Development of Multiplex Real-Time PCR Assays for the Detection and Identification of ESKAPE Pathogens and their Closely Related Species Complexes:

Toward Reducing the Occurrence of Healthcare-Associated Infection Using Enhanced Surveillance of Clinical and Environmental Samples

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by

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Declaration

I, the **Candidate**, certify that the Thesis is all my own work and that I have not obtained a degree in this University or elsewhere on the basis of any of this work.

Signed: Grainne Mc Andrew   Date: 27/04/2023

Grainne Mc Andrew

Summary of Thesis

Microorganisms have the ability cause a wide range of infections in humans - from superficial skin infections to invasive pneumonia and sepsis. Advancements in medical technology, as well as an expanding global population, mean that increasing numbers of people receive life-saving treatment and interventions each year. However, the increased use of invasive medical procedures and antimicrobial treatments can result in an increased risk of patient infection.

Infections which occur during or after contact with healthcare facilities are known as healthcare-associated infections (HAI). HAI are a significant cause of morbidity and mortality worldwide. A small number of bacterial species cause the majority of HAI: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter*. These species have been grouped together under the acronym ESKAPE due to their ability to "eskape" conventional treatments. They frequently cause severe, multidrug-resistant infections in patients who are already seriously ill, immunocompromised, or undergoing invasive treatment such as mechanical ventilation. Relative to other pathogenic bacteria, the ESKAPE pathogens are particularly adept at survival in healthcare settings. In addition to displaying high levels of antimicrobial resistance, the ESKAPEs have been shown to persist on inanimate surfaces in the hospital environment.

Currently, routine diagnostics for ESKAPE HAI and for hospital environmental surveillance are culture-based. These methods lack speed, sensitivity, and accuracy. This study describes the development of a series of real-time multiplex PCR assays for specific identification of each ESKAPE, as well as methodology for environmental sample collection. This enables the rapid and sensitive identification of pathogens from clinical and environmental samples from healthcare settings.

In addition to identifying the primary causes of HAI - the ESKAPE pathogens - this study includes methods to detect the often-misdiagnosed species complexes which are closely related to the ESKAPEs. Namely, the species contained in the *A. baumannii* group (*A. baumannii, Acinetobacter pittii, Acinetobacter nosocomialis, Acinetobacter seifertii* and *Acinetobacter lactucae*) and the *K. pneumoniae* species complex (*K. pneumoniae, Klebsiella quasipneumoniae, Klebsiella variicola, Klebsiella*

quasivariicola, and *Klebsiella africana*). *A. baumannii* and *K. pneumoniae* are considered critically important human pathogens. However, they exist within closely related, and poorly understood species complexes. Currently, there are no rapid real-time PCR diagnostics to accurately identify these species. Existing data pertaining to the clinical relevance of these species is limited to a small number of studies which have identified isolates to the species level using sequence-based techniques. These data have shown that *A. baumannii* group and *K. pneumoniae* complex members have inherently different antimicrobial resistance profiles which affects disease prognosis and patient outcomes.

As such, the aim of this study was to create novel real-time PCR diagnostics which would provide rapid and accurate information on the occurrence of ESKAPE pathogens with improved species identification capabilities.

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Chapter 1: Introduction

1.1 The ESKAPE Pathogens: Origins and Taxonomy

In 2008, while commenting on the US Centres for Disease Control and Prevention (CDC) National Institute for Allergies and Infections Disease (NIAID) research agenda, Rice [1] coined the ESKAPE acronym to describe a group of bacteria which displayed remarkable abilities to cause invasive, antimicrobial resistant infections in hospitalised patients. These species: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* have continued to be leading causes of morbidity and mortality. What underpins the ESKAPE pathogens is their ability to cause healthcare-associated infections (HAI) with high burdens of disease and multidrug resistance. Each has been included in the World Health Organisation (WHO) priority pathogen list for research and development of new antibiotics [2] and the US Centres for Disease Prevention and Control (CDC) list of antibiotic resistance threats [3].

1.1.1 *Enterococcus faecium*

Enterococci are commonly found as commensals of the human and animal gastrointestinal tract. The species *E. faecium* and *Enterococcus faecalis* are responsible for the majority of human infection: [4]. However, *E. faecium* causes a much higher burden of disease due to high levels of antimicrobial resistance (AMR) [5]. *E. faecium* is Gram-positive, catalase-negative and facultatively anaerobic [6]. It tolerates high bile, NaCl and alcohol concentrations and can readily grow between 10°C and 45°C [7, 8], which enables it to survive in a diverse range of habitats, from mammalian host to hospital environments [9, 10].

The number of infections caused by *E. faecium* and *E. faecalis* has shifted considerably. In the past, *E. faecalis* was the most frequently isolated species, outnumbering *E. faecium* infections approximately 10 to 1 [11, 12]. The emergence of the vancomycin-resistant phenotype saw *E. faecium* levels increase, almost equalling *E. faecalis* in some regions [13, 14]. *E. faecalis* still cause a high number of infections but tend to be susceptible to most antimicrobials, including vancomycin [8, 15].

1.1.2 *Staphylococcus aureus*

S. aureus are Gram-positive, non-motile, mannitol-fermenting and coagulase-positive [16]. Coagulase production distinguishes them from other Staphylococci, such as

Staphylococcus epidermidis and *Staphylococcus lugdunensis* - referred to as coagulase negative Staphylococci (CoNS) [17].

S. aureus is perhaps the most well-known and extensively studied member of the ESKAPE pathogens. It was among the first species displaying antimicrobial resistance to be isolated in the clinical setting [18], and continues to cause a high burden of disease today. The discovery of methicillin resistant *S. aureus* (MRSA) in 1961 [19] led to extensive research into this pathogen, which was spurred by an increase in cases in the 1990s [20, 21]. MRSA was the first major AMR challenge faced by global healthcare providers and resulted in extensive political and mainstream media attention [22, 23]. The lessons learned from the early 2000's when MRSA levels were at their highest have shaped our response to AMR and infection prevention and control practices today [24, 25]

1.1.3 *Klebsiella pneumoniae*

K. pneumoniae was first described as a causative agent of pneumonia in 1882 by Friedlander [26]. It is a Gram-negative, non-motile rod, which is indole and oxidase negative, and positive for lactose and adonitol fermentation, citrate utilisation and the urease test. It forms distinctive pink mucoid colonies when grown aerobically on MacConkey agar [27].

Within the genus, there are several other well-characterised human pathogens, including *Klebsiella oxytoca*, and *Klebsiella aerogenes* (formerly *Enterobacter aerogenes*) [28, 29]. These species are differentiated by routine clinical diagnostics, unlike the more recently described and lesser-known members of the *Klebsiella pneumoniae* Species Complex (KpSC).

K. pneumoniae forms part of a bacterial species complex with other closely related *Klebsiella* (**Figure 1.1**). These species were previously classified as phylogroups of *K. pneumoniae* until advances in sequencing resolved the phylogenetics of this complex [30-32]. Currently, there are 7 members of the *Klebsiella pneumoniae* species complex (KpSC): *Klebsiella pneumoniae* (Kp1), *Klebsiella quasipneumoniae* subsp. *quasipneumoniae* (Kp2), *Klebsiella variicola* subsp. *variicola* (Kp3), *Klebsiella quasipneumoniae* subsp. *similipneumoniae* (Kp4), *Klebsiella variicola* subsp. *tropica (Kp5)*, *Klebsiella quasivariicola* (Kp6), and *Klebsiella africana* (Kp7). The KpSC species are very closely related and share only 90% Average Nucleotide Identity

(ANI) with other members of the genus, such as *K. aerogenes*, *K. oxytoca*, *Klebsiella michiganensis* and *Klebsiella grimontii*.

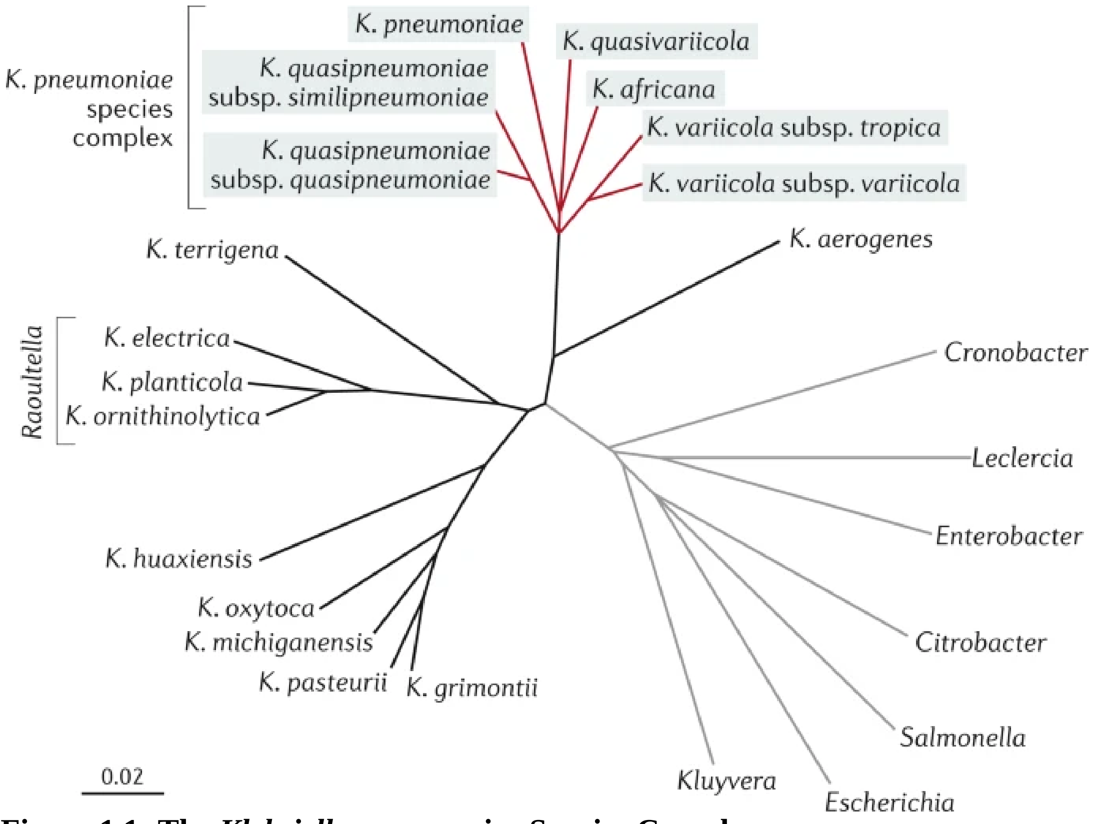

Figure 1.1: The *Klebsiella pneumoniae* Species Complex
Phylogenetic tree constructed from Whole Genome Sequence data demonstrating the relationship between *K. pneumoniae*, the KpSC (red branches), other *Klebsiella* (black branches) and Enterobacteriaceae (grey branches). *Image reprinted from Wyres et al.* **[33]** *with permission from Springer Nature*

1.1.4 *Acinetobacter baumannii*

Acinetobacter are non-fermenting, Gram-negative bacilli in the family Moraxellaceae, and are commonly isolated from soil, water and sludge [34]. They are oxidase-negative, catalase-positive, and generally non-motile [35]. The genus *Acinetobacter* was initially proposed by Brisou and Prevot in 1954 and has since undergone a number of taxonomic changes [36]. In the 1980s DNA-DNA hybridisation techniques led to the description 15 genospecies, including *A. baumannii* [37, 38]. Similar to *K. pneumoniae*, *A. baumannii* exists within a species complex of closely related species, which cannot be differentiated by culture-based methods [39].

The Acinetobacter calcoaceticus-baumannii (Acb) complex consists of *A. baumannii, Acinetobacter calcoaceticus, Acinetobacter pittii, Acinetobacter nosocomialis, Acinetobacter lactucae* and *Acinetobacter seifertii* [40].

Table 1.1: Species of the *Acinetobacter calcoaceticus-baumannii* complex and their previous designations

Species	Pseudonyms/ Historical Nomenclature	Reference
A. calcoaceticus	Genospecies 1	[38]
A. baumannii	Genospecies 2	[38]
A. pittii	Genospecies 3	[40]
A. nosocomialis	Genospecies 13TU	[40]
A. seifertii	Genospecies ‘close to 13TU’	[41]
A. lactucae	*A. dijkshoorniae*	[42]

More recent genetic analysis has confirmed that the Acb complex clusters independently from other *Acinetobacter* species, and that *A. baumannii* is also a distinct monophyletic group [43, 44]. While each species within the Acb now has a validly published name (**Table 1.1**), some retained their historical genospecies as recently as 2015 [41].

1.1.5 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative, motile, oxidase-positive, lactose non-fermenter. It forms rod-shaped cells which produce the characteristic blue-green pigments pyocyanin and pyoverdine [45]. It is primarily an aerobic organism, although it can also grow anaerobically by reduction of nitrate rather than true fermentation [46]. Its metabolic diversity allows *P. aeruginosa* to survive in a diverse range of environments, including soils and water, with very low nutritional requirements [47].

P. aeruginosa is a normal part of the gut microbiome, but does not adhere well to normal, intact epithelial cells, and so does not typically cause infection in otherwise healthy individuals [45]. However, in hospitalised patients and people with underlying illnesses such as cystic fibrosis and Chronic Obstructive Pulmonary Disease (COPD), *P. aeruginosa* can cause persistent invasive infection [48, 49]. Infections caused by other *Pseudomonas* species, such as *Pseudomonas putida* and *Pseudomonas fluorescens* have been reported in severely immunocompromised patients. They remain a rare cause of opportunistic infection, with lower AMR levels when compared to *P. aeruginosa* [50-52].

1.1.6 *Enterobacter*

The *Enterobacter* genus falls within the family Enterobacteriaceae, along with other important pathogens such as *Klebsiella*, *Escherichia* and *Citrobacter* [53]. The genus and its type-strain, *Enterobacter cloacae*, were first described by Hormaeche and Edwards in 1960 [54]. *Enterobacter* species are facultatively anaerobic, motile rods, which are ornithine decarboxylase-positive, urease-negative, and positive for the fermentation of sucrose and adonitol [55]. They inhabit a diverse range of environments, from human commensals to plant pathogens, marine environments, and soils [56-58].

Our understanding of the epidemiology and population structure of the genus is limited, as many *Enterobacter* species are not differentiated by routine diagnostics [59, 60]. Likewise, the differentiation of *Enterobacter* species from other closely related genera such as *Lelliottia*, *Leclercia*, *Kluyvera*, *Pluralibacter* and *Pantoea* is not achieved using routine clinical diagnostics [29, 55, 61].

There have been a number of important human pathogens reclassified from *Enterobacter*, most notably *Enterobacter aerogenes* (now *K. aerogenes*) and *Enterobacter sakazakii* (now *Cronobacter sakazakii*). These species can be differentiated from other *Enterobacter* species using phenotypic methods or matrix-assisted light desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) [29, 62].

K. aerogenes is phenotypically more similar to *Enterobacter*, being motile, ornithine decarboxylase-positive and urease-negative [55, 63]. Genotypically it is closest to *K. pneumoniae* [64, 65]. Its description as a *Klebsiella* predates the genomics era, with the description of *Klebsiella mobilis* in 1971 [66, 67]. Whole Genome Sequence (WGS) analysis has confirmed its placement in the genus *Klebsiella* [64, 68] and in this study it is treated as such.

Since 2005, 14 species have been both declared as novel *Enterobacter*, and subsequently revised and assigned to other genera (**Supplementary Table S1.1**). The majority of these species are environmental isolates, or former heterotypic synonyms such as *Enterobacter mulleri* (now *Enterobacter asburiae*) and *Enterobacter tabaci* (now *Enterobacter mori*).

This taxonomic upheaval makes accurate surveillance and diagnosis of *Enterobacter* infection difficult. Advances in phylogenetic analysis have led to the status of many clinically relevant Enterobacter being revised and challenged [66, 69-71].

1.1.7 Taxonomic Uncertainty in the ESKAPE Pathogens

Although the ESKAPEs have been described as important human pathogens, several species are poorly defined, both taxonomically and from a diagnostics perspective (**Figure 1.2**). This is primarily due to the difficulty in differentiating closely related species [69, 72, 73]. Genomic analysis has shown that both *K. pneumoniae* and *A. baumannii* form species complexes. Many of the species which form the KpSC and the Acb complex have only recently been given species designation [32, 74]. In the case of *Enterobacter*, the genus has undergone a considerable amount of species reclassification in recent years (**Supplementary Table S1.1**). Therefore, many of the diagnostics which target these organisms predate the taxonomic updates. This has resulted in the potential for the misidentification of ESKAPE pathogens, particularly KpSC, Acb and *Enterobacter* species [75-80]. This in turn has the led to a lack of accurate data on the occurrence, prevalence and clinical significance of “true” ESKAPE species, which are considered to be the most burdensome bacterial pathogens [81]. In order to improve our understanding of their epidemiology, diagnostics which have the ability to accurately identify each ESKAPE, which can be applied in the clinical setting are needed.

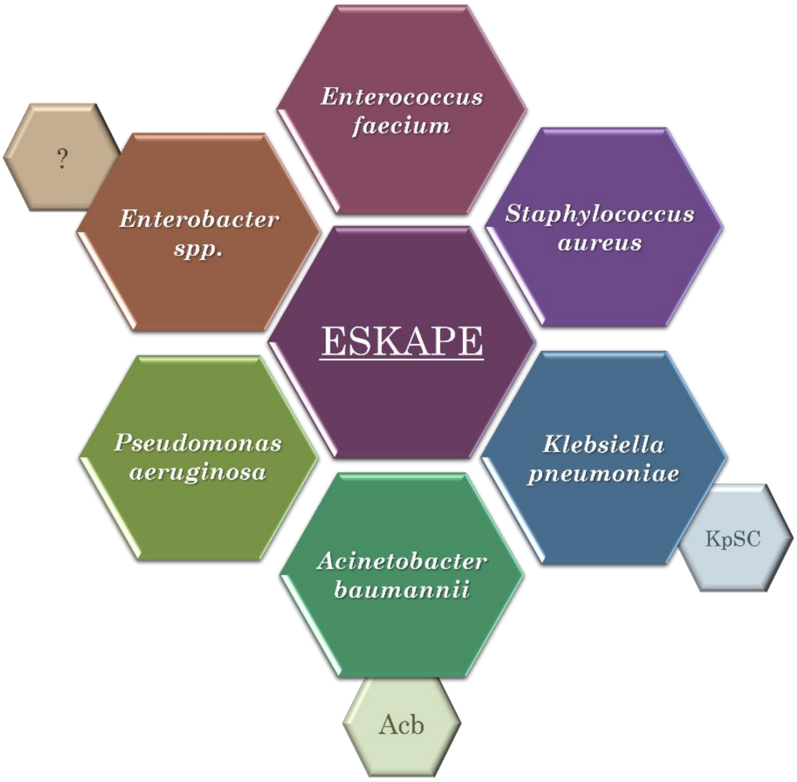

Figure 1.2: An expanded definition of the ESKAPE pathogens
Our current understanding of ESKAPE pathogen taxonomy and epidemiology is flawed due to imprecise means of identification. When considering the ESKAPEs, members of the *Klebsiella* pneumoniae Species Complex (KpSC) and the *Acinetobacter calcoaceticus-baumannii* complex (Acb) should be included as they have the potential to be misdiagnosed as *K. pneumoniae* and *A. baumannii*, respectively. In addition, the current lack of precise diagnostics for the *Enterobacter* genus has left a number of questions yet to be answered surrounding their contribution to infection. These ESKAPE members are in particular need of accurate diagnostics to resolve their role in human infection and healthcare associated infections (HAI).

1.2 Clinical Significance of the ESKAPE Pathogens

1.2.1 HAI and Antimicrobial Resistance

1.2.1.1 E. faecium

Globally, *E. faecium* is associated with an estimated 219,000 deaths per annum [82]. It causes a range of infections including bloodstream infection (BSI), urinary tract infection (UTI), endocarditis and neonatal sepsis and meningitis [83, 84]. In healthy persons, *Enterococcus* species form part of the normal gut microbiome. When exposed to antimicrobials the resulting dysbiosis can cause the number of Enterococci to increase. Following extended periods of antimicrobial treatment, intestinal overgrowth can occur, leading to further systemic infection [85, 86]. Enterococcal cells can translocate the intestinal epithelial wall and enter the bloodstream or are shed in faeces which can result in skin or environmental contamination [87, 88]. Central lines and catheters are the primary route of exogenous entry for *E. faecium* in the hospital setting [3].

E. faecium displays high levels of antimicrobial resistance. In the most recent data reported to European Antimicrobial Resistance Surveillance Network (EARS-Net), 52.4% of *E. faecium* isolates were resistant to two or more classes of antimicrobials [89], while in the US, 70% of *E. faecium* isolated from central line-associated bloodstream infections (CLABSIs) are vancomycin resistant [3]. The global dissemination of vancomycin resistant Enterococci is of particular concern; it was included in the CDC's list of serious antibiotic resistant threats in 2019 [3].

Vancomycin resistance in *E. faecium* is conferred by the van operon with the *vanA* gene being the most commonly detected, followed by *vanB* and sporadic reports of *vanD* [90-92]. Vancomycin-resistant Enterococci (VRE) emerged in the early 1990s and stemmed from high levels of ampicillin resistance, leading to increased use of cephalosporins and vancomycin [93, 94]. By 2008, 30.9% of *E. faecium* BSI isolates in Ireland were vancomycin-resistant [95]. Ireland had the highest rate of VRE in Europe between 2008 and 2015, when it peaked at 45.8%. While some reductions have been seen in recent years, VRE continues to cause a higher burden of disease in Ireland than it does in other European countries [96].

1.2.1.2 S. aureus

S. aureus causes a large proportion of globally reported bacterial infections, and was found to be the leading cause of death due to bacterial infections in 135 countries, according to the 2019 Global Burden of Disease study [82, 97]. It causes a range of infections in humans, including pneumonia, BSI, endocarditis, and skin and soft tissue infections (SSTI) [98].

S. aureus asymptomatically colonises 20%-30% of the population [20, 99] and is predominantly isolated from the anterior nares, but can also be found in the pharynx, axillae and groin [100]. Colonisation increases the risk of infection, particularly when epithelial and mucosal barriers are disrupted due to injury or invasive medical devices [16, 101].

In Europe, the population-weighted mean of *S. aureus* isolates resistant to methicillin was found to be 15.7% with countries such as Cyprus, Romania, and Greece reporting percentages of over 40%. In Ireland this level was 12.1%, down from 42.3% in 2006 [89, 95]. However, some data suggest an increase of methicillin sensitive *S. aureus* (MSSA) causing BSI in the same time period [102, 103]. Although the reduction of MRSA is a positive development, *S. aureus* remains a threat both in the hospital and community setting. The emergence of hypervirulent strains [104, 105] and recent increases in BSI caused by MSSA means it continues to cause a high burden of disease globally [5, 102].

1.2.1.3 K. pneumoniae

K. pneumoniae causes a variety of human infections, including UTI, BSI, pneumonia, and neonatal sepsis. Due to its opportunistic nature, it poses an increased risk to vulnerable populations such as immunocompromised persons, the elderly and neonates [106, 107]. In Europe, *K. pneumoniae* is responsible for an estimated 9-11.3% of the overall number of HAI and 7,440 deaths per annum [96, 97, 108]. *K. pneumoniae* can also cause community acquired (CA) infections such as liver abscesses, endophthalmitis, septicaemia, meningitis, and enterocolitis [109, 110]. CA *K. pneumoniae* infections are predominantly caused by hypervirulent clones [111-113]. These hypervirulent *K. pneumoniae* strains have a number of virulence factors which can be chromosomally encoded, or plasmid mediated [114, 115].

K. pneumoniae and other *Klebsiella* species have played a key role in the emergence and dissemination of AMR [116]. *K. pneumoniae* is intrinsically resistant to ampicillin due to the chromosomally encoded bla_{SHV}, an Ambler Class A β-lactamase. Mutations in the amino acid sequence of BLA and TEM enzymes resulted in extended-spectrum β-lactamase (ESβL) activity which was first detected in 1983 [117, 118]. The incorporation of mutated versions of $bla_{\mathrm{SHV\text{-}1}}$ into plasmids resulted in dissemination of ESβLs to other species [119, 120].

Carbapenem-resistant *Klebsiella* isolates have increasingly been reported since their initial discovery in North Carolina in 1996, with 7.9% of *Klebsiella* isolates in Europe displaying carbapenem resistance [97, 121]. Concerningly, carbapenem resistance has been estimated to be as high as 34.8% of isolates in low and middle-income countries (LMICs) [122], with a study in Malawi reporting that 92% of the *Klebsiella* BSI isolates tested were multidrug resistant (MDR) [123].

The numbers of invasive *K. pneumoniae* infections reported in Ireland rose by 36% from 2014 to 2018 [124]. AMR levels amongst *K. pneumoniae* isolates in Ireland is lower than that of other European countries, however the percentage of AMR isolates has increased slightly in recent years [125]. MDR rates have remained relatively steady at 5-9% since 2013, as has the number of carbapenemase-producing *K. pneumoniae* isolates [124].

The surveillance data described above was collected using routine clinical diagnostics and does not take into account the members of the KpSC which have the potential to be misidentified as *K. pneumoniae* [76, 126]. While *K. pneumoniae* (*sensu stricto*, Kp1) is recognised as causing the majority (85%) of infections [127, 128] there is growing evidence to suggest that other KpSC species cause serious human infection. *K. variicola* has been described as an emerging human pathogen, capable of causing serious human infections and producing hypervirulent strains [129, 130].

Due to the inability of routine diagnostics to differentiate between the species, the relative burden of *K. pneumoniae* and other KpSC members in HAI is not fully understood [28, 129-131]. This has been addressed in **Chapter 3** of this study, where a real-time multiplex polymerase chain reaction (PCR) to detect each KpSC member, and to differentiate between *K. pneumoniae sensu stricto*, *K. quasipneumoniae* and *K. variicola* was developed.

1.2.1.4 A. baumannii

A. baumannii is frequently implicated in BSI, pneumonia, SSTI, and UTI [79, 132]. It causes severe invasive infections with a high mortality rate, estimated to be between 12-40% [133, 134]. It is predominantly found within the hospital setting, although it has been occasionally shown to cause CA infections [135-137]. Recent exposure to healthcare facilities and antimicrobials is strongly correlated with development of MDR *A. baumannii* infection. It is particularly associated with critical care units such as intensive care units (ICU), where invasive treatments such as intravenous catheters and ventilators are frequently used. [132, 138]. A combination of intrinsic and acquired AMR genes, and its ability to resist desiccation has led to the global emergence of MDR *A. baumannii* as a hospital-associated pathogen [139-141].

Carbapenem-resistant *A. baumannii* has been classified as an urgent AMR risk and a priority pathogen by the CDC and the WHO [2, 3]. Treatment options for carbapenem-resistant *A. baumannii* are limited [142].

In 2019 *A. baumannii* was associated with over 400,000 deaths, particularly in Southeast Asia, East Asia, and Oceania [5]. In the European Union/European

Economic Area (EU/EEA), the number of infectious caused by AMR *A. baumannii* was estimated to be over 27,000 in 2015 [143]. The population-weighted mean of isolates displaying combined resistance to fluroquinolones, aminoglycosides and carbapenems in the EU/EEA between 2016-2020 was 34.1%. This figure varies greatly between countries, with Southern Europe and Eastern Europe showing levels of combined resistance over 90%. Ireland, the UK and Scandinavian countries on the other hand, have rates below 2% [89, 97]. Globally, pooled estimates of carbapenem resistant *A. baumannii* in LMICs are also high, at 72.4% [122]. Infections due to *A. baumannii* should therefore be carefully monitored to prevent the emergence of high rates of AMR seen in other parts of Europe and the world.

Careful monitoring is limited by the inability of routine diagnostics to differentiate *A. baumannii sensu stricto* (genospecies 1) from members of the Acb complex. This is further confounded by the fact that *A. calcoaceticus* is not considered to be a human pathogen, unlike the other Acb species, leading to the use of the updated term "*A baumannii* group" to describe the clinically relevant species of *A. baumannii, A. pittii, A. nosocomialis*, *A. seifertii* and *A. lactucae* [44, 74, 144]. *A. baumannii sensu stricto* comprises of the majority (70%) of clinical isolates [141, 145-150], although *A nosocomialis* has been found at levels as high as 47% [151]. These levels can vary by study location (geographically) and design (infection type and patient comorbidities). An assay to detect the *A. baumannii* group was included in the multiplex real-time PCR described in **Chapter 2** of this study, in addition to an assay to detect *A. baumannii sensu stricto.*

1.2.1.5 P. aeruginosa

P. aeruginosa causes a range of infection types. In addition to causing acute invasive infections such as pneumonia, SSTIs and BSI, *P. aeruginosa* is also associated with chronic, persistent infection of the lungs of people with cystic fibrosis [48, 152, 153]. It does not typically cause CA infection in immune-competent hosts. In the hospital setting however, it is a leading cause of device-associated respiratory infection such as ventilator-associated pneumonia (VAP). Detection of *P. aeruginosa* in patients with VAP range between 12%-16%, and it is often the 2nd most frequently detected organism after *S. aureus* [108, 154]. It was the 4th most frequent cause of all HAI types in the US between 2015-2017, causing an estimated 28,513 infections [108].

Carriage of *P. aeruginosa* has been linked to increased likelihood of developing an infection during a hospital stay with one study showing a significantly higher likelihood of developing infection [155]. However, the main risk factors for development of *P. aeruginosa* infection are hospital environmental contamination and prior antibiotic therapy which is not anti-pseudomonal [155, 156].

In the EU/EEA, 12.1% of all *P. aeruginosa* infections have a combined resistance phenotype [89], (defined as being resistant or intermediate to at least 3 of the following classes of antimicrobials: extended spectrum cephalosporins, fluoroquinolones, aminoglycosides, carbapenems and piperacillin/tazobactam [157, 158]). Whilst in the US, 14.2% of device associated infections were found to be MDR [108].

1.2.1.6 Enterobacter

Enterobacter form part of the normal gastrointestinal microbiome in humans [59]. They cause a range of infections such as pneumonia, UTI, SSTI, and BSI [159]. They have been implicated in hospital outbreaks [58], particularly in neonatal units [160-163]. Until recently, *E. cloacae* was thought to cause the majority of human infection. In reality, *Enterobacter* infections are caused by a diverse range of species within the genus [71, 161]. Their close genetic relatedness, and the lack of a single prominent pathogen (such as *K. pneumoniae* in the genus *Klebsiella*), means the relative burden of individual *Enterobacter* species remains unresolved [164-166].

Enterobacter have varied resistance patterns and a high level of genetic diversity [167]. They are intrinsically resistant to first generation cephalosporins, amoxicillin and cefoxitin through constitutive low-level expression of the β-lactamase AmpC [168]. They are the 3rd most frequently isolated Enterobacteriaceae, after *Escherichia coli* and *Klebsiella* [5, 169]. However, the relative rates of carbapenem resistance have been shown to be highest amongst *Enterobacter* [170, 171].

Enterobacter species were the second most frequently isolated carbapenemase-producing Enterobacteriaceae in Ireland after *E. coli* (*E. coli* 27%, n=155; *E. cloacae* 24%, n=132; *K. pneumoniae* 21%, n=118) [124]. These isolates have been identified at hospital laboratories using standard clinical diagnostics, leading to them being described as "*E. cloacae*" and "Other *Enterobacter* species". When these *Enterobacter* taxa are combined, the number increases to 150 isolates, making it almost as frequently isolated as carbapenemase-producing *E. coli*. [124, 125]. A paper which used WGS to investigate isolates of OXA-48 producing Enterobacteriaceae in an Irish hospital also found *Enterobacter* spp. to be the most prevalent (*n*=35/56) [172]. It is this level of antimicrobial resistance that makes them a formidable nosocomial pathogen, rather than intrinsic virulence [159]. For this reason, the rapid and accurate differentiation of *Enterobacter* from other Enterobacteriaceae is necessary for improved patient outcomes and epidemiological studies [70, 165, 166].

1.2.2 The Role of the Hospital Environment in the Transmission of HAI Caused by ESKAPE Pathogens

It has been estimated that 29,000 patients in Irish healthcare facilities will develop at least one HAI per annum [173]. Many of these infections have an endogenous route following invasive procedures and treatments [156, 174, 175], or are as a result of direct contact with contaminated hands or aerosolised pathogens [176-178]. As well as the well-established routes of person-to-person transmission, fomites and the built environment play an important role in HAI (**Figure 1.3**)[179-181].

The built environment can act as a reservoir for pathogenic bacteria [174, 182, 183]. In addition to displaying high levels of antimicrobial resistance, many of the ESKAPEs are particularly adept at surviving outside a host. They display a remarkable range of survival mechanisms which enables them to resist eradication efforts, including resistance to desiccation [184] and disinfectants [10], as well as the metabolic capacity to survive in low nutrient conditions [185, 186]. Environmental reservoirs have been identified in many documented outbreaks of ESKAPE species in healthcare facilities, including bedside furniture, medical equipment, sinks, and other plumbing fixtures [187-192].

E. faecium is frequently isolated from the hospital environment and is increasingly resistant to alcohol-based hand sanitisers [10, 87, 193]. *S. aureus* is heavily implicated in both wet and dry surface biofilms in hospitals [194]. *A. baumannii* has demonstrated an ability to survive on dry surfaces for up to 49 days [195-198]. *K. pneumoniae* has been isolated from sinks [199, 200] and cleaning detergent [201]. *P. aeruginosa* is a well-known contaminant of hospital water systems, where it forms biofilms in sinks, faucets, showers, and drains, leading to patient infection [202, 203]. *Enterobacter* species have been found on surfaces in ICUs [162] and in the relatively sterile conditions of the International Space Station [204].

The routine monitoring of the hospital environment for pathogenic contamination is not currently recommended [205]. This is due to the low correlation of culture-based detection methods with patient infection and outbreak levels. Environmental surveillance of the healthcare environment is generally conducted after an outbreak is suspected, or for the purpose of research [179, 206]. The introduction of more sensitive methods including nucleic acid diagnostics would greatly increase the quality of the data generated from environmental surveillance. This would lead to a better understanding of pathogen transmission and the development of improved infection control strategies to prevent HAI from ESKAPE pathogens [207, 208].

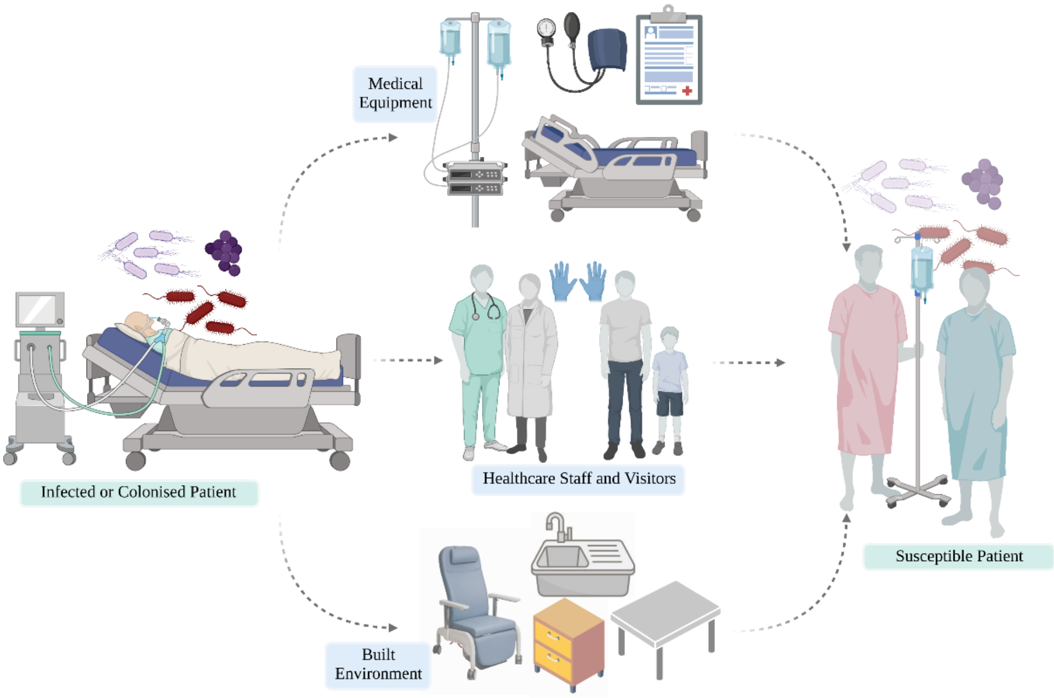

Figure 1.3: Schematic detailing potential routes of transmission for HAI
In addition to person-to-person contact with healthcare workers and visitors (centre) the hospital environment is also implicated in pathogen transmission. Colonised or infected patients (left) have the potential to spread pathogenic organisms to the medical equipment surrounding the patient treatment area (top) or to the built environment of the hospital such as plumbing and fixtures (bottom). This creates an indirect route of transmission from patient to patient in hospitals (right). *Image created with BioRender.com*

1.3 Accurate ESKAPE Identification: Current Landscape and Deficiencies

1.3.1 *E. faecium, S. aureus* and *P. aeruginosa*

The ESKAPE pathogens are frequent causes of HAI and are detected using a large number of culture, molecular and nucleic acid diagnostic (NAD) based methods. However, the ability to accurately identify ESKAPE pathogens to the species level varies [73, 78, 209]. *E. faecium*, *S. aureus,* and *P. aeruginosa* are readily differentiated from closely related species using standard methods.

Identification of *E. faecium* involves a combination of biochemical and antimicrobial susceptibility analysis. Commercial chromogenic media identifies vancomycin resistance but does not accurately differentiate *E. faecium* from *E. faecalis* [210]. Culture-based differentiation involves assessing acid formation in mannitol, bile esculin, pyruvate and arabinose over several days [211]. Misidentification using automated biochemical profiling systems has been reported [7, 212, 213]. Definitive identification to the species level cannot be performed without molecular techniques [212, 214]. *S. aureus* is distinguishable from other Firmicutes and *Staphylococci* using traditional culture-based methods, including the coagulase test, mannitol salt agar and Baird Parker agar [98, 215]. Molecular diagnostics using real-time PCR have been described and *S. aureus* is included in many commercial NAD panels discussed in **Section 1.4.4** [216, 217]. *P. aeruginosa* is identified using cetrimide agar at 42°C, or commercial biochemical tests [218]. Molecular and nucleic acid-based diagnostics for *P. aeruginosa* have also been described [219-222] .

1.3.2 *A. baumannii, K. pneumoniae* and *Enterobacter*

For the remainder of the ESKAPE pathogens, accurate diagnostics are less readily available. As discussed in **Section 1.1**, recent genetic advancements have placed *A. baumannii* and *K. pneumoniae* in species complexes. These species cannot be differentiated using routine culture-based methods, commercially available biochemical tests or MALDI-TOF MS. Nor are they differentiated using the sample-to-answer NADs discussed in **Section 1.4.4**, with the FilmArray PNplus instead describing them as the Acb complex and the *K. pneumoniae* group [223].

1.3.2.1 A. baumannii

MALDI-TOF MS has been shown to have low specificity for *A. baumannii*, particularly for species outside the Acb [78]. Although NAD based methods to delineate *Acinetobacter* species have been described, they have been shown to lack specificity. For example, a PCR targeting *bla*$_{OXA-51}$ [224] which was once thought to be specific to *A. baumannii*, may no longer be fully reliable due to the emergence of *bla*$_{OXA-51}$ genes in *A. nosocomialis* and *A. seifertii*, likely due to horizontal gene transfer [225]. A conventional, multiplex PCR targeting *gyrB* has been shown to be highly accurate but does not detect newer members of the Acb complex such as *A. seifertii* or *A. lactucae* [226, 227].

Sequencing of a 350 base pair (bp) hypervariable region of the *rpoB* gene can discriminate between *Acinetobacter* species [228]. However, a study which compared some of the most frequently used methods for *Acinetobacter* identification found that partial *rpoB* sequencing misidentified 3 strains *of A. nosocomialis* [146].

1.3.2.2 K. pneumoniae

The ability to differentiate *Klebsiella pneumoniae* phylogroups by MALDI-TOF MS has been demonstrated [128, 229], however this is limited by the updating of manufacturer databases, and the requirement for prior culturing of isolates. Conventional PCR for the differentiation of certain KpSC members have been described but predate the description of new KpSC species and so, have not been shown to be totally specific [230, 231].

A real-time PCR assay which used SYBR® Green fluorescence chemistry has recently been described for the detection, but not differentiation of the KpSC [232]. Although *gyrB* sequencing was initially used to study the phylogroups of *K. pneumoniae* [233], due to their close genetic relationship, WGS is required to fully discriminate between all KpSC members [33, 77]

1.3.2.3 Enterobacter

Enterobacter are difficult to differentiate from other closely related Enterobacteriaceae, and generally require sequence-based analysis to accurately identify isolates. Misidentification of *Enterobacter* at the genus level has been reported using MALDI-TOF MS [80, 234]. A conventional PCR which detects and differentiates *E. cloacae, E. hormaechei, E. roggenkampii,* and *E. kobei* has been described [235], however it does not detect other clinically relevant *Enterobacter* such as *E. asburiae, E. ludwigii* or *E. bugandensis* which cause considerable levels of *Enterobacter* infection [80, 236-238]. The most frequently used method of

Enterobacter identification is sequencing of the *hsp60* gene [62, 163, 239]. A second gene which codes for Hsp40 (*dnaJ*) has also been recently proposed as a phylogenetic marker for *Enterobacter* [240].

1.3.3 Differing Clinical Outcomes Amongst Poorly Defined Members of the ESKAPE Pathogens

The lack of accurate diagnostics has meant that the epidemiology and patient outcomes for some ESKAPE pathogens is not fully understood. The identification of KpSC, *A. baumannii* and *Enterobacter* at the species level generally occurs retrospectively, using sequencing-based methods. In studies which also investigate disease manifestations and patient outcomes, important differences have been found between the "true" ESKAPE pathogens and their lesser recognised, closely related taxa.

1.3.3.1 A. baumannii

There is growing evidence to suggest that infections caused by *A. baumannii*, and the other *A. baumannii* group species (*A. pittii, A. nosocomialis, A. seifertii* and *A. lactucae*) are different clinical entities. Infections caused by species other than *A. baumannii* contribute approximately 30% of all *Acinetobacter* infections [141, 145, 150]. When clinical outcomes associated with each species are compared, *A. baumannii* has been found to be more associated with secondary BSI due to pneumonia and sepsis, while *A. nosocomialis* has been more frequently found in primary BSI [79, 145, 241].

A. baumannii has been shown to contribute a higher proportion of MDR isolates [148, 241, 242] and has been identified as an independent risk factor for mortality [145, 243, 244]. Importantly, these studies conducted multivariate analysis, adjusting for AMR and patient comorbidities, suggesting *A. baumannii* has higher intrinsic virulence than the other species. The differential diagnosis of *A. baumannii* group infections has been recommended to improve patient outcomes [245].

1.3.3.2 K. pneumoniae

The relative morbidity between members of the KpSC has also been shown to differ. A multivariate analysis which adjusted for patient comorbidities found that the 30-day mortality rate for patients with *K. variicola* BSI was higher than for other KpSC species [129]. A second study also found *K. variicola* to be more frequently implicated in bacteraemia than *K. pneumoniae*, but with considerably lower levels of AMR [246].

High levels of non-*pneumoniae* KpSC have been found in studies which investigated BSI (26.9%, *n*=119) [247] and UTI (32.8%, *n*=125) [248] in Japan.

1.3.3.3 Enterobacter

Conclusions on relative pathogenicity or disease prognosis are difficult to make when definitions of species are ever-changing. It is now clear that *E. cloacae sensu stricto* causes a small number of infections relative to *E. hormaechei* [71, 159, 165]. More work is needed to fully characterise the clinical characteristics of individual *Enterobacter* species, using multiple methods which can be replicated across studies. A study which used *hsp60* sequencing to investigated 157 cases of carbapenem non-susceptible *Enterobacter* found that Hoffman cluster III, VIII and VI (**Supplementary Table S1.1**) were the most prevalent (47.1%, 24.8%, and 19.1%, respectively) and that the resistance rates for other antimicrobials differed significantly ($p<0.001$) [165]. There is evidence to suggest that *E. bugandensis* possesses higher virulence relative to other *Enterobacter* species [162, 237]. Differentiation at the genus level is particularly important for infections caused by *Enterobacter* species, due to the high levels of AMR in *Enterobacter* [170, 171], relative to AMR levels found in closely-related genera such as *Lelliottia*, *Leclercia* and *Pantoea* [29, 55, 61].

1.4 Nucleic Acid Based Diagnostics: Background and Advantages

1.4.1 The Limitations of Culture Based Diagnostics

The limitations of culture-based diagnostics have been long recognised; however, they remain the gold standard method to diagnose the majority of bacterial infection [249]. Culture of bacterial isolates is slow and laborious, sometimes taking several days to identify the pathogen present in a sample [250]. The initial culturing of a clinical specimen generally provides insufficient information to identify the species present without further biochemical testing [251, 252]. For many infection types, they lack sensitivity. Culture negative infections have been reported in 31% of infective endocarditis cases [253, 254], 30% of hospital acquired pneumonia episodes [255, 256], and 25% of prosthetic joint infections [257-259]. This is due to a variety of factors including fastidious organisms which may not be cultivable using routine methods, and viable but non-culturable (VBNC) cells, including those found in biofilms [260, 261].

Several studies which have directly compared culture and NAD have found that a considerably higher number of samples are positive for bacterial pathogens when NAD assays are utilised. A study which investigated *P. aeruginosa* and *S. aureus* in 327 respiratory samples from 40 patients with cystic fibrosis found the mean positivity rates across age groups ranged from 3.3-44.5% using traditional culture, and 60.9-74.1% using real-time PCR [262]. A similar study which compared culture and quantitative real-time PCR-based detection in sputum samples (*n*=5,003) showed that culture-based positivity rates for *Haemophilus influenzae* and *Moraxella catarrhalis* ranged from 10-24% and 4-6%, respectively, while qPCR detected the same organisms in 33-47% and 13-19% of samples [263]. For Group B *Streptococcus*, the positivity rate across 500 vaginal-rectal swabs was found to be 21.6% using routine culture and 31% using commercial PCR-based methods, which suggests that culture missed over 25% of true positive results [264].

A comprehensive investigation of the performance of culture and NAD for the detection of pathogens in respiratory samples was performed by Enne *et al.* [154]. Pathogens were detected in 44.2% of samples (*n*=652) using routine culture and in 74.2% of samples (*n*=620) using a commercially available nested PCR platform. In addition to comparative analysis, Bayesian latent class modelling was used to further estimate the sensitivity of the two diagnostic methods. This statistical approach is used in the absence of a gold-standard and uses latent class analysis to estimate the unobservable "latent" samples in a population, in this case the false negatives generated by culture. This enables a more accurate determination of culture-based sensitivity [265]. Using Bayesian latent class analysis, the estimated sensitivity of routine culture for the detection of ESKAPE pathogens was 38% for *K. pneumoniae*, 43% for *Enterobacter*, 58% for *A. baumannii*, 65% for *P. aeruginosa* and 65% for *S. aureus*. For the same species, the sensitivity of the PCR-based diagnostic was between 89.4% and 99.3%. This statistical approach further substantiates the argument that rapid NADs offer increased sensitivity of detection relative to culture.

In addition to low sensitivity, culture-based methods can also lack specificity. As discussed in **Section 1.1**, some species of ESKAPEs are not fully identifiable using biochemical profiling associated with routine culture diagnostics. While the use of molecular methods such as MALDI-TOF MS is a considerable improvement to biochemical profiling alone, it remains reliant on initial culture of the organism for identification. In addition, the identification of species using MALDI-TOF MS is reliant on the spectral database provided by the manufacturer, which may not be fully accurate [128, 266, 267].

The use of rapid NAD methods can improve patient outcome by identifying the causative agent of infection with increased speed, accuracy, and sensitivity [268, 269]. This allows clinicians to provide targeted treatment, reducing the need for empirical antimicrobial therapy, which increases the effectiveness of treatment and improves antimicrobial stewardship [249, 270].

1.4.2 Conventional PCR

Since its conception, PCR has become one of the most important techniques in molecular biology and has enabled the routine detection of microorganisms without the need for culture. The PCR method uses thermostable polymerases and a three-step thermal cycling approach of denaturation at ≈95°C, primer annealing at ≈60°C and primer extension at ≈72°C to exponentially amplify DNA [271]. During denaturation, double stranded DNA (dsDNA) separates to form single stranded DNA (ssDNA). In the annealing step, a pair of synthetic oligonucleotide primers hybridise to the complementary regions on opposing ssDNA strands. The primers span the region of DNA to be amplified and act as a substrate for the thermostable polymerase to extend the complementary DNA strand via the sequential addition of deoxynucleotides during extension. The cyclic amplification continues with the newly synthesised dsDNA strand serving as template for the next cycle, thus creating copies of the target segment exponentially [272, 273]. Analysis of the products of a conventional PCR reaction is performed after the thermal cycling is complete. This additional step uses independent visualisation techniques such as gel electrophoresis, bead-based hybridisation arrays, or an Enzyme Linked Immunosorbent Assay (ELISA) [274, 275]. Post-PCR analysis increases sample handling and possible routes to introduce contamination. It also increases the time taken for result acquisition, and generally provides only qualitative data [276].

1.4.3 Real-Time PCR

The introduction of real-time PCR enabled accurate and reproducible quantitative analysis [277] of nucleic acids. This is achieved by detecting fluorescence that is associated with the accumulation of newly amplified DNA as it occurs in each cycle using thermocyclers that are equipped with optical sensors. The principle of quantification is that the two-fold increase in fluorescence during each exponential amplification can be related to the starting concentration of template material. The number of cycles required to generate a fluorescence signal which passes a defined threshold above the background fluorescence is known as the quantification cycle, or "C_q" [278, 279]. In addition to this “relative” quantification approach which compares the C_q at which the target is detected, the use of calibration curves derived from samples of known concentration enabled the development of quantitative real-time PCR (qPCR) using “absolute” quantification [280, 281]. The two primary methods of fluorescent chemistries used in real-time PCR are non-specific such as intercalating dyes, and strand specific, which can further be divided into hydrolysis and hybridisation probes [273, 276].

1.4.3.1 Non-Specific Fluorescent Chemistries

Non-specific detection involves the use of dyes such as SYBR® Green-I, which fluoresce upon binding to the minor groove of dsDNA (**Figure 1.4**). During PCR, the amount of dsDNA will increase with target amplification and the increase in fluorescence between cycles can therefore be measured [5]. The non-specific nature of intercalating dyes means that the specificity of the assay is dependent on the primers. Melt curve analysis can assist in discriminating between desired and misprimed products and is based on the change in fluorescence when dsDNA melts at differing temperatures. The melting curve profile is dependent on the product length and nucleotide content, particularly the nucleotide bases guanine and cytosine (GC content) [276, 282]. However, the non-specific binding approach lacks multiplexing capability, i.e., is unable to detect and differentiate between multiple desired products in the same reaction.

1.4.3.2 Strand-Specific Detection: Hydrolysis and Hybridisation Probes

In contrast, strand-specific detection uses fluorescently labelled oligonucleotide probes to directly measure accumulation of the reaction product. The probe is complimentary to an internal region of one of the strands produced during amplification. These can be divided into hydrolysis and hybridisation-based chemistry, in which the hydrolysis probes known as TaqMan probes are the most frequently used.

Fluorescence detection of TaqMan hydrolysis probes is based on the hydrolysis of the bound probe by the 5′-3′ exonuclease activity of the DNA polymerase (**Figure 1.4**) [283]. Initially, hydrolysis probes were dual labelled with a reporter and a quencher dye, the latter of which absorbs the fluorescent emissions of the reporter dye through Fluorescence Resonance Energy Transfer (FRET) [284]. Once the oligonucleotide probe is hydrolysed by the polymerase, the unbound reporter dye is no longer in close proximity to the quencher and the increase in fluorescence can be detected. More recently, the background fluorescence levels of hydrolysis probes have been improved through the use of dark quenchers [285, 286]. Dark quenchers absorb the energy from the reporter fluorophore and release it as heat rather than fluorescence [276, 284, 286]. Other modifications to hydrolysis probes such as 3′ Minor Groove Binder (MGB) ligands have been developed to increase the binding affinity and specificity of the probe [287].

Hybridisation probes are also strand specific but do not become degraded in order to separate the fluorescent reporter dye from its quencher. One such method, known as Molecular Beacons, uses a hairpin shaped dual-labelled probe (**Figure 1.4**). The hairpin structure in the unbound form allows FRET to occur between the two fluorophores. Once annealed to its target sequence, the configuration of the probe changes. This is sufficient to reduce the effect of FRET by the quencher dye and the increase in fluorescence of the reporter dye can be measured [285, 286].

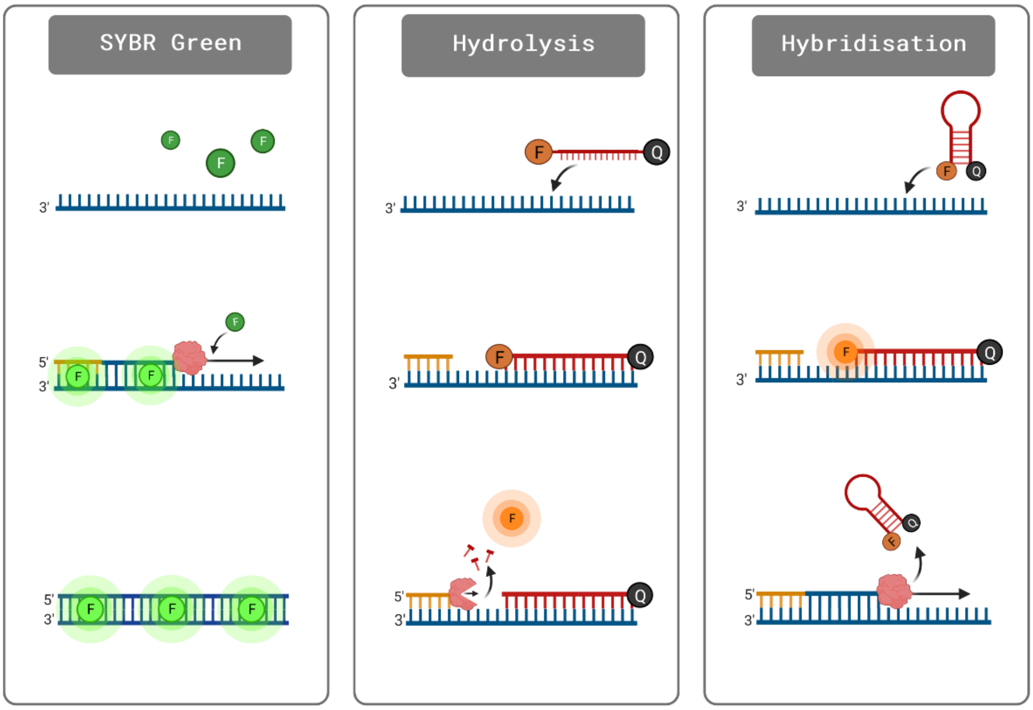

Figure 1.4: Fluorescence detection chemistries used in real-time PCR.
SYBR Green**:** Unbound fluorophore (top), does not bind to ssDNA and does not emit fluorescence. As dsDNA accumulates (middle) the fluorophore intercalates with the minor groove and emits fluorescence (bottom). Hydrolysis Probes: Fluorescence is not emitted from the unbound probe (top) or bound probe (middle) due to the proximity of the quencher to the fluorophore. The 5′-3′ exonuclease action of the polymerase hydrolyses the probe, releasing the fluorophore from the effects of the quencher molecule and enabling fluorescence (bottom). Hybridisation Probes: Form a hairpin structure in the unbound state which does not emit fluorescence (top). Fluorescence is emitted in the bound/hybridised state when the hairpin structure is disrupted, and the fluorophore is no longer adjacent to the quencher (middle). The probe is displaced by the polymerase during extension and returns to a hairpin configuration with no fluorescence emission (bottom). *Image created with BioRender.com*

1.4.4 Sample-to-Answer Diagnostic Systems for Infectious Disease

Nucleic acid amplification tests allow for rapid and specific identification of pathogens from a wide range of sample types. Laboratory-developed, or so-called in-house assays offer enormous flexibility in terms of customisation and choice of diagnostic target panels. The design and implementation of these assays, however, requires skilled laboratory personnel with extensive knowledge of molecular techniques. Real-time PCR technology is now advancing from in-house validated assays, into highly multiplexed, fully automated, 'sample-in result-out' platforms [288, 289]. These commercially available systems are targeted toward the clinical setting and generally used in hospital laboratories to rapidly diagnose pathogens from specific specimen types or clinical presentations. Sample preparation, nucleic acid amplification, detection and result analysis are performed automatically on a single device, which considerably reduces the hands-on time and expertise needed to test samples. Typically, all reagents for nucleic acid extraction and real-time PCR are contained in an individual cartridge, to which the sample is added, before the cartridge is loaded into the sample analyser. A number of such systems are commercially available and will be discussed below.

1.4.4.1 BioFire® FilmArray®

The BioFire® FilmArray® offers a series of diagnostic panels based on clinical manifestations (pneumonia, meningitis/encephalitis, bloodstream culture; respiratory, gastrointestinal, or joint infection) which are FDA (US Food and Drug Administration) cleared and CE-IVD (Conformité Européenne-*In Vitro* Diagnostics) marked [290-295]. The FilmArray® Pneumonia *Plus* Panel (PNplus) was used as a comparative gold-standard diagnostic in **Chapter 2** of this thesis to investigate respiratory specimen of hospitalised patients with suspected HAP or VAP [154]. The FilmArray PNplus has the ability to detect 18 bacterial pathogens, 9 viruses and 7 AMR genes [154].

Each panel is packaged as a disposable pouch, containing the lysis, amplification, and detection reagents in individual compartments (**Figure 1.5**). Nucleic acid is purified using mechanical bead beating as the lysis technique [288]. Nested real-time PCR then occurs in separate pouch compartments; first, a high-level multiplex PCR to enrich for target nucleic acids. The amplified material is then diluted and undergoes a second PCR using LCGreen® Plus intercalating dye which binds to dsDNA. A final melt curve analysis provides the fluorescence data to identify the target organism [154, 288, 289]. Instrument run time is approximately 1 hour 15 mins with a maximum number of 12 pouch modules [296].

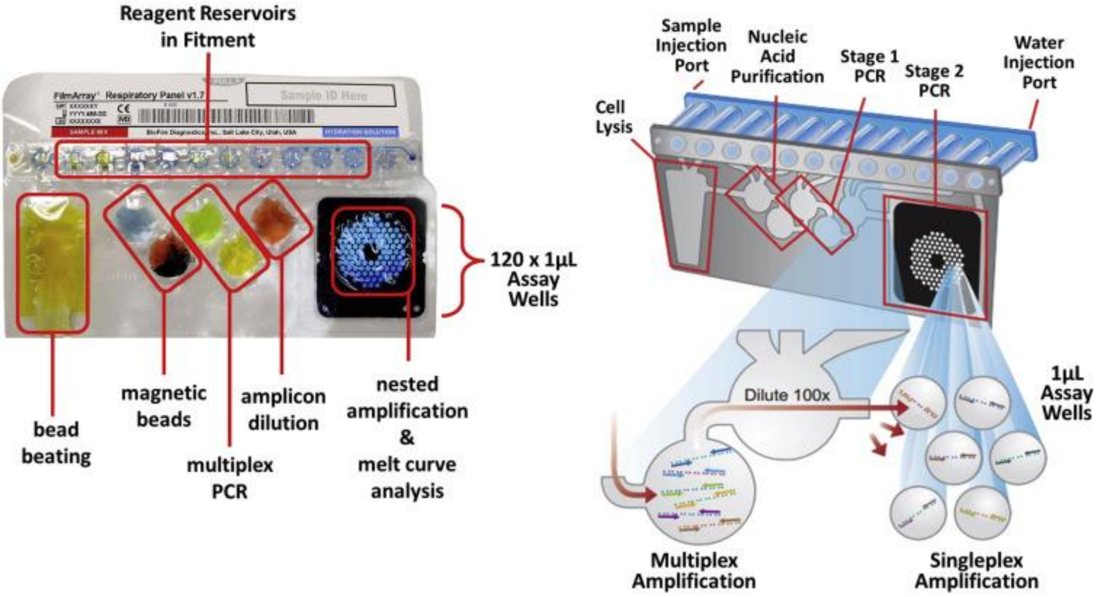

Figure 1.5: Photographic image (left) and illustration (right) of a BioFire® Film Array® pouch.
Individual compartments of the BioFire® FilmArray pouch contain the necessary reagents for nucleic acid purification and nested real-time PCR. Sample injection is followed by cell lysis via bead beating and nucleic acid purification using functionalised magnetic beads. Stage 1 of the nested PCR occurs as two separate multiplex reactions which each contain half of the target panel. The resulting amplicons are diluted and transferred to a multiwell array for Stage 2, where singleplex PCR and melt curve analysis occur in each of the 120 wells. *Image reprinted from Danielson et al. [289] with permission from Elsevier*

1.4.4.2 Cephid® GeneXpert®

The GeneXpert uses a cartridge system which incorporates sample preparation, nucleic acid purification, amplification, and detection. Each cartridge typically offers rapid and sensitive detection of individual pathogens and their associated antimicrobial resistances, rather than the highly multiplexed panels of bacterial, viral and AMR determinants seen in the BioFire®. However, the instrumentation available offers flexibility to run up to 80 cartridges on one machine [264]. The GeneXpert® system uses chemical and ultrasonic lysis for nucleic acid extraction, and molecular-beacon fluorescence chemistry in its real-time PCR. Instrument run time is typically under two hours, depending on the cartridge chosen [288, 289].

1.4.4.3 Cuertis® Unyvero®

Similar to the BioFire®, the Cuertis® Unyvero® uses high level multiplexing on a single cartridge to detect up to 38 pathogens and antimicrobial resistances. Cartridges are available for blood culture, hospitalised pneumonia, implant and tissue infection, and intra-abdominal infection [297-300]. Rather than the integrated sample preparation seen in the BioFire® and the GeneXpert®, the sample is first placed in the proprietary "lysator" instrument where mechanical bead beating occurs, before being transferred to the cartridge along with a second tube containing chemical reagents [256, 297]. The cartridge is then placed in the sample analyser where nucleic acid purification, multiplex PCR, and detection takes place. Multiplex PCR and target detection occurs in 8 chambers inside the cartridge. Each chamber houses an independent multiplex assay, which together make up the cartridge target panel. Amplicon detection occurs via array hybridisation. Overall turnaround time is longer than that of the BioFire® and GeneXpert®. The Unyvero® sample analyser can hold a maximum of two cartridges and takes approximately 5 hours from sample lysis to run completion [154, 301].

Although the above diagnostic platforms are highly useful in a clinical setting, none provide a targeted means of simultaneously identifying all of the ESKAPE pathogens. Therefore, this thesis aimed to provide a highly multiplexed real-time PCR assay which was tailored to detect and identify the ESKAPEs with increased specificity. The process of developing a hydrolysis probe-based real-time PCR assay from initial conception to final multiplex validation involves a combination of *in silico* and *in vitro* testing and will be discussed in general terms below.

1.4.5 Overview of the Multiplex Real-Time PCR Design and Validation Process

The development of a multiplex real-time PCR assay requires several considerations, from assay conception to final multiplex optimisation and validation (**Figure 1.6**). Each assay should detect and be specific for all species of interest. This involves an initial taxonomic investigation into the species of interest for potential subspecies, closely related species, and closely related genera [302]. This investigation forms the basis of the inclusivity (desired species) and exclusivity (undesired species) panels which are used to validate assay specificity. The groupings of each monoplex assay should be decided at this stage, such that the later design and optimisation steps performed for each monoplex assay are advantageous for their performance together as a multiplex.

The species divided between the inclusivity and exclusivity panels have sufficient genomic dissimilarity to be considered as separate taxa [303]. However, to differentiate them using real-time PCR it is necessary to identify a region of the genome which meets certain requirements. These regions, referred to as “NAD targets” must contain sufficient nucleotide conservation in desired species, while also having the nucleotide heterogeneity necessary to differentiate from undesired species. NAD targets share many properties with molecular markers used in phylogenetic studies. They are relatively short regions of the genome which can be used to differentiate species, but which also display evolutionary stability [304]. Nucleotide sequences of various NAD targets for members of the inclusivity and exclusivity panels are retrieved from nucleotide sequence databases such as GenBank [305] and aligned using multiple sequence alignment software [306]. Sequence alignments are used to identify which of the NAD targets under preliminary investigation warrant further analysis. The NAD targets used in this study are discussed in detail in **Section 1.4.6.**

The design of oligonucleotide primers and probes based on the sequence of the NAD target is arguably the most important part of the real-time PCR development process. In order to achieve efficient detection of the desired species, the primers and probes must display certain characteristics [284, 307]. The complimentary sequence of the forward and reverse primers should be fully conserved in all sequences of the inclusivity panel. However, the forward and reverse primers should not be complimentary to itself or to each other, so as to avoid primer dimers. Primers should be approximately 20 nucleotides in length and have a GC content of 50%. The melting temperature (T_m) of the primers – defined as the temperature at which 50% of the primer-template duplexes in the reaction have formed – should be approximately 58°C [308]. The distance between the primers (amplicon length) should be between 100-400 nucleotides [281].

The probe is located between the forward and reverse primer. The probe sequence must also be fully conserved in the complimentary sequences of the inclusivity panel. Crucially, for sequences of the exclusivity panel, the probe must contain mismatches to prevent annealing and detection. This forms the basis for detection and differentiation in a real-time PCR assay. Mismatched basepairs in the centre of the probe region are preferrable. The probe should be approximately 22 nucleotides in length and have a T_m of approximately 63°C [281, 307]}. To avoid unspecific annealing, the T_m of the probe should differ from the T_m of the complimentary exclusivity sequence by at least 10°C. This parameter is known as the ΔT_m and is influenced by the overall length and sequence of the probe, and by the nature and position of the mismatch(es) [281, 308]. Finally, the probes of each monoplex assay should be labelled with fluorophores which have emission spectra at differing wavelengths to facilitate differentiation in a multiplex reaction [276].

Assay specificity *in vitro* is not guaranteed based on *in silico* specificity. Validation of real-time PCR assays *in vitro* is necessary in all instances and requires high-quality template DNA from well-characterised, representative members of the inclusivity and exclusivity panels [309]. Culture collection isolates are an ideal source of such material. Genomic DNA (gDNA) isolated from culture collection isolates can be stored at -20°C and used as template in real-time PCR reactions. The gDNA can be quantified and standardised to the desired concentration.
Thermocycling parameters and fluorescence acquisition settings are determined by the polymerase and real-time PCR instrument used. The concentrations of the oligonucleotide are typically in the range of 0.2-0.6 µM/reaction for primers and 0.1-0.5 µM/reaction for probes [307, 310]. The gDNA from all members of the inclusivity panel should be detected by the assay, producing sigmoidal amplification curves with a C_q shift (ΔC_q) of 3.32 cycles between $\log_{10}$ dilutions of starting template [308]. No members of the exclusivity panel should be detectable. Adjustments such as increasing the annealing temperature of the PCR reaction between can be useful to eliminate minor cross reactions between the probe and members of the exclusivity panel.

Assuming the *in vitro* specificity of each assay in monoplex format, the assays can then be combined to form a single multiplex reaction. An internal amplification control (IAC) should be incorporated at this stage. This is an additional assay which is used to confirm the success of the multiplex PCR reaction [278, 311]. IAC template DNA is spiked at predetermined concentrations (typically 100-10,000 copies per reaction) into each test well during PCR set-up and must be detected for the result to be considered valid. IACs can be broadly categorised into competitive and non-competitive, meaning they either compete for the same primers as the primary assay, or have independent primers [311]. A non-competitive IAC targeting synthetic DNA is a convenient IAC format for infectious disease NADs as they are unlikely to reduce the performance of the primary assays, are highly customisable and provide consistent results [281]. A randomly generated nucleic acid sequence, not found in nature ("synthetic") is manufactured into a double-sided gene fragment to act as template for the IAC. Primers and a probe are designed following the same principles as above. Due to the limitations of currently available fluorescent chemistries and real-time PCR instrument optics, the maximum number of assays which can be reliably differentiated in a multiplex reaction is 5 [312]. This includes the IAC and should be accounted for during the initial planning of the multiplex assay.

The combination of several monoplex real-time PCR assays into a single multiplex can now proceed, although this also requires careful optimisation. Multiple amplification reactions with varying kinetics are now occurring simultaneously in the same reaction well. The final multiplex should retain 100% specificity for desired species and ideally have analytical sensitivity of <50 genomic equivalents for each target [281, 308].

The reaction efficiency of an assay in multiplex may be substantially lower than for the same assay in monoplex format. Each target template will be denatured, undergo primer and probe annealing, and extension at a different rate [309]. To overcome the effects of this, the kinetics of each reaction can be altered by adjusting the concentrations of the relevant oligonucleotides. For example, if the lower range of detection for one assay has increased considerably relative to its performance in monoplex format, the primer concentration for that assay may be increased in increments of 10% to improve the reaction kinetics. However, altering the concentration of one primer set may in turn affect the performance of another assay in the multiplex format [307, 310]. One method to determine the optimal concentration for all oligonucleotides in a multiplex is a concentration matrix approach, in which all permutations of oligonucleotide concentration in the expected working rage (e.g., 0.2-0.6μM) are tested against in a stepwise manner [309]. The conditions which provide the most favourable performance for all assays in the multiplex reaction – including the IAC – can then be selected. The limit of detection (LOD) for each assay in the optimised multiplex reaction should then be determined by testing replicate samples of a dilution series of low concentration template, and performing a probit regression analysis to calculate the LOD with 95% probability [281, 313, 314]

These fundamentals can be applied for the development of any real-time PCR assay. Once experimentally validated, real-time PCR can be used to detect nucleic acids from theoretically, any organism, from viruses [315, 316] to bacterial and fungal species [219, 317, 318], or for the detection of other characteristics such as antimicrobial resistance genes [319].

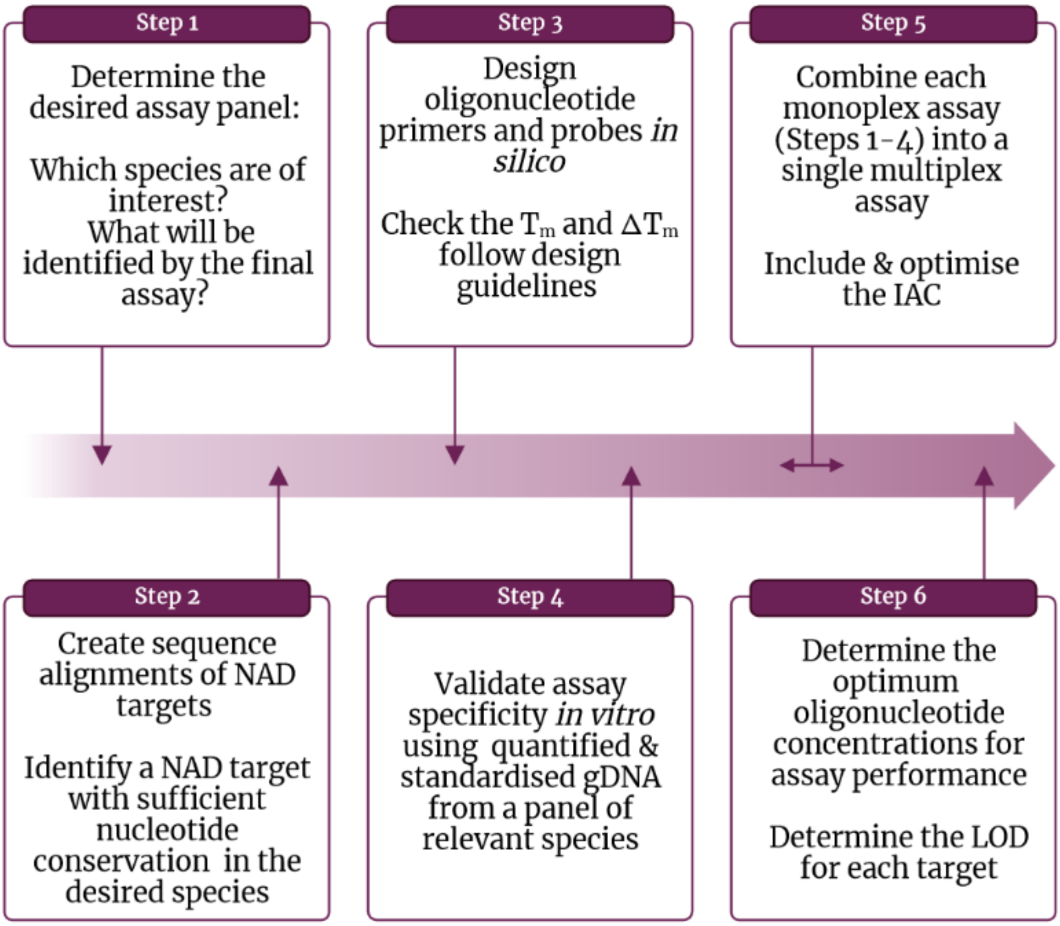

Figure 1.6: The process of designing a multiplex real-time PCR assay
Steps 1-3: Assay design is performed *in silico*, using sequence alignments of NAD targets to check for assay specificity. Steps 4-6: Confirmation of assay specificity, and optimisation of multiplex conditions are performed *in vitro. Image created with BioRender.com*

1.4.6 Nucleic Acid Diagnostic Targets Used in This Study

The premise of all real-time PCR diagnostic assays is the detection of a portion of the genome in which the nucleotide bases complimentary to the primer and probe sequences are conserved. Each real-time PCR assay described in this study has been shown to be specific to its intended target, differentiating individual species, genera, or species complexes. Here we will briefly discuss the functions and properties of the NAD targets utilised in this study.

1.4.6.1 Elongation Factor 4 (lepA)

The *lepA* gene codes for the highly conserved GTPase LepA, also known as Elongation Factor 4 (EF4). Approximately 599 amino acids (aa) in length, LepA is one of the most conserved proteins in bacteria with 55%-68% amino acid identity [320]. Despite several studies into the role of LepA its precise function has not been fully resolved. It was initially thought that LepA facilitated back translocation of the $tRNA_2$-mRNA complex in stalled ribosomes during the elongation phase of protein synthesis [320-322]. More recent studies have disproven this, instead suggesting that it is involved in 30S subunit biogenesis and assembly [323, 324].

The *lepA* gene is frequently used in multilocus sequence typing (MLST) schemes [325-327]. In this study, *lepA* proved to be a useful NAD target in four of the twelve novel real-time PCR assays designed, demonstrating sufficient nucleotide sequence heterogeneity to specifically detect *E. faecium, S. aureus, K. pneumoniae* and *K. variicola*.

1.4.6.2 RNA polymerase subunit Beta (rpoB)

The β subunit of the DNA-directed RNA polymerase is encoded by the *rpoB* gene and is ~1362aa in length [328]. It is found universally in bacteria and contains a mixture of conserved and variable regions [329]. These characteristics have led to it being used extensively as a gene marker in phylogenetic studies and as a NAD target in bacterial diagnostics [330].

Partial sequencing of the *rpoB* gene has been shown to provide higher resolution than the 16S rRNA hypervariable V3V4 region when evaluating the diversity of bacterial communities [331, 332]. In addition, and of most relevance to this study, *rpoB* sequencing has been extensively used to differentiate *Acinetobacter* species [146, 228, 333]. The *rpoB* gene was targeted in this study for a real-time PCR assay to detect the *A. baumannii* group, consisting of *A. baumannii, A. pittii, A. nosocomialis, A. seifertii* and *A. lactucae*, enabling differentiation from other closely related *Acinetobacter* species.

1.4.6.3 SsrA-binding protein / RNA binding protein small protein B (smpB)

The SsrA-binding protein, or small protein B (SmpB) is coded for by the gene *smpB* and is conserved universally in bacteria [334, 335]. When the 160aa SmpB binds to SsrA (tmRNA) it forms part of a ribonucleoprotein complex which is involved in rescuing of stalled ribosomes in a process known as trans-translation. The complex mimics tRNA in a stalled ribosome, eliminating the dysfunctional mRNA and tagging the nascent protein for proteolysis [336, 337]. It therefore plays a critical role in cellular function. The *smpB* gene has previously been shown to be a suitable NAD target for *H. influenzae*, [338] and was used in this study to detect and differentiate *A. baumannii sensu stricto*.

1.4.6.4 Ribosomal protein S7 (rpsG)

The *rpsG* gene codes for the 30S ribosomal protein S7, one of many ribosomal proteins which form the small (30S) subunit of bacterial ribosomes together with 16S rRNA [339]. Ribosomal protein S7 is involved in ribosome self-assembly of the head of the 30S subunit, where it binds to the 3′ major domain of 16S rRNA to initiate folding [340]. The nucleotide sequence of *rpsG* has been shown to be highly conserved and has been used in MLST schemes [341], and in a DNA hybridisation array for the detection of microbiological agents responsible for nosocomial pneumonia [342]. In the current study using real-time PCR, *rpsG* was targeted to specifically detect *P. aeruginosa*.

1.4.6.5 DNA repair protein RadA (radA)

DNA repair protein RadA is a 460aa protein that plays a role in branch migration during recombinational DNA repair [343-345]. It is universally conserved in bacteria and displays genetic synergy with other proteins involved in the repair of double stranded DNA breaks such as RecG, and its paralog RecA [346]. Mutations in *radA* have been shown to increase sensitivity to ultraviolet (UV) irradiation and ciprofloxacin [347].

Alnajar and Gupta studied 118 conserved proteins in members of the Enterobacteriaceae and found that the amino acid sequence of RadA contained a 1aa insertion uniquely conserved amongst the *Enterobacter*, *Leclercia* and *Lelliottia* genera [348]. In this study, we used the nucleotide sequence of *radA* to create a genus-specific assay for *Enterobacter*. Differentiation of *Enterobacter* species from other closely related Enterobacteriaceae without sequence-based analysis has not previously been reported [239, 240].

1.4.6.6 Zur-Khe Intergenic Region (ZKIR)

In addition to the conserved protein coding genes discussed in the previous sections, intergenic regions can serve as NAD targets [349]. A study which sought to uncover novel NAD targets for KpSC species [232] found that a region between the *khe* (*Klebsiella* haemolysin) and *zur* (zinc uptake regulator) genes was highly conserved in all five KpSC species (**Figure 1.1**). The *khe* gene has previously been used as a target for *Klebsiella* PCR diagnostics [350, 351], however the study by Barbier *et al.* [232] showed that the *khe* primers were unspecific and had the potential to cross react with *Raoultella* species.

In **Chapter 3**, the ZKIR region was used to detect each member of the KpSC as part of a multiplex real-time PCR assay which also specifically detects the species *K. pneumoniae* (*lepA*), *K. variicola* (*lepA*) and *K. quasipneumoniae* (*rapZ*).

1.4.6.7 Rnase adapter protein RapZ (rapZ)

The RNase adapter protein RapZ is a conserved protein (284 aa) which is involved in regulation of glucosamine-6-phosphate (GlcN6P) biosynthesis [352, 353]. GlcN6P is synthesised by GlmS and is a precursor of the cell envelope components peptidoglycan and lipopolysaccharide (LPS). RapZ controls the expression of *glmS* via RNase E mediated degradation of the small RNA GlmZ [354, 355].

The *rapZ* gene was targeted in this study to detect and differentiate the species *K. quasipneumoniae*, which includes the phylogroups Kp2 (*K. quasipneumoniae* subsp. *quasipneumoniae*) and Kp4 (*K. quasipneumoniae* subsp. *similipneumoniae*) from other KpSC members.

1.4.7 The Limitations of Nucleic Acid Diagnostics

The field of infectious disease diagnostics has been revolutionised since the introduction of PCR, due to its enhanced speed, sensitivity, and specificity. Although culture remains the gold-standard for the diagnosis of the majority of bacterial infections, NADs have been adopted as a routine diagnostic method for infections caused by viruses and fastidious bacteria [256, 288, 316]. However, NAD-based approaches are not without limitations, and careful consideration must be taken before their use in the clinical laboratory.

While NADs are highly specific, this can also be considered a limitation. Organism-specific assays are limited in scope and can only detect organisms present in the diagnostic panel, whilst culture may detect all cultivable bacteria. High level multiplexing of NADs such as real-time PCR is limited by the optical detection capabilities of platforms and fluorescence chemistries that are currently available [276, 312]. Popular real-time PCR platforms such as the Roche LightCycler® or the ThermoFisher QuantStudio™ 5 have maximum multiplexing capabilities of 6 targets [312, 356]. In other NAD platforms such as the BioFire® FilmArray®, the use of nested PCR and a multi-well microarray increases multiplexing capacity [289].

Similarly, the sensitivity of NADs relative to culture can also pose challenges. NADs can have a limit of detection as low as a single cell [357, 358]. Exponential amplification enables the detection of low concentrations of starting material, but also makes it more susceptible to contamination. NADs require stringent quality control measures to avoid the production of false positive results due to contamination [358]. In addition, the sensitivity of NADs means that positive results from complex clinical samples must be carefully interpreted to avoid false positives due to the detection of colonising bacteria which do not require treatment [288, 308].

NADs have decreased the turnaround time, typically generating results in hours, rather than the days required for culture-based methods. For example, culture-based antimicrobial susceptibility testing (AST) can take up to 72 hours [359]. Delays in the start of appropriate antimicrobial treatment has been shown to be associated with excess patient mortality [360]. NADs have the ability to detect genotypic resistance factors in as little as 2 hours, which reduces the time that a patient may receive empirical, potentially inappropriate antimicrobial therapy [291, 361]. However, the presence of resistance genes does not always equate to phenotypic resistance. This can be due to low expression levels of the resistance gene. Therefore, while NADs are useful as a rapid means of initial AST, confirmatory testing using culture is required to fully elucidate phenotypic antimicrobial susceptibility and additional information such as the Minimum Inhibitory Concentration (MIC) of the antimicrobial [359, 362].

Another limitation of NADs relative to culture is the inability to distinguish between viable, metabolically active versus inactive cells, which may have implications on result interpretation [363]. DNA from dead cells without an intact cell wall can be still detected due to the relative stability of the DNA molecule [364]. Depending on the application this may be considered a false positive result, which again highlights the need for careful analysis of results. To overcome this, NADs can be designed to target RNA, which rapidly degrades after cell death [365, 366]. Alternatively, the use of propidium monoazide (PMA) is an effective way of inhibiting the amplification of DNA from cells with damaged cell walls, thus only allowing the detection of DNA from intact cells. It requires the exposure of the PMA-treated sample to UV light to covalently bond the PMA to dsDNA. The cell wall of intact bacterial cells provides a sufficient barrier to prevent the PMA from binding to the DNA within, thus selecting for cells with damaged walls only. While effective, this method is laborious and expensive and is generally not used in clinical applications [364, 367].

Table 1.2: Advantages and disadvantages of nucleic acid diagnostics

Advantages	Disadvantages
Rapid turn-around-time from sample to results	Cost of instrument purchase and maintenance can be high
High sensitivity/low limit of detection: NADs have a low false negative rate and can detect target at low starting concentrations	Genotypic antimicrobial susceptibility results do not necessarily equate to phenotypic resistance and require culture-based confirmation
High specificity: NADs have a low false negative rate and can accurately differentiate between closely related species	Low scope of detection: The target panel is predetermined and is limited by multiplexing capabilities
Potential for automation and high throughput sample analysis	Differentiating live from dead cells requires assay targeting RNA or additional sample treatment

Overall, NADs offer a considerable number of advantages over culture-based methods (**Table 1.2**). The use of rapid and specific NADs for the detection of pathogens can reduce the time taken to obtain an accurate diagnosis and guide clinical decisions such as the optimal antimicrobial treatment regime (if any) [154, 250]. Furthermore, there is a clear need for novel NADs for the specific identification of ESKAPE species. As discussed in **Section 1.3,** both traditional culture and existing PCR-based assays can potentially misidentify infections caused by these important pathogens. This study sought to addresses these issues by developing multiplex real-time PCR assays for the ESKAPEs and closely related species.

1.5 Scope of Thesis

The overall aim of this study was to develop and optimise a suite of real-time multiplex PCR assays for the rapid and accurate identification of ESKAPE pathogens and their associated species complexes. The developed assays should show utility for use in both human clinical samples and environmental samples collected from hospital environments. This will allow for the full profiling of ESKAPEs will provide more accurate information on their epidemiology, role in HAI and transmission from the built environment.

Chapter 2 describes the development of two multiplex real-time PCR assays for the detection and differentiation of (i) *S. aureus*, *K. pneumoniae sensu stricto*, and *P. aeruginosa*, and (ii) *E. faecium*, *A. baumannii sensu stricto*, the *A. baumannii* group, and the *Enterobacter* genus. These assays were validated using a panel of lower respiratory tract specimens from hospitalised patients with suspected HAP/VAP.

Chapter 3 describes the development of a multiplex PCR assay to detect each member of the KpSC, and to differentiate between *K. pneumoniae sensu stricto*, *K. quasipneumoniae,* and *K. variicola*, as well as detect the remaining KpSC members *K. quasivariicola* and *K. africana.*

Diagnostics and monitoring of HAI was extended to the built environment in **Chapter 4** where a protocol for the detection of a Mock Microbial Community (MMC) comprising *A. baumannii*, *S. aureus* and the fungal species *Cryptococcus neoformans* was established. We used multiplex real-time PCR to investigate MMC recovery efficiency from surfaces using various gDNA extraction kits, swab materials and surface types.

Finally, **Chapter 5** discusses the overall conclusions of this research and suggests possible future research directions.

1.6 Supplementary Material

Table S1.1: Current and former taxonomic designations in the genus *Enterobacter*.

Current name	Former name	Synonyms (Heterotypic, Hoffman Clusters, Enteric Groups, orthographic)	Mainly Isolated From	Reference
"Pseudoenterobacter timonensis"	*“Enterobacter timonensis”*	mt20	Humans	[368-370]
Cronobacter dublinensis	*Enterobacter sakazakii*		Humans, Food Products	[371, 372]
Cronobacter muytjensii	*Enterobacter sakazakii*		Humans, Food Products	[371, 372]
Cronobacter sakazakii	*Enterobacter sakazakii*		Humans, Food Products	[371, 372]
Cronobacter turicensis	*Enterobacter sakazakii*		Humans, Food Products	[371, 372]
Enterobacter asburiae		*Enterobacter muelleri,* Hoffman Cluster I, CDC Enteric Group 17, Chavda Group J, Sutton Clade J	Humans	[373-375]
Enterobacter bugandensis		Hoffman Cluster IX, Chavda Group R, Sutton Clade R	Humans	[236]
Enterobacter cancerogenus	*Erwinia cancerogena*	*Enterobacter taylorae,* Sutton Clade U	Plants, Humans	[376-378]
Enterobacter chengduendsis			Humans	[379]
Enterobacter chuandaensis			Humans	[380]
Enterobacter cloacae subsp. *cloacae*	*Enterobacter cloacae*	Hoffman Cluster XI, Chavda Group G, Sutton Clade G	Humans	[373, 381]
Enterobacter clocae subsp. *dissolvens*	*Enterobacter dissolvens, Erwinia dissolvens*	Hoffman Cluster XII, Chavda Group H, Sutton Group H	Humans	[70, 370, 373, 381]
Enterobacter dykesii			Plants, Food Products	[382]

Enterobacter hormaechei subsp. *hoffmanii*	*Enterobacter hormaechei*	Hoffman Cluster III, Chavda Group D, Sutton Clade D	Humans	[70, 370, 375]
Enterobacter hormaechei subsp. *hormaechei*	*Enterobacter hormaechei*	Hoffman Cluster VII, CDC Enteric Group 75, Chavda Group E, Sutton Clade E	Humans	[70, 239, 370, 383]
Enterobacter hormaechei subsp. *oharae*	*Enterobacter hormaechei*	Hoffman Cluster VI, Chavda Group C Sutton Clade C,	Humans	[375, 384, 385]
Enterobacter hormaechei subsp. *steigerwaldii*	*Enterobacter hormaechei*	Hoffman Cluster VIII, Enteric Group 75, Chavda Group B, Sutton Clade B	Humans	[70, 239, 370, 383]
Enterobacter hormaechei subsp. *xiangfangensis*	*Enterobacter xiangfangensis*	Hoffman Cluster VI, Chavda Group A, Sutton Clade A	Humans	[70, 370, 375, 386]
Enterobacter huaxensis			Humans	[380]
Enterobacter kobei		Hoffman Cluster II, NIH Group 21, Chavda Group Q, Sutton Clade Q	Humans	[387]
Enterobacter ludwigii		Hoffman Cluster V, Chavda Group I, Sutton Clade I	Humans	[388]
Enterobacter mori		*Enterobacter tabaci*, Chavda Group F, Sutton Clade F	Plants	[57, 389, 390]
Enterobacter oligotrophicus		*Enterobacter oligotrophica*	Soil	[391]
Enterobacter quasihormaechei			Humans	[392]
Enterobacter quasimori			Humans	[370]
Enterobacter quasiroggenkampii			Humans	[370]
Enterobacter roggenkampii		Hoffman Cluster IV, Chavda Group M, Sutton Clade M	Humans, Plants	[375]
Enterobacter sichauanensis			Humans	[393]
Enterobacter soli		Sutton Clade V	Soil, Wastewater	[56]

Enterobacter vonholyi			Plants, Food Products	[382]
Enterobacter wuhouensis			Humans	[392]
Franconibacter helveticus	*Cronobacter helveticus, Enterobacter helveticus*		Plants, Fruit	[61, 394, 395]
Franconibacter pulveris	*Cronobacter pulveris, Enterobacter pulveris*		Fruit, Food Products	[61, 395, 396]
Klebsiella aerogenes	*Enterobacter aerogenes*	*Klebsiella mobilis, Klebsiella aeromobilis*	Humans	[64, 66, 68]
Kluyvera intermedia	*Enterobacter intermedius*	*Kluyvera cochleae, Enterobacter intermedium*	Humans	[397, 398]
Kosakonia arachidis	*Enterobacter arachidis*		Plants, Soil	[61, 399]
Kosakonia cowanii	*Enterobacter cowanii*		Plants, Humans	[61, 400]
Kosakonia oryzae	*Enterobacter oryzae*		Rice, Plants	[61, 401]
Kosakonia oryzendophytica	*Enterobacter oryzendophyticus*		Rice, Plants	[402, 403]
Kosakonia oryziphila	*Enterobacter orziphilus*		Rice, Plants	[402, 403]
Kosakonia radicincitans	*Enterobacter radicinitans*		Plants	[61, 404]
Kosakonia sacchari	*Enterobacter sacchari*		Plants	[386, 405]
Leclercia adecarboxylata	*Escherichia adecarboxylata*			[348, 406]
Leclercia pneumoniae			Humans	[348, 407]
Lelliottia amnigena	*Enterobacter amnigenus*		Soil, Humans	[61, 348, 408]
Lelliottia nimipressuralis	*Enterobacter nimipressuralis, Erwinia nimipressuralis*	Hoffman Cluster X	Plants	[61, 348, 373]
Pantoea agglomerans	*Enterobacter agglomerans*	*Erwinia herbicola, Erwinia milletiae*	Humans	[409-411]
Phytobacter massiliensis	*Enterobacter massiliensis, Metakosakonia massiliensis*		Humans	[348, 412, 413]
Pluralibacter gergoviae	*Enterobacter gergoviae*		Humans	[61, 414]
Pluralibacter lignolyticus	*Enterobacter lignolyticus*	SCF1	Plants, Soil	[348, 415]

Pluralibacter pyrinus	*Enterobacter pyrinus*	Plants, Soil	[61, 416]
Siccibacter turicensis	*Cronobacter zurichensis, Enterobacter turicensis*	Plants, Fruit	[61, 394, 395]

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Chapter 2: Development of Novel Multiplex Real-Time PCR Assays for Rapid and Specific Detection of ESKAPE Pathogens from Lower Respiratory Tract Specimens

Abstract

Healthcare associated infections (HAI) are a significant public health concern globally. Hospital acquired pneumonia and ventilator-associated pneumonia (HAP/VAP) contributes to over 33% of the total number of disability-adjusted life years (DALYs) lost to HAI. Among the bacterial species which cause HAI, the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii*, *Pseudomonas aeruginosa, Enterobacter* species) have emerged as the most burdensome and difficult to treat. Furthermore, the diagnosis of HAP/VAP is hindered by a lack of culture-independent, rapid molecular diagnostics. This study describes the development of two multiplex real-time PCR assays which have the ability to detect and differentiate each member of the ESKAPE pathogens. These assays were validated using genomic DNA from a panel of 181 culture collection isolates and found to be 100% specific to their intended target species, and had a lower limit of detection of between 6 and 39 genomic equivalents. A panel of 114 lower respiratory tract specimens from patients with suspected hospital acquired and/or ventilator associated pneumonia (HAP/VAP) was investigated using the ESKAPE multiplex real-time PCR assays. The results generated were compared to those obtained from a commercially available PCR diagnostic platform: the BioFire® FilmArray® Pneumonia plus panel. The ESKAPE multiplex real-time PCR assays developed in this study achieved full concordance with the BioFire® FilmArray® for 87.7% of samples. We additionally demonstrated the potential misidentification of *Enterobacter* species by the FilmArray® using confirmatory sequencing of the *hsp60* gene. The assays developed in this study enable the rapid and sensitive identification of the ESKAPE pathogens using genomic DNA extracted from complex clinical specimen. The timely and precise identification of the etiological agent of HAP/VAP will assist in the reduction of DALYs lost due to HAI and improve patient outcomes.

2.1 Introduction

Hospital acquired pneumonia (HAP) and ventilator associated pneumonia (VAP) are frequent causes of healthcare associated infection (HAI), particularly in intensive care units (ICU). They are the most commonly reported and burdensome nosocomial infection, resulting in increased patient mortality, duration of treatment and hospitalisation [1-5]. Lower respiratory tract infections (LRTI) caused by antimicrobial resistant organisms were associated with over 1.5 million deaths in 2019 [2]. The ESKAPEs (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, *Enterobacter*) cause a high number of multidrug resistant infections and are responsible for the majority of HAP/VAP infections [6-9].

The prevalence of HAP and VAP is highest in ICUs, where patients are at high risk of developing HAI. HAP in ICU patients can cause up to an 82% increase in 30-day mortality [10]. Patients with multiple co-morbidities undergoing invasive treatment, immunosuppressant, or antimicrobial therapy, are at a higher risk of developing HAP and VAP [11, 12]. Walter *et al.* [12] estimated the prevalence of HAP to be 1.3% of hospitalised patients in acute care hospitals, rising to 8.1% of patients in ICUs. The incidence rates of VAP in two recent European studies were between 6.6-18.3 episodes per 1000 patient days [13, 14]. The burden of HAP was estimated in terms of disability-adjusted life years (DALYs) by Cassini et al. [15] using 2011 data from the European Centre for Disease Prevention and Control (ECDC). In this study, while HAP was the 3rd most frequently reported HAI, it was the most contributing factor in DALYs, responsible for 33.7% of the total. These studies demonstrate the severe disease manifestation of HAP and VAP.

Infections associated with ESKAPE pathogens can have a considerable effect on patient outcome. This is in part due to their high rates of antimicrobial resistance (AMR) which impedes treatment options. Inappropriate initial antimicrobial therapy results in longer treatment duration and increased mortality [16, 17]. The ESKAPEs have been grouped based on their ability to evade biocidal activity through the acquisition of AMR determinants and virulence factors [18]. While *E. faecium* is not thought to be a prevalent lung pathogen, it has been occasionally reported [19-21]. It is found at rates of 1%-3% in HAP/VAP surveillance studies (**Table 2.1**). *S. aureus* is a well-documented and leading cause of HAP and VAP. Both methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) cause a large burden of infection [22, 23]. *Klebsiella* species, particularly *K. pneumoniae* are also frequent causes of nosocomial pneumonia. However, *K. pneumoniae* exists in a species complex (KpSC) which cannot be differentiated using routine methods. Few studies have distinguished between infections caused by *K. pneumoniae sensu stricto* [24, 25]. Similar to *K. pneumoniae, A. baumannii* exists within a complex: the *Acinetobacter calcoaceticus baumannii* (Acb) complex [26]. While Acb complex species are phenotypically indistinguishable, *Acinetobacter calcoaceticus* is not thought to be pathogenic [27]. In this study, the term "*A. baumannii* group" will be used to describe the pathogenic species *A. baumannii, Acinetobacter. pittii, Acinetobacter. nosocomialis, Acinetobacter. seifertii* and *Acinetobacter. lactucae* [28]. *P. aeruginosa* consistently causes high levels of HAP, VAP and other LRTI; together with *S. aureus,* they form the largest proportion of infection. Multidrug resistant *P. aeruginosa* is a particular cause for concern due its global dissemination and the difficulty in treating these infections [29, 30]. The final member of the ESKAPE pathogens, the *Enterobacte*r genus, contains a large number of species and subspecies which are in taxonomic disarray [31-34]. Many of these are clinically relevant and together they are implicated in HAP and VAP at rates of approximately 6%-8% (**Table 2.1**). Though the contribution of each ESKAPE toward the incidence of HAP/VAP varies, together they exemplify the difficulties in preventing and treating such infections.

The burden of illness attributed to HAP/VAP is exacerbated by the lack of accurate diagnostic tests. Diagnosis is based on a variety of clinical, microbiological, and radiological findings [35, 36]. In terms of microbiological diagnosis, culture-based techniques remain the gold standard [37, 38]. Sputum, bronchoalveolar lavage or endotracheal aspirates are cultured in microbiological media to determine the presence of potential respiratory tract pathogens [39]. This method is slow and lacks sensitivity. Negative cultures occur in 30% of suspected HAP cases [40, 41], while the determination of antibiograms takes additional time. Furthermore, a negative culture result does not reduce the probability of HAP prognosis [14, 38].

The pathogens associated with HAP and VAP are varied, with ESKAPEs forming the largest portion of cultured samples [11, 42, 43]. A large-scale point prevalence study conducted across the EU/EEA in 2017 reported that over 44% of all HAI were caused by ESKAPE species, with this figure increasing to 56% in pneumonia and LRTI [6]. Although the ESKAPEs have long been recognised as important causes of infection [9], many species remain poorly defined in terms of taxonomy and nomenclature [24, 31, 44]. Some are found in wider species complexes [26, 45] , while *Enterobacter* has seen several species recently reclassified to new genera [34, 46]. Culture-based diagnostics, even those using higher resolution methods such as matrix-assisted laser desorption ionization time-of-flight Mass Spectrometry (MALDI-TOF MS), are unable to differentiate between these species [47-50]. It is possible that infections attributed to *A. baumannii, K. pneumoniae* and *Enterobacter*, may be caused by alternative, closely related species such as *A. pittii, Klebsiella variicola* or *Klebsiella aerogenes* (formerly *Enterobacter aerogenes*), respectively [24, 51].

Nucleic acid diagnostics for ESKAPE pathogens have been described, using conventional [52-55] and real-time PCR [56-59]. However, few have been validated against the species complex members mentioned above. This may result in misidentification of the causative organism of infection, as has been seen in previous case reports [24, 49, 60]. The use of chromosomally encoded β-lactamases as a diagnostic target, specifically in *A. baumannii*, has reduced specificity since the discovery of plasmid-mediated *bla*$_{\text{OXA-51}}$-like enzymes in *A. nosocomialis and A. seifertii* [61, 62]. These methods also fail to include all species; no single method is capable of detecting and identifying each ESKAPE pathogen simultaneously. There is a distinct lack of rapid nucleic acid diagnostic methods to detect *Enterobacter* species. Renner *et al.* [63] describes a proprietary isothermal recombinase polymerase amplification method for each ESKAPE with the exception of *Enterobacter*, as the authors were unable to identify a nucleic acid diagnostic (NAD) target which had sufficient conservation in *Enterobacter* to achieve specificity. Currently, the ability to precisely identify these species relies upon sequencing-based approaches which takes time and are not useful in a routine diagnostic setting [64, 65]. There is a clear need for rapid and accurate methods to detect these important causes of HAP and other HAI.

Table 2.1: ESKAPE pathogens detected in HAP/VAP surveillance studies, expressed a percentage of all bacterial infections reported

Publication (Year)	Seligman (2013) [66]	Slavcovici (2015) [43]	Walter (2018) [12]	Yin (2021) [30]	Martin-Loeches (2015) [67]	Weiner-Lastinger (2020) [68]	Suetens (2018) [6]	ECDC (2019) [13]	Enne (2022) [42]		ENVIN-Helics Informe (2019) [69]	Ko (2021) [70]
Study Location	Brazil	Romania	EU/ EEA	China	Europe & S. America	US	EU/ EEA	EU/ EEA	UK		Spain	Korea
Clinical Manifestation	HAP	HAP & VAP	HAP	HAP & VAP	VAP	pVAP[a]	HAP & LRTI	VAP	HAP & VAP		VAP	HAP & VAP
Sample Size (Isolates)	140	221	1,403	2,930	369	9,266	2,188	14,033	652[b], 620[c]		460	236
Data Collection Period	2007 - 2009	2007 - 2013	2011 - 2012	2007 - 2016	2013 - 2014	2015 - 2017	2016 - 2017	2017	2016 - 2018		2019	2019
Microorganisms (%)												
Enterococcus spp.	3.6	1.4	2.0	-	-	-	2.6	-	0.1 [b]	- [c,d]	1.1	2.1
S. aureus	36.4	23.5	12.0	12.6	24.0	28.8	12.4	18.5	12.7 [b]	22.1 [c]	13.9	10.2
Klebsiella spp.	11.4	18.0	12.0	15.4	14.0	10.1	14.3	15.2	6.7 [b]	20.1 [c]	14.6	15.3
Acinetobacter spp.	7.1	27.6	10.0	25.6	7.0	3.2	8.2	4.5	0.9 [b]	1.5 [c]	2.4	28.8
P. aeruginosa	10.0	19.5	17.0	20.1	24.0	12.9	15.1	19.9	11.2 [b]	16.6 [c]	22.0	15.3
Enterobacter	12.9	2.3	5.0	4.1	12.0	8.4	6.0	10.4	1.8 [b]	8.2 [c]	7.6	4.2

[a]Possible Ventilator Associated Pneumonia as defined by CDC/NHSN reporting guidelines
[b]Detected via culture
[c]Detected via FilmArray PNplus
[d]Not included in FilmArray PNplus panel

As the number of HAP/VAP caused by ESKAPE pathogens result in significant costs, morbidity, and mortality, a clinical need remains to rapidly detect and identify these microorganisms [8, 10, 42]. This study aims to address this need, particularly with HAP/VAP presenting in ICUs which require invasive treatment for the most seriously ill patients. In this study we describe the development and validation of two novel real-time multiplex PCR assays for the detection and differentiation of each ESKAPE group: *E. faecium, S. aureus, K. pneumoniae sensu stricto, A. baumannii sensu stricto, A. baumannii* group*, P. aeruginosa* and the *Enterobacter* genus. Multiplex 1 detects *P. aeruginosa, S. aureus* and *K. pneumoniae,* while multiplex 2 detects *E. faecium, A. baumannii,* the *A. baumannii* group and *Enterobacter* genus. Hydrolysis probes tagged with differing fluorophores have been used in each multiplex, along with an internal amplification control. We developed this multiplex to enable the rapid, sensitive, and accurate detection of the primary causes of HAP and VAP. This method also reliably differentiates between closely related species, previously only possible through sequencing.

The two internally controlled multiplex real-time PCR assays developed in this study were designed to detect and identify the ESKAPE pathogens in accordance with minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines [71]. These assays were initially validated using culture collection isolates were subsequently applied to directly detect ESKAPE pathogens from respiratory samples, including sputum, bronchoalveolar lavage, and endotracheal aspirates.

2.2 Materials and Methods

2.2.1 Sample Collection and Ethics Statement

The collection and use of the samples in this study adheres to a University College London (UCL) and UK Health Research Authority (Reference: 16/HRA/3882, IRAS ID: 201977) ethics agreement relating to the use of specimens surplus to clinical needs. Surplus routine lower respiratory tract samples were collected and anonymised from patients with suspected HAP/VAP at 15 participating ICUs located across the UK, between September 2016 and May 2018. Samples were frozen at -80°C within 72 hours of collection.

2.2.2 Diagnostics Target Identification

Target genes which could be used as nucleic acid diagnostic markers for each of the ESKAPEs were identified. A number of housekeeping genes which have previously been shown to be suitable targets for nucleic acid diagnostics [72-76] were evaluated *in silico.* A novel diagnostic target for the specific detection of *Enterobacter* genus was also identified based on conserved signature insertions and deletions in members of the Enterobacteriaceae [77]. For each target gene under investigation, publicly available nucleotide sequence information (**Supplementary Material Table S2.1**) was retrieved from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Nucleotide sequence alignments of ESKAPE and closely related species were created using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) [78]. Regions of nucleotide sequence were evaluated for unique diagnostic motifs from which oligonucleotide primers and hydrolysis probes were designed (**Table 2.2, Table 2.3**).

2.2.3 Bacterial Strains, Culture Media, and Growth Conditions

A total of 181 bacterial strains were used in this study, comprising of 51 *Enterococcus*, 22 *Staphylococcus*, 23 *Klebsiella*, 23 *Acinetobacter*, 22 *Pseudomonas*, 11 *Enterobacter*, and 29 closely related or LRTI-associated species (**Supplementary Tables S2.2 and S2.3**). All strains were purchased from either the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany); the Belgian Co-ordinated Collections of Microorganisms bacterial collection (BCCM/LMG, Gent, Belgium); the National Collection of Type Cultures (NCTC, Public Health England, Salisbury, UK); or the American Type Culture Collection (ATCC, provided by Microbiologics, Saint Cloud, Minnesota, US). All ESKAPE species were cultured following recommendations from the relevant culture collection (medium, temperature, atmosphere) using Oxoid dehydrated culture medium (Basingstoke, UK) for approximately 12-18 h or until sufficient growth was observed (**Supplementary Tables S2.2 and S2.3**).

2.2.4 Genomic DNA Isolation and Quantification

Genomic DNA (gDNA) was extracted and purified from culture collection isolates using the Zymo Quick gDNA Miniprep kit (Zymo Research, Irvine, California, United States). The protocol for “Cell Suspensions and Proteinase K Digested Samples” was followed, using 1 mL of overnight culture [79]. Genomic DNA concentrations for all culture collection isolates was assessed using the Qubit HS dsDNA kit and the Qubit 1.0 fluorometer (ThermoFisher, Waltham, Massachusetts, US).

For clinical specimens, a modified procedure combining mechanical lysis (PowerBead® Pro. Qiagen, Germany) and purification (DNeasy® Blood & Tissue. Qiagen, Hilden, Germany) was developed. Samples were stored at -80°C prior to use. Liquidised sputum (200 µL) was combined with 180 µL Dneasy® Buffer ATL in a PowerBead® Pro tube. Tubes were processed at for a total of 7 mins at 3,000 oscillations/min on a Mini-BeadBeater 16 (Thistle Scientific, Glasgow, UK). Tubes were placed on ice for 60 s after an initial 4 mins of bead beating and again after a subsequent 3 mins of bead beating. Tubes were centrifuged at 15,000 x *g* for 2 mins, and lysate was transferred to a sterile microcentrifuge tube, to which 20 µL of Proteinase K was added. The procedure for “Soft Tissue Lysis Using Tissue Disruptor” was followed thereafter [80]. All genomic DNA was stored at -20°C prior to use.

2.2.5 Real-Time PCR Primers, Hydrolysis Probe, and Internal Amplification Control Design

Oligonucleotide primers and hydrolysis probes specific to each ESKAPE group (5 species, 1 species complex and 1 genus) were manually designed in accordance with general recommendations and guidelines [81, 82]. Primers were designed to have a melting temperature (T_m) of approximately 55.0-61°C. Probes were designed to be perfect matches for the species of interest and to span unique diagnostic motifs which contained mismatches in non-target species. Probes were designed to have a T_m of 5-10°C higher than primers which differed in T_m (ΔT_m) by >10°C to the complementary sequence of non-target species. MeltCalc© 3 (http://www.meltcalc.com) [83] and the online Integrated DNA Technologies OligoAnalyser™ tool (https://eu.idtdna.com/pages/tools/oligoanalyzer) were used to calculate the T_m and ΔT_m of all primers and probes.

A non-competitive internal amplification control (IAC) [84] targeting a 500bp synthetic construct was also developed. The synthetic IAC target was created using a random sequence generator [85] and manufactured as a double-sided gene fragment “gBlock®” (**Supplementary Material Table S2.4**). Primers and probe targeting the synthetic construct were designed following the same guidelines [81, 82]. All primers, probes, and the synthetic gene fragment used in this study were supplied by Integrated DNA Technologies (Coralville, Iowa, US).

Table 2.2: Oligonucleotide primers and hydrolysis probes used in ESKAPE multiplex 1

Name	Function	Target	Sequence (5′ to 3′)[a]	Final Conc. (µM)
Paer_F1	*P. aeruginosa* forward primer	*rpsG*	TCATGAACCACGTGATGGA	0.500
Paer_R1	*P. aeruginosa* reverse primer		ACCGGAACCTGGTAAGTC	0.500
Paer_Probe	P. aeruginosa probe		**FAM**/CCTTGCCGC/**ZEN**/GTTCCTTCAC/**Iowa Black FQ**	0.300
Saur_F1	*S. aureus* forward primer	*lepA*	TGCTTTTAGTGGTGCTTCTG	0.375
Saur_R1	*S. aureus* reverse primer		TAGGTTTAGACCAAGACGATGT	0.375
Saur_Probe	S. aureus probe		**ROX/**CCATCTGGAGCTGGCACAACT**/Iowa Black RQ**	0.150
Kpneu_F1	*K. pneumoniae* forward primer [b]	*lepA*	TCGGAATTTCATAGGTCAGC	0.625
Kpneu_F2	*K. pneumoniae* forward primer [b]		TCGGAATTTCGTAGGTCA	0.500
Kpneu_R1	*K. pneumoniae* reverse primer [b]		CCAAAGAAGTTATCTACGTCGA	0.625
Kpneu_P	K. pneumoniae probe [b]		**CYAN500/**CTCGACACACAAGGTAATCACG**/Iowa Black FQ**	0.300
IAC_F1	IAC forward primer	Synthetic Construct	GTCGTATCGCAAGTTTCG	0.500
IAC_R1	IAC reverse primer		CCGTACGACTAGGAAGC	0.500
IAC_Probe	IAC probe		**Cy5/**AGATCGAAG**/TAO/**TGAGCATCGCT**/Iowa Black RQ**	0.200

[a] Fluorophores and quenchers of hydrolysis probes are shown in **bold;** FAM = 6-carboxyfluorescein, ROX = carboxy-X-rhodamine, CYAN500 = CYAN500 N-hydroxysuccinimide ester, Cy5 = indodicarbocyanine 5; Iowa Black®, ZEN™ and TAO™ are non-abbreviated trademarks

[b] *K. pneumoniae sensu stricto*, including the subspecies *K. pneumoniae* subsp. *pneumoniae, K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis*

Table 2.3: Oligonucleotide primers and hydrolysis probes used in ESKAPE multiplex 2

Name	Function	Target	Sequence (5′ to 3′)[a]	Final Conc (µM)
Efaec_F1	*E. faecium* forward primer	*lepA*	CCAAGTGGAGACTTAGATGC	0.500
Efaec_R1	*E. faecium* reverse primer		CTAGCAACTGCTTTTGGC	0.500
Efaec_Probe	*E. faecium* probe		**FAM/**ATCACGGAC**/ZEN/**GGTATGGTCAAAC**/Iowa Black FQ**	0.200
Abaum_F1	*A. baumannii* forward primer[b]	*smpB*	ATGGCGAAAGCAACAGT	0.500
Abaum_F2	*A. baumannii* forward primer[b]		ATGGCGAAAGCGACAG	0.500
Abaum_R1	*A. baumannii* reverse primer[b]		TGACATAACTTTCTGTCAAACTC	0.500
Abaum_Probe	A. baumannii probe[b]		**ROX/**AAATCTTTACGTGCCGGTCGT**/Iowa Black RQ**	0.200
Abg_F1	*A. baumannii* group forward primer[c]	*rpoB*	AAGAAGCAGCTCGTGAG	0.500
Abg_F2	*A. baumannii* group forward primer[c]		AAGAAGCGGCTCGTGA	0.500
Abg_R1	*A. baumannii* group reverse primer[c]		TGAATACGACGTTTAACAGC	0.500
Abg_Probe	*A. baumannii* group probe[c]		**HEX/**AATGGCGGT**/ZEN/**GGTTCAACTA**/Iowa Black FQ**	0.200
Entero_F1	*Enterobacter* forward primer	*radA*	TTGCAGCAGCAGGGT	0.750
Entero_F2	*Enterobacter* forward primer		TTGCAGCAGCAGAGTC	0.500
Entero_F3	*Enterobacter* forward primer		AGAAACGCGGCAGC	0.500
Entero_R1	*Enterobacter* reverse primer		TGTAATGAATGTGGTGCG	0.750
Entero_Probe	*Enterobacter* probe		**CYAN500/**AAGCCGCCACACCACG**/Iowa Black FQ**	0.400
IAC_F1	IAC forward primer	Synthetic Construct	GTCGTATCGCAAGTTTCG	0.500
IAC_R1	IAC reverse primer		CCGTACGACTAGGAAGC	0.500
IAC_Probe	IAC probe		**Cy5/**AGATCGAAG**/TAO/**TGAGCATCGCT**/Iowa Black RQ**	0.200

[a] Fluorophores and quenchers of hydrolysis probes are shown in **bold;** FAM = 6-carboxyfluorescein, ROX = carboxy-X-rhodamine, HEX = 4,4,7,2,4,5,7-hexachloro-6-carboxyfluorescein, CYAN500 = CYAN500 N-hydroxysuccinimide ester, Cy5 = indodicarbocyanine 5; Iowa Black®, ZEN™ and TAO™ are non-abbreviated trademarks

[b] *A. baumannii sensu stricto* [c] *A. baumannii sensu stricto* and the species *A. pittii, A. nosocomialis, A. seifertii*, and *A. lactucae*

2.2.6 Real-Time PCR

A Roche LightCycler®480 System II and LightCycler®480 Probes Master kit (Roche Diagnostics, Basel, Switzerland) were used for all real-time PCR experiments. For both multiplex assays, 10 µL of LightCycler®480 Probes Master (6.4 mM $MgCl_2$); optimised concentrations of relevant primers and fluorescent-labelled hydrolysis probes (**Table2.2, Table 2.3**); template DNA and 1 x 10^4 copies of SIAC gene fragment were brought to a final volume of 20 µL using nuclease-free water (Ambion, ThermoFisher, MA, US). Specificity testing of each assay was performed using 1 x 10^4 genome equivalents of the culture collection strains described above. Following multiplexing, the concentration of primers and probes were optimised using a oligonucleotide concentration matrix approach, whereby a range of oligonucleotide concentrations were trialled to determine the combination which gave the most favourable results [86].

The cycling parameters consisted of initial enzyme activation for 10 mins at 95°C, 50 cycles of 95°C for 10 s and 60°C for 30 s, and a final single cooling step of 40°C for 10 s. The temperature transition rate was 4.4°C/s while heating, and 2.2°C/s while cooling. A colour compensation file was generated to avoid fluorescence leaking between channels prior to experimental analysis on the LightCycler®480, as per the manufacturer's instructions [87].

To determine the analytical sensitivity of the multiplex assays, genomic DNA of each target ESKAPE group (multiplex 1: DSM50071, DSM23622, DSM30104; multiplex 2: DSM 20477, ATCC13047, DSM30007) was tested in the relevant multiplex assay using the conditions outlined, with twelve replicates of eight DNA concentrations ranging from 15 – 1 genome equivalents [84]. A probit regression analysis was used to calculate the limit of detection with 95% confidence (Minitab version 19) [88].

2.2.7 Evaluation of ESKAPE Multiplex Performance using Clinical Specimens

The diagnostic capabilities of the real-time PCRs developed in this study were additionally evaluated using lower respiratory tract specimens. A subset of samples which had been collected as part of a larger study [89] were randomly selected for testing. These samples had previously been extensively characterised using the BioFire® FilmArray® Pneumonia *plus* (PNplus) panel (bioMérieux, Marcy-l'Étoile, France). Samples were blinded by University College London prior to receipt at University of Galway. Genomic DNA was isolated from 114 specimens as described above, after which 5 µL was directly used as template for the ESKAPE multiplex PCR assays developed in this study. Samples were tested in duplicate, following the PCR reaction conditions outlined, including the addition of SIAC template.

Following ESKAPE multiplex testing and unblinding, samples which had discordant results underwent confirmatory testing using alternative molecular methods. For *Enterobacter* species, PCR targeting the *hsp60* gene was carried out according to Hoffman *et al.* [90], with sequencing being performed externally (Sequiserv, Germany). Identity of the nucleotide sequences (342 bp) was examined using the online NCBI nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Confirmation of the presence of *K. pneumoniae* was performed using a recently described SYBR® Green-based assay which detects the KpSC [91].

2.3 Results

2.3.1 Diagnostics Target Identification

In silico analysis revealed nucleic acid diagnostic markers capable of distinguishing each ESKAPE group. Housekeeping genes previously shown to be suitable bacterial diagnostics targets (*lepA, smpB, rpoB, rpsG*) contained sufficient sequence heterogeneity for the design of specific probes for six of the targeted ESKAPE groups. Based on *in silico* analysis of 740 target sequences of interest (**Supplementary Material Table S2.1**) all diagnostic assays were 100% specific. For the *S. aureus* (*lepA*) assay, 43 sequences were retrieved for Staphylococcus species; the probe designed was inclusive for the 12 *S. aureus* strains retrieved. For the *K. pneumoniae* (*lepA*) assay, 153 sequences were retrieved for Klebsiella and closely related species; the probe designed was inclusive for the 31 *K. pneumoniae* strains. For the *P. aeruginosa* (*rpsG*) assay, 42 sequences from Pseudomonas and closely related species were retrieved; the probe designed was inclusive for the 10 *P. aeruginosa* strains. For the *E. faecium* (*lepA*) assay, 54 Enterococcus sequences were retrieved; the probe was specific for the 18 *E. faecium* strains. For the *A. baumannii senso stricto* (*smpB*) assay, 73 sequences were retrieved for Acinetobacter species; the probe designed was specific for the 10 *A. baumannii* strains. For the *A. baumannii* group (*rpoB*) assay, 114 sequences for Acinetobacter species were retrieved; the probe designed was specific for the 88 *A. baumannii* group strains. In order to distinguish the genus *Enterobacter*, a novel diagnostics gene target was used. The *radA* gene encodes for the DnaB-type helicase RadA, which promotes RecA-mediated DNA strand exchange during homologous recombination [92, 93]. The amino-acid sequence of RadA contains a Glycine insertion specific to the *Enterobacter*, *Lelliottia* and *Leclercia* genera [77]. Analysis of the nucleic acid sequence revealed a region conserved in *Enterobacter* spp. Finally, for the *Enterobacter* (*radA*) assay, 261 sequences from Enterobacter and closely related species were retrieved, and the probe designed was specific for the 163 Enterobacter species.

2.3.2 Specificity of ESKAPE Real-Time PCR Assays

The diagnostic specificity of each ESAKPE target was also empirically determined testing against a panel of 181 ESKAPE species and closely related or LRTI-associated species (**Supplementary Table S2.2 and Supplementary Table S2.3**). In both monoplex and multiplex formats, the specificities of each target were determined to be 100%. Using multiplex 1, the *S. aureus* (*lepA*) assay was specific for the 15 strains tested for. The *K. pneumoniae* (*lepA*) assay was specific for the 15 strains tested for. The *P. aeruginosa* (*rspG*) assay was specific for the 13 strains tested for. The IAC construct was also specific for the designed region demonstrating no cross reactivity.
Using multiplex 2, the *E. faecium* (*lepA*) assay was specific for the 14 strains tested for. The *A. baumannii sensu stricto* (*smpB*) assay was specific for the 8 strains tested for. The *A. baumannii* group (*rpoB*) assay was specific for the 16 (*A. baumannii n*=8, *A. pittii n*=4, *A. nosocomialis n*= 2, *A. seifertii n*=1, *A. lactucae n*=1) strains tested for. The *Enterobacter* genus assay (*radA*) was specific for 11 (*E. asburiae n*=1, *E. cancerogenus n*=1, *E. cloacae n*=3, *E. hormaechei n*=1, *E. kobei n*=1, *E. ludwigii n*=1, *E. mori n*=1, *E. soli n*=1, *E. xiangfangensis n*=1) strains tested for. The synthetic construct used for SIAC was also specific with no cross reactions observed (**Supplementary Figures S2.1 & S2.2**).
Interpretation tables (**Table 2.4, Table 2.5**) are outlined below to describe how to analyse results generated from both multiplex 1 and multiplex 2. If after performing multiplex 1, *K. pneumoniae*, *P. aeruginosa* or *S. aureus* are determined to not be present in the reaction, multiplex 2 should be performed to determine if remaining members of the ESKAPE pathogens are present (*E. faecium, A. baumannii, A. baumannii group* or Enterobacter genus). Where co-infections may be possible it may also be prudent to perform both multiplex 1 and multiplex 2 to fully determine the prevalence of each ESKAPE pathogen.

Table 2.4: Results interpretation for multiplex 1

Fluorophore Excitation-Emission [a]	**Cyan500** 440-488	**FAM** 498-580	**ROX** 533-610	**Cy5** [b] 618-660	**Result Interpretation**
	+	-	-	+/-	*K. pneumoniae* [c]
	-	+	-	+/-	*P. aeruginosa*
	-	-	+ [d]	+/-	*S. aureus* [d]
	-	-	-	-	Invalid SIAC (must be repeated)
	-	-	-	+	Negative [e]

[a] Wavelength in nanometres
[b]In the presence of high concentrations of primary target, the SIAC may become inhibited
[c]*K. pneumoniae sensu stricto,* including the subspecies *K. pneumoniae* subsp. *pneumoniae, K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae* subsp. *rhinoscleromatis*
[d]A diagnostic cut-off of 30.0 C_q was used for *S. aureus*
[e] Not an ESAKPE pathogen detected by ESKAPE multiplex 1

Table 2.5: Results interpretation for multiplex 2

Fluorophore Excitation-Emission [a]	**Cyan 500** 440-488	**FAM** 498-580	**HEX** 533-580	**ROX** 533-610	**Cy5** [b] 618-660	**Result Interpretation**
	+	-	-	-	+/-	*Enterobacter*
	-	+	-	-	+/-	*E. faecium*
	-	-	+	+	+/-	*A. baumannii* [c]
	-	-	+	-	+/-	*A. baumannii* group [d]
	-	-	-	-	-	Invalid SIAC must be repeated
	-	-	-	-	+	Negative [e]

[a] Wavelength in nanometres
[b] In the presence of high concentrations of primary target, the SIAC may become inhibited
[c] *A. baumannii sensu stricto*
[d] *A. baumannii sensu stricto* and the species *A. pittii, A. nosocomialis, A. seifertii,* and *A. lactucae*
[e] Not an ESAKPE pathogen detected by ESKAPE multiplex 2

2.3.3 Analytical Sensitivity of the ESKAPE Real-Time PCR Assays

The lower limit of detection (LOD) of each ESKAPE target group was determined using a probit regression analysis (95% confidence). In multiplex 1 the LOD of the *P. aeruginosa* (*rspG*), *S. aureus* (*lepA*), *K. pneumoniae* (*lepA*) and *P. aeruginosa* assays were empirically determined to be 6, 20, and 7 genome equivalents, respectively. In multiplex 2, the *E. faecium*, *A. baumannii*, *A. baumannii* group and *Enterobacter* assay LOD were empirically determined to be 39, 1, 7 and 15 genome equivalents. In both multiplex 1 and multiplex 2, 1 x 10^4 copies of SIAC template were consistently detected at 30-31 C_q, indicating the performance of the SIAC was reliable in the presence of varying primary target concentrations.

2.3.4 Evaluation of Multiplex Performance on Clinical Samples

DNA isolated from a collection of lower respiratory tract specimen was tested blindly, using ESKAPE multiplex 1 & 2 under the reaction conditions described. 61/114 samples (53.5%) were positive for ESKAPE species. From the 114 samples, *S. aureus* (*n*=22, 19.3%) was the most frequently detected, followed by *P. aeruginosa* (*n*=18, 15.8%), *K. pneumoniae* (*n*=15, 13.2%), *E. faecium* (*n*=10, 8.8%), *A. baumannii sensu stricto* (*n*=3, 2.6%) and *Enterobacter spp*. (*n*=2, 1.8%). Of the 61 positive samples, 9 samples were positive for two ESKAPEs (7.9%).

The results obtained via the ESKAPE multiplex PCRs were then compared to those from the originating study which used the FilmArray PNplus [89]. Full agreement between the FilmArray PNplus and the ESKAPE multiplex PCRs developed in this study was achieved for 87.7% of samples (100/114). For the 14 samples with discordant results (**Table 2.6**), some samples contained mixed pathogens. If minor discordance (1 pathogen missed by either PCR method) was considered, concordance is raised to 98.2% (112/114).

Following unblinding, additional confirmatory tests were performed on samples with discordant results. The FilmArray PNplus detected *Enterobacter* in 9 samples which were not detected using the ESKAPE multiplex. Sequence analysis of the *hsp60* gene revealed that 5 of the 9 samples positive for *Enterobacter* using the FilmArray PNplus had >99% sequence identity for other species (*Escherichia coli* (*n*=3), *Klebsiella aerogenes* (*n*=1) and *Serratia liquefaciens* (*n*=1)) (**Table 2.6**). This suggests that the FilmArray PNplus is not 100% specific for *Enterobacter* and the negative result obtained using ESKAPE multiplex 2 was correct. However, the remaining 4 samples that were not detected by the ESKAPE real-time PCRs were confirmed to be *Enterobacter* and were as such were false negative results.

The FilmArray PNplus detected 4 additional *Klebsiella pneumoniae* complex isolates which were not detected by the ESKAPE real-time PCRs. These samples were confirmed to contain *K. pneumoniae* using a real-time PCR confirmatory assay [91].

Although the confirmatory assay does not specifically detect *K. pneumoniae sensu stricto*, this assay was chosen due to the thorough validation using representative KpSC strains described by the authors [91]. Currently, there are no other real-time PCR based methods to detect *K. pneumoniae sensu stricto*, or indeed the KpSC, which have been validated in this manner. For the purpose of this study, we have determined that the four samples which were negative by the ESKAPE real-time PCR for *K. pneumoniae* were false negatives, based on the concordance of the other two methods. Full confirmatory testing to identify *K. pneumoniae sensu stricto* would require metagenomic next-generation sequencing of the sample [94, 95], which was beyond the scope of this study.

Table 2.6: Samples which produced discordant results for the ESKAPE multiplex real-time PCR assays and the FilmArray PNplus

Sample ID	ESKAPE real-time PCR	BioFire FilmArray PNplus [a]	Confirmatory testing	Description of discordance
CS006	*A. baumannii*, *P. aeruginosa*	*K. pneumoniae*, Acb, *P. aeruginosa*	*K. pneumoniae* present [b]	Sample contained high levels of *P. aeruginosa* (C_q <17) and *A. baumannii* (C_q <18). ESKAPE false negative for *K. pneumoniae*
RS006	*Enterobacter*	*K. pneumoniae*, *Enterobacter*	*K. pneumoniae* present [b]	ESKAPE false negative for *K. pneumoniae*
RS022	*E. faecium*	*Enterobacter*	*Enterobacter* not present [c]	BioFire false positive for *Enterobacter*: *hsp60* sequence returned as *Klebsiella aerogenes* HF955027.1 (100%)
RS051	Negative	*Enterobacter*	*Enterobacter* not present [c]	BioFire false positive for *Enterobacter: hsp60* sequence returned as *Escherichia coli* CP082835.1 (100%)
TS009	*S. aureus*	*P. aeruginosa*, *S. aureus*		Mixed sample - contained high levels of *S. aureus* (C_q = 21). ESKAPE false negative for *P. aeruginosa*
US001	*S. aureus*	*Enterobacter*, *S. aureus*	*Enterobacter* not present [c]	BioFire false positive for *Enterobacter: hsp60* sequence returned as *Escherichia coli* CP110996.1 (100%)
US003	*K. pneumoniae*	*Enterobacter*, *K. pneumoniae*	*Enterobacter* present [c]	ESKAPE false negative for *Enterobacter*: *hps60* sequence returned as *Enterobacter hormaechei* CP051132.1 (99.4%)
US013	*S. aureus*	*Enterobacter*, *S. aureus*	*Enterobacter* not present [c]	BioFire false positive for *Enterobacter: hsp60* sequence returned as *Serratia liquefaciens* CP006252.1 (97.1%) [d]

Table continued on next page

Table 2.6 continued: Samples which produced discordant results for the ESKAPE multiplex real-time PCR assays and the FilmArray PNplus

Sample ID	ESKAPE real-time PCR	BioFire FilmArray PNplus [a]	Confirmatory testing	Description of discordance
US021	*S. aureus*	*Enterobacter*, *S. aureus*	*Enterobacter* present [c]	ESKAPE false negative for *Enterobacter*: *hsp60* sequence returned as *Enterobacter ludwigii* MW988544.1 (99.1%)
US042	*S. aureus*	Acb, *Enterobacter*, *S. aureus*	*Enterobacter* present [c]	Sample contained high levels of S. *aureus* (C_q <17). ESKAPE false negative for Acb and *Enterobacter*: *hsp60* sequence returned as *Enterobacter mori* CP084692.1 (97.7%) [e]
US059	*P. aeruginosa*	*Enterobacter*, *K. pneumoniae*, *P. aeruginosa*	*K. pneumoniae* present [b]	Sample contained high levels of *P. aeruginosa* (C_q <16). BioFire false positive for *Enterobacter*: *hsp60* sequence returned as *E. coli* CP118558.1 (100%) ESKAPE false negative for *K. pneumoniae*
US073	*P. aeruginosa*, *S. aureus*	*K. pneumoniae*, *P. aeruginosa*, *S. aureus*	*K. pneumoniae* present [b]	Sample contained high levels of *P. aeruginosa* (C_q <19) and *S. aureus* (C_q = 23). ESKAPE false negative for *K. pneumoniae*.
US096	Negative	*Enterobacter*	*Enterobacter* present [c]	ESKAPE false negative for *Enterobacter*: *hsp60* sequence returned as *Enterobacter hormaechei* CP033102.1 (99.4%)
US097	Negative	*K. pneumoniae*	*K. pneumoniae* present [b]	ESKAPE false negative for *K. pneumoniae*.

[a] Detection of ESKAPE pathogens by the BioFire FilmArray PNplus panel
[b] Using the ZKIR *Klebsiella pneumoniae* species complex assay by Barbier et al. [87]
[c] Using a standard NCBI Nucleotide BLAST of *hsp60* sequence
[d] NCBI Whole Genome Shotgun Contig BLAST of *hsp60* sequence returned the higher value of 99.4% for *Serratia grimesii* JAOXCL010000009.1
[e] NCBI Whole Genome Shotgun Contig BLAST of *hsp60* sequence returned the higher value of 99.7% for *E. mori* QZDQ02000018.1

2.4 Discussion

HAP and VAP are the most frequently reported and burdensome HAI, with the majority caused by the group of bacterial species known as the ESKAPE pathogens. In its national call to action, the US National Organisation to Prevent Hospital Acquired Pneumonia (NOHAP) expressed the need for new diagnostic technologies to "elucidate the roles of specific pathogens" [4]. Our understanding of the microbial aetiology of HAP/VAP is limited by the specificity and sensitivity of culture-based diagnostics. Recent developments in molecular diagnostics have excellent potential but need further validation [37, 96]. Many lack precise species identification for important pathogens, including members of the ESKAPEs.

Several real-time PCR assays to detect ESKAPEs and other pathogens associated with HAP and VAP have been described [56-58]. However, the clinical usefulness of these assays is limited as they lack specificity, sufficient amplification controls, and thorough validation of non-target species. Specific detection of all ESKAPE pathogens using molecular methods has not yet been described. Accurate differentiation of ESKAPE species is important for several reasons. They cause a high burden of disease and DALYs, yet from a diagnostic perspective some ESKAPE pathogens are poorly defined. *K. pneumoniae* and *A. baumannii* form part of wider species complexes in which AMR, disease prognosis, and overall mortality may differ [24, 97-100]. There are few reliable methods to detect *Enterobacter* species which cause HAP and VAP with high mortality [101]. Further studies are required to improve our understanding of the epidemiology of these species in HAI, HAP, and VAP.

This study describes the development of two novel real-time multiplex PCR assays to simultaneously detect and differentiate each member of the ESKAPEs with 100% specificity, and an LOD of 6 – 39 genome equivalents for each target. First, individual monoplex real-time PCR assays were designed and validated using a panel of 181 culture collection isolates. The assays were then grouped into two multiplex reactions on the basis of frequency of ESKAPE pathogen association with HAP/VAP. Multiplex 1 detects *S. aureus*, *K. pneumoniae* and *P. aeruginosa*, which are the most prevalent causes of HAP/VAP (**Table 2.1**). Multiplex 2 detects *E. faecium, A. baumannii,* the *A. baumannii* group, and *Enterobacter*. These species are less frequently reported in HAP/VAP than the species targeted in multiplex 1 but remain important causes of AMR infections which lack accurate diagnostics. This configuration allows the use of multiplex 1 as a standalone, rapid diagnostic for the most prevalent causes of HAP/VAP. Multiplex 2 can be run in parallel or after multiplex 1 to provide comprehensive profiling of ESKAPE pathogens.

Subsequently, 114 clinical specimens were blindly tested to provide further insight into the true rates of ESKAPE pathogens in HAP/VAP, and to demonstrate the clinical utility of the developed assays. Using the diagnostic assays developed, 53.5% (61/114) of samples were positive for at least 1 ESKAPE, in line with other studies [6, 12].
S. aureus was the most frequently detected organism (19.3%), followed by *P. aeruginosa* (15.8%), and *K. pneumoniae* (13.2%). These figures are similar to those of existing etiological studies of HAP/VAP described in **Table 2.1**. *E. faecium* was detected in 8.8% of samples, which was higher than expected relative to current literature for HAP/VAP (**Table 2.1**). However, previously published studies were performed using culture-based diagnostics, or molecular methods which do not include *E. faecium* in their target panel. The true rates of *E. faecium* in HAP/VAP may differ, although its role remains unclear and warrants further investigation. The aspiration of gastrointestinal contents has been suggested as a possible route of entry for *E. faecium* to the lung which may explain the identification of this microorganism in this study. [102, 103]. The multiplex PCR developed in this study is capable of detecting and differentiating *A. baumannii sensu stricto* and the wider *A. baumannii* group (*A. baumannii, A. pittii, A. nosocomialis, A. lactucae,* and *A. seifertii*). While only *A. baumannii* was identified in this current study (2.6%), an assay which has the capacity to also detect other members of the genus is useful to help further investigate the role these microorganisms play in HAP/VAP. A number of studies [97, 99, 104] have implicated *A. pittii, A. nosocomialis, A. seifertii* and *A. lactucae* in human infections and as such should also be tested for. *Enterobacter* formed 1.8% of positive samples in our study. The rates of infection caused by true *Enterobacter* species are largely unknown due to current limitations in differentiating *Enterobacter* from species in closely related genera [31, 34, 105]. This assay is highly specific for *Enterobacter*, based on the targeting of a conserved sequence insertion in the *radA* gene. Due to the lack of accurate diagnostics, it is possible that some cases of HAP/VAP reported in the literature to be caused by *Enterobacter* have been misidentified, thus increasing the estimated rates of occurrence.

We compared our findings to the results obtained using a commercially available molecular diagnostic platform: the BioFire FilmArray PNplus. As part of an earlier study, the same panel of clinical specimen were tested using the FilmArray PNplus, and thus provided a direct comparison to the results obtained using the ESKAPE multiplex assays [89, 106-108]. A high level of concordance (87.7%; 100/114) was found between the ESKAPE multiplex PCRs and the FilmArray PNplus. This concordance is raised to 98.2% if only minor discordance is considered. However, 9 samples which the FilmArray PNplus determined to contain *Enterobacter* were negative using the ESKAPE multiplex. Further sequence analysis of the *hsp60* gene which is used as a gold standard for *Enterobacter* identification was performed to determine which results were correct [90]. This analysis suggested that 5/9 samples did not contain *Enterobacter* and may represent false positives by the FilmArray PNplus.

Four samples were determined to contain *Enterobacter* and as such were false negative results with the ESKAPE multiplex developed in this study. In these four samples (**Table 2.6**), three contained mixed samples with high concentrations of other ESKAPE pathogens and as such, the levels of target DNA may have inhibited the PCR reaction. There were 7 additional samples (5 *K. pneumoniae,* 1 *P. aeruginosa and* 1 *A. baumannii*) which were detected by the FilmArray PNplus which were not detected by the ESKAPE multiplex PCR assays. As highlighted in **Table 2.6**, the majority of these samples also were mixed samples containing other ESKAPE pathogens at high concentrations. This was likely overcome in the FilmArray PNplus owing to the nested PCR approach the platform uses, and as such we hypothesise that the sensitivity of the FilmArray PNplus platform was superior for the detection of pathogens present at low concentrations in a mixed sample which are otherwise inhibited. In addition to the presence of high concentrations of target DNA in a mixed sample, the duration between sample collection and testing using the FilmArray PNplus (6 months) was substantially shorter than the 48 months of storage before testing using the ESKAPE multiplex PCRs. Sample degradation during this time may have impacted the ability of the ESKAPE multiplex PCR to detect pathogens present in low abundance.

This study has demonstrated the applicability of the ESKAPE multiplex real-time PCR assays for use on culture isolates and complex clinical samples. In settings where ESKAPE pathogens are considered a likely cause of infection, the real-time PCR assays described in this study offer rapid and reproducible alternatives to culture based diagnostics. The assays are limited to their current format of a 4-plex and 5-plex real-time PCR using hydrolysis probes. The ability to combine them into a single multiplex, or to add additional targets is limited by fluorescent detection capabilities of the real-time PCR instrument used. Future work may investigate the optimisation of these assays for use with real-time PCR instruments which offer higher multiplexing levels. Alternatively, the use of nested real-time PCR such as the format used in the FilmArray® could be investigated to increase the sensitivity of the assays for mixed pathogen samples. This, however, is limited by the availability of devices which can perform microfluidic nested-PCR, and which can be customised with lab-developed assays.

We describe the development of two novel real-time PCR assays capable of detecting and differentiating between each of the ESKAPE pathogens, which cause the majority of HAI. These are the first described multiplex diagnostics assays which can simultaneously detect and differentiate between all ESKAPEs. These diagnostic assays have been validated against a large panel of respiratory pathogens and have been determined to be 100% specific with sensitivities of below 40 cell equivalents for all assays tested for. Furthermore, these diagnostics assays have been used directly on clinical specimens. The method developed has been demonstrated to be highly effective for the rapid detection of ESKAPE pathogens in clinical specimens from patients with suspected HAP/VAP, displaying 87.8% concordance with an FDA approved, CE-IVD marked molecular diagnostic, the BioFire Film Array PNplus. These multiplex assays should now be deployed in further studies as they will complement new and existing testing technologies to elucidate the role of ESKAPE pathogens in infection and human health.

2.5 Supplementary Material

Table S2.1: NCBI accession numbers of the sequences used to design and validate each novel real-time PCR assay *in silico*

Sequences listed in **bold font** are detected using that assay based on *in silico* analysis

Sequences used to perform *in silico* analysis for *E. faecium* assay targeting *lepA*		
NBCI Accession Number	**Species**	**URL**
CP034169.1	*Enterococcus avium*	https://www.ncbi.nlm.nih.gov/nuccore/CP034169.1
CP024590.1	*E. avium*	https://www.ncbi.nlm.nih.gov/nuccore/CP024590.1
AP019814.1	*E. avium*	https://www.ncbi.nlm.nih.gov/nuccore/AP019814.1
DYYB01000006.1	*Enterococcus casseliflavus*	https://www.ncbi.nlm.nih.gov/nuccore/DYYB01000006.1
JXKK01000007.1	*E. casseliflavus* DSM20680	https://www.ncbi.nlm.nih.gov/nuccore/JXKK01000007.1
UGJD01000001.1	*E. casseliflavus* NCTC12361	https://www.ncbi.nlm.nih.gov/nuccore/UGJD01000001.1
CP065535.1	*Enterococcus durans* KCTC13289	https://www.ncbi.nlm.nih.gov/nuccore/CP065535.1
CP012384.1	*E. durans*	https://www.ncbi.nlm.nih.gov/nuccore/CP012384.1
CP116579.1	*E. durans*	https://www.ncbi.nlm.nih.gov/nuccore/CP116579.1
CP088203.1	*E. durans*	https://www.ncbi.nlm.nih.gov/nuccore/CP088203.1
LR962775.1	*Enterococcus faecalis*	https://www.ncbi.nlm.nih.gov/nuccore/LR962775.1
CP100596.1	*E. faecalis*	https://www.ncbi.nlm.nih.gov/nuccore/CP100596.1
LR962843.1	*E. faecalis*	https://www.ncbi.nlm.nih.gov/nuccore/LR962843.1
CP110054.1	*E. faecalis*	https://www.ncbi.nlm.nih.gov/nuccore/CP110054.1
CP110074.1	*E. faecalis*	https://www.ncbi.nlm.nih.gov/nuccore/CP110074.1
CP118962.1	*E. faecalis* DSM20478	https://www.ncbi.nlm.nih.gov/nuccore/CP118962.1
CP082231.1	*E. faecalis*	https://www.ncbi.nlm.nih.gov/nuccore/CP082231.1
LR594051.1	*E. faecalis* NCTC8732	https://www.ncbi.nlm.nih.gov/nuccore/LR594051.1
AP017623.1	*E. faecalis*	https://www.ncbi.nlm.nih.gov/nuccore/AP017623.1
OD940440.2	*E. faecalis*	https://www.ncbi.nlm.nih.gov/nuccore/OD940440.2
CP063549.1	***Enterococcus faecium***	https://www.ncbi.nlm.nih.gov/nuccore/CP063549.1
CP046077.1	***E. faecium***	https://www.ncbi.nlm.nih.gov/nuccore/CP046077.1
CP066473.1	***E. faecium***	https://www.ncbi.nlm.nih.gov/nuccore/CP066473.1
LN999844.1	***E. faecium***	https://www.ncbi.nlm.nih.gov/nuccore/LN999844.1
CP038996.1	***E. faecium.***	https://www.ncbi.nlm.nih.gov/nuccore/CP038996.1
CP091221.1	***E. faecium***	https://www.ncbi.nlm.nih.gov/nuccore/CP091221.1
AP026628.1	***E. faecium***	https://www.ncbi.nlm.nih.gov/nuccore/AP026628.1
CP050255.1	***E. faecium***	https://www.ncbi.nlm.nih.gov/nuccore/CP050255.1
CP101669.1	***E. faecium***	https://www.ncbi.nlm.nih.gov/nuccore/CP101669.1
CP102534.1	***E. faecium***	https://www.ncbi.nlm.nih.gov/nuccore/CP102534.1
CP042834.1	***E. faecium***	https://www.ncbi.nlm.nih.gov/nuccore/CP042834.1
CP053704.1	***E. faecium***	https://www.ncbi.nlm.nih.gov/nuccore/CP053704.1
CP032308.1	***E. faecium***	https://www.ncbi.nlm.nih.gov/nuccore/CP032308.1
CP033041.1	***E. faecium***	https://www.ncbi.nlm.nih.gov/nuccore/CP033041.1

AP022341.1	***E. faecium***	https://www.ncbi.nlm.nih.gov/nuccore/AP022341.1
CP116520.1	***E. faecium***	https://www.ncbi.nlm.nih.gov/nuccore/CP116520.1
CP006030.1	***E. faecium***	https://www.ncbi.nlm.nih.gov/nuccore/CP006030.1
CP040368.1	***E. faecium***	https://www.ncbi.nlm.nih.gov/nuccore/CP040368.1
CP050485.1	*Enterococcus gallinarum*	https://www.ncbi.nlm.nih.gov/nuccore/CP050485.1
CP078505.1	*E. gallinarum*	https://www.ncbi.nlm.nih.gov/nuccore/CP078505.1
CP014067.2	*E. gallinarum*	https://www.ncbi.nlm.nih.gov/nuccore/CP014067.2
CP030932.1	*Enterococcus gilvus*	https://www.ncbi.nlm.nih.gov/nuccore/CP030932.1
ASWH01000001.1	*E. gilvus* ATCC BAA-350	https://www.ncbi.nlm.nih.gov/nuccore/ASWH01000001.1
JXKO01000005.1	*E. gilvus* DSM15689	https://www.ncbi.nlm.nih.gov/nuccore/JXKO01000005.1
AISM01000009.1	*Enterococcus hirae*	https://www.ncbi.nlm.nih.gov/nuccore/AISM01000009.1
VOEC01000001.1	*E. hirae*	https://www.ncbi.nlm.nih.gov/nuccore/VOEC01000001.1
CP003504.1	*E. hirae* ATCC9790	https://www.ncbi.nlm.nih.gov/nuccore/CP003504.1
LR134297.1	*E. hirae* NCTC12368	https://www.ncbi.nlm.nih.gov/nuccore/LR134297.1
JXKU01000023.1	*Enterococcus malodoratus* DSM20681	https://www.ncbi.nlm.nih.gov/nuccore/JXKU01000023.1
UFXU01000001.1	*E. malodoratus* NCTC12365	https://www.ncbi.nlm.nih.gov/nuccore/UFXU01000001.1
CP104764.1	*Enterococcus raffinosus*	https://www.ncbi.nlm.nih.gov/nuccore/CP104764.1
CP104762.1	*E. raffinosus* ATCC49464	https://www.ncbi.nlm.nih.gov/nuccore/CP104762.1
AP022822.1	*Enterococcus saigonensis*	https://www.ncbi.nlm.nih.gov/nuccore/AP022822.1
AP025690.1	*Enterococcus* sp PLM3	https://www.ncbi.nlm.nih.gov/nuccore/AP025690.1
Sequences used to perform *in silico* analysis for *S. aureus* assay targeting *lepA*		
NCBI Accession Number	**Species**	**URL**
NZ_LEOS01000001.1	*Mammaliicoccus sciuri* DSM20345	https://www.ncbi.nlm.nih.gov/nuccore/NZ_LEOS01000001.1
AP018562.1	*Staphylococcus argenteus*	https://www.ncbi.nlm.nih.gov/nuccore/AP018562.1
CP015758.1	*S. argenteus*	https://www.ncbi.nlm.nih.gov/nuccore/CP015758.1
CP012119.2	***Staphylococcus aureus***	https://www.ncbi.nlm.nih.gov/nuccore/CP012119.2
CP018629.1	***S. aureus* MRSA107**	https://www.ncbi.nlm.nih.gov/nuccore/CP018629.1
LS483324.1	***S. aureus* NCTC10344**	https://www.ncbi.nlm.nih.gov/nuccore/LS483324.1
LS483308.1	***S. aureus* NCTC13137**	https://www.ncbi.nlm.nih.gov/nuccore/LS483308.1

LS483319.1	***S. aureus* NCTC13140**	https://www.ncbi.nlm.nih.gov/nuccore/LS483319.1
LS483301.1	***S. aureus* NCTC13394**	https://www.ncbi.nlm.nih.gov/nuccore/LS483301.1
LS483316.1	***S. aureus* NCTC13395**	https://www.ncbi.nlm.nih.gov/nuccore/LS483316.1
LS483314.1	***S. aureus* NCTC3761**	https://www.ncbi.nlm.nih.gov/nuccore/LS483314.1
NC_007795.1	***S. aureus* NCTC8325**	https://www.ncbi.nlm.nih.gov/nuccore/NC_007795.1
LS483310.1	***S. aureus* NCTC9752**	https://www.ncbi.nlm.nih.gov/nuccore/LS483310.1
CP032051.1	***S. aureus***	https://www.ncbi.nlm.nih.gov/nuccore/CP032051.1
CP014426.1	***S. aureus* USA300-*SUR18***	https://www.ncbi.nlm.nih.gov/nuccore/CP014426.1
NZ_CP007601.1	*Staphylococcus capitis*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP007601.1
AP018587.1	*Staphylococcus caprae* JMUB898	https://www.ncbi.nlm.nih.gov/nuccore/AP018587.1
NZ_LATV01000007.1	*Staphylococcus cohnii*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_LATV01000007.1
NZ_CP015114.1	*Staphylococcus condiment* DSM11674	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP015114.1
CP020463.1	*Staphylococcus epidermidis* 1457	https://www.ncbi.nlm.nih.gov/nuccore/CP020463.1
AE015929.1	*S. epidermidis* ATCC12228	https://www.ncbi.nlm.nih.gov/nuccore/AE015929.1
NC_004461.1	*S. epidermidis* ATCC12228	https://www.ncbi.nlm.nih.gov/nuccore/NC_004461.1
CP030246.1	*S. epidermidis* CSF41498	https://www.ncbi.nlm.nih.gov/nuccore/CP030246.1
CP014132.1	*S. epidermidis*	https://www.ncbi.nlm.nih.gov/nuccore/CP014132.1
CP028282.1	*S. epidermidis*	https://www.ncbi.nlm.nih.gov/nuccore/CP028282.1
NZ_CP013114.1	*Staphylococcus equorum*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP013114.1
CP025031.1	*Staphylococcus haemolyticus*	https://www.ncbi.nlm.nih.gov/nuccore/CP025031.1
CP025396.1	*S. haemolyticus*	https://www.ncbi.nlm.nih.gov/nuccore/CP025396.1
LT963441.1	*S. haemolyticus*	https://www.ncbi.nlm.nih.gov/nuccore/LT963441.1
NC_007168.1	*S. haemolyticus* JCSC1435	https://www.ncbi.nlm.nih.gov/nuccore/NC_007168.1
NZ_GL545260.1	*Staphylococcus hominis*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_GL545260.1
CP027846.1	*Staphylococcus kloosii* ATCC43959	https://www.ncbi.nlm.nih.gov/nuccore/CP027846.1
NC_013893.1	*Staphylococcus lugdunensis*	https://www.ncbi.nlm.nih.gov/nuccore/NC_013893.1
CP017463.1	*Staphylococcus pasteuri*	https://www.ncbi.nlm.nih.gov/nuccore/CP017463.1

NC_014925.1	*Staphylococcus pseudintermedius* HKU10-03	https://www.ncbi.nlm.nih.gov/nuccore/NC_014925.1
NC_007350.1	*Staphylococcus saprophyticus* ATCC15305	https://www.ncbi.nlm.nih.gov/nuccore/NC_007350.1
NZ_CP022046.2	*Staphylococcus sciuri*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP022046.2
LT906460.1	*Staphylococcus simiae* NCTC13838	https://www.ncbi.nlm.nih.gov/nuccore/LT906460.1
NZ_CP014016.2	*Staphylococcus simulans*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP014016.2
NC_020164.1	*Staphylococcus warneri* SG1	https://www.ncbi.nlm.nih.gov/nuccore/NC_020164.1
CP003668.1	*S. warneri* SG1	https://www.ncbi.nlm.nih.gov/nuccore/CP003668.1
NZ_CP007208.1	*S. xylosus*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP007208.1
CP022881.1	*Staphylococcus* sp M0911	https://www.ncbi.nlm.nih.gov/nuccore/CP022881.1
Sequences used to perform *in silico* analysis for *K. pneumoniae* assay targeting *lepA*		
NCBI Accession Number	**Species**	**URL**
CP020448.2	*Citrobacter braakii*	https://www.ncbi.nlm.nih.gov/nuccore/CP020448.2
CP024819.1	*Citrobacter freundii*	https://www.ncbi.nlm.nih.gov/nuccore/CP024819.1
CP018810.1	*C. freundii*	https://www.ncbi.nlm.nih.gov/nuccore/CP018810.1
CP012266.1	*Cronobacter dublinensis*	https://www.ncbi.nlm.nih.gov/nuccore/CP012266.1
CP003312.1	*Cronobacter sakazakii*	https://www.ncbi.nlm.nih.gov/nuccore/CP003312.1
CP012257.1	*Cronobacter universalis*	https://www.ncbi.nlm.nih.gov/nuccore/CP012257.1
CP046618.1	*Enterobacter asburiae*	https://www.ncbi.nlm.nih.gov/nuccore/CP046618.1
CP056474.1	*Enterobacter cloacae*	https://www.ncbi.nlm.nih.gov/nuccore/CP056474.1
AP019007.1	*Enterobacter oligotrophicus*	https://www.ncbi.nlm.nih.gov/nuccore/AP019007.1
CP058253.1	*Enterobacter roggenkampii*	https://www.ncbi.nlm.nih.gov/nuccore/CP058253.1
CP002824.1	*Klebsiella aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP002824.1
CP024880.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP024880.1
CP028951.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP028951.1
CP023963.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP023963.1
CP011574.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP011574.1
CP026722.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP026722.1
CP033817.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP033817.1
CP026756.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP026756.1
CP031756.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP031756.1

CP014029.2	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP014029.2
CP014748.2	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP014748.2
CP024885.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP024885.1
CP011539.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP011539.1
FO203355.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/FO203355.1
LR134475.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/LR134475.1
CP024883.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP024883.1
LR134122.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/LR134122.1
NC015663.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/NC015663.1
NZ_CP059391.1	*Klebsiella africana*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP059391.1
NZ_CP084874.1	*K. africana*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP084874.1
NZCP044527.1	*Klebsiella grimontii*	https://www.ncbi.nlm.nih.gov/nuccore/NZCP044527.1
CP067433.1	*K. grimontii*	https://www.ncbi.nlm.nih.gov/nuccore/CP067433.1
NZ_LR607336.1	*K. grimontii*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_LR607336.1
CP023185.1	*Klebsiella michiganensis*	https://www.ncbi.nlm.nih.gov/nuccore/CP023185.1
CP004887.1	*K. michiganensis*	https://www.ncbi.nlm.nih.gov/nuccore/CP004887.1
CP003218.1	*K. michiganensis*	https://www.ncbi.nlm.nih.gov/nuccore/CP003218.1
NZAP022547.1	*K. michiganensis*	https://www.ncbi.nlm.nih.gov/nuccore/NZAP022547.1
CP073236.1	*K. michiganensis*	https://www.ncbi.nlm.nih.gov/nuccore/CP073236.1
CP027426.1	*Klebsiella oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP027426.1
CP020358.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP020358.1
CP026285.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP026285.1
CP011636.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP011636.1
JAIFQY010000013.1	*Klebsiella pasteurii*	https://www.ncbi.nlm.nih.gov/nuccore/JAIFQY010000013.1
CP089403.1	*K. pasteurii*	https://www.ncbi.nlm.nih.gov/nuccore/CP089403.1
NZUGLV01000001.1	***Klebsiella pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/NZUGLV01000001.1
ACZD01000034.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/ACZD01000034.1
CP026160.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP026160.1
CP026178.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP026178.1
CP038002.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP038002.1
CP036300.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP036300.1
LR133964.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/LR133964.1
CP028816.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP028816.1
CP015130.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP015130.1
CP031817.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP031817.1
CP021696.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP021696.1
CP014008.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP014008.1
CP036190.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP036190.1
CP034082.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP034082.1
CP035383.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP035383.1
CP030269.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP030269.1
CP027189.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP027189.1
CP025080.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP025080.1

CP025639.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP025639.1
CP025631.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP025631.1
CP025090.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP025090.1
CP019047.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP019047.1
CP016813.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP016813.1
CP014010.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP014010.1
CP003785.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP003785.1
AP006725.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/AP006725.1
CP026021.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP026021.1
CP021955.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP021955.1
CP025211.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP025211.1
CP016159.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP016159.1
CP028806.1	***K. pneumoniae***	https://www.ncbi.nlm.nih.gov/nuccore/CP028806.1
CP023478.1	*Klebsiella quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP023478.1
CP014156.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP014156.1
CP014155.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP014155.1
CP014154.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP014154.1
CP012300.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP012300.1
CP012252.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP012252.1
CP031257.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP031257.1
CP029597.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP029597.1
CP014071.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP014071.1
CP014696.2	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP014696.2
CP034678.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP034678.1
AP019687.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/AP019687.1
CP039791.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP039791.1
AP022142.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/AP022142.1
CP084803.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP084803.1
CP030171.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP030171.1
CP029437.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP029437.1
CP029443.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP029443.1
CP029432.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP029432.1
UJYW01000004.1	*Klebsiella quasivariicola*	https://www.ncbi.nlm.nih.gov/nuccore/UJYW01000004.1

VLPG01000016.1	*K. quasivariicola*	https://www.ncbi.nlm.nih.gov/nuccore/VLPG01000016.1
WHZK01000014.1	*K. quasivariicola*	https://www.ncbi.nlm.nih.gov/nuccore/WHZK01000014.1
UJYZ02000002.1	*K. quasivariicola*	https://www.ncbi.nlm.nih.gov/nuccore/UJYZ02000002.1
UJZG01000020.1	*K. quasivariicola*	https://www.ncbi.nlm.nih.gov/nuccore/UJZG01000020.1
CP022823.1	*K. quasivariicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP022823.1
LR134235.1	*Klebsiella variicola*	https://www.ncbi.nlm.nih.gov/nuccore/LR134235.1
LR130538.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/LR130538.1
CP008700.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP008700.1
CP018307.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP018307.1
CP000964.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP000964.1
CP030173.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP030173.1
CP010523.2	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP010523.2
CP001891.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP001891.1
CP013985.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP013985.1
LR130543.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/LR130543.1
LR130544.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/LR130544.1
LR130539.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/LR130539.1
CP016344.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP016344.1
CP028555.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP028555.1
CP009274.2	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP009274.2
CP020847.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP020847.1
CP017289.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP017289.1
CP017849.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP017849.1
CP017284.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP017284.1
CP032354.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP032354.1
CP027064.2	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP027064.2
CP033631.1	*Klebsiella* sp P1CD1	https://www.ncbi.nlm.nih.gov/nuccore/CP033631.1
CP037441.1	*Klebsiella* sp PO552	https://www.ncbi.nlm.nih.gov/nuccore/CP037441.1
AP022665.1	*Kluyvera ascorbata*	https://www.ncbi.nlm.nih.gov/nuccore/AP022665.1
CP045843.1	*Kluyvera intermedia*	https://www.ncbi.nlm.nih.gov/nuccore/CP045843.1
CP022114.1	*Kluyvera* genomosp.3	https://www.ncbi.nlm.nih.gov/nuccore/CP022114.1
LR699009.1	*Pantoea gergoviae*	https://www.ncbi.nlm.nih.gov/nuccore/LR699009.1
CP009450.1	*P. gergoviae*	https://www.ncbi.nlm.nih.gov/nuccore/CP009450.1
CP002272.1	*Pluralibacter lignolyticus*	https://www.ncbi.nlm.nih.gov/nuccore/CP002272.1
CP079752.1	*Raoultella ornithinolytica*	https://www.ncbi.nlm.nih.gov/nuccore/CP079752.1
CP008886.1	*R. ornithinolytica*	https://www.ncbi.nlm.nih.gov/nuccore/CP008886.1
CP038281.1	*R. ornithinolytica*	https://www.ncbi.nlm.nih.gov/nuccore/CP038281.1
CP004142.1	*R. ornithinolytica*	https://www.ncbi.nlm.nih.gov/nuccore/CP004142.1
CP012555.1	*R. ornithinolytica*	https://www.ncbi.nlm.nih.gov/nuccore/CP012555.1
LR134195.1	*R. ornithinolytica*	https://www.ncbi.nlm.nih.gov/nuccore/LR134195.1

CP054471.1	*R. ornithinolytica*	https://www.ncbi.nlm.nih.gov/nuccore/CP054471.1
CP029752.1	*Raoultella planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CP029752.1
CP044121.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CP044121.1
CP040183.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CP040183.1
CP019899.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CP019899.1
CP082168.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CP082168.1
CP069815.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CP069815.1
CABDVR010000001.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CABDVR010000001.1
SUPN01000010.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/SUPN01000010.1
DADZWO010000013.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/DADZWO010000013.1
PDLC01000002.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/PDLC01000002.1
CP023877.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CP023877.1
CP023874.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CP023874.1
MPYJ04000036.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/MPYJ04000036.1
NZ_JMPP01000022.1	*R. planticola* CIP100751	https://www.ncbi.nlm.nih.gov/nuccore/NZ_JMPP01000022.1
CP016032.1	*Serratia marcescens*	https://www.ncbi.nlm.nih.gov/nuccore/CP016032.1
MH460883.1	*S. marcescens*	https://www.ncbi.nlm.nih.gov/nuccore/MH460883.1
CP038662.1	*Serratia nematodiphila*	https://www.ncbi.nlm.nih.gov/nuccore/CP038662.1
Sequences used to perform *in silico* analysis for *A. baumannii sensu stricto* assay targeting *smpB*		
NCBI Accession Number	**Species**	**URL**
CP001921.1	***Acinetobacter baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/CP001921.1
NZ_AEOZ01000060.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/NZ_AEOZ01000060.1
NZ_AEOY01000060.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/NZ_AEOY01000060.1
NZ_ADGZ01000204.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/NZ_ADGZ01000204.1
NZ_ADHA01000098.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/NZ_ADHA01000098.1
NZ_AEOX01000002.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/NZ_AEOX01000002.1
NZ_AHAI01000029.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/NZ_AHAI01000029.1
CP000521.1	***A. baumannii* ATCC17978**	https://www.ncbi.nlm.nih.gov/nuccore/CP000521.1
CP001937.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/CP001937.1
CP002522.2	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/CP002522.2
NZ_AIEI01000038.1	*Acinetobacter bereziniae* LMG1003	https://www.ncbi.nlm.nih.gov/nuccore/NZ_AIEI01000038.1

CP018259.1	*A. bereziniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP018259.1
BBTI01000002.1	*Acinetobacter brisouii* DSM18516	https://www.ncbi.nlm.nih.gov/nuccore/BBTI01000002.1
NZ_CP020000.1	*Acinetobacter calcoaceticus*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP020000.1
AIEC01000263.1	*A. calcoaceticus* DSM30006	https://www.ncbi.nlm.nih.gov/nuccore/AIEC01000263.1
BBTM01000009.1	*A. calcoaceticus* KCTC2357	https://www.ncbi.nlm.nih.gov/nuccore/BBTM01000009.1
GG704949.1	*A. calcoaceticus* RUH2202	https://www.ncbi.nlm.nih.gov/nuccore/GG704949.1
AMIW01000022.1	*A. calcoaceticus*	https://www.ncbi.nlm.nih.gov/nuccore/AMIW01000022.1
AMIX01000007.1	*A. calcoaceticus*	https://www.ncbi.nlm.nih.gov/nuccore/AMIX01000007.1
AMIY01000022.1	*A. calcoaceticus*	https://www.ncbi.nlm.nih.gov/nuccore/AMIY01000022.1
CP029397.1	*Acinetobacter defluvii*	https://www.ncbi.nlm.nih.gov/nuccore/CP029397.1
AP014630.1	*Acinetobacter guillouiae* NBRC110550	https://www.ncbi.nlm.nih.gov/nuccore/AP014630.1
CP032135.1	*Acinetobacter haemolyticus*	https://www.ncbi.nlm.nih.gov/nuccore/CP032135.1
ADMT01000116.1	*A. haemolyticus* ATCC19194	https://www.ncbi.nlm.nih.gov/nuccore/ADMT01000116.1
CP018260.1	*A. haemolyticus*	https://www.ncbi.nlm.nih.gov/nuccore/CP018260.1
CP024620.2	*Acinetobacter indicus*	https://www.ncbi.nlm.nih.gov/nuccore/CP024620.2
CP022298.1	*Acinetobacter johnsonii*	https://www.ncbi.nlm.nih.gov/nuccore/CP022298.1
CP031011.1	*A. johnsonii*	https://www.ncbi.nlm.nih.gov/nuccore/CP031011.1
GG704965.1	*A. johnsonii*	https://www.ncbi.nlm.nih.gov/nuccore/GG704965.1
CP010350.1	*A. johnsonii*	https://www.ncbi.nlm.nih.gov/nuccore/CP010350.1
CP024632.1	*Acinetobacter junii*	https://www.ncbi.nlm.nih.gov/nuccore/CP024632.1
CP028800.1	*A. junii*	https://www.ncbi.nlm.nih.gov/nuccore/CP028800.1
GG705011.1	*A. junii*	https://www.ncbi.nlm.nih.gov/nuccore/GG705011.1
KB976991.1	*Acinetobacter lactucae* ANC4052	https://www.ncbi.nlm.nih.gov/nuccore/KB976991.1
LJPG01000061.1	*A. lactucae*	https://www.ncbi.nlm.nih.gov/nuccore/LJPG01000061.1
CP020015.1	*A. lactucae*	https://www.ncbi.nlm.nih.gov/nuccore/CP020015.1
GG705061.1	*Acinetobacter lwoffii*	https://www.ncbi.nlm.nih.gov/nuccore/GG705061.1
NZ_AIEL01000030.1	*A. lwoffii* NCTC5866	https://www.ncbi.nlm.nih.gov/nuccore/NZ_AIEL01000030.1
ACPN01000095.1	*A. lwoffii*	https://www.ncbi.nlm.nih.gov/nuccore/ACPN01000095.1
AFQY01000005.1	*A. lwoffii*	https://www.ncbi.nlm.nih.gov/nuccore/AFQY01000005.1

NZ_CP014019.1	*Acinetobacter nosocomialis*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP014019.1
NZ_CP010368.1	*A. nosocomialis*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP010368.1
NGIH01000031.1	*A. nosocomialis*	https://www.ncbi.nlm.nih.gov/nuccore/NGIH01000031.1
NZ_CP020588.1	*A. nosocomialis*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP020588.1
DJJM01000028.1	*A. nosocomialis*	https://www.ncbi.nlm.nih.gov/nuccore/DJJM01000028.1
CP002080.1	*Acinetobacter oleivorans*	https://www.ncbi.nlm.nih.gov/nuccore/CP002080.1
NZ_CP015145.1	*Acinetobacter pittii*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP015145.1
NZ_CP026089.1	*A. pittii*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP026089.1
NZ_CP027254.1	*A. pittii*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP027254.1
NZ_CP017938.1	*A. pittii*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP017938.1
NZ_AGFH01000026.1	*A. pittii*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_AGFH01000026.1
NZ_CP021428.1	*A. pittii*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP021428.1
NC_016603.1	*A. pittii*	https://www.ncbi.nlm.nih.gov/nuccore/NC_016603.1
NZ_CP018909.1	*A. pittii*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP018909.1
BAGY01000014.1	*Acinetobacter radioresistens* DSM6976	https://www.ncbi.nlm.nih.gov/nuccore/BAGY01000014.1
GG705131.1	*A. radioresistens*	https://www.ncbi.nlm.nih.gov/nuccore/GG705131.1
NZ_ACVR01000020.1	*A. radioresistens*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_ACVR01000020.1
CP015615.1	*Acinetobacter schindleri*	https://www.ncbi.nlm.nih.gov/nuccore/CP015615.1
CP025618.2	*A. schindleri*	https://www.ncbi.nlm.nih.gov/nuccore/CP025618.2
PGPD01000019.1	*Acinetobacter seifertii*	https://www.ncbi.nlm.nih.gov/nuccore/PGPD01000019.1
PHFF01000094.1	*A. seifertii*	https://www.ncbi.nlm.nih.gov/nuccore/PHFF01000094.1
PHFG01000191.1	*A. seifertii*	https://www.ncbi.nlm.nih.gov/nuccore/PHFG01000191.1
APCT01000020.1	*A. seifertii*	https://www.ncbi.nlm.nih.gov/nuccore/APCT01000020.1
LYQI01000076.1	*A. seifertii*	https://www.ncbi.nlm.nih.gov/nuccore/LYQI01000076.1
QAYP01000094.1	*A. seifertii*	https://www.ncbi.nlm.nih.gov/nuccore/QAYP01000094.1
APOO01000022.1	*A. seifertii* NIPH973	https://www.ncbi.nlm.nih.gov/nuccore/APOO01000022.1
CP016896.1	*Acinetobacter soli*	https://www.ncbi.nlm.nih.gov/nuccore/CP016896.1
AP018824.1	*Acinetobacter ursingii*	https://www.ncbi.nlm.nih.gov/nuccore/AP018824.1

DGJA01000142.1	*A. ursingii*	https://www.ncbi.nlm.nih.gov/nuccore/DGJA01000142.1
NZ_AIEA01000015.1	*A. ursingii* DSM16037	https://www.ncbi.nlm.nih.gov/nuccore/NZ_AIEA01000015.1
DJJL01000012.1	*A. ursingii*	https://www.ncbi.nlm.nih.gov/nuccore/DJJL01000012.1
ABYN01000160.1	*Acinetobacter* sp. ATCC27244	https://www.ncbi.nlm.nih.gov/nuccore/ABYN01000160.1
BAEB01000021.1	*Acinetobacter* sp. NBRC100985	https://www.ncbi.nlm.nih.gov/nuccore/BAEB01000021.1
Sequences used to perform *in silico* analysis for *A. baumannii* group assay targeting *rpoB*		
NCBI Accession Number	**Species**	**URL**
KJ956450.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956450.1
EU477108.2	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/EU477108.2
KJ956452.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956452.1
HQ123415.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123415.1
KJ956448.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956448.1
HQ123413.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123413.1
KJ956451.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956451.1
HQ123414.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123414.1
KJ956455.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956455.1
KJ956449.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956449.1
KU167844.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167844.1
KJ956460.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956460.1
HQ123412.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123412.1
HQ123411.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123411.1
KU167837.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167837.1
KU167840.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167840.1
KU167842.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167842.1
HQ123410.2	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123410.2
KJ956447.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956447.1
KJ956454.1	***A. baumannii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956454.1
KC510989.1	*A. brisouii* DSM18516	https://www.ncbi.nlm.nih.gov/nuccore/KC510989.1
KJ956437.1	*A. calcoeceticus* ANC4104	https://www.ncbi.nlm.nih.gov/nuccore/KJ956437.1
HQ123423.1	*A. calcoeceticus* LUH12679	https://www.ncbi.nlm.nih.gov/nuccore/HQ123423.1
HQ123424.1	*A. calcoeceticus* ANC3680	https://www.ncbi.nlm.nih.gov/nuccore/HQ123424.1
HQ123422.1	*A. calcoeceticus* LUH3785	https://www.ncbi.nlm.nih.gov/nuccore/HQ123422.1
HQ123418.1	*A. calcoeceticus*	https://www.ncbi.nlm.nih.gov/nuccore/HQ123418.1
HQ123421.1	*A. calcoeceticus*	https://www.ncbi.nlm.nih.gov/nuccore/HQ123421.1
EU477149.2	*A. calcoeceticus*	https://www.ncbi.nlm.nih.gov/nuccore/EU477149.2
KY435935.1	*A. defluvii*	https://www.ncbi.nlm.nih.gov/nuccore/KY435935.1
KU961613.1	*A. haemolyticus*	https://www.ncbi.nlm.nih.gov/nuccore/KU961613.1
KU961612.1	*A. haemolyticus*	https://www.ncbi.nlm.nih.gov/nuccore/KU961612.1
CP032135.1	*A. haemolyticus*	https://www.ncbi.nlm.nih.gov/nuccore/CP032135.1
CP018260.1	*A. haemolyticus*	https://www.ncbi.nlm.nih.gov/nuccore/CP018260.1

KU961619.1	*A. junii*	https://www.ncbi.nlm.nih.gov/nuccore/KU961619.1
CP019041.1	*A. junii*	https://www.ncbi.nlm.nih.gov/nuccore/CP019041.1
CP028800.1	*A. junii*	https://www.ncbi.nlm.nih.gov/nuccore/CP028800.1
LRPE01000023.1	***A. lactucae***	https://www.ncbi.nlm.nih.gov/nuccore/LRPE01000023.1
RFEL01000039.1	***A. lactucae***	https://www.ncbi.nlm.nih.gov/nuccore/RFEL01000039.1
RFES01000006.1	***A. lactucae***	https://www.ncbi.nlm.nih.gov/nuccore/RFES01000006.1
KJ600793.1	***A. lactucae***	https://www.ncbi.nlm.nih.gov/nuccore/KJ600793.1
KU167885.1	***A. lactucae***	https://www.ncbi.nlm.nih.gov/nuccore/KU167885.1
KU167882.1	***A. lactucae***	https://www.ncbi.nlm.nih.gov/nuccore/KU167882.1
KJ600787.1	***A. lactucae***	https://www.ncbi.nlm.nih.gov/nuccore/KJ600787.1
KJ600788.1	***A. lactucae***	https://www.ncbi.nlm.nih.gov/nuccore/KJ600788.1
KU167880.1	***A. lactucae***	https://www.ncbi.nlm.nih.gov/nuccore/KU167880.1
KJ600790.1	***A. lactucae***	https://www.ncbi.nlm.nih.gov/nuccore/KJ600790.1
KJ600791.1	***A. lactucae***	https://www.ncbi.nlm.nih.gov/nuccore/KJ600791.1
MG564152.1	*A. lwofii*	https://www.ncbi.nlm.nih.gov/nuccore/MG564152.1
MG564146.1	*A. lwofii*	https://www.ncbi.nlm.nih.gov/nuccore/MG564146.1
MG564158.1	*A. lwofii*	https://www.ncbi.nlm.nih.gov/nuccore/MG564158.1
MG564142.1	*A. lwofii*	https://www.ncbi.nlm.nih.gov/nuccore/MG564142.1
KJ956466.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956466.1
KU167846.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/KU167846.1
KU167848.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/KU167848.1
KU167856.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/KU167856.1
HQ123404.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123404.1
HQ123392.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123392.1
HQ123393.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123393.1
KU167853.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/KU167853.1
KU167855.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/KU167855.1
KU167850.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/KU167850.1
KU167857.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/KU167857.1
HQ123396.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123396.1
EU477118.2	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/EU477118.2
KU167849.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/KU167849.1
HQ123390.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123390.1
KU167851.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/KU167851.1
HQ123391.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123391.1
HQ123395.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123395.1
KU167858.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/KU167858.1
KU167854.1	***A. nosocomialis***	https://www.ncbi.nlm.nih.gov/nuccore/KU167854.1
EF611399.1	*Acinetobacter parvus DSM16617*	https://www.ncbi.nlm.nih.gov/nuccore/EF611399.1
KU167873.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167873.1
KU167862.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167862.1
KU167868.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167868.1
KU167860.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167860.1
HQ123375.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123375.1
HQ123381.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123381.1
HQ123379.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123379.1
KU167861.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167861.1
KU167864.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167864.1

KU167865.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167865.1
EU477114.2	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/EU477114.2
KU167863.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167863.1
KU167867.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167867.1
KU167869.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167869.1
KU167871.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167871.1
KU167874.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167874.1
KU167875.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167875.1
KU167876.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167876.1
HQ123378.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123378.1
KJ956425.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956425.1
HQ123388.1	***A. pittii***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123388.1
KJ956427.1	***A. seifertii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956427.1
KJ956428.1	***A. seifertii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956428.1
KJ956465.1	***A. seifertii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956465.1
KJ956430.1	***A. seifertii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956430.1
KU726606.1	***A. seifertii***	https://www.ncbi.nlm.nih.gov/nuccore/KU726606.1
KU167888.1	***A. seifertii***	https://www.ncbi.nlm.nih.gov/nuccore/KU167888.1
KJ956442.1	***A. seifertii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956442.1
KJ956440.1	***A. seifertii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956440.1
KJ956456.1	***A. seifertii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956456.1
KJ956457.1	***A. seifertii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956457.1
KJ956464.1	***A. seifertii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956464.1
HQ123426.1	***A. seifertii***	https://www.ncbi.nlm.nih.gov/nuccore/HQ123426.1
KJ956461.1	***A. seifertii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956461.1
EU477126.2	***A. seifertii***	https://www.ncbi.nlm.nih.gov/nuccore/EU477126.2
KJ956453.1	***A. seifertii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956453.1
KJ956463.1	***A. seifertii***	https://www.ncbi.nlm.nih.gov/nuccore/KJ956463.1
KU961642.1	*Acinetobacter towneri* LUH10282	https://www.ncbi.nlm.nih.gov/nuccore/KU961642.1
KU961643.1	*A. towneri* LUH13865	https://www.ncbi.nlm.nih.gov/nuccore/KU961643.1
AP018824.1	*A. ursingii*	https://www.ncbi.nlm.nih.gov/nuccore/AP018824.1
EU742575.1	*A. ursingii* NIPH177	https://www.ncbi.nlm.nih.gov/nuccore/EU742575.1
EU742577.1	*A. ursingii* NIPH375	https://www.ncbi.nlm.nih.gov/nuccore/EU742577.1
Sequences used to perform *in silico* analysis for *P. aeruginosa* assay targeting *rpsG*		
NCBI Accession Number	**Species**	**URL**
CP028303.1	*Acidovorax avenae*	https://www.ncbi.nlm.nih.gov/nuccore/CP028303.1
CP028302.1	*A. avenae*	https://www.ncbi.nlm.nih.gov/nuccore/CP028302.1
NC_018708.1	*Acidovorax* sp KKS102	https://www.ncbi.nlm.nih.gov/nuccore/NC_018708.1
CP030075.1	***Pseudomonas aeruginosa***	https://www.ncbi.nlm.nih.gov/nuccore/CP030075.1
CP032552.1	***P. aeruginosa***	https://www.ncbi.nlm.nih.gov/nuccore/CP032552.1
CP028584.2	***P. aeruginosa***	https://www.ncbi.nlm.nih.gov/nuccore/CP028584.2
CP032257.1	***P. aeruginosa***	https://www.ncbi.nlm.nih.gov/nuccore/CP032257.1
CP031877.1	***P. aeruginosa***	https://www.ncbi.nlm.nih.gov/nuccore/CP031877.1

CP031876.1	***P. aeruginosa***	https://www.ncbi.nlm.nih.gov/nuccore/CP031876.1
CP031878.1	***P. aeruginosa***	https://www.ncbi.nlm.nih.gov/nuccore/CP031878.1
CP031879.1	***P. aeruginosa***	https://www.ncbi.nlm.nih.gov/nuccore/CP031879.1
NC_002516.2	***P. aeruginosa* PAO1**	https://www.ncbi.nlm.nih.gov/nuccore/NC_002516.2
CP016162.1	*Pseudonomas alcaliphila*	https://www.ncbi.nlm.nih.gov/nuccore/CP016162.1
CP009048.1	*Pseudonomas alkylphenolica*	https://www.ncbi.nlm.nih.gov/nuccore/CP009048.1
CP015878.1	*Pseudonomas citronellolis*	https://www.ncbi.nlm.nih.gov/nuccore/CP015878.1
CP014158.1	*P. citronellolis*	https://www.ncbi.nlm.nih.gov/nuccore/CP014158.1
NZ_CP009455.1	*Pseudonomas cremoricolorata*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP009455.1
NC_020829.1	*Pseudonomas denitrificans*	https://www.ncbi.nlm.nih.gov/nuccore/NC_020829.1
CP004143.1	*P. denitrificans*	https://www.ncbi.nlm.nih.gov/nuccore/CP004143.1
NC_008027.1	*Pseudonomas entomophila*	https://www.ncbi.nlm.nih.gov/nuccore/NC_008027.1
NC_016830.1	*Pseudonomas fluorescens*	https://www.ncbi.nlm.nih.gov/nuccore/NC_016830.1
CP013861.1	*Pseudonomas fragi*	https://www.ncbi.nlm.nih.gov/nuccore/CP013861.1
CP002727.1	*Pseudonomas fulva*	https://www.ncbi.nlm.nih.gov/nuccore/CP002727.1
HG322950.1	*Pseudonomas knackmussii*	https://www.ncbi.nlm.nih.gov/nuccore/HG322950.1
NZ_CP014947.1	*Pseudonomas koreensis*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP014947.1
NZ_JRMB01000003.1	*Pseudonomas lutea*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_JRMB01000003.1
NC_009439.1	*Pseudonomas mendocina*	https://www.ncbi.nlm.nih.gov/nuccore/NC_009439.1
CP023641.1	*P. mendocina*	https://www.ncbi.nlm.nih.gov/nuccore/CP023641.1
NZ_BDAI01000025.1	*Pseudonomas nitroreducens*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_BDAI01000025.1
LK391695.1	*Pseudonomas oleovorans*	https://www.ncbi.nlm.nih.gov/nuccore/LK391695.1
NZ_CP013987.1	*Pseudonomas oryzihabitans*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP013987.1
NZ_CP018758.1	*Pseudonomas psychrotolerans*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP018758.1
NC_002947.4	*Pseudonomas putida*	https://www.ncbi.nlm.nih.gov/nuccore/NC_002947.4
NZ_LUCV01000009.1	*P. putida*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_LUCV01000009.1
AP013068.1	*Pseudonomas resinovorans*	https://www.ncbi.nlm.nih.gov/nuccore/AP013068.1
NC_007005.1	*Pseudonomas syringae*	https://www.ncbi.nlm.nih.gov/nuccore/NC_007005.1
NC_004578.1	*Pseudonomas syringae*	https://www.ncbi.nlm.nih.gov/nuccore/NC_004578.1
NZ_MDEM01000018.1	*Pseudonomas xanthomarina*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_MDEM01000018.1

CP025229.1	*Pseudomonas* sp AK6U	https://www.ncbi.nlm.nih.gov/nuccore/CP025229.1
CP017432.1	*Pseudomonas* sp Lz4W	https://www.ncbi.nlm.nih.gov/nuccore/CP017432.1
NC_015740.1	*Stutzerimonas stutzeri*	https://www.ncbi.nlm.nih.gov/nuccore/NC_015740.1
Sequences used to perform *in silico* analysis for *Enterobacter* genus assay targeting *radA*		
NCBI Accession Number	**Species**	**URL**
CP007557.1	*Citrobacter freundii*	https://www.ncbi.nlm.nih.gov/nuccore/CP007557.1
CP026238.1	*C. freundii*	https://www.ncbi.nlm.nih.gov/nuccore/CP026238.1
CP012554.1	*Citrobacter portucalensis*	https://www.ncbi.nlm.nih.gov/nuccore/CP012554.1
CP029727.1	*Citrobacter* sp.	https://www.ncbi.nlm.nih.gov/nuccore/CP029727.1
CP026216.1	*Citrobacter* sp.	https://www.ncbi.nlm.nih.gov/nuccore/CP026216.1
LT556084.1	*Citrobacter* sp.	https://www.ncbi.nlm.nih.gov/nuccore/LT556084.1
LT556085.1	*Citrobacter* sp.	https://www.ncbi.nlm.nih.gov/nuccore/LT556085.1
CP019986.1	*Citrobacter werkmanii*	https://www.ncbi.nlm.nih.gov/nuccore/CP019986.1
CP021963.1	*Citrobacter youngae*	https://www.ncbi.nlm.nih.gov/nuccore/CP021963.1
CP028974.1	*Cronobacter sakazakii*	https://www.ncbi.nlm.nih.gov/nuccore/CP028974.1
CP003312.1	*C. sakazakii*	https://www.ncbi.nlm.nih.gov/nuccore/CP003312.1
CP011047.1	*C. sakazakii*	https://www.ncbi.nlm.nih.gov/nuccore/CP011047.1
CP027108.1	*C. sakazakii*	https://www.ncbi.nlm.nih.gov/nuccore/CP027108.1
BIGA01000089.1	***Enterobacter asburiae***	https://www.ncbi.nlm.nih.gov/nuccore/BIGA01000089.1
CP007546.1	***E. asburiae***	https://www.ncbi.nlm.nih.gov/nuccore/CP007546.1
CP011591.1	***E. asburiae***	https://www.ncbi.nlm.nih.gov/nuccore/CP011591.1
CP014993.1	***E. asburiae***	https://www.ncbi.nlm.nih.gov/nuccore/CP014993.1
RXSB01000005.1	***E. asburiae***	https://www.ncbi.nlm.nih.gov/nuccore/RXSB01000005.1
CP011863.1	***E. asburiae* ATCC35953**	https://www.ncbi.nlm.nih.gov/nuccore/CP011863.1
LT992502.1	***Enterobacter bugandensis***	https://www.ncbi.nlm.nih.gov/nuccore/LT992502.1
CP025225.1	***Enterobacter cancerogenus***	https://www.ncbi.nlm.nih.gov/nuccore/CP025225.1
QGAL01000004.1	***E. cancerogenus***	https://www.ncbi.nlm.nih.gov/nuccore/QGAL01000004.1
FYBA01000004.1	***E. cancerogenus* ATCC33241**	https://www.ncbi.nlm.nih.gov/nuccore/FYBA01000004.1
ABWM02000004.1	***E. cancerogenus* ATCC35316**	https://www.ncbi.nlm.nih.gov/nuccore/ABWM02000004.1
CAADIW010000001.1	***E. cancerogenus* NCTC12126**	https://www.ncbi.nlm.nih.gov/nuccore/CAADIW010000001.1
JAMFTT010000004.1	***Enterobacter chuandaensis***	https://www.ncbi.nlm.nih.gov/nuccore/JAMFTT010000004.1

JANWNR010000008.1	***E. chuandaensis***	https://www.ncbi.nlm.nih.gov/nuccore/JANWNR010000008.1
AAXRDX010000005.1	***Enterobacter cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/AAXRDX010000005.1
CP002886.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP002886.1
CP003737.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP003737.1
CP008823.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP008823.1
CP008897.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP008897.1
CP008905.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP008905.1
CP009756.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP009756.1
CP009850.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP009850.1
CP009854.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP009854.1
CP010512.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP010512.1
CP011572.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP011572.1
CP011581.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP011581.1
CP011584.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP011584.1
CP011650.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP011650.1
CP011798.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP011798.1
CP012162.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP012162.1
CP014280.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP014280.1
CP016906.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP016906.1
CP017475.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP017475.1
CP018785.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP018785.1
CP018814.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP018814.1
CP019839.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP019839.1
CP019889.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP019889.1
CP020053.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP020053.1
CP020089.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP020089.1
CP021162.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP021162.1
CP021167.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP021167.1
CP021749.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP021749.1
CP021776.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP021776.1
CP021851.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP021851.1
CP021896.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP021896.1
CP021902.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP021902.1
CP022148.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP022148.1
CP022532.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP022532.1
CP026719.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP026719.1
CP026850.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP026850.1
CP026975.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP026975.1
CP027604.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP027604.1
CP029716.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP029716.1
CP030076.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP030076.1
CP030347.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/CP030347.1
DACOJF010000031.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOJF010000031.1
DACOJH010000006.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOJH010000006.1
DACOJI010000002.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOJI010000002.1
DACOKG010000005.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOKG010000005.1

DACOKI010000002.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOKI010000002.1
DACOKL010000004.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOKL010000004.1
DACOKO010000003.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOKO010000003.1
DACOKQ010000005.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOKQ010000005.1
DACOKS010000002.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOKS010000002.1
DACOKT010000002.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOKT010000002.1
DACOKU010000005.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOKU010000005.1
DACOKW010000004.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOKW010000004.1
DACOLB010000003.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOLB010000003.1
DACOLE010000005.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOLE010000005.1
DACOLH010000003.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOLH010000003.1
DACONO010000009.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACONO010000009.1
DACOPB010000008.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOPB010000008.1
DACOPL010000030.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOPL010000030.1
DACOPM010000009.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOPM010000009.1
DACOPZ010000005.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOPZ010000005.1
DACRIU010000006.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACRIU010000006.1
DACZWF010000005.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACZWF010000005.1
DACZWZ010000005.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACZWZ010000005.1
DACZXV010000002.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/DACZXV010000002.1
LT840187.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/LT840187.1
NMPX01000032.1	***E. cloacae***	https://www.ncbi.nlm.nih.gov/nuccore/NMPX01000032.1
CP001918.1	***E. cloacae* ATCC13047**	https://www.ncbi.nlm.nih.gov/nuccore/CP001918.1
CP017990.1	***E. cloacae complex***	https://www.ncbi.nlm.nih.gov/nuccore/CP017990.1
CP027618.1	***E. cloacae complex***	https://www.ncbi.nlm.nih.gov/nuccore/CP027618.1
CP028950.1	***E. cloacae complex***	https://www.ncbi.nlm.nih.gov/nuccore/CP028950.1
LT991957.1	***E. cloacae complex***	https://www.ncbi.nlm.nih.gov/nuccore/LT991957.1

CP003678.1	***E. cloacae* ssp. *dissolvens***	https://www.ncbi.nlm.nih.gov/nuccore/CP003678.1
FP929040.1	***E. cloacae* NCTC9394**	https://www.ncbi.nlm.nih.gov/nuccore/FP929040.1
JAHEVX010000005.1	***Enterobacter dykesii***	https://www.ncbi.nlm.nih.gov/nuccore/JAHEVX010000005.1
CP010377.1	***Enterobacter hormaechei***	https://www.ncbi.nlm.nih.gov/nuccore/CP010377.1
CP011662.1	***E. hormaechei***	https://www.ncbi.nlm.nih.gov/nuccore/CP011662.1
CP028538.1	***E. hormaechei***	https://www.ncbi.nlm.nih.gov/nuccore/CP028538.1
CP029126.1	***E. hormaechei***	https://www.ncbi.nlm.nih.gov/nuccore/CP029126.1
CP031565.1	***E. hormaechei***	https://www.ncbi.nlm.nih.gov/nuccore/CP031565.1
CP031571.1	***E. hormaechei***	https://www.ncbi.nlm.nih.gov/nuccore/CP031571.1
CP031574.1	***E. hormaechei***	https://www.ncbi.nlm.nih.gov/nuccore/CP031574.1
CP031726.1	***E. hormaechei***	https://www.ncbi.nlm.nih.gov/nuccore/CP031726.1
CP017186.1	***E. hormaechei* DSM14563**	https://www.ncbi.nlm.nih.gov/nuccore/CP017186.1
CP017180.1	***E. hormaechei* DSM16687**	https://www.ncbi.nlm.nih.gov/nuccore/CP017180.1
CP017179.1	***E. hormaechei* DSM16691**	https://www.ncbi.nlm.nih.gov/nuccore/CP017179.1
CP027142.1	***E. hormaechei* ssp. *hoffmanii***	https://www.ncbi.nlm.nih.gov/nuccore/CP027142.1
CP010384.1	***E. hormaechei* ssp. *oharae***	https://www.ncbi.nlm.nih.gov/nuccore/CP010384.1
CP012165.1	***E. hormaechei* ssp. *oharae***	https://www.ncbi.nlm.nih.gov/nuccore/CP012165.1
DACOIF010000031.1	***E. hormaechei* ssp. *oharae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOIF010000031.1
DACOIN010000029.1	***E. hormaechei* ssp. *oharae***	https://www.ncbi.nlm.nih.gov/nuccore/DACOIN010000029.1
LEET01000002.1	***E. hormaechei* ssp. *oharae***	https://www.ncbi.nlm.nih.gov/nuccore/LEET01000002.1
CP012167.1	***E. hormaechei* ssp. *steigerwaltii***	https://www.ncbi.nlm.nih.gov/nuccore/CP012167.1
CP010376.2	***E. hormaechei* ssp. *steigerwaltii***	https://www.ncbi.nlm.nih.gov/nuccore/CP010376.2
LT991954.1	***E. hormaechei* ssp. *steigerwaltii***	https://www.ncbi.nlm.nih.gov/nuccore/LT991954.1
QMCW01000028.1	***E. hormaechei* ssp. *steigerwaltii***	https://www.ncbi.nlm.nih.gov/nuccore/QMCW01000028.1
CP024908.1	***E. hormaechei* ssp. *xiangfangensis***	https://www.ncbi.nlm.nih.gov/nuccore/CP024908.1
CP029246.1	***E. hormaechei* ssp. *xiangfangensis***	https://www.ncbi.nlm.nih.gov/nuccore/CP029246.1
CP030007.1	***E. hormaechei* ssp. *xiangfangensis***	https://www.ncbi.nlm.nih.gov/nuccore/CP030007.1
LDQR01000003.1	***E. hormaechei* ssp. *xiangfangensis***	https://www.ncbi.nlm.nih.gov/nuccore/LDQR01000003.1

LDQS01000029.1	***E. hormaechei* ssp. *xiangfangensis***	https://www.ncbi.nlm.nih.gov/nuccore/LDQS01000029.1
LDQT01000015.1	***E. hormaechei* ssp. *xiangfangensis***	https://www.ncbi.nlm.nih.gov/nuccore/LDQT01000015.1
LDQU01000047.1	***E. hormaechei* ssp. *xiangfangensis***	https://www.ncbi.nlm.nih.gov/nuccore/LDQU01000047.1
NEZA02000006.1	***E. hormaechei* ssp. *xiangfangensis***	https://www.ncbi.nlm.nih.gov/nuccore/NEZA02000006.1
PYBR01000005.1	***E. hormaechei* ssp. *xiangfangensis***	https://www.ncbi.nlm.nih.gov/nuccore/PYBR01000005.1
QMDB01000060.1	***E. hormaechei* ssp. *xiangfangensis***	https://www.ncbi.nlm.nih.gov/nuccore/QMDB01000060.1
RXSQ01000001.1	***E. hormaechei* ssp. *xiangfangensis***	https://www.ncbi.nlm.nih.gov/nuccore/RXSQ01000001.1
RXST01000001.1	***E. hormaechei* ssp. *xiangfangensis***	https://www.ncbi.nlm.nih.gov/nuccore/RXST01000001.1
CP017183.1	***E. hormaechei* ssp. *xiangfangensis* LMG27195**	https://www.ncbi.nlm.nih.gov/nuccore/CP017183.1
CP071178.1	***Enterobacter kobei***	https://www.ncbi.nlm.nih.gov/nuccore/CP071178.1
CP088119.1	***E. kobei***	https://www.ncbi.nlm.nih.gov/nuccore/CP088119.1
CP017181.1	***E. kobei* DSM13645**	https://www.ncbi.nlm.nih.gov/nuccore/CP017181.1
CP006580.1	***Enterobacter ludwigii***	https://www.ncbi.nlm.nih.gov/nuccore/CP006580.1
CP017279.1	***E. ludwigii***	https://www.ncbi.nlm.nih.gov/nuccore/CP017279.1
CP040527.1	***E. ludwigii***	https://www.ncbi.nlm.nih.gov/nuccore/CP040527.1
CP056119.1	***E. ludwigii***	https://www.ncbi.nlm.nih.gov/nuccore/CP056119.1
CP083824.1	***E. ludwigii***	https://www.ncbi.nlm.nih.gov/nuccore/CP083824.1
CP055276.1	***Enterobacter mori***	https://www.ncbi.nlm.nih.gov/nuccore/CP055276.1
CP071063.1	***E. mori***	https://www.ncbi.nlm.nih.gov/nuccore/CP071063.1
CP084692.1	***E. mori***	https://www.ncbi.nlm.nih.gov/nuccore/CP084692.1
CP091779.1	***E. mori***	https://www.ncbi.nlm.nih.gov/nuccore/CP091779.1
JAHCLV010000012.1	***Enterobacter oligotrophicus***	https://www.ncbi.nlm.nih.gov/nuccore/JAHCLV010000012.1
JALAGP010000145.1	***E. oligotrophicus***	https://www.ncbi.nlm.nih.gov/nuccore/JALAGP010000145.1
JAJAPJ010000002.1	***Enterobacter quasihormaechei***	https://www.ncbi.nlm.nih.gov/nuccore/JAJAPJ010000002.1
SJON01000004.1	***E. quasihormaechei***	https://www.ncbi.nlm.nih.gov/nuccore/SJON01000004.1
JAHEVU010000004.1	***Enterobacter quasimori***	https://www.ncbi.nlm.nih.gov/nuccore/JAHEVU010000004.1

JAHUUW010000002.1	***E. quasiroggenkampii***	https://www.ncbi.nlm.nih.gov/nuccore/JAHUUW010000002.1
JAMGKB010000003.1	***Enterobacter quasiroggenkampii***	https://www.ncbi.nlm.nih.gov/nuccore/JAMGKB010000003.1
JAKCKM010000003.1	***Enterobacter roggenkampii***	https://www.ncbi.nlm.nih.gov/nuccore/JAKCKM010000003.1
LVVI01000020.1	***E. roggenkampii***	https://www.ncbi.nlm.nih.gov/nuccore/LVVI01000020.1
VDAH01000013.1	***E. roggenkampii***	https://www.ncbi.nlm.nih.gov/nuccore/VDAH01000013.1
CP017184.1	***E. roggenkampii* DSM16690**	https://www.ncbi.nlm.nih.gov/nuccore/CP017184.1
JANFCY010000015.1	***E. roggenkampii* DSM16690**	https://www.ncbi.nlm.nih.gov/nuccore/JANFCY010000015.1
JACWFD010000027.1	***E. sichuanensis***	https://www.ncbi.nlm.nih.gov/nuccore/JACWFD010000027.1
JAHLTI010000002.1	***Enterobacter sichuanensis***	https://www.ncbi.nlm.nih.gov/nuccore/JAHLTI010000002.1
CP003026.1	***Enterobacter soli* LF7a**	https://www.ncbi.nlm.nih.gov/nuccore/CP003026.1
FYBB01000011.1	***E. soli***	https://www.ncbi.nlm.nih.gov/nuccore/FYBB01000011.1
JAGGDT010000007.1	***E. soli***	https://www.ncbi.nlm.nih.gov/nuccore/JAGGDT010000007.1
JARADK010000001.1	***E. soli***	https://www.ncbi.nlm.nih.gov/nuccore/JARADK010000001.1
LXES01000025.1	***E. soli* ATCCBAA-2102**	https://www.ncbi.nlm.nih.gov/nuccore/LXES01000025.1
JAMDPD010000017.1	***Enterobacter vonholyi***	https://www.ncbi.nlm.nih.gov/nuccore/JAMDPD010000017.1
JAMGKV010000003.1	***E. vonholyi***	https://www.ncbi.nlm.nih.gov/nuccore/JAMGKV010000003.1
JANIER010000004.1	***Enterobacter wuhouensis***	https://www.ncbi.nlm.nih.gov/nuccore/JANIER010000004.1
SJOO01000004.1	***E. wuhouensis***	https://www.ncbi.nlm.nih.gov/nuccore/SJOO01000004.1
CP020817.1	***Enterobacter* sp.**	https://www.ncbi.nlm.nih.gov/nuccore/CP020817.1
CP024812.1	***Enterobacter* sp. CRENT-193**	https://www.ncbi.nlm.nih.gov/nuccore/CP024812.1
CP021137.1	***Enterobacter* sp. DKU**	https://www.ncbi.nlm.nih.gov/nuccore/CP021137.1
CP012999.1	***Enterobacter* sp. E20**	https://www.ncbi.nlm.nih.gov/nuccore/CP012999.1
CP017087.1	***Enterobacter* sp. HK169**	https://www.ncbi.nlm.nih.gov/nuccore/CP017087.1
CP031101.1	*Enterobacteriaceae*	https://www.ncbi.nlm.nih.gov/nuccore/CP031101.1
CP031104.1	*Enterobacteriaceae*	https://www.ncbi.nlm.nih.gov/nuccore/CP031104.1
CP088744.1	*Escherichia coli*	https://www.ncbi.nlm.nih.gov/nuccore/CP088744.1
LR025101.1	*Escherichia coli*	https://www.ncbi.nlm.nih.gov/nuccore/LR025101.1

LR134221.1	*E. coli* NCTC11113	https://www.ncbi.nlm.nih.gov/nuccore/LR134221.1
CP057104.1	*Escherichia fergusonii*	https://www.ncbi.nlm.nih.gov/nuccore/CP057104.1
QISR01000003.1	*Franconibacter helveticus*	https://www.ncbi.nlm.nih.gov/nuccore/QISR01000003.1
AXSZ01000019.1	*Franconibacter pulveris*	https://www.ncbi.nlm.nih.gov/nuccore/AXSZ01000019.1
DNVC01000196.1	*Franconibacter pulveris*	https://www.ncbi.nlm.nih.gov/nuccore/DNVC01000196.1
DAMOMP010000032.1	*Kluyvera ascorbata*	https://www.ncbi.nlm.nih.gov/nuccore/DAMOMP010000032.1
JMPL01000044.1	*K. ascorbata* ATCC33433	https://www.ncbi.nlm.nih.gov/nuccore/JMPL01000044.1
UGNM01000003.1	*K. ascorbata* NCTC9737	https://www.ncbi.nlm.nih.gov/nuccore/UGNM01000003.1
CP022114.1	*Kluyvera* genomo sp.3	https://www.ncbi.nlm.nih.gov/nuccore/CP022114.1
JANEWK010000044.1	*Kluyvera georgiana*	https://www.ncbi.nlm.nih.gov/nuccore/JANEWK010000044.1
LSME01000011.1	*K. georgiana*	https://www.ncbi.nlm.nih.gov/nuccore/LSME01000011.1
LXEU01000067.1	*K. georgiana* ATCC51603	https://www.ncbi.nlm.nih.gov/nuccore/LXEU01000067.1
DACYQB010000006.1	*Kluyvera intermedia*	https://www.ncbi.nlm.nih.gov/nuccore/DACYQB010000006.1
BCYS01000001.1	*K. intermedia* NBRC102594	https://www.ncbi.nlm.nih.gov/nuccore/BCYS01000001.1
CP019445.1	*Kosakonia cowanii*	https://www.ncbi.nlm.nih.gov/nuccore/CP019445.1
CP022690.1	*Kosakonia cowanii*	https://www.ncbi.nlm.nih.gov/nuccore/CP022690.1
CP035129.1	*Kosakonia cowanii*	https://www.ncbi.nlm.nih.gov/nuccore/CP035129.1
LT799040.1	*Kosakonia oryzae*	https://www.ncbi.nlm.nih.gov/nuccore/LT799040.1
CP015113.1	*Kosakonia radicincitans*	https://www.ncbi.nlm.nih.gov/nuccore/CP015113.1
CP018016.1	*K. radicincitans*	https://www.ncbi.nlm.nih.gov/nuccore/CP018016.1
CP007215.3	*Kosakonia sacchari*	https://www.ncbi.nlm.nih.gov/nuccore/CP007215.3
CP016337.1	*K. sacchari*	https://www.ncbi.nlm.nih.gov/nuccore/CP016337.1
CP034225.1	*Kosakonia* sp	https://www.ncbi.nlm.nih.gov/nuccore/CP034225.1
JWJV01000011.1	*Leclercia adecarboxylata*	https://www.ncbi.nlm.nih.gov/nuccore/JWJV01000011.1
CP013990.1	*L. adecarboxylata*	https://www.ncbi.nlm.nih.gov/nuccore/CP013990.1
CP036199.1	*L. adecarboxylata*	https://www.ncbi.nlm.nih.gov/nuccore/CP036199.1
CP071383.1	*Leclercia pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP071383.1
CP026167.1	*Leclercia* sp LSNIH1	https://www.ncbi.nlm.nih.gov/nuccore/CP026167.1
PQKC01000005.1	*Leclercia* sp LSNIH2	https://www.ncbi.nlm.nih.gov/nuccore/PQKC01000005.1
CP026387.1	*Leclercia* sp LSNIH3	https://www.ncbi.nlm.nih.gov/nuccore/CP026387.1

PQKB01000006.1	*Leclercia*.sp LSNIH5	https://www.ncbi.nlm.nih.gov/nuccore/PQKB01000006.1
PQKA01000004.1	*Leclercia* sp. LSNIH6	https://www.ncbi.nlm.nih.gov/nuccore/PQKA01000004.1
PQKD01000003.1	*Leclercia* sp. LSNIH7	https://www.ncbi.nlm.nih.gov/nuccore/PQKD01000003.1
PQJY01000005.1	*Leclercia* sp. LSNIH8	https://www.ncbi.nlm.nih.gov/nuccore/PQJY01000005.1
DIIP01000062.1	*Leclercia* sp. UBA5667	https://www.ncbi.nlm.nih.gov/nuccore/DIIP01000062.1
PDDA01000001.1	*Lelliottia amnigena* LMG2784	https://www.ncbi.nlm.nih.gov/nuccore/PDDA01000001.1
BCNN01000008.1	*L. amnigena* NBRC10570	https://www.ncbi.nlm.nih.gov/nuccore/BCNN01000008.1
LR134135.1	*L. amnigena* NCTC12124	https://www.ncbi.nlm.nih.gov/nuccore/LR134135.1
RJVC01000005.1	*L. amnigena*	https://www.ncbi.nlm.nih.gov/nuccore/RJVC01000005.1
CP015774.2	*L. amnigena*	https://www.ncbi.nlm.nih.gov/nuccore/CP015774.2
CP023529.1	*L. amnigena*	https://www.ncbi.nlm.nih.gov/nuccore/CP023529.1
PQVR01000007.1	*Lelliottia aquatilis*	https://www.ncbi.nlm.nih.gov/nuccore/PQVR01000007.1
PQVT01000005.1	*L. aquatilis*	https://www.ncbi.nlm.nih.gov/nuccore/PQVT01000005.1
PQVU01000005.1	*L. aquatilis*	https://www.ncbi.nlm.nih.gov/nuccore/PQVU01000005.1
PQVV01000004.1	*L. aquatilis*	https://www.ncbi.nlm.nih.gov/nuccore/PQVV01000004.1
PQVW01000005.1	*L. aquatilis*	https://www.ncbi.nlm.nih.gov/nuccore/PQVW01000005.1
CP018628.1	*Lelliottia jeotgali*	https://www.ncbi.nlm.nih.gov/nuccore/CP018628.1
CP025034.1	*Leliottia nimipressuralis*	https://www.ncbi.nlm.nih.gov/nuccore/CP025034.1
SDDX01000013.1	*L. nimipressuralis*	https://www.ncbi.nlm.nih.gov/nuccore/SDDX01000013.1
CP028520.1	*Lelliottia* sp.	https://www.ncbi.nlm.nih.gov/nuccore/CP028520.1
PKFT01000003.1	*Lelliottia* sp.	https://www.ncbi.nlm.nih.gov/nuccore/PKFT01000003.1
PKFU01000004.1	*Lelliottia* sp.	https://www.ncbi.nlm.nih.gov/nuccore/PKFU01000004.1
PKFV01000005.1	*Lelliottia* sp.	https://www.ncbi.nlm.nih.gov/nuccore/PKFV01000005.1
PQVS01000005.1	*Lelliottia* sp.	https://www.ncbi.nlm.nih.gov/nuccore/PQVS01000005.1
CABKSF010000003.1	*Metakosakonia massiliensis*	https://www.ncbi.nlm.nih.gov/nuccore/CABKSF010000003.1
JFOK01000020.1	*Pantoea agglomerans*	https://www.ncbi.nlm.nih.gov/nuccore/JFOK01000020.
BCZA01000009.1	*P. agglomerans* NBRC102470	https://www.ncbi.nlm.nih.gov/nuccore/BCZA01000009.1
UGSO01000001.1	*P. agglomerans* NCTC9381	https://www.ncbi.nlm.nih.gov/nuccore/UGSO01000001.1

MLJI01000001.1	*Pantoea cypripedii* LMG2657	https://www.ncbi.nlm.nih.gov/nuccore/MLJI01000001.
SCKT01000009.1	*Pantoea dispersa* DSM30073	https://www.ncbi.nlm.nih.gov/nuccore/SCKT01000009.1
MLJJ01000002.1	*Pantoea septica* LMG5345	https://www.ncbi.nlm.nih.gov/nuccore/MLJJ01000002.1
CP009450.1	*Pluralibacter gergoviae*	https://www.ncbi.nlm.nih.gov/nuccore/CP009450.1
CP020388.1	*P. gergoviae*	https://www.ncbi.nlm.nih.gov/nuccore/CP020388.1
CP002272.1	*Pluralibacter lignolyticus*	https://www.ncbi.nlm.nih.gov/nuccore/CP002272.
CP012871.1	*P. lignolyticus*	https://www.ncbi.nlm.nih.gov/nuccore/CP012871.1
CP022139.1	*Salmonella enterica*	https://www.ncbi.nlm.nih.gov/nuccore/CP022139.
LR133910.1	*S. enterica* NCTC7300	https://www.ncbi.nlm.nih.gov/nuccore/LR133910.1
LR134150.1	*S. enterica* NCTC7306	https://www.ncbi.nlm.nih.gov/nuccore/LR134150.1
CP014017.2	*Serratia liquefaciens*	https://www.ncbi.nlm.nih.gov/nuccore/CP014017.
CP006252.1	*S. liquefaciens* ATCC27592,	https://www.ncbi.nlm.nih.gov/nuccore/CP006252.1
CP050960.1	*Serratia marcescens*	https://www.ncbi.nlm.nih.gov/nuccore/CP050960.1
CP118932.1	*S. marcescens*	https://www.ncbi.nlm.nih.gov/nuccore/CP118932.1
AP021873.1	*S. marcescens* ATCC274	https://www.ncbi.nlm.nih.gov/nuccore/AP021873.
LR134155.1	*Serratia rubidaea* NCTC9419	https://www.ncbi.nlm.nih.gov/nuccore/LR134155.
CP026845.1	*Shigella boydii* NCTC9733	https://www.ncbi.nlm.nih.gov/nuccore/CP026845.1
CP026875.1	*S. boydii* NCTC9734	https://www.ncbi.nlm.nih.gov/nuccore/CP026875.1
CP027027.1	*Shigella dysenteriae*	https://www.ncbi.nlm.nih.gov/nuccore/CP027027.
CP026840.1	*S. dysenteriae* ATCC9753	https://www.ncbi.nlm.nih.gov/nuccore/CP026840.1
CP014768.1	*Shigella* sp.	https://www.ncbi.nlm.nih.gov/nuccore/CP014768.1
JMSQ01000004.1	*Siccibacter colletis*	https://www.ncbi.nlm.nih.gov/nuccore/JMSQ01000004.
AWFZ01000050.1	*Siccibacter turicensis* LMG23730	https://www.ncbi.nlm.nih.gov/nuccore/AWFZ01000050.

Table S2.2: Growth conditions and culture collection identification numbers of strains tested *in vitro* as part of inclusivity panels for ESKAPE multiplex 1 and 2

Species	Culture Collection ID	Medium [a]	Temp	Atmosphere [b]
Acinetobacter baumannii [c,d]	DSM 30007	TS	37°C	Aerobic
Acinetobacter baumannii [c,d]	DSM 30008	TS	37°C	Aerobic
Acinetobacter baumannii [c,d]	DSM 30011	TS	37°C	Aerobic
Acinetobacter baumannii [c,d]	LMG984	TS	37°C	Aerobic
Acinetobacter baumannii [c,d]	LMG994	TS	37°C	Aerobic
Acinetobacter baumannii [c,d]	NCTC 13302	TS	37°C	Aerobic
Acinetobacter baumannii [c,d]	NCTC 13305	TS	37°C	Aerobic
Acinetobacter baumannii [c,d]	ATCC 51432	TS	37°C	Aerobic
Acinetobacter lactucae [d]	LMG 29605	TS & 12mg/L imipenem	37°C	Aerobic
Acinetobacter nosocomialis [d]	LMG993	TS	30°C	Aerobic
Acinetobacter nosocomialis [d]	LMG 10619	TS	30°C	Aerobic
Acinetobacter pittii [d]	DSM9312	TS	30°C	Aerobic
Acinetobacter pittii [d]	DSM9318	TS	30°C	Aerobic
Acinetobacter pittii [d]	DSM9341	TS	30°C	Aerobic
Acinetobacter pittii [d]	LMG1035	TS	30°C	Aerobic
Acinetobacter seifertii [d]	DSM 102854	BHI	37°C	Aerobic
Enterobacter asburiae [e]	DSM 17506	TS	37°C	Aerobic
Enterobacter cancerogenus [e]	DSM 17580	TS	30°C	Aerobic
Enterobacter cloacae [e]	ATCC 13047	TS	37°C	Aerobic
Enterobacter cloacae [e]	NCTC 11933	TS	37°C	Aerobic
Enterobacter cloacae subsp. *dissolvens* [e]	DSM 16657	TS	37°C	Aerobic
Enterobacter hormaechei [e]	DSM 12409	TS	37°C	Aerobic
Enterobacter kobei [e]	DSM 13645	TS	37°C	Aerobic
Enterobacter ludwigii [e]	DSM 16688	TS	37°C	Aerobic
Enterobacter mori [e]	DSM 26271	N	30°C	Aerobic
Enterobacter soli [e]	LMG 25861	TS	30°C	Aerobic
Enterobacter xiangfangensis [e]	DSM 101093	N	30°C	Aerobic
Enterococcus faecium [f]	DSM 20477	BHI	37°C	Microaerophilic
Enterococcus faecium [f]	ATCC 700221	BHI	37°C	Microaerophilic
Enterococcus faecium [f]	DSM2146	BHI	37°C	Microaerophilic
Enterococcus faecium [f]	DSM2918	BHI	37°C	Microaerophilic
Enterococcus faecium [f]	DSM6177	BHI	37°C	Microaerophilic
Enterococcus faecium [f]	DSM 13589	BHI	37°C	Microaerophilic
Enterococcus faecium [f]	DSM 13590	BHI	37°C	Microaerophilic
Enterococcus faecium [f]	LMG 16003	BHI	37°C	Microaerophilic
Enterococcus faecium [f]	LMG 16004	BHI	37°C	Microaerophilic
Enterococcus faecium [f]	LMG 16170	BHI	37°C	Microaerophilic
Enterococcus faecium [f]	LMG 16192	BHI	37°C	Microaerophilic
Enterococcus faecium [f]	LMG 16474	BHI	37°C	Microaerophilic
Enterococcus faecium [f]	NCTC 12202	BHI	37°C	Microaerophilic
Enterococcus faecium [f]	NCTC 12204	BHI	37°C	Microaerophilic
Klebsiella pneumoniae [g]	DSM681	N	37°C	Aerobic
Klebsiella pneumoniae [g]	DSM789	N	37°C	Aerobic

Klebsiella pneumoniae subsp. *ozaenae* [g]	DSM 16358	N	37°C	Aerobic
Klebsiella pneumoniae subsp. *pneumoniae* [g]	ATCC 13883	N	37°C	Aerobic
Klebsiella pneumoniae subsp. *pneumoniae* [g]	DSM1620	N	37°C	Aerobic
Klebsiella pneumoniae subsp. *pneumoniae* [g]	DSM2026	N	37°C	Aerobic
Klebsiella pneumoniae subsp. *pneumoniae* [g]	DSM5758	N	37°C	Aerobic
Klebsiella pneumoniae subsp. *pneumoniae* [g]	DSM9377	N	37°C	Aerobic
Klebsiella pneumoniae subsp. *pneumoniae* [g]	DSM 16609	N	37°C	Aerobic
Klebsiella pneumoniae subsp. *pneumoniae* [g]	DSM 30102	N	37°C	Aerobic
Klebsiella pneumoniae subsp. *pneumoniae* [g]	DSM 30104	N	37°C	Aerobic
Klebsiella pneumoniae subsp. *pneumoniae* [g]	DSM9376	N	37°C	Aerobic
Klebsiella pneumoniae subsp. *pneumoniae* [g]	DSM11678	N	37°C	Aerobic
Klebsiella pneumoniae subsp. *pneumoniae* [g]	DSM12059	N	37°C	Aerobic
Klebsiella pneumoniae subsp. *rhinoscleromatis* [g]	DSM16231	N	37°C	Aerobic
Pseudomonas aeruginosa [h]	DSM288	TS	37°C	Aerobic
Pseudomonas aeruginosa [h]	DSM1117	TS	37°C	Aerobic
Pseudomonas aeruginosa [h]	DSM1128	TS	37°C	Aerobic
Pseudomonas aeruginosa [h]	DSM1253	TS	37°C	Aerobic
Pseudomonas aeruginosa [h]	DSM1299	TS	37°C	Aerobic
Pseudomonas aeruginosa [h]	DSM1707	TS	37°C	Aerobic
Pseudomonas aeruginosa [h]	DSM3227	TS	37°C	Aerobic
Pseudomonas aeruginosa [h]	DSM 11148	TS	37°C	Aerobic
Pseudomonas aeruginosa [h]	DSM 24599	TS	37°C	Aerobic
Pseudomonas aeruginosa [h]	DSM 46349	TS	37°C	Aerobic
Pseudomonas aeruginosa [h]	DSM 46358	TS	37°C	Aerobic
Pseudomonas aeruginosa [h]	DSM 50071	TS	37°C	Aerobic
Pseudomonas aeruginosa [h]	DSM 50073	TS	37°C	Aerobic
Staphylococcus aureus [i]	DSM799	TS	37°C	Aerobic
Staphylococcus aureus [i]	DSM2569	TS	37°C	Aerobic
Staphylococcus aureus [i]	DSM6236	TS	37°C	Aerobic
Staphylococcus aureus [i]	DSM6571	TS	37°C	Aerobic
Staphylococcus aureus [i]	DSM9144	TS	37°C	Aerobic
Staphylococcus aureus [i]	DSM15676	TS	37°C	Aerobic
Staphylococcus aureus [i]	DSM17091	TS	37°C	Aerobic
Staphylococcus aureus [i]	DSM18827	TS	37°C	Aerobic
Staphylococcus aureus [i]	DSM21979	TS	37°C	Aerobic
Staphylococcus aureus [i]	DSM46320	TS	37°C	Aerobic
Staphylococcus aureus [i]	LMG16217	TS	37°C	Aerobic
Staphylococcus aureus [i]	NCTC 11963	TS	37°C	Aerobic
Staphylococcus aureus [i]	NCTC 11965	TS	37°C	Aerobic
Staphylococcus aureus [i]	DSM 23622	TS	37°C	Aerobic

Staphylococcus aureus subsp. *anaeroubis* [i]	DSM 20714	TS	37°C	Microaerophilic

[a] TS=Tryptone Soy; BHI=Brain Heart Infusion; N=Nutrient
[b] Microaerophilic conditions were achieved using Oxoid AnaeroJar 2.5L # AG0025A and CampyGen 2.5L #CN0025A (ThermoFisher, MA, US)
[c] Detected in multiplex 2 ROX
[d] Detected in multiplex 2 HEX
[e] Detected in multiplex 2 CYAN500
[f] Detected in multiplex 2 FAM
[g] Detected in multiplex 1 CYAN500
[h] Detected in multiplex 1 FAM
[i] Detected in multiplex 1 ROX

Table S2.3: Growth conditions and culture collection identification numbers of strains tested *in vitro* as part of exclusivity panels for ESKAPE multiplex 1 and 2

Species	Culture Collection ID	Medium [a]	Temp	Atmosphere [b]
Acinetobacter calcoaceticus	LMG 1046	gDNA [c]		
Acinetobacter lwoffii	DSM 2403	TS	37°C	Aerobic
Acinetobacter lwoffii	LMG 1004	TS	37°C	Aerobic
Acinetobacter lwoffii	LMG 1029	TS	37°C	Aerobic
Acinetobacter parvus	DSM 16617	TS	30°C	Aerobic
Acinetobacter soli	DSM 22956	TS	30°C	Aerobic
Acinetobacter ursingii	DSM 16037	TS	30°C	Aerobic
Chlamydophila pneumoniae	ATCC 53592	gDNA [c]		
Citrobacter freundii	DSM 30039	TS & 0.3% YE	30°C	Aerobic
Cronobacter sakazakii	NCTC 11467	TS	37°C	Aerobic
Enterococcus avium	DSM 20063	BHI	37°C	Microaerophilic
Enterococcus avium	DSM 20679	BHI	37°C	Microaerophilic
Enterococcus avium	LMG 12900	BHI	37°C	Microaerophilic
Enterococcus avium	LMG 16310	BHI	37°C	Microaerophilic
Enterococcus casseliflavus	DSM 4841	BHI	37°C	Microaerophilic
Enterococcus casseliflavus	DSM 7370	BHI	37°C	Microaerophilic
Enterococcus casseliflavus	NCTC 12362	BHI	37°C	Microaerophilic
Enterococcus durans	DSM 20633	BHI	37°C	Microaerophilic
Enterococcus durans	NCTC 662	BHI	37°C	Microaerophilic
Enterococcus durans	NCTC 8129	BHI	37°C	Microaerophilic
Enterococcus durans	NCTC 8307	BHI	37°C	Microaerophilic
Enterococcus faecalis	DSM 2570	BHI	37°C	Microaerophilic
Enterococcus faecalis	DSM 2981	BHI	37°C	Microaerophilic
Enterococcus faecalis	DSM 6134	BHI	37°C	Microaerophilic
Enterococcus faecalis	DSM 8629	BHI	37°C	Microaerophilic
Enterococcus faecalis	DSM 8630	BHI	37°C	Microaerophilic
Enterococcus faecalis	DSM 12956	BHI	37°C	Microaerophilic
Enterococcus faecalis	DSM 13591	BHI	37°C	Microaerophilic

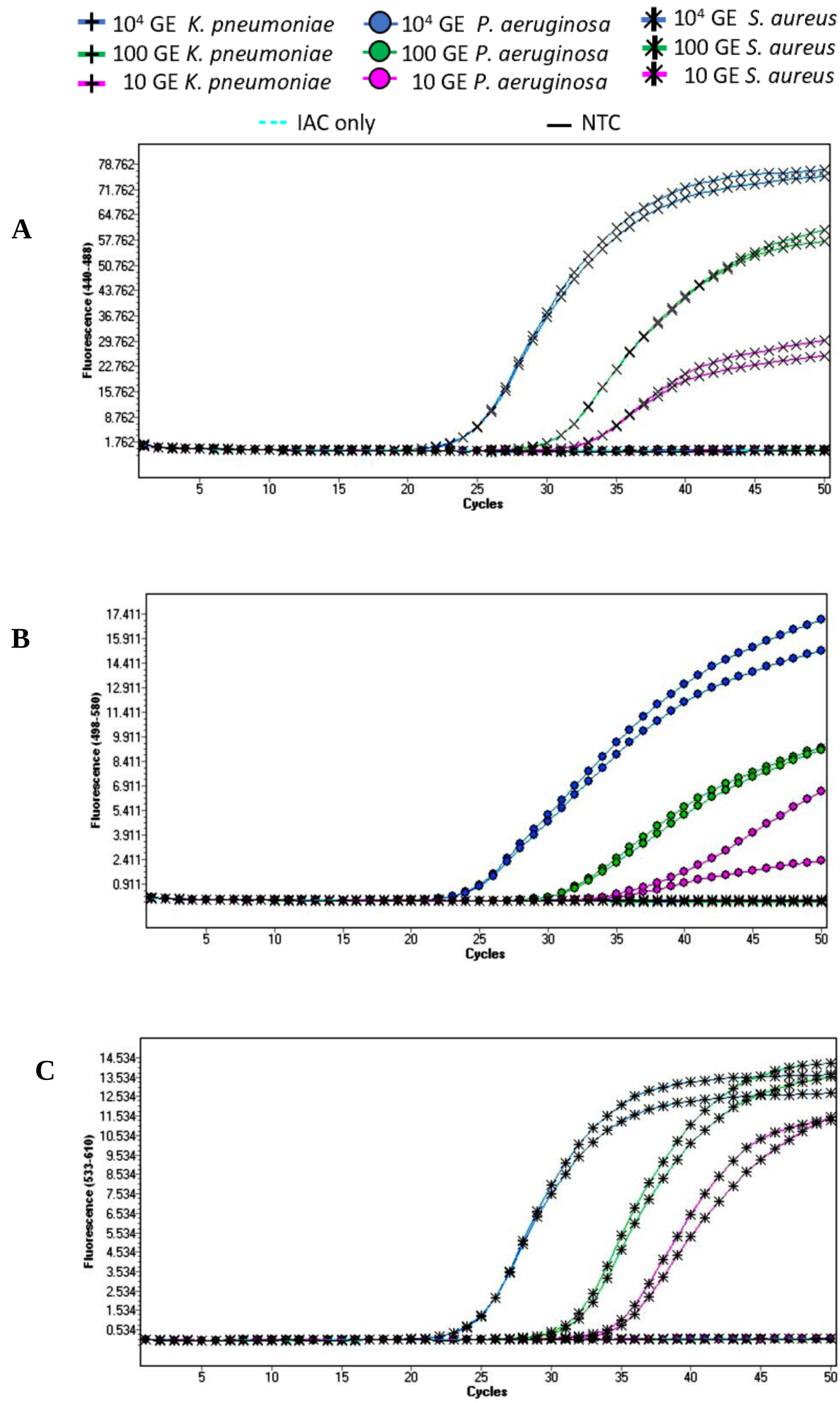
Enterococcus faecalis	DSM 20371	BHI	37°C	Microaerophilic
Enterococcus faecalis	DSM 20376	BHI	37°C	Microaerophilic
Enterococcus faecalis	DSM 20380	BHI	37°C	Microaerophilic
Enterococcus faecalis	DSM 20409	BHI	37°C	Microaerophilic
Enterococcus faecalis	DSM 20478	BHI	37°C	Microaerophilic
Enterococcus faecalis	LMG 17122	BHI	37°C	Microaerophilic
Enterococcus faecalis	LMG 17123	BHI	37°C	Microaerophilic
Enterococcus faecalis	NCTC 12201	BHI	37°C	Microaerophilic
Enterococcus faecalis	NCTC 12203	BHI	37°C	Microaerophilic
Enterococcus gallinarum	DSM 20628	BHI	37°C	Microaerophilic
Enterococcus gallinarum	DSM 20718	BHI	37°C	Microaerophilic
Enterococcus gallinarum	NCTC 12360	BHI	37°C	Microaerophilic
Enterococcus gilvus	LMG 13600	BHI	37°C	Microaerophilic
Enterococcus malodoratus	CIP 105126	BHI	37°C	Microaerophilic
Enterococcus malodoratus	LMG 12905	BHI	37°C	Microaerophilic
Enterococcus malodoratus	LMG 15718	BHI	37°C	Microaerophilic
Enterococcus raffinosus	DSM 5633	BHI	37°C	Microaerophilic
Enterococcus raffinosus	LMG 12172	BHI	37°C	Microaerophilic
Enterococcus raffinosus	LMG 12888	BHI	37°C	Aerobic
Escherichia coli	DSM 30083	TS	37°C	Aerobic
Haemophilus influenzae	DSM 24049	gDNA [c]		
Klebsiella aerogenes	DSM 12058	TS	37°C	Aerobic
Klebsiella aerogenes	DSM 30053	TS	37°C	Aerobic
Klebsiella aerogenes	LMG 2969	TS	37°C	Aerobic
Klebsiella aerogenes	NCTC 10006	TS	37°C	Aerobic
Klebsiella oxytoca	ATCC 43086	N	37°C	Aerobic
Klebsiella oxytoca	DSM 5175	N	37°C	Aerobic
Klebsiella variicola	ATCC 31488	TS	37°C	Aerobic
Klebsiella variicola	DSM 15968	N	37°C	Aerobic
Leclercia adecarboxylata	DSM 5077	N	37°C	Aerobic
Legionella pneumophila subsp. *pneumophila*	DSM 7513	gDNA [c]		
Lelliottia amnigena	DSM 4486	TS & 0.3% YE	30°C	Aerobic
Lelliottia nimipressuralis	DSM 18955	TS	37°C	Aerobic
Moraxella catarrhalis	DSM 11994	gDNA [c]		
Mycoplasma pneumoniae	ATCC 29342	gDNA [c]		
Pantoea agglomerans	NCTC 9381	TS	37°C	Aerobic
Pluralibacter gergoviae	ATCC 33028	TS	37°C	Aerobic
Proteus mirabilis	DSM 4479	gDNA [c]		
Proteus vulgaris	DSM 2140	gDNA [c]		
Pseudomonas alcaligenes	DSM 50342	TS	30°C	Aerobic
Pseudomonas chlororaphis	DSM 50083	TS	30°C	Aerobic
Pseudomonas fluorescenes	DSM 50090	TS	30°C	Aerobic
Pseudomonas luteola	DSM 6975	TS	30°C	Aerobic
Pseudomonas oleovorans	DSM 1045	TS	30°C	Aerobic
Pseudomonas putida	DSM 291	TS	30°C	Aerobic
Pseudomonas resinovorans	LMG 2274	TS	30°C	Aerobic
Pseudomonas stutzeri	LMG 11199	TS	30°C	Aerobic
Pseudomonas stutzeri	DSM 5190	TS	37°C	Aerobic
Raoultella ornithinolytica	DSM 7464	TS	30°C	Aerobic
Raoultella planticola	NCTC 9528	N	37°C	Aerobic
Raoultella planticola	DSM 2688	N	30°C	Aerobic

Raoultella planticola	DSM 4617	N	30°C	Aerobic
Raoultella terrigena	DSM 2687	N	30°C	Aerobic
Raoultella terrigena	DSM 7331	N	30°C	Aerobic
Serratia marcescens	DSM 1608	N	30°C	Aerobic
Serratia marcescens	DSM 30126	N	30°C	Aerobic
Staphylococcus epidermidis	ATCC 35984	BHI	37°C	Aerobic
Staphylococcus epidermidis	Strain 1457 [d]	BHI	37°C	Aerobic
Staphylococcus epidermidis	CSF 41489 [e]	BHI	37°C	Aerobic
Staphylococcus epidermidis	DSM 20044	TS	37°C	Aerobic
Staphylococcus felis	DSM 7377	TS	37°C	Aerobic
Staphylococcus haemolyticus	DSM 20263	TS	37°C	Aerobic
Staphylococcus sp.	ATCC 27626	TS	37°C	Aerobic
Streptococcus agalactiae	DSM 2134		gDNA [c]	
Streptococcus pneumoniae	DSM 11865		gDNA [c]	
Streptococcus pneumoniae	DSM 11867		gDNA [c]	
Streptococcus pyogenes	DSM 20565		gDNA [c]	
Tetragenococcus solitarius	DSM 5634	BHI	37°C	Aerobic
Tetragenococcus solitarius	LMG 12890	BHI	37°C	Aerobic

[a] TS= Tryptic Soy; BHI=Brain Heart Infusion; N= Nutrient, YE = Yeast Extract
[b] Microaerophilic conditions were achieved using Oxoid AnaeroJar 2.5L # AG0025A and CampyGen 2.5L #CN0025A (ThermoFisher, MA, US)
[c] Purified genomic DNA from stocks held within this laboratory
[d] Galac *et al*. [109]
[e] Galac *et al*. [110]

Table S2.4: Sequence of synthetic double-sided DNA fragment (gBlock®) used as template for the IAC assay in ESKAPE multiplex 1 & 2

Sequence (5′-3′)	CATAAAAAAAAGGACGTCGACGGACCAATTTTAGGATGACACCACTAATC GATCGTCCTTCGTCGTATCGCAAGTTTCGCGCCAGATCGAAGTGAGCATC GCTGCCAGGCGGGGCAAAGGATAGACGAACTCGCATGTTTCGTTACAGCA TTGTTTTTGCTAAACGGCTTCCTAGTCGTACGGCGAAAATTTAAATCGTA CAGCTCTTTGGTGACCACGCCTTTACCCCTTAGGCAATATAAATTGTAGA TCTTCTGACTGCGCGGTCCTTAGGATTATACGAACTCGACTGCCCAACCC CAAACGTGACATCTCGTCGGGACACCGTATTGGACCAGGGGGCTGCGGGC TTTTCGCCGGTTCATCCCTCAAACGGCGTTGCCCTAGCTTTTCCAATTCG CGCATAATTTGTTAGCAAAGGACACGTTAAGCACGTTAGCTCGTGCCCGT AGCCCCCAGCTACCGCTCCTCCGTACATTATAAAAACGGATGGATTACTT



10^4 GE K. pneumoniae
100 GE K. pneumoniae
10 GE K. pneumoniae
10^4 GE P. aeruginosa
100 GE P. aeruginosa
10 GE P. aeruginosa
10^4 GE S. aureus
100 GE S. aureus
10 GE S. aureus
IAC only
NTC
A
Fluorescence (440-488)
78.762
71.762
64.762
57.762
50.762
43.762
36.762
29.762
22.762
15.762
8.762
1.762
5 10 15 20 25 30 35 40 45 50
Cycles
B
Fluorescence (498-580)
17.411
15.911
14.411
12.911
11.411
9.911
8.411
6.911
5.411
3.911
2.411
0.911
5 10 15 20 25 30 35 40 45 50
Cycles
C
Fluorescence (533-610)
14.534
13.534
12.534
11.534
10.534
9.534
8.534
7.534
6.534
5.534
4.534
3.534
2.534
1.534
0.534
5 10 15 20 25 30 35 40 45 50
Cycles

D

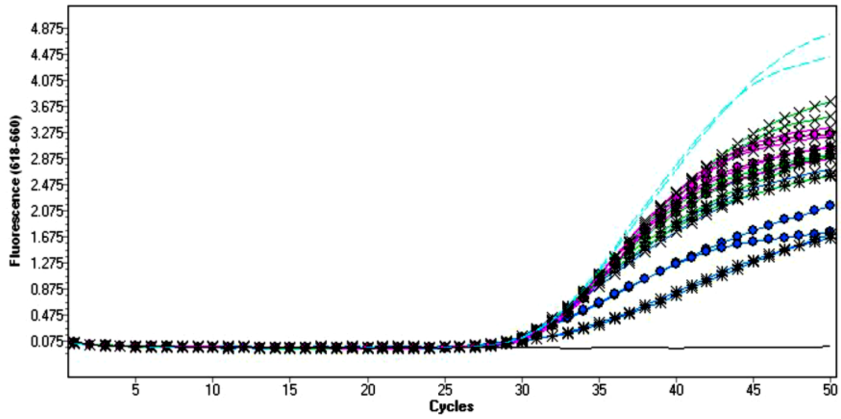
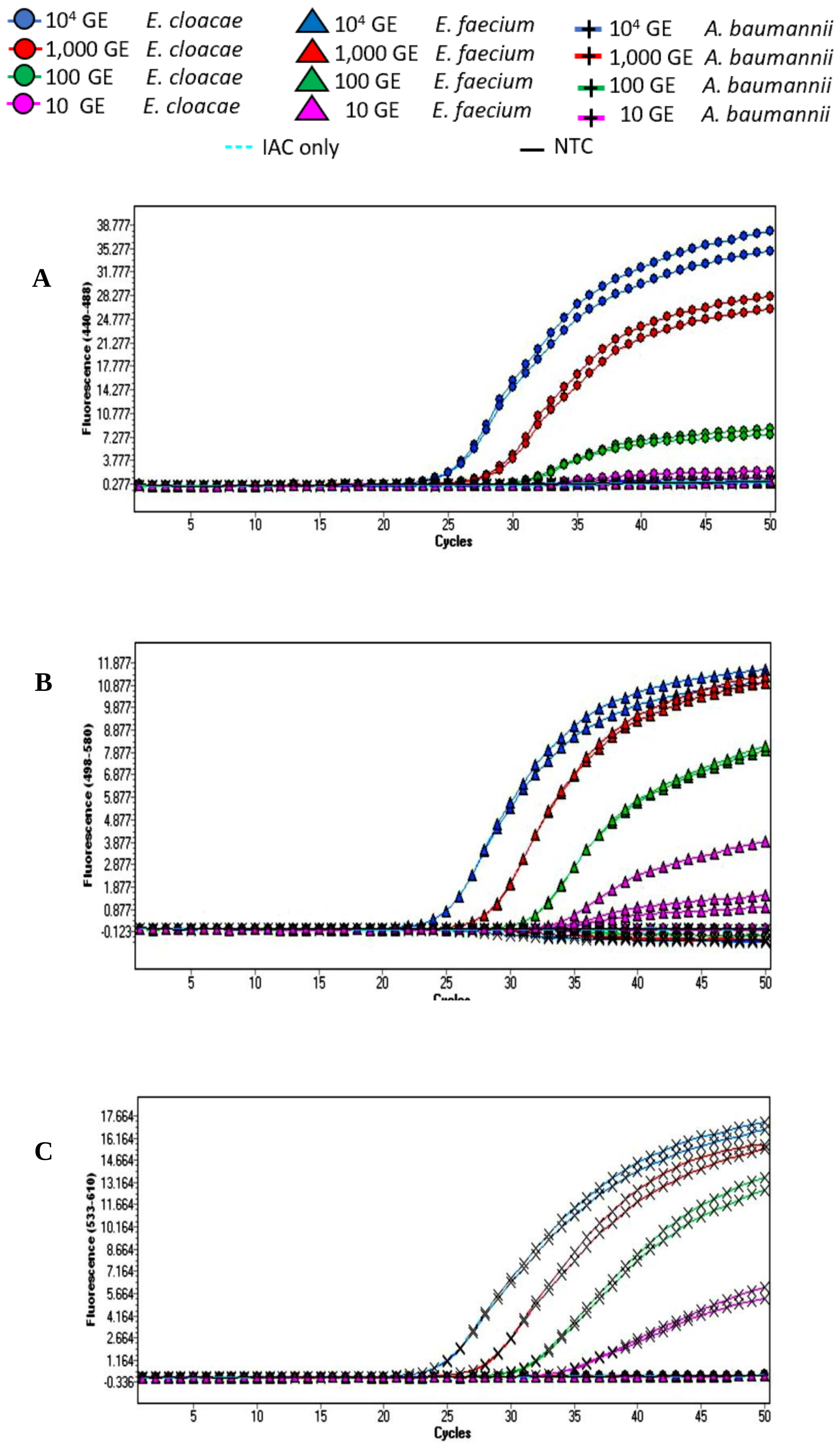
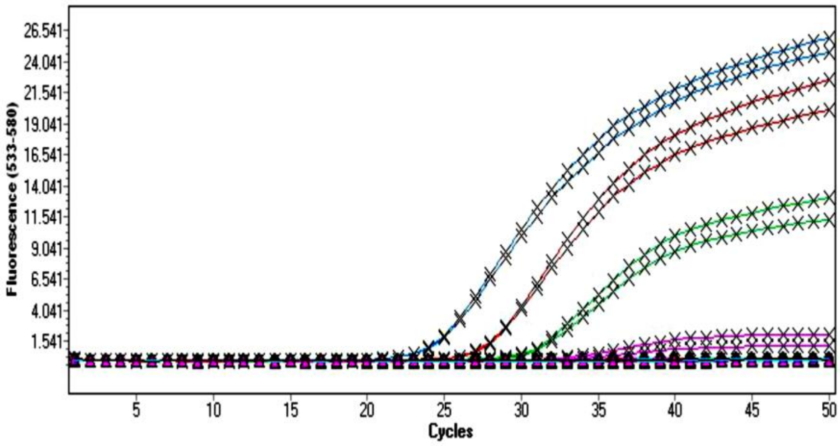


Figure S2.1: PCR amplification curves demonstrating the specific detection of target species at various concentrations (10,000 – 10 GE) in ESKAPE multiplex 1

Fig S2.1 (A) Amplification curves for *K. pneumoniae* DSM30104 (crosses) using the *lepA* assay in the CYAN500 channel (440-488 nm); (B) Amplification curves for *P. aeruginosa* DSM50071 (circles) using the *rpsG* assay in the FAM channel (498-580 nm); (C) Amplification curves for *S. aureus* DSM23622 (stars) using the *lepA* assay in the ROX channel (533-610 nm); (D) Amplification curves for the SIAC template spiked into each sample, and SIAC template only (dashed lines) using the IAC assay in the Cy5 (618-660 nm) channel. For all images (A-C): 10,000 GE = blue; 100 GE = green; 10 GE = pink, IAC template only = turquoise.


10⁴ GE E. cloacae
1,000 GE E. cloacae
100 GE E. cloacae
10 GE E. cloacae
10⁴ GE E. faecium
1,000 GE E. faecium
100 GE E. faecium
10 GE E. faecium
10⁴ GE A. baumannii
1,000 GE A. baumannii
100 GE A. baumannii
10 GE A. baumannii
IAC only
NTC
A
Fluorescence (440-488)
Cycles
B
Fluorescence (498-580)
C
Fluorescence (533-610)
Cycles

D



E

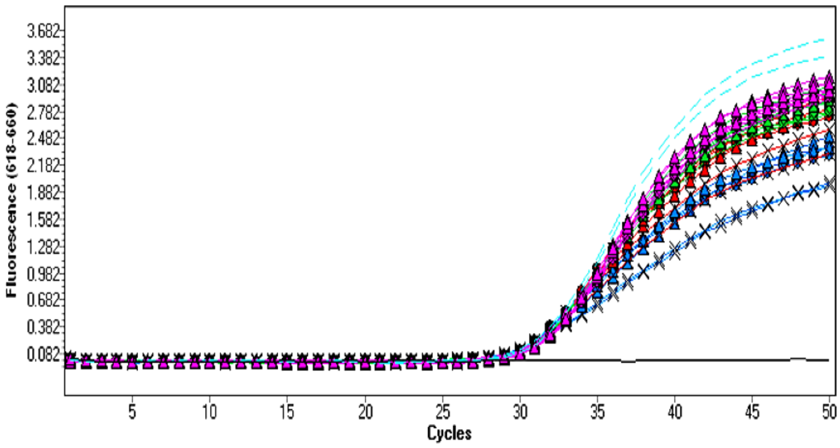


Figure S2.2: PCR amplification curves demonstrating the specific detection of target species at various concentrations (10,000 – 10 GE) in ESKAPE multiplex 2

Fig 2.2 (A) Amplification curves for *Enterobacter cloacae* ATCC13047 (circles) using the *radA* assay in the CYAN500 channel (498-550 nm); (B) Amplification curves for *Enterococcus faecium* DSM20477 (triangles) using the *lepA* assay in the FAM channel (498-580 nm); (C) Amplification curves for *A. baumannii* DSM30007 (crosses) using the *smpB A. baumannii*-specific assay in the ROX channel (533-610 nm); (D) Amplification curves for *A. baumannii* DSM30007 (crosses) using the *rpoB A. baumannii* group assay in the HEX channel (533-580 nm); (E) Amplification curves for the IAC template spiked into each sample, and IAC template only (dashed lines) using the IAC assay in the Cy5 channel (618-660 nm).

For all images (A-E) 10,000 GE = blue; 1,000 GE = red; 100 GE = green; 10 GE = pink, IAC-only = turquoise, negative template control (NTC) = black**.** No cross-reactions were observed against any non-target species tested for.

2.6 References

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Chapter 3: Detection and Differentiation of the *Klebsiella pneumoniae* Species Complex using Multiplex Real-Time PCR

Abstract

Klebsiella pneumoniae is an important human pathogen which has been shown to cause high levels of antimicrobial resistant infections worldwide. It is a frequent cause of healthcare-associated infection (HAI), resulting in severe invasive infections such as pneumonia, urinary tract, and bloodstream infections. However, despite its prominence as pathogen, the taxonomic status of the phylogroups of *K. pneumoniae* has only recently been resolved. The *Klebsiella pneumoniae* species complex (KpSC) is a group of closely related bacterial species that includes *Klebsiella pneumoniae, Klebsiella quasipneumoniae, Klebsiella variicola, Klebsiella quasivariicola,* and *Klebsiella africana.* The precise identification of these species is challenging, as they share many phenotypic and genotypic characteristics, which can lead to misidentification, even when molecular-based methods are used. There is a need for a rapid and accurate diagnostic method to distinguish between the closely related species of the KpSC. Here we describe the development of a novel multiplex real-time PCR assay for the detection of each of the KpSC members, and the specific identification of the species *K. pneumoniae*, *K. quasipneumoniae* and *K. variicola.* The assay has been shown to be 100% specific for its intended target species and is the first such rapid molecular diagnostic with the ability to differentiate and identify the most prominent KpSC species without the need for sequencing.

3.1 Introduction

Klebsiella species are the 3rd most frequently reported cause of healthcare associated infection [1-3]. They cause an estimated 7,440 deaths in the European Union/European Economic Area (EU/EEA) per year and are associated with 25% of the antimicrobial resistance-associated disability-adjusted life years (DALYs) [4]. The most frequent infection types due to *Klebsiella* are pneumonia, urinary tract infection and bacteraemia [5-7]. They can also colonise the gastrointestinal and nasopharyngeal tract of healthy humans and animals [6, 8-10]. *K. pneumoniae* is particularly associated with nosocomial infections displaying high levels of resistance to antimicrobials [11]. Although it is an important human pathogen [12], the methods used to routinely identify *K. pneumoniae* are imprecise [13]. There are several species which share 93%-95% Average Nucleotide Identity (ANI) to *K. pneumoniae*, forming a monophyletic clade known as the *K. pneumoniae* species complex (KpSC) [14, 15].

These species were initially recognised as phylogroups of *K. pneumoniae sensu stricto* [16]. Currently, the KpSC consists of *K. pneumoniae* (Kp1), *K. quasipneumoniae* subsp. *quasipneumoniae* (Kp2) and subsp. *similipneumoniae* (Kp4), *K. variicola* subsp. *variicola* (Kp3) *and* subsp. *tropica* (Kp5), *K. quasivariicola* (Kp6), and *K. africana* (Kp7). Advances in phylogenomics have allowed for the separation of these species and subspecies from *K. pneumoniae sensu stricto,* thus altering our understanding of the role of *K. pneumoniae* and its associated species complex in human infection [17].

The initial description of the KpSC was by Brisse *et al.* [16] when it was discovered that *K. pneumoniae* contained 3 distinct phylogroups, KpI, KpII, KpIII, based on *parC* and *gyrB* sequencing. Subsequently, KpII was split into two subgroups: KpII-A and KpII-B [18]. KpIII was transferred to a new species, *K. variicola* [19]. In the years that followed, KpII-A and KpII-B became *K. quasipneumoniae* subsp. *quasipneumoniae* and K. *quasipneumoniae* subsp. *similipneumoniae*, or Kp2 and Kp4 respectively [20]. The species designation for *K. pneumoniae*, *K. quasipneumoniae* and *K. variicola* has been in place for several years and is well established in literature and diagnostics [21, 22].

The phylogroups Kp5 and Kp6 were recognised as distinct taxa but not given species designation until more recently [23]. *K. quasivariicola* (Kp6) has yet to be validly recognised as a species [24], while the description of Kp5 as the *species K. variicola* subsp. *tropica* coincided with the discovery of a novel KpSC species: *K. africana* or Kp7 [14]. The changes to the KpSC phylogroups Kp5-Kp7 in recent years has led to some inconsistencies in nomenclature and has also raised questions on the accuracy of KpSC diagnostics developed prior to their discovery [25, 26].

The current taxonomic status of the KpSC has been supported by data from several studies which analysed the genomes of KpSC and other *Klebsiella*. This includes the position of the subspecies of *K. pneumoniae* (subsp. *pneumoniae, ozenae and rhinoscleromatis*) in the phylogroup Kp1 [27], and the species and subspecies status of the other KpSC members [15, 28] (**Table 3.1**). Whole genome sequencing has shown that the KpSC has 3%-4% nucleotide divergence across the core genome and shares approximately 90% ANI with other *Klebsiella* species [29, 30]. This was further demonstrated by Rodrigues [14] when they analysed ANI and *in silico* DNA-DNA hybridisation (isDDH) data, and generated phylogenetic trees based on recombination-purged core genomes, housekeeping genes and chromosomal β-lactamases. Taken together, these studies indicate that the KpSC consists of 5 closely related, but distinct species with diverse roles in infection, colonisation, and the environment.

Table 3.1: The *Klebsiella pneumoniae* species complex: subspecies and associated phylogroups

Species	Subspecies	Phylogroup	Reference
K. pneumoniae	*pneumoniae, ozaenae, rhinoscleromatis*	KpI / Kp1	[16]
K. quasipneumoniae	*quasipneumoniae*	KpII-A / Kp2	[20]
K. variicola	*variicola*	KpIII / Kp3	[19]
K. quasipneumoniae	*similipneumoniae*	KpII-B / Kp4	[20]
K. variicola	*tropica*	Kp5	[14]
K. quasivariicola		Kp6	[24]
K. africana		Kp7	[14]

The close genetic relatedness of the KpSC makes correct identification difficult. Culture-based diagnostics are unable to differentiate between species. *K. variicola* may be differentiated by a negative adonitol fermentation, but this is not routinely performed in the clinical laboratory and can vary between strains [31, 32]. Existing molecular methods predate many of the taxonomic updates in the KpSC and thus may not be accurate methods of identification [21, 22]. This has resulted in a lack of reliable data concerning their role in infection and overall epidemiology [33, 34].

Since the earliest studies of the KpSC, it has been suggested that *K. pneumoniae sensu stricto*, or Kp1 causes the majority (80%) of infections [35]. More recent studies using clinical isolates and publicly available sequence data range between 65%-94% for *K. pneumoniae*. *K. variicola* is typically the second most frequently isolated, at 4%-25%, followed by *K. quasipneumoniae* at 1.5%-8% [15, 22, 28, 36-39]. The level of *K. pneumoniae* has been found to be as high as 99.5% (n=939) amongst isolates which were submitted to the European Study on Carbapenemase-Producing Enterobacteriales (EuSCAPE) study as “suspected non-susceptible” to meropenem (*K. variicola* = 0.3%, n=3; *K. quasipneumoniae* = 0.2%, n=1). [40, 41]. This suggests that amongst European KpSC lineages, almost all isolates displaying meropenem resistance are *K. pneumoniae*.

There are other studies which suggest levels of Antimicrobial Resistance (AMR), disease prognosis and patient outcomes may differ amongst the KpSC. Several investigations into species-specific outcomes of KpSC infection have produced varying results. The first, conducted using multilocus sequence typing on bloodstream infection isolates in Sweden, showed that 30-day mortality was higher for patients infected with *K. variicola* in a multivariate analysis adjusted for patient comorbidities (29.4% Vs 13.5%; p= 0.037) [36]. A study which focused on extended-spectrum β-lactamase (ESβL)-producing isolates found no significant difference in virulence or mortality between *K. pneumoniae, K. quasipneumoniae* and *K. variicola* [34]. Imai *et al.* [37] found 27% (n=119) of bloodstream infections attributed to *K. pneumoniae* in Japan were other KpSC. *K. variicola* and *K. quasipneumoniae* were associated with older (>80 years) patients and those with malignancy, respectively. For military personnel with trauma-related injuries, it was found that *K. variicola* was associated with bacteraemia, and that levels of Multidrug Resistance (MDR) in *K. pneumoniae* were considerably higher [26]. There were no significant differences between clinical characteristics or disease complications in a study investigating KpSC isolates from urine [39], however they also found that *K. pneumoniae* had higher levels of resistance to several types of antimicrobials.

Relative rates of each KpSC, geographic location, patient comorbidities, infectious site, and local AMR patterns may all play a role in affecting the outcomes of these studies. Many studies into the KpSC or *K. pneumoniae* are restricted to bloodstream or MDR isolates [42, 43]. This may result in disproportionate representation of certain species-specific outcomes. There is therefore a need to investigate KpSC from a diverse range of clinical and environmental sample types [17]. The ability to differentiate members of the KpSC rapidly and accurately would improve our understanding of their epidemiology and resistance patterns.

Due to the challenges associated with identifying KpSC using culture and/or molecular-based methods, we sought to develop a rapid, culture-free diagnostic assay with improved accuracy for species identification. A series of novel real-time PCR assays were developed to specifically detect (i) the KpSC, (ii) *K. pneumoniae,* (iii) *K. variicola,* and (iv) *K. quasipneumoniae.* These assays were then combined to create a multiplex real-time PCR which can be used to simultaneously detect and differentiate between the most frequently reported members of the KpSC.

3.2 Materials & Methods

3.2.1 Nucleic Acid Diagnostic Target Identification

Nucleic acid diagnostic (NAD) targets for the real-time PCR assays developed in this study were identified by evaluating housekeeping genes which are conserved in *Klebsiella* or have previously been used in nucleic acid diagnostic assays [44-46]. For each target gene under investigation, nucleotide sequence information was retrieved from the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov) or from the American Type Culture Collection (ATCC) (https://genomes.atcc.org) for Klebsiella and other closely related genera. FASTA sequence alignments were created using the Clustal Omega tool from the European Bioinformatics Institute [47, 48]. Diagnostic motifs which would facilitate the design of real-time PCR assays specific for KpSC members were identified. If these features, such as insertions and deletions (indels), regions of nucleotide conservation, or Single Nucleotide Polymorphisms (SNPs) were uniquely present in KpSC, the NAD target was further evaluated.

3.2.2 Bacterial Strains & Culture Conditions

A panel of *Klebsiella*, *Raoultella*, and other closely related Enterobacteriaceae were used to validate the multiplex real-time PCR developed in this study. A total of 54 culture collection isolates were selected, including representative species from each KpSC phylogroup. Recommended growth conditions from the originating culture collection were followed (**Table 3.2**). Each strain was grown on nutrient agar (LabM, Lancashire, UK), tryptone soya agar (Oxoid, Basingstoke, UK), or tryptone soya agar with the addition of 0.3% (w/v) yeast extract (Oxoid), for 24 h or until sufficient growth was observed. A single colony was then transferred to liquid medium and incubated for a further 18 h with continuous shaking at 120 rpm.

Table 3.2: Growth conditions and details of culture collection strains used for specificity testing

Species	Subspecies	ID[a]	KpSC[b]	Temp (°C)	Medium[c]
Klebsiella pneumoniae	*pneumoniae*	CIP 104216	1	37	TS
Klebsiella pneumoniae	*pneumoniae*	DSM 681	1	37	N
Klebsiella pneumoniae	*pneumoniae*	DSM 2026	1	37	N
Klebsiella pneumoniae	*pneumoniae*	DSM 5758	1	37	N
Klebsiella pneumoniae	*pneumoniae*	DSM 9377	1	37	N
Klebsiella pneumoniae	*pneumoniae*	DSM 9376	1	37	N
Klebsiella pneumoniae	*pneumoniae*	DSM 11678	1	37	N
Klebsiella pneumoniae	*pneumoniae*	DSM 12059	1	37	N
Klebsiella pneumoniae	*pneumoniae*	DSM 16609	1	37	N
Klebsiella pneumoniae	*pneumoniae*	DSM 30102	1	37	N
Klebsiella pneumoniae	*pneumoniae*	DSM 30104	1	37	N
Klebsiella pneumoniae	*ozaenae*	DSM 16358	1	37	N
Klebsiella pneumoniae	*rhino-scleromatis*	DSM 16231	1	37	N
Klebsiella quasipneumoniae	*quasi-pneumoniae*	CIP 111839	2	37	TS
Klebsiella quasipneumoniae	*quasi-pneumoniae*	CIP 111852	2	37	TS
Klebsiella quasipneumoniae	*quasi-pneumoniae*	DSM 28211	2	37	N
Klebsiella variicola	*variicola*	ATCC 31488	3	37	N
Klebsiella variicola	*variicola*	CIP 108642	3	30	TS
Klebsiella variicola	*variicola*	DSM 15968	3	37	TS
Klebsiella quasipneumoniae	*simili-pneumoniae*	CIP 111862	4	30	TS
Klebsiella quasipneumoniae	*simili-pneumoniae*	CIP 111869	4	30	TS
Klebsiella quasipneumoniae	*simili-pneumoniae*	DSM 28212	4	37	N
Klebsiella quasipneumoniae	*simili-pneumoniae*	DSM 100662	4	37	N
Klebsiella variicola	*tropica*	CIP 111654	5	30	TS
Klebsiella variicola	*tropica*	CIP 111870	5	30	TS
Klebsiella variicola	*tropica*	CIP 111898	5	37	TS
Klebsiella quasivariicola		CIP 111871	6	30	TS
Klebsiella quasivariicola		CIP 111897	6	37	TS
Klebsiella africana		CIP 111653	7	30	TS
Klebsiella aerogenes		DSM 12058		37	TS
Klebsiella aerogenes		DSM 30053		37	TS
Klebsiella aerogenes		LMG 2969		37	TS
Klebsiella grimontii		CIP 111401		37	TS
Klebsiella michiganensis		CIP 110787		30	TS
Klebsiella oxytoca		ATCC 43086		37	N

Klebsiella oxytoca	DSM 5175	37	N
Klebsiella pasteurii	CIP 111696	37	TS
Klebsiella spallanzanii	CIP 111695	37	TS
Citrobacter freundii	DSM 30039	30	TS & 0.3% YE
Cronobacter sakazakii	DSM 11467	37	TS
Enterobacter cloacae	ATCC 13047	37	TS
Escherichia coli	DSM 30083	37	TS
Kluyvera intermedia	CIP 79.27	30	TS
Leclercia adecarboxylata	DSM 5077	37	N
Lelliottia amnigena	DSM 4486	30	TS & 0.3% YE
Pantoea agglomerans	NCTC 9381	30	TS
Pluralibacter gergoviae	ATCC3 3028	37	TS
Raoultella ornithinolytica	DSM 7464	30	N
Raoultella planticola	CIP 100751	30	TS
Raoultella planticola	DSM 2688	30	N
Raoultella planticola	DSM 4617	30	N
Raoultella terrigena	DSM 2687	30	N
Raoultella terrigena	DSM 7331	30	N
Serratia marcescens	DSM 30126	30	N

[a] Culture collection identification number
[b]*Klebsiella pneumoniae* species complex phylogroup
[c]Growth medium: TS = tryptone soya, N = nutrient, TS & 0.3% YE = tryptone soya & 0.3% w/v yeast extract

3.2.3 Extraction & Quantification of Genomic DNA

Genomic DNA (gDNA) was extracted from liquid culture using the Zymo Quick gDNA Miniprep kit (Zymo Research, Irvine, California, United States) following the manufacturer's protocol for "Cell Suspensions and Proteinase K Digested Samples"[49]. Liquid culture (1 mL) was centrifuged at 10,000 x *g* for 3 mins. The supernatant was discarded and 800 µL of Zymo Genomic Lysis buffer was added directly to the pellet and resuspended with vortexing. The solution was incubated at room temperature for 5 mins, vortexed briefly, and incubated for a further 5 mins at room temperature. The lysate was transferred to a Zymo-Spin IICR Column fitted with a collection tube and centrifuged at 10,000 x *g* for 1 min. The manufacturer's protocol was followed thereafter until the final elution step. gDNA was eluted using 50 µL nuclease-free water (Ambion, ThermoFisher, Waltham, Massachusetts, US) followed by incubation at room temperature for 2 mins and centrifugation for 30 s at 21,300 x *g*. A further 50 µL nuclease-free water was added to the spin column followed by centrifugation at 21,300 x *g* to give a final elution volume of 100 µL.

A Qubit 1.0 fluorometer and High Sensitivity dsDNA kit (ThermoFisher, Waltham, MA, US) were used to quantify eluted gDNA. Samples were stored at -20°C prior to use in PCR.

3.2.4 Design of Primers and Probes for Real-Time PCR

Nucleotide sequence alignments of NAD targets deemed suitable for KpSC differentiation were analysed for regions in which primers and probes complementary to the target sequence could be developed. The accession numbers of the 348 nucleotide sequences used for the final *in silico* diagnostic target alignments and test development can be found in **Supplementary Table S3.1**.

Novel real-time PCR assays were created to specifically detect the KpSC (Kp1-Kp7), *K. pneumoniae* (Kp1), *K. quasipneumoniae* (Kp2 & Kp4), and *K. variicola* (Kp3 & Kp5). Oligonucleotide primers and probes were designed manually in accordance with general recommendations and guidelines [50] (**Table 3.3**). Primers were designed to have a melting temperature (T_m) of 56-60°C. Probes were designed to have T_m approximately 5°C above the average primer T_m for that assay. The T_m for each oligonucleotide was determined using the online Oligo Analyser™ tool (https://eu.idtdna.com/calc/analyzer) Unspecific detection was investigated in silico by verifying the difference in melting temperature (ΔT_m) was >10°C for the probe sequence and the complementary sequence of non-target species using MeltCalc© 3 [51]

A non-competitive internal amplification control (IAC) [52] assay was designed and included in the multiplex. This assay targets a 500 bp synthetic gene fragment (**Supplementary Table S3.2**). The primers and probe for the IAC were manually designed following the same general guidelines as above.

All oligonucleotide primers and probes used in this study were manufactured by Integrated DNA Technologies (Coralville, Iowa, US).

3.2.5 Optimisation of Multiplex Real-Time PCR Reaction

Real-time PCR experiments were performed using a Roche LightCycler®480 System II and the LightCycler® 480 Probes Master kit (Roche Diagnostics, Basel, Switzerland). Assay performance was initially investigated in monoplex format. Prior to multiplexing, a colour compensation file was generated per the manufacturer's instructions [53] and applied to each subsequent multiplex PCR experiment.

Optimum reaction conditions (thermocycling parameters, oligonucleotide concentrations) for multiplex real-time PCR were determined empirically, using $1x10^4$ Genome Equivalents (GE) of gDNA as template. Genome size was calculated based on the genome size of *K. pneumoniae,* Kp1 (DSM30104^T); *K. quasipneumoniae* subsp. *quasipneumoniae* 01A030, Kp2 (DSM28211^T); Kp7 *K. variicola* subsp. *variicola* F2R9, Kp3 (DSM 15968^T); and *K. africana* 200023, (CIP111653^T) described in Rodrigues *et al.* [14] which equates to approximately 6.0 fg of DNA per cell. The optimised PCR master mix contained 2X LightCycler® 480 Probes Master; primers and probes as outlined in Table 3.3; template DNA (5µL); IAC template (500 copies), adjusted to a final volume of 20µL with nuclease-free water (Ambion, ThermoFisher). Following template addition, the sealed plates were incubated at 55°C with shaking at 180 rpm for 5 mins (Thermomixer® C, Eppendorf) prior to thermocycling. The thermocycling parameters used were as follows: initial enzyme activation for 10 mins at 95°C, 50 cycles of 95°C for 10 s and 63°C for 30 s, and a final single cooling step of 40°C for 10 s. The temperature transition rate was 4.4°C/s while heating, and 2.2°C/s while cooling.

Table 3.3: The sequence, target and optimised concentrations of oligonucleotide primers and probes developed for the KpSC real-time multiplex assay

Oligonucleotide	Function	Target	Sequence (5′-3′) [a]	Final Conc. (µM)
Kqp_F	*K. quasipneumoniae* forward primer	*rapZ*	TGTCGAGCAAGAATTTGTC	0.500
Kqp_P	*K. quasipneumoniae* probe		**HEX/**ATTGTCGAT**/ZEN/**ACCTCGGAGATGTC**/Iowa Black FQ**	0.200
Kqp_R	*K. quasipneumoniae* reverse primer		GAAGGATTCGAAGACCATG	0.500
Kpneu_F	*K. pneumoniae* forward primer	*lepA*	GCTGAACTTTATCGACACC	0.500
Kpneu_P	*K. pneumoniae* probe		**CYAN500/**AGATGGATCTCGAAGTGGTTCC**/Iowa Black FQ**	0.200
Kpneu_R1	*K. pneumoniae* reverse primer		CAGGTCGATCTTGTTGAG	0.250
Kpneu_R2	*K. pneumoniae* reverse primer		GCAGGTCGATCTTATTGAG	0.250
Kvarii_F1	*K. variicola* forward primer	*lepA*	TCAGCAGTTCGATATCGC	0.500
Kvarii_F2	*K. variicola* forward primer		AAGCTGCTGCAGAAGC	0.500
Kvarii_P	*K. variicola* probe		**FAM/**GGCAAATA**/ZEN/**ATTAAGGAGTTGG**/Iowa BlackFQ**	0.200
Kvarii_R	*K. variicola* reverse primer		CTGATTTTGGTCATTGCGA	0.500
ZKIR_F	Pan-KpSC forward primer	ZKIR	ATCTGTTACTTTATCACGTTCC	1.000
ZKIR_P1	Pan-KpSC probe 1		**ROX/**ATGCTATATCTGAAGTGTCTCATTTTCG**/Iowa Black RQ**	0.400
ZKIR_P2 [b]	Pan-KpSC probe 2		**ROX/**TGCTATATCTGAAATGTCTCATTTTCGG**/Iowa Black RQ**	0.400
ZKIR_R2	Pan-KpSC reverse primer		AGTGGCGAATGCAATCA	1.000
IAC_F	IAC primer	Synthetic Construct	ATGCCAGTCAGCATAAGGA	0.250
IAC_P	IAC probe		**Cy5/**TCGGCACTA**/TAO/**CCGACACGAAC**/Iowa Black RQ**	0.200
IAC_R	IAC primer		CAGACCTCTGGTAGGATGTAC	0.250

[a] Flurophores and quenchers are shown in **bold**; HEX = 4,4,7,2,4,5,7-hexachloro-6-carboxyfluorescein, CYAN500 = CYAN500 N-hydroxysuccinimide ester, FAM = 6-carboxyfluorescein, ROX = carboxy-X-rhodamine, Cy5 = indodicarbocyanine 5; Iowa Black®, ZEN™ and TAO™ are non-abbreviated trademarks
[b] Required for the detection of *K. africana* (Kp7)

3.2.6 Determination of Specificity and Analytical Sensitivity

In silico analysis of 348 diagnostic target sequences (**Supplementary Table S3.1**) were used to design primers and probes which were specific to the KpSC or KpSC member. Following the design of each assay, *in vitro* specificity was tested in both monoplex, and multiplex format using 1 x 10^4 GE of gDNA from each of the 54 strains outlined in **Table 3.2**. An additional dilution series of the type strains *K. pneumoniae* DSM30104, *K. quasipneumoniae* DSM28211 and *K. variicola* DSM15968 was created from 1 x 10^4 GE to 10 GE to determine the analytical sensitivity of each assay in the multiplex.

3.3 Results

3.3.1 Nucleic Acid Diagnostic Target Identification

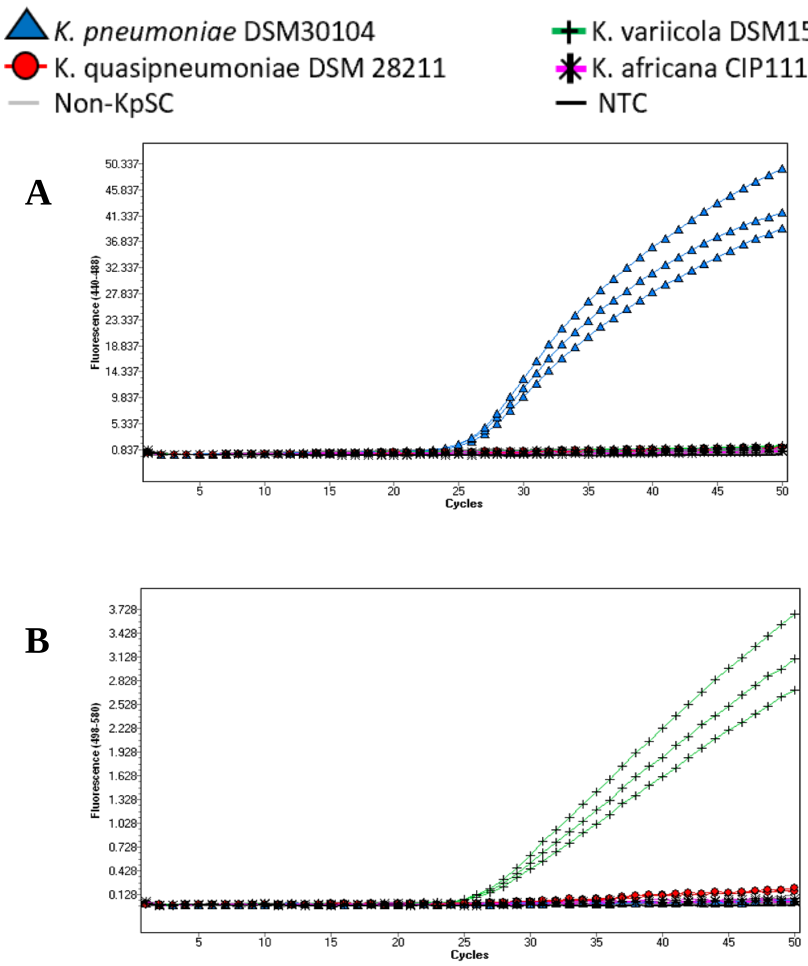
Regions of the *Klebsiella* genome which have previously been shown to be conserved, or to act as suitable NAD targets were evaluated. Nucleotide sequence alignments of these regions were assessed for the presence of unique diagnostic motifs (indels, SNPs) in the KpSC, *K. pneumoniae, K. quasipneumoniae* and *K. variicola*. For the assay to detect all KpSC members, an intergenic region referred to as the ZKIR (*zur-khe* intergenic region) was chosen [46]. This region is highly conserved amongst KpSC, making it a suitable target to differentiate KpSC members from other *Klebsiella*. Nucleotide conservation in ZKIR meant that it was not possible to design species-specific primers and probes in this region. Alternative NAD targets, namely the genes encoding elongation factor 4 (*lepA*) and the small RNA-binding protein RapZ (*rapZ*) [45, 54, 55] were found to contain sufficient nucleotide heterogeneity to differentiate *K. pneumoniae, K. quasipneumoniae* and *K. variicola*.

3.3.2 Design of Primers and Probes for Real-Time PCR

Diagnostic targets with which could be used to differentiate the KpSC, *K. pneumoniae, K. variicola,* and *K. quasipneumoniae* via a real-time PCR were identified. Nucleotide sequence alignments of ZKIR, *lepA* and *rapZ* from *Klebsiella* and other Enterobacteriaceae (**Supplementary Table S3**.1) were used to design primers and probes which would specifically detect the KpSC or desired KpSC member. The assays to specifically detect *K. pneumoniae* and *K. variicola* both target the *lepA* gene, however the primers and probes are independent of each other and anneal at different regions of the *lepA* gene. The *rapZ* gene was targeted for the final species-specific assay to detect *K. quasipneumoniae*. This is the first description of *rapZ* as a diagnostic marker. The sequences for the oligonucleotide primers and fluorescently labelled hydrolysis probes developed in this study are listed in **Table 3.3**.

3.3.3 Validation of Assay Specificity *in vitro*

The specificity of each real-time PCR assay was initially determined *in silico* using nucleotide sequence alignments of the diagnostic target for that assay. A total of 348 FASTA sequences from *Klebsiella*, *Raoultella* and other *Enterobacteriaceae* were retrieved from the NCBI GenBank or ATCC genomes collection. Additional *in vitro* validation using gDNA extracted from a panel of culture collection isolates (**Table 3.2**) was performed in monoplex and multiplex formats. This panel contains representative strains from each KpSC phylogroup and from closely related species. The assays were found to be 100% specific for the 54 strains tested.



C

D

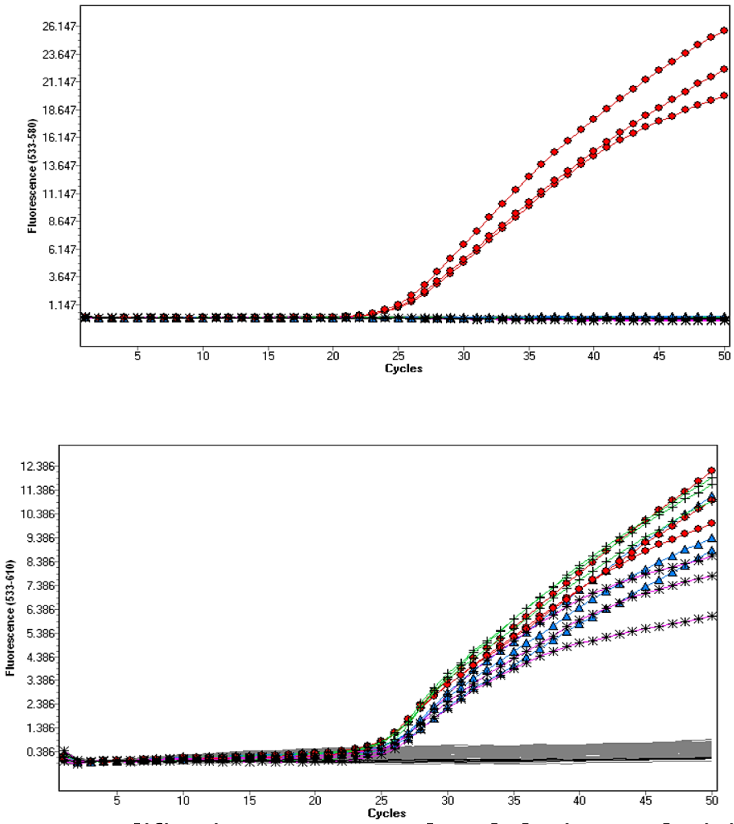


Figure 3.1: PCR amplification curves produced during exclusivity testing for KpSC multiplex using 10,000 GE of each species as template

Fig S3.1 (A) Amplification curves for *K. pneumoniae* DSM30104 (triangles) using the *lepA* assay in the CYAN500 channel (440-448 nm); (B) Amplification curves for *K. variicola* DSM15968 (circles) using the *lepA* assay in the FAM channel (498-580 nm); (C) Amplification curves for *K. quasipneumoniae* DSM28211 (crosses) using the *rapZ* assay in the HEX channel (533-580 nm); (D) Amplification curves for KpSC members (*K. pneumoniae* DSM30104 (triangles) *K. variicola* DSM15968 (circles) *K. quasipneumoniae* DSM28211 (crosses) and *K. africana* CIP111653 (stars) using the ZKIR-2 assay in the ROX channel (533-610 nm).

This multiplex real-time PCR assay can be used to detect all KpSC members (**Figure 3.1 D**), and to specifically detect *K. pneumoniae* (**Figure 3.1 A**), *K. variicola* (**Figure 3.1 C)** and *K. quasipneumoniae* (**Figure 3.1B**). The emission wavelength of the fluorophores used to label the hydrolysis probe in each assay allows for differentiation in multiplex format (**Table 3.4**). This format enables the simultaneous detection and differentiation of these important Klebsiella species and will enhance current species identification methods. The detection matrix for the discrimination of *K. pneumoniae, K. variicola* and *K. quasipneumoniae,* and for the presumptive identification of *K.*

quasivariicola and *K. africana* is shown in **Table 3.4**. A positive result in the pan-KpSC channel and the Cy5 IAC channel must be observed for the result to be considered valid. Additional positive channels in CYAN500, FAM, or ROX indicate that the sample is positive for *K. pneumoniae, K. variicola,* or *K. quasipneumoniae,* respectively.

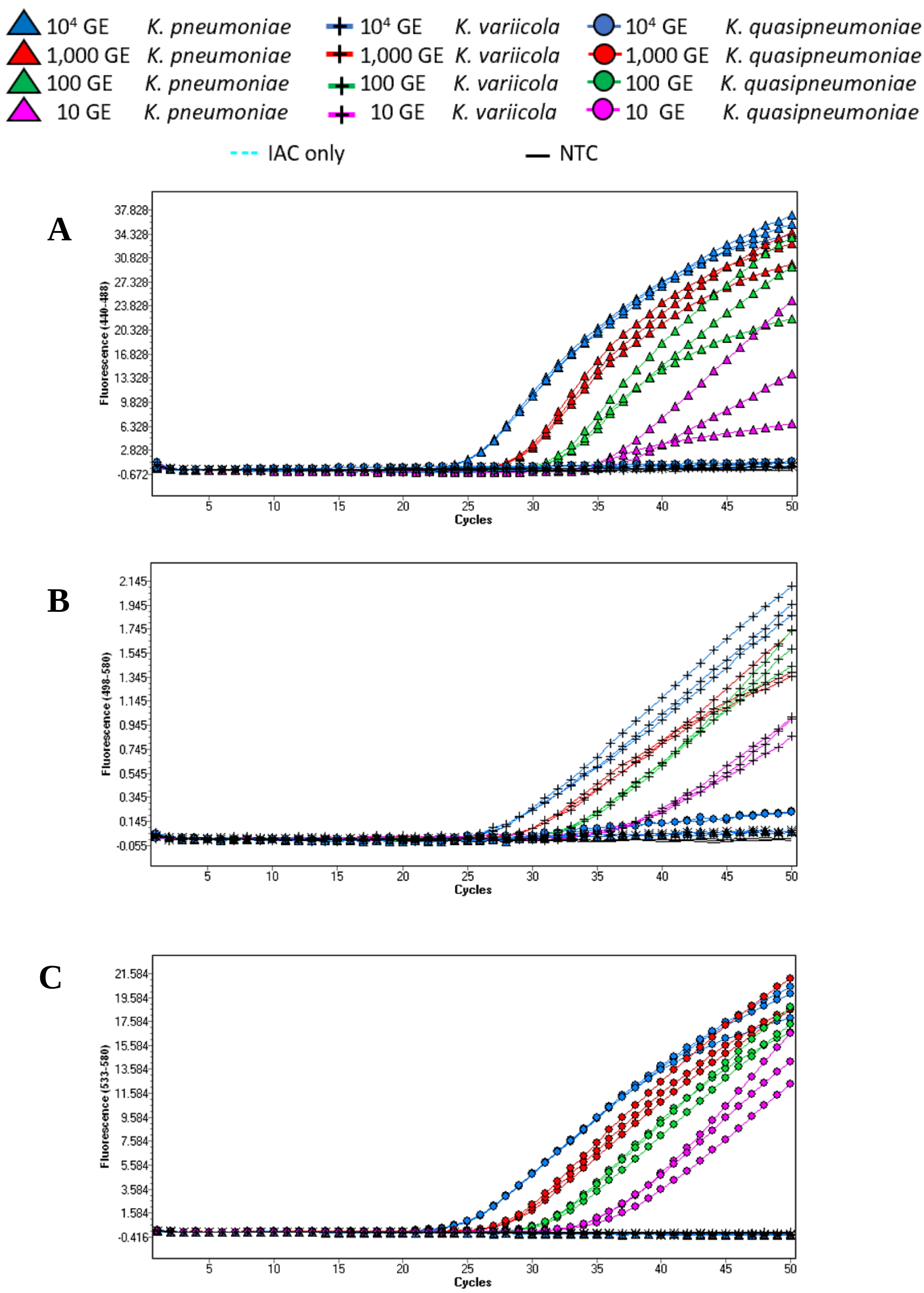
Table 3.4: Multiplex results interpretation

Fluorophore	**CYAN 500**	**FAM**	**HEX**	**ROX** [b]	**Cy5** [c]	
Excitation - Emission [a]	440-488	498-580	533-580	533-610	618-660	**Species (Phylogroup)**
	+	-	-	+	+	*K. pneumoniae* (Kp1)[b]
	-	+	-	+	+	*K. variicola* (Kp3 / Kp5)[b]
	-	-	+	+	+	*K. quasi-pneumoniae* (Kp2 / Kp4)[b]
	-	-	-	+	+	KpSC other than Kp1-Kp5 [d]
	-	-	-	-	-	Invalid IAC (must be repeated)
	-	-	-	-	+	Negative [e]

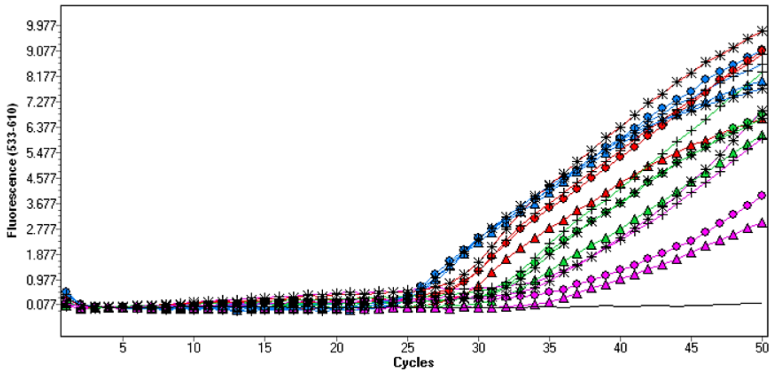
[a]Wavelength in nanometres
[b]Detection in both the identifying channel (Cyan 500, FAM, or HEX) and in ROX is required for the result to be considered valid
[c]In the presence of high concentrations of primary target, the IAC may become inhibited
[d]Indicative of *K. quasivariicola* (Kp6) or *K. afriana* (Kp7). Requires confirmatory testing
[e]No KpSC detected in sample

3.3.4 Experimental Determination of Analytical Sensitivity

The analytical sensitivity was assessed using a serial dilution of quantified gDNA from the type strain of each target species. Using the optimised reaction conditions described, analytical sensitivity of 10 GE was achieved for each target strain tested (**Figure 3.2**).



D



E

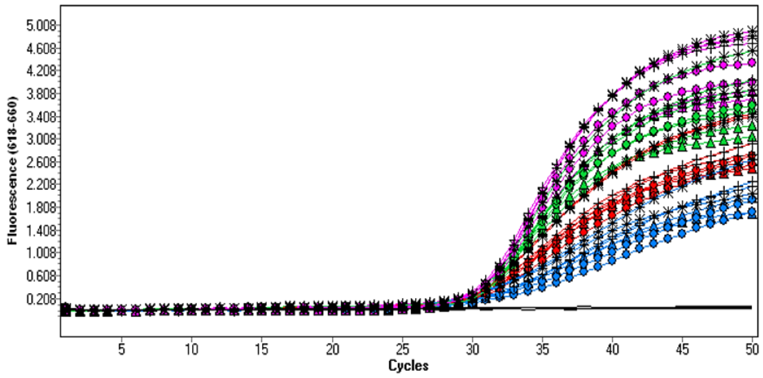


Figure 3.2: PCR amplification curves demonstrating detection of target species to 10 GE

Figure S3.2 (A) Amplification curves for *K. pneumoniae* DSM30104 dilution series (triangles) using the *lepA* assay in the CYAN500 channel (440-448 nm); (B) Amplification curves for *K. variicola* DSM15968 (circles) using the *lepA* assay in the FAM channel (498-580 nm); (C) Amplification curves for *K. quasipneumoniae* DSM28211 (crosses) dilution series using the *rapZ* assay in the HEX channel (533-580 nm). Amplification curves for a dilution series of all KpSC members (*K. pneumoniae* DSM30104 (triangles) *K. variicola* DSM15968 (circles) *K. quasipneumoniae* DSM28211 (crosses) using the ZKIR assay in the ROX channel (533-610 nm) with all non-KpSC members labelled with grey lines; (E) Amplification curves for IAC assay targeting the synthetic construct with the no template control visible as a solid black line in the Cy5 channel (618-660 nm). For all assays (A-D) 10,000 GE = blue; 1,000 GE = red; 100 GE = green; 10 GE = pink

3.4 Discussion

In the global burden of bacterial AMR study, *K. pneumoniae* was the second most reported pathogen in deaths associated with AMR [56]. It is frequently associated with MDR healthcare associated infections [33, 57, 58]. Although it is recognised as a major cause of AMR infection, methods used to identify *K. pneumoniae* are imprecise [13, 59]. Recent advances in phylogenetics have revealed several new species which form a complex with *K. pneumoniae*, and which can only be differentiated using WGS [11, 14]. Each member of the KpSC has been implicated in human infection and colonisation [60, 61]. There is also evidence to suggest that the various members of the KpSC display differing levels of antimicrobial resistance, leading to differing clinical outcomes [36, 39]. This highlights the requirement for rapid and accessible diagnostics which can accurately identify members of the KpSC.

We have developed a real-time multiplex PCR which enables pan-KpSC detection and the simultaneous differentiation of the most clinically relevant species: *K. pneumoniae, K. quasipneumoniae*, and *K. variicola.* This is the first multiplex real-time PCR assay with the ability to distinguish between members of the KpSC, and which has been validated *in vitro* using all 7 KpSC phylogroups. This assay has been tested *in silico* using 348 unique nucleotide sequences (**Supplementary Table S3.1**) and *in vitro* using a panel of 54 *Klebsiella* and *Enterobacteriaceae* (**Table 3.2**). Representative species from each KpSC phylogroup have been used to confirm the specificity of this assay, as well as closely related *Klebsiella* such as *K. aerogenes* and *K. oxytoca*.

For the assay to detect all KpSC, we utilised the *zur-khe* intergenic region (ZKIR). The ZKIR has previously been targeted for pan-KpSC detection, using KpSC-specific primers and SYBR® Green fluorescence chemistry [46]. They found the ZKIR was conserved in KpSC, and that it was superior to other genes used for KpSC identification such as *khe* and *tyrB* [62, 63]. To facilitate the development of a multiplex assay as part of this study, alternative primers and novel hydrolysis probes targeting the ZKIR were designed. Due to sequence heterogeneity in the preferred probe region, it was necessary to include two ROX-labelled hydrolysis probes. The probe ZKIR_P2 (**Table 3.3**) was required for the efficient detection of *K. africana*. This resulted in a pan-KpSC assay which detected all members with similar analytical sensitivity.

For the assays to detect *K. pneumoniae* and *K. variicola* the *lepA* gene was targeted as it displayed sufficient nucleotide heterogeneity to create species-specific assays. The lepA gene is highly conserved in bacteria [54] has previously been used as a diagnostic target for a variety of bacterial species [64, 65]. A novel diagnostic target (*rapZ*) was used to differentiate *K. quasipneumoniae* [66]. This gene has also been shown to be highly conserved in bacteria, as it plays an essential role in the regulation of amino acid metabolism [67, 68].

This multiplex assay can also be used for the presumptive identification of *K. quasivariicola* and *K. africana*, based on a positive KpSC result and a negative result for the assays specific for *K. pneumoniae, K. variicola,* or *K. quasipneumoniae* (**Table 3.4**). While this study focused on developing a method for the detection of the KpSC members most frequently associated with human infection, *K. quasivariicola* and *K. africana* have been found in infection and colonisation isolates [10, 24].

Rapid, culture-free diagnostics are an essential tool in reducing the spread of healthcare associated infections and AMR [69, 70]. Accurate and reliable species identification is also required to understand local epidemiology of infections. Currently, accurate KpSC differentiation relies on whole genome sequence analysis [28]. Sequencing-based pathogen identification remains unavailable in the majority of clinical laboratories due to the cost, expertise, and equipment required [71]. Existing molecular-based methods have not been validated using all KpSC members, due to more recent taxonomic updates in the group. Additionally, some have been shown to produce inaccurate results when these species are encountered [13, 72].

Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) is a popular method for bacterial species identification in the hospital setting due to the ease-of-use and high accuracy in identifying most culture isolates [73]. However, as well as relying on culture-based enrichment prior to identification, it is unable to differentiate between KpSC species. This is primarily due to the lack of species data in the accompanying spectral database. In many cases, the spectral database misidentifies the KpSC isolate as *K. pneumoniae sensu stricto* [61, 74]. Some studies have demonstrated that unique mass spectra are produced, which could allow for more accurate differentiation in the future [38, 75, 76].

Conventional PCR has been used to discriminate between some members of the KpSC. One such method targets the chromosomal β-lactamases *bla*$_{\mathrm{SHV}}$, *bla*$_{\mathrm{OKP}}$, and *bla*$_{\mathrm{LEN}}$ which are typically found in *K. pneumoniae, K. quasipneumoniae* and *K. variicola*, respectively [22]. However, it has recently been shown that *K. quasivariicola* (Kp6) populations contain a mixture of *bla*$_{\mathrm{LEN}}$ and *bla*$_{\mathrm{OKP}}$ genes which would cause these species to be misidentified [14]. Long *et al.* [34] also found a non-*K. quasipneumoniae* isolate carrying *bla*$_{\mathrm{OKP}}$, which they reported as a *K. variicola*. An alternative end-point PCR which detects *K. pneumoniae, K. variicola* and the *Klebsiella* genus using a triplex PCR system has also been described [21]. This assay predates many of the taxonomic updates to the KpSC and may not be specific for the species targeted. A more recent triplex end-point PCR which detects *K. pneumoniae, K. variicola* and *K. quasipneumoniae* through targeting of the genes encoding diguanylate phosphodiesterase, phosphodiesterase mutase and the tripartite tricarboxylate transporter (*tctA*), respectively was tested using a panel of *Klebsiella* which did not include Kp5-Kp7 or *Klebsiella aerogenes* [77].

Real-time PCR for the detection of KpSC members has been described, but these studies have similar issues arising from the use of incomplete strain panels for specificity testing. One such assay which targets the 16S rRNA gene was developed prior to the description of KpSC as distinct species. [78]. A more recent real-time PCR assay to detect but not differentiate the species *K. pneumoniae, K. quasipneumoniae* and *K. variicola*, targeting the *fiu* gene was described by Sun *et al.* [79]. However, no other KpSC species were included in the exclusivity panel, and *K. oxytoca* and *K. aerogenes* were the only other *Klebsiella* species used.

The simultaneous detection of all KpSC members using real-time PCR is possible through the use of a SYBR® Green-based assay targeting an intergenic region between the zinc uptake regulator (*zur*) and a putative haemolysin (*khe*) referred to as “ZKIR” [46]. Although the ZKIR assay provides useful means of detecting all KpSC, it does not differentiate between species and cannot be highly multiplexed as with assays using hydrolysis probes.

The burden of disease caused by *K. pneumoniae* has been well-documented [4, 80]. Less recognised are the species which make up KpSC, which have varying clinical characteristics, and are often misidentified as *K. pneumoniae* due to their close genetic relatedness [36, 37, 39]. *K. pneumoniae sensu stricto*, and the wider KpSC have diverse ecological niches, ranging from plant-associated endophytes to MDR human pathogens [81-83]. Clinical isolates displaying high levels of antimicrobial resistance from species other than *K. pneumoniae,* such as *K. variicola* and *K. quasipneumoniae* have been reported [84, 85]. They have also been implicated in animal infection and plant disease [7, 59]. The functional and ecological diversity displayed by KpSC highlights their importance from a global, One Heath perspective [81, 82].

Improved methods of KpSC identification would increase our understanding of their relative roles in human infection and the environment, and contribution to AMR. As such we developed a real-time multiplex PCR to detect each member of the KpSC, and differentiate between *K. pneumoniae sensu stricto, K. quasipneumoniae,* and *K. variicola.* The specific detection of these three species will enable the rapid and accurate identification of the most prominent members of the KpSC. This is combined with the pan-KpSC assay which also has the ability to detect *K. quasivariicola* (Kp6) and *K. africana* (Kp7). In the event of a real-time PCR result which suggests a sample contains *K. quasivariicola* or *K. africana,* further testing would be required to identify these less-frequently encountered KpSC to the species level.

This assay has been validated using a large panel of nucleotide sequences and a well characterised panel of culture collection isolates (**Table 3.2**) and was found to be 100% specific for the target species. The ability to detect 10 GE has been demonstrated for each KpSC species, in both the species-specific and pan-KpSC assays. This multiplex has the ability to specifically identify the three most clinically relevant members of the KpSC, while the inclusion of a pan-KpSC assay allows for detection of all species. This assay's diagnostic performance now warrants further investigation in a clinical laboratory to characterise isolates from patients to determine the local epidemiology of members of the KpSC circulating in various patient cohorts.

3.5 Supplementary Material

Table S3.1: NCBI accession numbers of the sequences used to design and validate each novel real-time PCR assay *in silico*

Sequences used to perform *in silico* analysis for assays targeting *lepA*		
NCBI Accession Number	**Species**	**URL**
CP020448.2	*Citrobacter braakii*	https://www.ncbi.nlm.nih.gov/nuccore/CP020448.2
CP024819.1	*Citrobacter freundii*	https://www.ncbi.nlm.nih.gov/nuccore/CP024819.1
CP018810.1	*Citrobacter freundii*	https://www.ncbi.nlm.nih.gov/nuccore/CP018810.1
CP012266.1	*Cronobacter dublinensis*	https://www.ncbi.nlm.nih.gov/nuccore/CP012266.1
CP003312.1	*Cronobacter sakazakii*	https://www.ncbi.nlm.nih.gov/nuccore/CP003312.1
CP012257.1	*Cronobacter universalis*	https://www.ncbi.nlm.nih.gov/nuccore/CP012257.1
CP046618.1	*Enterobacter asburiae*	https://www.ncbi.nlm.nih.gov/nuccore/CP046618.1
CP056474.1	*Enterobacter cloacae*	https://www.ncbi.nlm.nih.gov/nuccore/CP056474.1
CP058253.1	*Enterobacter roggenkampii*	https://www.ncbi.nlm.nih.gov/nuccore/CP058253.1
AP019007.1	*Enterobacter oligotrophicus*	https://www.ncbi.nlm.nih.gov/nuccore/AP019007.1
LR134122.1	*Klebsiella aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/LR134122.1
CP002824.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP002824.1
NC015663.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/NC015663.1
CP024880.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP024880.1
CP028951.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP028951.1
CP023963.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP023963.1
CP011574.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP011574.1
CP026722.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP026722.1
CP033817.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP033817.1
CP026756.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP026756.1
CP031756.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP031756.1
CP014029.2	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP014029.2
CP014748.2	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP014748.2
CP024885.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP024885.1
CP011539.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP011539.1
FO203355.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/FO203355.1
LR134475.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/LR134475.1
CP024883.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP024883.1
NZ_CP059391.1	*Klebsiella africana*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP059391.1
NZ_CP084874.1	*K. africana*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP084874.1
CAAHGQ010000005.1	*K. africana* Kp7 CIP111653	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGQ010000005.1

NZCP04452 7.1	*Klebsiella grimontii*	https://www.ncbi.nlm.nih.gov/nuccore/NZCP044527.1
CP067433.1	*K. grimontii*	https://www.ncbi.nlm.nih.gov/nuccore/CP067433.1
NZ_LR6073 36.1	*K. grimontii*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_LR607336.1
CP023185.1	*Klebsiella michiganensis*	https://www.ncbi.nlm.nih.gov/nuccore/CP023185.1
CP004887.1	*K. michiganensis*	https://www.ncbi.nlm.nih.gov/nuccore/CP004887.1
CP003218.1	*K. michiganensis*	https://www.ncbi.nlm.nih.gov/nuccore/CP003218.1
NZAP02254 7.1	*K. michiganensis*	https://www.ncbi.nlm.nih.gov/nuccore/NZAP022547.1
CP073236.1	*K. michiganensis*	https://www.ncbi.nlm.nih.gov/nuccore/CP073236.1
CP027426.1	*Klebsiella oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP027426.1
CP020358.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP020358.1
CP026285.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP026285.1
CP011636.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP011636.1
JAIFQY0100 00013.1	*Klebsiella pasteurii*	https://www.ncbi.nlm.nih.gov/nuccore/JAIFQY010000013.1
CP089403.1	*K. pasteurii*	https://www.ncbi.nlm.nih.gov/nuccore/CP089403.1
CABGGT01 0000018.1	*K. pasteurii* CIP111696	https://www.ncbi.nlm.nih.gov/nuccore/CABGGT010000018.1
CP026160.1	*Klebsiella pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP026160.1
CP026178.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP026178.1
CP038002.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP038002.1
CP036300.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP036300.1
CP016159.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP016159.1
LR133964.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/LR133964.1
CP021955.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP021955.1
CP025211.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP025211.1
CP028816.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP028816.1
CP015130.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP015130.1
CP031817.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP031817.1
CP021696.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP021696.1
CP014008.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP014008.1
CP036190.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP036190.1
CP034082.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP034082.1
CP035383.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP035383.1
CP030269.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP030269.1
CP027189.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP027189.1
CP025080.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP025080.1
CP025639.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP025639.1
CP025631.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP025631.1
CP025090.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP025090.1
CP019047.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP019047.1
CP016813.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP016813.1
CP014010.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP014010.1
CP003785.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP003785.1
AP006725.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/AP006725.1
CP026021.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP026021.1
N/A	*K. pneumoniae* ATCC4352	https://www.atcc.org/products/4352

NZUGLV01000001.1	*K. pneumoniae* ssp. *rhinoscleromatis*	https://www.ncbi.nlm.nih.gov/nuccore/NZUGLV01000001.1
ACZD01000034.1	*K. pneumoniae* ssp. *rhinoscleromatis*	https://www.ncbi.nlm.nih.gov/nuccore/ACZD01000034.1
CP028806.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP028806.1
CP023478.1	*Klebsiella quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP023478.1
CP014156.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP014156.1
CP014155.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP014155.1
CP014154.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP014154.1
CP012300.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP012300.1
CP012252.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP012252.1
CP031257.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP031257.1
CP029597.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP029597.1
CP014071.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP014071.1
CP014696.2	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP014696.2
CP034678.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP034678.1
AP019687.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/AP019687.1
CP039791.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP039791.1
AP022142.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/AP022142.1
CP084803.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP084803.1
CP030171.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP030171.1
CP029437.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP029437.1
CP029443.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP029443.1
CP029432.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP029432.1
CAAHGF010000008.1	*K. quasipneumoniae* Kp2 CIP111839	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGF010000008.1
CAAHFU010000124.1	*K. quasipneumoniae* Kp2 CIP111852	https://www.ncbi.nlm.nih.gov/nuccore/CAAHFU010000124.1
CAAHFY010000006.1	*K. quasipneumoniae* Kp4 CIP111862	https://www.ncbi.nlm.nih.gov/nuccore/CAAHFY010000006.1

CP084770.1	*K. quasipneumoniae* Kp4 CIP111869	https://www.ncbi.nlm.nih.gov/nuccore/CP084770.1
UJYW01000004.1	*Klebsiella quasivariicola*	https://www.ncbi.nlm.nih.gov/nuccore/UJYW01000004.1
VLPG01000016.1	*K. quasivariicola*	https://www.ncbi.nlm.nih.gov/nuccore/VLPG01000016.1
WHZK01000014.1	*K. quasivariicola*	https://www.ncbi.nlm.nih.gov/nuccore/WHZK01000014.1
UJYZ02000002.1	*K. quasivariicola*	https://www.ncbi.nlm.nih.gov/nuccore/UJYZ02000002.1
UJZG01000020.1	*K. quasivariicola*	https://www.ncbi.nlm.nih.gov/nuccore/UJZG01000020.1
CAAHGS010000079.1	*K. quasivariicola* Kp6 CIP111871	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGS010000079.1
NZ_CAAHGB010000003.1	*K. quasivariicola* Kp6 CIP111879	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CAAHGB010000003.1
CP022823.1	*K. quasivariicola* KPN1705	https://www.ncbi.nlm.nih.gov/nuccore/CP022823.1
NZ_CABEJC010000004.1	*Klebsiella spallanzanii* CIP111695	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CABEJC010000004.1
LR130538.1	*Klebsiella variicola*	https://www.ncbi.nlm.nih.gov/nuccore/LR130538.1
LR134235.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/LR134235.1
CP008700.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP008700.1
CP018307.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP018307.1
CP000964.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP000964.1
CP030173.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP030173.1
CP010523.2	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP010523.2
CP001891.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP001891.1
CP013985.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP013985.1
LR130543.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/LR130543.1
LR130544.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/LR130544.1
LR130539.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/LR130539.1
CP016344.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP016344.1
CP028555.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP028555.1
CP009274.2	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP009274.2
CP020847.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP020847.1
CP017289.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP017289.1
CP017849.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP017849.1
CP017284.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP017284.1
CP032354.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP032354.1
CP027064.2	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP027064.2
CP084767.1	*K. variicola* Kp5	https://www.ncbi.nlm.nih.gov/nuccore/CP084767.1
CAAHGN010000007.1	*K. variicola* Kp5 CIP111654	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGN010000007.1
CAAHGO010000006.1	*K. variicola* Kp5 CIP111898	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGO010000006.1
CAAHGL010000007.1	*K. variicola* Kp5 CIP111870	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGL010000007.1
CP033631.1	*Klebsiella* sp. P1CD1	https://www.ncbi.nlm.nih.gov/nuccore/CP033631.1

CP037441.1	*Klebsiella* sp. PO552	https://www.ncbi.nlm.nih.gov/nuccore/CP037441.1
AP022665.1	*Kluyvera ascorbata*	https://www.ncbi.nlm.nih.gov/nuccore/AP022665.1
CP022114.1	*Kluyvera* genomosp.3	https://www.ncbi.nlm.nih.gov/nuccore/CP022114.1
CP045843.1	*Kluyvera intermedia*	https://www.ncbi.nlm.nih.gov/nuccore/CP045843.1
LR699009.1	*Pluralibacter gergoviae*	https://www.ncbi.nlm.nih.gov/nuccore/LR699009.1
CP009450.1	*Pluralibacter gergoviae*	https://www.ncbi.nlm.nih.gov/nuccore/CP009450.1
CP002272.1	*Pluralibacter lignolyticus*	https://www.ncbi.nlm.nih.gov/nuccore/CP002272.1
CP079752.1	*Raoultella ornithinolytica*	https://www.ncbi.nlm.nih.gov/nuccore/CP079752.1
CP008886.1	*R. ornithinolytica*	https://www.ncbi.nlm.nih.gov/nuccore/CP008886.1
CP038281.1	*R. ornithinolytica*	https://www.ncbi.nlm.nih.gov/nuccore/CP038281.1
CP004142.1	*R. ornithinolytica*	https://www.ncbi.nlm.nih.gov/nuccore/CP004142.1
CP012555.1	*R. ornithinolytica*	https://www.ncbi.nlm.nih.gov/nuccore/CP012555.1
LR134195.1	*R. ornithinolytica*	https://www.ncbi.nlm.nih.gov/nuccore/LR134195.1
CP054471.1	*R. ornithinolytica*	https://www.ncbi.nlm.nih.gov/nuccore/CP054471.1
CP029752.1	*Raoultella planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CP029752.1
CP044121.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CP044121.1
CP040183.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CP040183.1
CP019899.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CP019899.1
CP082168.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CP082168.1
CP069815.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CP069815.1
CABDVR010000001.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CABDVR010000001.1
SUPN01000010.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/SUPN01000010.1
DADZWO010000013.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/DADZWO010000013.1
PDLC01000002.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/PDLC01000002.1
CP023877.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CP023877.1
CP023874.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CP023874.1
MPYJ04000036.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/MPYJ04000036.1
NZ_JMPP01000022.1	*R. planticola* CIP100751	https://www.ncbi.nlm.nih.gov/nuccore/NZ_JMPP01000022.1
CP016032.1	*Serratia marcescens*	https://www.ncbi.nlm.nih.gov/nuccore/CP016032.1
MH460883.1	*Serratia marcescens*	https://www.ncbi.nlm.nih.gov/nuccore/MH460883.1
CP038662.1	*Serratia nematodiphila*	https://www.ncbi.nlm.nih.gov/nuccore/CP038662.1
Sequences used to perform *in silico* analysis for assay targeting ZKIR		
NCBI Accession Number	**Species**	**URL**
CP020448.2	*Citrobacter braakii*	https://www.ncbi.nlm.nih.gov/nuccore/CP020448.2

CP024819.1	*Citrobacter freundii*	https://www.ncbi.nlm.nih.gov/nuccore/CP024819.1
CP012253.1	*Cronobacter sakazakii*	https://www.ncbi.nlm.nih.gov/nuccore/CP012253.1
CP012257.1	*Cronobacter universalis*	https://www.ncbi.nlm.nih.gov/nuccore/CP012257.1
CP045769.1	*Enterobacter cancerogenus*	https://www.ncbi.nlm.nih.gov/nuccore/CP045769.1
CP025225.1	*Enterobacter cancerogenus*	https://www.ncbi.nlm.nih.gov/nuccore/CP025225.1
CP056776.1	*Enterobacter cloacae*	https://www.ncbi.nlm.nih.gov/nuccore/CP056776.1
CP041925.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP041925.1
CP055904.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP055904.1
LR134475.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/LR134475.1
CP035466.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP035466.1
LR134126.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/LR134126.1
CP047669.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP047669.1
CP045870.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP045870.1
CP044214.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP044214.1
AP022108.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/AP022108.1
WMHU01000001.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/WMHU01000001.1
NEYW02000018.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/NEYW02000018.1
PXKX01000023.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/PXKX01000023.1
JAKCJA010000021.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/JAKCJA010000021.1
CP059391.1	*K. africana* Kp7	https://www.ncbi.nlm.nih.gov/nuccore/CP059391.1
CP084874.1	*K. africana* Kp7	https://www.ncbi.nlm.nih.gov/nuccore/CP084874.1
CAAHGQ010000004.1	*K. africana* Kp7 CIP111653	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGQ010000004.1
CP044527.1	*K. grimontii*	https://www.ncbi.nlm.nih.gov/nuccore/CP044527.1
LR607363.1	*K. grimontii*	https://www.ncbi.nlm.nih.gov/nuccore/LR607363.1
LR594038.1	*K. grimontii*	https://www.ncbi.nlm.nih.gov/nuccore/LR594038.1
CP056150.1	*K. grimontii*	https://www.ncbi.nlm.nih.gov/nuccore/CP056150.1
CP079754.1	*K. grimontii*	https://www.ncbi.nlm.nih.gov/nuccore/CP079754.1
CP067433.1	*K. grimontii*	https://www.ncbi.nlm.nih.gov/nuccore/CP067433.1
CP036175.1	*Klebsiella huaxiensis*	https://www.ncbi.nlm.nih.gov/nuccore/CP036175.1
CP003218.1	*K. michiganensis*	https://www.ncbi.nlm.nih.gov/nuccore/CP003218.1
CP048108.1	*K. michiganensis*	https://www.ncbi.nlm.nih.gov/nuccore/CP048108.1
LR607380.1	*K. michiganensis*	https://www.ncbi.nlm.nih.gov/nuccore/LR607380.1
CP071393.1	*K. michiganensis*	https://www.ncbi.nlm.nih.gov/nuccore/CP071393.1
LR607366.1	*K. michiganensis*	https://www.ncbi.nlm.nih.gov/nuccore/LR607366.1
CP026269.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP026269.1
CP008788.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP008788.1
CP018362.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP018362.1
CP026275.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP026275.1
CP026715.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP026715.1
CP056581.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP056581.1
CP056453.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP056453.1

CP026285.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP026285.1
CP020358.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP020358.1
CP011636.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP011636.1
CP056418.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP056418.1
CP089403.1	*K. pasteurii*	https://www.ncbi.nlm.nih.gov/nuccore/CP089403.1
JAIFQY010000015.1	*K. pasteurii*	https://www.ncbi.nlm.nih.gov/nuccore/JAIFQY010000015.1
CABGGT010000016.1	*K. pasteurii*	https://www.ncbi.nlm.nih.gov/nuccore/CABGGT010000016.1
AP023148.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/AP023148.1
LR130541.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/LR130541.1
CP075552.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP075552.1
CP075275.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP075275.1
NZ_JAAVSQ010000016.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_JAAVSQ010000016.1
NZ_SOMK01000022.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_SOMK01000022.1
CP044393.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP044393.1
CP028716.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP028716.1
FO203501.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/FO203501.1
CP046939.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP046939.1
CP040593.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP040593.1
LR133964.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/LR133964.1
CP024874.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP024874.1
CP028176.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP028176.1
NZ_SIZK01000003.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_SIZK01000003.1
NZ_CP065833.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP065833.1
N/A	*K. pneumoniae ATCC4352*	https://www.atcc.org/products/4352
NZ_LXMI01000022.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_LXMI01000022.1
CP030171.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP030171.1
NZ_LGAL01000005.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_LGAL01000005.1
NZ_CP030171.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP030171.1
AP022142.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/AP022142.1
LT898450.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/LT898450.1
CP043928.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP043928.1
NZ_JAFFTJ010000001.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_JAFFTJ010000001.1
NZ_SGRL01000050.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_SGRL01000050.1
CP068237.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP068237.1

NZ_CP068237.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP068237.1
CP034136.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP034136.1
CP014696.2	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP014696.2
NZ_CP014696.2	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP014696.2
NZ_UGLL01000001.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_UGLL01000001.1
NZ_JVVC01000134.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_JVVC01000134.1
NZ_JAAOBL010000006.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_JAAOBL010000006.1
LR134196.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/LR134196.1
CP023478.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP023478.1
LR588411.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/LR588411.1
LR134202.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/LR134202.1
CP045641.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP045641.1
CP073660.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP073660.1
CAAHGF010000053.1	*K. quasipneumoniae* Kp2 CIP111839	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGF010000053.1
CAAHFU010000030.1	*K. quasipneumoniae* Kp2 CIP111852	https://www.ncbi.nlm.nih.gov/nuccore/CAAHFU010000030.1
CAAHFY010000023.1	*K. quasipneumoniae* Kp4 CIP111862	https://www.ncbi.nlm.nih.gov/nuccore/CAAHFY010000023.1
CP084770.1	*K. quasipneumoniae* Kp4 CIP111869	https://www.ncbi.nlm.nih.gov/nuccore/CP084770.1
CP022823.1	*K. quasivariicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP022823.1
CAAHGS010000093.1	*K. quasivariicola* CIP111871	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGS010000093.1
CAAHGB010000003.1	*K. quasivariicola* Kp6 CIP111879	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGB010000003.1
CABEJC010000038.1	*K. spallanzanii*	https://www.ncbi.nlm.nih.gov/nuccore/CABEJC010000038.1
CP063932.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP063932.1
CP010523.2	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP010523.2
CP064319.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP064319.1
LR607362.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/LR607362.1
CP000964.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP000964.1
CAAHGJ010000003.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGJ010000003.1
CP050958.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP050958.1

CP017289.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP017289.1
CP017849.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP017849.1
CP065162.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP065162.1
NZ_SZND01000011.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_SZND01000011.1
NZ_UJZF01000014.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_UJZF01000014.1
NZ_JAFHRV010000018.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_JAFHRV010000018.1
NZ_BIHE01000005.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_BIHE01000005.1
CP063867.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP063867.1
NZ_LR130538.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_LR130538.1
CP013985.1	*K. variicola* Kp3.CIP108642	https://www.ncbi.nlm.nih.gov/nuccore/CP013985.1
CAAHGN010000047.1	*K. variicola* Kp5 CIP111654	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGN010000047.1
CAAHGL010000054.1	*K. variicola* Kp5 CIP111870	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGL010000054.1
CAAHGO010000055.1	*K. variicola* Kp5 CIP111898	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGO010000055.1
CP020657.1	*Klebsiella* sp. M5al	https://www.ncbi.nlm.nih.gov/nuccore/CP020657.1
CP056483.1	*Klebsiella* sp. RHBSTW-00464	https://www.ncbi.nlm.nih.gov/nuccore/CP056483.1
CP055481.1	*Klebsiella* sp. RHBSTW-00484	https://www.ncbi.nlm.nih.gov/nuccore/CP055481.1
CABGGW010000050.1	*Klebsiella* sp. SB6422	https://www.ncbi.nlm.nih.gov/nuccore/CABGGW010000050.1
CABGGQ010000013.1	*Klebsiella* sp. SB6421	https://www.ncbi.nlm.nih.gov/nuccore/CABGGQ010000013.1
CP050321.1	*Kluyvera* gensp. 3	https://www.ncbi.nlm.nih.gov/nuccore/CP050321.1
LR134138.1	*Kluyvera intermedia*	https://www.ncbi.nlm.nih.gov/nuccore/LR134138.1
LR699009.1	*P. gergoviae*	https://www.ncbi.nlm.nih.gov/nuccore/LR699009.1
LR134195.1	*R. ornithinolytica*	https://www.ncbi.nlm.nih.gov/nuccore/LR134195.1
LR134230.1	*R. ornithinolytica*	https://www.ncbi.nlm.nih.gov/nuccore/LR134230.1
AP022438.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/AP022438.1
CP019899.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CP019899.1
CP040183.1	*R. planticola*	https://www.ncbi.nlm.nih.gov/nuccore/CP040183.1
JMPP01000009.1	*R. planticola* CIP100751	https://www.ncbi.nlm.nih.gov/nuccore/JMPP01000009.1
LR595855.1	*R. terrigena*	https://www.ncbi.nlm.nih.gov/nuccore/LR595855.1
Sequences used to perform *in silico* analysis for assay targeting *rapZ*		
NCBI Accession Number	**Species**	**URL**
NZ_JAIRBE010000019.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_JAIRBE010000019.1
NZ_JAFHXN010000001.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_JAFHXN010000001.1

CP049600.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP049600.1
CP002824.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/CP002824.1
NZ_CP041925.1	*K. aerogenes*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP041925.1
N/A	*K. aerogenes* ATCC13048	https://www.atcc.org/products/13048
N/A	*K. aerogenes* ATCC49469	https://www.atcc.org/products/49469
LR134124.1	*K. aerogenes* NCTC10006	https://www.ncbi.nlm.nih.gov/nuccore/LR134124.1
LR134475.1	*K. aerogenes* NCTC9735	https://www.ncbi.nlm.nih.gov/nuccore/LR134475.1
CP059391.1	*K. africana*	https://www.ncbi.nlm.nih.gov/nuccore/CP059391.1
CP084874.1	*K. africana*	https://www.ncbi.nlm.nih.gov/nuccore/CP084874.1
CAAHGQ010000012.1	*K. africana* Kp7 CIP111653	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGQ010000012.1
VNUQ01000020.1	*K. grimontii*	https://www.ncbi.nlm.nih.gov/nuccore/VNUQ01000020.1
QFVM01000025.1	*K. grimontii*	https://www.ncbi.nlm.nih.gov/nuccore/QFVM01000025.1
WMOY01000015.1	*K. grimontii*	https://www.ncbi.nlm.nih.gov/nuccore/WMOY01000015.1
VNVV01000008.1	*K. grimontii*	https://www.ncbi.nlm.nih.gov/nuccore/VNVV01000008.1
VNUP01000027.1	*K. grimontii*	https://www.ncbi.nlm.nih.gov/nuccore/VNUP01000027.1
LR594038.1	*K. grimontii* NCTC9146	https://www.ncbi.nlm.nih.gov/nuccore/LR594038.1
CP054159.1	*K. michiganensis*	https://www.ncbi.nlm.nih.gov/nuccore/CP054159.1
CP089395.1	*K. michiganensis*	https://www.ncbi.nlm.nih.gov/nuccore/CP089395.1
CP073236.1	*K. michiganensis*	https://www.ncbi.nlm.nih.gov/nuccore/CP073236.1
NZ_UGMJ01000003.1	*K. michiganensis* NCTC10261	https://www.ncbi.nlm.nih.gov/nuccore/NZ_UGMJ01000003.1
NZ_UGJV01000001.1	*K. michiganensis* NCTC8167	https://www.ncbi.nlm.nih.gov/nuccore/NZ_UGJV01000001.1
NZ_CP033844.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP033844.1
CP017928.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP017928.1
CP011618.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP011618.1
CP089399.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP089399.1
CP089411.1	*K. oxytoca*	https://www.ncbi.nlm.nih.gov/nuccore/CP089411.1
LS483483.1	*K. oxytoca* NCTC11355	https://www.ncbi.nlm.nih.gov/nuccore/LS483483.1
LR133932.1	*K. oxytoca* NCTC11356	https://www.ncbi.nlm.nih.gov/nuccore/LR133932.1
LR134333.1	*K. oxytoca* NCTC13727	https://www.ncbi.nlm.nih.gov/nuccore/LR134333.1
CABGGT010000019.1	*K. pasteurii* CIP111696	https://www.ncbi.nlm.nih.gov/nuccore/CABGGT010000019.1
CABGHD010000018.1	*K. pasteurii* SB6407	https://www.ncbi.nlm.nih.gov/nuccore/CABGHD010000018.1
CABGGP010000012.1	*K. pasteurii* SB6409	https://www.ncbi.nlm.nih.gov/nuccore/CABGGP010000012.1

CABGGO010000012.1	*K. pasteurii* SB6410	https://www.ncbi.nlm.nih.gov/nuccore/CABGGO010000012.1
CABGGV010000001.1	*K. pasteurii* SB6413	https://www.ncbi.nlm.nih.gov/nuccore/CABGGV010000001.1
CABGHE010000054.1	*K. pasteurii* SB6417	https://www.ncbi.nlm.nih.gov/nuccore/CABGHE010000054.1
CABGHB010000003.1	*K. pasteurii* SB6423	https://www.ncbi.nlm.nih.gov/nuccore/CABGHB010000003.1
CP045015.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP045015.1
CP082290.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP082290.1
NZ_SSYO01000004.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_SSYO01000004.1
NZ_FKYN01000008.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_FKYN01000008.1
NZ_FLCV01000006.1	*K. pneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_FLCV01000006.1
CDOT01000035.1	*K. pneumoniae* ATCC13884	https://www.ncbi.nlm.nih.gov/nuccore/CDOT01000035.1
ACZD01000132.1	*K. pneumoniae* ATCC13884	https://www.ncbi.nlm.nih.gov/nuccore/ACZD01000132.1
0cdd86090c324424.	*K. pneumoniae* ATCC4352	https://www.ncbi.nlm.nih.gov/nuccore/0cdd86090c324424.
CP009208.1	*K. pneumoniae* ATCC43816	https://www.ncbi.nlm.nih.gov/nuccore/CP009208.1
CP064352.1	*K. pneumoniae* ATCC43816	https://www.ncbi.nlm.nih.gov/nuccore/CP064352.1
UGMQ01000003.1	*K. pneumoniae* NCTC5046	https://www.ncbi.nlm.nih.gov/nuccore/UGMQ01000003.1
UGLV01000001.1	*K. pneumoniae* NCTC5048	https://www.ncbi.nlm.nih.gov/nuccore/UGLV01000001.1
NZ_CABDVJ010000002.1	*K. pneumoniae* NCTC9150	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CABDVJ010000002.1
LR588410.1	*K. pneumoniae* NCTC9171	https://www.ncbi.nlm.nih.gov/nuccore/LR588410.1
CP084860.1	*K. pneumoniae* SB1067	https://www.ncbi.nlm.nih.gov/nuccore/CP084860.1
CP084859.1	*K. pneumoniae* SB1139	https://www.ncbi.nlm.nih.gov/nuccore/CP084859.1
CP084843.1	*K. pneumoniae* SB611	https://www.ncbi.nlm.nih.gov/nuccore/CP084843.1
CP084829.1	*K. pneumoniae* SB615	https://www.ncbi.nlm.nih.gov/nuccore/CP084829.1
NZ_CP065838.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP065838.1
CP023478.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP023478.1
CP078761.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP078761.1
CP031257.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP031257.1
CP066173.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP066173.1

LR607330.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/LR607330.1
CP082785.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP082785.1
CP084818.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP084818.1
LT898450.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/LT898450.1
CP043928.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP043928.1
CP026368.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP026368.1
CP045641.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP045641.1
CP063874.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP063874.1
CP030171.1	*K. quasipneumoniae*	https://www.ncbi.nlm.nih.gov/nuccore/CP030171.1
CP029597.1	*K. quasipneumoniae* ATCC700603	https://www.ncbi.nlm.nih.gov/nuccore/CP029597.1
CP014696.2	*K. quasipneumoniae* ATCC700603	https://www.ncbi.nlm.nih.gov/nuccore/CP014696.2
CAAHGF010000089.1	*K. quasipneumoniae* Kp2 CIP111839	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGF010000089.1
CAAHFU010000035.1	*K. quasipneumoniae* Kp2 CIP111852	https://www.ncbi.nlm.nih.gov/nuccore/CAAHFU010000035.1
CP034136.1	*K. quasipneumoniae* Kp4	https://www.ncbi.nlm.nih.gov/nuccore/CP034136.1
CAAHFY010000004.1	*K. quasipneumoniae* Kp4 CIP111862	https://www.ncbi.nlm.nih.gov/nuccore/CAAHFY010000004.1
CP084770.1	*K. quasipneumoniae* Kp4 CIP111869	https://www.ncbi.nlm.nih.gov/nuccore/CP084770.1
CP084803.1	*K. quasipneumoniae* Kp4 SB98	https://www.ncbi.nlm.nih.gov/nuccore/CP084803.1
LR134196.1	*K. quasipneumoniae* NCTC11357	https://www.ncbi.nlm.nih.gov/nuccore/LR134196.1
LR588411.1	*K. quasipneumoniae* NCTC9170	https://www.ncbi.nlm.nih.gov/nuccore/LR588411.1
LR134202.1	*K. quasipneumoniae* NCTC9180	https://www.ncbi.nlm.nih.gov/nuccore/LR134202.1
CAESCD010000008.1	*K. quasivariicola* Kp6	https://www.ncbi.nlm.nih.gov/nuccore/CAESCD010000008.1

VLPG01000003.1	*K. quasivariicola* Kp6	https://www.ncbi.nlm.nih.gov/nuccore/VLPG01000003.1
UJYW01000007.1	*K. quasivariicola* Kp6	https://www.ncbi.nlm.nih.gov/nuccore/UJYW01000007.1
CAAHGS010000091.1	*K. quasivariicola* Kp6 CIP111871	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGS010000091.1
CAAHGB010000003.1	*K. quasivariicola* Kp6 CIP111879	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGB010000003.1
CABEJC010000037.1	*K. spallanzanii* CIP111695	https://www.ncbi.nlm.nih.gov/nuccore/CABEJC010000037.1
CP050958.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP050958.1
LR607362.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/LR607362.1
CP047360.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP047360.1
CP008700.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP008700.1
CP001891.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP001891.1
CP087978.1	*K. variicola*	https://www.ncbi.nlm.nih.gov/nuccore/CP087978.1
CP087979.1	*K. variicola* Kp3	https://www.ncbi.nlm.nih.gov/nuccore/CP087979.1
CP092632.1	*K. variicola* Kp3	https://www.ncbi.nlm.nih.gov/nuccore/CP092632.1
CP088956.1	*K. variicola* Kp3	https://www.ncbi.nlm.nih.gov/nuccore/CP088956.1
CP013985.1	*K. variicola* Kp3 LMG23571	https://www.ncbi.nlm.nih.gov/nuccore/CP013985.1
CP084767.1	*K. variicola* Kp5 CDC4241-71	https://www.ncbi.nlm.nih.gov/nuccore/CP084767.1
CAAHGN010000002.1	*K. variicola* Kp5 CIP111654	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGN010000002.1
CAAHGL010000004.1	*K. variicola* Kp5 CIP111870	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGL010000004.1
CAAHGO010000005.1	*K. variicola* Kp5 CIP111898	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGO010000005.1
CAAHGJ010000045.1	*K. variicola* Kp5 SB94	https://www.ncbi.nlm.nih.gov/nuccore/CAAHGJ010000045.1
LR134235.1	*K. variicola* NCTC9668	https://www.ncbi.nlm.nih.gov/nuccore/LR134235.1
LR588409.1	*K. variicola* NTC9178	https://www.ncbi.nlm.nih.gov/nuccore/LR588409.1

Table S3.2: Sequence of synthetic double-sided DNA fragment (gBlock®) used as template for the IAC

Sequence (5′-3′)	AGCACCTCTAAGTAAGTGAGCGGTCGTGACATTATCCCTGATTTTCTCACTACTATTAGTACTCACGGCGCAATTCCACCACAGCCTTGTCTCGCCAGAATGCCAGTCAGCATAAGGAAGAGCTCAAGGCAGGTCAACTCGCACTGTGAGGGTCACATGGGCGTTCGGCACTACCGACACGAACCTCAGTTAGCGTACATCCTACCAGAGGTCTGTGGCCCCGTGGTCAAAAGTGCGGGTTTCGTATTTGCTGCTCGTCAGTACTTTCAGAATCATGACCTGCACGGCAAAGAGACGCTTATTATGGAGCTCGACATGGCAATAACGCGACGAATCTACGTCACGACGAGAATAGTGTAAACGAAGCTGCTGACGGCGGAAGCGTCAAAGGGGTCTGTGAATTGTTATTCGCGAAAAACATCCGTCCCCGTGGGGGATAGTCACCGACGCCGTTTTATAGAAGCCTAGGGGAACAGGTTGGTTTAACTAGCTTAAGAA

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Chapter 4: Towards Enhanced Hospital Environmental Monitoring: A Protocol to Detect a Mock Microbial Community of Clinically Relevant Pathogens from Surfaces Using Real-Time Multiplex PCR

Preface: At its inception, this project aimed to use the real-time multiplex PCR assays described in Chapter 2 to investigate the role of the hospital environment in HAI. Environmental monitoring was due to be conducted at regular intervals to determine if ESKAPE pathogens persisted in the hospital environment using DNA extracted from swab samples and tested using multiplex real-time PCR.

The COVID-19 pandemic meant that sample collection from ICU surfaces in a tertiary (HSE Model 4) hospital was not carried out as originally planned. It was decided that the aims of the project should be revised to investigate ESKAPEs and their closely related species complexes in HAP and VAP using clinical specimens. The sample collection protocol outlined in this chapter provides an important insight into the benefits and limitations of surface swabs for environmental surveillance using molecular methods. In order to reduce HAI and improve patient outcomes, enhanced surveillance should be conducted not only from direct clinical specimen but from the surrounding hospital environment.

Abstract

This chapter outlines the development of a protocol for the recovery and molecular detection of pathogens from the built environment. The aim of this study was to create a method which would recover and detect a range of microorganisms from solid surfaces, which could subsequently be applied to environmental surveillance of healthcare facilities. A literature review was conducted to provide further insight into current methods and limitations of environmental surface sampling.

A mock microbial community (MMC), containing representative bacterial (*Staphylococcus aureus, Acinetobacter baumannii*) and fungal (*Cryptococcus neoformans)* species, was used to mimic contamination of surfaces. Nucleic acid was recovered from these surfaces using two commercially available DNA extraction kits, in combination with two sampling materials and two test surface types sampling and extraction protocols. The recovery of DNA from this MMC was determined using a novel multiplex real-time PCR which specifically detected each organism in the MMC.

A sampling and extraction protocol was optimised to enable the detection of MMC DNA from cell concentrations between 10 – 50 CFU/cm^2. Recovery of mock microbial DNA was highest when using the Qiagen DNeasy® PowerSoil® Pro extraction kit, sampled using polyester Dacron swabs, from textured surfaces. These findings are placed in context of existing research into the hospital environmental microbiome, infection control practices, and recent insights from metagenomic studies of the hospital environment.

4.1 Introduction

An estimated 8.9 million healthcare-associated infections (HAI) occur annually in Europe [1] leading to increased patient mortality, duration of hospital stay and antimicrobial treatment [2, 3]. The acquisition rate of HAIs is highest in critical care wards such as intensive care units (ICUs) [4]. This is despite extensive precautionary measures to prevent such infections [5, 6]. Pathogenic microorganisms can be shed by patients into their surroundings, or transferred via staff to surfaces, potentially creating an environmental route of transmission [7]. Many methods have been explored to reduce HAI, such as enhanced cleaning, improved hand-hygiene measures, patient isolation and even building design [5, 8]. The vast majority of HAIs are caused by a small number of organisms which are particularly adapted to the hospital environment, often displaying high levels of antimicrobial resistance [9]. There is considerable overlap between pathogens which cause high levels of HAI, and pathogens which have been shown to survive on surfaces for long periods. All of the ESKAPE species have been recovered from surfaces in healthcare facilities [10-14]. Of these, *Enterococcus faecium*, *Staphylococcus aureus*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* are particularly associated with long-term persistence in the hospital environment. These species display enhanced levels of biofilm formation and resistance to desiccation on surfaces [15-17].

Routine surveillance of the hospital environment is currently not recommended [18, 19]. This guideline stems from the poor correlation observed between culture-based surveillance and HAI levels in healthcare facilities. In their 2003 report, the CDC and the Healthcare Infection and Control Practises Advisory Committee (HICPAC) described routine or “random, undetected sampling” as “neither cost effective nor warranted” [20]. They noted that hospital environmental cultures were conducted as standard practice through to the 1970’s before being discontinued on the advice of the CDC and the American Hospital Association [21]. Thus, no standard protocol exists for collection and processing of hospital environmental surface samples.

However, in the years since the CDC and the HIPAC made these recommendations, our understanding of HAI transmission and of the potential role of the environment has changed considerably [22-24]. The emergence of multi-drug resistant organisms (MDROs) has transformed our approach to infection prevention and control practices. The availability and cost of molecular-based detection have also become more accessible in recent years. While culture-based methods lack the necessary sensitivity, molecular-based surveillance of the hospital environment may offer a viable alternative. Future investigations and surveillance of pathogens found on inanimate surfaces using molecular methods may offer new insights and help reduce HAI [25].

Several studies have demonstrated a clear link between prior room occupancy, and HAI acquisition [26-28]. This indicates the potential for surface to patient transmission of pathogens. A recent systematic review and metanalysis showed that the likelihood of infection acquisition increased by a factor of 2.14 if the previous room occupant was infected or colonised with the same species [28].

The timely identification of reservoirs and potential fomites may reduce the likelihood of HAI [6, 29]. There have been several documented cases of outbreaks which were not resolved until the complete removal or replacement of an environmental source of contamination. This is perhaps most widely seen in association with wet-surface biofilms, with the replacement of sinks, showers and faucets [30, 31], although outbreak elimination has also been documented for “dry surface” fomites such as neonatal ICU incubators [32]. This evidence supports the hypothesis that the environment can act not only as a reservoir, but also as a source of infection.
Currently, the CDC guidelines describe four situations in which microbiological sampling is appropriate: outbreak investigations, research, hazard/biohazard threat assessment, and quality-assurance. The latter of which includes exceptions such as the routine culturing of water used in haemodialysis, and to confirm sterilisation processes for medical equipment. Routine monitoring of hospital environments can also be useful to identify deficiencies in cleaning procedures [33, 34]. However, the majority of outbreak investigation and research studies continue to rely on culture-based detection which lacks sensitivity [30, 35-37].

Despite the evidence to support the role of the hospital environment in HAI, there remains no clear consensus on sampling methodology for hospital environmental surveillance. In the absence of standardisation, a large number of experimental variables exist in the current literature, leading to inconsistencies in the conclusions drawn [38-40]. The collection method, area sampled, processing time and detection method all affect the outcomes of such studies. As such, our aim was to create a sensitive and reproducible method to detect microbial contamination on solid surfaces. During the experimental design process for this study, we reviewed existing literature to determine the most appropriate testing parameters.
Two DNA extraction kits were chosen based on their suitability to extract DNA from mixed community samples: the Qiagen DNeasy® PowerSoil® Pro Kit and the ZymoBIOMICS™ DNA Miniprep. Each are tailored and have been used for DNA extraction from environmental and mixed community samples (**Table 4.1**). Both use mechanical lysis in the form of bead-beating (BB), and spin-column based solid-phase purification. Our aim was to evaluate their ability to extract high quality DNA from an MMC which contains Gram-positive and Gram-negative bacterial cells, and fungal cells. Mechanical lysis is particularly useful for mixed community samples such as

environmental samples as it enables the rapid and efficient lysis of the thick cell walls found in fungi and Gram-positive cells [41, 42]. To capture the full microbial diversity of a mixed community sample, it is necessary to optimise the lysis protocol. The duration of sample processing (e.g. bead beating) will vary based on the type and volume of the sample, bead composition and instrument used [43]. Various durations of BB should be investigated changed to ensure thorough cell lysis has occurred without degrading the nucleic acids of easier to lyse, Gram-negative bacteria.

One of the most diverse variables in environmental surface sampling is the collection format. The composition, size and adsorption properties of swabs, sponges and wipes all differ enormously [39, 44]. When reporting the results of environmental sampling studies, information on the type and manufacturer should be provided [45]. Swabs are useful for sampling irregularly shaped (e.g. door handles) and difficult-to-reach areas. They can be made from natural materials such as cotton or calcium alginate, or synthetic materials such as polyester, nylon and macrofoam [46]. Proprietary swabs such as the Copan eSwab® and FLOQSwabs®, and the Isohelix™ DNA buccal swab are also available [47-49]. Sponges are more commonly used for recovery from larger surface areas [36, 50]. They can be used alone with gloved hands, or are available on the end of a stick, such as the 3M™ Sponge-Stick. Their size makes them unsuitable for direct sample processing; they must be transferred to liquid and rinsed, which risks loss of the collected sample. Wipes and gauze are subject to the same issues as sponges for direct processing, although have been used in several recent metagenomics studies including environmental surveillance of the International Space Station [51, 52]. In the present study, we chose to include only swabs in our comparison due to their ease of use and reduced cost relative to some of the other collection methods discussed. As our overall aim was to create a simple and reproducible method to conduct hospital environmental surveillance, the other collection formats were determined to be unsuitable.

When considering test surfaces to be evaluated in this study, we chose two non-porous surfaces which are used for countertop and table finishes in laboratory and healthcare facilities. Trespa® Toplab® is a form of high-pressure compact laminate, with a smooth, hardened finish, while the melamine-faced chipboard is finished with a textured wood-grain effect laminate. These structures have been found to be contaminated with pathogenic bacteria in healthcare environments [34, 53] and so, similar surface materials were used to replicate this. They additionally offered opposing textures, which has been shown to influence recovery efficiency [54, 55].

In this study, we report on the development of an optimised protocol for the molecular detection of a wide range of pathogens from the built environment. The design and testing parameters of this study have been chosen to replicate a typical sampling scenario in a healthcare facility [18].

4.2 Methods

4.2.1 Overview

Recovery of mock microbial community (MMC) DNA using a variety of methods was investigated. These included: two commercially available DNA extraction kits (ZymoBIOMICS™ DNA Miniprep and Qiagen DNeasy® PowerSoil® Pro); two types of swab material (cotton and Dacron); and two types of surface material ("smooth" Trespa® laboratory bench panel and "grained" melamine-faced chipboard with a textured wood-grain effect) (**Table 4.1**). A multiplex real-time PCR was developed to detect each member of the MMC from swab samples.

Table 4.1: Experimental design was based upon a review of current literature for hospital environmental sampling.
For each category (DNA extraction kit, swab material and test surface) two test conditions were selected for based on their previous use and performance in studies which conducted environmental monitoring.

Experimental Conditions	Permutations	Notes	References
DNA Extraction Kit	Qiagen DNeasy® PowerSoil® Pro	Popular for microbiome studies of the hospital and built environment	[45, 55-58]
	ZymoBIOMICS™ DNA Miniprep	Shown to produce comparable results for mixed community samples	[42, 58, 59]
Swab Material	Cotton	Amongst the most frequently used materials in surface sampling studies	[40, 60, 61]
	Dacron [a]	Synthetic materials have been shown to release collected biomass more readily	[11, 46, 54]

Test Surface	Trespa® "smooth"	Non-porous material, finite composition for reproducibility	[47, 62]
	Melamine "grained"	Non-porous, textured surface used for non-medical furniture	[6, 63]

[a] polyethylene terephthalate (PET)

4.2.2 Microbial Strains and Growth Conditions

C. neoformans IHEM 4158 was streaked on Sabouraud dextrose agar (SDA) (LabM, UK) and incubated at 30°C for 48 h. A single colony was transferred to a sterile 20 mL polypropylene tube (universal) containing 15 mL of Sabouraud dextrose broth (SDB) (LabM, UK) and incubated at 30°C, 120 rpm for 16 h. *S. aureus* DSM 23622 and *A. baumannii* DSM 30007 were streaked on individual tryptic soy agar (TSA) (Oxoid, UK) plates and incubated at 37°C for 24 h. A single colony of each was transferred to individual universals containing 15 mL brain heart infusion broth (BHIB) (Oxoid, UK) and incubated at 37°C, 120 rpm for 16 h.

4.2.3 Estimation of the Concentration of Microbial Cultures using Absorbance Readings and Plate Counts

From these 16-h liquid cultures, the optical density at 600 nm (OD_{600}) was measured in duplicate for 1 mL of neat, 1:2, 1:5, 1:10, 1:100 and 1:1000 dilutions, made with sterile broth (BHIB or SDB). Immediately after reading the OD_{600} values, the neat liquid culture contained in the universal was serially diluted to 1:10^8. Dilutions between 10^{-3} (1:1000) to 10^{-8} were plated (100 µL) in triplicate for each organism, using the growth conditions described above. The colonies were counted after 24 h and again at 48 h for the bacterial species, and at 48 h for *C. neoformans* due to its slower growth rate. The triplicate colony counts for each dilution set were averaged and used to calculate the CFU/mL for a 16-h culture under the specified growth conditions. This experiment was repeated twice more, using fresh cultures. These values were plotted in Microsoft Excel to create linear calibration curves. The slope-intercept equation and R^2 values of the trendline of each graph were also calculated using Microsoft Excel. The calibration curve, resultant trendline, and the slope-intercept equation of the trendline was then used to select an OD_{600} value for each species, which equated to a known cell concentration [64].

4.2.4 Preparation of MMC

In the subsequent experiments, cultures of *C. neoformans*, *S. aureus* and *A. baumannii* were grown under the conditions described in **Section 4.2.2** and adjusted to the desired OD_{600} value, which once selected, remained consistent between experiments. This served as a standardised starting concentration from which the CFU/mL could be estimated. Each standardised culture broth (1 mL) was pelleted, resuspended in phosphate buffered saline (PBS) (Oxoid, UK) with gentle vortexing, and serially diluted to the required concentration using PBS. These were then pelleted, resuspended in a smaller volume of PBS, and combined to give an MMC suspension containing each of the three species at equal cell concentrations. To ensure uniformity between experimental test conditions, a single MMC of sufficient volume was created for each experiment, rather than multiple suspensions for each test condition or replicate.

4.2.5 Determination of Swab Absorbance

The mass of liquid absorbed by both the cotton (#TS/6-A. Technical Service Consultants, UK) and Dacron (#TS/19-G. Technical Service Consultants, UK) swab heads was investigated using methods adapted from existing literature [44, 65]. A tube containing 1 mL of PBS was weighed. A swab was then submersed in the liquid for 5-10 seconds. The swab was pressed against the sides of the tube to remove excess liquid, before being discarded. The tube was reweighed to determine the mass of liquid remaining. The difference in mass between the two values was used to estimate the volume of PBS absorbed by each swab ($m = d.v$) where m = mass, d = density and v = volume.

4.2.6 Evaluation of Direct Swab Processing for MMC DNA Extraction

To ensure that direct swab processing did not negatively affect lysis efficiency, MMC DNA extracted directly from an inoculated swab head was compared to DNA extracted from a liquid MMC suspension of the same concentration. DNA eluted from both samples was used as template in a multiplex real-time PCR, and the quantification cycles (C_q) of each were compared.

MMC (100 µL) was pipetted directly on to a Dacron swab. An equivalent volume (100 µL) of sterile PBS was pipetted directly on to the head of a second Dacron swab to act as a negative extraction control (NEC). The swab heads were placed in individual

ZymoBIOMICS^{TM} (herein referred to as "Zymo") ZR BashingBead^{TM} lysis tubes (#D4300. Zymo Research, US). A sterile Halsted forceps and Mayo scissors were used to cut the shaft of the swabs at the base, so that only the swab head remained in the tube. Finally, a second portion of the MMC suspension (100 µL) was transferred to a fresh Zymo lysis tube.

Each tube was processed on a bead-beater (#BSP-112011. Thistle Scientific, UK) for a total of 10 mins at 3,500 oscillations/min using the following method: 5 mins processing, 1 min rest on ice, 5 mins processing, 1 min rest on ice. The Zymo purification protocol was then followed [66]. DNA was eluted in 50 µL of nuclease-free water provided in the Zymo kit. In a deviation from the standard protocol, the eluted DNA was reloaded on to the Zymo-Spin^{TM} IICR column and centrifuged for a second time. The eluted DNA was then passed through the Zymo-Spin^{TM} III-HRC filter as described in the manufacturer's protocol. The resulting "filtered" DNA samples were evaluated using the multiplex real-time PCR outlined in **Table 4.2** and **Section 4.2.12**, with 5uL of the eluted MMC DNA used as template.

4.2.7 Comparison of Extraction Kit Performance using MMC Applied Directly to Swabs

The MMC DNA extraction protocol was initially optimised using MMC suspension applied directly to swab heads rather than test surfaces. MMC suspension (100 µL) was applied directly to swab heads. Alternatively, 100 µL of sterile PBS was applied to swab heads to act as a negative control. Swab heads were transferred to individual Zymo, or Qiagen DNeasy® PowerSoil® Pro lysis tubes. BB was performed for both lysis tube types in parallel, for either 5 or 10 mins, using the method described in **Section 4.2.6.** Following lysis, the relevant manufacturers protocol (either Zymo or Qiagen) was followed [66, 67].

For DNA extracted using Zymo, elution was performed as described in **Section 4.2.6.** The DNA extracted using the Qiagen kit (#47014. Qiagen, Germany) was eluted using 50 µL of Qiagen Solution C6, before being reloaded on to the Qiagen MB Spin column and centrifuged for a second time. MMC DNA eluted from all samples was evaluated using multiplex real-time PCR.

4.2.8 Cleaning and Preparation of Test Surfaces

Test surfaces were cleaned thoroughly before and after each experiment to ensure no detectable level of microbial DNA remained, following the method outlined by Jansson *et al.* [68]. Surface cut-outs with a surface area of approximately 400 cm^2 were placed inside a biological safety cabinet. Both sides of the surface cut-outs were first washed with hot water containing dish soap and a clean bristle-brush. The bristle brushes were sprayed with 70% (v/v) ethanol and autoclaved between experiments. The cut-outs were placed upright to air-dry inside the bacterial safety cabinet, sprayed with 70% (v/v) ethanol, and again allowed to air-dry. Finally, the ultraviolet sterilisation cycle of the bacterial safety cabinet was run for 15 mins while the surface cut-outs remained upright.

MMC suspensions of a known concentration were applied to the test surfaces. The surface cut-outs were rotated from their upright position to lay flat on the base of the bacterial safety cabinet. A sterile, disposable sampling stencil (SWA3096. Scientific Laboratory Supplies, Ireland) measuring 10 cm x 10 cm was used to guide the placement of the cell suspension. A pipette was used to “dot” 200 μL of MMC suspension evenly on the surface in a spiral pattern, spaced evenly apart. The blower fan of the safety cabinet was turned off, the sash was closed, and the surface cut-outs remained in place until swabbing occurred.

4.2.9 Sampling of Test Surfaces Using Swabs

After the desired drying time (2-24 h) had passed, the surfaces were sampled using swabs moistened with PBS. The swab was submerged in a 1.5 mL microcentrifuge tube containing sterile PBS. The swab head was pressed against the side of the microcentrifuge tube to remove excess liquid. The swab was rubbed on the 10 x 10cm section of the surface cut-out to be sampled, in slalom (zigzag) pattern with firm pressure in each direction (top to bottom, right to left, bottom to top, left to right). This gave a total of four slalom “passes” for each area, with swab rotation by 90° between each pass (**Supplementary Figure S4.1).**

For the negative extraction controls, a 10 cm x 10 cm surface section which had not been seeded with MMC was swabbed using the same technique. For DNA extraction, swab heads were processed as described in **Section 4.2.6**, with the resulting eluted DNA being immediately evaluated using multiplex real-time PCR.

4.2.10 Culture-Based Comparison

A liquid suspension of *S. aureus* was used to compare the recovery efficiency of culture-based sampling, based on the method outlined by Hedin *et al.* [63]. *S. aureus* culture was standardised and diluted as before. Liquid suspensions of *S. aureus* (200 µL) equating to 1 x 10^4 CFU and 1 x 10^5 CFU, were applied to test surfaces as described and allowed to dry for 24 h. Dacron swabs moistened with PBS were used to sample the test surface with the same technique as used for previous sampling experiments, described in **Section 4.2.9.** Swab heads were transferred to sterile 5 mL polypropylene tubes containing 1 mL PBS and vortexed vigorously for 1 min before being incubated, stationary at room temperature for 5 mins. The PBS contained in the polypropylene tube was then plated (100 µL) on to TSA plates in triplicate. Plates were incubated for 48 h at 37°C and then inspected for growth. Experiments were performed in duplicate.

4.2.11 Design of Multiplex Real-Time PCR Assay to Detect MMC DNA

A multiplex real-time PCR assay was developed to specifically detect each member of the MMC: *C. neoformans*, *S. aureus* and *A. baumannii*. The individual species-specific assays had been developed in this laboratory as part of separate studies into infectious disease and human health. They have each been validated for performance and target specificity using genomic DNA (gDNA) from pure culture isolates of their respective, closely related species.

For this study, the monoplex assays were combined to create a multiplex real-time PCR assay which could simultaneously detect all three MMC species (**Table 4.2**). A non-competitive internal amplification control targeting a 500 bp double sided synthetic construct (gBlock®) (**Supplementary Table S4.1**) was also incorporated into the multiplex assay. Different fluorophores used to label each hydrolysis probe (**Table 4.2**) allowed for the specific detection and relative quantification of each organism. All primers, probes, and the synthetic gene fragment used in this study were supplied by Integrated DNA Technologies (Coralville, IA, US).

Table 4.2: Sequence of oligonucleotide primers and probes used in the multiplex real-time PCR assay to detect MMC DNA extracted from swabs

Name	Function	NAD Target	Sequence (5′-3′) [a]	Oligo Conc (µM)
Cryp F2	*C. neoformans* forward primer	60S acidic ribosomal protein P2	CAACAGGGTGGCAACG	0.625
Cryp R1	*C. neoformans* reverse primer		GCTGAGGGTTCTTCCAAG	0.625
Cryp P1	*C. neoformans* probe		**FAM/**CTCTGCCGCTGACATCAAG**/Iowa Black FQ**	0.300
Saur F1	*S. aureus* forward primer	*lepA*	TGCTTTTAGTGGTGCTTCTG	0.375
Saur R1	*S. aureus* reverse primer		TAGGTTTAGACCAAGACGATGT	0.375
Saur P	S. aureus probe		**ROX/**CCATCTGGAGCTGGCACAACT**/Iowa Black RQ**	0.200
Abg F1.1	*A. baumannii* forward primer	*rpoB*	AAGAAGCAGCTCGTGAG	0.500
Abg F1.2	*A. baumannii* forward primer		AAGAAGCGGCTCGTGA	0.500
Abg R3	*A. baumannii* reverse primer		TGAATACGACGTTTAACAGC	0.500
Abg P	*A. baumannii* probe		**HEX/**AATGGCGGT**/ZEN/**GGTTCAACTA**/Iowa Black FQ**	0.200
SIAC F2	IAC forward primer	Synthetic Construct	GTCGTATCGCAAGTTTCG	0.500
SIAC R2	IAC reverse primer		CCGTACGACTAGGAAGC	0.500
SIAC P	IAC probe		**5Cy5/**AGATCGAAG**/TAO/**TGAGCATCGCT**/Iowa Black RQ**	0.200

[a]Fluorophores and quenchers of hydrolysis probes are shown in **bold**; FAM = 6-carboxyfluorescein, ROX = carboxy-X-rhodamine, HEX = 4,4,7,2,4,5,7-hexachloro-6-carboxyfluorescein, Cy5 = indodicarbocyanine 5; Iowa Black®, ZEN™ and TAO™ are non-abbreviated trademarks

4.2.12 Colour Compensation and Multiplex Real-Time PCR Conditions

A Roche LightCycler®480 System II and LightCycler®480 Probes Master kit (Roche Diagnostics, Switzerland) were used for all real-time PCR experiments. A colour compensation file was generated to avoid the detection of overlapping hydrolysis probe emission spectra, as per the manufacturer's instructions [69]. Briefly, a master mix was prepared for each individual assay using the oligonucleotides described in **Table 4.2** in monoplex (*n*=4). The generation of a colour compensation file requires increased probe concentrations relative to the concentrations used in an optimised multiplex assay. Each reaction of each monoplex master mix contained 10uL of LightCycler®480 Probes Master (Roche Diagnostics, Switzerland), hydrolysis probe (400 nM) and the relevant primers (500 nM), made to a final volume of 15 µL/reaction. An additional "blank" master mix was prepared which contained 10 µL of LightCycler®480 Probes Master per reaction, but which substituted nuclease-free water for oligonucleotides, to a final volume of 15 µL/reaction. Master mix was combined with 5 µL of the appropriate gDNA as template in replicates of five. Template input concentration was $1x10^6$ genomic equivalents (GE) for *A. baumannii*, *S. aureus* and *C. neoformans*; and $1x0^4$ GE for the synthetic internal amplification control (SIAC). Nuclease-free water was substituted for template in the "blank" master mix.

The thermocycling parameters for colour compensation, and for all subsequent real-time PCR experiments consisted of initial enzyme activation for 10 mins at 95°C, 50 cycles of 95°C for 10 s and 60°C for 30 s with one fluorescence acquisition per cycle, and a final single cooling step of 40°C for 10 s. The temperature transition rate was 4.4°C/s while heating, and 2.2°C/s while cooling. The filter settings used in each real-time PCR experiment to detect the fluorescent probes were as follows: HEX = 533-580 nm, ROX = 533-610 nm, FAM = 465-510 nm, Cy5 = 618-660nm [69]. The run protocol for colour compensation included a period of continuous fluorescence acquisition during a gradient temperature increase from 40°C to 70°C. This followed the PCR thermocycling but occurred prior to cooling, as described in the Roche LightCycler®480 manual.

4.2.13 Optimisation of Oligonucleotide and SIAC Template Concentration in Multiplex PCR

Optimisation of oligonucleotide concentration was carried out to improve the analytical sensitivity of the multiplex real-time PCR assay. The optimal concentration of the oligonucleotides (**Table 4.2**) and of the SIAC template (1,000 copies/reaction) was determined empirically, using a concentration matrix approach [70]. This was based on the performance of the assay and relative C_q values obtained when quantified gDNA from pure culture isolates was used as template. Briefly, gDNA from *C. neoformans*, *S. aureus*, and *A. baumannii* was quantified using a Qubit 1.0 fluorometer and the Qubit High Sensitivity double-stranded DNA kit (ThermoFisher Scientific, MA, US). A dilution series was prepared to give PCR template concentrations of 1 x 10^4 GE - 10 GE per reaction for each species. PCR master mixes were prepared with varying concentrations of oligonucleotide and SIAC template. Performance of each master mix was evaluated based on the lower limit of detection, relative C_q values, and consistency of SIAC detection, until the optimal concentration was determined.

The optimised master mix to detect DNA extracted from MMC samples used 10 µL of LightCycler® Probes Master, the oligonucleotide concentrations outlined in **Table 4.2**, and 1,000 copies of SIAC template (**Supplementary Table 4.1**) per reaction, and was adjusted to a final volume of 15 µL/reaction using nuclease-free water.

4.3 Results

4.3.1 Standardisation of Microbial Cultures for MMC

To accurately compare the recovery of MMC from surfaces, it was necessary to first quantify and standardise the MMC applied to the surfaces. Standardisation of the MMC was achieved by creation of a linear calibration curve for each species. This was based on the average CFU/mL obtained from colony counts, and OD_{600} values from three culture replicates. An OD_{600} value was selected for each species and used to standardise the starting concentration of the MMC for subsequent experiments.

The average CFU/mL for *A. baumannii*, *S. aureus* and *C. neoformans* was 3.824 x 10^8, 1.199 x 10^9, and 2.153 x 10^6, respectively. These values were used to estimate the cell concentration which equated to the OD_{600} dilution series shown in **Table 4.3**. Direct correlation was not possible due to the fact that as the OD_{600} value approached zero, the colony numbers of the equivalent dilution remained too numerous to count.

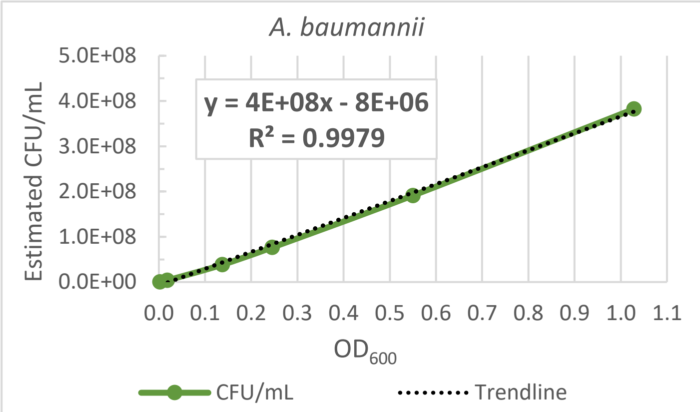
Table 4.3: Average OD_{600} values of replicate cultures, and the estimated CFU/mL of the equivalent dilution factor, were used to create linear calibration curves

Species	Dilution	Average OD_{600}	Estimated CFU/mL[a]
A. baumannii	1	1.029	**3.824E+08**
	1/2	0.550	1.912E+08
	1/5	0.246	7.649E+07
	1/10	0.137	3.824E+07
	1/100	0.018	3.824E+06
	1/1000	0.002	3.824E+05
S. aureus	1	1.963	**2.027E+09**
	1/2	1.396	1.014E+09
	1/5	0.670	4.055E+08
	1/10	0.371	2.027E+08
	1/100	0.042	2.027E+07
	1/1000	0.004	2.027E+06
C. neoformans	1	0.689	**7.698E+06**
	1/2	0.402	3.849E+06
	1/5	0.180	1.540E+06
	1/10	0.094	7.698E+05
	1/100	0.010	7.698E+04
	1/1000	0.001	7.698E+03

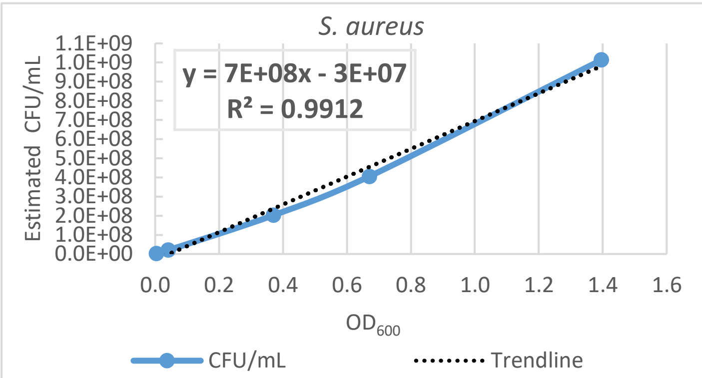
[a]The estimated CFU/mL for the dilution range was extrapolated from the average CFU/mL of neat culture determined from plate counts.

The estimated CFU/mL was plotted against the average OD_{600} values, to produce linear calibration curves for each species (**Figure 4.1**). The trendline for each calibration curve and its associated slope-intercept line equation (in the form $y = mx + c$) was used to estimate the OD_{600} value which would give the desired cell concentration (**Table 4.4**).

A



B



C

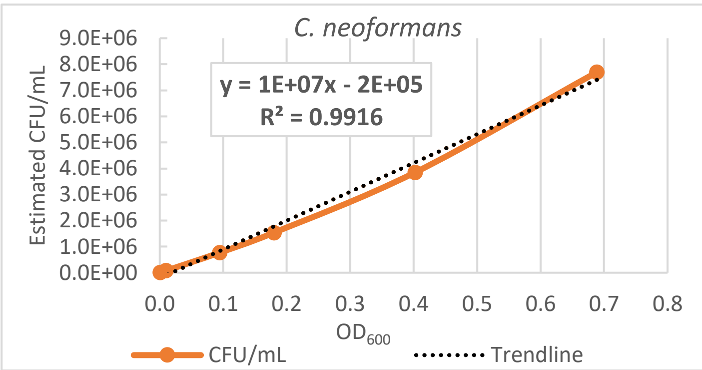


Figure 4.1: Linear calibration curves of average OD_{600} Vs estimated CFU/mL
Calibration curve with slope-intercept equation and R^2 value shown for 16-h cultures of (A) *A. baumannii*, (B) *S. aureus* and (C) C. *neoformans*. Data points are based on colony counts and OD_{600} readings from three culture replicates.

Target cell concentrations which had an OD_{600} value of less than 0.5 were selected for culture standardisation. An OD_{600} value in this range is generally recommended, as at higher OD_{600} values the graph loses linearity, thereby reducing accuracy [71].

The target cell concentrations chosen (which are represented in the slope-intercept equation as "y" were 1×10^8 CFU/mL for *A. baumannii*, 1×10^8 CFU/mL for *S. aureus*, and 3×10^6 CFU/mL *C. neoformans* (**Table 4.4**). This resulted in OD_{600} values (represented in the slope-intercept equation as "x") of 0.270, 0.186 and 0.321 respectively.

Table 4.4: Required OD_{600} values to obtain selected cell concentrations

Species	Equation of Trendline [a]	Target CFU/mL [b] (y)	Required OD_{600} (x)
A. baumannii	y = 4E+08x – 8E+06	1.00E+08	**0.270**
S. aureus	y = 7E+08x - 3E+07	1.00E+08	**0.186**
C. neoformans	y = 1E+07x – 2E+05	3.00E+06	**0.321**

[a]Slope-intercept equation $y = mx + c$, where "m" (slope) and "c" (y-intercept) are constants based on the data points used to plot the line.
[b]Target concentration in CFU/mL is represented by the variable y. The required OD_{600} (x) can be calculated when a desired concentration (y) is inputted to the equation.

In subsequent experiments, fresh cultures were brought to the predetermined OD_{600} values shown in **Table 4.4**, prior to dilution and pooling to create an MMC of the required concentration.

4.3.2 Determination of Swab Absorbance

Although both swab heads (cotton, Dacron) were of similar dimensions while dry, excess retention of PBS by one type may affect cell recovery during surface sampling. The Dacron and cotton swabs retained approximately 149 mg and 135 mg of PBS, respectively. Given the density of 1X PBS at 20°C is equal to 1.005 g/cm^3 this equates to 150 µL of PBS for the Dacron swab and 136 µL for the cotton swab. The similar volume of PBS absorbed by the two materials tested suggests they have comparable absorbance properties.

4.3.3 Recovery of MMC DNA is Not Inhibited by Direct Swab Processing

If the MMC DNA extracted directly from inoculated swabs were detected later than DNA from liquid samples, it may suggest that the presence of the swab inside the lysis tube was inhibiting the shearing action of the lysis beads. The C_q of DNA extracted directly from swabs inoculated with MMC, and from a liquid MMC suspension of the same concentration were compared using multiplex real-time PCR. Extraction of MMC DNA from swabs, as opposed to a liquid solution, produced an average C_q shift of +0.36 (**Table 4.5**).

Table 4.5: Comparison of C_q values when MMC DNA is extracted from a liquid suspension, or directly from an inoculated swab head

Species (Fluorophore)	*A. baumannii* (HEX)	*S. aureus* (ROX)	*C. neoformans* (FAM)
1 x 10^6 CFU/species			
Liquid Suspension	22.57	24.02	22.87
Swab Application	23.57	24.98	24.03
C_q Shift [a]	*+1.00*	*+0.96*	*+1.16*
1 x 10^5 CFU/species			
Liquid Suspension	26.21	28.39	26.92
Swab Application	26.13	27.83	26.59
C_q Shift [a]	*-0.09*	*-0.57*	*+0.33*

[a] The difference in C_q value (C_q direct swab – C_q liquid suspension)

These results do not indicate disproportionate lysis inhibition and suggest that the relative yield of DNA was not negatively impacted by the presence of the swab inside the lysis tube. This facilitates the direct extraction of DNA from swab samples rather than incorporating an additional rinse step to release cells from the swab material into the DNA extraction buffer.

4.3.4 Determination of Lysis Requirements using MMC Applied Directly to Swabs

The performance and lysis requirements of both extraction kits was initially evaluated using MMC suspension applied directly to swab heads. DNA isolation kit and BB duration were compared for an MMC suspension of 1,000 CFU per species applied directly to a swab head, followed by DNA extraction and real-time PCR of eluted DNA.

Table 4.6: Comparison of average C_q values obtained using differing BB durations for each DNA extraction kit

Extraction Kit	BB Duration (min)	Species (Fluorophore): *A. baumannii* (HEX)	*S. aureus* (ROX)	*C. neoformans* (FAM)
Qiagen	5	30.02	32.23	ND [a]
	10	31.57	33.84	32.78
Zymo	5	31.50	35.21	32.80
	10	32.09	36.25	33.11

[b]Not Detected for >50% of replicates (*n*=4)

The earliest C_q values for *A baumannii* and *S. aureus* were achieved using the Qiagen kit and 5 mins of BB. However, *C. neoformans* DNA was not reliably detected using this approach (**Table 4.6**), suggesting that 5 mins is insufficient to lyse the fungal cell wall of *C. neoformans* when using the Qiagen DNeasy® PowerSoil® Pro beads.

MMC DNA extracted using the Qiagen kit was consistently detected earlier than for samples extracted using the Zymo kit for each species, but most notably for *S. aureus*. *S. aureus* DNA was detected an average of 2.69 cycles earlier when extracted using the Qiagen kit (**Table 4.6**). We proceeded with 10 mins of BB for both extraction kits to further evaluate MMC recovery from surfaces.

4.3.5 Comparison of Extraction Kits and Swab Material for the Recovery of MMC DNA from Test Surfaces

To determine the most suitable MMC recovery method by comparing the swab material, surface texture and DNA isolation kit used. MMC suspension was applied to test surfaces at a concentration of approximately 10 CFU/cm^2 for each species. Trespa® laboratory bench panel was used to mimic “smooth” surfaces, and melamine-faced chipboard with a textured wood-grain effect provided a textured “grained” surface. Following air drying time of 2 h, the surfaces were sampled with swabs (cotton, Dacron) moistened with sterile PBS.

The data in **Table 4.7** shows the average C_q from all permutations of kit type, swab material and test surface when MMC was applied to surfaces at 10 CFU/cm^2 for each species.

Similar to the results seen in **Section 4.3.4**, samples extracted using the Qiagen kit were detected earlier in the multiplex PCR, relative to samples extracted using Zymo.

This was again most apparent for *S. aureus*, which was detected an average of 1.93 cycles earlier (**Table 4.7**).

Table 4.7: Average C_q values obtained for extraction kits, swab material, and test surfaces. MMC was applied to surfaces at 10 CFU/cm^2 for each species

	Species (Fluorophore)	*A. baumannii* (HEX)	*S. aureus* (ROX)	*C. neoformans* (FAM)
Kit	Qiagen	32.52	35.91	33.49
	Zymo	33.81	37.84	34.69
Swab	Dacron	32.71	36.07	33.63
	Cotton	33.62	37.23	34.55
Surface	Grained	32.78	36.45	33.92
	Smooth	33.55	37.12	34.44

Increased MMC recovery using Qiagen was also evident from the C_q values for individual sampling efforts (**Table 4.8**). The C_q values for *A. baumannii* and *S. aureus* were consistently earlier when MMC DNA was extracted using the Qiagen kit.

Table 4.8: Average C_q values obtained for MMC DNA recovered using differing swab materials on test surfaces. MMC was applied to surfaces at 10 CFU/cm^2 for each species

	Species (Fluorophore) **Swab / Surface**	*A. baumannii* (HEX)	*S. aureus* (ROX)	*C. neoformans* (FAM)
Qiagen	Dacron / Grained	31.46	34.20	32.50[a]
	Cotton / Grained	32.81	36.06	34.03
	Dacron / Smooth	32.10	35.99	33.94[a]
	Cotton / Smooth	33.71	37.38	ND
Zymo	Dacron / Grained	33.18	38.04	34.46
	Cotton / Grained	33.67	37.49	34.67[a]
	Dacron / Smooth	34.11	ND	ND
	Cotton / Smooth	34.27	38.00	34.94[a]

[a]1/2 technical replicates not detected; ND = 2/2 technical replicates not detected.

However, the positivity rate of *C. neoformans* detection was 50% (n=8) for both Qiagen (**Figure 4.2C**) and Zymo kits (**Figure 4.3C**). This low recovery rate suggests that 10 CFU/cm^2 is below the threshold for reliable detection of *C. neoformans* cells using this method.

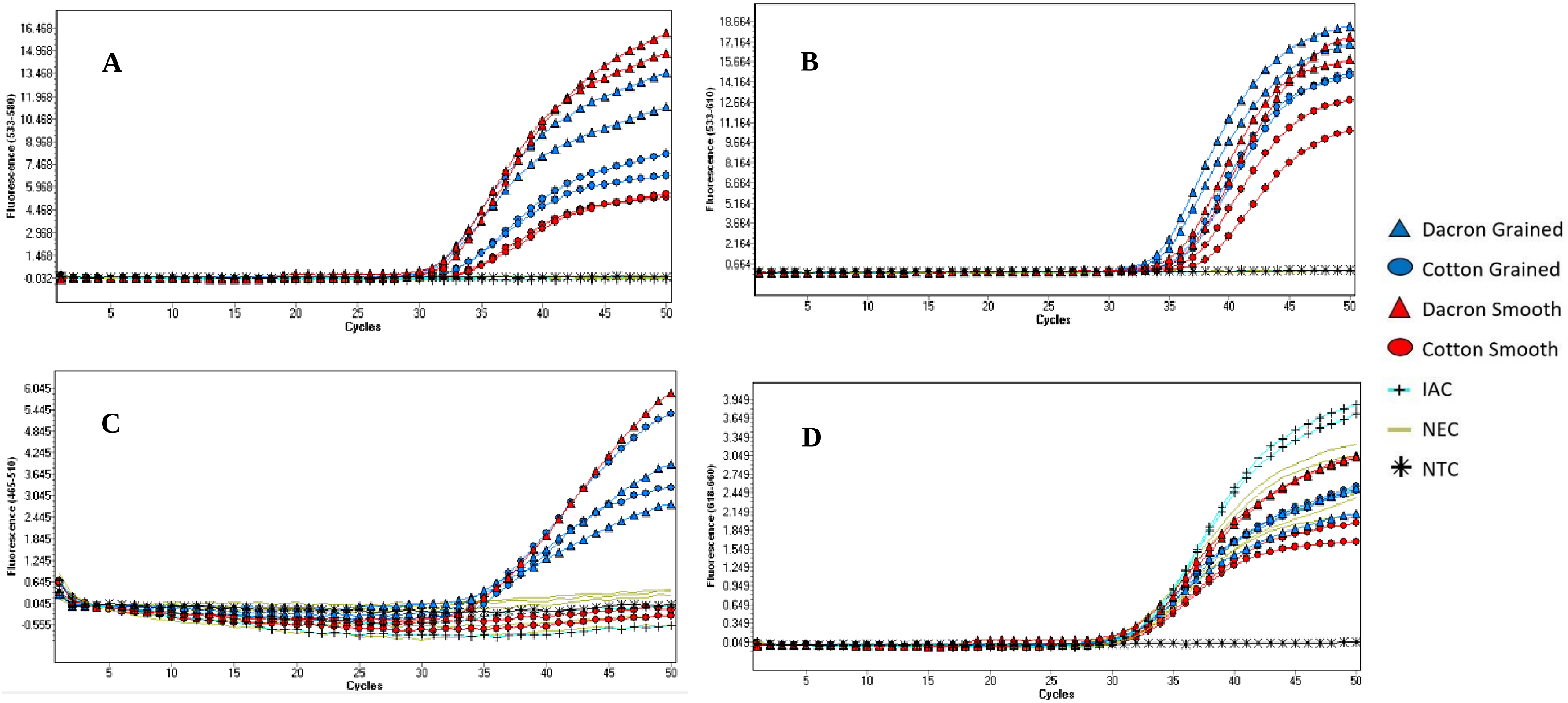


Figure 4.2: PCR amplification curves of MMC DNA extracted using Qiagen PowerSoil Pro. MMC was applied to surfaces at 10 CFU/cm^2 for each species

(A) *A. baumannii* detected in 100% (n=8) of samples, (B) *S. aureus* detected in 100% (n=8) of samples, (C) *C. neoformans* detected in 50% (n=8) of samples, (D) SIAC detected in 100% of samples.

Dacron swabs (Triangle) and Cotton swabs (Circle) were used on Grained (Blue) and Smooth (Red) test surfaces. NEC (Gold) = Negative Extraction Control, NTC (Black) = Negative Template Control, SIAC (Turquoise) = synthetic internal amplification control template only.

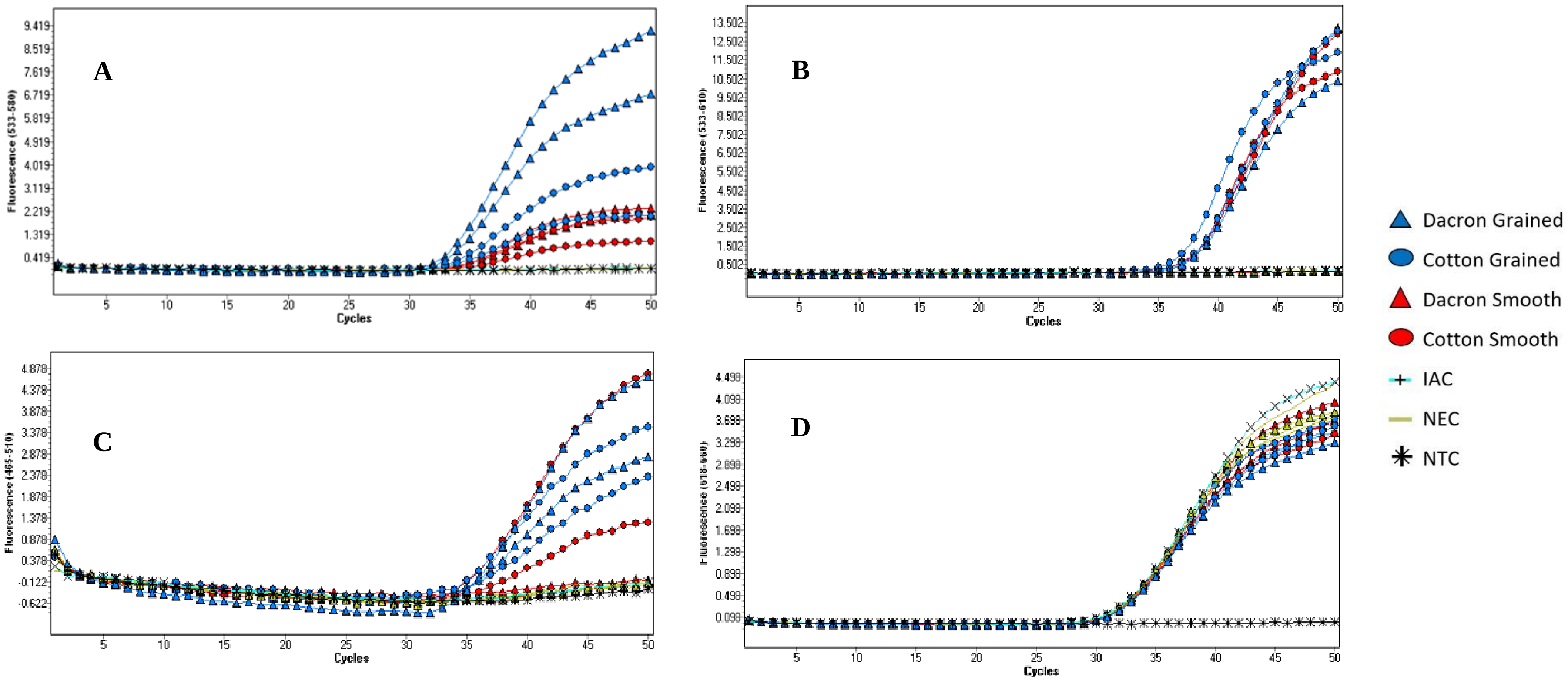


Figure 4.3: PCR amplification curves of MMC DNA extracted using ZymoBIOMICS. MMC was applied to surfaces at 10 CFU/cm^2 for each species

(A) *A. baumannii* detected in 100% (*n*=8) of samples, (B) *S. aureus* detected in 75% (*n*=8) of samples, (C) *C. neoformans* detected in 50% (*n*=8) of samples, (D) SIAC detected in 100% of samples.

Dacron swabs (Triangle) and Cotton swabs (Circle) were used on Grained (Blue) and Smooth (Red) test surfaces. NEC (Gold) = Negative Extraction Control, NTC (Black) = Negative Template Control, SIAC (Turquoise) = Synthetic Internal Amplification Control template only.

Based on these results, the Qiagen kit was selected to further evaluate swab material and surface texture on MMC recovery. For this purpose, an increased MMC concentration was used. By increasing the starting MMC concentration, the true effect of the swab material and surface texture was determined, without the introduction of other limiting factors such as lower limit of detection of the assay.

4.3.6 Grained Surfaces and Dacron Swabs Increase Recovery of MMC DNA

The Qiagen kit was used to further investigate the impact of test surface texture and swab material on the recovery efficiency of MMC DNA. MMC suspensions were applied to test surfaces at concentrations of 100 CFU/cm^2 and 50 CFU/cm^2 for each species and allowed to air-dry for 24 h. Swab sampling occurred as described previously. DNA was extracted from swabs using the Qiagen kit with BB duration of 10 mins. MMC DNA was consistently detected with earlier C_q values when Dacron swabs were used to sample a grained surface (**Table 4.9**).

Table 4.9: Average C_q values for each MMC species detected in multiplex real-time PCR at 100 CFU/cm^2 and 50 CFU/cm^2

CFU/ cm^2	Species (Fluorophore) / Swab / Surface	*A. baumannii* (HEX)	*S. aureus* (ROX)	*C. neoformans* (FAM)
100	Dacron / Grained	28.03	31.02	29.94
	Cotton / Grained	28.82	31.62	30.66
	Dacron / Smooth	28.76	31.06	30.22
	Cotton / Smooth	29.72	32.11	31.21
50	Dacron / Grained	29.07	31.54	30.06
	Cotton / Grained	29.84	32.50	30.73
	Dacron / Smooth	30.91	34.35	32.02
	Cotton / Smooth	30.77	33.79	31.64

This finding applied to both the MMC concentrations tested and for each species. The results achieved using Dacron swabs on grained surfaces for recovery of 100 CFU/cm^2 MMC (**Figure 4.4**) and for 50 CFU/cm^2 MMC (**Figure 4.5**) were indicative of enhanced recovery over the other methods tested.

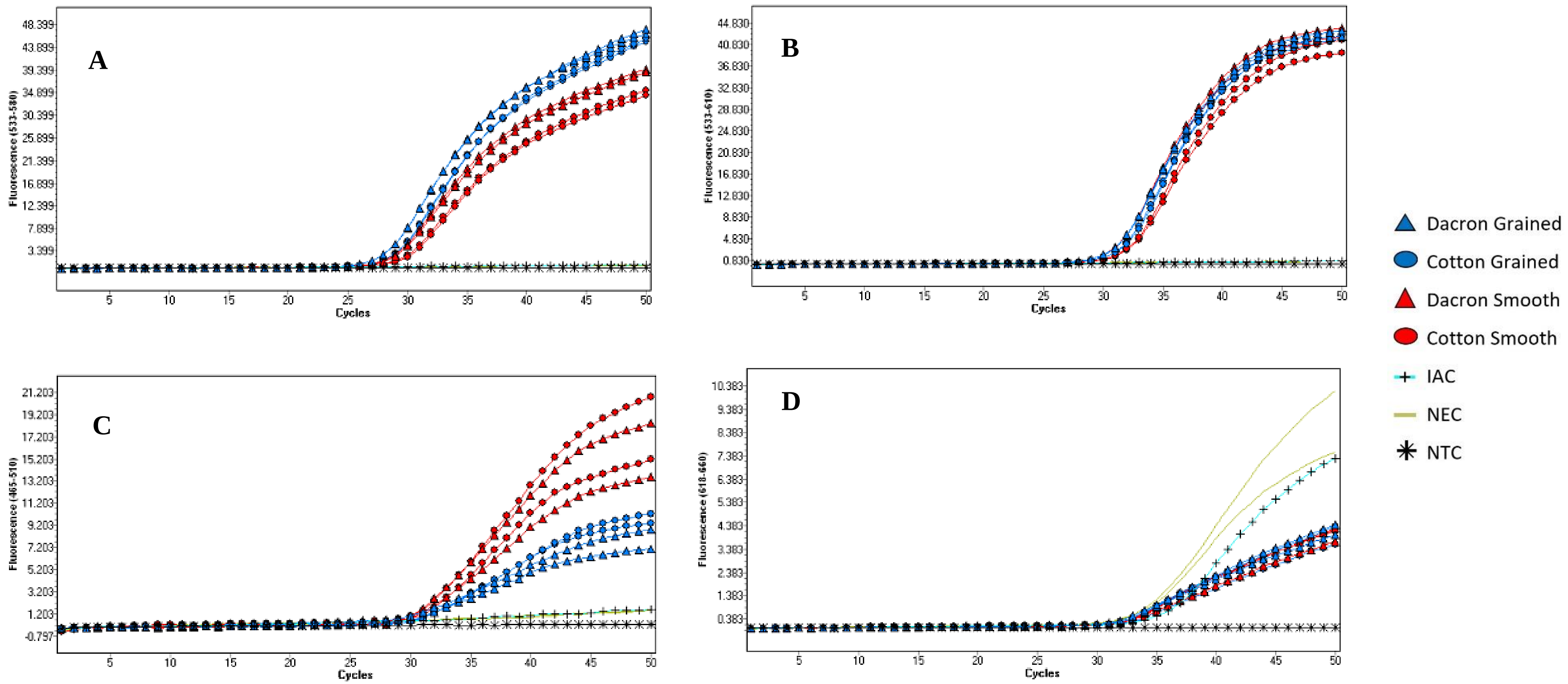


Figure 4.4: PCR amplification curves of MMC DNA recovered from surfaces spiked with 100 CFU/cm^2 for each species and extracted from swabs using Qiagen PowerSoil Pro

(A) *A. baumannii* detected in 100% (*n*=10) of samples, (B) *S. aureus* detected in 100% (*n*=10) of samples, (C) *C. neoformans* detected in 100% (*n*=10) of samples, (D) SIAC detected in 100% of samples.

Dacron swabs (triangle) and cotton swabs (circle) were used on grained (blue) and smooth (red) test surfaces. NEC (gold) = negative extraction control, NTC (black) = negative template control, SIAC (turquoise) = synthetic internal amplification control template only

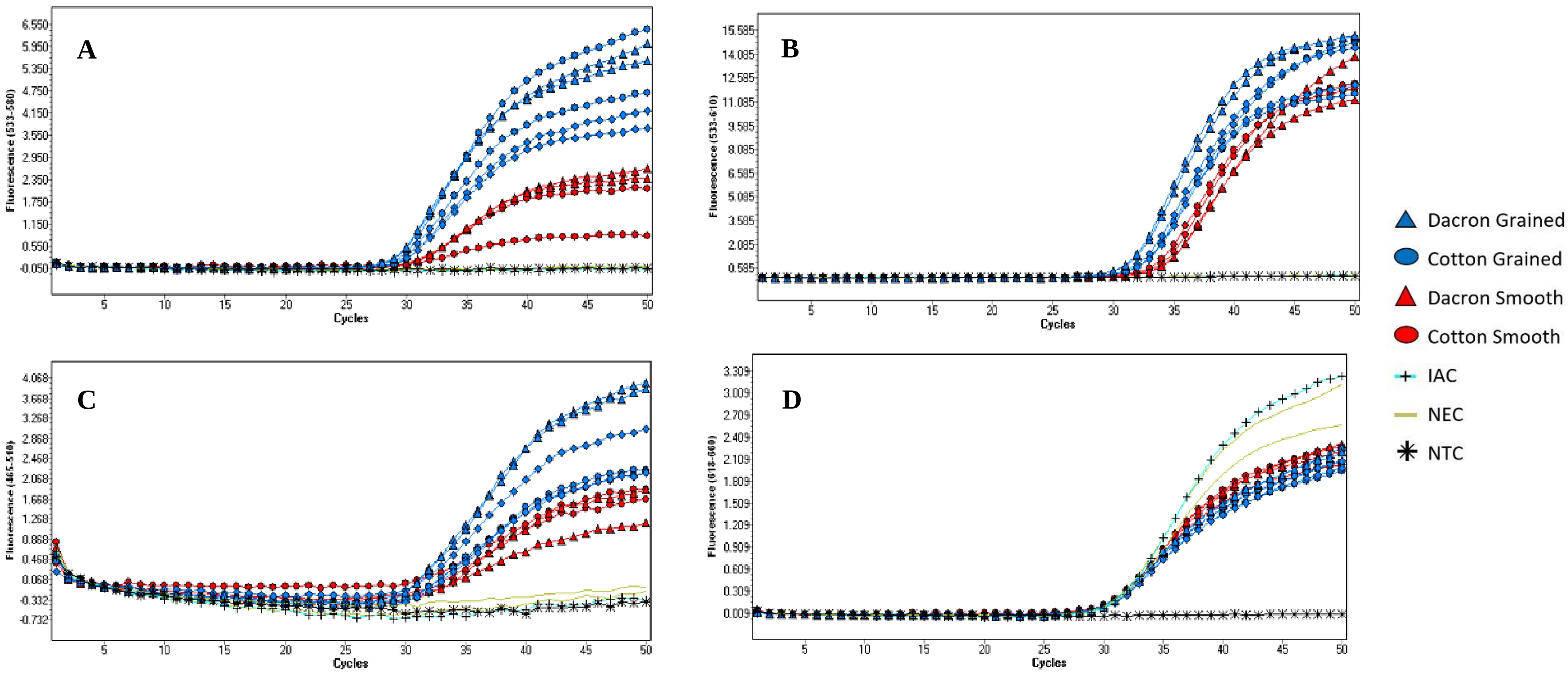


Figure 4.5: PCR amplification curves of MMC DNA recovered from surfaces spiked with 50 CFU/cm² for each species and extracted from swabs using Qiagen PowerSoil Pro

(A) *A. baumannii* detected in 100% (*n*=8) of samples, (B) *S. aureus* detected in 100% (*n*=8) of samples, (C) *C. neoformans* detected in 88% (*n*=8) of samples, (D) SIAC detected in 100% of samples. Dacron swabs (triangle) and cotton swabs (circle) were used on grained (blue) and smooth (red) test surfaces.

NEC (gold) = negative extraction control, NTC (black) = negative template control, SIAC (turquoise) = synthetic internal amplification control template only.

Detection of 50 CFU/cm^2 was achieved using each of the swab and surface permutations tested (**Table 4.9**). Based on the relative C_q values for each sampling approach, the recovery of MMC DNA was highest when Dacron swabs were used on grained surfaces. This finding was consistent with the results presented in **Table 4.8** when this sampling combination also produced the earliest C_q values.

4.3.7 *S. aureus* was Not Detectable in a Culture-Based Comparison

S. aureus was chosen as the representative organism to conduct a culture-based comparison for surface swab recovery. No growth was observed on plates inoculated with sampling liquid (100 µL) obtained from surfaces which *S. aureus* cell suspensions of 1 x 10^4 CFU (100 CFU/cm^2) and 1 x 10^5 CFU (1,000 CFU/cm^2) had been applied. This result is in contrast to the molecular-based method which detected 10 CFU/cm^2 of *S. aureus* (**Figure 4.2B, Figure 4.3B**).

4.3.8 Multiplex Real-Time PCR to Detect MMC DNA

The multiplex real-time PCR used in this study was created from monoplex real-time PCR assays which were previously designed and validated in this laboratory as part of other studies. The sequence and concentration of the oligonucleotide primers and probes used in each of these assays are shown in **Table 4.2.**

The hydrolysis probes which detect *C. neoformans, S. aureus*, *A. baumannii* and the SIAC template were labelled with the 5′ fluorophores FAM, ROX, HEX and Cy5, respectively. Each of the four hydrolysis probes used 3′ Iowa Black® quenchers, with the appropriate modification (FQ or RQ) corresponding to the emission spectra of the associated fluorophore. The *A. baumannii* and SIAC probe also contain internal quenchers between the 9th and 10th nucleotide base of TAO™ and ZEN™, respectively [72].

The sequence of the 500 bp double sided gene fragment (**Supplementary Table S4.1**) used as the SIAC template was generated using a random nucleotide sequence generator [73]. The gBlock® was diluted and added to the PCR master mix to give the desired template concentration per reaction.

4.3.9 Optimisation of Multiplex Real-Time PCR Assay

Optimisation of the multiplex PCR reaction conditions took place to improve the analytical sensitivity of the assay. The relative concentrations of the oligonucleotide primers and probes, and of the SIAC template were adjusted based on the C_q values obtained for a dilution series of gDNA for each species.

Analytical sensitivity of 10 GE for each target species in the multiplex was achieved using the oligonucleotide concentrations outlined in **Table 4.2**. It was necessary to use an increased concentration of primers targeting *C. neoformans*, relative to the other assays in the multiplex to ensure detection to 10 GE (**Figure 4.6C, Table 4.10**).

Table 4.10: Average C_q values obtained using optimised oligonucleotide concentrations

Species (Fluorophore)	*A. baumannii* (HEX)		*S. aureus* (ROX)		*C. neoformans* (FAM)	
Template Concentration [a]	Avg. C_q	Std. Dev.	Avg. C_q	Std. Dev.	Avg. C_q	Std. Dev.
100 GE	31.34	0.07	32.20	0.07	32.96	0.03
10 GE	33.29	0.08	35.81	0.34	35.31	0.21

[a]Quantified gDNA extracted from pure culture isolates

The optimised SIAC template concentration was determined to be 1,000 copies per reaction, which gave C_q values between 31.04 and 31.94 (**Figure 4.6D**). The results in **Figure 4.6** additionally demonstrate the specificity of each assay in the multiplex for its target. For example, **Figure 4.6A** which shows the HEX 533-580nm channel, *A. baumannii* (labelled with triangles) was detected, while *S. aureus* (squares) and *C. neoformans* (circles) were not, confirming that no cross-reaction between species, or fluorophore leakage between channels occurred.

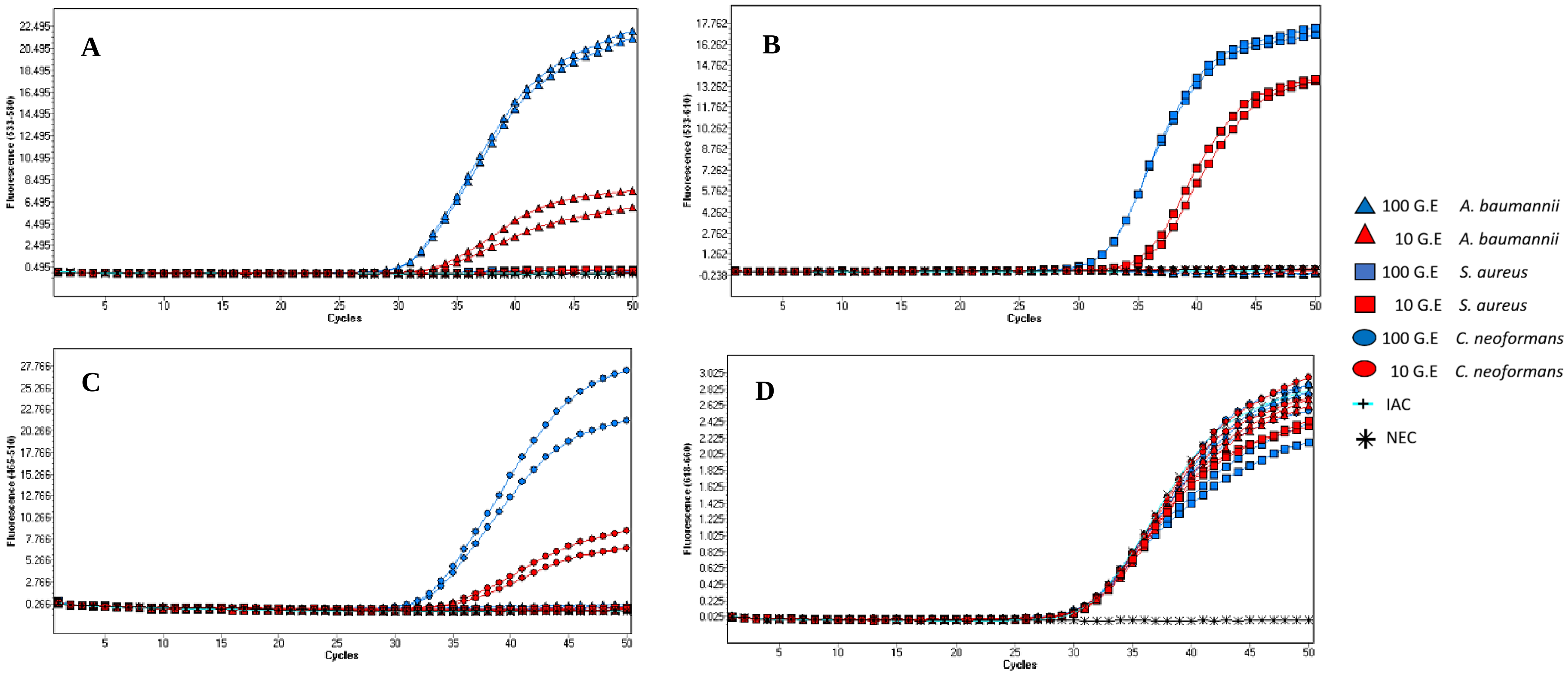


Figure 4.6: PCR amplification curves of pure culture gDNA demonstrating target specificity, and analytical sensitivity to 10 GE for each species

(A) *A. baumannii* detection in HEX 533-580nm. Concentrations shown are 100 GE (blue triangles) and 10 GE (red triangles). (B) *S. aureus* detection in ROX 533-610nm. 100 GE (blue squares) and 10 GE (red squares). (C) *C. neoformans* detection in FAM 465-510nm. 10 GE (blue circles) and 10 GE (red circles). (D) SIAC detection in Cy5 618-660nm with input concentration of 1,000 copies/reaction. Detection of SIAC occurred in gDNA samples spiked with SIAC template (blue, red) and in the SIAC template only control reaction (turquoise).

4.4 Discussion

The precise role of the hospital environment in the transmission of HAI remains unknown [12, 24, 37]. Our understanding microbial surface contamination in healthcare facilities has been limited by the use of insensitive, culture-based techniques. Routine surveillance of the hospital environment is currently not recommended, and therefore little has been done to standardise the techniques involved [20, 38]. Existing research using both culture-based and molecular-based detection methods have yielded varying results on the most effective and sensitive approach [36, 39, 62, 63, 68]. A literature review was conducted to determine which experimental variables should be included in this study for comparison (**Table 4.1**), and to assess the limitations of existing studies. In this study, we have described a simple and easily reproducible method to conduct environmental surface sampling. A mock microbial community (MMC) consisting of bacterial and fungal species was used to simulate contamination. Reliable detection of 50 CFU/cm^2 for each species was achieved using Dacron swabs and the Qiagen DNeasy® PowerSoil® Pro DNA extraction kit with 10 mins bead beating.

There is no single sampling method which can recover all biomass types (DNA, viral, bacterial, fungal cells) from all surface textures with equally high efficiency. For this reason, the experimental design of this study was made to resemble the surfaces and pathogens most likely to be found in a hospital environment. Several studies have created mock environmental conditions with the purpose of evaluating the efficiency of recovery methods. The results of these studies are varied, due in part to the enormous number of variables involved in experimental design such as inoculum, surface material and area, sampling material, and detection method. When interpreting the results from existing studies, it is important to remember that the most appropriate method for one condition may not be as effective in others [40]. Often, the superior collection material (e.g., swab, sponge, wipe) will differ based on the detection method used. Culture-based detection requires viable cells to be collected and released intact into culture medium, while molecular methods do not. Therefore, the results of existing studies will be discussed below in terms of detection method, i.e., molecular, or culture-based detection.

A limited number of studies employ molecular-based detection in combination with surface sampling. PCR and quantitative (qPCR) approaches have largely been

reserved for investigations into the recovery of viral pathogens [54, 74-77] or free DNA [49, 65, 78]. Overall, there remains no clear consensus on the most efficient method for surface recovery of any of these biological sample types.
Jansson *et al.* [68] studied the recovery of *Listeria monocytogenes* on glass and ridged plastic at concentrations of between 1 x 10^6 CFU/cm^2 (4 cm^2) and 4 x 10^4 CFU/cm^2 (100 cm^2) using qPCR. They found that cotton swabs performed better than flocked nylon, and that recovery was higher on glass surfaces rather than ridged plastic. They also investigated the recovery of Mengovirus using the same range of swab and surface materials. Importantly, the authors concluded that the recovery efficiency of differing swab materials is highly dependent on the target biomass (bacteria, virus, free DNA) and of the surface area and texture. However, the use of frozen *L. monocytogenes* cells may introduce higher levels of free DNA than anticipated due to cell lysis upon thawing, which may in turn affect the DNA extraction process. In contrast to the approach used by Janssen *et al.*, this study used freshly cultured MMCs and lower cell concentrations in the range of 10-100 CFU/cm^2 for each MMC species (**Section 4.2.4**), to more closely mimic hospital environmental contamination from "persistor" organisms.

An in-depth study into the factors influencing microbial recovery efficiency was conducted by Gomez-Silvan *et al.* [55]. This study focused on the domestic built environment and used an MMC which included *Pseudomonas syringae*, *Bacillus subtilus*, *Saccharomyces cerevisiae* and *Aspergillus nidulans*. The authors outlined the role of nucleic acid extraction kits, swab material, surface type and sample storage conditions on the recovery of nucleic acid, using qPCR. Of the surface types investigated they found a high degree of inter-surface variability, resulting in similar overall recovery averages for the metal, plastic and wooden surfaces tested. Similarly, there was no significant difference between recovery efficiency for the flocked nylon eSwab®, polyurethane BBL™ CultureSwab™ EZ, or the sponge BiSKit™.
However, the composition and concentration of the MMC used in this study may have resulted in some of the variability seen. *P. syringae* consistently displayed poor surface recovery (<25%), thereby reducing the overall sample averages. The Gram-negative bacteria was seeded at a concentration of approximately 116 cells/cm^2 (35% of an MMC containing 1 x 10^4 cells applied to 30 cm^2). This was not the case when MMC was applied directly to a swab head, which indicated the loss was during the seeding

and recovery process. This highlights the need for careful consideration of MMC preparation, lysis conditions and the lower limit of detection when conducting tests to evaluate recovery efficiency. During protocol optimisation in the current study, it was possible to detect bacterial species applied to surfaces at 10 CFU/cm^2 (**Table 4.9**). However, the final conclusions on swab material and surface type were made using higher MMC concentrations to avoid introducing the lower limit of detection of the multiplex real-time PCR assay as an additional factor (**Table 4.10**). This ensured that the conclusions formed were based on true recovery levels for all cell types.

An earlier study [50] which compared qPCR and culture found that that smooth surfaces (glass, metal) produced the highest recovery rates for *Pantoea agglomerans* (formerly *Enterobacter agglomerans, Erwinia herbicolata* [79]). The recovery efficiency, using 2.1 x 10^6 “templates”/cm^2 did not exceed 53% in qPCR (6.8 x 10^8 templates applied to 317 cm^2). Crucially, the recovery efficiency for culture did not exceed 0.006% (6.8 x 10^5 CFU/cm^2; 6.35 x 10^8 CFU applied to 930 cm^2). Due to the low recovery efficiency of culture, no meaningful comparison of collection material could be made. For the qPCR analysis, the authors found that macrofoam swabs produced higher recovery efficiencies than sponge, although commented that sponge may be useful for large, non-porous surfaces. The macrofoam swabs and sponges were subject to a rinse step to remove the collected cells, followed by filter concentration and either DNA extraction or culture. As discussed previously, this may lead to sample loss, including on the filter membrane used for sample concentration. A major advantage of using swabs (or collection materials with a similar dimension) is their ability to fit into standard microcentrifuge tubes, thereby allowing them to be directly processed during DNA extraction.

Both Jansson *et al.* [68] and Buttner *et al.* [50] found recovery was higher when sampling from smooth surfaces, while Gomez-Silvan *et al.* [55] reported large amounts of inter-surface variability with no surface providing superior recovery. In contrast, our results suggested that the “grained” surface consistently produced higher recovery of MMC DNA, inferred via the relative C_q value in a real-time PCR. The swabs used in these studies are not directly comparable to our finding of Dacron swabs having increased recovery relative to cotton. The ability of the technique developed in this study to detect 10 – 50 CFU/cm^2 in a mixed community of bacterial and fungal species has not yet been demonstrated in existing literature.

Despite the limitations of culture-based methods, it's low cost and relative simplicity to perform means that it remains the most frequently used detection method for both hospital, and laboratory-centred research [37, 62, 80]. Contributions from the findings of culture-based research have informed us on important aspects such as which hospital surfaces are likely to have an increased bioburden, and techniques for enhanced cell collection [12, 46, 63, 81]. With regard to collection methods which have yielded the best results in simulated laboratory trials, the results are again, varied. A review of several such papers carried out by Rawlinson *et al.* [39] determined that cotton and macrofoam swabs were in general, the most effective swab material, while sponges were advantageous for *Clostridium difficile* collection and for large uneven surfaces. They also discuss the advantages of direct collection methods such as contact plates and dipslides for sampling textiles, and for collection of methicillin-resistant *S. aureus* (MRSA) from non-porous surfaces.

The low sensitivity of culture based sampling was highlighted in the review by Galvin *et al.* [40] which found recovery efficiencies for indirect collection methods such as swabs and sponges to be 10% - 71%, with a median value of approximately 30%. Although cotton-tipped swabs were the most frequently used in their qualitative analysis of studies, the authors recommend flocked nylon swabs. They concluded that a standardised approach to hospital environmental sampling would not only allow for meaningful comparisons between the results of studies, but also create a starting point for introducing guidelines on acceptable levels of hospital surface contamination.
One aspect which existing literature agrees upon, is the advantage of pre-wetting the collection matrix before sampling dry surfaces [46, 47, 63]. This can be performed with a range of sterile liquids, including culture broth [11, 60], water [82], NaCl solution [57, 83], neutralising buffer [47, 62] or, as used in this study, PBS [45, 53, 55]. Neutralising buffer is necessary to counteract the effect of disinfecting compounds when culturing samples taken from treated surfaces, and is part of the International Standards for sampling from food preparation areas [61]. Although necessary for culture-based recovery, PBS was chosen to avoid the use of additional detergents which could impact cell lysis and affect the results of our study [63].

Similar to the results of our culture-based comparison, studies which directly compared detection methods found considerable increase in sensitivity when molecular approaches such as PCR were used [11, 50, 83]. In this study it was not possible to detect 1,000 CFU/cm^2 of *S. aureus* using a swabbing technique based on the one outlined by Hedin *et al.* [63] (**Section 4.3.7**). Comparatively, Hedin *et al.* demonstrated a recovery efficiency of 16% using similar surface and swab materials: “plastic laminated MDF-board surface” and Rayon swabs moistened with PBS. However, a higher starting inoculum of 1.76 x 10^4 cells/cm^2 was used with the aim of mimicking contamination of surfaces from spillage of biological fluids. These concentrations were not in the range of interest for our study, which aimed to simulate typical hospital ICU surveillance conditions [10].

This study provides a framework for the sensitive detection of pathogens from surfaces in the hospital environment. We have developed this protocol with the aim of improving and standardising microbial sampling, using simple and readily available methods. For this study, fresh MMC suspension were cultured, quantified, and standardised prior to each experiment. A linear calibration curve was created to calculate the OD_{600} value required for each species to achieve a standardised CFU/mL (**Table 4.4**). This approach enabled accurate MMC standardisation without the need for additional colony counting experiments or freeze-thawing of previously quantified samples [47, 68]. For the sampling material, a number of factors were considered, such as swab composition, swab moistening, and swab absorbency. These conditions have been shown to affect the results of previous studies investigating the sampling efficiency of swabs for bacterial cells and DNA [44, 46, 65]. Both the cotton and Dacron swabs used were shown to absorb similar volumes of PBS (within 10% of each other) and thus were considered comparable. The swab material chosen should be efficient not only at collecting microbial cells from a surface, but also at releasing them into the sample processing matrix, in this case, the gDNA lysis buffer. For this purpose, the effect of extracting MMC DNA directly from a swab was investigated by extracting MMC DNA directly from an MMC liquid suspension, and from a swab to which MMC suspension had been applied. The C_q shift observed between the two samples (**Table 4.5**) indicated minimal loss of MMC DNA due to sample retention on the swab, or due to the swab material inhibiting MMC cell lysis. The ability to perform cell lysis directly from the swab material increases cell recovery and reduces hands-on sample preparation time. The lysis protocol for the DNA extraction kits selected was also optimised prior to conducting surface sampling experiments. The lysis conditions required for mixed community samples, such as the MMC used in this study can vary based on cell-wall composition [43]. Two BB durations of 5 and 10 mins were investigated. The results suggested that 5 mins of BB was insufficient to lyse the fungal cell wall, while 10 mins of BB resulted in detection of each MMC species without degradation of DNA from Gram-negative cells (**Table 4.6**). The data generated during this initial pre-surface seeding phase provides insight into the performance of the sampling collection methods chosen and can be overlooked in other studies which investigate surface sampling [38, 40].

This work aimed to simulate contamination of surfaces in a healthcare environment by using two materials commonly used to make desks, tables, and work surfaces in healthcare facilities (**Table 4.1**). Standardised MMC was applied to two test surfaces and allowed to dry for periods of up to 24 h. Surface seeding was conducted in parallel, using the same MMC suspension to ensure equivalent treatment of samples. The Qiagen kit was chosen to further evaluate the effect of swab material and surface texture on MMC recovery, as it was determined to have a higher overall recovery efficiency (**Table 4.7**). The results also suggested that this protocol approached its lower limit of detection when MMC was applied to surfaces at 10 CFU/cm^2 per species, as detection in the real-time PCR was possible, but inconsistent (**Table 4.8**). There are currently no other studies which have demonstrated the ability to detect, or indeed have conducted surface sampling at this level [48, 62]. While the ability to detect such a low level of MMC from surfaces was promising, a higher starting concentration was chosen for the remaining surface sampling experiments so that the results were detected with consistency and could be reliably compared. Therefore, the conclusions on swab material and surface texture were drawn from the data generated using surfaces spiked with 50 and 100 CFU/cm^2 for each MMC species. In MMC applied at 50 CFU/cm^2 for each species, *A. baumannii* and *S. aureus* were detected in 100% of swab samples, and *C. neoformans* was detected in 88% of swab samples (**Figure 4.5**). All three MMC species were detected in 100% of swab samples when MMC was applied at 100CFU/cm^2 for each species (**Figure 4.4**). At each concentration tested, MMC DNA was detected at an earlier C_q value when Dacron swabs were used to sample a textured surface (**Table 4.8, Table 4.9**), indicating higher recovery efficiency. These results were finally compared to a culture-based surface sampling method described by Hedin *et al.* [63], which was unable to detect 1,000 CFU/cm^2 of *S. aureus*, further highlighting the superior sensitivity of molecular-based detection.

The protocols developed in this work were standardised so that comparative data could be generated in future studies using alternative MMC species, DNA extraction kits, swab material or test surfaces. The limitations of this study include the exclusive use of a flat 100cm^2 area. Future work would include spiking and sampling a range of surface sizes and irregular shapes such as buttons and rails, which are frequent reservoirs of pathogens in the hospital environment [25, 84, 85].

We have demonstrated that this method can be used to detect low starting concentrations of pathogens from surfaces using an MMC and simulated environmental conditions. Reliable detection of 50 CFU/cm^2 of each MMC species was achieved using surface swabbing, direct swab processing, and real-time PCR. The data generated indicates that the Qiagen DNeasy PowerSoil Pro kit, Dacron swabs and surfaces with a textured, wood-grain effect produced the highest recovery efficiency, based on the C_q values obtained in a real-time multiplex PCR assay. The optimised methodology developed in this study could be used in hospitals to monitor for cleaning deficiencies and identify sources of contamination when outbreaks occur. It could serve as a useful research tool to understand the role the built environment plays in the spread of HAIs, or to assess biohazard threat and quality-assurance. Improved methods that improve environmental surveillance will increase our understanding of HAI transmission, and guide future policy on infection prevention and control.

4.5 Supplementary Material

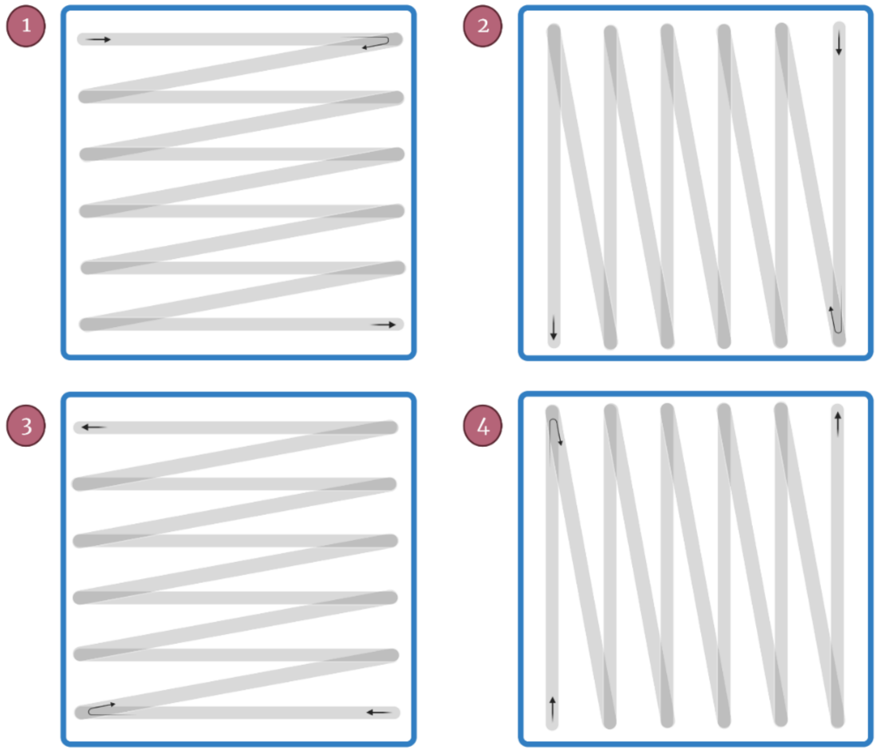


Figure S4.1: Slalom swabbing pattern used to sample test surfaces
An area of 10cm x 10cm was sampled using four passes of the same swab: (1) top to bottom (2) right to left, (3) bottom to top, (4) left to right. *Image created with BioRender.com*

Table S4.1: Sequence of synthetic double-sided DNA fragment (gBlock®) used as template for the IAC assay

Sequence (5′-3′)	5′-CATAAAAAAAAGGACGTCGACGGACCAATTTTAGGATGACACCA CTAATCGATCGTCCTTCGTCGTATCGCAAGTTTCGCGCCAGATCGAA GTGAGCATCGCTGCCAGGCGGGGCAAAGGATAGACGAACTCGCATGT TTCGTTACAGCATTGTTTTTGCTAAACGGCTTCCTAGTCGTACGGCG AAAATTTAAATCGTACAGCTCTTTGGTGACCACGCCTTTACCCCTTA GGCAATATAAATTGTAGATCTTCTGACTGCGCGGTCCTTAGGATTAT ACGAACTCGACTGCCCAACCCCAAACGTGACATCTCGTCGGGACACC GTATTGGACCAGGGGGCTGCGGGCTTTTCGCCGGTTCATCCCTCAAA CGGCGTTGCCCTAGCTTTTCCAATTCGCGCATAATTTGTTAGCAAAG GACACGTTAAGCACGTTAGCTCGTGCCCGTAGCCCCCAGCTACCGCT CCTCCGTACATTATAAAAACGGATGGATTACTT-3′

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Chapter 5: Conclusions and Future Research

5.1 General Remarks

5.1.1 The Clinical Relevance of the ESKAPEs

The ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp*.) represent the most difficult to treat and burdensome causes of hospital acquired infection (HAI). The selection and grouping of these particular species followed the release of the research agenda of the US National Institute of Allergy and Infectious Disease, where their propensity to cause HAI and "eskape" conventional treatment was highlighted [1]. Since then, the ESKAPEs continue to cause the majority of HAI and remain the primary targets of efforts from the WHO and CDC to reduce infections caused by antimicrobial resistant bacteria [2-4]. The ESKAPE pathogens are set apart from other nosocomial pathogens by a combination of antimicrobial resistance and virulence factors, and their ability to cause HAI with high morbidity [5].

5.1.1.1 Antimicrobial Resistance

Each of the ESKAPE pathogens display high levels of antimicrobial resistance (AMR) and cause a large proportion of the overall deaths associated with AMR worldwide [5]. Vancomycin-resistant *E. faecium*, methicillin-resistant *S. aureus*, and carbapenem-resistant *K. pneumoniae, A. baumannii*, *P. aeruginosa* and *Enterobacter* are of particular concern. In Ireland, these resistance phenotypes were reported in 38.4%, 12.6%, 17.6%, 1.6%, 6.5% and 25.8% of ESKAPE isolates in 2019, respectively [6, 7]. These figures are much higher in certain geographic regions such as Eastern Europe and Southeast Asia, which is of great concern owing to the limited treatment options caused by bacteria resistant to these antimicrobials [8].

5.1.1.2 Resistance to Chemical and Environmental Stress

Many of the ESKAPE pathogens display an ability to survive stressful conditions which typically cause bacterial cell death. This enables them to survive outside the host in the hospital environment. *E. faecium* is noted for its ability to tolerate to heat and disinfectants [9]; *S. aureus* and *P. aeruginosa* produce thick biofilm on inanimate surfaces [10, 11]; and *A. baumannii* has been shown to resist desiccation on dry surfaces for up to 30 days [12, 13]. These traits are not unique to the individual species, and considerable overlap occurs, particularly with the production of biofilm. However, amongst the ESKAPEs, these species can be considered to be paradigms of environmental stress survival mechanisms.

5.1.1.3 Metabolic Adaptability and Genomic Plasticity

The ability of the ESKAPE pathogens to resist antimicrobials and to tolerate environmental stressors stems from perhaps, their most important feature. ESKAPE species are highly adaptable and display an incredible ability to uptake and regulate genetic information. Metabolically, they use multiple respiratory and metabolic pathways to ensure survival in low nutrient conditions. Genetically, virulence and resistance determinants are readily shared on mobile genetic elements such as plasmids and integrons [14]. Each ESKAPE has evolved to establish itself in the hospital setting and to maximise its success as a nosocomial pathogen [15-20].

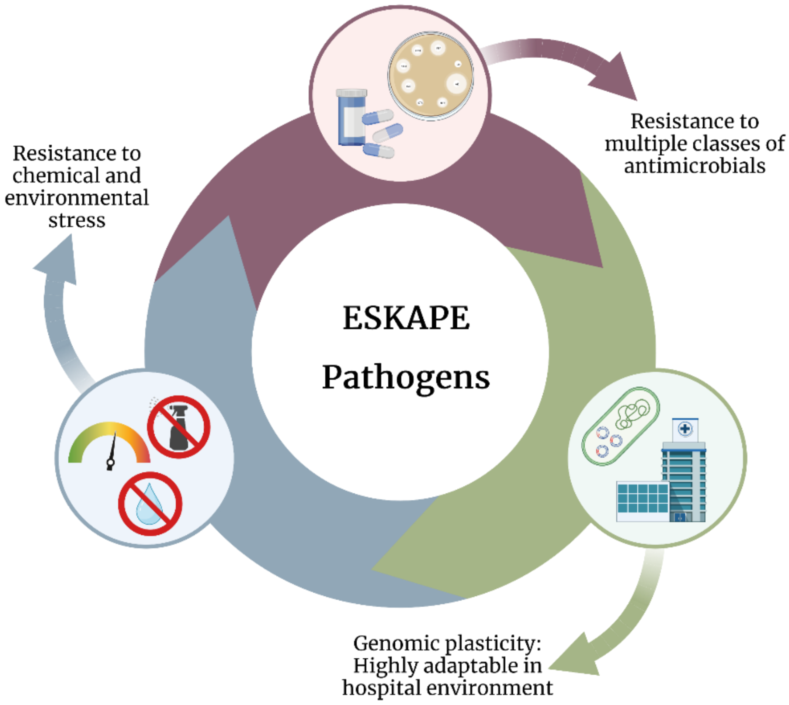

Figure 5.1: No single characteristic enables the ESKAPE pathogens to cause high levels of burdensome HAI. Rather, a combination of resistance and virulence factors influence and enhance each property. *Image created with BioRender.com*

5.1.2 Current Limitations in ESKAPE Diagnostics

The accurate and sensitive detection of ESKAPE pathogens from clinical and environmental samples plays a central role in controlling their ability to cause disease [21]. Culturing of samples continues to be the gold standard for bacterial detection and identification. Prevention of HAI and control of pathogen outbreaks relies on appropriate surveillance measures, and the prompt identification of infection [22-24]. The use of culture-based diagnostics inhibits this. For example, the sensitivity of culture-based methods for hospital acquired and ventilator associated pneumonia (HAP, VAP) has been shown to be as low as 37.8% for ESKAPE pathogens and is even lower for slow-growing and fastidious organisms [25]. The low sensitivity and time required for bacterial culture can negatively affecting patient outcomes. Rapid nucleic acid diagnostics (NAD) offer a faster, more accurate and sensitive alternative to culture-based methods. The use of rapid NAD for the detection and surveillance of HAI caused by ESKAPE species will improve our understanding of their epidemiology, resistance patterns, and transmission.

The ESKAPE pathogens pose an increased threat to human health and are frequent causes of HAI. As such, diagnostics for individual members of the ESKAPEs have been developed with some receiving CE-IVD certification for use in clinical laboratories [26-30]. However, the specificity of existing methods to identify certain ESKAPE species is low. Advancements in whole genome sequence (WGS) analysis had unveiled closely related species which are distinct from the true ESKAPE pathogens [31-33]. These species are phenotypically indistinguishable from the ESKAPE species to which they are closely related, and until recently remained unrecognised as independent entities. Some can be found in complexes (the *A. baumannii* group, the *K. pneumoniae* Species Complex), or in the case of *Enterobacter* are contained in differing genera [34-37].

Rapid and sensitive methods to detect HAI will not only improve patient outcomes but also provide us with more information on their incidence and transmission.

5.2 General Conclusions

The work outlined in this thesis provides a suite of methods to accurately identify the ESKAPE pathogens, and other closely related species from clinical, and environmental samples. A series of real-time multiplex PCR assays were designed which have the ability to not only detect the ESKAPE species, but also to detect and differentiate species complexes which are closely related to the ESKAPEs such as members of the *A. baumannii* group and the *K. pneumoniae* species complex (KpSC). Due to the imprecision of existing diagnostics, the additional species within these complexes are typically misidentified as the primary ESKAPE pathogen [38-41]. This undermines the collection of accurate clinical data for ESKAPE species. The assays described in this study provide an improved means of identification and include to our knowledge, the first description of real-time PCR methodologies which can detect the *Enterobacter* genus, the *A. baumannii* group, and the KpSC with full specificity. It is also the first real-time PCR assay with the ability to simultaneously identify each of the ESKAPE pathogens.

To further understand the causes of HAI, we developed a protocol to conduct environmental surveillance using surface swabs and NAD and validated its performance using a mock microbial community. This provides a holistic approach to the detection and surveillance of pathogens which cause HAI from both clinical and environmental samples.

Two multiplex real-time PCR assays were developed in **Chapter 2** to identify each of the ESKAPEs with increased specificity relative to currently available molecular detection methods. Multiplex 1 detects and differentiates *S. aureus, P. aeruginosa* and *K. pneumoniae*, while multiplex 2 discriminates *E. faecium*, the *Enterobacter* genus, *A. baumannii sensu stricto,* and the *A. baumannii* group. The assays were validated using a panel of 210 culture collection isolates and were found to be 100% specific for each target organism. Sensitivities for the multiplex assays were found to be between 1.2 and 39.5 genomic equivalents.

The clinical utility of these assays was demonstrated by blind testing of a panel of 114 respiratory specimen from patients with suspected HAP/VAP. It was determined that 53.5% of the clinical specimen (n=61) contained at least one ESKAPE species. The results using the novel real-time PCR assays were compared to those obtained from the BioFire® FilmArray® Pneumonia panel *plus* (PNplus). Full agreement between the results of the FilmArray PNplus and the ESKAPE real-time multiplex PCRs was reached in 87.7% (n=100) of samples. Amongst the discordant results were 5 samples deemed by the BioFire to contain *Enterobacter*. Further analysis using *hsp60* sequencing determined that these were in fact false positives. This highlights the specificity of our *Enterobacter* assay, and the potential for commercially available diagnostics to misidentify even the most clinically important pathogens.

In addition to detecting the ESKAPE species, we included an assay to differentiate *A. baumannii sensu stricto* from the closely related species of the *A. baumannii* group. The FilmArray PNplus is unable to differentiate *A. baumannii sensu stricto* from the *A. baumannii* group, which consists of the species A. *pittii, A. nosocomialis, A. seifertii* and *A. lactucae*. These species are thought to cause up to 30% of *Acinetobacter* infections and are increasingly recognised as different clinical entities with variable AMR rates and morbidity. Although only *A. baumannii sensu stricto* was found in the HAP/VAP clinical specimen (*n*=3), the addition of the *A. baumannii* group assay enables the detection of these species and improves diagnostic accuracy.

Chapter 3 describes an assay to fully characterize the members of the *Klebsiella pneumoniae* species complex (KpSC). *K. pneumoniae* causes severe invasive infections and are the third most frequently reported HAI worldwide [42]. They cause a quarter of Disability Adjusted Life Years (DALYs) associated with antimicrobial resistance, and as such are considered critically important pathogens [43]. Presently, the lack of methods to differentiate members of the KpSC severely limits our understanding of their epidemiology. They are phenotypically indistinguishable from *K. pneumoniae sensu stricto* using routine methods. Moreover, molecular methods such as MALDI-TOF and commercially available NAD fail to accurately discriminate the KpSC, and isolates are typically misidentified as *K. pneumoniae sensu stricto [39, 44]*.

The KpSC is formed by 5 species (*K. pneumoniae, K. quasipneumoniae, K. variicola, K. quasivariicola, K. africana*), and were until recently, considered to be phylogroups of *K. pneumoniae* rather than distinct taxa. Differentiation of species is currently only possible using WGS [32, 45]. When accurate speciation of clinical isolates has been performed using, approximately 15% of isolates have been found to be non-*pneumoniae* KpSC, although this number varies considerably. Furthermore, some studies have demonstrated that infections caused by KpSC can have differing clinical prognoses. This highlights the need for accurate species differentiation within the KpSC.

We have developed a multiplex real-time PCR assay which detects all 5 members of the KpSC, and which includes assays to specifically detect the three most prominent pathogens *K. pneumoniae, K. quasipneumoniae* and *K. variicola.* A positive result in the pan-KpSC assay and a negative result in the remaining three species specific assays enables the presumptive identification of the remaining two species *K. quasivariicola* and *K. africana*. An internal amplification control targeting a synthetic dsDNA gene fragment was included in the multiplex to further ensure the validity of results.

The multiplex was validated using a panel of culture collection isolates of each KpSC member and closely related *Klebsiella* and Enterobacteriaceae. It is the first assay with the ability to differentiate the KpSC members with full specificity. The limit of detection for each assay was ~ 10GE.

In **Chapter 4**, the capacity for NAD to conduct environmental surveillance of healthcare facilities was investigated. Environmental contamination was simulated by the application of a microbial mock community to test surfaces at low concentrations in a laboratory environment. Prior to the COVID-19 pandemic, it was intended that prospective environmental surveillance of an ICU would be conducted using the ESKAPE multiplex real-time PCR assays described in **Chapter 2**. Nonetheless, the sample collection and processing technique which was developed provides an enhanced and reproducible method to conduct environmental surveillance [46].

The increased availability of molecular diagnostics in the clinical laboratory has enabled a shift away from traditional culture-based methods for infectious disease diagnostics. It has greatly improved our understanding of pathogen transmission, and infection prevention and control. This is not true however for the surveillance of the hospital environment. The role of the environment in HAI remains unresolved, mainly due to the limited use of environmental surveillance and a lack of standardised approaches to do so. Currently, routine monitoring of surfaces in the hospital is not recommended. This is because the correlation between pathogens identified from surfaces using culture-based methods, and incidence of HAI is low [47, 48]. However, the potential for pathogens to colonise and persist in the hospital environment has been demonstrated. Outbreaks of HAI have been linked to environmental sources of contamination and can be difficult to eradicate once established [49-51].

In this chapter, a standardised protocol for sample collection and nucleic acid extraction was created and optimised under laboratory conditions. A mock microbial community of bacterial (*S. aureus, A. baumannii*) and fungal (*Cryptococcus neoformans*) species was used to ensure accurate representation of surface contamination in the extracted nucleic acid sample. These species are known to persist in the environment and represent the varying lysis requirements of Gram positive, Gram negative and fungal cell walls. The optimised protocol enabled the sensitive detection of pathogens from test surfaces, at concentrations as low as 10 CFU/cm^2. This offers a markedly more sensitive method to detect microbial contamination on surfaces. This in turn may improve the quality of data generated from environmental surveillance in healthcare facilities and in the future, guide infection prevention and control practices.

5.3 Future Research

The chief aims of this project were to improve the quality and accuracy of rapid diagnostics for ESKAPE pathogens. The diagnostic approaches developed in this thesis have been validated using a combination of clinical specimen and culture collection isolates. Further application of these assays in the clinical setting would increase our understanding of the prevalence and transmission of ESKAPE pathogens. The clinical utility of the ESKAPE multiplex assays was demonstrated on respiratory specimen from patients with suspected HAP/VAP in **Chapter 2**. ESKAPEs are known to cause a large proportion of HAP/VAP, and this was confirmed in our findings with 53.5% of samples being positive for at least one ESKAPE species. While this supports the status of the ESKAPEs as important pathogens, future work will endeavour to investigate their role in the hospital environment. Using the methods developed in **Chapter 4**, prospective surveillance of the surfaces in an Intensive Care Unit will be conducted. This will provide longitudinal data on the existence and persistence of ESKAPEs in the built environment of healthcare facilities. Improved surveillance and timely identification of potential reservoirs could help to prevent HAI caused by ESKAPEs.

The assay described in **Chapter 3** enables the differentiation of KpSC species. This is the first description of a rapid diagnostic for KpSC and as such, would be a useful tool in the clinical setting. Further validation of this assay will use clinical isolates from a range of infection types (bloodstream, respiratory, urinary tract) to investigate the true occurrence of KpSC members and their influence on patient outcomes.

Similarly, the development of an assay to fully profile the *A. baumannii* group species would increase our understanding of their epidemiology and role in HAI. The differentiation of *A. baumannii sensu stricto* from other members of the *A. baumannii* group is facilitated in the ESKAPE multiplex described in **Chapter 2** of this thesis. Future work will establish a diagnostic method to specifically identify each of the *A. baumannii* group species to include the clinically relevant *A. nosocomialis, A. pittii, A. seifertii* and *A. lactucae*.

Finally, as technology evolves the application of point-of-use genome sequencing will be investigated to improve the diagnosis of HAI from a One Health perspective. The untargeted approach offered by WGS methods will enable the full profiling of clinical and environmental isolates. The ultimate goal being the establishment of a WGS diagnostic pipeline to rapidly profile the microbiome and resistome of a given sample. This could be used not only to investigate the species present in a clinical sample, but to conduct environmental profiling and potentially link environmental contamination to patient infection. Metagenomic profiling of clinical and environmental samples has been used to investigate HAI [52, 53]. However, the current limitations of sensitivity and cost associated with WGS based analysis would first need to be overcome to facilitate the transition from real-time PCR based NAD [54, 55]. Rather, WGS could be used as a complimentary approach to further investigate samples which have been first screened using the multiplex real-time PCR assays described in this study.

5.4 Final Conclusions

Human, animal, and environmental health are under increasing threat from antimicrobial resistant pathogens [5, 56]. Globalisation in the form of increased travel and population density, as well as an aging population in need of invasive medical treatments contribute to the burden of infectious disease. If we are to avoid the reality of a post-antimicrobial era, urgent action must be taken to reduce the consumption of antimicrobials and infections arising from contact with healthcare facilities [57]. Improved infectious disease diagnostics can alleviate the need for empirical antimicrobial treatment and improve patient outcomes [58, 59]. The group of pathogens responsible for the majority of HAI are known as the ESKAPEs due to their ability to "eskape" treatment and eradication efforts. The species which form the ESKAPE pathogens - *E. faecium, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa* and the *Enterobacter* genus – represent the most burdensome causes of HAI and are associated with high levels of patient morbidity [14].

Although the ESKAPE pathogens have been designated as critically important pathogens to human health, the methods used to identify them currently are outdated and lack precision. Culture based diagnostics are slow and insensitive, while existing molecular methods for the ESKAPEs have the potential to misdiagnose closely related species.

The focus of this thesis, therefore, was to develop highly multiplexed, real-time PCR diagnostic assays to facilitate the rapid and accurate detection of ESKAPE pathogens and closely related species from clinical and environmental samples. We have developed a series of NAD assays which have been validated using clinical and culture collection isolates. These assays enable the simultaneous detection and differentiation of each ESKAPE. In addition, assays to identify the closely related species of the A. baumannii group (*A. baumannii, A. nosocomialis, A. pittii, A. seifertii, A. lactucae*) and the K. pneumoniae Species Complex (*K. pneumoniae, K. quasipneumoniae, K. variicola, K. quasivariicola, K. africana*) were developed. These clinically relevant species are currently indistinguishable without the use of sequencing and are misrepresented in clinical data pertaining to the ESKAPEs. Finally, the development of a method to detect pathogens from the hospital environment using NAD offers a holistic approach to the detection, and ultimately reduction of HAI.

5.5 References

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