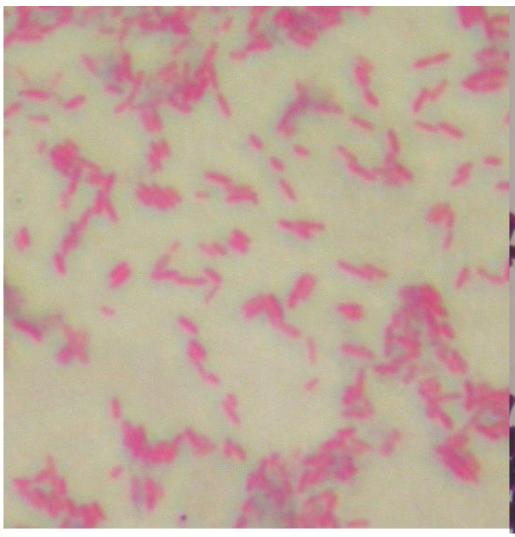


Figure 3.3: R042 Gram negative cocci.

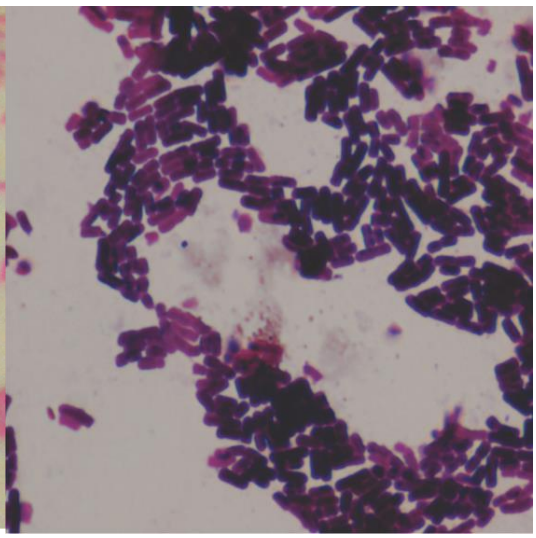
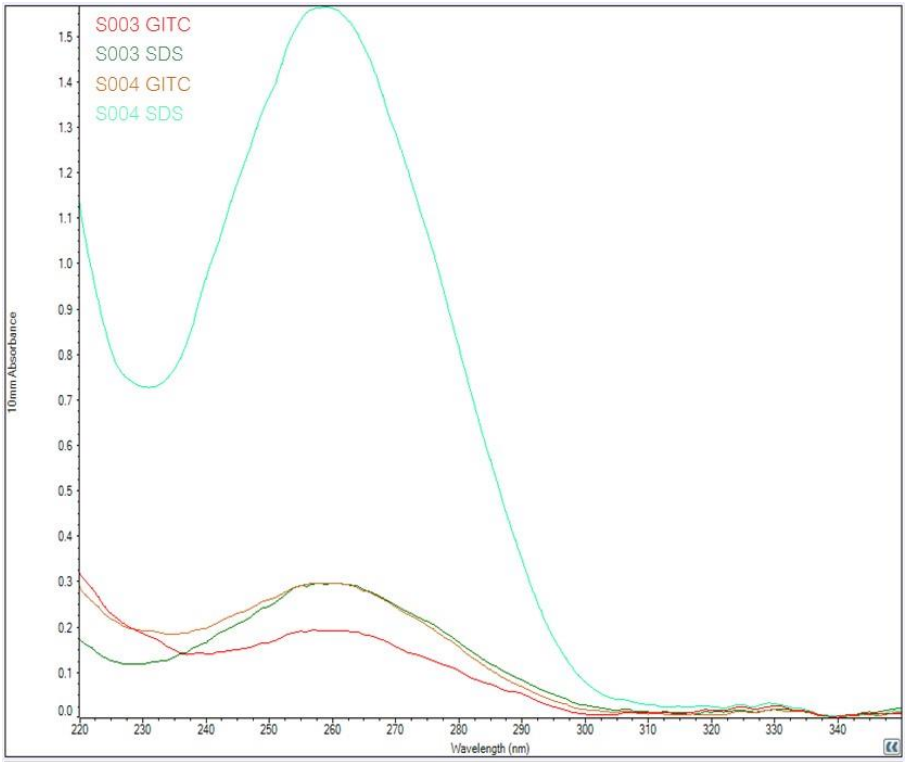


Figure 3.4: R039 Gram positive rods with spores.

3.3 Comparison of blood DNA isolation methods

Initial samples were run in tandem to compare different DNA extraction methods. A spectrophotometer was used to determine the efficiency of extraction methods by observing the purity and yield of DNA obtained. On average, the SDS lysis extraction method worked best and had increased DNA yields compared to the GITC extraction method (**Figure 3.5**). DNA purity measured by the NanoDrop™ 2000 also showed the SDS lysis extraction method had absorbance ratios at 260nm/280nm and 260nm/230nm which indicated little contamination. Samples with 260nm/280nm ratios between 1.8 and 2.0 and 260nm/230nm ratios between 2.0 and 2.2 were accepted as sufficient quality indicating little to no contamination from proteins or solvents used in the DNA extraction process.



#	Sample ID	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type	Factor
1	S003 GITC	9.4	ng/µl	0.188	0.101	1.86	1.03	DNA	50.00
2	S003 SDS	14.6	ng/µl	0.292	0.164	1.79	2.55	DNA	50.00
3	S004 GITC	14.6	ng/µl	0.293	0.154	1.90	1.55	DNA	50.00
4	S004 SDS	78.1	ng/µl	1.561	0.816	1.91	2.15	DNA	50.00

Figure 3.5: Spectrophotometer results comparing the GITC and SDS lysis DNA extraction methods. DNA quantity was established in ng/µl and measured absorbance ratios at 260nm/280nm and 260nm/230nm were indicative of purity.

3.4 Sterile sample microbe identification

Sterile samples were obtained from veterinarians within the Greater Waikato Region and around Auckland, New Zealand which had information on the age group and clinical details that presented (**Table 3.3**). Each of these samples had the DNA extracted and were screened by PCR to determine which microbial species or genus returned positive.

Table 3.3: Results from PCR test on sterile samples. Showing details of age, clinical details, and species returning PCR positive (indicated by "+").

Sample ID	Age group	Clinical details	Gram positive	Gram negative	*Staphylococcus* spp.	*S. aureus*	*Streptococcus* spp.	*Enterococcus* spp.	*E. coli*	Coliforms	Panfungal	*Pseudomonas* spp.	*Bacillus* spp.	*R. equi*	VAP	EHV-1	EHV-4
S001	Newborn	N/A	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
S002	Newborn	Low immunoglobulin G initially, cyanotic, hypovolemic collapsed with sudden onset diarrhoea	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
S003	Newborn	Mare's placenta thick and red. Foal weak and did not suckle. Dilated pupils which were non-responsive to light. Treated, bottle fed, antibiotic treatment. At 2 days foal became weaker, white blood count decreased. Mare had come from Australia where they had a few chlamydial abortions. Swab from mare's uterus grew *Streptococcus* spp. and *E. coli*	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-

Sample ID	Age group	Clinical details	Gram positive	Gram negative	*Staphylococcus* spp.	*S. aureus*	*Streptococcus* spp.	*Enterococcus* spp.	*E. coli*	Coliforms	Panfungal	*Pseudomonas* spp.	*Bacillus* spp.	*R. equi*	VAP	EHV-1	EHV-4
S004	Newborn	Meconium stained placentitis	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-
S005	Newborn	2 weeks overdue, small dummy foal	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-
S006	N/A	N/A	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-
S007	Newborn	Mare toxic/intestinal (caesarean)	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
S008	N/A	N/A	+	+	+	-	+	+	+	+	+	-	-	-	-	-	-
S009	Newborn	Diarrhoea	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
S010	N/A	N/A	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
S011	N/A	N/A	+	-	+	-	-	+	+	-	-	-	-	-	-	-	-
S012	Newborn	Septic joints	-	+	-	-	-	-	+	-	-	+	-	-	-	-	-
S013	Foal	Right front pastern swollen. Horse lame and febrile. NSFm radiographs and ultrasound showed heteroechoic fluid pocket subcutaneous but also possibly communicating with coffin joint. Incised area pocket, purulent material confirmed joint involvement	+	+	+	+	-	-	-	-	-	+	-	-	-	-	-
S014	Foal	Failure passive transfer (<200mg/dL) and neutrophilia, lame left front coffin. Started on systemic antibiotics and non-steroidal anti-inflammatory drugs which significantly improved lameness. Required 3L plasma to	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-

Sample ID	Age group	Clinical details	Gram positive	Gram negative	*Staphylococcus* spp.	*S. aureus*	*Streptococcus* spp.	*Enterococcus* spp.	*E. coli*	Coliforms	Panfungal	*Pseudomonas* spp.	*Bacillus* spp.	*R. equi*	VAP	EHV-1	EHV-4
		get immunoglobulin G over 800. Effusion right carpus, fluid returned no growth. Ultrasound consistent with moderate pneumonia and omphalophlebitis. Developed patent urachus. Systemic antibiotics changed from penicillin/gentamicin to ceftazidime. Joints treated with lavage and intraarticular aminoglycosides. Cell counts increased to 180. Gentamicin added back in systemically. Cell count then plateaued around 80. More aggressive lavage and debridement of carpus did not improve cell count. Never lame on knee. Intermittent moderately lame on coffin. Synovial fluid had degenerative neutrophils at first but now nondegenerate. Ultrasound lungs and umbilicus and bloodwork relatively unremarkable. Now on chloramphenicol. Foal has every reason to have developed septic joints, but I have not been able to culture anything. No changes on radiographs. The cell count and appearance of cells (and history) support septic arthritis, though degree of lameness																

Sample ID	Age group	Clinical details	Gram positive	Gram negative	*Staphylococcus* spp.	*S. aureus*	*Streptococcus* spp.	*Enterococcus* spp.	*E. coli*	Coliforms	Panfungal	*Pseudomonas* spp.	*Bacillus* spp.	*R. equi*	VAP	EHV-1	EHV-4
		does not															
S015	Foal	Acutely lame, right leg stifle effusion, started on systemic penicillin and gentamicin. Infection progressed to pneumonia, *R. equi* detected via tracheal wash sample	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
S016	Newborn	Pneumonia and diarrhoea - on antibiotics	-	-	+	-	-	-	-	-	-	+	-	+	-	-	-
S017	N/A	N/A	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
S018	N/A	Potbelly, swollen LH distal limb, temp: 38.7°C. RH distal limb swelling and temp: 38.7°C. Treated with penicillin and gentamicin for 3 days and it resolved. Serum amyloid A:3000, white blood count: 13.5X10g, Fibrogen:6, low total protein and albumin, chest and abdominal ultrasound found free fluid in both. Increased amount of comet tails. No thickened intestines	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-
S019	Yearling	Swab from burst submandibular abscess. Potential Strangles. Mild temperature elevation (38.1°C vs normal 37.6°C)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S020	Yearling	Submandibular abscess	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
S021	Yearling	Temperature at 38.6°C, swelling under jaw with draining fluid. No nasal discharge.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Sample ID	Age group	Clinical details	Gram positive	Gram negative	*Staphylococcus* spp.	*S. aureus*	*Streptococcus* spp.	*Enterococcus* spp.	*E. coli*	Coliforms	Panfungal	*Pseudomonas* spp.	*Bacillus* spp.	*R. equi*	VAP	EHV-1	EHV-4
		Bright and responsive															
S022	Yearling	Abscessed lymph node under jaw	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S023	Neonate	Acute diarrhoea 17.2.17, temp was 40°C. Treated with calefour. 18.2.17 temp at 39.2°C	+	+	-	-	-	-	-	-	-	-	+	+	+	-	-

From the 23 animals screened (**Table 3.3**), 86.96% of samples returned positive for one or more PCR result, indicating successful amplification and confirmation of the presence of microbes. These positive samples were analysed to determine trends of infection types (**Figure 3.6**). Of the 23 sterile sample types received and analysed by PCR, Gram positive pathogens (n=16) had the highest occurrence rate, with the potential to cause systemic or localised infection to horses of all age groups. Gram negative pathogens were the next most frequent (n=11) within the sample group. At the genus level it was *Staphylococcus* spp. that were observed to have the highest frequency (n=10). There were no observed PCR positives for either EHV-1 or EHV-4.

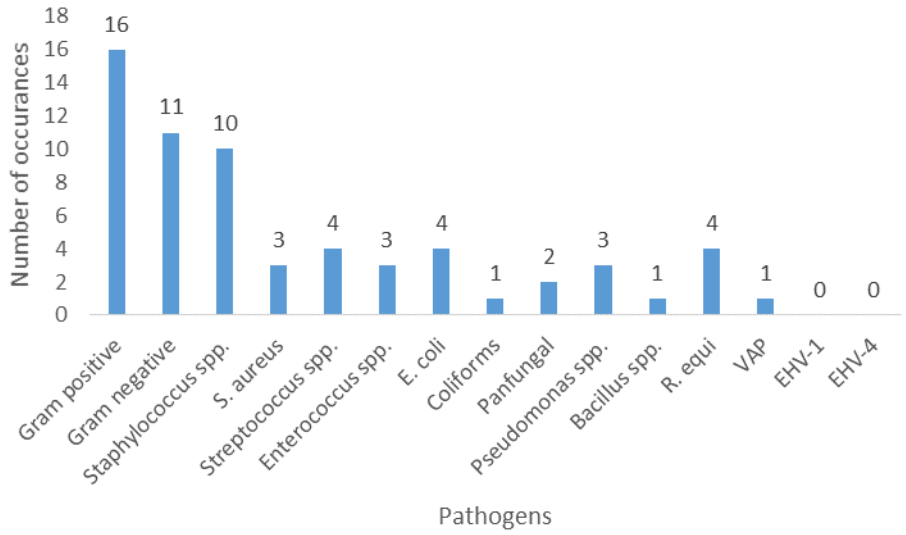


Figure 3.6: Sterile pathogens PCR results. Occurrences represent the frequency of infection across samples received (n=23).

To assess the frequency of polymicrobial infections, further analysis of **Table 3.3** showed 43.48% of tested sterile samples returned PCR positive for both Gram positive and Gram negative infections. Excluding PCR results for Gram positive and Gram negative primers, the frequency PCR positives was assessed from information in **Table 3.3** and presented in **Figure 3.7**, showing 10/23 (43.48%) sterile samples returned PCR positive for 2 or more genus or species specific and a maximum of 6 unique PCR positives within a single sample.

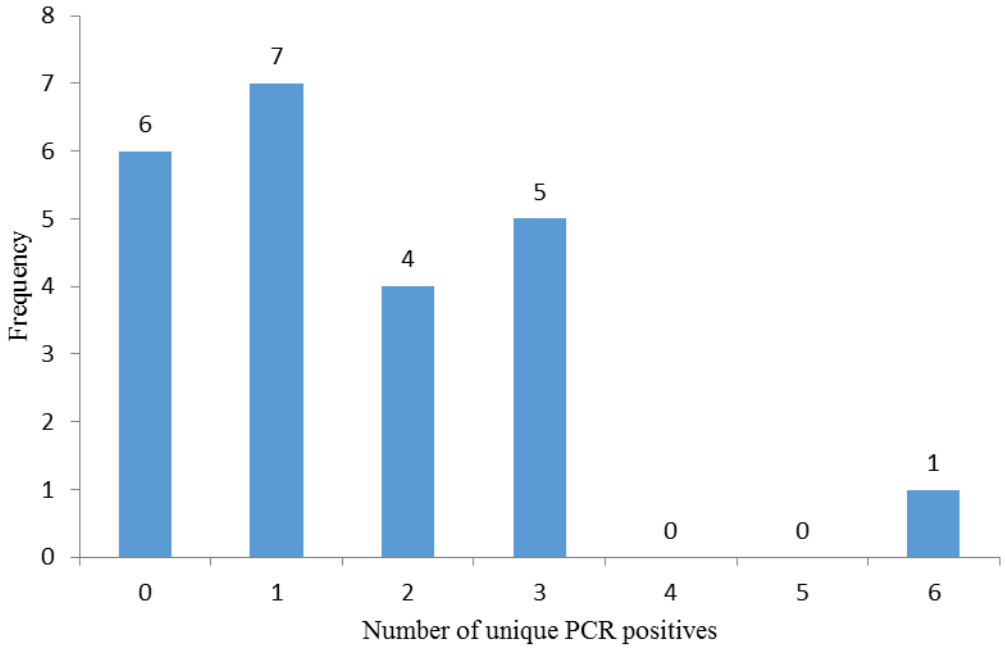


Figure 3.7: Frequency of polymicrobial infections within sterile sample group where 1 equals PCR positive for 1 pathogen specific primer and 6 equals PCR positive for 6 pathogen specific primers. Data excludes PCR positives for Gram positive and Gram negative bacterial species.

The largest age group represented within the sterile samples was newborns (n=9), where 88.89% of these returned a positive result for one of the primer sets used in the study (**Table 3.4**). Yearlings had the lowest percent positive results for a single age group at only 25%.

Table 3.4: Frequency of PCR positive results by age in sterile grouped samples.

Age Group	Occurrence of positive PCR	Total count of age group (n=23)	% Positive
Newborn	8	9	88.89
Neonate	1	1	100.00
Weanling	0	0	0.00
Foal	2	3	66.67
Yearling	1	4	25.00
2+	0	0	0.00
N/A	5	6	83.33

This data was presented showing the total distribution of infection with age groups (**Figure 3.8**). No strong observable trends correlating with age to certain infection types were seen.

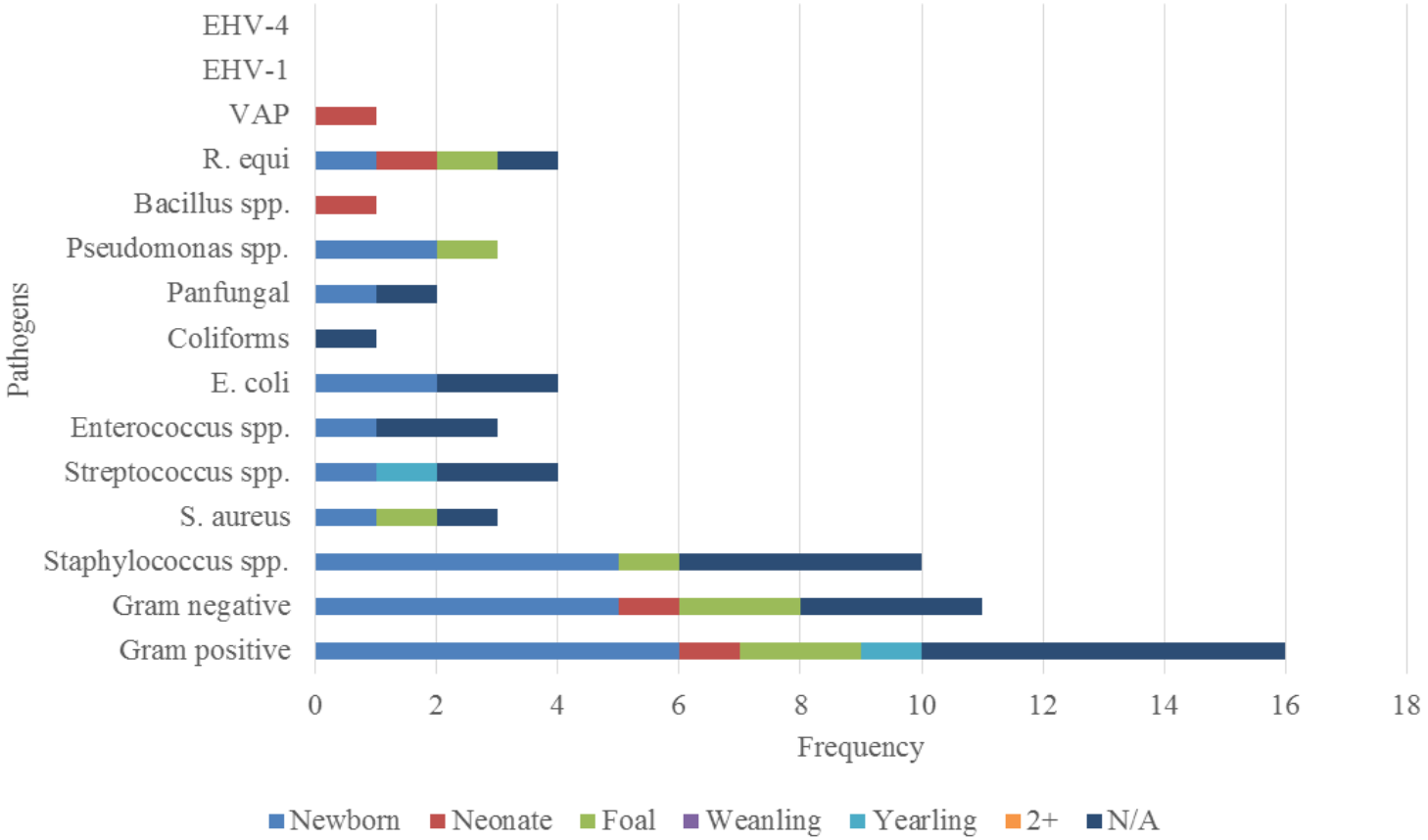


Figure 3.8: Total distribution of microorganisms within sterile samples by age group.

3.5 Respiratory sample microbe identification

Respiratory samples were obtained from veterinarians within the Greater Waikato Region and around Auckland, New Zealand which had information on the age group and clinical details that presented with suspected infection (**Table 3.5**). Each of these samples had the DNA extracted and were screened by PCR to determine which microbial species or genus returned positive.

Table 3.5: Results from PCR test on respiratory samples, excluding PCR positive 16S which returned positive for all samples due to natural respiratory flora. Details of age, clinical details, and microbial species returning PCR positive (indicated by "+") are shown.

Sample ID	Age group	Clinical details	*S. equi equi*	*S. equisimilis*	*S. zooemidemicus*	EHV-1	EHV-4	*R. equi*	VAP
R001	N/A	Exposed to horse with possible Strangles	-	-	-	-	-	-	-
R002	N/A	Exposed to horse with possible Strangles	-	-	-	-	+	-	-
R003	N/A	Exposed to horse with possible Strangles	-	-	-	-	+	-	-
R004	N/A	Exposed to horse with possible Strangles	-	-	-	-	-	-	-
R005	N/A	Exposed to horse with possible Strangles	-	-	-	-	-	-	-
R006	N/A	Exposed to horse with possible Strangles	-	-	-	+	-	-	-
R007	N/A	Exposed to horse with possible Strangles	-	-	+	-	-	-	-
R008	N/A	Exposed to horse with possible Strangles	-	-	-	-	-	-	-
R009	N/A	Exposed to horse with possible Strangles	-	-	+	-	-	-	-

Sample ID	Age group	Clinical details	*S. equi equi*	*S. equisimilis*	*S. zooemidemicus*	EHV-1	EHV-4	*R. equi*	VAP
R010	N/A	Exposed to horse with possible Strangles	-	-	+	-	-	-	-
R011	Foal	Snotty nose	-	-	+	-	-	-	-
R012	2+	Exposed to horse with possible Strangles	-	-	-	-	-	-	-
R013	N/A	N/A	-	-	+	-	-	-	-
R014	N/A	Possible Strangles	-	-	-	-	+	-	-
R015	N/A	Possible Strangles	-	-	-	-	+	-	-
R016	N/A	Possible Strangles	-	-	-	-	+	-	-
R017	N/A	Foal with purulent nasal discharge	-	-	+	+	-	-	-
R018	2+	Nasal discharge	-	+	-	-	-	-	-
R019	Yearling	Nasal discharge	-	-	-	-	-	-	-
R020	Yearling	Bilateral epistaxis, tachycardia, neurological (Mini breed)	-	+	+	-	-	-	-
R021	N/A	Normal foal, due to go home and want to check all is fine before it leaves since damn has nasal discharge	-	+	+	-	-	-	-
R022	2+	Nasal discharge and inflamed larynx	-	-	-	-	-	-	-
R023	2+	Nasal discharge	-	+	-	-	-	-	-
R024	N/A	Swollen right side of face - came from sales	-	+	-	-	-	-	-
R025	2+	Nasal discharge	-	+	-	-	-	-	-
R026	N/A	Suspected *S. equi equi*	-	-	-	-	-	-	-
R027	N/A	Suspected *S. equi equi*	-	-	-	-	-	-	-

Sample ID	Age group	Clinical details	*S. equi equi*	*S. equisimilis*	*S. zooemidemicus*	EHV-1	EHV-4	*R. equi*	VAP
R028	N/A	Suspected *S. equi equi*	-	-	-	-	-	-	-
R029	2+	Swollen nares and mild nasal discharge	-	+	+	-	-	-	-
R030	2+	Lymphadenopathy - submandibular and nasal discharge	-	+	-	-	-	-	-
R031	Yearling	Previous positive for *S. equi equi* - has now had 1x negative guttural pouch wash and 1x negative swab - this is the 3rd test	-	-	-	-	-	-	-
R032	2+	N/A	-	+	-	-	-	-	-
R033	2+	Nasal discharge	-	-	+	-	-	-	-
R034	N/A	Purulent nasal discharge, slightly increased respiratory sounds, otherwise bright and responsive, recently weaned	-	-	+	-	-	-	-
R035	N/A	Severe serious to mucoid bilateral nasal discharge. Swollen submandibular lymph nodes. Owner reports coughing. Rest of clinical within normal limits	-	-	-	-	-	-	-
R036	2+	Horse had bad respiratory infection in early January. Lingering bilateral nasal discharge. Endoscopic exam showed inflammation of pharynx and mucopus. Guttural pouches clean	-	-	-	-	-	-	-
R037	2+	Clear nasal discharge and cough, normal temperature and lung sounds	-	-	-	-	-	-	-
R038	2+	Seropurulent nasal discharge temperature and lung sounds normal	-	-	-	-	-	-	-
R039	N/A	Coughing, bilateral purulent nasal discharge.	-	+	-	-	-	+	-
R040	2+	Suspected *S. equi equi*	-	-	-	-	-	-	-
R041	N/A	Pyrexia, purulent nasal discharge and cough	-	-	+	-	+	+	-
R042	N/A	Pyrexia, purulent nasal discharge and cough. Tracheal wash - Pneumonia, nasal swab previously submitted	-	-	+	-	+	+	-
R043	2+	Bilateral nasal discharge neutropenia and no fever	+	-	-	-	-	+	-

Sample ID	Age group	Clinical details	*S. equi equi*	*S. equisimilis*	*S. zooemidemicus*	EHV-1	EHV-4	*R. equi*	VAP
R044	N/A	Swollen submandibular lymph nodes and pyrexia	-	-	-	-	-	+	-
R045	N/A	Copious nasal discharge, no temperature	+	-	-	-	-	+	+
R046	2+	N /A	-	-	+	-	-	-	-
R047	N/A	N/A	-	-	+	-	-	-	-
R048	Weanling	Has nasal discharge and increased respiratory sounds	-	-	-	-	-	+	-
R049	Weanling	Has nasal discharge and increased respiratory sounds. Has been coughing	-	-	-	-	+	-	-
R050	Weanling	Coughing, mild nasal discharge and increased lung sounds	-	-	+	-	-	+	-
R051	Weanling	Snotty nose, increased chest sounds, just been weaned, some are coughing	-	-	+	-	-	-	-
R052	Weanling	Snotty nose, increased chest sounds, just been weaned, some are coughing	-	-	+	-	-	-	-
R053	Weanling	Snotty nose, increased chest sounds, just been weaned, some are coughing	-	-	+	-	-	-	-
R054	Weanling	Snotty nose, increased chest sounds, just been weaned, some are coughing	-	-	+	-	-	-	-
R055	Weanling	Snotty nose, increased chest sounds, just been weaned, some are coughing	-	-	+	-	+	-	-
R056	Weanling	Snotty nose, increased chest sounds, just been weaned, some are coughing	-	-	+	-	-	-	-
R057	2+	Febrile, nasal discharge, no cough, painful pharynx, 39.5°	-	-	-	-	-	+	-

From the 57 animals screened (**Table 3.5**), 71.93% of samples returned positive for one or more PCR result, indicating successful amplification and confirmation of the presence of microbes. These positive samples were analysed to determine trends of infection types (**Figure 3.9**). Of the 57 respiratory sample types received and analysed by PCR, *S. zooepidemicus* (n=22) had the highest occurrence rate, with the potential to cause systemic or localised infection to horses of all age groups. *S. equisimilis* (n=11) was the next most frequent, with EHV-4 (n=9) and *R. equi* (n=9) both having the third most frequent rate.

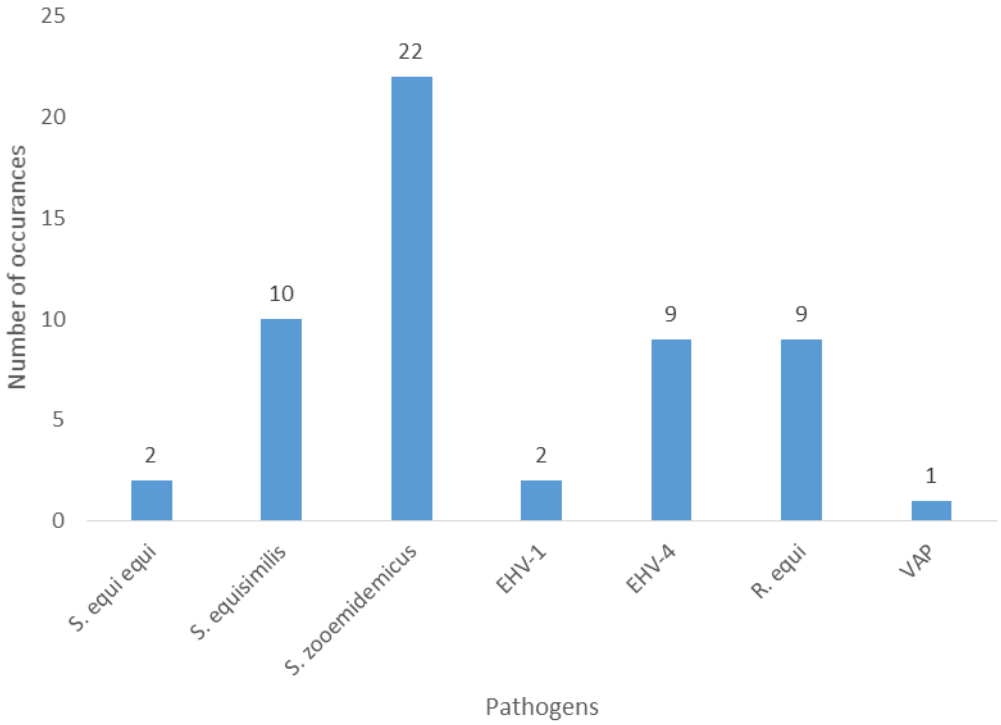


Figure 3.9: PCR results from respiratory clinical samples. Occurrences represent the frequency of infection across samples received (n=57).

To assess the frequency of polymicrobial infections, further analysis showed 19.3% of the tested respiratory samples returned PCR positive for multiple pathogens (**Table 3.5**). Excluding PCR results for the 16S primers, the frequency of the PCR positives showed 11/57 (19.29%) respiratory samples returned PCR positive for 2 or more genus or species specific microorganisms and a maximum of 3 unique PCR positives within a single sample (**Figure 3.10**).

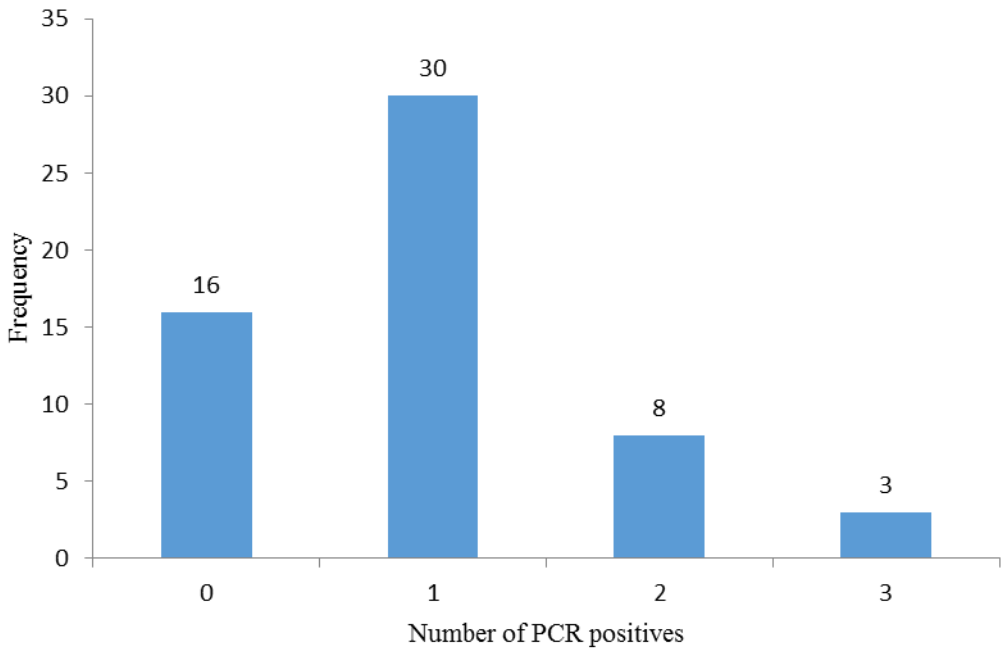


Figure 3.10: Frequency of polymicrobial infections within the respiratory sample group. Data excludes PCR positives for 16S primers.

The largest age group represented within the respiratory samples was animals with uncategorised ages (n=28), where 71.43% of these returned a positive result for at least one of the primer sets used in the study (**Table 3.6**). Yearlings had the lowest percent positive results for a single age group at only 33.33%.

Table 3.6: Frequency of PCR positives observed in each age group for respiratory samples.

Age Group	Total count of age group (n=57)	Occurrence of positive PCR	% Positive
Newborn	0	0	0.00
Neonate	0	0	0.00
Weanling	9	9	100.00
Foal	1	1	100.00
Yearling	3	1	33.33
2+	16	10	62.50
N/A	28	20	71.43

This data was presented showing the total distribution of infection with age groups (Figure 3.11). No strong observable trends correlating with age to certain infection types were seen.

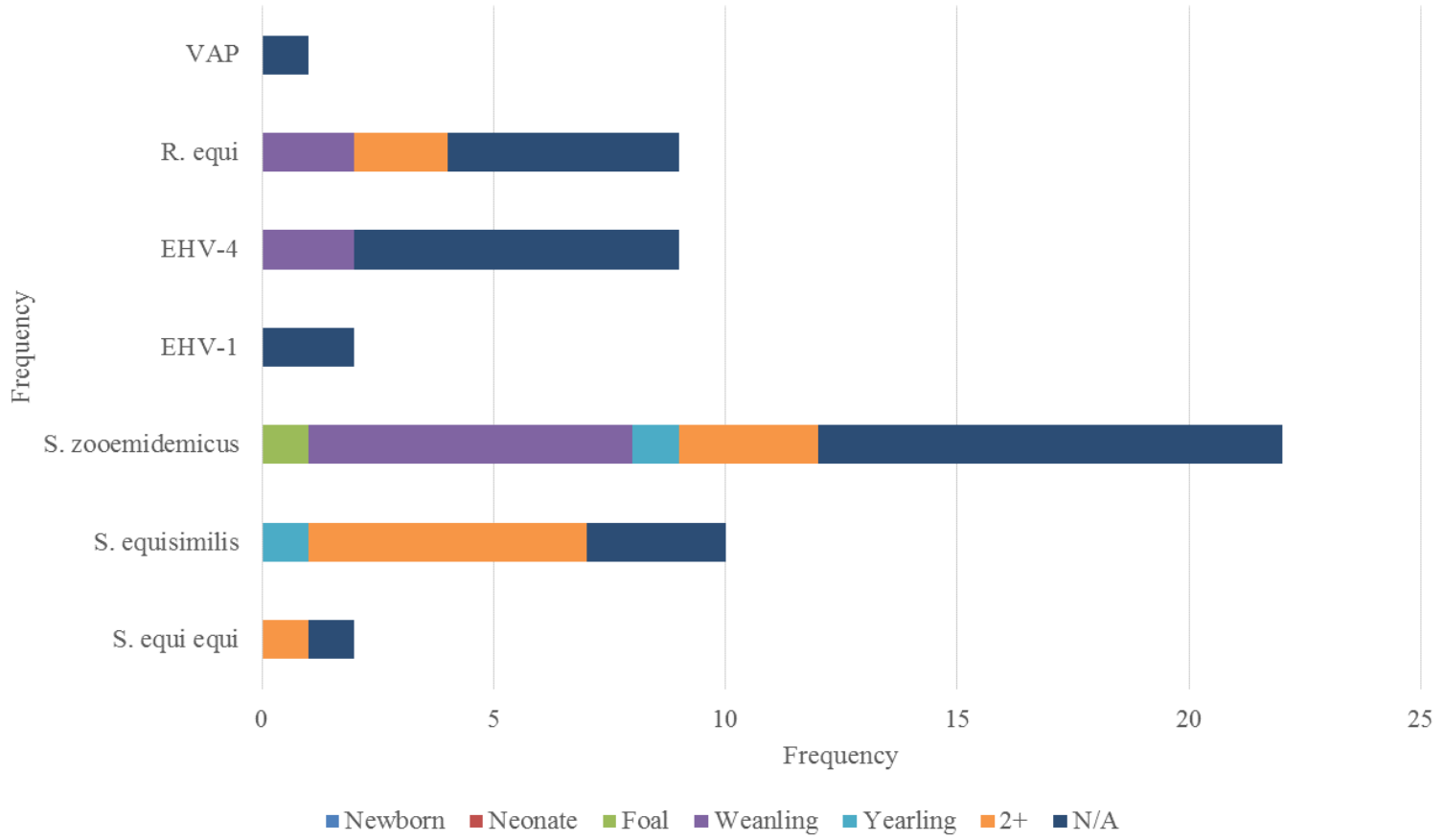


Figure 3.11: Total distribution of microorganisms within respiratory samples by age group.

3.6 Gastrointestinal sample microbe identification

Gastrointestinal samples were obtained from veterinarians within the Greater Waikato Region and around Auckland, New Zealand which had information on the age group and clinical details that presented (**Table 3.7**). Each of these samples had the DNA extracted and were screened by PCR to determine which microbial species of genus returned positive.

Table 3.7: Results from PCR testing on gastrointestinal samples. Details of age, clinical details, and microbial species returning PCR positive (indicated by "+") are shown.

Sample ID	Age group	Clinical details	*Salmonella* spp.	*L. intracellularis*	*C. difficile*	TcdA	TcdB	*C. sordellii*	*C. perfringens*	C. net	*R. equi*	VAP
G001	N/A	N/A	-	-	+	+	+	-	-	-	-	
G002	N/A	N/A	-	-	-	-	-	-	+	-	+	
G003	N/A	N/A	-	-	-	-	-	-	+	-	+	
G004	Neonate	Diarrhoea at 2 weeks old, one of a number affected between 2-10 weeks old.	-	-	-	-	-	-	-	-	-	
G005	2+	Colitis, suspected Salmonellosis.	-	-	-	-	-	-	+	-	-	
G006	Yearling	Yearlings with diarrhoea. Bright, eating, non febrile.	-	-	-	-	-	-	-	-	-	
G007	Yearling	Yearlings with diarrhoea. Bright, eating, non febrile.	-	-	-	-	-	-	-	-	-	
G008	Foal	Previous positive for *C. difficile* A + B.	-	-	+	+	+	-	+	-	-	
G009	Foal	Previous positive for *C. difficile* A + B.	-	-	+	+	+	-	-	-	-	

Sample ID	Age group	Clinical details	*Salmonella* spp.	*L. intracellularis*	*C. difficile*	TcdA	TcdB	*C. sordellii*	*C. perfringens*	C. net	*R. equi*	VAP
G010	Foal	Previous positive for *C. difficile* A.	-	-	+	+	-	-	-	-	-	
G011	Yearling	Previous positive for *C. difficile* A + B.	-	-	+	+	+	-	-	-	-	
G012	N/A	N/A	-	-	-	-	-	-	-	-	-	
G013	N/A	Previous positive for *C. difficile* B.	-	-	+	-	+	-	-	-	-	
G014	N/A	Negative for previous tests.	-	-	-	-	-	-	-	-	-	
G015	N/A	Negative for previous tests (Update 16/12/2015: *R. equi*).	-	-	-	-	-	-	-	-	+	
G016	N/A	Previous positive for *C. perfringens* PLC.	-	-	-	-	-	-	+	-	-	
G017	N/A	Negative for previous tests.	-	-	-	-	-	-	-	-	-	
G018	N/A	Negative for previous tests.	-	-	-	-	-	-	-	-	-	
G019	2+	Recurrent smelly diarrhoea.	-	-	-	-	-	-	-	-	-	
G020	N/A	Positive for *Salmonella.*	+	-	-	-	-	-	-	-	-	
G021	N/A	Previous positive for *R. equi.*	-	-	-	-	-	-	+	-	-	
G022	N/A	Negative for previous tests.	-	-	-	-	-	-	-	-	-	
G023	N/A	Negative for previous tests.	-	-	-	-	-	-	-	-	-	
G024	N/A	Negative for previous tests.	-	-	-	-	-	-	-	-	-	
G025	N/A	Previous positive for *R. equi.*	-	-	-	-	-	-	-	-	+	
G026	N/A	Negative for previous test, suspected inhibitory.	-	-	-	-	-	-	-	-	-	
G027	N/A	Previous positive for *Salmonella.*	+	-	-	-	-	-	-	-	-	
G028	N/A	Previous positive for *R. equi.*	-	-	-	-	-	-	-	-	+	

Sample ID	Age group	Clinical details	*Salmonella* spp.	*L. intracellularis*	*C. difficile*	TcdA	TcdB	*C. sordellii*	*C. perfringens*	C. net	*R. equi*	VAP
G029	N/A	Previous positive for *R. equi*. Negative for VAP.	-	-	-	-	-	-	-	-	+	
G030	N/A	Previous positive for *L. intracellularis.*	-	+	-	-	-	-	-	-	-	
G031	N/A	N/A	-	-	-	-	-	-	-	-	-	
G032	N/A	N/A	-	-	-	-	-	-	-	-	-	
G033	N/A	N/A	-	-	-	-	-	-	-	-	-	
G034	N/A	N/A	-	-	-	-	-	-	-	-	-	
G035	N/A	N/A	-	-	-	-	-	-	+	-	-	
G036	N/A	N/A	-	-	-	-	-	+	+	-	-	
G037	N/A	N/A	-	-	-	-	-	-	+	-	-	
G038	N/A	N/A	-	-	-	-	-	-	-	-	-	
G039	N/A	N/A	-	-	-	-	-	-	+	-	-	
G040	N/A	N/A	-	-	-	-	-	-	-	-	-	
G041	N/A	N/A	-	-	-	-	-	-	+	-	-	
G042	N/A	Previous positive for *R. equi* and *Salmonella.*	+	-	-	-	-	-	-	-	+	
G043	N/A	Previous positive for *Salmonella.*	+	-	-	-	-	-	-	-	-	
G044	N/A	Previous positive for *R. equi.*	-	-	-	-	-	-	+	-	+	
G045	N/A	N/A	-	-	-	-	-	-	-	-	-	
G046	N/A	N/A	-	-	-	-	-	-	-	-	-	-
G047	N/A	N/A	-	-	-	-	-	-	-	-	-	-

Sample ID	Age group	Clinical details	*Salmonella* spp.	*L. intracellularis*	*C. difficile*	TcdA	TcdB	*C. sordellii*	*C. perfringens*	C. net	*R. equi*	VAP
G048	N/A	Negative for previous tests.	-	-	-	-	-	-	-	-	-	-
G049	N/A	*Rhodococcus.*	-	-	-	-	-	-	+	-	+	-
G050	N/A	Previous positive for *R. equi.*	-	-	-	-	-	-	-	-	-	-
G051	N/A	N/A	-	-	-	-	-	-	+	-	-	-
G052	N/A	Previous positive for *R. equi.*	-	-	-	-	-	-	+	-	+	-
G053	N/A	N/A	-	-	-	-	-	-	-	-	-	-
G054	N/A	N/A	-	-	-	-	-	-	-	-	-	-
G055	N/A	N/A	-	-	-	-	-	-	+	-	-	-
G056	N/A	N/A	-	-	-	-	-	-	-	-	-	-
G057	N/A	Previous positive for *Salmonella.*	+	-	-	-	-	-	-	-	-	-
G058	N/A	Negative for previous tests.	-	-	-	-	-	-	-	-	-	-
G059	N/A	Previous positive for *C. difficile* B and *Salmonella*.	+	-	+	-	+	-	-	-	-	-
G060	Yearling	Chronic scours.	-	-	-	-	-	-	-	-	-	-
G061	Neonate	Diarrhea, losing protein.	-	-	-	-	-	-	-	-	-	-
G062	Neonate	Acute diarrhoea 17.2.17, temp was 40°C. Treated with calefour. 18.2.17 temp at 39.2°C.	-	-	-	-	-	-	+	-	+	+
G063	N/A	Enteritis.	-	-	-	-	-	-	-	-	+	-
G064	N/A	Diarrhoea, poor doer, fibrogen of 8.	-	-	-	-	-	-	-	-	-	-
G065	Weanling	Watery diarrhoea. *Rhodococcus* on farm. Suspected *Rhodococcus* or Rotavirus.	-	-	-	-	-	-	-	-	+	-

Sample ID	Age group	Clinical details	*Salmonella* spp.	*L. intracellularis*	*C. difficile*	TcdA	TcdB	*C. sordellii*	*C. perfringens*	C. net	*R. equi*	VAP
G066	Newborn	3 days old, diagnosed with pneumonia, has been on antibiotics for 48 hrs. PCR and culture requested. Suspicious of *L. intracellularis* from ultrasound. Diarrhoea reported.	-	-	-	-	-	+	+	-	-	-

From the 66 animals screened, (**Table 3.7**) 54.5% of samples returned positive for one or more PCR result, indicating successful amplification and confirmation of the presence of microbes. These positive samples were analysed to determine trends of infection types (**Figure 3.12**). Of the 66 respiratory sample types received and analysed by PCR, *C. perfringens* (n=18) had the highest occurrence rate, with the potential to cause systemic or localised infection to horses of all age groups. *R. equi* (n=13) was the second most frequent, with one sample returning a positive for *R. equi* VAP. There were no observed PCR positives for netF.

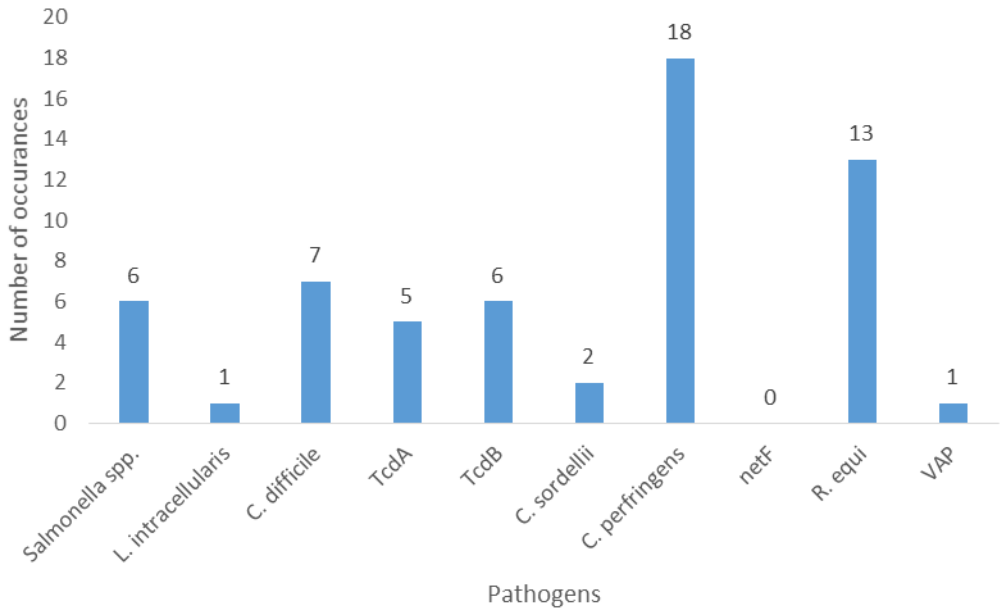


Figure 3.12: PCR results from gastrointestinal clinical samples. Occurrences represent the frequency of infection across samples received (n=66).

To assess the frequency of polymicrobial infections, further analysis showed 24.24% of the tested gastrointestinal samples returned PCR positive for multiple pathogens (**Table 3.7**). The frequency of the PCR positives showed 16/66 (24.24%) gastrointestinal samples returned PCR positive for 2 or more genus or species specific microorganisms and a maximum of 4 unique PCR positives within a single sample (**Figure 3.13**).

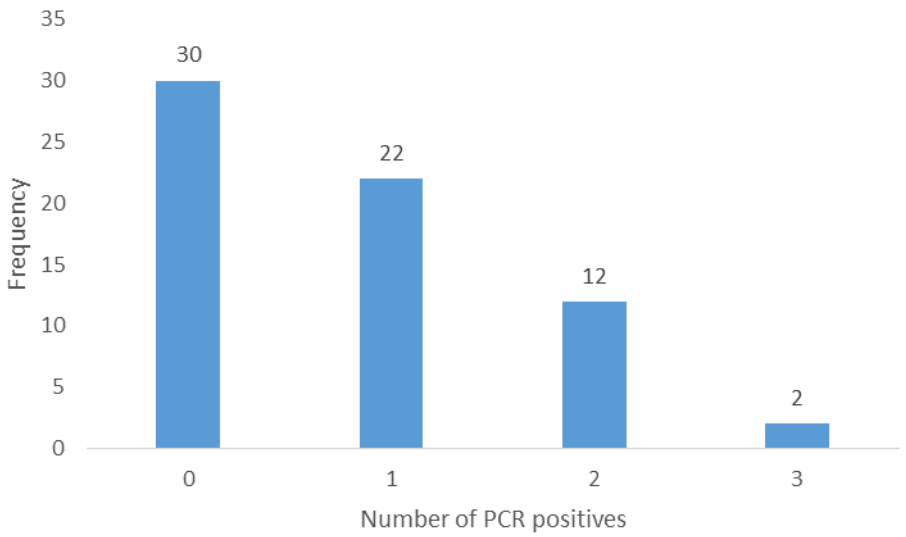


Figure 3.13: Frequency of polymicrobial infections within the gastrointestinal sample group (Excluding *C. difficile*; TcdA and TcdB counted independently).

The largest age group represented within the gastrointestinal samples was animals with uncategorised ages (n=52), where 53.85% of these returned a positive result for one of the primer sets used in the study (**Table 3.8**). Yearlings had the lowest percent positive results for a single age group at only 25%. Newborns, neonates, and foals all had a positive frequency of 100% (n=1).

Table 3.8: Gastrointestinal age distribution of positive results.

Age Group	Number	Occurrence of PCR Positive	Positives frequency
Newborns	1	1	100.00
Neonates	3	1	33.33
Weanlings	1	1	100.00
Foals	3	3	100.00
Yearlings	4	1	25.00
2+	2	1	50.00
N/A	52	28	53.85

This data was presented showing the total distribution of infection with age groups (**Figure 3.14**). No strong observable trends correlating with age to certain infection types were seen.

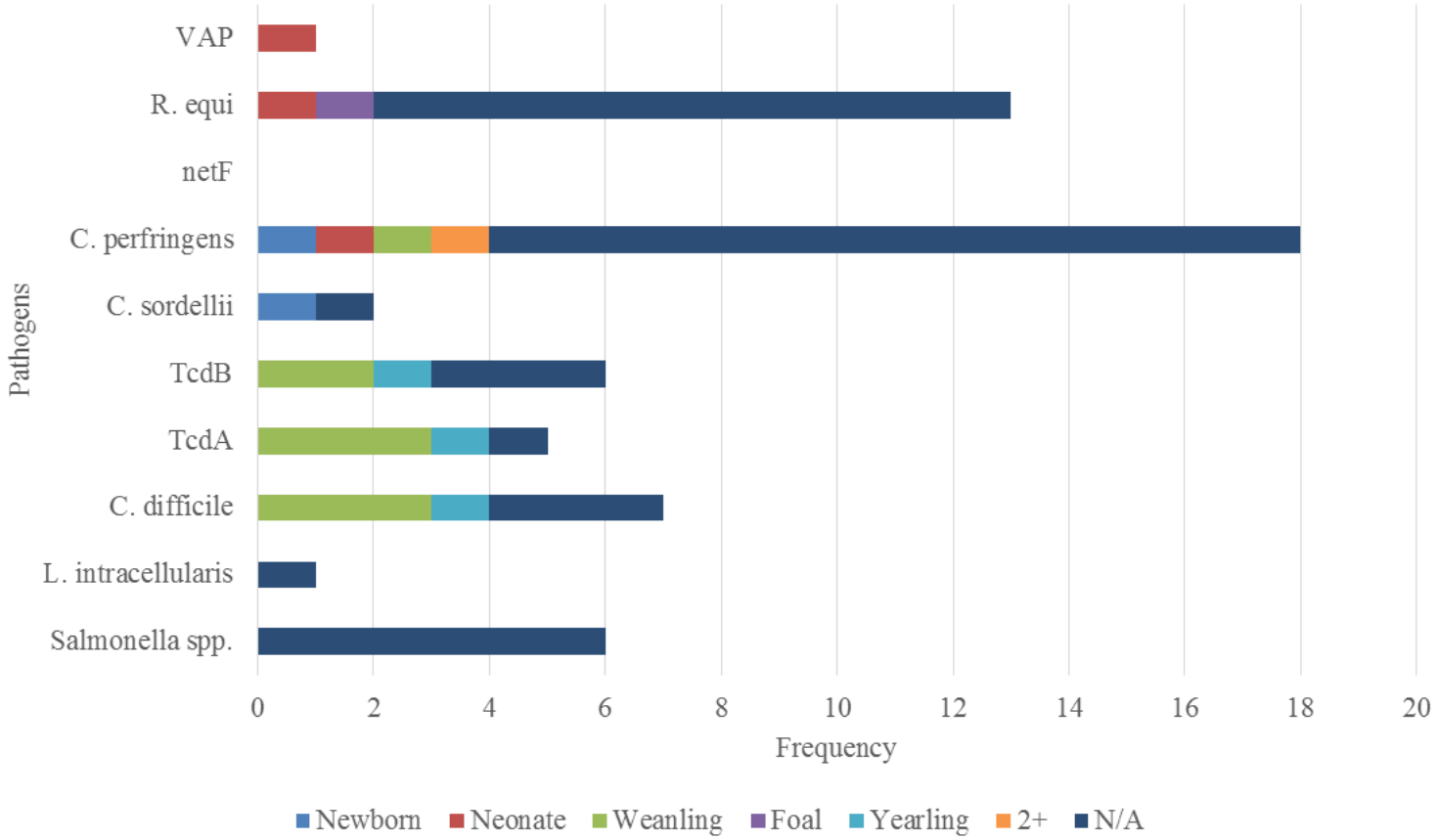


Figure 3.14: Total distribution of microorganisms within gastrointestinal samples by age group.

3.7 Sequencing results

To confirm the accuracy of each PCR primer set and validate the results, amplified PCR products from clinical samples were sent for sequencing. Some sequencing returned indeterminable results (**Table 3.9**), whereas other sequencing was able to be imported into NCBI BLAST to confirm amplification or the correct product and in some cases allowed microbe detection at a species level.

Table 3.9: Table of PCR sequencing results that were of poor quality and unable to be analysed using BLAST.

Sample ID	Genus primers	Quality	Length (nucleotides)
G063	VAP	1%	193
R003	EHV-4 (TK)	3.30%	275
S003	*Staphylococcus* spp.	0.00%	5
	Gram positive	0.00%	307
S004	Gram negative	0.50%	409
	Panfungal	0.20%	468
S005	Coliforms	0%	63
	Gram negative	0.60%	328
S006	*Staphylococcus aureus*	0%	5
S008	Gram negative	1.40%	418
	Gram positive	9.50%	317
	Panfungal	0.30%	470
S009	Gram positive	0.70%	282
S011	Gram positive	0.00%	199

Out of the 14 low quality sequencing results received, 7/14 (50%) of samples were for 16S bacterial primers (**Table 3.9**). Despite this, some of these samples were successfully sequenced (G063, R003, S004, S006, S008, S011) when different microbial primers were used (**Table 3.10**). BLAST analysis of these sequences showed species identification similar to what was expected from the amplicon (**Table 3.10**). However, irregularities did occur during the BLAST analysis of S015, which presented with high quality sequencing, but the BLAST analysis returned DNA for a tescalcin gene from a Przewalski's horse, and not virulence associated plasmid genes as expected. This would suggest non-specific amplification of a product had occurred.

Table 3.10: Table of PCR sequencing results following successful NCBI BLAST analysis.

Sample ID	Genus sequenced	Length	Alignment species	Max score	Total score	Query cover	E value	Identity %	Accession number
G037	*C. perfringens* PLC	265	*Clostridium perfringens*	490	490	100	8E-135	100	CP023410.1
	C. perfringens CPE	59	*Clostridium perfringens*, enterotoxin (cpe)	110	110	100	3E-21	100	MF326933.1
	C. sordellii (Forward)	100	*Clostridium sordellii*	134	134	100	3E-28	100	KM657127.1
G063	CHO (R)	89	*R. equi*, cholesterol oxidase (choE) gene	165	165	100	1E-37	100	KF670817.1
	C. perfringens PLC	262	*C. perfringens*, phospholipase C precursor (plc) gene,	479	479	100	2E-131	99	HM625066.1
	TraA	680	*R. equi* virulence plasmid	1254	1254	100	0E+00	100	AF116907.2
	VAP	181	*R. equi* virulence plasmid	335	335	100	3E-88	100	AF116907.2
G064	VAP	180	*R. equi*	333	333	100	9E-88	100	KT443893.1
R003	EHV-4	270	*Equid herpesvirus* 4 glycoprotein gB gene	497	497	99	5E-137	100	M26171.1
R015	EHV-1	319	*Equine herpesvirus* type 1 glycoprotein 14 (gp14) gene	590	590	100	1E-164	100	M34861.1
R017	EHV-1	319	*Equine herpesvirus* type 1 glycoprotein 14 (gp14) gene	590	590	100	1E-164	100	M34861.1
R039	CHO	199	*R. equi*	350	350	94	1E-92	100	FN563149.1
R041	*Streptococcus* spp.	196	*Streptococcus acidominimus*	351	351	100	3E-91	99	AY266992.1
R042	*Streptococcus* spp.	195	*Streptococcus pneumoniae*	294	294	100	5E-76	94	AP018043.1
	S. pneumoniae	314	*Streptococcus pneumoniae*	575	575	100	3E-160	99	AP018391.1

Sample ID	Genus sequenced	Length	Alignment species	Max score	Total score	Query cover	E value	Identity %	Accession number
	POL.								
S002	*Staphylococcus* spp.	355	*Staphylococcus cohnii*	640	640	100	1E-179	99	KU883202.1
	Staphylococcus spp.	355	*Staphylococcus equorum*	634	634	97	5E-178	99	CP013980.1
S004	*Staphylococcus* spp.	351	*Staphylococcus equorum*	625	625	98	3E-175	99	KX198025.1
S006	*Staphylococcus* spp.	330	*Staphylococcus epidermidis*	534	534	95	5E-148	97	CP022247.1
	Streptococcus spp.	195	*Streptococcus pneumoniae*	342	342	99	2E-90	98	AP018043.1
S007	*S. aureus*	191	*Staphylococcus aureus*	84.2	84.2	31	1E-12	92	CP020741.1
	Staphylococcus spp.	367	*Staphylococcus epidermidis*	617	617	99	5E-173	97	CP022247.1
	Streptococcus spp.	191	*Streptococcus pseudopneumoniae*	348	348	100	4E-92	99	GU326209.1
S008	Coliforms	228	*Escherichia coli*	422	422	100	3E-114	100	CP023388.1
	E. coli	162	*Escherichia coli*	289	289	100	2E-74	99	CP023383.1
	Staphylococcus spp.	298	*Staphylococcus epidermidis*	545	545	100	2E-151	99	CP022247.1
	Streptococcus spp.	189	*Streptococcus pneumoniae*	333	333	100	1E-87	98	AP018043.1
	Enterococcus spp. (Culture)	342	*Enterococcus gallinarum*	632	3139	100	2E-177	100	CP023515.1
	Streptococcus spp. (Culture)	191	*Streptococcus pneumoniae*	331	331	100	4E-87	98	AP018043.1
S011	*Staphylococcus* spp.	339	*Staphylococcus epidermidis*	621	621	100	4E-174	99	CP022247.1

Sample ID	Genus sequenced	Length	Alignment species	Max score	Total score	Query cover	E value	Identity %	Accession number
S013	Gram negative	399	Uncultured *Staphylococcus* sp. clone	737	737	100	0E+00	100	MF377707.1
S015	VAPA (Forward)	136	*Equus przewalskii* tescalcin gene	226	226	94	1E-55	98	XM_008515476.1
S016	CHO (R)	64	*R. equi*	119	119	100	6E-24	100	KF670817.1
	Staphylococcus spp.	368	*Staphylococcus equorum*	676	676	99	0E+00	100	CP013980.1
	Stapyhlococcus spp. (Acetone)	348	*Staphylococcus equorum*	643	643	100	0E+00	100	CP013980.1
S023	*Bacillus* spp.	208	*Bacillus cereus*	351	351	94	3E-93	99	KY675383.1
	GmPos	333	*Bacillus cereus*	604	8403	100	4E-169	99	CP017060.1
	CHO (culture)	185	*R. equi*	342	342	100	2E-90	100	FN563149.1

3.8 Electropherograms

Sequencing results analysed in Geneious showed varying levels of efficiency (**Figure 3.15** and **Figure 3.16**). Electropherograms show the quality of sequencing returned and demonstrates the accuracy of the PCR and the products amplified. A full list of electropherogram sequencing quality for associated primers can be found in Appendix 3.

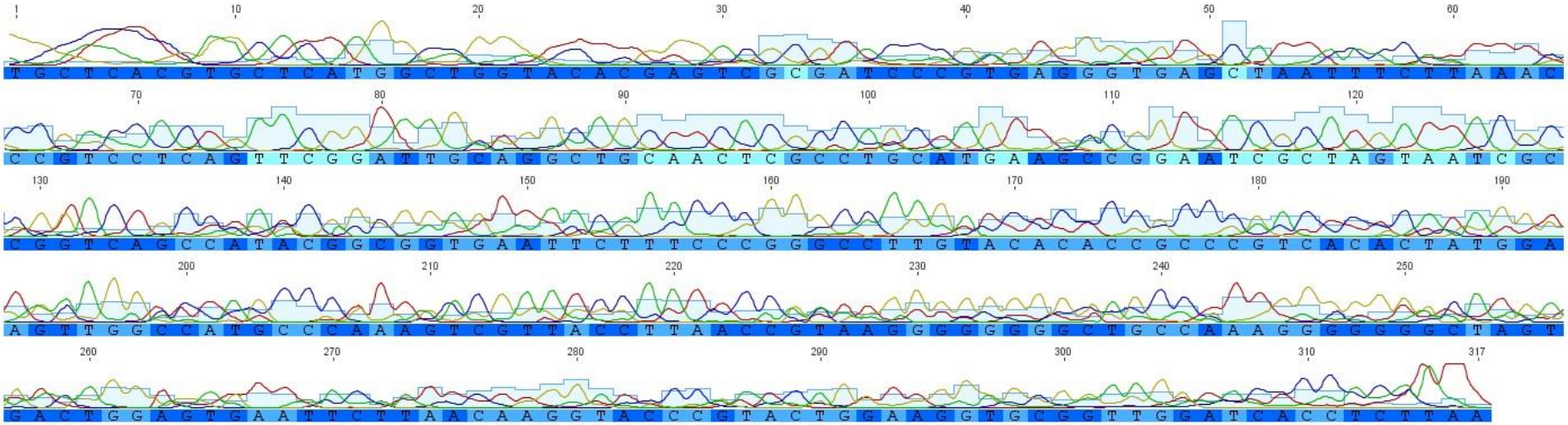


Figure 3.15: Example of low quality sequencing of the products obtained for the Gram positive 16S primers from sample S008.

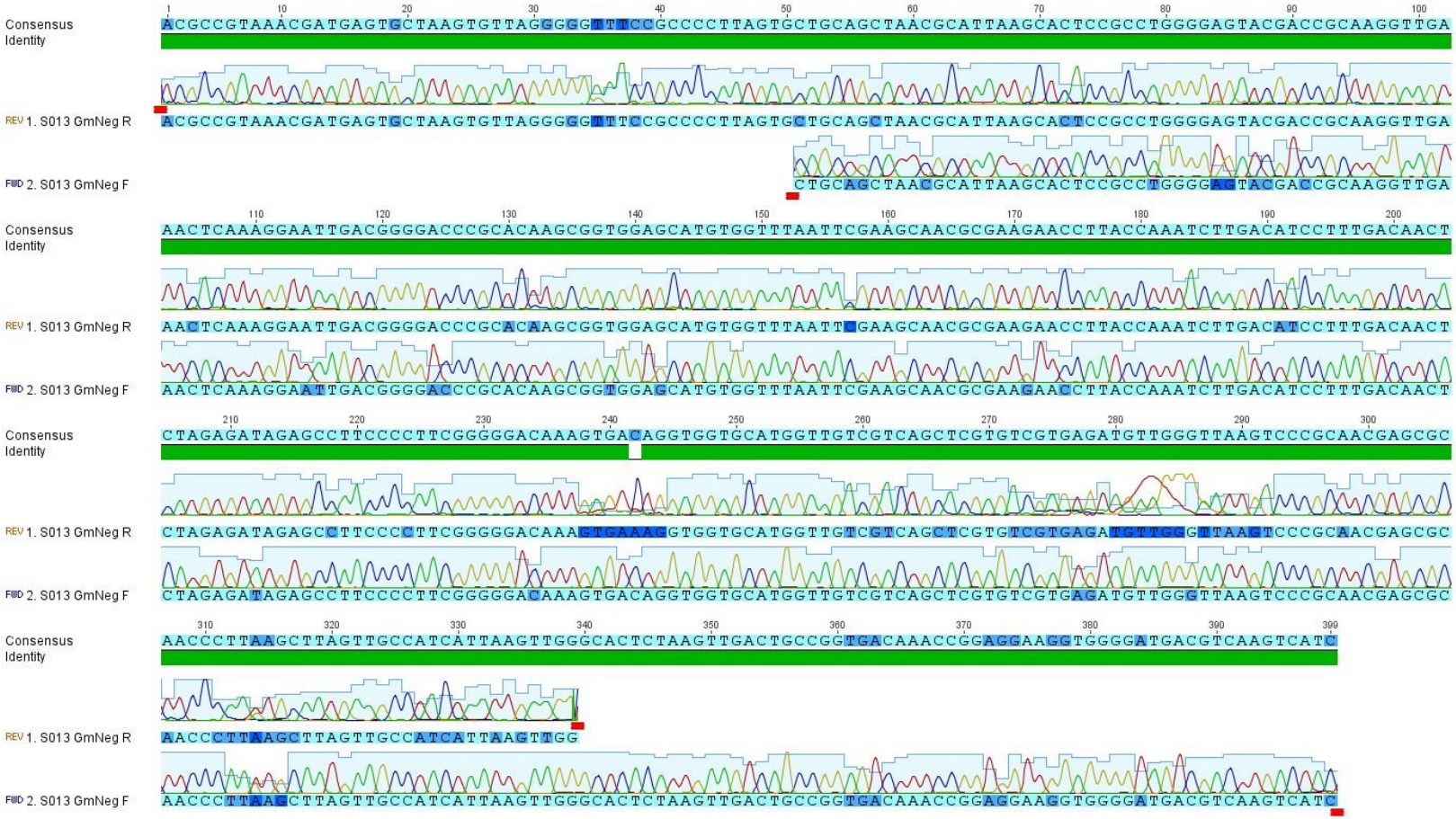


Figure 3.16: Example of high quality sequencing and alignment for the products obtained for the Gram negative 16S primers from sample S013.

3.9 Confirmation of primer efficiency

Primers were initially tested to ensure they were able to generate amplicons of the correct size. Amplified products were compared to a 100bp molecular weight ladder to ensure product size matched the expected product size described by the literature primers were retrieved from.

3.10 PCR products amplified from sterile samples

DNA from sterile samples was tested with primers for Gram positive 16S, Gram negative 16S, Staphylococcus genus, *S. Aureus*, Streptococcus genus, *E. coli*, Coliforms, Enterococcus genus, Bacillus genus, Pseudomonas genus, *R. equi* (CHO), Panfungal genus, EHV-1 and EHV-4 (**Figure 3.17**). Amplicons of the correct size were obtained for each set of primers. However, primer dimers could be observed in some of the amplified products.

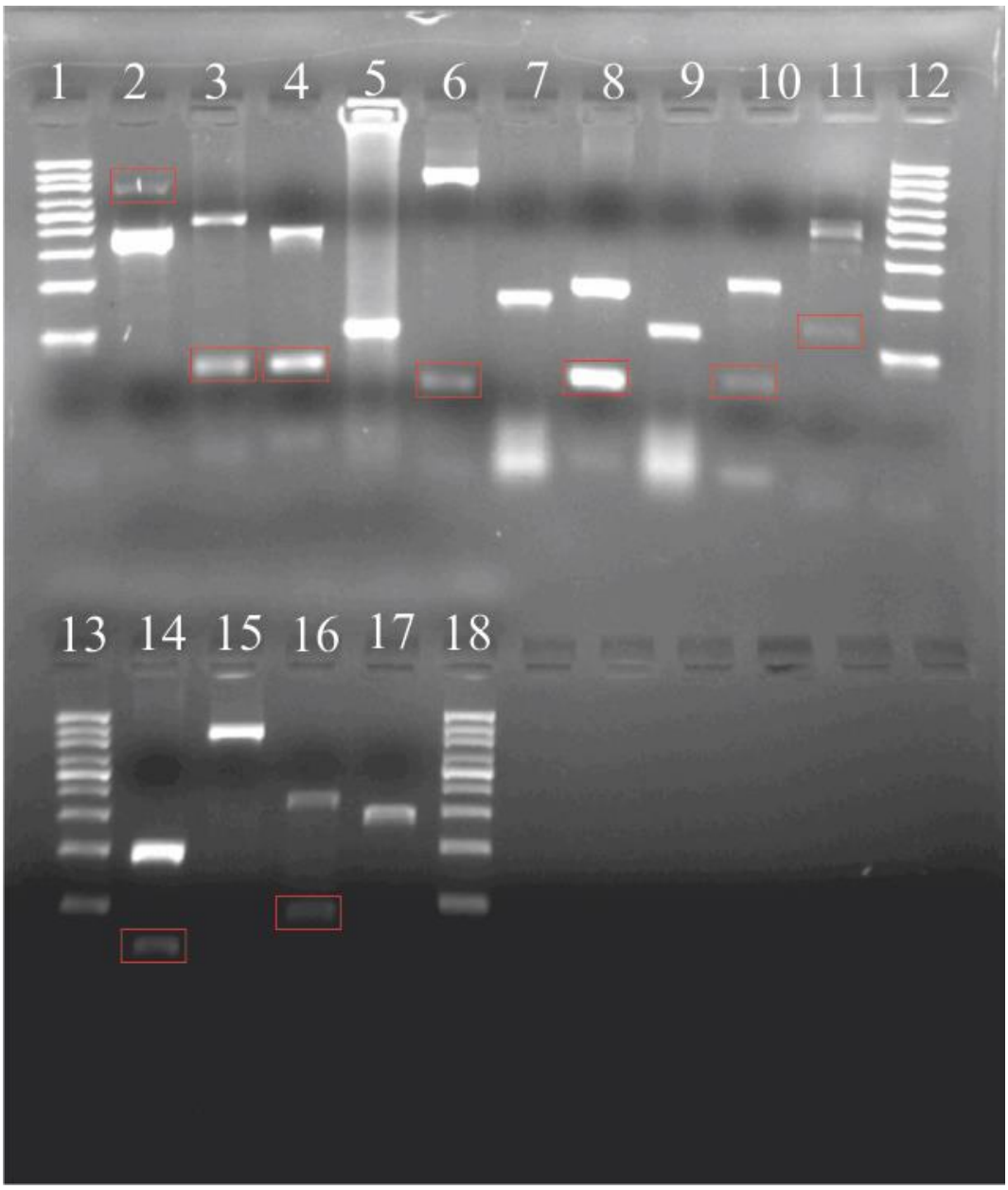


Figure 3.17: Gel electrophoresis image of amplified bacterial, fungal, and viral products using primers with DNA extracted from various sterile clinical samples. Lane 1: Genscript 100bp ladder; 2: Gram positive 16S; 3: Gram negative 16S; 4: Staphylococcus genus; 5: *S. Aureus*; 6: Streptococcus genus; 7: *E. coli*; 8: Coliforms; 9: Enterococcus genus; 10: Bacillus genus; 11: Pseudomonas genus; 12: Genscript 100bp ladder; 13: Genscript 100bp ladder; 14; *R. equi* (CHO); 15: Panfungal genus; 16: EHV-1; 17: EHV-4; 18: Genscript 100bp ladder. Primer dimers (boxed) can be observed in some lanes.

3.11 PCR products amplified from respiratory samples

DNA from respiratory samples was tested with primers for *S. equi equi*, *S. equi* (EqbE), Gram positive 16S, *S. equisimilis* (equisim) and *S. zooepidemicus* (sodA) (**Figure 3.18** and **Figure 3.19**). Amplicons of the

correct size were obtained for each set of primers. However, primer dimers could be observed in some of the amplified products.

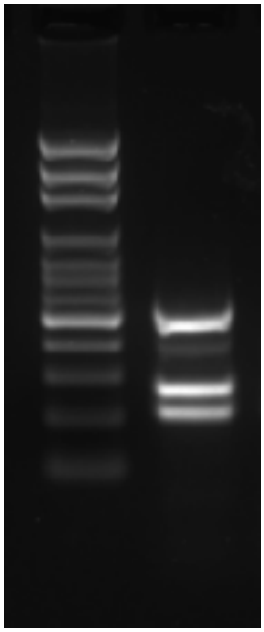


Figure 3.18: Gel electrophoresis image of amplified bacterial products with DNA extracted from a respiratory clinical sample. Lane 1: Solis Biodyne 100bp ladder; 2: *S. equi equi* (seel) at 520bp, 16S positive control at 410bp, *S. equisimilis* (equisim) at 279bp, *S. zooepidemicus* (sodA) at 235bp.

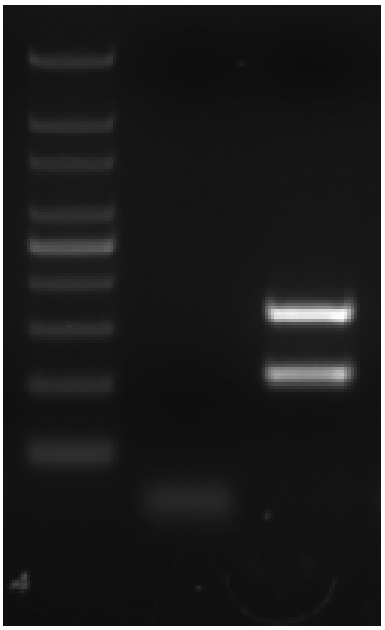


Figure 3.19: Gel electrophoresis image of amplified bacterial products with DNA extracted from a respiratory clinical sample. Lane 1: Genscript 100bp ladder; 2: *S. equi* (EqbE) at 324bp, sorD at 220bp.

3.12 PCR products amplified from gastrointestinal samples

DNA from gastrointestinal samples was tested with primers for 16S rRNA, *Salmonella*, *L. intracellularis*, TcdA, TcdB, *C. perfringens*, *C. sordellii*, *R. equi* and associated virulence markers (**Figure 3.20** - **Figure 3.26**). Amplicons of the correct size were obtained for each set of primers. Primer dimers could be observed in some of the amplified products. PCR positive image of *L. intracellularis* from sample G030 could not be obtained for imaging due to lack of species specific reagents.

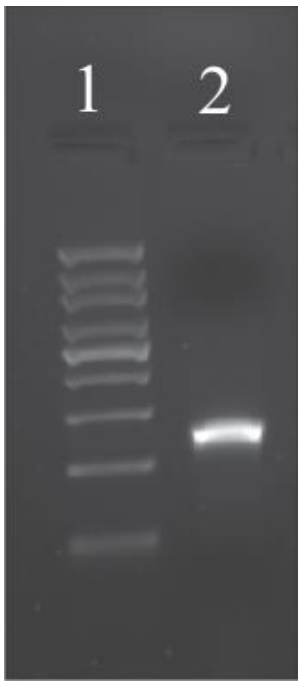


Figure 3.20: Gel electrophoresis image of amplified bacterial 16S PCR products with DNA extracted from a gastrointestinal sample. Lane 1: Genscript 100bp ladder; 2: 16S.

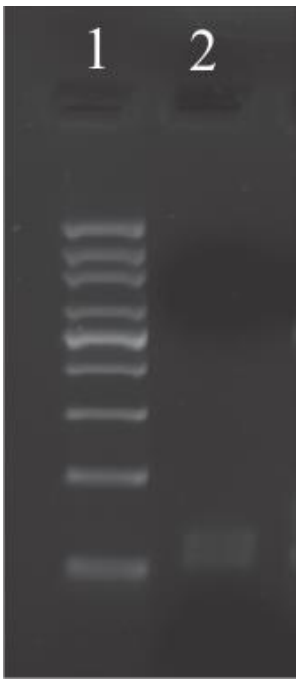


Figure 3.21: Gel electrophoresis image of amplified bacterial PCR products with DNA extracted from a gastrointestinal sample. Lane 1: Genscript 100bp ladder; 2: *Salmonella*.

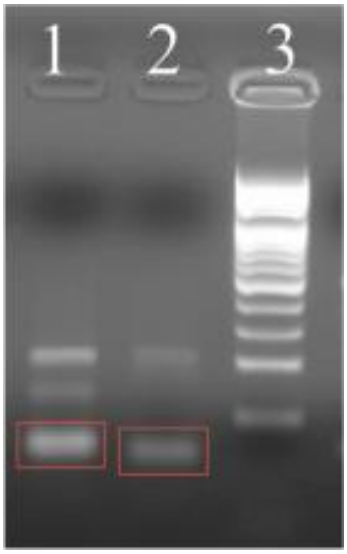


Figure 3.22: Gel electrophoresis image of amplified bacterial PCR products with DNA extracted from a gastrointestinal sample. Lane 1: TcdA and TcdB; 2: *R. equi* CHO positive; 3: SolisBiodyne 100bp ladder; Primer dimers (boxed) can be observed in some lanes.

Figure 3.23: Gel electrophoresis image of amplified bacterial PCR products with DNA extracted from a gastrointestinal sample. Lane 1: Genscript 100bp ladder; 2: *C. perfringens* PLC positive 3: *C. sordellii* negative 4: *C. perfringens* netF negative; 5; *C. perfringens* PLC positive; 6: *C. sordellii* positive. Primer dimers (boxed) can be observed in some lanes.

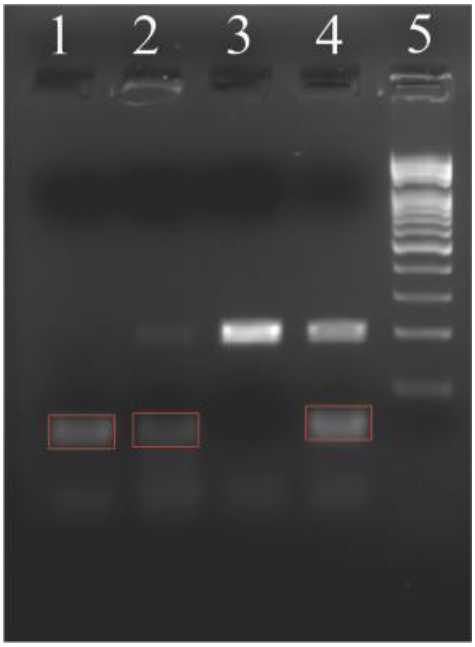


Figure 3.24: Gel electrophoresis image of amplified bacterial PCR products with DNA extracted from a gastrointestinal sample. Lane 1: Negative control. Lane 2: CHO positive. Lane 3: CHO Positive. Lane 4: CHO positive. Lane 4: Genscript 100bp ladder. Primer dimers (boxed) can be observed in some lanes.

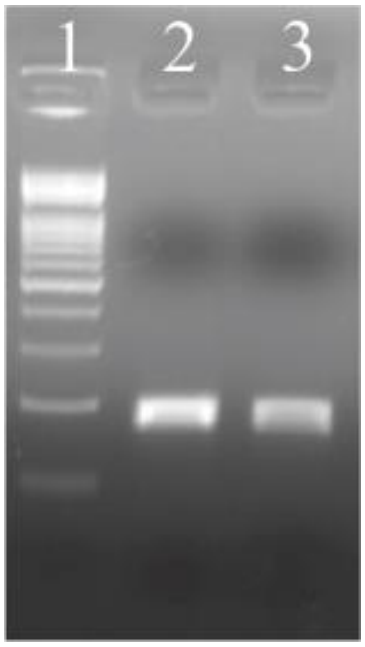


Figure 3.25: Gel electrophoresis image of amplified bacterial 16S PCR products with DNA extracted from a gastrointestinal sample. Lane 1: Genscript 100bp ladder; 2. VAP positive; 3. VAP positive.

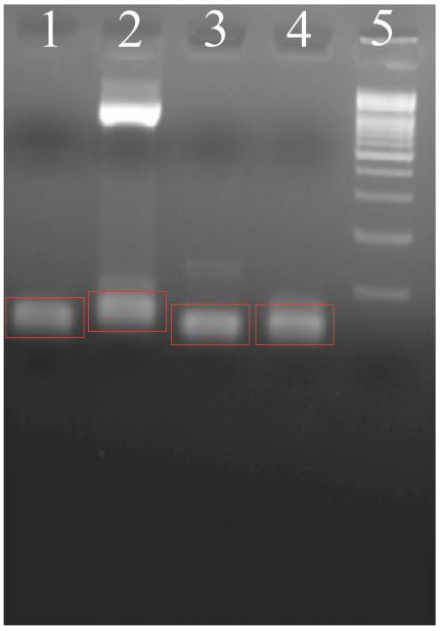


Figure 3.26: Gel electrophoresis image of amplified bacterial 16S PCR products with DNA extracted from a gastrointestinal sample. Lane 1: TraA negative; 2: TraA positive; 3: TraA negative; 4: TraA; 5: Genscript 100bp ladder. Primer dimers (boxed) can be observed in some lanes.

3.13 Restriction digests

Due to the clinical significance of Equine herpesvirus, restriction digests were used to confirm the presence of EHV-1 and EHV-4 to mitigate false positives occurring from mispriming. The changes seen following a restriction digest of the PCR product with the relevant restriction enzyme allowed the confidence to conclude the presence of EHV-1 and EHV-4 within clinical samples.

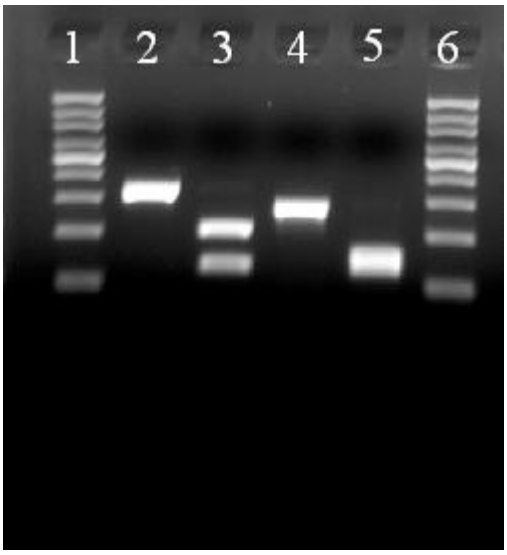


Figure 3.27: Gel electrophoresis image of a PCR restriction digest for the confirmation of Equine herpesvirus. Lane 1: Genscript 100bp ladder; 2: EHV-1 PCR amplicon; 3: EHV-1 amplicon digested with HincII; 4: EHV-4 PCR amplicon; 4; 5: EHV-4 amplicon digested with HindIII.

Chapter Four

DISCUSSION

Culture based diagnosis is a limited diagnostic technique, as only organisms suited to a particular media will grow and has the potential to produce a false negative result. To achieve accurate culturing many selective or enriched media must be used, particularly when detecting microbes in non-sterile samples, such as faeces. The use of additional selective media significantly increases the costs associated with culturing, and the trial-and-error approach of culturing with many different media increases the time for diagnosis. In addition to the shortfall of media specificity, culturing can also be inhibited following the application of antimicrobials as therapeutic treatments leave the pathogenic organism undetected. The accurate identification of these organisms is invaluable, allowing easier detection within groups of animals that may be vulnerable to infection and for the administration of the correct therapeutic approach that will mitigate the spread of infection.

Due to the relative low sensitivity, expense, and extensive diagnostic time required, there is a pressing need for alternative equine diagnostic methods. Compared to traditional methods, PCR is rapid, bypasses the need for selective media, lowers expense, and provides same day results. It still requires specialist skills, as DNA must be extracted from the sample and added to specific reactions. Also, critical for any PCR is the correct primer selection, which can be overcome over time, through the collection of further information on common infections present. The additional use of known universal primers are important, as they can be used as indicators of successful amplification and determine the presence of infection within normally sterile samples. However, it is important to note, that there is the potential for these primers to react with any bacterial DNA that is present within TAQ polymerase being used and they may not be reliable with the occurrence of polymicrobial infections as often these cannot be accurately sequenced afterwards.

The overall aim of this project was to compare the efficacy of molecular and traditional diagnostic techniques for the identification of microbes responsible for infection in equines.

4.1 Traditional culture method efficiency

Out of 13 cultures, 6 were able to be identified to a genus level using simple Columbia broth and blood agar approaches. This method took on average 48 hours, where microbes were initially grown in broth, streaked onto agar, and allowed to grow into single colonies. Identification then used morphological characteristics and Gram staining.

Not all sterile samples received from veternarians (n=23) were able to be cultured in media, due to the sample type. For example, swab based samples could be used for either PCR *or* culture, not both. Due to this the results section on culture material ended up being relatively low. This does however highlight another limiting factor, the volumes required for diagnostics to be carried out. Due to PCR amplifying DNA exponentially with each cycle, a minimum of one DNA template strand at the first round of PCR has the potential to be amplified to over 34 billion amplicons following a standard 35 cycle program. Our DNA extraction technique used 0.2 ml of equine blood, compared to a mean of ~30ml of blood, that is used to detect bacterial infection using culture in medical pathology (Bouza *et al.*, 2007). This comparison clearly shows a distinct advantage molecular diagnostic techniques have over traditional methods.

Identifying microbial organisms in a culture was also more challenging overall when compared to PCR. Determining the causative pathogen using traditional culturing is a multifactorial process, requiring the use of multiple growth media, plates, various stains, and a knowledge of the potential pathogens involved. Traditional methods have proven useful for a rough guidance to treatment, however, the time require to obtain these results needs to be taken into consideration. Gram stains, which determine if an

organism is Gram positive or Gram negative take mere minutes, however, the blood or sample of infection takes a minimum of 12-24 hours to begin growing to a stage where identification using the morphological characteristics of a pathogen are possible. It must also be noted that bacterial culturing will only identify the presence of bacteria. Additional culturing methods must be undertaken to identify any fungal or viral microbes that may also be present.

4.2 Shortfalls of traditional culture methods

The known shortfalls of bacteriological culturing methods that have driven this project were recognised during our attempts at culturing. Growing bacteria in a basic culture broth took a minimum of 12 hours, and another 12 hours to grow identifiable colonies on blood agar. These basic culturing methods were insufficient for detecting fungi and viruses, and were only suitable for identifying select bacterial species. In addition to this low sensitivity we were unable to detect polymicrobial infections using blood agar and Columbia broth alone, indicating that if this was used as the primary diagnostic technique, many results received would have been incomplete or provided false negatives. Culture based diagnosis requires a high level of expertise, firstly to determine what culture medias to use, and secondly to interpret the result. This leaves this technique prone to human error and subjectivity. Although once considered the gold standard, for the detection of pathogenic microbes in horses bacteriological culturing is not a sufficient diagnostic tool, due to difficulty, and high rates of potential false negatives (Furr, 2003).

4.3 Efficacy of DNA extraction techniques

Nanodrop spectrophotometer results showed varying levels of DNA template purity following extraction from clinical samples. DNA quantification and qualification results from Nanodrop spectroscopy showed the SDS lysis method consistently produced higher quality and quantity DNA from clinical blood samples compared to the GITC extraction

methods. However, optimisation of these extraction techniques still requires further investigation, as a more rapid extraction technique could further speed the diagnostic time. In addition to this, alternative DNA extraction techniques such as PEG/KOH or kit based methods have the potential to produce high quality DNA from multiple clinical samples using a single procedure, further simplifying mass sample processing, and potentially decreasing the time required from sample reception to analysis of PCR.

4.4 Development of molecular approach

By developing PCR protocols sufficient to amplify multiple products related to clinical pathogens we were able to provide diagnostic information for the guidance of treatment. TD-PCR which was used due to variable manufacturer recommended melt temperatures from different primers, provided a sufficient method of tandem amplification of multiple PCR products, however, the efficiency of amplification for some primers was lowered due to the generalized PCR protocols. This could be mitigated by alternative primer design, however, artefacts like non-specific amplification and low resolution bands can still be used for diagnostic purposes.

The use of virulence associated primers proved useful in discriminating environmental and pathogenic species, and this could be applied further in primer development. By designing pathogen specific primers for other species, such as potential commensal gut species PCR diagnosis may have increased efficiency in discriminating commensal and pathogenic species.

Multiplex primers were used in some instances so simplify PCR protocols, increasing the throughput, and lowering risk of human error. Although convenient, occasionally DNA extraction artefacts such as organic inhibitors from clinical samples could interfere with the efficacy of multiplex PCR and cause false positives or false negatives, therefore uniplex reactions were used for the majority of testing to ensure this did not occur. With further replication and optimisation the introduction of multiplex primers for most

species specific primers would prove useful for increasing throughput and decreasing rates of potential human error.

4.5 PCR efficiency

The PCR protocols used in this project all use TD-PCR to decrease non-specific binding, which allowed for the use of multiple sets of pathogen primers within one thermal cycler run. Using known isolated specimens as positive controls, each PCR produced high quality bands. However, the use of DNA template derived from clinical samples resulted in the quality of PCR products and specificity to be lowered. Despite this, these PCR products were still of sufficient quality for analysis and diagnostics (**Figure 3.17 - Figure 3.26**).

One of the biggest advantages of PCR in diagnosis is the ability to exploit genes related specifically to the virulence of a pathogen. By using CPE, netF, and VAP it was possible for us to differentiate between commensal and pathogenic forms of a microbe using the same technology in tandem with the initial diagnostic process. DNA sequencing proved the accuracy of these additional primer sets, and were sufficient to differentiate between virulent and non-virulent strains, necessary to guide treatment. Unfortunately, netF did not show any positive results, and therefore primers efficiency cannot be validated, however, netF has yet to be reported in New Zealand. The acquisition of a purified netF sample would allow for the validation of these primer sets, allowing for the use of this to monitor and identify when different pathogens enter New Zealand as performed in Gohari *et al.* (2015) for the testing of cows and foals.

The appearance of non-specific bands forming with clinical samples created the need for a secondary molecular procedure to confirm the accuracy of amplification. In one case, two restriction enzymes were used to run conclusive restriction digest experiments to confirm the amplified bands were the expected product. This method was similar to diagnostic methodology used in detecting human adenovirus (Allard *et al.*, 2001). The

combination of PCR and restriction enzyme (PCR-RE) allowed rapid and reliable identification of subgroups of adenovirus. This technique can be applied to pathogen detection confirmation, offering a reliable result faster than DNA sequencing. Although these additional digests extended the diagnostic time by a minimum of 4 hours they allowed a reliable confirmation that was further clarified using DNA sequencing (EHV-1 & EHV-4 electropherograms, see Appendix 3).

DNA sequencing was used to confirm both the accuracy of primer amplification and identify the pathogen at a species level by using BLAST to analyse sequence data. The quality of DNA sequencing was highly variable, and can be attributed to multiple factors. On occasion, non-specific binding and primer dimers during PCR amplification had to potential to reduce the quality or length of DNA sequencing (Rychlik, 1995). Also, DNA sequencing electropherograms frequently showed multiple peaks, indicating multiple, non-identical DNA sequences were being sequenced and read simultaneously by the capillary mechanism, a phenomenon recognised from the DNA sequencing of multiple pathogens (Hartmeyer & Justesen, 2010). This irregularity was seen strongly in sequencing results from 16S rRNA primers and can be attributed to the presence of multiple microbes causing multiple amplicons from different species. For non-16S rRNA genes, better DNA sequencing results could be obtained by using a different PCR product purification method, such as a gel extraction, cloning of the PCR product, or through more specific primer design.

Overall, the confidence gained from sequencing the PCR products meant it was possible to provide a provisional diagnostic result within 6-24 hours and confirm with sequencing within 5 days. Returning provisional results back within a short time frame, meant that veterinarians were able to make modifications to any ongoing treatment. Restriction digests of PCR products were extremely useful as they confirmed the result without sequencing and could be incorporated into testing procedures to decrease any false positives.

4.6 Use of PCR to diagnose horse infections

Rapid diagnostic techniques have an important place in veterinary medicine, helping to mitigate otherwise avoidable spread of diseases and infections. Of the 146 total samples received for testing, 66.4% returned a PCR positive for one or more of the infection indicative primers. In contrast, culturing methods only succeeded in identifying 46% of samples cultured to a species level. All positive PCRs were sequenced to prove the correct amplification of products and to help in the diagnosis of the causative microbe.

In most cases, the sequencing results confirmed the reliability of PCR primers for equine diagnostics with all but one high quality sequencing data returning a BLAST result suitable to the gene sequenced. This provided good evidence that the primers used within the study were appropriate for the amplification of these pathogenic genes, and therefore for diagnostics. Species level data obtained through BLAST analysis reflected previous information on suspected pathogens found in literature (McKenzie III & Furr, 2001; Toombs-Ruane *et al.*, 2016), with *S. pneumoniae*¸ *S. epidermidis*, and *S. equorum* occurring within multiple clinical samples.

It is important to note that *R. equi* primers CHO consistently produced low quality sequencing with lowered lengths and poor alignment, resulting in either the forward or reverse sequence being available for sequence analysis. Another irregularity noticed during BLAST analysis was within the forward sequence for vapA from S015, which at a trimmed length of 136 nucleotides returned a strong identity score to a tescalcin gene from *Equus przewalskii* and was not related to the *R. equi* virulence associated proteins. Through further investigation it was discovered that the 136bp PCR product **Figure A3.18** shared a 94.9% similarity to *Equus przewalskii* tescalcin gene (NCBI accession: XM_008515476.1). This amplified area **Figure A3.19** then shared a 56.6% similarity when mapped to the *R. equi* virulence plasmid (NCBI accession: AF116907.2). **Figure A3.20** shows the areas of the *Equus przewalskii* tescalcin gene in which the vapA primers bound, producing amplicons falsely identified as *R. equi*. This irregularity was noted and use of vapA primers was discontinued and replaced with VAP, which produced high quality sequencing specific to either *R. equi* vapA or vapB.

Generally, 16S rRNA Gram positive and Gram negative primers returned low quality results, however their use within diagnostics should not be discontinued, with generalised primers incorporated for validating procedures. Generalised 16S rRNA primers provide a good positive control in sample testing, ensuring the PCR works, and identifying the presence of abnormal pathogens within normally sterile sample types. Additionally, in non-sterile samples, the use of 16S rRNA primers can assist in the detection of inhibitory compounds that may cause false negatives in testing which otherwise would have been previously diagnosed as an absence of infection.

Using a panel of primers to a variety of microbes the following samples were screened:

i) Sterile samples

86.96% of all sterile samples returned PCR positive and 73.9% of results were identified at a genus level or higher, signifying the sensitivity of molecular technique. In comparison, 84.62% of culturing results produced a result (defined as growing within growth media), but only 46.15% returned a result that could be identified at a genus level. Additionally, methods needed to culture and return a result were more variable than methods used for PCR, which were more consistent following DNA extraction.

Within sterile samples, high levels of Gram positive infections and significantly higher occurrences of *Staphylococcus* spp. were seen which follow the trends expected and reported in the literature (Toombs-Ruane *et al.*, 2016). These trends were also seen within the age divisions, with nearly 50% of sterile samples coming from newborn animals, which is the age bracket of vulnerability that is associated with increased risk of systemic infection. It appears that prevalent systemic pathogens follow trends expected from the limited literature on NZ sepsis, however this could also be due to our primer choices being influenced around pathogenic species previously identified within the available literature.

The specificity and efficacy of detection using PCR in sterile samples could be significantly increased, as these sample groups are not affect by the presence of environmental pathogens that may cause false positives. This could be achieved by designing additional primers at a genus or species level for species known or suspected to cause infections within sterile sites. Determining these likely species can be achieved by retrospectively analysing culture results received from these or similar sites within the same geographical location, or through literature searching. With the introduction of extra primers into this PCR panel, sensitivity, and the ability to exactly identify organisms causing infection would increase, however, the time required to set up more reactions would also increase. This could be mitigated by the introduction of multiplex PCR which would use these primers, within one tube, lowering the time needed to set reactions up. This also has the potential to lower error rates associated with pipetting into many individual tubes and they can be set up and optimised through the use of multiple concentration and temperature gradients before use with clinical samples.

ii) Respiratory samples

By using established PCR protocols, DNA extraction techniques, and PCR primers derived from a previous study (Patty, 2012; Patty & Cursons, 2014), respiratory samples produced high quality results within a short time frame. As respiratory samples were mostly received as dry swabs or swabs in saline solution, the DNA extraction process was faster, compared to gastrointestinal or sterile samples as there was no need for extensive treatment to remove potentially inhibitory compounds. It is important to note that a limited number of swabs were delivered in an agar media which carried over contamination of an agar substance into the extracted DNA, which proved inhibitory in PCR, giving false negative samples. These false negatives were noticed due to the use of a universal 16S primer set with the respiratory samples, used to ensure correct amplification in all samples.

Results for respiratory disease were limited, due to the non-sterile nature of the nasal tract, which negated the use of broad primer sets that would amplify commensal bacteria in the upper respiratory tract. The sensitivity of

this primer assay could be improved by the addition of more species specific primers against organisms, such as *S. pneumoniae, Pasturella* spp., and Equine rhinovirus (Dunowska *et al.*, 2002b; Wood *et al.*, 1993). This same methodology could also be implemented for the detection of Equine influenza control if this virus became present within NZ as accurate PCR primers have already been validated for virus identification (Foord *et al.*, 2009).

iii) Gastrointestinal samples

The molecular analysis of gastrointestinal infection showed high detection rates, however, the range of primers available limit the number of species that were able to be detected. Also, due to the absence of clinical information, it was not possible to make any conclusions about age trends in the Waikato region and infection.

Improvement of gastrointestinal molecular diagnosis requires the introduction of more specific primer sets and of methodology that can assess the level of the pathogen and its amplification. Similar to the respiratory tract, over colonisation of the gastrointestinal tract with commensal bacteria can cause gastrointestinal infection in animals, and these infections are rarely detected due to the regular presence of these microbes within the gut. To achieve this level of detection it would be necessary to use a more quantitative molecular method to detect the abnormality of pathogens within a faecal sample. qPCR or digital PCR would allow this, by obtaining the actual levels of certain pathogenic species, such as *E. coli*. Early amplification compared to the control would be indicative of an overabundance of that microbe within the gastrointestinal tract. Such technology has already been used in the detection of *Salmonella* spp. which are not part of the normal gastrointestinal flora of horses, but may be present in some animals carrying infection (Pusterla *et al.*, 2010). Detecting non-commensal gastrointestinal pathogens that may also be found in a healthy gut could also be achieved using primers associated with virulence, such as the VAP primer used in distinguishing facultative and pathogenic *R. equi* species. While incorporating additional primers it would be of value to identify primers suitable for the detection of the RNA virus, equine

rotavirus as it's prevalence in NZ equines warrants rapid detection and should not be overlooked (Horner, 1989). Primers to accomplish this detection have been used for the identification of Group A rotaviruses and provide a valuable starting point (Collins *et al.*, 2008).

These results from this investigation show varying efficiencies of PCR detection, however, with further development, and the widening of pathogen search primers this method could provide an accurate and high throughput method of pathogen identification in NZ veterinary settings.

4.7 Shortfalls of molecular approach

There are a number of things that can affect the efficiency of PCR diagnosis, including determining appropriate and unique target sequences, primer design, type of PCR technology being used, DNA extraction methods and PCR product detection that are suitable for a mass panel of detectable species (Yamamoto, 2002). However, these are often able to be mitigated through the optimization of PCR protocols. Current PCR panels do not include all potential pathogens that could be present causing infection, meaning selected pathogenic species are often overlooked (Lehmann *et al.*, 2008). Inhibitory compounds present in the PCR template such as enzymes or reagents can affect the amplification of PCR making it difficult with non-pure clinical samples, such as those from tissue (Stefańska *et al.*, 2016). In addition, some pathogenic species may be more difficult to be lysed or extracted from the sample meaning they can be missed (Stefańska *et al.*, 2016). The availability of multiple DNA extraction techniques, depending on sample type, often means the DNA template used with any kind of molecular technique can be vastly different and can affect the accuracy of downstream applications. An absence of standardisation of these extraction methods can confound results, particularly as poorly purified DNA can be inhibitory to PCR (Schrader *et al.*, 2012).

When testing for a panel of infectious organisms PCR conditions need to be similar in order to run multiple samples in tandem. Unfortunately different

primers for different genes can require vastly different PCR cycling conditions. To mitigate this issue an example of specific PCR technology is the use of TD-PCR. PCR is also subject to the risk of carry-over contamination from reagents, workers, tools, and other samples which have the potential to contaminate subsequent samples leading to false positives in DNA amplification diagnosis, therefore, extreme care must be taken to avoid this in order to maintain the accuracy of PCR diagnosis.

4.8 Molecular versus traditional diagnostic approaches

From our investigation we have seen that molecular diagnostic techniques are faster, and more sensitive than traditional culturing methods, and these advantages make PCR diagnostics a valuable addition to veterinary care. PCR took between 6-12 hours to complete following sample reception, which included a maximum of 2.5 hours to extract DNA, 3 hours for PCR, and 30 minutes for agarose gel electrophoresis and visualisation. Culturing methods often took 48 hours for analysis following sample reception, which included 12-24 hours for the blood sample to grow in culture broth, another 12-24 hours for single colony streaks to form on the agar, and 15 minutes to visualise a single colony with Gram staining.

In terms of the frequency of positive results, PCR using sterile samples identified the most pathogens from each sample, and this is linked to the fact that sterile samples will not normally have any commensal microbial species present, allowing us to search for any pathogens. Comparatively, the variety of identified pathogens in respiratory and gastrointestinal samples was lower due to the restrictions on primer choice as a result of the presence of commensal microbial species in these areas.

The results received using traditional culturing methods were limited by the use of a single growth medium. Although there was evidence of bacterial growth in 85% of cultured samples, due to the presence of turbid media, we were unable to identify all microbes to a genera level in all samples. In addition, using culture we were unable to identify any viral or fungal species,

whereas PCR identified both viral and fungal species using identical methods that were used for the identification of bacteria.

A big advantage that bacterial, viral, and fungal culturing does hold over both PCR based methods is the ability to differentiate between live and dead microbes. Due to the high specificity of molecular techniques it is possible for them to amplify and return positive results to microbes which may have been present but not necessarily responsible for infection. This overly sensitive feature may actually cause veterinarians to overlook possible causes of infection and therefore misdiagnose and apply inappropriate treatment. Due to the nature of cultures, only live microbes will be transferred from the animal and grow within the correct growth media (Kralik & Ricchi, 2017). Another advantage of culturing is the ability to perform antibiotic sensitivities on the colonies isolated, which proves useful in prescribing therapeutic treatment.

Despite this, molecular methods are clearly useful in identifying bacteria and other pathogenic microbes alongside or instead of typical culturing methods. PCR exhibits higher sensitivity and specificity, and can help reduce subjectivity when interpreting the morphological aspects involved with traditional culturing methods (Settanni & Corsetti, 2007).

4.9 Future of PCR in diagnostics

Worldwide PCR is being used as a detection technique for infection in human and veterinary settings. Predominantly in equine husbandry PCR is currently being used to identify viruses, such as Equine herpesvirus, or as a rapid identification of particularly virulent infections such as Strangles (McBrearty *et al.*, 2013; Patty & Cursons, 2014). To our knowledge at this point PCR is not being used as a sole diagnostic testing method in NZ equines. Based on results across the three sample groups, PCR detected microbes matched similar trends that had been previously identified in NZ literature discussed in chapter one, which provides confidence in the use of this methodology.

However, before introducing an entirely PCR based diagnostic scheme into commercial veterinary laboratories it would be wise to amend some of the protocols used in this investigation to increase the efficiency, accuracy, and usability of the PCR assays. Extending the primer range of each group would increase the diagnostic sensitivity, particularly including primers for pathogens that pose the most significant risk to the equine industry, such as equine influenza. To aid in the ease of replication, and to reduce the risk of pipetting error causing false negatives it would be advisable to develop primers that could be adapted for multiplex PCR, combining related genes using primers to *R. equi* along with a primer set that identifies VAP that allow the identification of the presence and virulence of a selected pathogen. It would also be important to develop accurate primers for detecting RNA viruses, such as those associated with equine rotavirus as it may be possible to identify these using identical methods.

4.10 Alternative molecular approaches in diagnostics

Due to the time sensitivity associated with equine diagnostics, particularly in the case of sepsis there is the opportunity to exploit technology that speed up the diagnosis outcome. For example, column based extraction kits, and the use of platforms such as quantitative PCR (qPCR) to identify pathogens within a sample would help to lessen the time it takes to identify using the current chloroform based extraction and PCR, used in this investigation. Kits, such as Promega Wizard® SV96 kit or Bilatest bead DNA 2 kit, tested for efficiency and throughput using mammalian blood cells are capable of producing high quality DNA with ease, and in a short timeframe (<30 minutes) required for diagnostic medicine (Smith *et al.*, 2003). Developing a multiplex qPCR procedure following a rapid kit DNA extraction would allow the identification of pathogens rapidly and more effectively, such as those seen in human LightCyclerSeptiFast which has a sensitivity at 95% (Chang *et al.*, 2013).

Despite the small number of positive results obtained from this investigation the trends did follow those reflected within the literature, allowing us to confirm the current trends surrounding the causes of infection in New Zealand equines. These epidemiological trends can be used to drive the development of other highly sensitive and effective molecular approaches, such as loop-mediated isothermal amplification (LAMP) or ELISA (Nemoto *et al.*, 2010). Using the findings from this study and previous ones, complimentary antibodies could be developed to the most prominent infection causing microbes and an ELISA assay developed, specific for each infection type. This would allow for not only rapid assay based testing for common equine infections, it would also allow for these detection methods to be undertaken with ease on the farm, avoiding the time wasted with sample postage and delivery that often slows down diagnostic times. Although no current literature describes the use of ELISA in on farm pathogen detection in horses, this methodology has been used in the measurement and detection of other serological samples and other animals such as measuring progesterone in dairy cattle milk, demonstrating the viability of testing without a wet lab (Rajamahendran *et al.*, 1990).

Lastly with the development of new molecular technology there is now the potential to use next generation sequencing (NGS) for the detection of microbes in equine samples. In clinical trials identifying bacteraemia-caused sepsis in human patients NGS identification was successful in identifying high levels of bacterial DNA fragments allowing for detection of Gram positive and Gram negative bacteria from blood samples in around 30 hours (Grumaz *et al.*, 2016). This NGS diagnostic approach that provides high quality results from patient blood samples, could easily be introduced into veterinary medicine, with NGS providing a non-invasive and rapid diagnostic method that could be incorporated into equine diagnostics (Grumaz *et al.*, 2016; Van Borm *et al.*, 2015). Since its development, the price of NGS has continued to drop, allowing its consideration as a price effective option for veterinary medicine due to the noted high throughput and the capacity to generate high levels of information useful for routine diagnostics (Van Borm *et al.*, 2015). It is being applied to veterinary medicine for the detection of infectious disease, and has the potential to

revolutionise detection and understanding of infectious diseases. However, this technology is not currently widely used within commercial laboratories due to the requirement for expertise and the price for equipment and reagents, which remains higher than currently available methods (Van Borm *et al.*, 2015). The continued advances in molecular biology have the potential to increase diagnostic capabilities and the health and welfare involved with animal husbandry and infection management. It is an area that should be continually investigated and implemented into diagnosis as the technology evolves and cheapens over time (Cunha & Inácio, 2015). Using NGS also allows us to explore the microbiome of equines, which in a diseased state can differ significantly (Costa *et al.*, 2012). Further exploration of normal and diseased microbiota provides a unique and detailed diagnostic method, particularly in the detection of gastrointestinal pathogens which may occur in pathogenic and commensal states (Costa & Weese, 2012). Current microbiome disease research by Costa *et al.* (2012) has demonstrated changes of relative abundance of Fusobacteri, Actinobacteria, and Spirochaetes within the gut in association with colitis. Of particular interest there is suggestions that some horse's microbiota leaves them more susceptible to gastrointestinal disease, providing another avenue for future research (Julliand & Grimm, 2016).

Chapter Five

FUTURE EXPERIMENTS AND RECOMMENDATIONS

Despite showing the potential of PCR as a standalone diagnostic test, due to limitations on the clinical samples received it was not possible to completely investigate the trends of equine infectious disease within New Zealand. It was possible to identify infectious microbes within clinical samples, however the information collected was not sufficient to be used as proof of common, temporal, or significant infection types present in New Zealand horses. Additionally, with a staggered and gradually broadened sample collection and mix of sample types it was difficult to subject each sample to the same strict diagnostic criteria. Therefore, it cannot be said with certainty the absolute cause of infection, but the information could be used to guide treatment within a veterinary setting.

With long term collection of samples overtime and standardisation of testing it would be possible to obtain sufficient information to develop a broad understanding of infection trends within New Zealand horses among different age groups, conditions, and temporal stages. Below are a number of considerations for future study design.

5.1 Extended sample collection

Determining trends and causes of infection in horses is an important part of equine diagnostic development. With sufficient information about mechanisms and trends of infection there will be better guidance allowing more accuracy with primary treatments while waiting for information from diagnostic facilities. To achieve this the following is recommended.

5.1.1 Collection of temporal samples

It is known that the causes of infections can vary throughout the year, and these temporal changes can have significant effects on routes of treatments required due to differences in infectious pathogens. Collecting samples through an entire year would allow diagnostic information to be obtained for analysis of the temporal trends observed, which can then be used for subsequent first lines of treatment.

5.1.2 Analysis by age groups

Analysis of trends of infection in varying age groups may also provide substantial information that can guide future treatment in horses. By obtaining sufficient samples across all age groups, it would be possible to identify potential links between age or development status, and the potential risk of certain types of infection. This level of analysis can again guide the first line of treatment, reducing the risk of applying the wrong treatment while waiting for diagnostic support from PCR or cultures.

5.1.3 Expand primer range

Biosecurity within the New Zealand equine industry is strict, however the industry is still vulnerable to infection from non-endemic pathogens. Equine influenza is abundant in many countries, but it is yet to affect New Zealand. By designing and incorporating primers for high risk pathogens such as equine influenza into established PCR detection processes it would be possible to screen for high biosecurity risks before infections spread.

5.2 Standardisation of methods

During the course of this investigation, there was no time to standardize the methodology of sample reception, DNA extraction, and PCR. Solidified and standardised methods are important for reproducible and reliable results that would allow this methodology to be readily incorporated into a diagnostic

facility with ease. Standardised methods could be achieved through experimenting with multiple DNA extraction methods to determine a single method that provided sufficient DNA quantity and purity for each sample type. Additionally, by outlining clear procedures for each sample group diagnosis could be achieved on a large scale by firstly extracting the DNA from all samples with the same methodology, and then a flow through to a standardised PCR tests for each sample division. By further altering PCR conditions and reagents it may also be possible to optimise all reactions to work to sufficient quality within one machine with a single PCR programme.

5.3 Multiplex PCR

The development of multiplex PCR across all sample groups also has the potential to increase the ease of a standalone PCR diagnostic system. By optimising multiplex reactions for each sample group it would be possible to further decrease the time required for molecular diagnosis, and likely reduce error rates that may occur during PCR from pipetting error resulting in false negatives. Multiplex PCR assays have been successfully developed for the detection of bacteria and virus in humans following optimisation. The use of nested primers, and enzymes were tested to increase specificity of multiplex PCR and were found to produce positive results, however, nested PCR increased risk of contamination and reduced automation (Elnifro *et al.*, 2000)

5.4 qPCR

qPCR is a molecular procedure based on PCR that uses fluorescent tags to produce quantitative and real-time results, allowing the identification of amplification faster, and with higher sensitivity when compared to conventional PCR (Deepak *et al.*, 2007). The use of fluorescence allows fast detection of low levels of DNA, such as that in some clinical samples, and has demonstrated utility in the detection of virus, bacteria, and fungi in various clinical samples (Deepak *et al.*, 2007). Through the use of qPCR it is possible to quantify the levels of amplified bacterial genes, and therefore detect the presence of over colonised pathogens within the gut which may

be seen as normal. Detection of pathogens within gastrointestinal infection using regular PCR is inhibited by the inability to detect changes to regular gut flora, and there is an inability to detect changes such as over colonisation by a single bacteria that may cause gastrointestinal infection.

qPCR technology has been utilised for the detection of specific pathogens in equine samples with high rates of success. Pusterla *et al.* (2010) utilised qPCR for the detection of *Salmonella* using the invA gene with 100% success rate in the 40 clinical samples, demonstrating the potential of qPCR as an accurate and rapid method of pathogen identification.

Chapter Six

CONCLUSIONS

The aim of this investigation was to determine the efficacy of PCR as a diagnostic tool for the identification of microbial infection in horses. PCR was found to be effective at detecting bacteria, fungi, and viruses associated with equine infection rapidly and was a reproducible methodology within a variety of sample types. The DNA based approach of PCR allowed for the identification of microbes from multiple sample types, proving successful in identifying pathogens in blood, faecal, respiratory, pus, and synovial fluid samples.

Identification rates from sterile samples with PCR were 87%, compared to genus level identification from traditional culturing methods at 46.1%. PCR also demonstrated higher sensitivity compared to cultures, allowing identification of pathogens from samples following empiric antibiotic therapy. PCR diagnosis was also faster than bacteriological culturing (6 hours vs 48 hours).

Despite small sample sizes retrieved from this project it is still possible to make estimates about prevalent pathogens affecting the New Zealand Equine Industry. The pathogens recorded from this project did align with those previously reported in the NZ equine literature.

A number of improvements can be made to the molecular methodology to increase throughput, reproducibility, and reduce error rates. Regardless of this, PCR has the potential to increase diagnostic efficiency, comparable to traditional culturing techniques increasing the health and welfare involved with animal husbandry and infection management.

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Appendix One

SOLUTIONS AND MEDIA

1. DNA Extraction Solutions

Plant CTAB (Used for faecal extractions)

- 5ml 1M Tris, pH 8.0
- 2ml 5mM EDTA
- 1.0g CTAB
- 4.15g NaCl
- 1.0g PVP-40
- 0.0445g DEICA
- 0.5ml 2-Mercaptopethanol

SDS Lysis

- 5ml Tris
- 5ml EDTA
- 5ml SDS
- 0.1ml NaCl

Lithium Chloride

- 10.6G LiCl
- Make up to 50ml with MQ water

10% CTAB

- 0.79g Tris-HCl
- 4.09g NaCl
- 0.37g EDTA
- 1g CTAB
- Make up to 50ml with MQ water

TE buffer

- 0.079g Tris-HCl
- 0.019g EDTA
- Make up to 50ml with MQ water
- Adjust pH to 8.0

PEG/KOH

- 27 ml Polyethylene glycol (PEG) 200 (Sigma)
- 465 µl 2M KOH (Riedel-de Haën)
- Make up to 50 ml with MQ water
- Adjust pH to 13.3-13.5

Na isopropanol

- 5.5ml 2M NaOH
- 0.25ml 5m EDTA
- 35ml Isopropanol

Viral extraction mix (GITC)

- 31.9g GITC in 20ml H_2O
- 9.55g GuHCl
- 6.0g Urea
- 0.35g of INCI
- 0.368g $Na_3C_6H_5O_7$
- 1.0ml 2-Mercaptoethanol

2. Agarose Gel Electrophoresis solutions

Super Buffer (50x)

- 8g NaOH
- 45G Boric Acid
- Make to 400ml in MQ water, pH8

Super Buffer (1x)

- 40ml 50x Super Buffer
- Bring to 2 litres with MQ water

2% Super Buffer Agarose Gel

- 0.800g of HydraGene LE Agarose
- 40ml 1x Super Buffer
- Add 3µl dilute EtBr (10mg/ml H_2O)

DNA loading buffer (6x)

- 3mL 30% (v/v) Glycerol
- 25mg 0.25% (w/v) Bromophenol Blue
- 25mg 0.25% (w/v) Xylene Cyanol FF
- Make up to 10ml with MQ water

Genscript ready to load 100bp molecular weight ladder - Ready to load (0.25µg/µl) forming 9 bands with a size of 100-1,500 bp. 5µl loaded in a 2% gel.

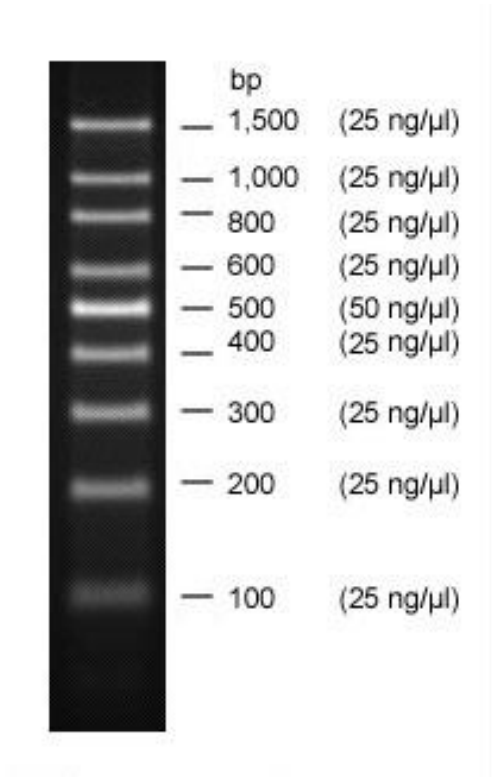

Figure A1.1: Genscript 100 bp DNA ladder run on a 2.0% agarose gel.

Solis BioDyne 100bp DNA Ladder - Ready to load (0.1µg/µl) forming 13 discrete bands with a size of 100-3,000 bp. 3µl loaded in a 2% gel.

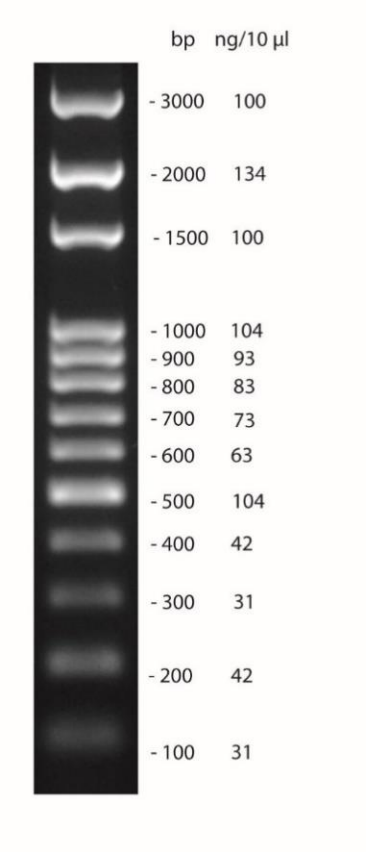

Figure A1.2: Solis BioDyne 100 bp DNA ladder run on a 2% agarose gel.

Bacteriological culturing

Columbia broth

- 35g of Columbia broth media
- 1 litre demineralised water
- Heat to boiling to dissolve media
- Aliquot 5ml into a 25ml culture flask
- Sterilize at 121°C for 15 minutes

Blood Agar Plates (10% sheep blood)

- 400ml of DEPC water
- 20.4g Columbia blood agar (Difco)
- 50ml sheep blo006Fd

Amikacin Agar Plates

- 40µl per 500ml Amikacin (250mg/ml stock)
- 400ml of DEPC water
- 20.4g Columbia blood agar (Difco)
- 50ml sheep blood

Appendix Two

PRIMERS AND PCR REAGENTS

Primers

Primers were obtained from IDT or Sigma Aldrich. Tubes were centrifuged at 16,600g for 10 seconds and resuspended in TE buffer following manufacturer's directions to a concentration of 200µM or 100µM. Stock solution was divided into 20µL aliquots and frozen at -20°C until required.

20µM working stock primers

10µl of forward primer (200µM) and 10µl of reverse primer (200µM) were combined in a 0.6ml DNase/RNase free microcentrifuge tube and diluted with 80µl TE buffer and stored at 4°C to be used as primers for working stock.

10µM working stock primers

10µl of forward primer (100µM) and 10µl of reverse primer (100µM) were combined in a 0.6ml DNase/RNase free microcentrifuge tube and diluted with 80µl TE buffer and stored at 4°C to be used as primers for working stock.

Degenerate primers bases and non-standard bases

Some primers were degenerate or used non-standard bases. Listed dictates are the nucleotides associated with each single-letter code:

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
I	Inosine
B	C or G or T
R	A or G

N A or C or G or T

D A or G or T

2mM PCR master mix

Table A2.1: Components in 200µl 2mM PCR master mix.

Components	**Final Concentration**	**Volume**
10x Buffer B1	1x	20µl
$MgCl_2$ (25mM)	2mM	16µl
dNTP mix (20mM each)	0.25mM	2.5µl

4mM PCR master mix

Table A2.2: Components in 200µl 4mM PCR master mix.

Components	**Final Concentration**	**Volume**
10x Buffer B1	1x	20µl
$MgCl_2$ (25mM)	4mM	32µl
dNTP mix (20mM each)	0.25mM	2.5µl

Appendix Three

SEQUENCING ELECTROPHEROGRAMS

DNA sequencing of PCR products was carried out during this project to determine the accuracy of microbial primers. In addition to BLAST analysis, electropherogram reports were examined to confirm the accuracy of the actual sequencing and confirm the primers were suitable for diagnostic testing (**Figure A3.1** – **Figure A3.20**).

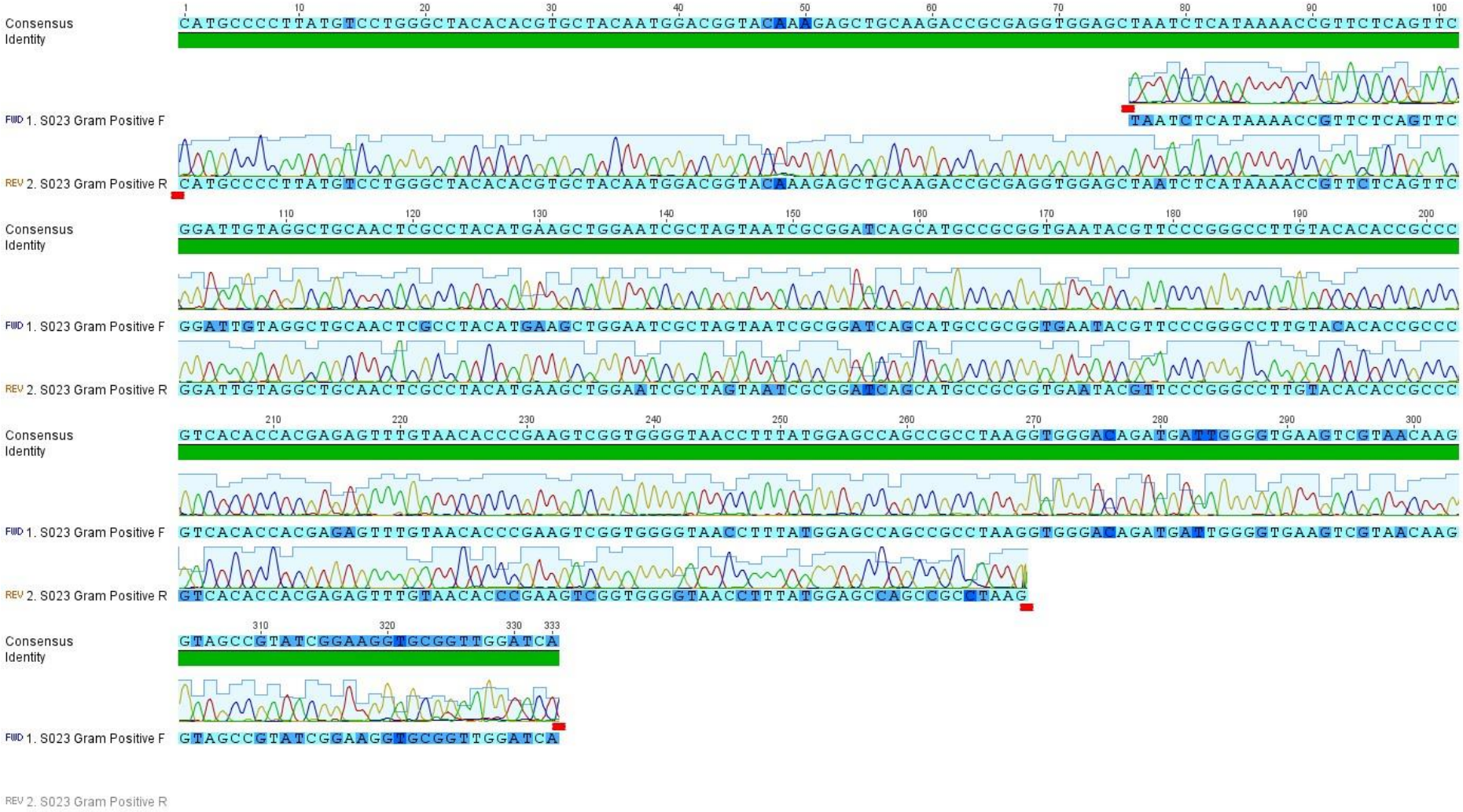

Figure A3.1: High quality electropherogram output from Gram positive 16S DNA sequencing of sample S023.

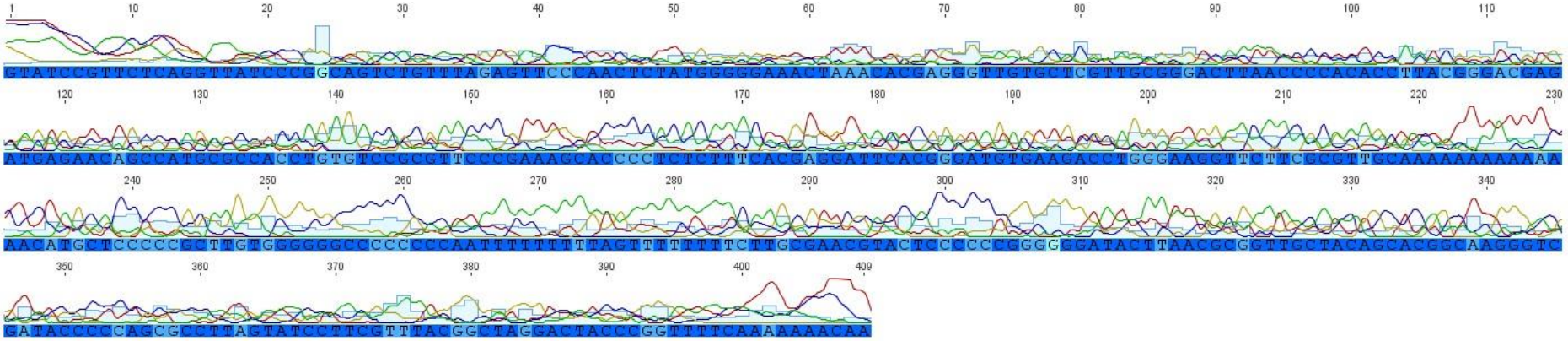

Figure A3.2: Low quality electropherogram output from Gram negative 16S DNA sequencing of sample S008 (Reverse sequence only).

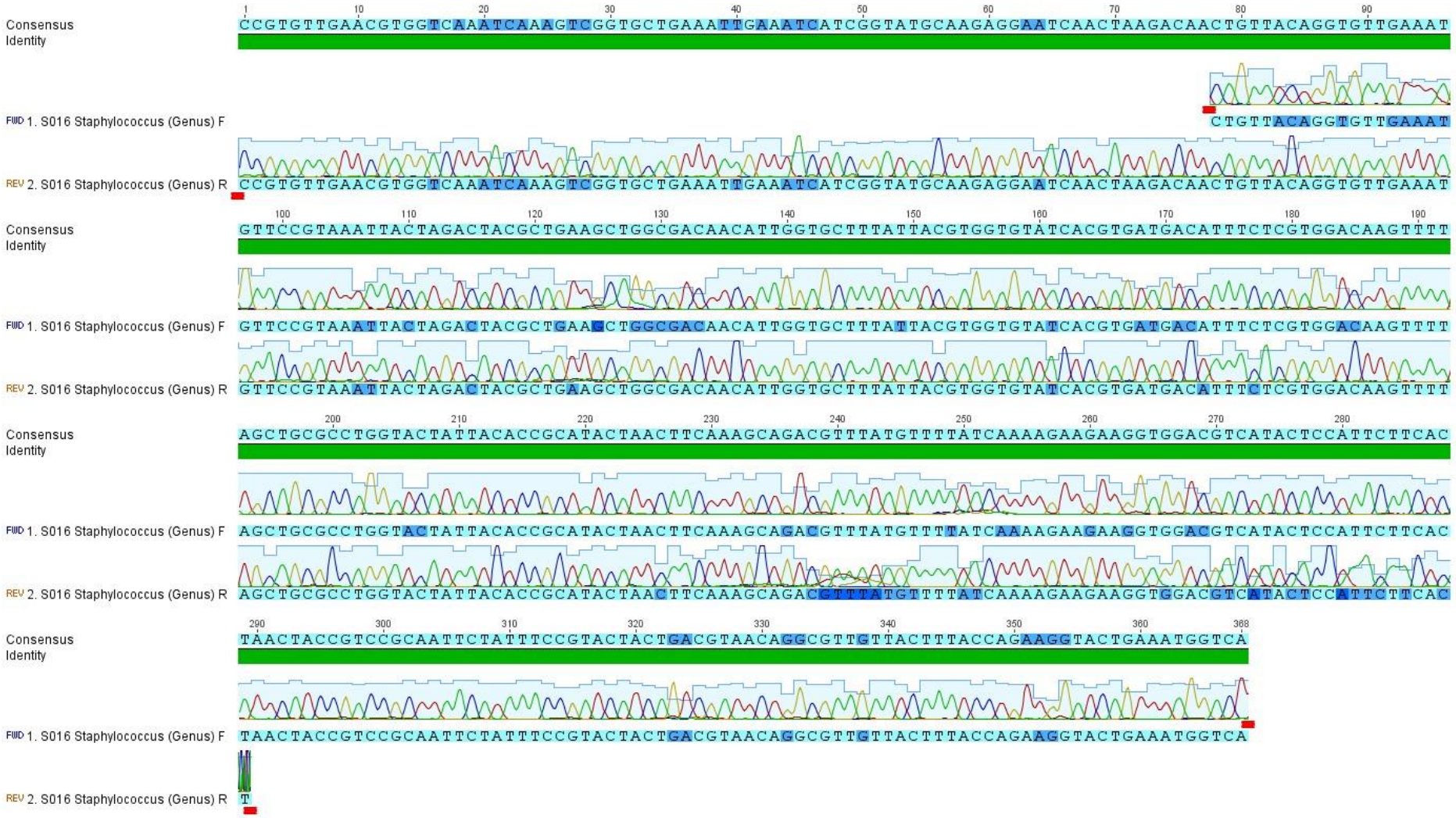

Figure A3.3: High quality electropherogram output from Staphylococcus (Genus) DNA sequencing of sample S016.

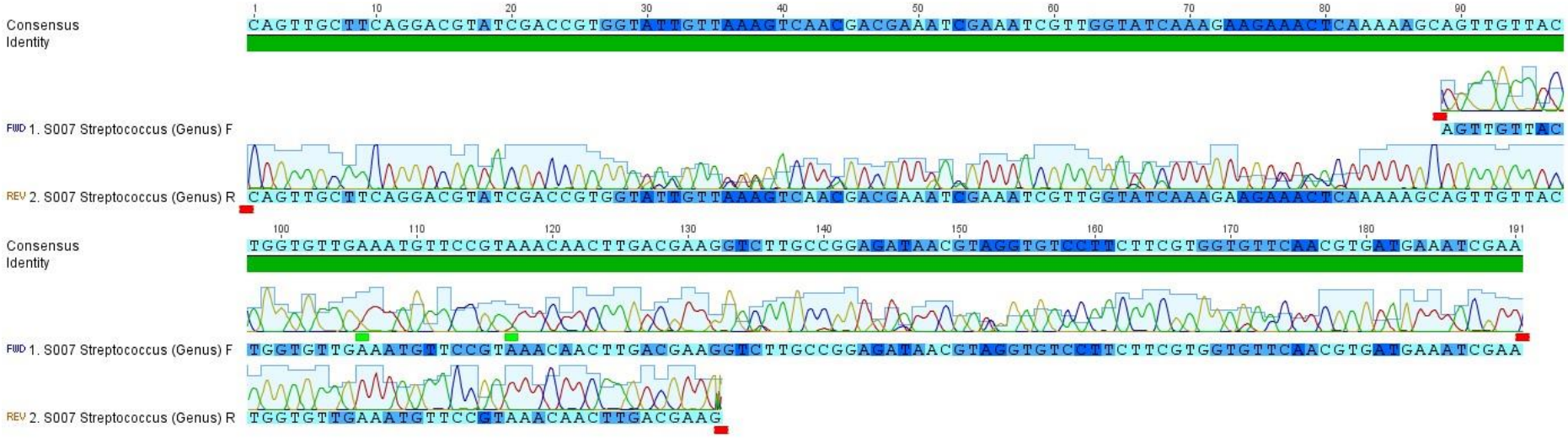

Figure A3.4: High quality electropherogram output from Streptococcus (Genus) DNA sequencing of sample S007.

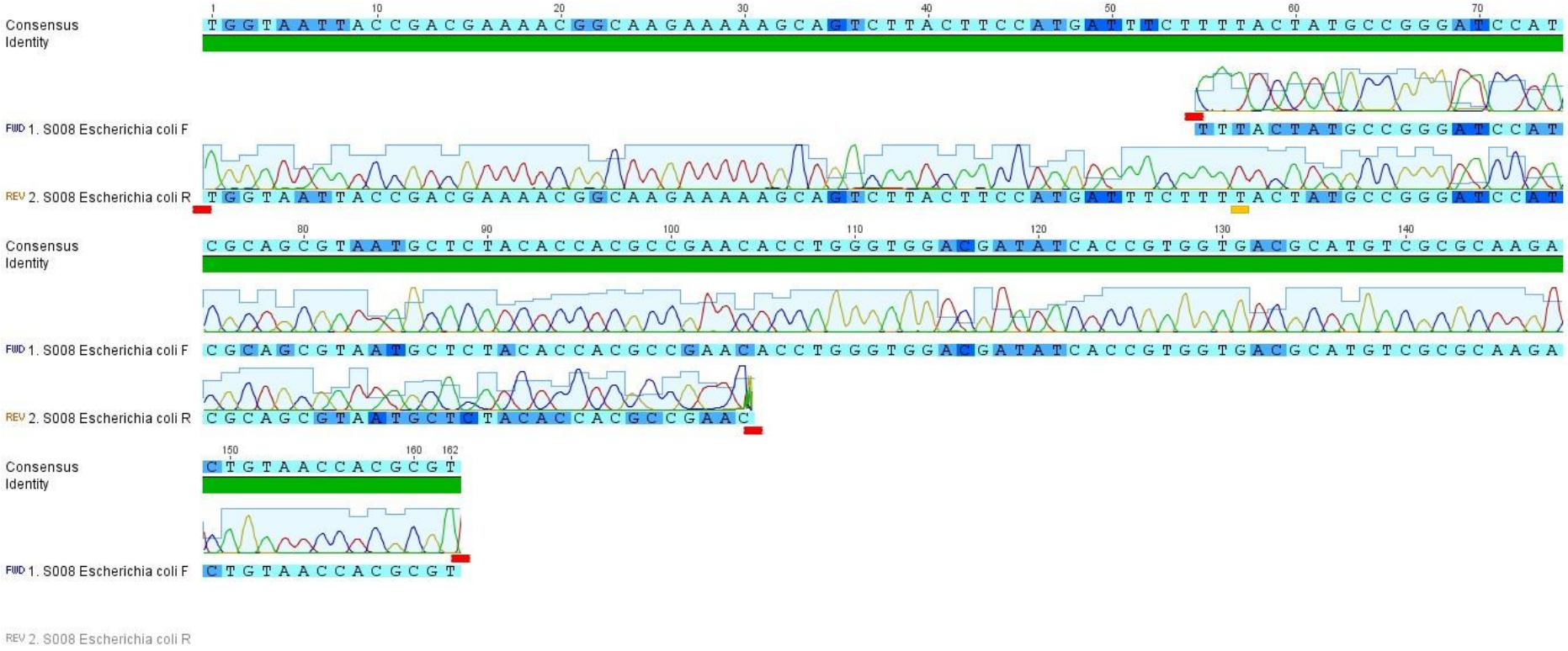

Figure A3.5: High quality electropherogram output from *Escherichia coli* DNA sequencing of sample S008.

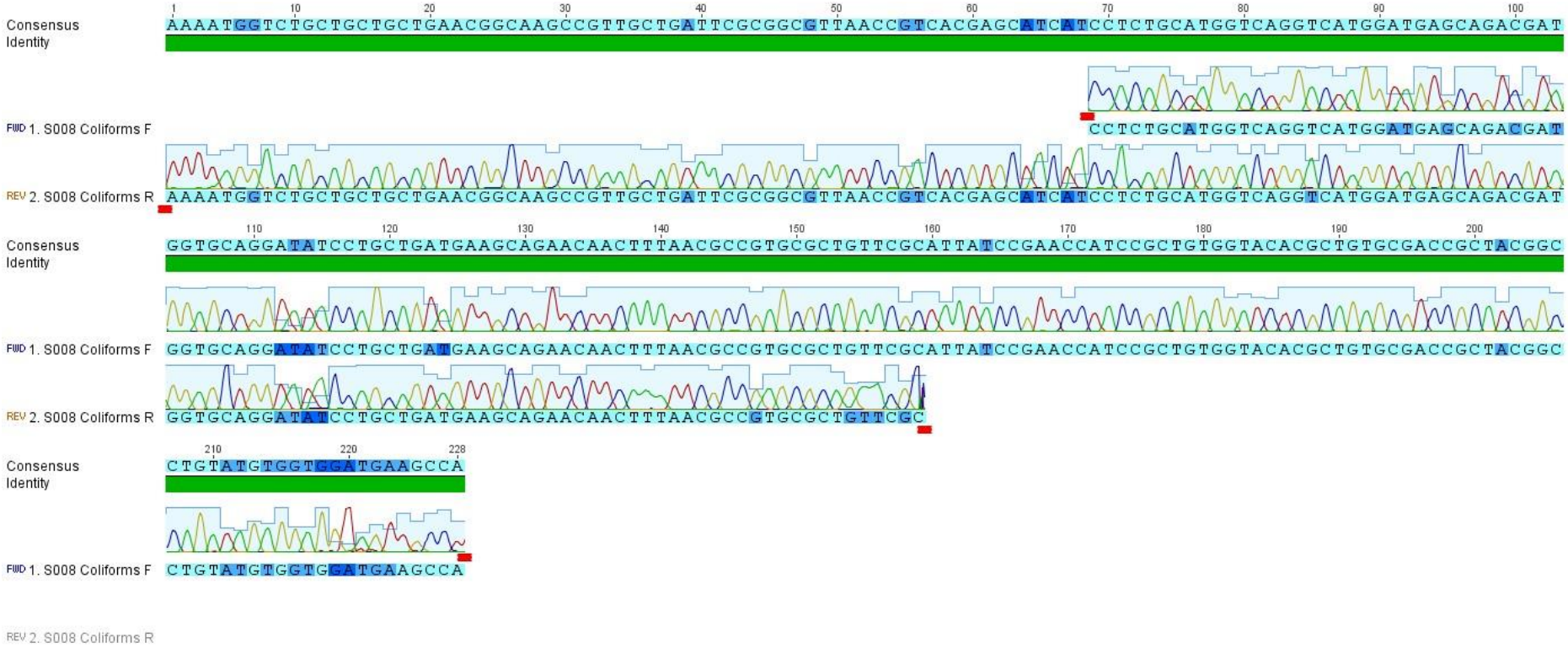

Figure A3.6: High quality electropherogram output from Coliforms DNA sequencing of sample S008.

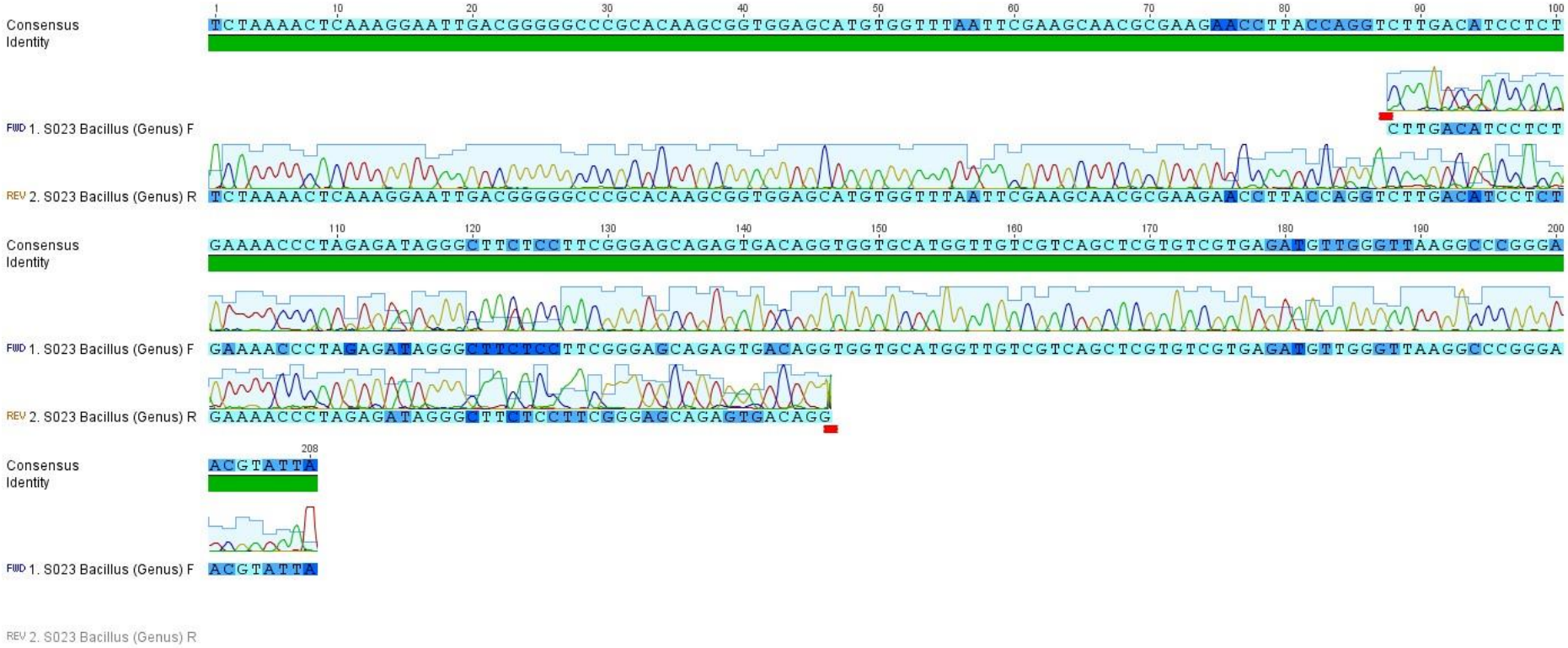

Figure A3.7: High quality electropherogram output from Bacillus (Genus) DNA sequencing of sample S023.

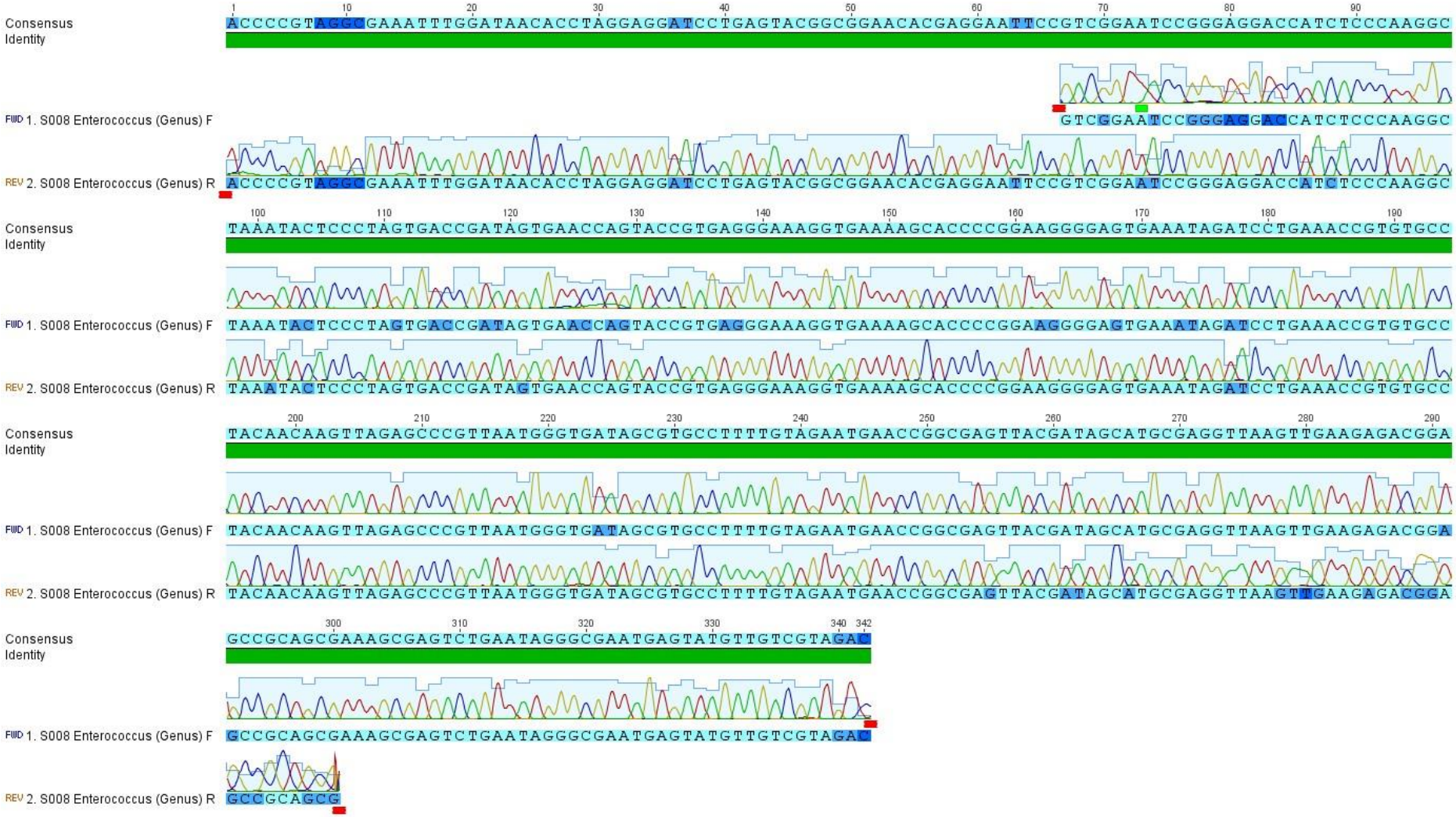

Figure A3.8: High quality electropherogram output from Enterococcus (Genus) DNA sequencing of sample S008.

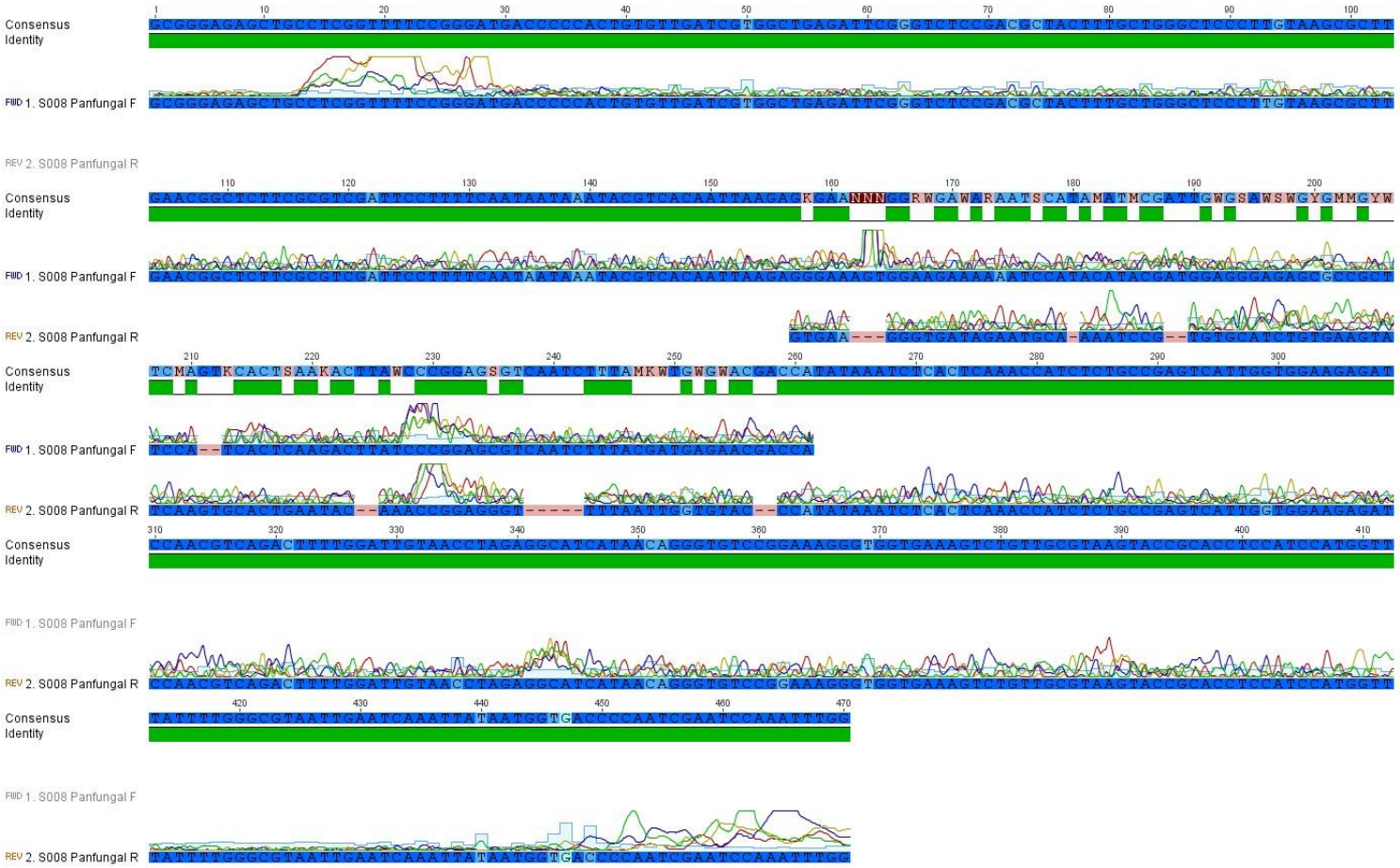

Figure A3.9: Low quality electropherogram output from Panfungal DNA sequencing of sample S008.

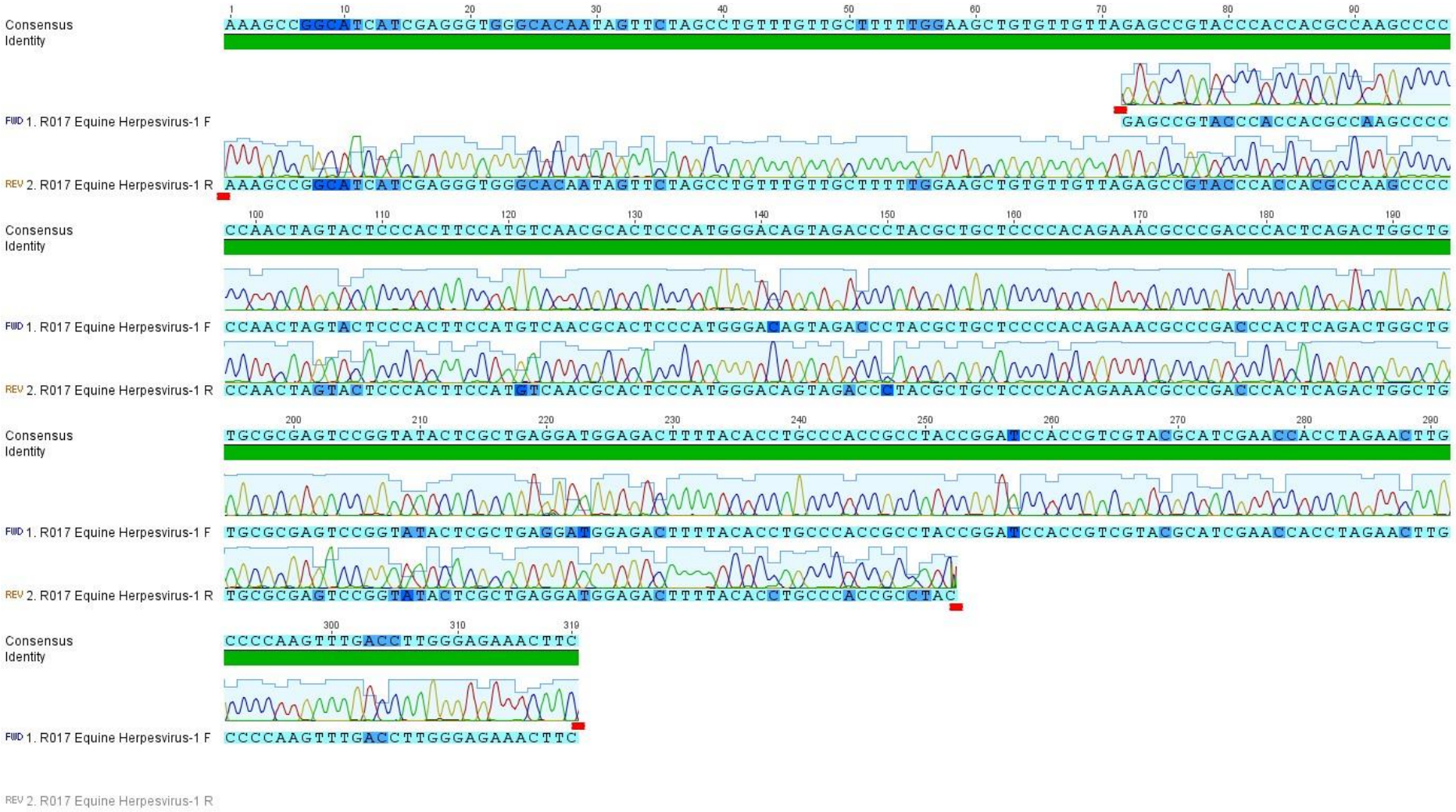

Figure A3.10: High quality electropherogram output from EHV-1 DNA sequencing of sample R017.

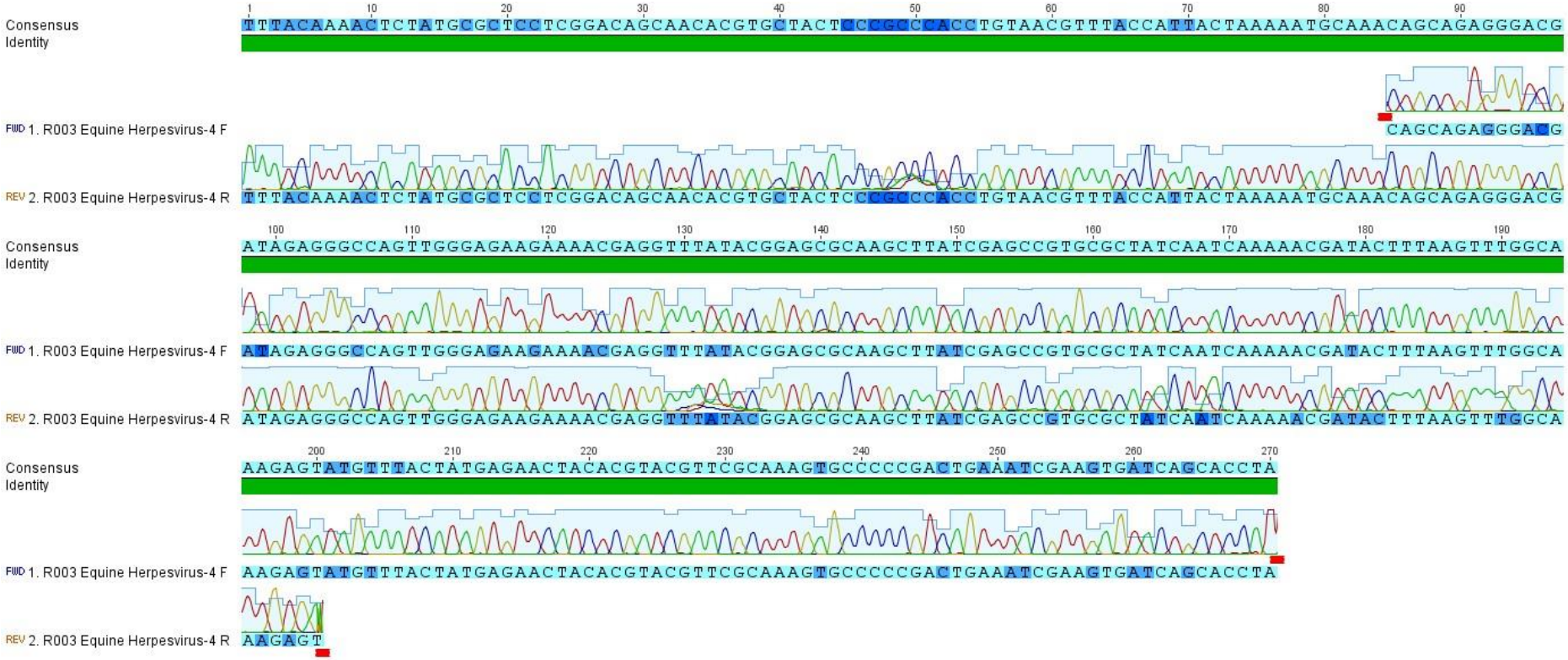

Figure A3.11: High quality electropherogram output from EHV-4 DNA sequencing of sample R003.

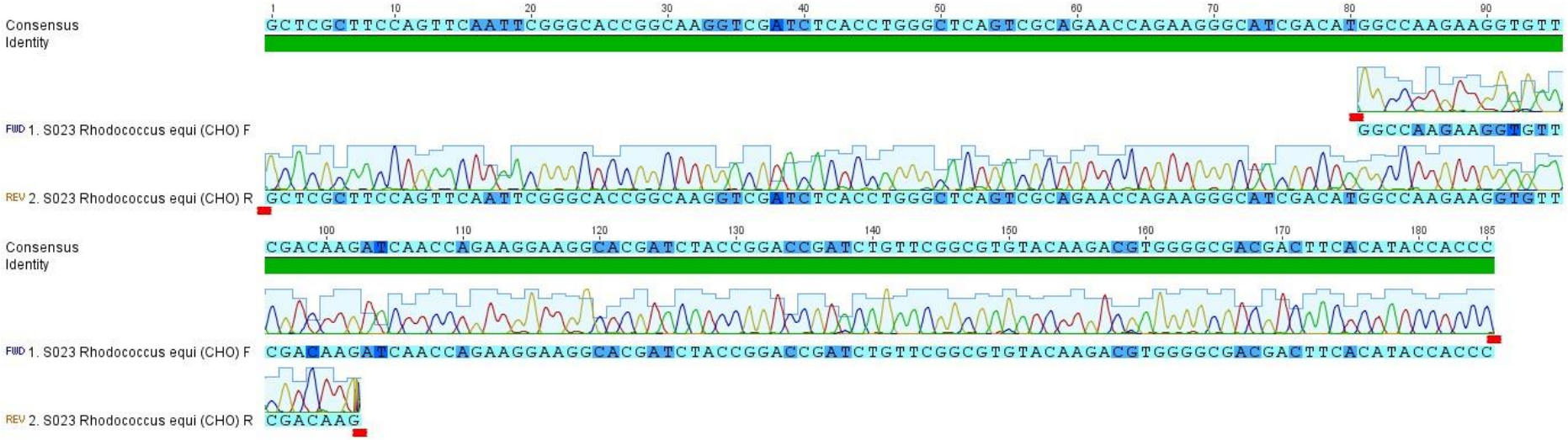

Figure A3.12: High quality electropherogram output from *R. equi* (CHO) DNA sequencing of sample S023 (DNA extracted from cultured material).

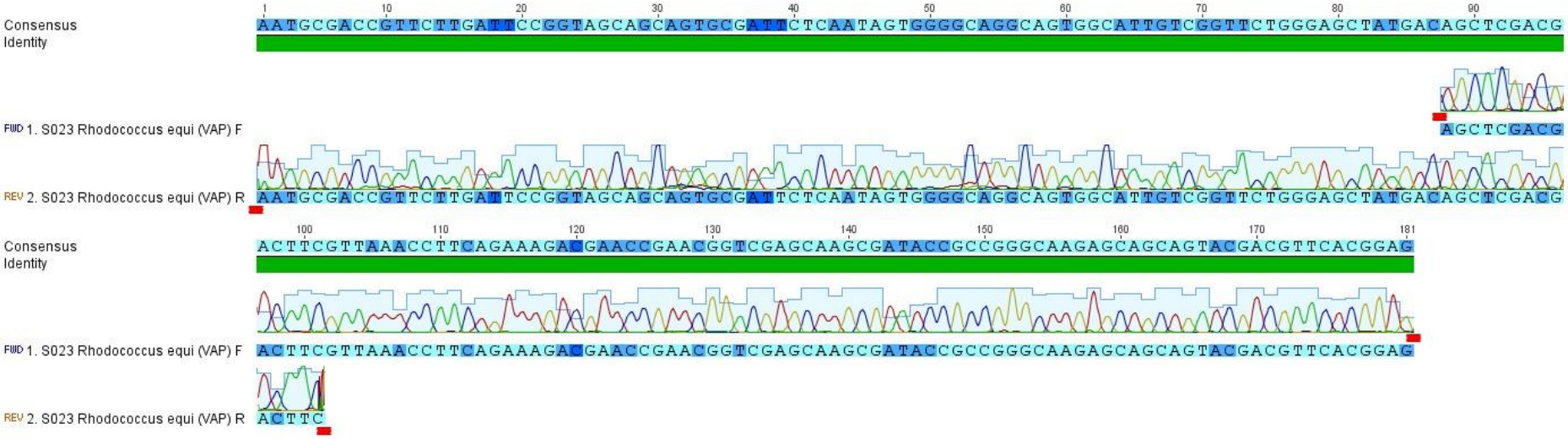

Figure A3.13: High quality electropherogram output from *R. equi* (VAP) DNA sequencing of sample S023.

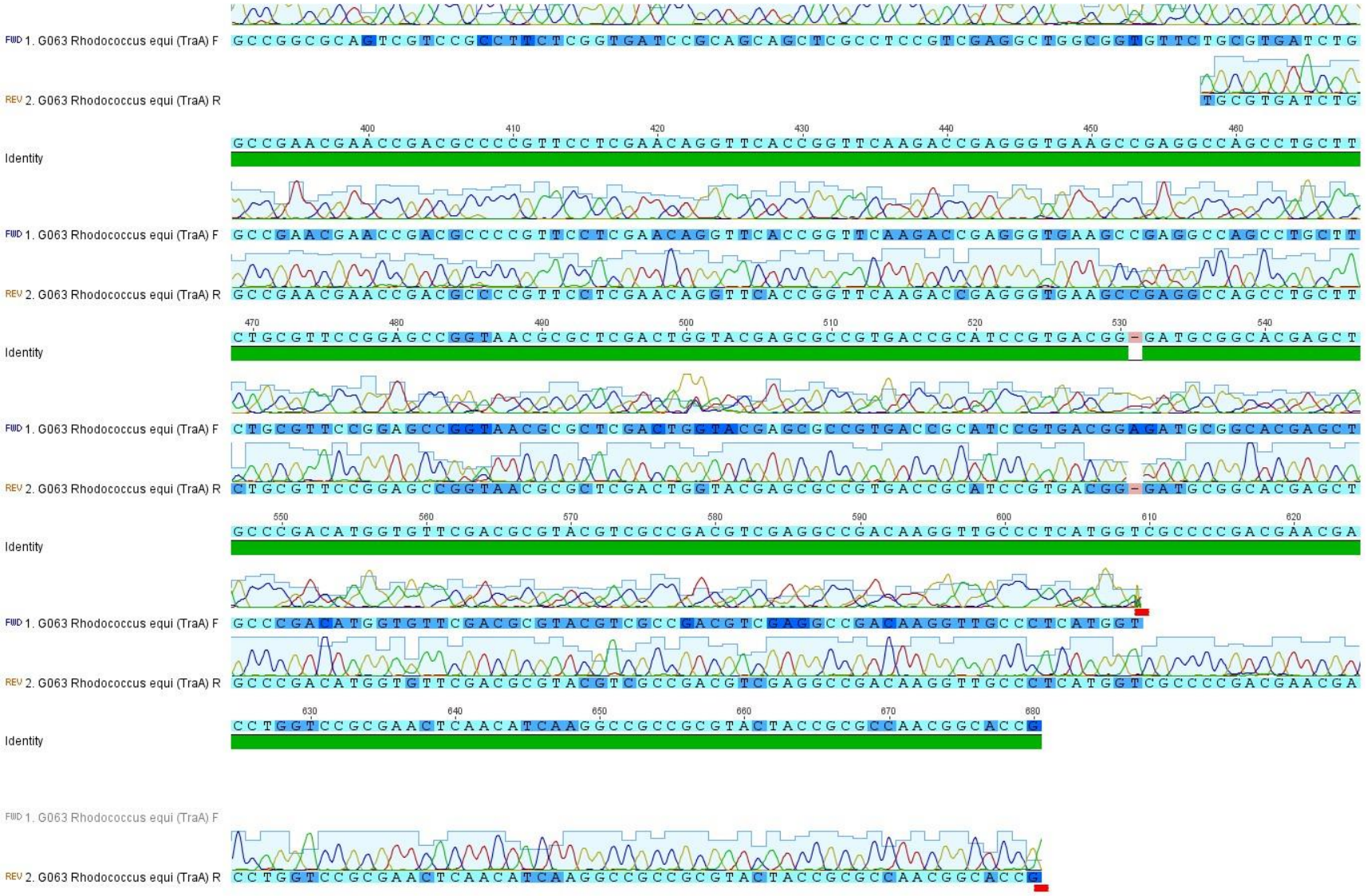

Figure A3.14: High quality electropherogram output from *R. equi* (TraA) DNA sequencing of sample G063.

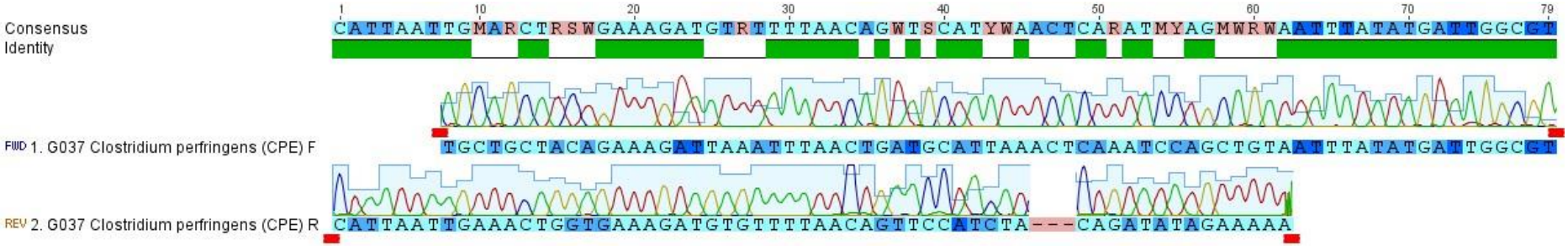

Figure A3.15: Moderate quality electropherogram output from *C. perfringens* (CPE) DNA sequencing of sample G037.

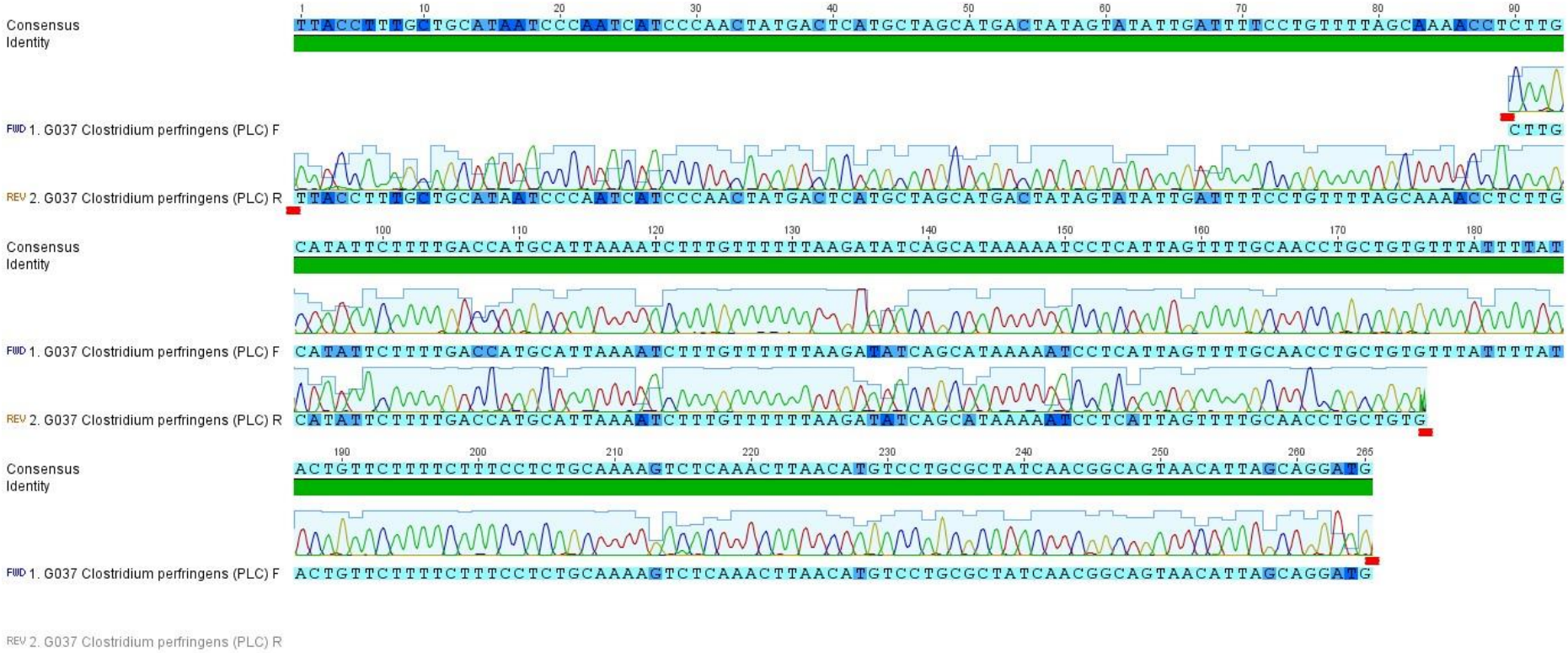

Figure A3.16: High quality electropherogram output from *C. perfringens* (PLC) DNA sequencing of sample G037.

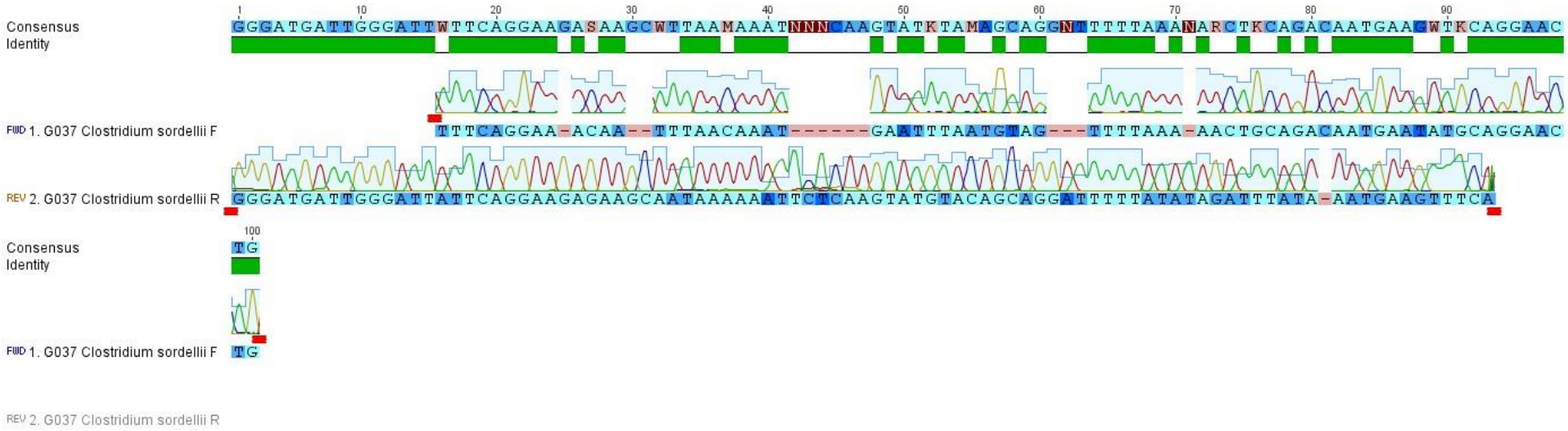

Figure A3.17: Moderate quality electropherogram output from *C. sordellii* DNA sequencing of sample G037.

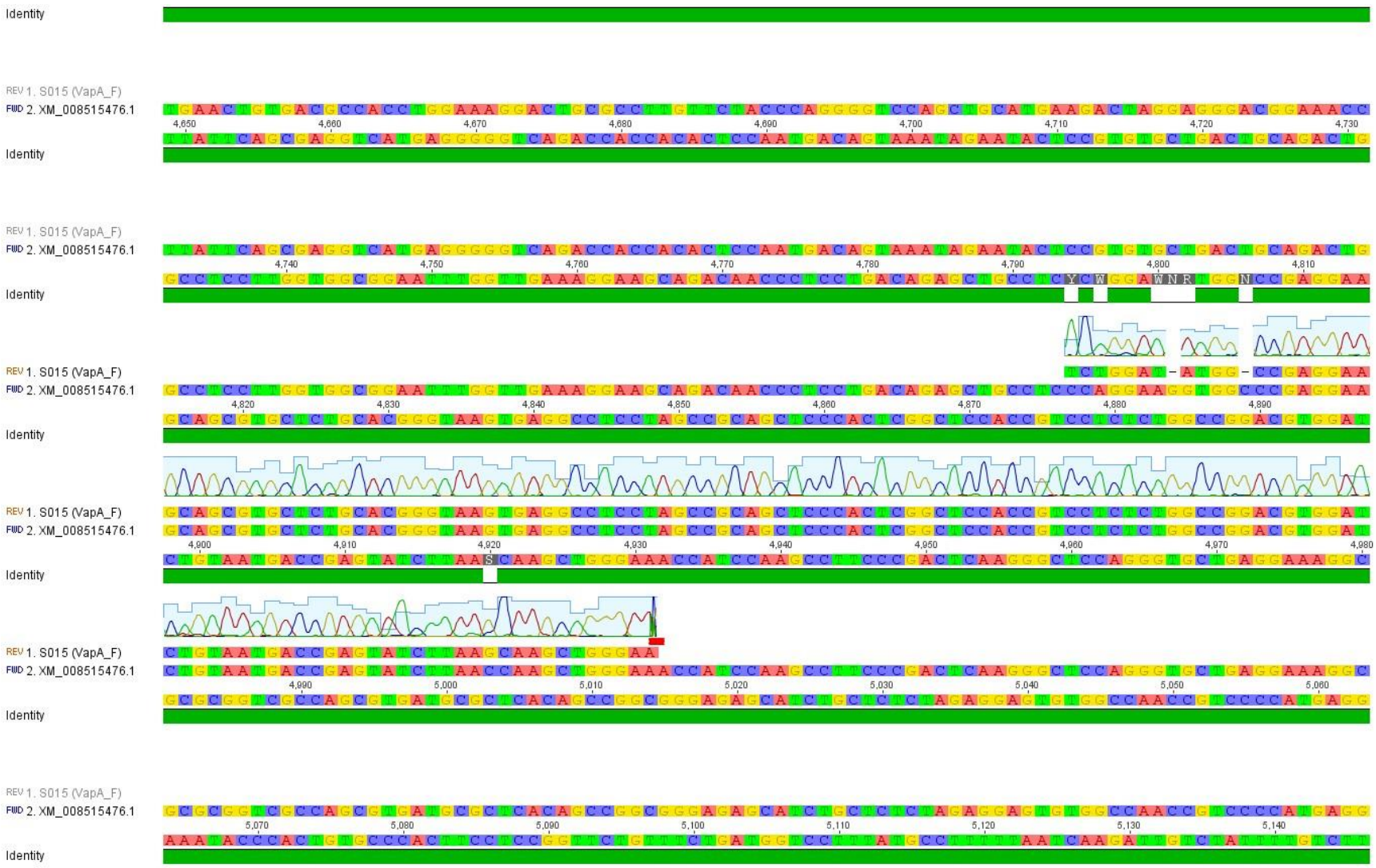

Figure A3.18: Alignment of S015 PCR product to *Equus przewalkskii* tescalcin gene (NCBI accession: XM_008515476.1).

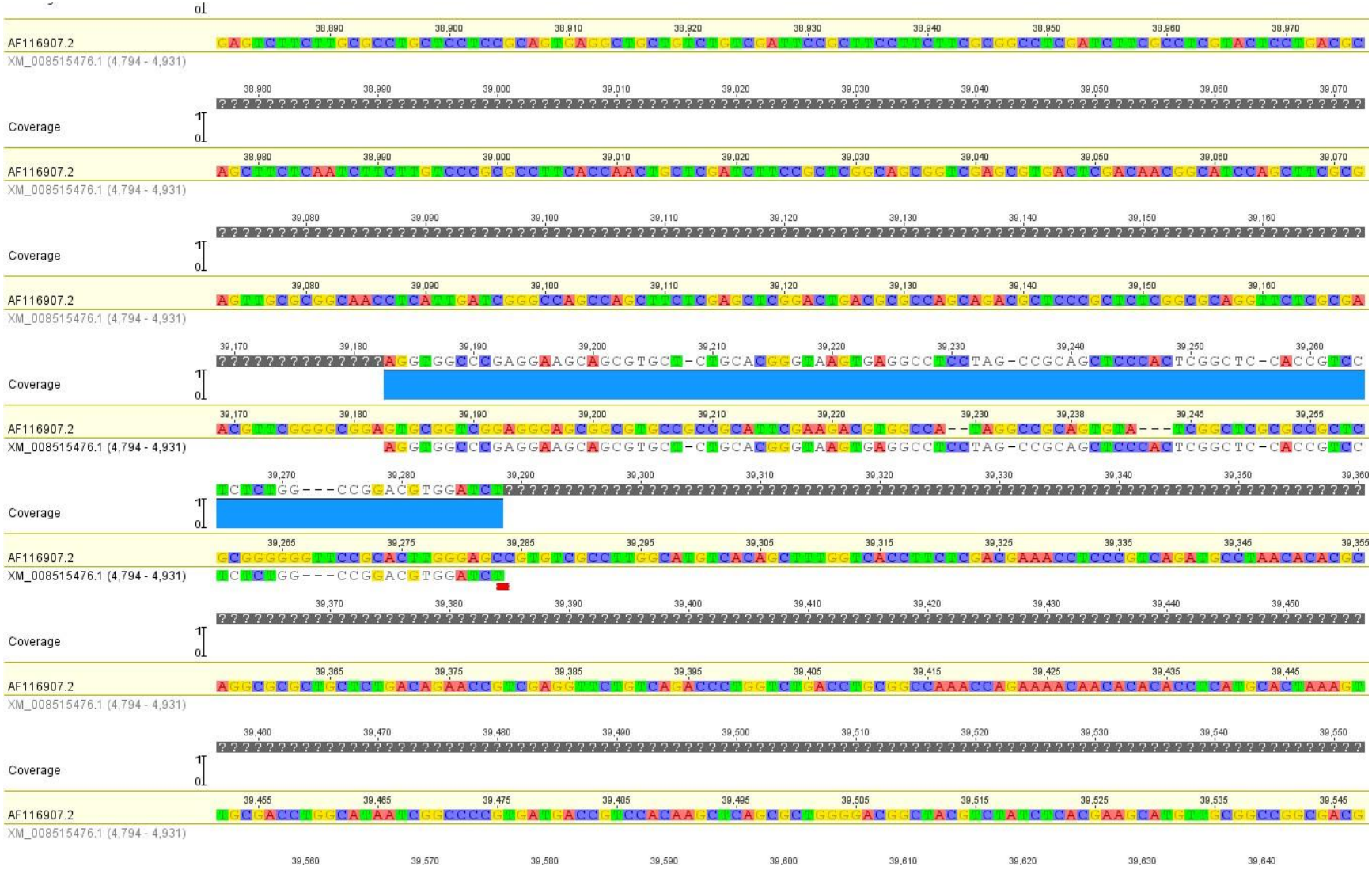

Figure A3.19: 137bp fragment from position 4,794 – 4,931 of *Equus przewalkskii* tescalcin gene (NCBI accession: XM_008515476.1) mapped to *R. equi* virulence plasmid compete sequence (NCBI accession: AF116907.2).

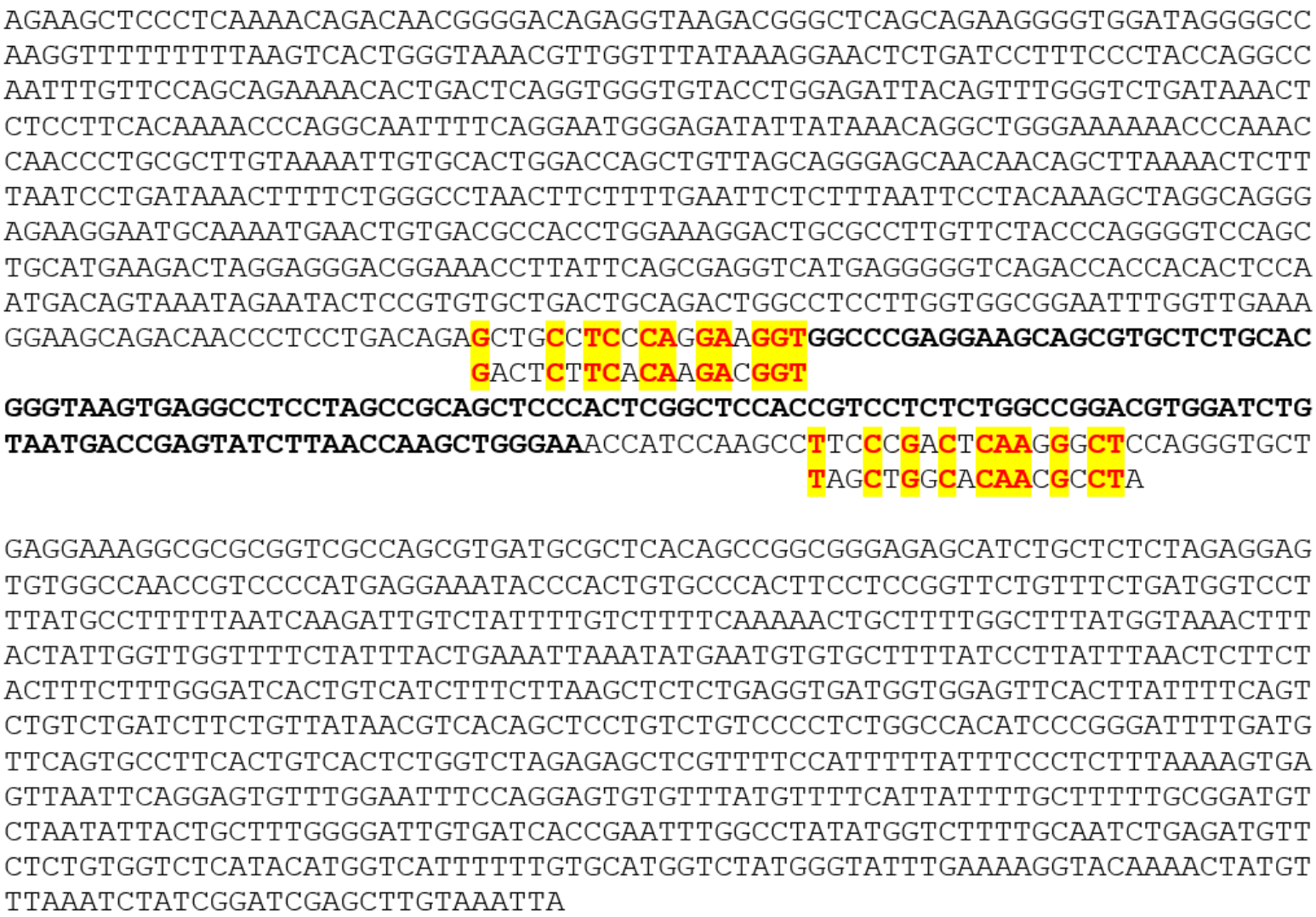

Figure A3.20: Alignment of vapA primers to *Equus przewalkskii* tescalcin gene (NCBI accession: XM_008515476.1) showing approximately 50% similarity. Amplified PCR product from sample S015 is shown in bold. Complimentary forward and reverse PCR primers for vapA are shown in yellow highlight.