Investigating the associations between oral colonisation with respiratory commensal pathogens, oral hygiene and hospital acquired pneumonia in older patients with lower limb fracture.

Thesis submitted for the degree of Doctor of Philosophy, Newcastle University

November 2012

Victoria Ewan

MRCP, BMBS, BMedSci

Institute for Ageing and Health

Newcastle University

Abstract

Hospital acquired pneumonia (HAP) occurs in 1% of all hospital in-patients, and in around 10% of patients with lower limb fracture, with a mortality of 18-43%. HAP arises from interactions between three main risk factor groups: resident oral microbiota, aspiration potential (dysphagia, reduced conscious level) and host factors (age, frailty, comorbidity). In this work novel multiplex real time PCR assays were used to study prospectively the oral colonisation dynamics of seven major commensal pathogens over the first fortnight after hospital admission in relation to oral health variables, medical variables and subsequent development of HAP. Of the 93 patients recruited, 10% developed HAP and 60% of in-hospital deaths after lower limb fracture were due to HAP. Persistent oral colonisation with *E. coli* or *S. aureus* was significantly associated with HAP or HAP/lower respiratory tract infection in older patients with lower limb fracture. In turn, *S. aureus* was associated with increased dental plaque at admission and with increased xerostomia indices at 14 days. Other factors such as witnessed aspiration and post-operative cough were also strongly associated with subsequent development of HAP. HAP was associated with increased risk of death and increased length of hospital admission. These findings suggest several potentially modifiable clinical risk factors, and a high risk population for HAP, to whom interventions could be targeted. Given the rise in the older population and the increased costs associated with HAP, early detection and prevention will become increasingly important. Further work is needed to understand the relationships between dental plaque, *S. aureus* and xerostomia, and also to identify microbial biomarkers which could be used at the start of hospital admission to stratify patients' risk of HAP.

Dedication

To my parents for making me believe that I could do anything I put my mind to.

To Dave for his love, support and for telling people his wife has "a high-flying research career"! And for listening to me drone on about oral microbiology, often when he is actually asleep.

To Julia who would have given me the job over the phone. Thank you for believing in me.

To Angus for introducing me to the world of dentistry and civilized meetings with coffee and chocolate.

To Andy for his patience and teaching and for not depriving me of the struggle!

To Barbara, Gary and all of the HPA Newcastle for allowing me to work with them, and for all their patience with me when I was new to the laboratory.

To the study patients who took part- I have carried your stories with me and hope I will do them justice.

To Steve, Mark, Matt and Andy and others at the Biology modeling group who became my third home in the final year- for all the fantastic lunchtime conversation, R headaches and google addiction.

To Joe West for helping me define the research question and suggesting I delete most of the original proposal- thank goodness…

To the MRC for funding the project and allowing me to be paid for having fun.

To Fiona, Linda and Goulton Grange for looking after John so well.

And to Alistair Gascoigne for taking an interest in a fed up Microbiology trainee on an ITU ward round.

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Abbreviations

%	Percent
˚C	Degrees celsius
AGNB	Aerobic gram negative bacteria
AP	Aspiration pneumonia
ARDS	Adult respiratory distress syndrome
ATS	American Thoracic Society
BAL	Bronchoalveolar lavage
BLAST	Basic Local Alignment Software Tool
bp	Base pairs
CAP	Community acquired pneumonia
CLRN	Comprehensive Local Research Network
COPD	Combined obstructive pulmonary disease
CVD	Cerebrovascular disease
df	Degrees of freedom
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetracetic acid
EMBL	Database of all downloaded DNA sequences (European)
FEV1	Forced expiratory volume in 1 second
GenBank	Database of all downloaded DNA sequences (US)
GUM	Genito-urinary medicine

HABAM	Hierarchical assessment of balance and mobility
HAP	Hospital acquired pneumonia
HCAI	Healthcare associated infection
HCAP	Healthcare acquired pneumonia
HPA	Health Protection Agency
ICU	Intensive care unit
IMD	Indices of multiple deprivation (United Kingdom)
KES	Klebsiella- Enterobacter- Serratia
LRTI	Lower respiratory tract infection
MMSE	Mini-mental state examination
MRC	Medical Research Council
MRSA	Meticillin resistant *Staphylococcus aureus*
NHAP	Nursing home acquired pneumonia
NHS	National Health Service
NICE	National Institute of Health and Clinical Excellence
NP	Nosocomial pneumonia
NUTH	Newcastle upon Tyne Hospitals
ORF	Open reading frame
PCR	Polymerase chain reaction
PRPM	Potential respiratory pathogens in the mouth
RNA	Ribonucleic acid

SIGN	Scottish Intercollegiate Guideline Network
SNP	Single Nucleotide Polymorphism
UK	United Kingdom
US	United States of America
VAP	Ventilator associated pneumonia
WCC	White cell count

Chapter 1 Introduction

1.1 Pneumonia

Over 100 years ago, Sir William Osler described pneumonia as "the most widespread and fatal of all acute diseases… now Captain of the men of death" [1]. Since then the mortality from pneumonia has decreased considerably over the 20th century [2, 3], mainly due to antibiotics and vaccination. However pneumonia remains the leading causing of death globally in children under five years [4], and influenza and pneumonia remain the fifth commonest cause of death in England and Wales in 2010 [5]. In 2011, 87% of the 25,696 deaths in England and Wales from pneumonia were in persons over age 75 [6]. In England, the number of people over age 75 is projected to increase from 4.9 million in 2010 to 6.1 million in 2020, and to 8.1 million by 2030 [7]. The proportions of persons with pneumonia would be expected to rise accordingly, but the incidence in older people may also be rising independently of demographic trends [8].

Pneumonia is characterised by the accumulation of bacteria and inflammatory exudate in the alveolar spaces of the lung, which can be visualised on chest radiography as consolidation (or a non-lucent "white" area) when sufficiently widespread. Pneumonia is caused by bacteria [9-12], and viruses [13-15] which are either inhaled or aspirated (or more rarely spread by the blood from a distant site) and which provoke an inflammatory immune response by the lung. Bacteria and viruses adhere to and replicate in the oro- and nasopharynx first before moving to the lung.

The commonest bacterial causes of pneumonia are *Streptococcus pneumoniae*, *Haemophilus influenzae* and Morhaxella cattarhalis, while the commonest viral pathogens are influenza and respiratory syncytial virus [9]. In older adults *S. pneumoniae* accounts for 20-60% of cases of community acquired pneumonia [14-16], however, other bacteria such as *Staphylococcus aureus*, and gram negative aerobic bacilli are also identified from pulmonary secretions [17, 18], the latter probably coinciding with the onset of routine use of antibiotics [12] .

Pneumonia may also occur in hospital, after admission with a separate problem, and this is known as hospital acquired pneumonia (HAP). HAP refers to a respiratory infection associated with new infiltrates on chest radiography which develops after 48 hours or more in a hospital environment [10, 19].

Respiratory infections are now the commonest healthcare acquired infection in England [20], and the mortality associated with HAP is high, and ranges from 18-43% depending on the group studied [21-26]. National Health Service (NHS) patients on general wards are not currently screened or stratified for risk of nosocomial respiratory infections, and no preventative measure are undertaken, in contrast with other healthcare associated infections. By screening and treating MRSA carriers, improving awareness about hand-washing and reducing unnecessary intravenous devices, government strategies have reduced the rates of MRSA bacteraemias according to Health Protection agency figures (http://www.hpa.org.uk/hpr/infections/hcai.htm). However these strategies have not affected rates of HAP, and respiratory infections have become proportionally more common since the 2006 hospital infection prevalence survey was undertaken [27].

Commonly identified risk factors for HAP include difficulty with feeding or oral secretions [21, 28-31], episodes of disorientation [21, 28] or decreased conscious level [24], aspiration [24, 28, 32] and oral colonisation with respiratory commensal pathogens [21, 29, 33]. The term "commensal pathogen" will be used in this work, and refers to organisms which may either be carried asymptomatically or act as pathogens (see 1.5.1), described in relation to *S. pneumoniae* by Henriques-Normark et al.[34] . Oral colonisation with respiratory commensal pathogens may represent a potential target for interventions to prevent HAP. Respiratory commensal pathogens have also been identified from the oral cavity in community dwelling persons without respiratory infection [35-37], but appear to increase during illness, proportional to illness severity [36, 38]. There remains confusion about the significance of potential pathogens being identified from the oropharynx in both community dwelling persons and those in hospitals or institutions. Several reports have

suggested that the organism causing pneumonia was isolated first from the oropharynx [39-42]. However relatively few studies have analysed prospectively the association between prior oral colonisation with respiratory commensal pathogens and HAP in non-ventilated patients [21, 33, 43]. Increased dental plaque has also been linked with respiratory tract infection [44, 45], and it has been proposed that dental plaque might modulate the risk of HAP either by inflammatory means, or by acting as a possible stable reservoir for opportunistic HAP pathogens [46, 47]. Further investigation is warranted as the oropharynx could be a target for preventative therapy.

1.1.1 Hospital acquired pneumonia in older people with lower limb fracture

Surgical patients are prone to developing HAP, and surgical wards are the second commonest location for HAP to be diagnosed, after intensive care units [20, 27, 48]. Patients with lower limb fracture (which requires urgent surgery) appear to be at particular risk of HAP, with an incidence of 8.6-10% [23, 49, 50]. Preventing HAP in older patients with hip fracture or other fractures of the lower limb is important because approximately a third of these patients will die within a year of hip fracture [51]. Pneumonia is one of the commonest postoperative complications, and the second commonest cause of death in these patients after cardiac disease and 43% of patients with HAP after hip fracture die [23]. Preventing HAP in a proportion of patients with lower limb fracture could potentially have a large impact on overall mortality and quality of life.

1.1.2 Epidemiology

Respiratory infections are now the commonest healthcare associated infection in England, with a prevalence of 1.5% (95% confidence intervals 1.4-1.6) across all hospital in-patients [20]. The prevalence of HAP has been estimated at 1.1-1.8% [52-54] in general ward patients, 1-31% in surgical patients [33, 48, 55-65], 18-22% in stroke patients [66, 67], 3-44% in long term care facilities [29, 30, 68, 69], and 4-5% in geriatric hospitals [53, 70]. Respiratory tract infections are the second commonest healthcare acquired infection in a variety of countries [71-73]. Assuming a rate of 1% of hospital patients developing HAP, of the 12,976273 admissions to hospital in 2005-2006 [74], nearly 130,000 patients will

have developed HAP. Infections due to *Streptococcus pneumoniae* and several respiratory viruses are more common in winter months [75, 76], and pneumonia is therefore more common overall in winter months.

1.1.3 Cost

A recent study of culture–positive pneumonia conducted in the United States (US) between 2002-2003 calculated the median cost of HAP as $31,220, compared with community acquired pneumonia (CAP) which had a median cost of $13,358 and ventilator asscociated pneumonia (VAP) which cost $ 98,192 [11]. Length of stay was greater in HAP (median 11 days versus 5 days in CAP) which may account for some of the increased costs. A 1993 study estimated the cost of one person developing HAP in the US to be $14,000 [77], which was extrapolated to suggest that overall cost for the 1.5 million US nursing home residents developing HAP was approximately 8 billion dollars. Thompson et al. studied a database of 618,495 patients undergoing intra-abdominal surgery of whom 13,292 developed HAP (2.2%) and found a greater length of stay (17 days versus 6 days) in those with HAP, a four-fold increase in the likelihood of transfer to an institution and a 75% increase in mean healthcare cost [78]. Other studies showed that HAP lengthened hospital stay by up to 12 days [22, 48, 79-81]. Antibiotics, bed days and increased need for rehabilitation are the most likely explanatory factors for increased cost associated with HAP.

1.2 Diagnosis of HAP

1.2.1 Clinical diagnosis

Pneumonia is diagnosed by a combination of clinical symptoms (cough, green or yellow sputum, fevers and chills, chest pain, malaise), new radiographic infiltrates on chest X-ray (CXR) and signs of inflammation (fever, raised white cell count). Despite the well recognised clinical syndrome, there is currently no gold standard diagnostic test to diagnose pneumonia, because new radiological infiltrates on chest radiograph alone could represent a number of pathologies, most notably pulmonary oedema. Indeed pneumonia and cardiac compromise resulting in pulmonary oedema can coexist. The best diagnostic criteria are a

combination of clinical signs in conjunction with new infiltrates on chest radiograph. Definitions used in research studies, and those from national guidelines are described in Table 1, and there is considerable variation both in criteria employed and the cut-offs for diagnostic values such as white cell count. The American Thoracic Society (ATS) and British Society for Antimicrobial Chemotherapy (BSAC) have both formulated guidelines for diagnosis [10, 19], the former semi-evidence based (no formalised quality assessment made of studies used) and the latter evidence-based (using Scottish Intercollegiate Guideline Network (SIGN) methodology). However both criteria involve the inflammatory response. There is conflicting evidence as to whether the inflammatory response may be absent or reduced in older people with HAP, with studies showing both a reduction [14, 82] and no reduction [83] in inflammatory response during episodes of CAP or blood stream infection with *P. aeruginosa* or Enterococcus sp. [84]. In addition, older people may present with delirium or decreased mobility [14, 28] or other non-respiratory symptoms such as falls, incontinence or diarrhoea.

Table 1. Definitions of HAP in national guidelines and selected journal articles relevant to HAP in older people

Type	*Major criterion*	**Minor criteria**
Semi-evidence based guidelines (US)[19]	New infiltrates on CXR	Plus two of Fever: over 38°C, Secretions: purulent, Leukocytosis/leukopenia
Evidence based guidelines (UK)[10]	New infiltrates on CXR or Secretions: purulent	Fever: over 38.3°C , White cells: > 10 or < 4, Increased oxygen requirement
Journal article[11]	ICD-9 code	
Journal article[70]	New infiltrates on CXR	Plus 2 of: Fever: over 38°C. Secretions: purulent, Chest pain, Auscultatory findings, Respiratory rate >20 breaths per min , Purulent sputum
Journal article[29]	Opinion of pulmonary physician	In conjunction with new infiltrates on CXR, Fever: over 99.5^{o}F, Secretions: purulent, White cells: rise of 5 or more, Attending physician opinion, Observed aspiration, Cough with fever, Dyspnoea, Pleuritic chest pain, Positive respiratory culture
Journal article[22]	New infiltrates on CXR	Fever: over 38 ^{o}C, Secretions: purulent, White cells: >12 or <3, Matching blood/respiratory cultures or gram stain of sputum
Journal article [18]	New infiltrates on CXR	Plus two of, Fever: over 38 ^{o}C, Secretions: purulent or change in character and increasing arterial-alveolar gradient, White cells: >11 or neutrophils <3.5
Journal article[54]	New infiltrates on CXR or bronchial breathing on	Plus all of, Fever: over 38 ^{o}C, Positive sputum/tracheal culture, Secretions: purulent , White cells: >12 or neutrophils <3
Journal article[85]	New infiltrates on CXR	Fever: over 38.5 ^{o}C, White cells: >15 48 hours before death, Positive sputum culture, Worsening gas exchange
Journal article[86]	New infiltrates on CXR	Plus one of, Fever: over 37.8 ^{o}C, Cough, Dyspnoea

Note: White cell count is measured in cells/metre^{3}

1.2.2 Histological diagnosis

Ideally, pulmonary histology would be used as a gold standard to further refine clinical and microbiological criteria. However studies have demonstrated poor inter-rater agreement [87], poor correlation with clinical diagnosis [85, 88], and a recent UK systematic review did not recommend that histology was used as a gold standard [10]. It seems highly probable that histological diagnosis will become the most accurate diagnostic method in the future, and thus allow correlation with clinical diagnosis. Other diagnostic approaches might include the use of metabolomics [89] or assaying immunological responses (such as tumour necrosis factor or immunoglobulins) along with microbiological analysis of invasive respiratory samples [90, 91]. However research is needed into both of these approaches before their adoption into clinical practice could be considered.

1.2.3 Aetiology

Differing rates of bacterial species have been identified from microbiological samples according to where the patient acquired the infection, hence the terminology community acquired pneumonia (CAP), hospital acquired pneumonia (HAP), ventilator associated pneumonia (VAP), post-operative pneumonia or nursing home acquired pneumonia (NHAP). In this work, HAP will be used to refer to those who are not or have not been mechanically ventilated and VAP for those who have been ventilated (excluding intra-operative ventilation). Community acquired pneumonia is caused most commonly by *S. pneumoniae*, Morhaxhella cattarhalis and *Haemophilus influenzae* [11, 14, 92, 93], whereas HAP, VAP and NHAP are also commonly caused by *S. aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and other gram-negative organisms such as *Klebsiella pneumoniae* [11, 17, 18, 26] (see Table 2). HAP which occurs within four days of hospital admission is said to more often resemble CAP in terms of bacterial aetiology [19]. However, a large French study of postoperative HAP found the bacterial aetiology more similar to that of ventilated patients [26] (see Table 2). Studies from the UK and Germany have

reported lower rates (1-1.3%) of gram negative bacilli as causative organisms of CAP than those found in the US (Table 2) [14, 94, 95], however there are no more recent UK studies with which to compare. The 2011 English National Point Prevalence Survey of Healthcare Associated Infections captured culture results from patients with HAP and VAP [20], but detailed results from this survey are yet to be published.

The concept of infections being of polymicrobial aetiology is now accepted [55, 96-99], a departure from the traditional view that monomicrobial cultures signify infection and polymicrobial cultures represent contamination [14]. Polymicrobial cultures were reported in 29% of patients with HAP in one study [26], with the commonest combinations of two organisms including either *S. aureus* and Enterobactericeae (24%) or Enterobactericeae and Streptococci (17%). In other studies of HAP, polymicrobial infections were identified in 13.3-28% of patients [17, 55, 99].

Recently it has been noted that persons who frequent healthcare environments as day patients (e.g. for renal dialysis) develop pneumonias from bacteria which are more like the hospital group than the community group, and pneumonia in this group is now known as healthcare associated pneumonia [11, 100] (HCAP).

Table 2. Bacterial aetiology of pneumonia in UK community setting versus hospital settings in the UK, US and France expressed in mean %

	Hospital acquired pneumonia, non-ventilated[11]	*Ventilator associated pneumonia[11]*	*Hospital acquired pneumonia (ventilated and non-ventilated)[26]*	*Community acquired pneumonia, UK[9]*	*Community acquired pneumonia, US[11]*
S. pneumoniae	3.1	5.8	10	39	16.6
H. influenzae	5.6 *	12.2*	19	5.2	16.6*
S. aureus	47.1	42.5	27	1.9	25.5
Gram negative enteric bacilli	21.8	29.6	33	1	22.9
Pseudomonas	18.4	21.2	17	n/a	17.1

1US 2002-2003, respiratory culture positive from Atlas database 2 France 1997-1998

*Haemophilus species recorded only

1.2.4 Aspiration pneumonia

Aspiration pneumonia (AP) is used to describe persons who develop pneumonia following witnessed or suspected episodes of aspiration of food, drink, saliva or any combination of these. Many studies do not distinguish between aspiration pneumonia and HAP [86, 101], and it is difficult to know whether to consider aspiration pneumonia as a distinct entity. The main differences between micro- and macro-aspiration are summarised in Table 3. It may be better to consider a spectrum between no aspiration, microaspiration and macroaspiration. HAP, NHAP, VAP and even sometimes CAP [102, 103] may be associated with aspiration (albeit micro-aspiration) as will be discussed below. In addition, those who are diagnosed with HAP may have experienced an episode of unwitnessed moderate-large volume aspiration. The bacterial aetiology of AP is broadly the same as HAP and anaerobes appear to be less common than previously thought [18, 104, 105].

Table 3. Comparing micro- and macro-aspiration

Parameter	*Microaspiration*	*Macroaspiration*
Cause of aspiration	Decreased conscious level	Neurological disease or decreased conscious level
Visibility of aspiration	Silent	May or may not be witnessed
Contents of aspirated fluid	Saliva	Food, drink, saliva
Volume of aspirated material	Small	Large

1.3 Antibiotic therapy

The main modality of treatment of HAP is antibiotic therapy; inadequate antibiotic therapy is associated with higher mortality (OR 2.6 95% CI 1.4-23.9) [17, 24, 106]. However no causative organism was identified in 14-60% of patients in studies specifically considering aetiology of pneumonia [17, 26, 92, 107], despite invasive respiratory sampling. Age over 70 was found to be associated with unknown aetiology of CAP despite adequate respiratory sampling, along with cardiac and renal comorbidity and non-alveloar infiltrates on chest radiography in one study [92]. In clinical practice in the UK, sputum samples are more routinely sent for analysis, and invasive respiratory sampling is rare unless patients are undergoing mechanical ventilation. However in a study of 73 patients over age 65 with CAP, 37% could not spontaneously produce sputum [14]. The sputum sample may contain a mix of material from upper and lower respiratory tracts as well as the mouth, and may not reflect the infecting organism from the lung [108].

Both diagnosis and treatment of HAP are more difficult without a positive microbiological culture, particularly in patients who do not get better after initial therapy. In the Eole study, 62% of patients needed a change in antibiotic therapy, and of these, 33%were because of persistent infection not responding to treatment, and 34% due to the organism being resistant to antibiotic therapy [26]. Inadequate treatment of HAP in non-ventilated patients may account in part for its high mortality.

1.4 Risk factors for HAP

Otherwise "healthy" hospital patients do not generally develop HAP; it is a disease of the compromised patient. Pneumonia overall affects those at the extremes of ages, pointing to a common immunological explanation for infection. Few studies investigating the occurrence of HAP in patients with lower limb fracture have been undertaken. Therefore observational studies from both older patients and patients exposed to surgery were considered in order to extrapolate risk factors which might be pertinent to older patients with lower limb fracture. Observational studies of HAP in older persons are

summarised in Table 4, and these and other potential risk factors are discussed below, and risk factors in surgical patients are summarised in Table 5 and discussed in 1.4.10.

Table 4. Observational studies of HAP in older patients

Authors/year published	*Participants*	*Incidence/ prevalence of pneumonia*	*Significant associations (multivariate analysis reported where possible)*		**Comments**
Terpenning et al. 2001 [101]	358 Veterans from inpatient, outpatient and day centre attendees	14% (AP)	*S. aureus* in saliva Needing help with feeding COPD	(OR=8.3) (OR=4.7) (OR= 2.5)	Retrospective diagnosis of AP by expert panel opinion (pulmonary physician) after ten years.
Michel et al. 1991 [53]	1919 French and Swiss geriatric hospital in-patients	5% (HAP)	Swallowing disorders Chronic bronchitis Intravenous catheter Urethral catheter Nutrition abnormalities	(OR= 5.74) (OR 5.00) (OR=5.49) (OR=3.33) (OR=3.18)	One day prevalence study
Rothan-Tondeur et al. 2003 [70]	2142 French geriatric hospital in-patients	4% (HAP)	HAP in previous 6 months Oxygen therapy Low albumin level Recent antibiotic therapy	(OR=4.5) (OR=16.15) (OR=4.81) (OR=3.20)	Case control study Mortality 12.2% at 30 days

Authors/year published	*Participants*	*Incidence/ prevalence of pneumonia*	*Significant associations (multivariate analysis reported where possible)*		**Comments**
Merchant et al. 1998 [54]	1886 patients Bombay hospital, India	1% (HAP)	Age over 50 GCS < 8 Tracheostomy Nasogastric tube feeding Histamine 2 receptor antagonists	(OR=4.49) (OR=10.5) (OR=30.28) (OR=75.5) (OR=19.1)	88% mortality Includes medical ICU patients and general ward patients
Harkness et al. 1990 [28]	740 bed acute-care hospital and 640-bed chronic disease hospital in New York State.	1.7 per 1000 days (acute care, HAP)	Neurologic disease Renal disease Deteriorating health (acute) Decreased consciousness Disorientation Aspiration Difficulty with oral secretions	(OR 4.4) (OR 10.0) (OR 2.7) (OR 2.6) (OR 3.0) (OR 17.0) (OR 5.8)	Case-control study, patients >age 65 Using logistic regression, best predictors in acute care setting was difficulty with oropharyngeal secretions and nasogastric tube.
		0.74 per 1000 days (long-term care, HAP)	Deteriorating health (acute) Malnourished appearance Recent weight change Decreased consciousness Disorientation Agitation Aspiration Nasogastric tube	(OR 24) (OR 15) (OR 5.7) (OR 20) (OR 10.5) (OR 120) (OR 130) (OR 120)	In long term care setting was difficulty with oropharyngeal secretions, deteriorating health and occurrence of unusual event.

Authors/year published	*Participants*	*Incidence/ prevalence of pneumonia*	*Significant associations (multivariate analysis reported where possible)*	**Comments**
Quagirello et al. 2005 [69]	613 residents of nursing homes in Connecticut, US	18% (NHAP)	Inadequate oral care (HR=1.55) Swallowing difficulty (HR=1.61)	Prospective study, radiographic pneumonia. Inadequate oral care was defined as absence of dental examination. Swallowing difficulty was defined as cough during swallow.
Hanson et al. 1992 [21]	600-bed university hospital, North Carolina, US.	N/A	Albumin <3.0 g/dl (OR 14.7) Neuromuscular disease (OR 24.8) Endotracheal intubation (OR 5.2) Oropharyngeal colonisation present in 29% cases versus 3% controls (p<0.001) but samples taken in <50% cases.	Case-control, prospective case acertainment. Cases over age 65 compared to cases between age 25 and 50. Did not distinguish aspiration pneumonia; large volume aspiration noted in 24% patients who developed HAP.
Alvarez et al. 1988 [31]	Nursing home care unit (61 beds) and intermediate care unit (60 and 59 beds) in Johnson City, Tennessee, US, 1980 -1983.	4 per 1000 days (HAP and NHAP)	Needs feeding p<0.01 Urinary incontinence p<0.5 Faecal incontinence p<0.5 Unstable medical condition p<0.5 No pneumococcal vaccine p<0.5 Nasogastric feeding p<0.01	Prevalence varied depending on unit studied Ward A 7.5% (unstable, mainly self-caring) Ward B 14.5% (unstable, needing care) Ward C 2.4% (stable nursing home patients) Multiple sources for case finding

Authors/year published	*Participants*	*Incidence/ prevalence of pneumonia*	*Significant associations (multivariate analysis reported where possible)*	**Comments**
Celis et al. 1988 [24]	118 patients with HAP and 120 controls in Spain	N/A	Tracheal intubation Depressed level of consciousness Thoracic or upper abdominal surgery Prior episode large volume aspiration Age over 70	Case-control, mortality 36% Controls appear to be paediatric and neutropaenic patients with NP. Did not distinguish between AP, VAP and HAP.
Redelmeier et al. [48]	Clinical database review, Canada	1% (HAP)	No association with gastric acid suppression	No microbiological parameters measured. Very large study over 16 years. Postoperative patients age>65
Oliveira et al. 2011 [109]	46 cases, 165 controls	21.8% HAP/VAP	Hypertension Stay greater than 5 days Not using dental floss or mouthwash	Case control Mean age 41
Van der Maarel-Wierink et al. [110]	Meta-analysis	AP	Dyspahgia (OR 9.84)	Included 4 cohort studies, 1 case control, 1 case cohort study.
Vergis et al. [32]	104 case controls pairs in long term care facility	HAP	Witnessed aspiration (OR 13.9) Sedative medication (OR 2.6) Comorbidity score (OR 1.2)	Mortality from HAP 23% after 14 days

Authors/year published	*Participants*	*Incidence/ prevalence of pneumonia*	*Significant associations (multivariate analysis reported where possible)*	**Comments**
Sellars et al. [67]	412 Stroke patients	18.9% HAP	Age >65 Dysarthria/aphasia Modified Rankin score >=4 Abbreviated mental test score <8 Failed swallow test	
Walter et al. [66]	236 acute stroke patients admitted to neurointensive care	22% VAP/HAP	Dysphagia (RR 9.92) National Institute for Health Stroke Score >=10 (RR 6.57) Non lacunar basal ganglia infarction (RR 3.10) Other infection at admission (RR 3.78)	
Langmore et al. [68]	102,842 Nursing home patients, US	3% NHAP	Suctioning needed COPD, heart failure Feeding tube Bedfast, delirium Weight loss Swallowing problems Urinary tract infection Dependence for eating	Univariate analysis only

Authors/year published	*Participants*	*Incidence/ prevalence of pneumonia*	*Significant associations (multivariate analysis reported where possible)*	**Comments**
Langmore et al. [30]	189 Clinic, ward and nursing home residents	21.7%AP (NH=44%)	All patients (no dental variables): Dependent for feeding or oral care Tube feeding Dentate patients only (n=101) Number of decayed teeth More than one medical diagnosis Dentate oral feeders only (n=90): Dependent for feeding Multiple medical diagnoses	Pneumonia diagnosed by consensus panel, most weight given to chest radiograph AP significantly commoner in NH patients with radiologically/endoscopically confirmed dysphagia, and in ward patients with prior tube feeding.

ACE- inhibitor= Angiotensin converting enzyme inhibitor, AP=Aspiration pneumonia, HAP=Hospital acquired pneumonia, OR=Odds ratio, HR= Hazards ratio, NHAP= Nursing home acquired pneumonia, COPD= Chronic obstructive pulmonary disease, NH= Nursing home

1.4.1 Age and gender

Age over 65 or 70 years has been consistently found to be a risk factor for HAP [24, 48, 54, 67, 81, 111] and one study found that people over the age of 65 were twice as likely to develop HAP compared to younger people aged 25-50 [28]. Importantly, older patients are more likely to die [24, 111], and less likely to regain pre-morbid functional status at discharge than their younger counterparts after HCAI [80], leading to change of residence at discharge, or loss of independent living. Impaired neutrophil chemotaxis, declining IgM activity and production of naïve B cells, and impaired memory T cell formation may be contributing factors [112-114], however it is not yet well understood how immunosenescence contributes to the development HAP. Interestingly, risk factors for HAP in older people appear similar to those in younger persons [28]. HAP appears to occur more frequently in males [50, 71, 115] for reasons which are unknown.

1.4.2 Mechanical ventilation

Mechanical ventilation is one the most important risk factors for developing pneumonia [24, 54], and intensive care units are the commonest location for pneumonias to occur in England [20], with incidences of VAP quoted at 8-36% [40, 116, 117]. Mortality may exceed 50% [116]. During mechanical ventilation, the presence of the endotracheal tube means both patients' mouths and tracheas are open, allowing continual access of microorganisms to the lungs. The tube itself also becomes colonised with bacteria which produce and reside within a biofilm [118]. While the risk of contracting HAP while undergoing mechanical ventilation is high, non-ventilated patients who acquire HAP on hospital wards represent over 75% of the total group who develop pneumonia in hospital [10, 119].

1.4.3 Chronic obstructive pulmonary disease (COPD)

Patients with COPD are more likely to develop community acquired pneumonia [120] and HAP [30, 53, 61, 102, 121]. The exact pathophysiological mechanism is unknown, but is likely to be related to persistent bacterial

presence in the lungs and impaired host immune function in patients with COPD [122]. Oral colonisation with opportunistic respiratory pathogens increases as COPD severity increases [38]. In addition, the TORCH and INSPIRE studies, which recruited large cohorts of COPD patients, showed that the use of inhaled corticosteroids such as fluticasone was associated with a higher risk of pneumonia despite reducing exacerbations [123, 124]. While pneumonia was not a predefined endpoint in the larger TORCH study, risk factors for pneumonia included increased age, more severe COPD, any exacerbation of COPD in the year preceding the study, lower body mass index and worse Medical Research Council (MRC) dyspnoea score. Interestingly smoking status (current versus ex) did not influence incidence of pneumonia in this group, in contrast with results from other studies of HAP [59, 61-63], which may have been because the prevalence of smoking was high overall in the cohorts.

1.4.4 Proton pump inhibitors (PPI) and bacterial overgrowth

Several studies have suggested that gastric acid suppression may be associated with HAP [125, 126] via bacterial overgrowth. Bacterial overgrowth (measured by counting bacteria per ml of gastric fluid) is commoner in those taking PPIs [127], and appears to be inversely proportional to gastric pH [127, 128]. The bacteria cultured were mainly oral bacteria (Streptococci, Staphylococci, Neisseriae) as well as *E. coli*, Lactobacilli and Aeromonads. However a large Canadian database study refuted the association between HAP and PPI via a population-wide case control study using a large healthcare database [48]. Redelmeier et al. also concluded that bacterial colonisation may be a less important risk factor than hypoalbuminaemia, and nasogastric tube placement (amongst other factors), because gastric acid suppression was not found to be associated with HAP. However several of the potential risk factors found to be associated with pneumonia are also risk factors for bacterial colonisation, and no microbiological parameters were examined in this study.

1.4.5 Reduced level of consciousness and microaspiration

Decreased level of consciousness and disorientation have been noted previously as risk factors for HAP [24, 28, 54], and use of antipsychotic drugs (which can cause both of these signs) has also been found to increase admissions to hospital with both pneumonia and hip fracture [129]. A common underlying mechanism to these risk factors may be microaspiration. Microaspiration or silent aspiration can occur during sleep in those with certain patterns of stroke [130] and occurs in a proportion of older patients with CAP [102]. Nakagawa et al. [130] attached a radio-labelled paste to the inner surface of participants' teeth which melted over a night. The participant's thorax was scanned the following day and the radiolabel was detected lateral to the midline in the thorax demonstrating the occurrence of aspiration. Silent aspiration (as evidenced by radiolabelled material) also occurs commonly in healthy people during sleep [131, 132] and is therefore likely to occur at other times of reduced consciousness such as hypoactive delirium or anaesthetic.

1.4.6 Dysphagia, nasogastric tubes and dependence on others for feeding and oral care

Dysphagia [53, 66, 69, 110] and aspiration [24, 28, 32] have been found to be important risk factors for HAP, the former probably because of the increased risk of the latter. Odds ratios for aspiration and dysphagia are consistently high in observational studies (see Table 4). Other proxies for aspiration and dysphagia may include the presence of a nasogastric tube for feeding, and dependence on others for oral care and feeding, and HAP has been associated with all of these factors [21, 28, 29]. Nasogastric tubes inserted for feeding put patients at greater risk of HAP both because of underlying dysphagia (and consequent aspiration risk) [110] and by being a source of respiratory commensal pathogens (especially *P. aeruginosa*, and *K. pneumoniae*) [133, 134]. Biofilms form on nasogastric tubes within 24 hours of insertion [135], and the colonised tube is situated in close proximity to the entrance to the trachea. Oral flora is also altered by enteral feeding tubes to include organisms such as Corynebacteria, Peptostreptococci and Fusobacteria more commonly [136].

1.4.7 Gastro-oesophageal reflux

Gastro-oesophageal reflux has also been associated with histological changes in the lungs of rats [137], and could play a role in the development of HAP. Indeed, gastro-oesophageal reflux of gastric contents colonised with respiratory pathogens is one of the proposed mechanisms for the development of VAP, and selective gut decontamination has been found to reduce rates of VAP [138], though oropharygeal colonisation may be a more important route of transmission [40]. However a study of 189 clinic, ward and nursing home patients suggested that nuclear scintigraphy confirmed gastro-oesophageal reflux was commoner in patients who did not develop pneumonia [30], and further work is needed in this area in non-ventilated patients.

1.4.8 Colonisation with respiratory commensal pathogens at sites proximal to the lungs

Culture based techniques have been used to link HAP with presence of respiratory commensal pathogens in both oro-pharyngeal [21], salivary (*S. aureus* only) [29] and bronchial samples [33] in non-ventilated patients in longitudinal studies. Studies in ventilated patients also found that nosocomial infection was commoner in patients with colonised dental plaque [42], or oropharyngeal samples [40]. Identical organisms were found in bronchoalveolar lavage samples and dental plaque from 8/14 patients with VAP [139] using pulsed-field gel electrophoresis. In a similar study, Heo et al. found genetic similarities (>95%) between bacteria found in dental plaque and those from bronchoalveolar lavage in 30 ICU patients suspected of having VAP [140]. A range of infecting pathogens were identified in the above studies including *S. aureus*, MRSA, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *H. influenzae* and Morhaxhella cattarhalis. Studies predominantly classified patients into "colonised" or "uncolonised" groups rather than considering the influence of acquisition versus colonisation, and this may also be important in determining risk of HAP.

1.4.9 Dental risk factors

Number of teeth and number of decayed teeth were identified as risk factors for aspiration pneumonia (AP) in dentate patients only in two studies [29, 30]. In addition, Terpenning et al. identified that AP was associated with *S. aureus* in saliva and P. gingivalis in dental plaque. Few of the other studies above investigated dental risk factors. However one study investigating nosocomial infection in ventilated patients found associations with caries and colonised dental plaque (mainly with aerobic gram negative bacilli) [42]. These findings suggest that HAP may be associated with dental factors, but further research is needed.

1.4.10 Risk factors associated with surgery

Post-surgical patients have additional risk factors for HAP, including pain preventing both adequate expansion of the lungs and adequate cough, anaesthesia with endotracheal intubation, and potential post-surgical complications local to the site of operation. Observational studies of post-operative patients are summarised in Table 5. The commonest risk factors identified in surgical patients include blood transfusion [22, 50, 55, 57, 64, 65], nasogastric tube insertion [48, 55, 56, 58], duration of anaesthetic or surgery [48, 56, 58, 59], and hypoalbuminaemia [48, 59, 141]. Other risk factors identified were pain or increased dose of opiates [22, 33] and cough or presence of sputum pre-operatively [56, 58]. Nasogastric tubes are usually inserted peri-operatively to manage ileus, vomiting or bowel obstruction rather than feeding, and this suggests that nasogastric tubes are a risk factor for HAP independent of the patient's ability to swallow. Redelmeier et al. conducted a highly comprehensive cohort study from a large Canadian database of post operative patients, and found that the risk factor with highest odds ratio was post-operative hypoalbuminaemia (odds ratio 5.74) [48]. Thoracic surgery and nasogastric tube insertion were the next most important risk factors (odds ratio 2.72 and 1.24 respectively). Unfortunately, they did not investigate blood transfusion as a risk factor, which appears to be one of the more important risk factors identified. It is unclear whether peri-operative blood loss or transfusion

itself is important, though renal ischaemia-reperfusion injury in mice has been shown to induce lung inflammation, without blood product transfusion [142].

Table 5. Summary of observational studies investigating post-operative pneumonia

Authors	*Type of surgery*	*Study type*	*Sample size*	*Significant risk factors*	*Notes*
Garcia-Alvarez et al. 2010 [50]	Thompson's hemi-arthoplasty for hip fracture	Prospective	208	Blood transfusion Male sex Fever during admission Died within 1 year of study Time to operation	9% (HAP)
Redelmeier et al. 2010 [48]	All, >age 65	Retrospective cohort	593,265	Low albumin Thoracic surgery Nasogastric tube	HAP 1% incidence
Lead-Noval et al. [55].	Cardiac	Case control	Cases n=42, controls n=90	Reintubation, nasogastric tube, > 4units blood transfused, empirical broad spectrum antibiotics used	13.3% HAP polymicrobial, 77.3% of bacteria isolated were gram negative rods.
El Solh et al. [22]	Cardiac	Case control	73 case control pairs	Charlson index >2, reintubation, transfusion more than 4 units blood, mean daily dose of morphine	Mean time to pneumonia 7.2 +-4.9 days
Belda et al. [33]	Lung cancer	Cohort	N=78	Higher postoperative pain, colonisation with potential respiratory microorganisms	31% incidence pneumonia
McAlister et al. [56]	Elective non-thoracic	Cohort	N=1055	Age over 65 Positive cough test*, perioperative nasogastric tube, duration of anaesthesia >2.5 hours	2.7% had postoperative pulmonary complication

Authors	*Type of surgery*	*Study type*	*Sample size*	*Significant risk factors*	*Notes*
Mohri et al. [57]	Gastric cancer	Cohort	N=529	Male gender, blood transfusion	HAP in 3.6%
Mitchell [58] et al.	Elective non-thoracic	Cohort	N=148	Nasogastric tube placement, longer duration of anaesthetic, preoperative sputum production	Postoperative pulmonary complication in 11%
Garibaldi et al. 1981 [59]	Elective thoracic/ abdominal	Prospective	N=520	Low albumin High American Society of Anaesthetists score Smoking history Longer preoperative stay Longer duration of operation Thoracic or upper abdominal surgery	Incidence 17.5% Classified pneumonia into 3 classes 1= good diagnosis with positive microbiology, 2= good diagnosis with no microbiology or 3= features suggestive of HAP Daily monitoring for 5 days after operation but no mention of further follow up
Dilworth et al. [43]	Upper abdominal		N=127	Smokers *H. influenzae* on pre-operative oropharyngeal sample	Incidence 4%
Lee et al. 2011 [64]	Lung cancer		N=417	Blood transfusion (OR 4.67) Age >70 (OR 3.56) Other complication (OR 3.03) Obstructive airways (OR 3.90)	Incidence 6.2% Mortality 27%

Authors	*Type of surgery*	*Study type*	*Sample size*	*Significant risk factors*	*Notes*
Blank et al. 2011 [65]	Pneumonectomy		N=129	Transfusion of blood product (OR 1.47)	Incidence 21%
Riera et al. 2010 [60]	Cardiac surgery		N=1600	Left ventricular ejection fraction <30% Chronic renal impairment Urgent surgery	Incidence 1.2% Mortality 42% in HAP patients Semi-quantitative endotracheal tube aspirates used
Agostini et al. 2010 [61]	Thoracic surgery		N=234	Body mass index >30 Smoking history COPD Age >75 ASA score >3	14.5% incidence
Tsubosa et al. 2010 [62]	Eosophagectomy		N=191	No significant risk factors found	Incidence 10%, even after oral care, chest physiotherapy, rehabilitation for swallowing disorders
Huang et al. 2010 [141]	Gastroinestinal		N= 215	Albumin (OR 0.27) Physical disability (OR 3.8)	10 year study- few patients

*Cough test is defined as patient taking deep breath and coughing once- any further coughing is deemed a positive test

COPD= Chronic Obstructive Pulmonary disease, ASA= American College of Anaesthesiologists

1.4.11 Putative risk factors for HAP in patients with lower limb fracture

Based on the findings above, important risk factors for older patients with lower limb fracture might include the presence of nasogastric tube, blood transfusion, COPD, aspiration, oral colonisation with respiratory pathogens, dental factors, duration of anaesthetic, reduced conscious level or disorientation and pain or analgesia use. Of these, colonisation with respiratory pathogens and dental factors are potentially the most amenable to modification. Reducing the bioburden in the oral cavity could be a potential method of preventing pneumonia in older patients prone to aspiration. Given that HAP is the second commonest complication after hip fracture and nearly half of these patients die[23], preventing HAP may have an important impact on survival after hip fracture.

1.5 Literature search strategy

In order to investigate what was known about the significance of oral colonisation with respiratory commensal pathogens and dental factors in the development of HAP, a literature search was conducted using Medline 1950-2010 and EMBASE 1980-2010. The search strategy "Nosocomial pneumonia" OR "Pneumonia AND Cross-infection" OR "nursing home acquired pneumonia" or "hospital acquired pneumonia" was used. Aspiration pneumonia was not specifically included in the search strategy, however relevant papers retrieved relating to aspiration pneumonia were considered. Studies conducted on patients in both hospitals and nursing homes were retrieved. A further search of the same databases was conducted using a variety of terms including "potential respiratory pathogens in the mouth" and "aerobic gram negative bacteria", "respiratory commensal pathogens", "oropharyngeal colonisation", "Oral microbiota", "Oral microbiome" and the MESH terms "gram negative bacteria" and "gram negative bacterial infections". Extensive iterative bibliography searching of retrieved papers was also undertaken. Regular email alerts were established in June 2009 using key words "pneumonia" and "nosocomial pneumonia" in Ovid and Scopus

respectively, and a citation alert for a key paper [86] was set up. The literature search was initially carried out in June 2009, updated in October 2011 and updated again in October 2012.

1.5.1 Observational studies investigating oral colonisation with respiratory commensal pathogens

Next generation sequencing techniques have revealed that the core or normal oral flora of adults is composed predominantly of 13 phyla including Acintobacteria, Bacteroidetes, Firmicutes, Chloroflexi, Tenericutes, Fusobacteria, Proteobacteria, Spichochaetes, Synergisteles, and two unnamed phyla [143]. While respiratory commensal pathogens (e.g. *E. coli*, *P. aeruginosa*) are identified in a percentage of "healthy" adults (Table 6), they are not commonly identified in studies of the normal oral flora [144]. Persons in whom these organisms were detected were not obviously unwell and therefore carriage was assumed to be asymptomatic. However, in certain groups of patients (E.g those on intensive care), it has been shown that oral carriage of commensal pathogens is related to subsequent nosocomial infection with the same organisms [42]. It is hypothesised that the same mechanism also occurs in hospital patients not on intensive care and those in nursing homes or even community dwelling patients. The term "commensal pathogen" has previously been coined in relation to *S. pneumoniae* which can cause disease in otherwise healthy persons but may also be carried asymptomatically [34]. It should also be noted that the terms colonization and carriage are used interchangably in this work, and assume that an organism has adhered to and multipled on the mucosa. Given that the mouth is essentially open (via the nasopharynx), repeated transient acquisition may occur rather than colonization in senso strictu.

The prevalence of oral aerobic gram negative bacilli (AGNB), including *H. influenzae*, *P. aeruginosa*, *E. coli*, and other coliforms, and *A. baumannii*, has been investigated in a variety of observational studies (Table 6). These organisms appear to occur more commonly in those who are more unwell, and those who are fed via nasogastric tube. An important study of healthy volunteers showed

that counts of gargled *E. coli*, *K. pneumoniae* and Proteus mirabilis reduced rapidly within three hours, even when using piliated *E. coli* [145]. Counts fell most rapidly at the buccal surface and least rapidly on the tongue. These data imply that healthy humans are able to clear these bacteria from their mouths. It is difficult to compare results between studies in Table 6 as both sampling sites and microbiological techniques vary (e.g. whether enrichment culture was used) leading to a wide variation in rates of both acquisition and colonisation/carriage. In addition, some of the variation may be explained because investigators were testing for several bacteria rather than a single bacterium. Mobbs et al. summarised results from 45 studies investigating AGNB in healthy volunteers dating back to 1921 and found rates of up to 61% [35]. Again, different sampling techniques such as swabs, oral rinses and saliva samples were used in different studies. Most of these 45 studies did not distinguish between acquisition and carriage which may have led to the high incidence of colonisation in some groups. The effects of diurnal variation, prior food or drinks and sleep on colonisation and acquisition of AGNB is unknown, and may also explain some of the variation seen.

Table 6. Observational studies of oral acquisition or colonisation with Gram negative bacilli

Population/setting	*Sample size*	*Sampling technique; frequency*	*Prevalence in healthy controls*	*Prevalence in patients*	*Acquisition (A) or colonisation (C)*
Hospital patients, varying types, hospital workers, fire fighters[36]	253	Area sampling device; 1 sample	0-2%	Orthopaedic 16% Moribund 57%	A
Nurses, medical students and laboratory staff[35]	120	Oral rinse (n=2), two days apart	Colonisation 6% Acquisition 35.8%	N/A	Both
COPD patients[38]	40	Oral rinse (n=2), two days apart	N/A	Mild 0% Moderate 7.7% Severe 29.7%	C
Hospital patients and after discharge[146]	183 ICU 228 general ward	Oropharyngeal swabs	N/A	Admission 1.1% 7 days 3.4% Discharge 12.4% 1 month after discharge 19.4% 3 months after discharge 20%	A
Medical admission ward patients[41]	28	Palatal swabs	N/A	43%	A
Older chronic care facility patients and controls (dental outpatient)[147]	28 (30 controls)	Dental plaque	0% (>1% cultivable flora)	14.3%	A

Population/setting	*Sample size*	*Sampling technique; frequency*	*Prevalence in healthy controls*	*Prevalence in patients*	*Acquisition (A) or colonisation (C)*
Male residents of two long term care wards [148]	68	Pharyngeal swabs	37-43% (three samples)	N/A	A
Nasogastric fed patients, PEG patients and orally fed patients from nursing and skilled nursing facilities [149]	215	Oropharyngeal sample	N/A	NG fed 81%* PEG fed 51% Orally fed 17%	A
Older patients in long term care, NG fed or orally fed[134]	90	Saliva	N/A	NG 73% Orally fed 13%	A
Patients waiting in hospital for cardiac revascularisation [150]	30	Saliva and dental plaque	N/A	*P. aeruginosa* in dental plaque 12% *Acinetobacter* spp 63% Saliva 59% Dental plaque	A
Older adults needing nursing care Healthy older adults Healthy adults <20 [151]	54, 21,22	Oral rinse	*S. pneumoniae* 76% Staphylococcal sp. 24% MRSA 0% *P. aeruginosa* 4.8% C. albicans 66%	*S. pneumoniae* 63% Staphylococcal sp. 37% MRSA 14.8% *P. aeruginosa* 5.6% C. albicans 66.7%	A

COPD= Combined obstructive pulmonary disease PEG= Percutaneous endoscopic gastrostomy NG=Nasogastric

*Also tested for *S. aureus*

1.5.2 Respiratory commensal pathogens and oral or dental factors

A number of studies have investigated the relationship between respiratory tract infection and dental factors such as plaque, caries, and periodontitis. Unfortunately, it is difficult to synthesise the results from these studies into a coherent picture to explain the relationships between these variables because of inconsistent results and varying endpoints. Dentate persons appear to have higher rates of respiratory infection [44, 86], and AP appears to be associated with increased tooth number in dentate patients [29]. One study found similar rates of pneumonia in dentate versus edentulous (no remaining dentition) patients, though rates in the latter were lower [152]. However, other studies either found no relationship with tooth number [152], or have not reported the relationship between HAP and number of teeth [30, 42], inferring that no relationship was found. Edentulous patients were more likely to be colonised with *S. pneumoniae*, MRSA or C. albicans in one study[152], but samples were taken from buccal mucosa in edentulous patients and form dental plaque in dentate patients, which may explain this finding. AP has been associated both with number of decayed teeth [29, 30], and salivary S. sobrinus [29] in dentate patients. Nosocomial infections in ICU patients have also been associated with caries [42]. In contrast, presence of respiratory pathogens was not associated with number of teeth [37, 147] or caries [37] in other studies.

Respiratory tract infections have been associated with heavier dental plaque deposits [44, 45] and with presence of respiratory commensal pathogens in dental plaque [147]. However, no relationship was found between presence of respiratory commensal pathogens in dental plaque and heavier dental plaque [42, 153]. Aspiration pneumonia has also been shown to be associated with P. gingivalis in dental plaque, and diabetes mellitus in dentate persons [29], but was not associated with increased gingival bleeding index. Presence of respiratory commensal pathogens in community dwelling older Japanese persons was associated with positive occult salivary blood [37]. In persons with learning difficulties, pneumonia was associated with an increased Oral

Assessment Guide (OAG) score, with individual components of "tongue" and "swallow" being significantly higher, and "gingiva" trending towards significance in those with pneumonia [152]. However, few studies have included measures of gingival health, and it is difficult to draw conclusions because of this.

Table 7. Observational studies linking pneumonia or presence of respiratory pathogens in oral cavity with dental factors

Setting	*Sample size*	*Findings*
Clinic, ward and institution, US [29]	357	AP in dentate patients associated with: Number of teeth, number of decayed units, S. sobrinus in saliva, P. gingivalis in dental plaque, diabetes mellitus, periodontal disease.
Clinic, ward and nursing home patients, US [30]	189	AP associated with number of decayed teeth and multiple medical diagnoses in dentate patients. No link with xerostomia.
Patients with learning difficulties	63	Pneumonia associated with microorganisms (*S. pneumoniae*, MRSA, P. melaninogenica, C. albicans by PCR) at baseline, worse OAG score, increased age and enteral feeding. Only OAG score associated in multivariate analysis (OR 1.6). No patient had caries.
Nursing home, Switzerland [44]	302	Respiratory tract infection associated with being dentate or needing to visit the dentist in emergency. Dentate patients with RTI had significantly higher plaque scores.
ICU patients, France [42]	57	Dental plaque increased (non-significantly) over time in ICU. High caries score (but not plaque score) associated with nosocomial infection. No correlation between plaque score and caries score. No correlation between plaque colonisation and plaque score. Dental plaque colonisation with AGNB but not *S. aureus* associated with subsequent infection. Plaque colonisation at day 5 associated with subsequent nosocomial infection (RR 9.6)
ICU patients, US [153]	34	Colonisation with respiratory pathogens (MRSA, *P. aeruginosa*, AGNB) in 22/34 patients was associated with antibiotic therapy but not with increased dental plaque score or illness severity.
Independent living patients, Japan [37]	265	Opportunistic respiratory pathogens (*P. aeruginosa*, *S. marcescens*, *K. pneumoniae*,C. albicans) detected by culture of single tongue sample more common if occult blood in saliva and increased age. No association with salivary flow (stimulated or unstimulated), caries, denture wearing or salivary pH.

Setting	*Sample size*	*Findings*
Nursing home, US [147]	28	Colonisation with *S. aureus*, *E. cloacae*, *P. aeruginosa*, *K. pneumoniae* or *E. coli* in 7/28, >1% of aerobic flora in 4/28. Cultures of dental and denture plaque, and buccal mucosa samples. Presence of respiratory organisms commoner if high plaque score or COPD, no difference in number of teeth or if dentures present.
Institutionalised and long term hospitalised patients, Japan [154]	343	HAP associated with Clusters C and D (Prevotella, Treponema, Veillonella) compared with cluster A (Streptococcus) detected by Terminal Restriction Fragment length polymorphism
Nursing home residents, health older and younger adults, Japan [151]	54,21,22	Higher Staphylococcal species but lower *P. aeruginosa* seen in those with poorer oral hygiene. Professional oral hygiene only reduced C. albicans significantly.
Nursing home residents, Japan [155]	71	Pneumonia commoner in those with higher tongue plaque. Higher salivary bacterial counts seen in those with higher tongue plaque.
Hospitalised patients awaiting cardiac revascularisation [150]	30	*P. aeruginosa* commoner in patients with <14 teeth Co-colonisation of *P. aeruginosa* and *Acinetobacter* spp. observed

RTI= respiratory tract infection, ICU=Intensive Care Unit, AGNB=Aerobic gram negative bacilli, RR=relative risk, COPD= Chronic obstructive pulmonary disease, OAG= Oral assessment guide

Despite the contradictory and incomplete nature of the above findings, it does appear that there is a relationship between respiratory tract infection and oral health, but the relationship may be indirect and complex. These findings have led some authors to hypothesise that dental plaque may be a stable reservoir of respiratory commensal pathogens, and that removal of plaque [47, 156-161] or disinfection of the oral surfaces might reduce the risk of respiratory infection. A number of oral hygiene intervention studies have been undertaken in ventilated and non-ventilated persons, and are described below.

1.5.3 Intervention trials to prevent HAP, VAP and AP

A summary of studies investigating oral hygiene interventions to prevent HAP are shown in Table 8. The majority of trials took place in Japan and contained small numbers of patients. Successful intervention trials in non-ventilated persons all included weekly dental hygienist attention, and interestingly there appeared to be a cumulative benefit over a number of months of professional oral hygiene [162]. Interventions took place over 2 years in successful studies in nursing home patients, and it was noted that no benefit was seen at six months [162], possibly due to temporal spacing in the occurrence of pneumonias, or because of cumulative effects of oral care. It has been noted that patients being uncooperative is one of the major difficulties in undertaking mechanical oral hygiene interventions in older people, and use of chlorhexidine may be more acceptable to older patients with challenging behaviour [163]. However, Bourigault et al. did not find a reduction in NHAP after tooth-brushing three times daily and use of chlorhexidine mouthwash (though frequency of use was unspecified) [164]. The majority of studies have used 0.12% chlorhexidine solution or 0.2% topical chlorhexidine, though some trials in ventilated persons have used 2% chlorhexidine solution to good effect, but one noted a 10% incidence of oral mucosal irritation [165].

Several groups have found strong relationships between VAP and prior presence of potentially pathogenic oral bacteria [40, 42]. Intervention studies conducted in ventilated patients have had mixed results, with some initial successful studies using chlorhexidine [166-169] or decontamination with topical antibiotic therapy [170], followed by several studies which did not show significantly reduced rates of VAP. In two such unsuccessful trials, *P. aeruginosa*, *A. baumannii* [171, 172] and MRSA [172] were not eradicated by chlorhexidine which was the main intervention. A meta-analysis in 2006 was unable to show that use of chlorhexidine reduced VAP [173]. However several more recent meta-analyses have revealed reductions in the incidence of VAP from using antiseptic decontamination with chlorhexidine [165, 174-177] or topical antibiotic therapy [176], though no reduction in mortality was seen [116, 176].

Several systematic reviews investigated interventions other than oral hygiene to prevent AP (though definitions are very similar to HAP). These have included studies investigating pharmacological methods [178, 179], and interventions to reduce aspiration [178]. However none of the methods (which included use of anti-thrombotic agents, amantadine, and feeding programmes) could be recommended. The anti-thrombotic agent, cilastazol, was found to be effective in preventing AP but caused bleeding which outweighed the benefits. The authors concluded that the studies retrieved were too few and of too poor quality to make robust conclusions.

Table 8. Summary of oral hygiene intervention trials to prevent HAP in non-ventilated persons

Setting	*Study type*	*N control: N intervention*	*Intervention*	*Findings*	*Quality issues*
Nursing home, Japan [86]	Randomised	182:184	Brushing teeth, tongue and buccal mucosa without dentifrice after each meal plus 1% povidone iodine in some. Weekly hygienist intervention.	Significant reduction in pneumonia (RR 1.67) and febrile days	51 patients excluded as died from other causes
Nursing home, Japan [180]	Crossover	21:25	Daily oral care from dentist and hygienist, povidone iodine applied to mouth after each meal	No reduction in febrile days	Small sample, pneumonia not an endpoint
Nursing home, US [181]	Non-randomised	65:78	Nursing assistant providing oral hygiene care- toothbrushing, antiseptic mouthwash (unspecified), oral and denture cleaning in edentulous, dilute hydrogen peroxide used in patients with decreased conscious level with 30^{o} head up.	Incidence of pneumonia same. With adjustment for comorbidity and frailty, death in control group higher OR 3.57	Intervention group significantly more dependent and cognitively impaired at baseline
Nursing home residents, Japan [182] [183]	Randomised	48:40	Professional oral hygiene care by dental hygienist including electric toothbrushing (with water supply), sponge brush and interdental brush to clean teeth, buccal mucosa, tongue and dentures. Normal care was sponge brushing and denture cleaning	Reduction in fever and fatal aspiration pneumonia. No significant reduction in Staphylococci or Pseudomonas sp. in oral care group	No definition aspiration pneumonia, randomisation not described

Setting	*Study type*	*N control: N intervention*	*Intervention*	*Findings*	*Quality issues*
Gastrointestinal surgery patients, Japan [184]	Randomised	15:15	Daily nurse led povidone iodine gargles (0.02%), toothbrushing, denture and tongue cleaning versus just iodine gargles in control group.	No reduction in fever, significant reduction in number of patients with pulmonary rales heard. Reduction in number of species of bacteria identified with oral care	Pneumonia not measured as an endpoint. Study underpowered
Nursing home residents, Japan [162]	Cluster randomised	A n=62 B n=59 C n=41	A= once weekly hygienist 6/12 B=nil 2/12, weekly hygienist 3/12 C= 0.35% povidone iodine gargles 2/12, weekly hygienist 3/12	No reduction in febrile days or aspiration pneumonia Significant reductions in bacterial species after hygienist intervention, cumulative over 6 months.	Small sample-pneumonia incidence compared to preceding 6/12 during summer period
Nursing home residents, Japan [185]				Reports findings from [151, 183]	

Setting	*Study type*	*N control: N intervention*	*Intervention*	*Findings*	*Quality issues*
Maxillofacial surgery patients, Japan [186]	Randomised	16:16	Electric toothbrush with 0.5% povidone iodine supply twice daily before and after surgery	Reduction in Methicillin sensitive Staphylococcal species, *H. influenzae* and *S. pneumoniae* by oral care, but not MRSA or *P. aeruginosa*. No cases of pneumonia found	Small study, not blinded
Geriatric facility patients, France [164]	Cluster randomised	868:1645	Annual visit to dentist, brushing of teeth, mucosa and tongue 3 times daily, use of chlorhexidine mouthwash (unclear when)	No significant difference between rates of first pneumonia or mortality	Control group had higher mortality and higher incidence of first episode of HAP

1.5.4 The case for investigating HAP in relation to oral respiratory commensal pathogens and oral hygiene

Although there is evidence that HAP may be preventable by improving oral hygiene, the body of evidence is limited and mainly of suboptimal quality. The majority of oral hygiene intervention trials have not investigated oral bacteria or dental factors, which appear to be the most modifiable risk factors, as outcome measures. Accurate data regarding the dynamics of oral colonization with opportunistic respiratory pathogens in hospital patients are currently lacking, and "dose" of organism may be important and has not been investigated [187]. Future intervention studies in older patients at risk of HAP are likely to be more robust if more information is known about some of these issues.

However traditional bacterial culture requires a minimum of 48 hours for a reliable result, and relies on timely delivery of samples to the laboratory to detect viable organisms. If the presence of certain oral bacteria were a biomarker of future risk of HAP, then more rapid testing would facilitate earlier intervention and theoretically prevent more disease. In addition, non-viable organisms can be detected by molecular methods thus reducing false-negative results. Many bacteria within the mouth are contained within biofilms and it is unclear how this may influence growth in laboratory conditions. Detection rates appear to be higher using molecular methods such as PCR compared with culture in clinical studies of CAP [188-190], especially after antibiotic treatment has been started [191]. Therefore molecular methods of detecting oral organisms may be superior to culture for this purpose.

1.6 Part 2 Molecular detection of respiratory commensal pathogens in the oral environment

It has been estimated that 1ml of saliva contains 100 million bacterial cells [143], and a highly specific molecular assay is therefore required to avoid high levels of false positive results. A variety of molecular assays could be used for the purpose of detecting a number of specific organisms from the oral cavity, such

as low density arrays [192, 193], PCR-luminex [194], and nested PCR [195]. However for logistical reasons, the real-time polymerase chain reaction (PCR) was most appropriate for this study, due to its availability in NHS and Health Protection Agency laboratories (because it is rapid, reliable, and quantitative, theoretically detecting a single copy of target DNA within a sample).

1.6.1 The polymerase chain reaction

The discovery of the PCR reaction was attributed to Kary Mullis in 1993 [196], and remains, with modifications, one of the most important and commonly used techniques in molecular biology [197]. PCR can be used to detect DNA in a range of samples, including clinical (e.g. blood or sputum) [198-202] or environmental (e.g. water) samples [203-205]. The concept of PCR is well established, and a summary of the reaction follows. If a specific organism is sought, the investigator identifies DNA which is well conserved within that species, and not seen in other organisms by comparing DNA sequences from national databases. Short single-strands of DNA complimentary to the regions flanking the double stranded target DNA are chosen and manufactured, and are known as primers. One primer is designed to flank the target area on the sense strand of DNA, and one primer to flank the other side of the target DNA on the anti-sense strand. The target DNA can then be amplified (or copied) using a heating block, the primers, and reagents which can synthesise new DNA. Reagents used include Taq polymerase, magnesium sulphate and each of the four nucleotide bases (A, G, C, T) of which DNA is made. Taq polymerase is a heat-stable enzyme whose function is to create new DNA polymers that are complimentary to a template strand of DNA using nucleotides in solution within a reaction. The nucleotides incorporated into the new DNA polymer are complimentary to the nucleotides on the template strand. During the PCR reaction, heat is used to anneal and denature double stranded DNA, in order to amplify target DNA sought within a sample. When the temperature is elevated to 95°C, all double stranded DNA denatures. As the temperature is lowered to 55°C, DNA is able to anneal once more, but primers may now anneal to any complimentary segments of target DNA. As the temperature rises again to 65°C, the DNA remains annealed, and the Taq

polymerase actively extends the primers (and other DNA segments) where there is a complimentary strand using the nucleotides included in the reaction. This sequence of temperature changes is known as a cycle, and a standard PCR reaction comprises 40 cycles. During each cycle, exponentially increasing numbers of copies of any target DNA present within the sample are generated. The final sample needs to be stained, and DNA products separated by gel electrophoresis and compared to known DNA products of different sizes under ultraviolet light. Products of the anticipated base pair length are considered to be the target DNA sought. Further analysis such as sequencing may be undertaken.

1.6.2 Disadvantages of PCR

Probably the greatest disadvantage of PCR is that the user chooses the target segment of gene to assay, and will be unaware of other organisms present within the sample. Inappropriate choice of gene target could lead to false positive and negative results, via detection of other species or the exclusion of relevant target species. Good sequence data is required for a number of strains of bacteria in order that primers can be designed confidently. GenBank and EMBL are the US and European banks of DNA sequences respectively, but sequence data is not validated so care is needed to align as many different sequences as possible to overcome any quality issues.

In addition, unless sequencing is undertaken, the user cannot be sure that the PCR product seen on gel electrophoresis is the target DNA rather than another similarly size segment of DNA (e.g. due to mispriming). Amplification of all DNA, not just of target DNA occurs during the PCR reaction, and non-specific products may occur [206]. However these issues can be overcome by the use of real-time PCR.

1.6.3 Real-time PCR

Real-time PCR is so called because data is generated during the PCR reaction (around 90 minutes), which obviates the need for further processing. Real-time PCR also allows you to quantify the amount of nucleic acid target in samples which is known as quantitative PCR (qPCR). These improvements are

engendered by the incorporation of fluorescent dyes into the PCR reaction. Two types of approach are used which are the incorporation of a DNA binding dye which will bind to any double stranded DNA and then fluoresce at a specific wavelength (e.g. SYBR Green), and probes labelled with reporter dye molecules) [207]. The former approach lacks specificity due to the dye being able to bind to any double stranded DNA generated in the PCR reaction, therefore the majority of diagnostic assays used in microbiology utilise the latter technique using probes labelled with fluorescent reporter dyes .

1.6.4 TaqMan 5′ Nuclease Real-time PCR chemistry

During the real time PCR reaction, as the temperature decreases from 95°C, the probe anneals to any complimentary strand of DNA at approximately 10°C higher than the primers. So the probe always anneals before the primers. The probe anneals to the same (anti-sense) strand of DNA as the forward primer, and includes two fluorescent dyes, one at either end. One dye is a reporter dye or fluorophore at the 5′ end, the other a quencher fluorophore at the 3′ end. While the two dyes are in close proximity, the quencher dye prevents the reporter from emitting the majority of its fluorescence. This is achieved by Fluorescence Resonance Energy Transfer (FRET). The reporter dye molecule is excited by a light source in the real-time PCR platform, and emits a "virtual photon" which is immediately absorbed by the nearby quencher dye molecule [207]. However, as the primer is elongated by the Taq DNA polymerase enzyme it eventually encounters the end of the annealed probe. In addition to its DNA polymerase activity, Taq polymerase has 5′-3′ exonuclease activity, meaning it breaks the bond between bases at the end of a nucleotide chain. Each base of the probe is broken off in turn- both broken from its neighbour and also separated from the strand of target DNA which allows the Taq polymerase to continue to extend the DNA primer. The two dyes are therefore separated by this process, the reporter dye (donor) then fluoresces without being quenched, and this emission is detected by the real time PCR platform. The emission spectrum of the donor (fluorescer) molecule needs to overlap with the absorption spectrum of the acceptor (quencher) molecule for FRET to occur [208] and thus only certain combinations of fluorescers and quenchers can be

used. Each copy of DNA which is amplified causes a rise in detected fluorescence, and as amplification is exponential, higher numbers of target DNA sequences in the original sample result in earlier fluorescence. In this way, real time PCR can be quantitative. The probe adds high specificity to the real time PCR result, as the probe should only bind to the target DNA sought.

1.6.5 Applications of real time PCR

The speed of real time PCR and lack of post-PCR processing mean that it is often chosen over traditional PCR. Real time PCR is used in a variety of clinical situations, including detecting organisms causing community acquired pneumonia [209-212], bacterial and viral respiratory pathogens [192], meningitis [213], calculating bacterial loads in diseases such as cystic fibrosis [214] and joint infection [215]. The utility of real time PCR can be further increased when it is multiplexed to allow simultaneous detection of several targets due to the use of multiple probe dye combinations active at different wavelengths.

1.6.6 Applications of real-time multiplex PCR

Clinical applications of multiplex real-time PCR include conditions where several organisms may be responsible for a single disease process or where only small volumes of sample are available, for example in meningitis [216]. In addition, laboratory time is saved in terms of sample preparation. Other real time multiplex PCR assays include those for respiratory viruses [190], for atypical organisms causing CAP [190, 201, 217], and other bacteria of clinical relevance [203, 218-221].

1.6.7 Disadvantages of real time multiplex PCR

There are three main issues with real time multiplex PCR. Firstly, due to the limited number of fluorescent dyes instruments can detect, only four or five targets can be detected in a single reaction. This means that different panels are needed for each disease under consideration, and some diseases would require several panels. Secondly, because PCR is a enzymatic process if two different targets are present in the sample in differing quantities, the target at the higher concentration may be amplified preferentially compared to the target at lower

concentration [222]. Thirdly, some samples yield a weakly positive result, when the fluorescence only occurs between cycles 35 and 40 [223]. In this case, clinicians must make a decision as to the management of the patient, based on the patient's condition and the pathogen involved. There is as yet little research on the distribution of cycle numbers of positive results in different infections, but it appears that this may be pathogen specific [187, 224].

1.6.8 Using real-time PCR to identify respiratory commensal pathogens in the oral cavity

Real-time multiplex PCR assays were suitable for the detection of respiratory commensal pathogens from the oral cavity. While several multiplex PCR assays have been developed for use in respiratory infections [195, 201, 213, 216-218, 225], only a few recent assays address the group of bacteria common to HAP, which include *S. aureus* and MRSA, *S. pneumoniae*, *H. influenzae*, *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, *A. baumannii*, and other coliform bacteria [193, 195]. Therefore a set of novel assays were designed and multiplexed for this purpose.

1.7 Aims of this work

The overall aim of this project was to determine whether characterising the presence of respiratory commensal pathogens within the oral bacterial community in patients with lower limb fracture at the start of hospital admission could stratify future risk of HAP. Individual aims are listed below.

1. Design a multiplex real time PCR assay for common respiratory bacterial pathogens in hospital acquired pneumonia.

2. Recruit a cohort of hospital in-patients with lower limb fracture and collect a series of oral samples over the first 14 days of admission, along with demographic data, plaque scores, xerostomia scores and identify cases of pneumonia and other complications prospectively.

3. Analyse the oral samples using the multiplexed real-time PCR assays after the completion of follow up.

4. Describe colonisation dynamics of respiratory commensal pathogens within the oral cavity in hospitalised older persons.

5. Investigate whether cases of HAP were associated with the presence of particular organisms or combinations of organisms within the mouth, oral hygiene variables, patient demography, operative factors or events in hospital.

6. Make recommendations about future research in this area and about changes to clinical practice which should be considered as a result of this work.

1.8 Chapter 2 Materials and methods

2.1 Bacterial isolates

Bacterial isolates used in this study are listed in Appendix 4. Isolates were kindly provided by the Health Protection Agency Public Health Laboratory Newcastle; Professor John Perry, Microbiology department, Freeman Hospital, Newcastle; and Professor Andrew Smith, Glasgow University.

2.2 Microbiological media and molecular biological enzymes and buffers

See Appendix A.

2.3 Storage of bacterial cultures

Bacterial cultures were stored at -20°C in STGG (skimmed milk, tryptone, glucose and glycerine) in 2ml microtubes.

2.4 Maintenance of working cultures

PCR assays were validated using bacterial DNA extracted from clinical isolates which had been identified in the local microbiology laboratory (Freeman Hospital, Newcastle upon Tyne). Bacteria were stored in STGG after identification. Aerobic bacteria were cultured on Columbia blood agar (or chocolate agar for *H. influenzae*) and incubated at 37°C in aerobic conditions, with additional 5% CO_2 for *S. pneumoniae* and *H. influenzae*. Anaerobic bacteria were cultured on Columbia blood agar, placed in an anaerobic jar with a negative control plate (inoculated with *P. aeruginosa*) and a sachet of Anaerogen™ (Oxoid Ltd., Basingstoke, Hampshire, England), and incubated at 37°C for 2-5 days.

2.5 Manual extraction of nucleic acids from bacterial cultures

2.5.1 Gram negative bacteria

The Qiagen DNeasy kit (Crawley, UK) was used to manually extract DNA from bacterial cultures (see 2.4) as per manufacturer instructions. Extraction of nucleic acids was undertaken using bacteria which had been cultured for 24-36 hours. Bacteria were emulsified in a 2ml microtube containing 180µl of buffer ATL using a 1µl plastic loop. 20µl of proteinase K was added. The suspension

was mixed by vortex mixer and incubated on a heating block at 56°C until the suspension became clear. The sample was mixed by vortexing for 15 seconds and 200µl of buffer AL was added followed by further mixing by vortex. 200µl ethanol was added and the sample was mixed by vortex again. The sample was transferred into a spin column by pipette and centrifuged at 6000xg for one minute. The tube containing flow-through was discarded, and the spin column was placed into a new 2ml collection tube and 500µl of buffer AW1 was added to the column. The sample was further centrifuged at 6000xg for one minute. The collection tube was discarded and the spin column placed into a new collection tube, and 500µl buffer AW2 was added. The sample was then centrifuged at 20,000xg for three minutes to remove any residual wash buffer. The spin column was transferred to a clean tube and 200µl buffer AE was added to the column to elute DNA. The sample was incubated at room temperature for one minute and then centrifuged for one minute at 6000xg to elute the extracted DNA off the spin column.

2.5.2 Gram positive bacteria

Gram positive bacteria were pre-treated with a lysis buffer containing 20mM Tris-Cl pH 8.0, 2mM Sodium EDTA, 1.2% Triton x100 and lysozyme 20 mg/ml. Bacteria were transferred to a microtube containing 180µl of the above lysis buffer using a 1µl loop, and incubated at 37°C on a heating block for 30 minutes. 25µl proteinase K and 200µl buffer AL were added and the sample was mixed by vortex mixer and incubated for a further 30 minutes at 56°C. 200µl ethanol was added to the sample and mixed by vortex mixer. The sample was then pipetted into spin columns and the procedure was continued as in 2.5.1.

2.6 Automated extraction of bacterial DNA from study samples and bacterial isolates

Automated DNA extraction was performed on the NucliSENS® easyMAG™ platform (bioMerieux, France) with off-board lysis as per manufacturer instructions. Automated extraction was used for all study samples and the majority of the known bacterial isolates used in validation studies. Swab

samples taken during the patient study were transferred manually into tubes containing lysis buffer using sterile forceps. A cotton-tipped swab was used to transfer and emulsify bacteria cultured on agar plates (for validation studies) into a tube containing lysis buffer. Samples were incubated in lysis buffer for a minimum of ten minutes at room temperature (21°C), transferred into the instrument plastic wells by pipette and loaded onto the easyMAG instrument. Added to each sample were 100µl of magnetic silica beads, prepared according to manufacturer instructions. The remainder of the process was carried out by the easyMAG instrument. The DNA bound to the magnetic silica beads, which were subsequently magnetised to the side of the plastic well. Lysis buffer at the bottom of the well was removed, and the magnetic silica was then released into guanidine thiocyanate. This process was repeated with a second wash buffer, and the DNA eluted into an elution buffer. The silica beads were magnetised midway up the wall of the sample well leaving a clear eluate. The eluates were transferred into sterile 2ml microtubes by pipette and stored at -80°C prior to testing with the PCR assays.

2.7 Amplification of DNA from bacterial isolates using standard PCR

2.7.1 Reaction conditions

PCR reactions were carried out in 50µl volumes and contained water, 5µl 10x buffer, dNTPs (0.125mM), Taq Gold polymerase (1.25 units), magnesium chloride (2.5mM) (Applied Biosystems, Warrington UK), primers (final concentration 20mM) and 5µl template DNA. PCR amplification was carried out in a GeneAmp 9700 thermal cycler (Applied Biosystems, Warrington, UK). Thermal cycling conditions were as follows: 94°C for 15 minutes, 40 cycles of 94°C for 30s, 55°C for 30s, then 72°C for 1 min. Then followed one hold at 72°C for 7 minutes and samples were then held at 4°C.

2.7.2 Gel electrophoresis for detecting PCR products

Agarose gel electrophoresis was used to visualise PCR products. The agarose gel was made by adding 100 mls of 1x Tris base/Boric acid/ EDTA (Ethylenediaminetetraacetic acid) buffer, (TBE buffer), to 1.2g of agarose gel (Microsieve 3:1, Flowgen). A solution was obtained by heating in a microwave

on full power (800 watts) for 90 seconds, and 10µl of 10mg/ml of ethidium bromide (Sigma-Aldrich laboratories, UK) was added. The solution was incubated in a water bath (55 °C) for 15 minutes and poured into a gel tray. The gel tray was placed into an electrophoresis bath (Amersham Pharmacia Biotech Inc., Sweden, via electrophoresis power supply EPS 301). Electrophoresis buffer contained 600mls 1x TBE buffer and 60µl ethidium bromide. 10µl of DNA molecular weight marker (Superladder- Low 100bp ladder, Thermo Fisher Scientific, Surrey, UK) was inoculated into the two most lateral wells. 10µl of gel loading buffer (BlueJuice from Invitrogen, Life technologies Ltd., Paisley, UK) was added to 10 µl of PCR products and mixed by pipetting, and 10µl of dyed sample was loaded into the gel. The gel was subjected to 80 volts for 70 minutes.

Electrophoresed DNA was visualised under ultraviolet light (302nm) using a Bio-Rad Universal Hood II (Bio-Rad laboratories Hercules, California) camera and photographed using the Molecular Imager Gel Doc XR System and the Quantity One 1-D software (version 4.6.2) from the same company.

2.8 Amplification of bacterial DNA from study samples and bacterial isolates using real-time PCR

Real time PCR assays were carried out in 50µl volumes and contained Universal PCR master mix (Applied Biosystems, Warrington, UK), primers (final concentration 20µM), probe (final concentration 10µM) and 5µl of DNA template. Thermal cycling and data analysis were conducted on a Taqman 7500 instrument (Applied Biosystems). Thermal cycling conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15s then 60°C for 1 min. Results were analysed using the auto-analysis feature.

2.9 Positive and negative controls used in PCR reactions and bacterial DNA extractions

PCR grade water (Sigma-Aldrich Company Ltd, Gillingham UK) and non template wells were used as negative controls for PCR reactions. Positive controls included a mixture of salmon sperm DNA (0.5mg/ml, Invitrogen, Paisley UK), DNA extracted from all target bacteria and human DNA

containing the gap gene. Approximately 20 samples of extracted DNA from each bacterial target were analysed using real time PCR to determine a mean Ct value. The Ct values differed by the organism tested, and accordingly different concentrations of each target bacteria were added to the positive control mix to aim for a final Ct value of 30-35 for each organism included. The same positive control mixture (100µl) was also used during each bacterial DNA extraction run, in addition to a dry swab as a negative extraction control.

2.10 Standardisation of sample testing

Samples were anonymised at the bedside and assigned a laboratory number at DNA extraction. A standard operating procedure was developed for testing study samples. The five primer and probe combinations were pre-prepared, and stored at -20C˚. The assays were undertaken on two 7500 Real-time PCR instruments between July 2010 and April 2012, after completion of patient follow up. The assays were processed by a single laboratory technician. Results were analysed using the Taqman auto-analysis feature and stored in a database (Microsoft Access, Office 2007) on the Newcastle upon Tyne Hospitals Trust server. This database also contained clinical details from the study patients and the addition of PCR results to the database un-blinded the study.

Chapter 3 Development of a real time multiplex PCR assay for detecting oral colonisation with potential respiratory pathogens

3.1 Introduction

3.1.1 Detecting potential respiratory pathogens in the oral environment

Oral colonisation with respiratory commensal pathogens may represent a risk factor for HAP [21, 33] in older persons. In the hospital setting, rapid identification of these organisms may be beneficial in order to implement timely interventions e.g. improved oral hygiene, given that median time to HAP onset may be as early as 7-11 days [21, 22]. Standard culture techniques require a minimum of 48 hours for full identification, compared with PCR which may be able to produce results within the working day. However, identifying individual pathogens within the densely populated oral environment requires a highly specific assay. Compared with standard endpoint PCR, real-time PCR has increased specificity due to the addition of a probe. Real-time PCR is therefore likely to be well suited to identifying organisms within a dense bacterial population, and is also frequently used in routine microbiology laboratories in the United Kingdom (UK).

3.2 Methods

3.2.1 Real-time PCR assay development: Literature review

The commonest bacterial causes of HAP in non-ventilated patients in the United States (US) [11] are shown in 1.2.3 (no similar data existed for patients in the UK). These bacteria first adhere to the oropharynx before aspiration or micro-aspiration into the lungs to cause HAP. Therefore the commonest ten bacterial pathogens were chosen as real-time PCR assay targets to represent the majority of cases of HAP, which included *S. aureus*, MRSA, *E. coli*, *P. aeruginosa*, *S. pneumoniae*, *H. influenzae*, *Acinetobacter* spp., *K. pneumoniae*, *S. marcescens* and *E. cloacae*. Satisfactory previously published assays existed for GAPDH (targeting the gap gene) [226] and *S. aureus*/MRSA assays (targeting femB and mecA respectively) [227], and further literature searching was not undertaken for these targets. A literature review of real time and standard PCR assays

targeting the other organisms was conducted in October 2008 using Medline 1950-present (criteria described in Table 9). Iterative searching of bibliographies of each paper was performed until no further papers were found. Papers relating to typing rather than primary identification were not retrieved. Where little was found for a particular organism, an additional "Google" search was undertaken. The results of this search are shown in Appendix B.

Table 9. Medline search strategy for literature search of previously published PCR assays for target organisms

Target Pathogen	*Search Strategy*	*Hits*	**Papers****
Pseudomonas aeruginosa	[*Pseudomonas aeruginosa* OR Pseudomonas infections] AND Polymerase chain reaction(expl)	619	24
Klebsiella pneumoniae	[Klebsiella(expl) OR Klebsiella infections] AND Polymerase chain reaction(expl)	301	6
Enterobacter spp.	Enterobacter(expl) AND Polymerase chain reaction(expl)	140	8
Escherichia coli	[Escherichia(expl) OR *E. coli* infections] AND Polymerase chain reaction.ti,ab.*	2196	36
Acinetobacter spp.	[*Acinetobacter*(expl) OR *Acinetobacter* infections] AND Polymerase chain reaction(expl)	255	10
Haemophilus influenzae	[*Haemophilus influenzae* OR Haemophilus infections] AND Polymerase chain reaction(expl)	305	38
Serratia marcescens	[Serratia infections OR Serratia(expl)] AND Polymerase chain reaction(expl)	86	2
Streptococcus pneumoniae	[*Streptococcus pneumoniae* OR Pneumococcal infections] AND Polymerase chain reaction(expl)	563	36

*search strategy amended as usual search gave 7122 citations.

** excluding further papers found by bibliography searching

3.2.2 Assay Design

The literature review yielded promising candidate genes for *E. coli* (uidA), *P. aeruginosa* (ecfX), *S. pneumoniae* (cps), *H. influenzae* (P6), *Acinetobacter* spp (16s-

23s ITS), and coliforms (16s). Insufficient data were available on the *S. marcescens* and Enterobacter genomes in 2009 which precluded assay design.

A table containing all assays retrieved from this search can be found in Appendix B. In silico analysis of sections of shortlisted genes or primers was undertaken using the basic alignment search tool (BLAST) against all the nucleotide database in GenBank, with the aim finding conserved genes within species and of avoiding genes/sequences present in other target organisms, common oral bacteria or human DNA.

Genes conserved within the species were found for *P. aeruginosa*, *E. coli*, *H. influenzae* and *S. pneumoniae*. However none of the previously published assays were both suitable for the Taqman instrument and sufficiently specific for use on oral samples. Therefore novel assays were designed for these targets. No gene from previously published assays distinguished the closely related Enterobacteriaceae, and further comparative genomic searching was undertaken using the Comprehensive Microbial Resource database (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi), but this was unsuccessful. Therefore a generic coliform assay was designed targeting the 16s gene.

For each target, all sequence data relating to the candidate gene were downloaded from GenBank and exported into Editseq software (Lasergene, DNA STAR incorporated, Madison, Wisconsin). Sequences were aligned using MegAlign (Lasergene, DNA STAR inc., Madison, Wisconsin) using clustal W analysis. Conserved regions were compared with the same gene from other organisms. Automated primer design was undertaken using the consensus sequence in PrimerExpress software (Applied Biosystems, Warrington, UK), and secondary structures were checked using NetPrimer (free software, http://www.premierbiosoft.com/netprimer). Primers or probes which formed secondary structures with $-\Delta G > 6$ were abandoned and redesigned. Specificity of final primers and probes was confirmed using a BLAST search against the nucleotide database in GenBank. No set of primers yielded any unexpected matches. The characteristics of novel assays are shown in Table 10, sequence

data for all assays is shown in Table 11 and function of all target genes are shown in Table 12.

Primers were manufactured by Eurogentec Ltd (Southampton, UK). Detection of known bacterial isolates was confirmed using endpoint PCR (see 2.7.1) and agarose gel electrophoresis (described in 2.7.2) prior to primer optimisation for real-time PCR.

Table 10. Locations, optimised reaction concentrations and melting temperatures of primers and probes for real time PCR assays designed in this study

Target	*Strain (GenBank Accession no)*	*Gene*	*Primer locations (length)*	*Probe location*	*Length of amplicon*	*Primer concs** F/R*	*Probe conc*	*Tms*** of primers and probe*
*E. coli**	K12 DH10B (CP000948 1783245-1785056)	uidA	F 1699-1714 (16) R 1765-1750 (16)	1722-1748 (27)	67	0.9/0.9	0.25	F 59°C, R 58°C Pr 68°C
P. aeruginosa	PA01 (AE004091.2 1409949-1410476)	ecfX	F 410-428 (19) R 474-454 (21)	Pr 431-451 (21)	65	0.05/0.9	0.25	F 59°C, R 59°C Pr 69°C
*S. pneumoniae**	G54 (CP001015 303775-304674)	cps A-D	F 344-368 (25) R415-398 (18)	Pr 374-392 (19)	48	0.3/0.3	0.25	F 59°C, R 58°C Pr 70°C
H. influenzae	86-028NP (CP000057.2 470967-471428)	P6	F 309-334 (26) R 431-409 (23)	Pr 336-364 (29)	123	0.9/09	0.25	F 59°C, R 58°C Pr 68°C
Acinetobacter spp.	AB 307-0294 (CP001172.1 19883-20482)	16s	F 119-140 (22) R 260-267 (24)	Pr 205-226 (22)	142	0.9/0.9	0.25	F 58°C, R 58°C Pr 69°C
Coliform	NTUH-K2044 (16108-17564)	16s	F 76-95 (20) R 159-139 (21)	Pr 100-116 (17)	84	0.9/0.9	0.25	F 59°C, R 58°C Pr 69°C

F= forward, R=reverse, *Minor groove binder probes designed ** concs= Concentration ***Tm= Melting temperature

Table 11. Primer and probe sequences for assays used in this study

Target organism	*Gene target*	*Primer sequences*	**Probe sequence and dye label (5′-3′)**
S. aureus	femB	F GAC ATT TGA TAG TCA ACG TAA ACG TAA ACG TAA TAT T R GCT CTT CAG TTT CAC GAT ATA AAT CTA AGA	**VIC**- TCA TCA CGT TCA AGG AAT CTG ACT TTA ACA CCA TAG T- **TAMRA**
MRSA	mecA	F CAT TGA TCG CAA CGT TCA ATT T R TGG TCT TTC TGC ATT CCT GGA	**CY5**-TGG AAG TTA GAT TGG GAT CAT AGC GTC AT- **DDQII**
E. coli	uidA	F CGC GCT TTC CCA CCA A R CGG CCT GTG GGC ATT C	**CY5**-CAA TTC CAC AGT TTT CGC GAT CCA GAC-**DDQII**
P. aeruginosa	ecfX	F GCC TGT CCC AGG TCG AAG T R GAT GTG CTT TTC CAC CAT GCT	**VIC**-CCG AGC GCA TGG GAA TCT CCC- **TAMRA**
S. pneumoniae	cps	F GTG TCG CTG TTT TAG CAG ATA GTG A R TCC CAG TCG GTG CTG TCA	**VIC-** AAA ATG TTA CGC AAC TGA C-**MGB**
H. influenzae	P6	F AAA CGG TAT TGT AAC GTT GTT GAA GA R CAG GTT CTG TAG CTG CAT TAG CA	**FAM**-CAG CAA CAG AGT AAC CGC CAA AAG TTT GA- **BHQ1**
Acinetobacter spp	16s	F TCA GAC CCA CCA TGA CTT TGA C R GGT GGA GAC TAG GAG AGT CGA ACT	**CY5**-TAG AGC GCC TGC TTT GCA CGC A-**DDQII**
Coliform	16s	F GCG GAC GGG TGA GTA ATG TC R GCG ACG TTA TGC GGT ATT AGC	FAM- AAA CTG CCT GAT GGA GG- MGB
Human cells	GAPDH	F CTC CCC ACA CAC ATG CAC TTA R CCT AGT CCC AGG GCT TTG ATT	**VIC**- AAA AGA GCT AGG AGG GAC AGG CAA CTT GGC **TAMRA**

F=Forward R=Reverse

Table 12. Functions of target genes in final PCR assays

Target	*Gene target*	**Gene function**
S. aureus	femB	Codes for fem protein. Involved in meticillin susceptibility, impaired cell separation, formation of peptaglycine interpeptide bridge, cell wall turnover.
MRSA	mecA	Meticillin resistance, encodes penicillin binding protein 2A
E. coli	uidA	Codes for beta-glucuronidase enzyme which cleaves methylumbelliferyl-β-D glucuronide (MUG)
P. aeruginosa	ecfX	Codes for extracytoplasmic function sigma factor. May be involved with virulence and haem uptake[228].
S. pneumoniae	cps	Capsular polysaccharide synthesis
H. influenzae	P6	Codes for outer membrane protein
Acinetobacter spp	16s-23s ITS	Internal transcribed spacer (ITS) between 16s and 23s ribosomal subunits
Coliform	16s	16s ribosomal RNA, a component of the 30s subunit of the prokaryotic ribosome, stabilises protein synthesis
GAPDH	gap	Codes for glyceraldehyde 3-phosphate dehydrogenase, an enzyme which catalyses the 6th step in glycolysis. Constitutively expressed in all human cells.

3.2.3 Real time PCR reaction

Real time PCR assays were carried out in 50µl volumes (as described in 2.8) and contained 25µl Universal PCR master mix (Applied Biosystems, Warrington, UK), primers (final concentration 20µM), probe (final concentration 10µM), 5µl of DNA template and PCR grade water. Thermal cycling and data analysis were conducted on two Taqman 7500 instruments (Applied Biosystems). Thermal cycling conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15s then 60°C for 1 min.

3.2.4 Controls

PCR grade water (Sigma-Aldrich Company Ltd, Gillingham UK) and non-template wells were used as negative controls. Positive controls included a mixture of salmon sperm DNA (0.5mg/ml (Invitrogen, Paisley UK)), all target bacterial DNA plus human DNA containing the gap gene (as described in 2.9). Approximately 20 samples of extracted DNA from each bacterial target were analysed using real time PCR to determine a mean Ct value. The Ct values differed by the organism tested, and accordingly different concentrations of each target bacteria were added to the positive control mix to aim for a final Ct value of 30-35 for each organism included.

3.2.5 Optimisation of primer concentrations for real time PCR

Primer concentrations were optimised by analysing standard concentrations of known bacterial isolates with a checkerboard of primer concentrations as shown in Table 13, according to manufacturer instructions. The combination of concentrations which produced the highest ΔRN value (level of fluorescence) was chosen.

Table 13. Primer concentration checkerboard used for optimisation of real-time PCR assays

		Forward Primer (µM)		
		0.05	0.3	0.9
Reverse Primer (µM)	0.05	0.05/0.05	0.3/0.05	0.9/0.05
	0.3	0.05/0.3	0.3/0.3	0.9/0.3
	0.9	0.05/0.9	0.3/0.9	0.9/0.9

3.2.6 Optimisation of probe concentration for real time PCR

Probe concentrations were optimised by analysing standard concentrations of known bacterial isolates with varying probe concentrations (50nM - 250nM in 50nM increments), according to manufacturer instructions. The concentration which yielded the lowest Ct value was chosen (250nM for all assays).

3.2.7 Multiplexing the PCR assays

Assays were initially multiplexed together in groups of three, with commonly identified pathogens separated to maximise sensitivity. Combinations of single, duplex and triplex assays were tested, in triplicate, against three known target bacterial isolates (singly, in three groups of two and a group of three bacterial isolates). Provided there was no increase in Ct value more than three cycles between each of the single assays and the triplex assay, the multiplex was considered satisfactory. Data from multiplex experiments are shown in Appendix D. The sensitivity of GAPDH and *Acinetobacter* spp assays decreased more than three Ct cycles after multiplexing in a variety of assay combinations, and therefore these were left as single assays.

3.2.8 Automated extraction of total nucleic acids from clinical bacterial isolates for sensitivity and specificity testing

Automated extraction of total nucleic acids from clinical bacterial isolates was performed using a NucliSens® easyMAG™ platform (bioMerieux, France) with off-board lysis, according to manufacturer instructions as described in 2.6. The extracted DNA samples were stored in 96-well plates at -80°C prior to analysis using real-time PCR.

3.3 Results

3.3.1 Inclusivity testing of the real-time PCR assays

Each assay was tested against a bank of clinical bacterial isolates which had been identified in a local microbiology laboratory to species level. Full results are available in Appendix E and a summary of results is included in Table 14. The assays identified the majority of clinical isolates tested.

3.3.2 Specificity testing of multiplexed real-time PCR assays

The multiplexed real-time PCR assays were tested against DNA extracted from a panel of 63 freshly cultured previously identified bacteria, including 43 oral bacterial species and target organisms. Standard reaction conditions were used as described in 3.2.3. The panel of bacteria are listed in Appendix E. All assays had satisfactory control results and identified target organisms correctly. When a threshold of Ct<27 was applied, the coliform assay correctly identified *E. coli*, *K. pneumoniae*, *E. cloacae* and *S. marcescens*. However, positive coliform real-time PCR results were also obtained when testing four of the 63 panel organisms (a haemolytic *Streptococcus* (group B), S. constellatus, Enterococcus faecium and S. epidermidis). The *E. coli* assay cross-reacted with a haemolytic *Streptococcus* (group B), S. constellatus, and E. faecium. The *S. aureus* assay cross-reacted with S. constellatus and a beta-haemolytic *Streptococcus* Group C (1).

Table 14. Inclusivity testing of final multiplex real-time PCR assays against known clinical isolates

Assay	*Number of organisms correctly identified*		*% correctly identified*
S. aureus	15/16		94
MRSA	17/17		100
*E. coli**	26/30		87
P. aeruginosa	25/28		89
S. pneumoniae	14/14		100
H. influenzae	16/17		94
Acinetobacter spp*	14/14		100
Coliform**	8/8	(*E. coli*)	100
	16/17	(*E. cloacae*)	100
	20/23	(*K. pneumoniae*)	87
	13/13	(*S. marcescens*)	100
	4/4	(M. morganii)	100

*Using threshold of Ct<35 **Using threshold of Ct<27

3.3.3 Detection of possible contamination of PCR reagents during testing of patient study samples

A total of 816 samples of DNA extracted from tongue or throat swabs were tested with the multiplex real-time PCR assays described above and in 2.10, and no inhibition occurred. Results from individual patients, converted from Ct

values to presence or absence, are shown in Appendix F. However, during the testing of study samples, it was noted that some negative controls from the *E. coli*, *Acinetobacter* spp and coliform assays yielded positive results.

During testing of the *E. coli* assay, water and non-template controls produced positive results, sometimes consistently within a particular PCR run and sometimes more sporadically, with Ct values >33. Further testing with sterile plastics, an unopened batch of PCR-grade water and preparing fresh primers and probe from unused stock excluded these as the source of contamination. It was noted that PCR runs which took place after a particular date exhibited very few negative controls with positive results, and one batch of universal master mix in particular appeared to almost eradicate the problem. The universal master mix was implicated as the source. Therefore this specific uncontaminated batch was used to retest samples which had been tested with potentially contaminated master mix (approximately half of the samples). Occasional sporadic positive results for water and non-template controls still occurred, albeit with Ct values >39. All Ct values from negative or water controls were >38 and a positive detection threshold of <35 was applied to all results from the *E. coli* assay, to differentiate between negative controls with positive results and positive samples.

Testing of samples with the *Acinetobacter* spp. assay demonstrated that only extraction negative controls produced positive results. The individual swabs and PCR water had both tested individually as negative controls during routine DNA extraction initially, but both had produced positive *Acinetobacter* spp assay results, suggesting that they were not the source of contamination. A previous report suggested that *A. baumannii* contamination of the buffer and line fluids from the easyMAG platform had previously been detected when testing HIV-1 viral load samples [229]. Buffer fluid samples from the easyMAG platform used in extracting study samples were tested using the *Acinetobacter* spp assay after PCR results were obtained (one year after DNA extractions were undertaken), but produced negative results. Buffer fluids had been on the instrument for 48 hours at the time of testing, and this may have been too early

for contamination to have developed. Claasen et al. noted that buffer fluids became contaminated after 1 week on the instrument. Ct values of extraction negative controls were all >36, so a threshold of <35 was used to determine positive results for study samples.

Positive results were obtained for all samples tested with the generic coliform assay, including all controls, with mean Ct values for negative controls of 31.26 (standard deviation 3.20). Retesting of samples using the less contaminated batch of master mix was not undertaken because it was presumed that even a less contaminated batch of master mix would not make sufficient difference to the Ct values on re-testing (given a mean Ct of 31.26) to rely on the results produced. Instead, two products were investigated to determine whether they could eradicate contamination within reagents.

The first was a DNA-free Taq polymerase (Moltaq 16s DNA Polymerase, Molzym, Bremen, Germany) which was tested using conventional PCR. The primers from the generic coliform assay were used to assay nucleic acids extracted from Klebsiella spp. isolates and water controls. The microtube containing master mix was placed in a previously frozen metal block, and DNA polymerase was added last. The PCR assays were carried out in 50µl volumes and contained Moltaq 16s DNA polymerase (2units), primers (final concentration 20µM), 5µl of 10x buffer, dNTPs 0.125mM and 5µl of DNA template. PCR amplification was carried out in a GeneAmp 9700 thermal cycler (Applied Biosystems, Warrington, UK). Thermal cycling conditions were as follows: 94ºC for 15 minutes, 40 cycles of 94ºC for 30s, 55ºC for 30s, 72ºC for 1 min. Then followed one hold at 72ºC for seven minutes and the samples were then held at 4ºC as described in 2.7.1. PCR products were separated using agarose gel electrophoresis, stained using ethidium bromide, and visualised under ultraviolet light as described in 2.7.2. However bands consistent with specific Klebsiella spp product were still detected in the water controls indicating that Klebsiella spp was present within reagents or water. Given the results detailed above, these results were consistent with Taq polymerase being the likeliest source of contamination.

The second approach investigated was the addition of 10 units of exonuclease III (Promega, Southampton, UK) to the real-time PCR reaction. Cherkaoui et al. successfully eradicated bacterial contamination from PCR of 144 clinical samples from normally sterile sites using this method [230]. The enzyme was added to the universal master mix and incubated at 37 ^{o}C for 30 minutes, then 70 ^{o}C for 20 minutes in water baths, before the addition of primers and probe. Thermal cycling took place in the Taqman 7500 instrument with automated analysis of results as described in 3.2.3. However no amplification was detected on the Taqman instrument when DNA from clinical isolates was tested, suggesting inhibition of the PCR reaction had occured, possibly from probe degradation by the exonuclease.

Both approaches to eliminate contamination from real-time PCR reactions were unsuccessful. The range of results from negative controls (lowest Ct value= 26.04) meant that a threshold could not be reliably applied to interpret study results from this assay. Of 167 negative controls, 164 (97%) results showed Ct>30, and the three results <30 had Ct value of 0, 26.04 and 26.5. Therefore a threshold of Ct<27 was used to view initial results from the coliform assay (available in Appendix F), but these data were not analysed further.

3.4 Discussion

Novel real time multiplex PCR assays were designed for five major bacterial respiratory pathogens. Inclusivity and specificity testing validated these assays and no inhibition problems were encountered during subsequent testing on oral samples, suggesting that the assays performed well in this bacterially dense population. Use of the Taqman probe (which binds only to the desired amplicon) rather SYBR-green probes (which bind to any double stranded DNA) led to high specificity.

Assays for genes specific to individual bacteria were designed rather than using universal bacterial primers coupled with specific probes because of the perceived difficulties surrounding 16s PCR, particularly in the oral environment. In actual fact, the 16s assay designed for *Acinetobacter* spp,. in the absence of other potential gene targets, functioned well. This suggests that real

time PCR is sufficiently specific even when testing bacterially rich samples where the target is in low copy number.

The coliform assay demonstrated consistently positive results when testing water and non-template controls at high levels, which precluded its further use. It has been previously documented that bacterial 16s PCR assays yielded positive results from water and non-template controls when identifying ubiquitous environmental organisms, owing to non-sterility of reagents at manufacturing level [230-232]. In addition, *E. coli* is used to produce recombinant Taq polymerase and some components of this organism may remain present in the Taq polymerase [233]. Companies such as Applied Biosystems and others buy Taq polymerase to make Universal master mix from a number of other companies and differences therefore exist between batches. Results from the negative controls during testing with the *E. coli* assay were much improved by the use of a single less contaminated batch of master mix, although sporadic false positive results with Ct values>39 were still observed. DNA free reagents tested in this work were not effective in reducing contamination in water and non-template controls, and use of endonucleases was not compatible with the Taqman platform. Other methods trialled have been use of ultraviolet light [231], DNase I [231], and ethylenediaminetetracetic acid (EDTA), but reductions in PCR sensitivity were observed due to inhibition. One group successfully used DNase I in combination with dithiothreitol (DTT) treatment and heat inactivation prior to PCR to remove residual DNase [234], however we were unable to replicate this. Regardless, these solutions are too time-consuming for routine use in a clinical laboratory. Resolving contamination issues at manufacturer level appears to be the most appropriate solution.

Contamination with *Acinetobacter* spp. is likely to have occurred on the easyMAG extraction platform, despite negative PCR results when testing buffer fluids later. A literature search revealed a previous study which described contamination with *A. baumannii* in Buffer 2 of the easyMAG system, which had been resolved by flushing buffer lines with 70% and storage of buffers at

4°C[229]. Buffer fluids were tested from the easyMAG platform used in the study in duplicate, albeit one year after the problems had occurred due to time lag between DNA extraction and PCR, but all tested negative. Given that only extraction controls were positive, and so consistently throughout testing, it remains likely the easyMag instrument was the source of contamination.

A small number of organisms were not detected by the *E. coli*, coliform and *S. aureus* assays. These included a S. constellatus, haemolytic *Streptococcus* group B and E. faecium (common to both *E. coli* and coliform assays), S. epidermidis (coliform assay only), and S. constellatus and group C *Streptococcus* (*S. aureus* assay only). There are several possible explanations for this. The design of the assays relies on the DNA sequences downloaded to GenBank being sufficiently representative of most strains of the target organisms. However some organisms (eg. S.aureus) exhibit a clonal population structure and therefore some strains may not have been detected, others may demonstrate a non-clonal population structure with frequent recombination of DNA [235], and there may not be adequate descriptions of these phenomena attached to sequence data (or simply inadequate sequence data). A further explanation could be that laboratory identification, although adequate for clinical purposes, may be inaccurate at a molecular level. Given that the cross-reacting organisms were common to both the *E. coli* and coliform assays, either the organisms were misidentified at laboratory level (and further sequencing of these isolates could confirm this) or these organisms possess genetic sequences identical to 16s and uidA genes of *E. coli*. In silico testing (BLAST) of the primers and probe from both assays against GenBank's nucleotide database did identify homology with some Streptococci for one of the primers or probe, but failed to identify streptococcal sequences for both primers and probe, even when the search was narrowed to include testing against only streptococcal sequences. Sequencing the relevant clinical bacterial isolates would reveal whether cross-reactivity occurred due to similarities in genetic sequences.

Assays could not be designed for *S. marcescens* or Enterobacter due to lack of sequence data at the time of design, which accounted for <3.7% and 4.3% of 835

cases of HAP respectively [11]. The pathogens covered in the designed assays still represent the majority of known bacterial aetiological agents, and the overall assay remains viable without the inclusion of these two targets. Now that next generation sequencing is becoming both faster and more affordable, it is likely that the volume of sequence data will rise exponentially in the next few years, and assay design for *S. marcescens* and *E. cloacae* will be achievable. Other important pathogens not sought in this study included Morhaxhella catarrhalis and viruses such as influenza. M. catarrhalis is the third commonest cause of community acquired pneumonia (CAP) [9], and it is likely that this organism would have been present in the mouths of study patients. However, it is not an important cause of HAP, and an assay for this organism was therefore not included. Viruses are also important causes of (1-23% of cases of CAP [236]), and were not sought in this study. Testing for viruses would have increased the cost of the project to an unacceptable level, as multiple viruses would need to be sought, however this could be an area for future work on the duplicate samples taken.

A possible criticism of the overall aims of the assays might be that relevant (and possibly uncultured) pathogens may have been omitted from this assay, given that an aetiology was discovered in <60% cases of pneumonia in older adults in one study [17], and the role of uncultured bacteria in HAP is unclear. A metagenomic approach could have been used instead of PCR to characterise oral samples, circumventing this issue. However at the time of conception of this study, next generation sequencing techniques were insufficiently advanced to allow timely and affordable analysis of the required number of oral samples. However further work is needed to characterise the "normal" flora of the oral cavity and to assess organisms present in bronchoalveolar lavage samples in patients with proven HAP to identify novel pathogens in this context.

3.4.1 Conclusions

Multiplex real-time PCR assays were successfully designed for five major bacterial respiratory pathogens, and were able to identify target bacteria from oral samples. Some reagent contamination issues were resolved by the

substitution of a particular batch of master mix, however designing bacterial 16s assays remains problematic due to contamination of reagents at manufacturing level.

Chapter 4

Dynamics of oral colonisation with respiratory commensal pathogens, oral health status and incidence of hospital acquired pneumonia in older patients with lower limb fracture.

4.1 Introduction

Hospital acquired pneumonia carries a mortality of 12-43% in older people [23, 24, 32, 70], and is one of the commonest complications following lower limb fracture in older adults, with an incidence of 8.6-10% [23, 49, 50]. The majority of lower limb fractures in the older person requiring hospital admission affect the neck of the femur (hip fracture); fractures of the ankle are less common [237]. Currently around 70,000-75,000 people sustain a hip fracture in the UK annually [238], and this figure is likely to rise further with the changing demography of the UK population. The crude one month mortality rate after hip fracture is approximately 10% and the one year mortality rate is around a third of patients, mainly from other illnesses or complications, rather than arising from the fracture itself [51]. Hospital acquired pneumonia (HAP) contributes significantly to the mortality of patients with hip fracture [121, 239, 240], and has been quoted as the second most common cause of death after operative treatment for hip fracture [23]. The prevention of accidental falls in older people is multi-factorial [241], and falls resulting in hip fractures are likely to pose continuing demands on NHS service provision in years to come. Understanding risk factors for HAP may lead to improved strategies for prevention, which may in turn reduce mortality, length of stay in hospital, and may improve functional outcome at discharge in those who would have developed HAP.

The most obvious modifiable risk factors to modulate the risk of HAP appear to be oral colonisation with respiratory commensal pathogens [21, 29, 33] and dental factors [29, 30, 44, 69], and both are incompletely understood as yet. Other risk factors such as dependence on others for feeding or managing oral secretions [21, 28, 30, 68], dysphagia [110], tendency to aspirate [28, 32], age [24,

48, 54, 67, 81, 111] and COPD [29, 30, 53, 61] may be less amenable to intervention. Two successful oral hygiene intervention studies of moderate quality have been conducted with a view to reducing pneumonia in nursing home and long term care facility patients[86, 181]. However, the target of intervention remains unclear, and if robust large-scale interventions studies are to be designed, understanding whether the target of intervention is removal of dental or denture plaque, eliminating colonisation of potential aetiological agents on dental or buccal surfaces, or tackling other issues, such as dry mouth, is crucial. It is assumed that colonisation with organisms associated with HAP begins after hospital admission, but the timing of the start of colonisation, and thus the optimal time to institute oral hygiene prevention measures, is unclear.

This study sought to investigate the occurrence of HAP in a cohort of older patients with lower limb fracture in relation to putative risk factors, including dental factors such as number of teeth, oral dryness, presence of dentures, dental and denture plaque, and also the extent of oral colonisation with the seven most common respiratory pathogens, using purpose designed multiplex real time PCR assays. The dynamics of oral colonisation with respiratory commensal pathogens were investigated. It was hypothesised that colonisation events would be associated with length of stay (i.e. an epidemiological explanation). "Community" pathogens such as *S. pneumoniae*, *H. influenzae* and *S. aureus* were expected to decline over time in hospital, and "hospital" pathogens (the remainder) were expected to increase.

The demographics, dentition, oral microbiology and outcomes of the study cohort, will be described in Chapter 4 and the relationships between HAP and the above variables will be investigated in Chapter 5.

4.2 Methods

4.2.1 Patient recruitment and consent

Patients were recruited from two orthopaedic admissions wards at Newcastle General Hospital between April 2009 and July 2010. All patients with lower limb fracture admitted to the Newcastle Hospitals were discussed at the next

daily trauma meeting at 0800, and potential participants were identified here. Recruitment occurred preoperatively where possible, but patients were usually operated the day after admission, and where preoperative recruitment was not possible, patients were recruited on the following day. Details of participation in the study were explained verbally, and potential participants were also given a leaflet to read. Written consent was obtained from patients who had the capacity to make the decision. Every effort was made to allow the patient to make their own decision including repetition of information, interviews at different times of day, and use of spectacles and hearing aids where needed. When patient consent was not possible, relatives were invited to act as personal consultees on behalf of the patient. A professional consultee was sought for patients who could not consent who had no relatives. Patient follow up was undertaken on destination wards provided they were within the Newcastle Upon Tyne Hospitals Trust.

Inclusion criteria included age > 65 and lower limb fracture. Exclusion criteria included recent immunosupression from chemotherapy or other immunosuppressive drugs (including any prescription of >=10mg prednisolone), acute illness, palliative care and community acquired pneumonia.

4.2.2 Recording of demographic data

Demographic data were recorded from the hospital notes by the study investigator (VE) or the study nurses (JB, SE) on paper study files. Active study files were stored with the patient notes on the ward, and other study files were kept in a locked filing cabinet at the Health Protection Agency Newcastle. Data were later entered into a study database, (Microsoft Access) which was held on the Newcastle upon Tyne Hospitals (NUTH) server to store patient data. Caldicott approval was granted.

Age, gender, weight, place of residence prior to admission, comorbidities, prescribed drugs at admission, operative and anaesthetic details, MRSA admission swab results, and smoking status were noted from the admission documentation. Operative details recorded included time of operation, type of

operation performed, length of operation as defined by start and end of anaesthetic recorded on anaesthetic chart and peri-operative antibiotics. All patients (apart from two who were treated without operation) received peri-operative antibiotics (three doses of Cefuroxime 750mg 12 hourly until August 2009, three doses of Teicoplanin 400mg 12 hourly thereafter). Therefore approximately 20 patients received the former and 73 the latter regimens. All patients received low molecular weight heparin in the form of 4500 units tinzaparin subcutaneously, unless already anticoagulated on warfarin. The postoperative analgesia used routinely was co-codamol 30/500mg four times daily and any increase from this was noted. When no weight had been recorded, patients were asked for their last known weight, or in 6 cases a last known weight was noted from the General Practitioner records. In addition, presence of associated visual impairment (glasses) and hearing impairment (hearing aid) were recorded. Functional scores including the Barthel index and the Clinical Frailty scale were calculated. The Hierarchical Assessment of Balance and Mobility (HABAM) score was also calculated at the time of consent. A Charlson comorbidity index was calculated from the above information using an online calculator found at www.medal.org.

4.2.3 Study variables

Ordinal and categorical variables used in the study and their definitions are described in Table 15. Three functional and complementary variables were chosen for use in the study. The Barthel index (scored out of 20) is recorded routinely in older patients in hospital in the UK, and is included in the admission proforma for patients with hip fracture in Newcastle upon Tyne Hospitals. However this score only decreases once patients are unable to independently attend to an activity of daily living, e.g. toileting or grooming. It does not account for earlier signs of frailty, such as fatigue, or needing help with higher order tasks such as shopping or dealing with money. The clinical frailty scale was developed to allow the clinician to quickly and easily distinguish the fittest from the frailest patients using nine categories, and has been validated on older Canadians to predict death and move to institutional care [242]. This scale has three categories for fitter patients who would not yet

be classified as frail, and one category for borderline frailty. While exercise tolerance is mentioned within the categories of the clinical frailty scale, it is not explicitly measured, and the independent patient who is able to walk 100 yards only would not necessarily be distinguished from the independent patient who could walk a mile. Therefore a mobility scale was also used in the study, and was developed by the same group as the clinical frailty scale [243]. This score was highly positively correlated with the clinical frailty score in a convenience sample of geriatric in and out patients, and had good test-retest and inter-rater reliability.

The Charlson comorbidity score is an age-weighted score of serious comorbidities, and its outcome predicts likelihood of ten-year survival. Some advocate simply counting number of comorbidities, including visual and hearing impairment [244]. However, the Charlson comorbidity index has been widely used and validated in a number of populations, and it was included for this reason.

Table 15. Ordinal and categorical variables used in the study and their definitions

Variable	*Explanation*	*Orientation*
Residence	3 categories according to site of residence pre admission,	1=own home 2=institution 3=hospital
Barthel score	Score 1-20 based on dependence on others for activities of daily living, e.g. toileting, stairs	High score means more independent.
Clinical frailty scale	Score 1-9 based on frailty category	High score means more frail
HABAM	Mobility score based on ability to standing balance, transfers between bed and chair and exercise tolerance /63	High score means more mobile
Consent	Two categories, able to consent for self (includes mild cognitive impairment) and unable to consent for self	1= self consent 2=help for consent
Charlson comorbidity index	An age weighted score of severity of significant illnesses; relates to 10 year survival	High score means low or zero probability of survival at 10 years
Smoking status	3 groups	1=current smoker 2= ex-smoker 3=never smoker

4.2.4 Recording of oral hygiene variables

The PI was trained in dental examination and plaque scoring at the Dental Hospital, Newcastle University, and attended two dental clinics in advance of the start of the study. The PI had undertaken dental plaque scoring in a previous study [245]. The number and position of teeth or teeth on dentures was recorded for each patient. Both dental and denture plaque (full mouth) were scored by a single investigator (VE) using the modified Quigley Hein index [246, 247], described below in Figure 1. This score was used because it was a visual score (and therefore did not necessitate the removal of plaque) and because of its ability to discriminate between larger and smaller plaque loads. Other possible plaque scoring systems required the use of a dental probe or did not distinguish between heavier loads of plaque (e.g. Silness and Loe or a dichotomous scoring system).

Plaque was scored on days 1, 7 and 14 at the bedside, with the aid of a head-torch and dental mirror. Dentures were removed for scoring, and were not cleaned after scoring. Only dentures that were being worn were scored. At the start of the study, it was decided that plaque or debris between but not on denture "teeth" was given a score of 3. In order to create a single plaque score per patient, a quartile score was assigned to each modified Quigley Hein index. Where scores were discordant in dentate denture wearers, the higher of the two quartiles was used in analysis. Midway through the study, intra-rater (VE) calibration using 138 surfaces gave kappa scores of 80.9% (good). On day 14 of admission, a xerostomia inventory questionnaire was undertaken [248]. This was a subjective measure of dry mouth using an 11 point questionnaire with responses increasing in frequency between "never" and "very often". Patients were questioned on their sensation of dry mouth for the preceding two weeks only. No objective measures of xerostomia were taken.

There was no specific oral hygiene policy on the orthopaedic admissions ward at the time of the study. Dentures were placed in denture pots overnight, where available. Those with teeth attended to their own oral hygiene.

Figure 1. Diagram of modified Quigley Hein scoring system

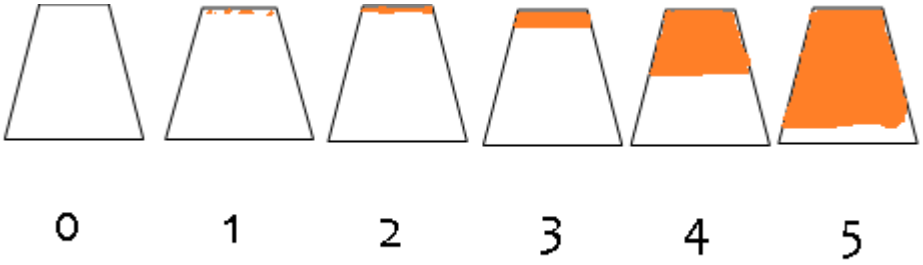


Table 16. Modified Quigley-Hein scoring system

Score	*Description*
0	No visible plaque
1	Plaque visible but not continuous around tooth border
2	Plaque continuous around tooth but less that 1mm in height
3	Plaque continuous around tooth and over 1mm but less than one third of tooth
4	Plaque covers between one and two thirds of tooth surface
5	Plaque covers more than two thirds of tooth surface

4.2.5 Collection of oral samples

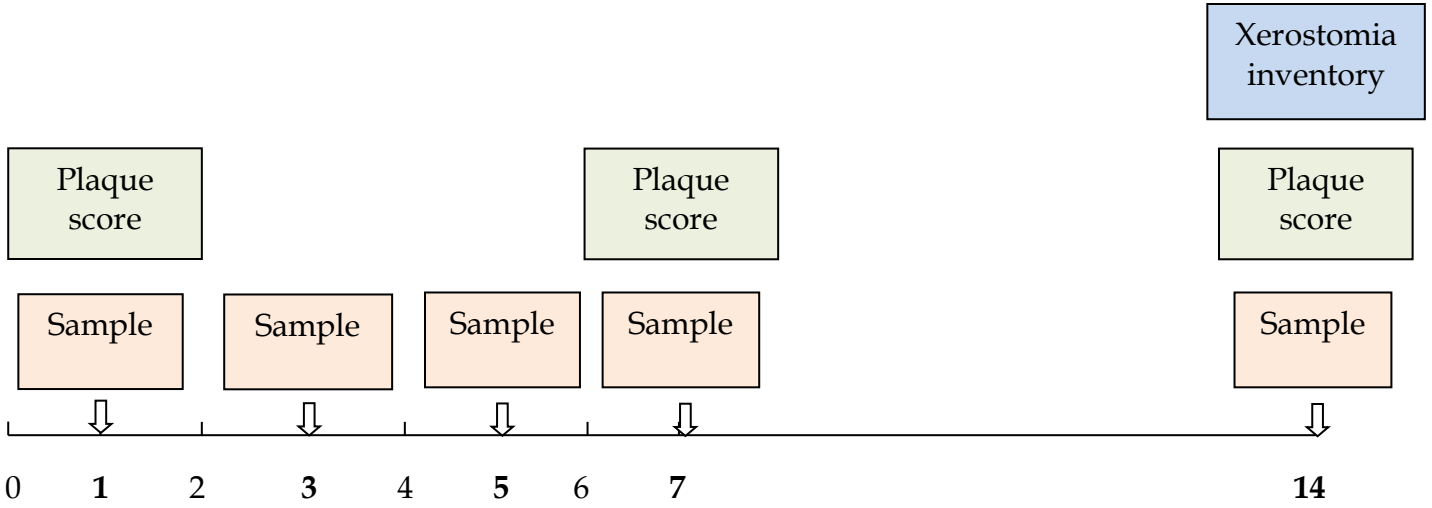
Dry flocked swabs (suitable for use with PCR assays [249, 250]) were used to sample the tongue and throat in duplicate at time points described in Figure 2. It was hypothesised that most changes in the structure of the oral bacterial community would occur within the first 5 days of admission because similar changes had been observed in patients after admission to intensive care [42]. In addition, time to HAP was quoted as up to 9 days after surgery, and 61% of pneumonias occurred within 5 days of operation [26]. Therefore, the sampling time-points were chosen to try and observe these changes over the first few days of admission.

The combination of tongue and throat samples was previously shown to be the minimally inclusive combination achievable in those with cognitive impairment in detecting coliforms, Pseudomonads and *S. aureus* in orthopaedic patients [245]. The day of admission was considered to be day 0. Where possible the first set of samples was taken pre-operatively (usually day 1), otherwise samples were taken the following morning. Where swab collection fell on weekend days, the sample was taken on the nearest working day. All samples were taken between 8.30am and 12 pm, or 1-4pm. No special instructions were given to patients regarding food or drink prior to sample collection. If patients were discharged before 14 days no further samples were taken.

Two throat swabs were taken from right and left anterior faucial pillars, using a back-and-forth motion three times. Two tongue swabs were also taken by rubbing three strokes posterior-anterior, then rotating the swab 180^{o} and making a further three strokes in the same way. Swab tips were transferred into 2ml microtubes which were labelled with the patient's hospital number, date, swab and day number, and the type of swab. The two swabs from each site were labelled "A" or "B" at random. "A" swabs were used for PCR analysis and "B" swabs were subjected to DNA extraction and stored at -80^{o}C as a duplicate.

Samples were transported to the Health Protection Agency within four hours of collection, and stored in a single plastic specimen bag at 2-8ºC until DNA extraction within 48 hours. Samples were anonymised by allocation of a laboratory number at DNA extraction, and stored at -80°C at Health Protection Agency Newcastle. After patient follow- up was complete, extracted DNA samples were analysed using the multiplex real-time PCR assays (described in 2.8 and 2.10) between September 2010 and December 2011 by a single clinical scientist (GE).

Figure 2. Sampling and observation schedule for study



4.2.6 Study patient follow up

After the initial sampling period, study participants were visited three times per week (Monday, Wednesday, Friday) until discharge to detect complications and to ensure complete ascertainment of cases of HAP. During the follow up visit, participants were asked an open question about general health, asked about presence of cough, and last recorded observations (blood pressure, pulse, temperature, oxygen saturation and respiratory rate) were noted. When asking about cough, patients were asked to report a new cough or a change to an existing cough. Glasgow Coma Score, an AVPU score (alert, alert to voice only, alert to pain, unresponsive) and any delirium were also noted. Delirium was diagnosed clinically by VE, based on comparison with previous visits or conversations with relatives or nursing staff. Complications such as new drugs, deep vein thrombosis, or acute illness were recorded on a weekly basis using case notes review.

HAP was diagnosed when antibiotics were started for pneumonia by the responsible clinician after 48 hours in hospital. Episodes of lower respiratory tract infection (i.e. symptoms and/or signs of respiratory tract infection without visible changes on chest radiograph) were also noted. The most abnormal observations and blood tests in the 48 hours prior to diagnosis were recorded. The diagnosis of HAP was further characterised as "probable" or "possible" case using American Thoracic Society and British Society for Antimicrobial Chemotherapy (Table 17 and Table 18).

After discharge, two sets of follow up phone calls were made at one and three months, firstly to the GP and secondly to the patient themselves or their care home to detect later cases of healthcare associated respiratory tract infection. Any prescriptions of antibiotics and their underlying indication were noted.

Table 17. Diagnostic criteria for hospital acquired pneumonia used in this study

British Society for Antimicrobial Chemotherapy guidelines [10]	*American Thoracic Society Guidelines[19]*
New infiltrates on chest radiograph Plus two of: Purulent respiratory secretions Temperature >38.3^{o}C White cell count>10 or <4 Increased oxygen requirement	New infiltrates on chest radiograph Plus two of: Purulent respiratory secretions Temperature >38^{o}C Leukocytosis or leucopoenia

Table 18. Criteria to distinguish probable and possible cases of hospital acquired pneumonia

Criteria	*Diagnosis*
Consolidation on chest radiograph* plus 2 or more minor criteria	Probable HAP
Consolidation on chest radiograph* plus 1 minor criterion	Possible HAP
Pneumonia as 1A on death certificate	Possible HAP

*as per report by radiologist

4.2.7 Ethic al considerations

Ethical approval was granted by the Newcastle and North Tyneside 2 research ethics committee. The Newcastle Upon Tyne Hospitals trust agreed to act as sponsor for the research and Research and Development approval was granted. Minor amendments were granted in 2009 in order to allow five swab samples rather than three, to sample tongue and throat rather than just throat, and to use two swabs at each time point. A second minor amendment was approved later in 2009, after the study had started to allow recruitment of other fracture types to the lower limb in order to boost recruitment numbers.

4.2.8 Statistical analyses

Microsoft Excel (versions 2007-2010) was used to store and manipulate anonymised data prior to analysis. All analysis was undertaken in R (R: A language and environment for Statistical computing, Vienna, Austria). Generalised linear models with a binomial error structure were used to analyse some variables. For repeated measures, generalised linear modelling with penalised quasi likelihood was used.

4.2.9 Funding

This project was funded by the Medical Research Council as part of a Clinical Research Training Fellowship in June 2008.

4.3 Results

4.3.1 Recruitment of study cohort

The final study cohort consisted of 93 patients recruited between April 2009 and July 2010 (consort diagram shown in Figure 3). Recruitment was difficult for many reasons. Consent needed to be coordinated around anaesthetist, surgeon and nursing visits, and obviously the operation itself. Patients were frequently in pain or scared and some were very tired. Many refused immediately, stating that they didn't wish to undertaken any extra unnecessary burdens at such a difficult time. Others accepted initially, but then declined when they realised they needed to sign a form. Interestingly, it was relatively easy to recruit those

with cognitive impairment because their relatives could appreciate the non-intrusive nature of the research and readily agreed for their relative to take part.

Reasons for exclusion and withdrawal are summarised in Table 19. More females declined to participate than males (54% versus 42%). The cohort includes three patients who withdrew but consented to have their data used, but these patients were excluded for analyses relating to HAP. Two patients recruited did not have an operation and one patient had two admissions separated by three days for the same fracture which had become unstable on discharge. Demographic data was taken from the first admission in this case. One patient could not be contacted during the post-discharge period for follow up, but his General practitioner was contacted.

Recruitment varied by month and between the same month in different years (Figure 4) and was proportional to the numbers of patients screened for each month.

Figure 3. Consort diagram showing fates of screened patients

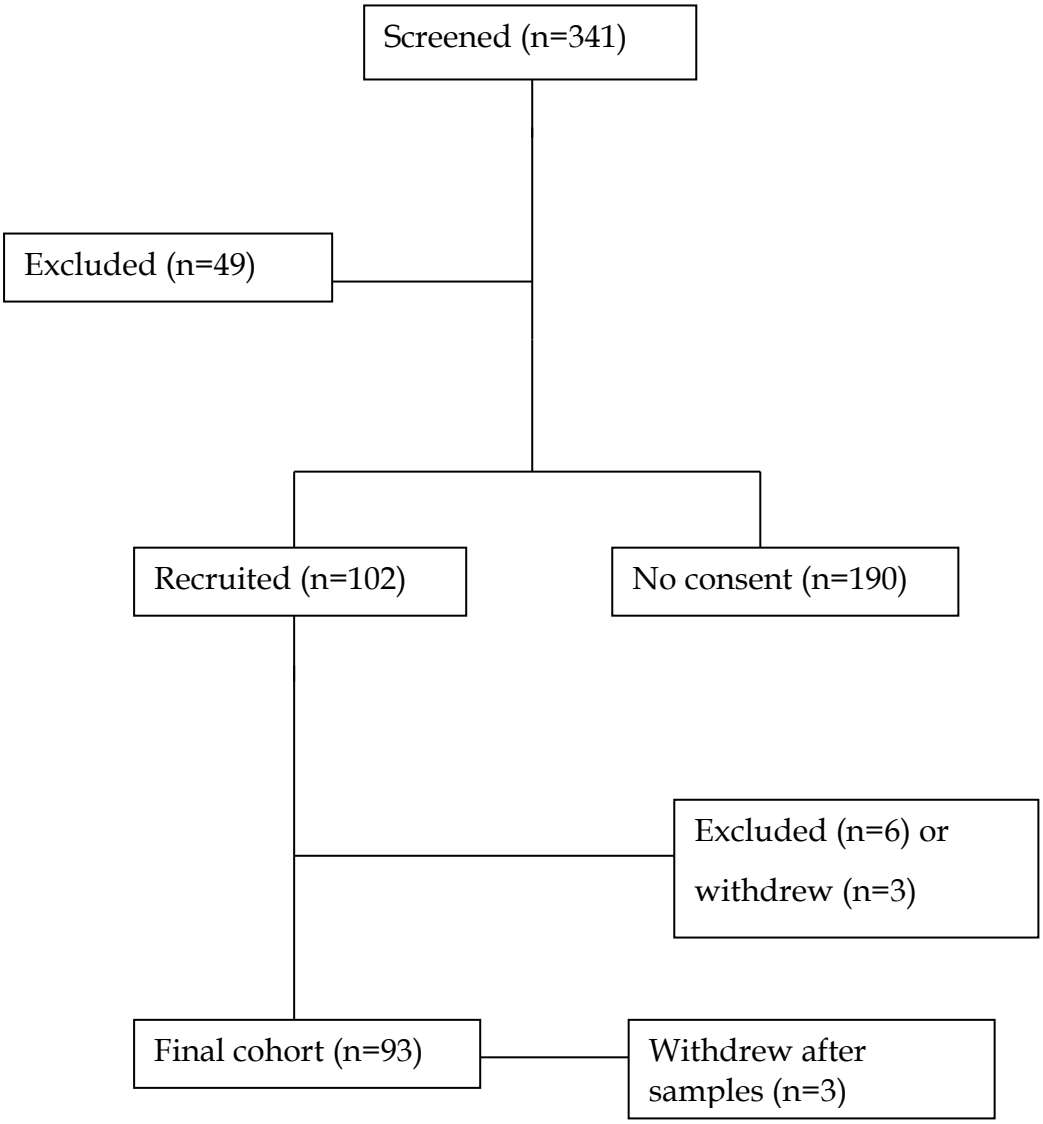
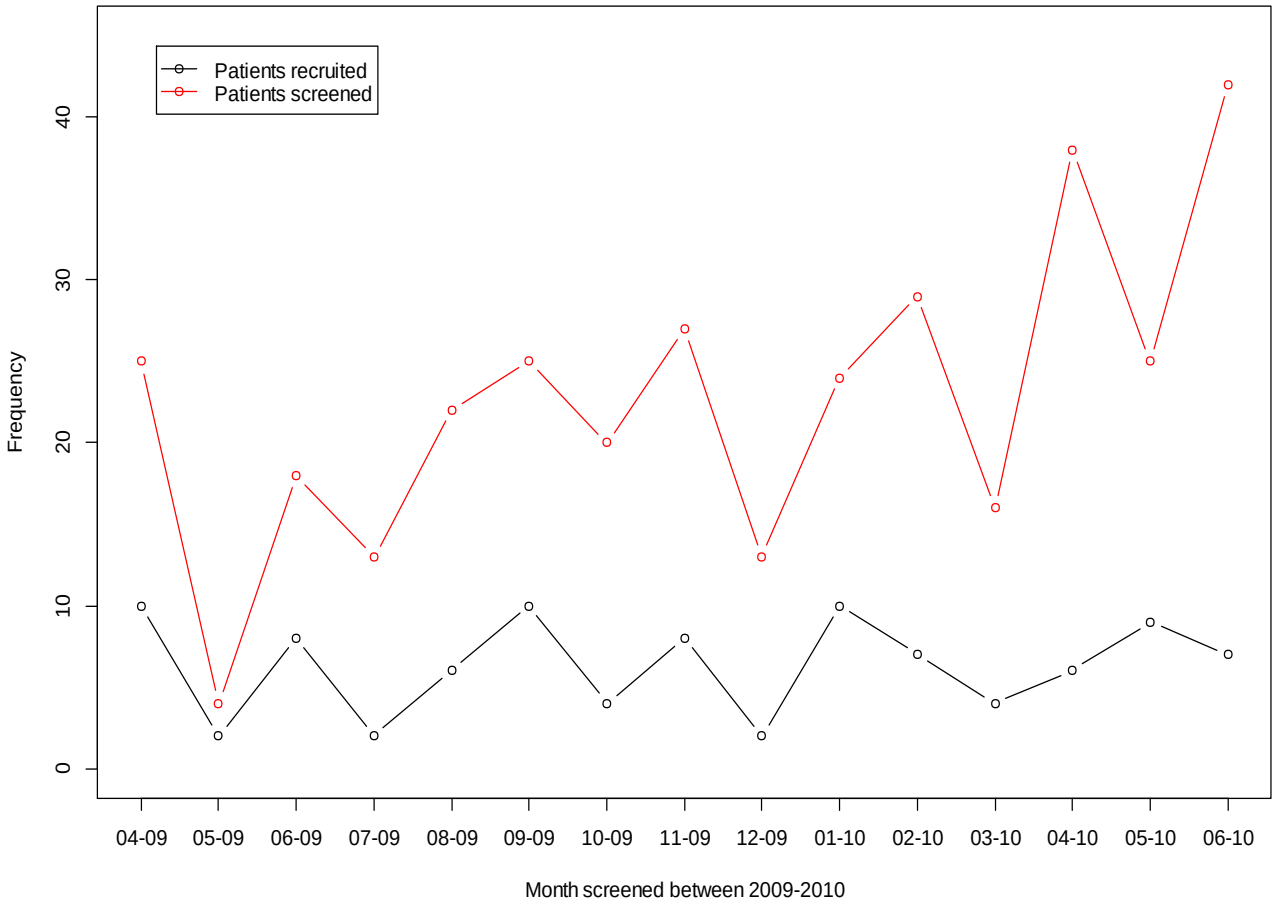


Table 19. Reasons for exclusion, withdrawal and refusal to participate in the study

Non-recruitment category	*Reasons for non-recruitment*	
Excluded at screening	Community acquired pneumonia	(n=16)
	Miscellaneous	(n=14)
	Unwell, transferred to other hospital	(n=13)
	Taking oral steroids	(n=6)
No consent obtained	No reason stated	(n=83)
	Logistical problems	(n=52)
	Moribund or aggressive	(n=35)
	Too tired or in too much pain	(n=20)
Withdrew or excluded after initial recruitment	Community acquired pneumonia	(n=4)
	Patient wish	(n=3)
	Transfer to other hospital	(n=2)

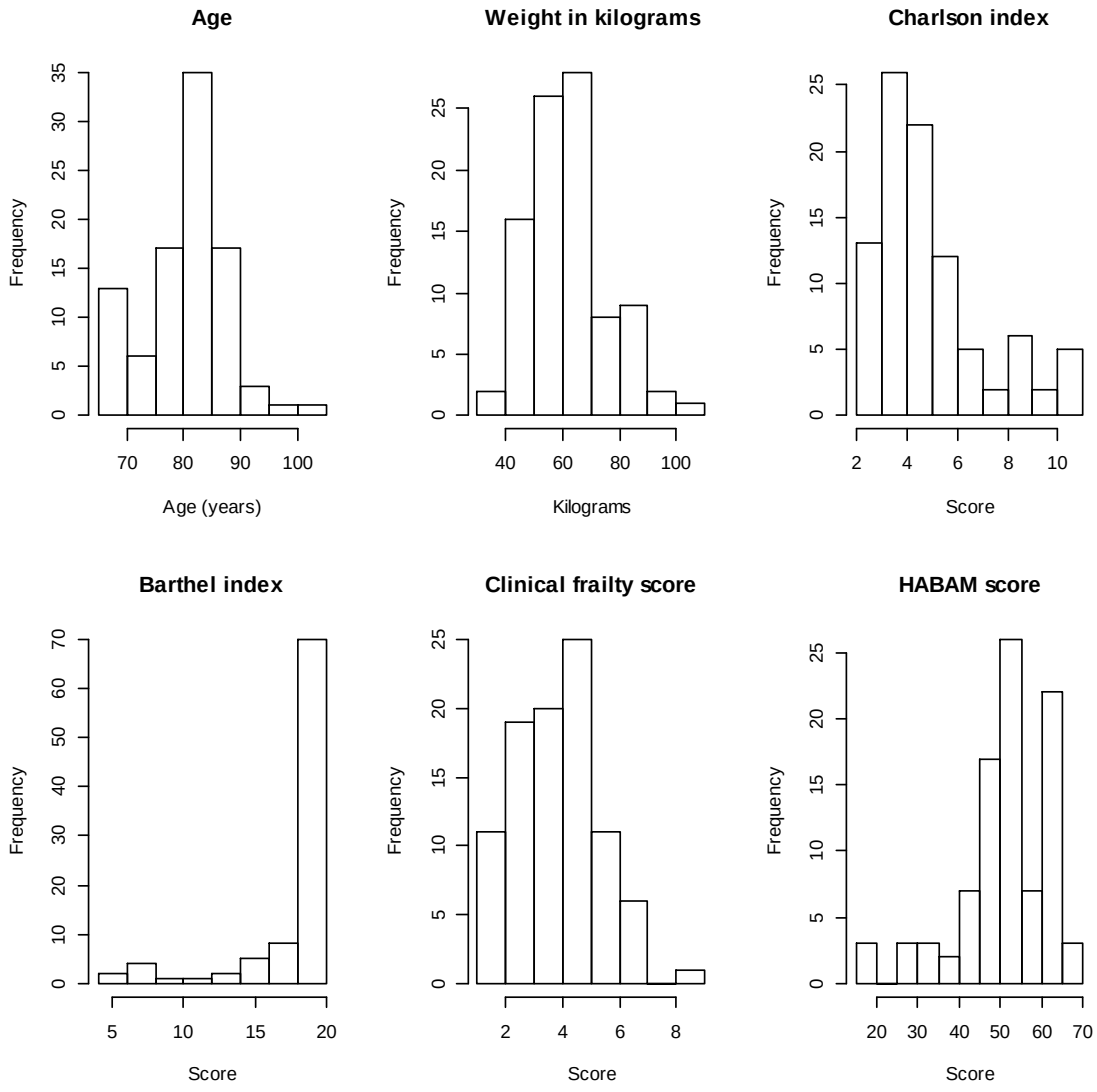
Figure 4. Numbers of patients screened and recruited by month during study period



4.3.2 Demography of the study cohort

Of the final cohort of 93 study patients, 29 (31%) were male and 64 (69%) were female. The majority of patients were admitted from their own homes (78/93, 84%), with 11 (12%) residing in nursing or residential care prior to admission, and four (4%) patients being transferred from other hospitals after a fall and subsequent fracture. Distributions of other demographic data are shown graphically in Figure 5.

Figure 5. Distributions of demographic and functional data from study patients.



Note: One patient had no weight recorded

.

Figure 5 describes a cohort with a mean age of 81, who were functionally independent (median Barthel score 20) but beginning to feel fatigued (median clinical frailty score of 4) and who were mobile but with some limit to their exercise tolerance (median HABAM score of 53). The Charlson index ranged from 2-11, with a median index of 5 (a score of 5 translates to a 21% risk of death at 10 years). The median weight was 63 kilograms (kg) with a range from 36 to 102 kg.

Visual impairment was common (75/93, 81%), and hearing impairment was less common (27/93, 29%). Patients were prescribed a range of 0-19 drugs at admission, with a median of 6 medications. Drugs that have been previously associated with pneumonia were prescribed frequently to the study patients and are summarised in Table 82, Appendix G. In terms of number of comorbidities at admission, the median number of comorbidities was 6, with a range from 0-15. In total, 17 (18%) patients had chronic obstructive airways disease (COPD). The majority of patients (n=76, 82%) were non-smokers at the time of the study which included 47 (51%) ex smokers and 31 (31%) never smokers.

When patients were divided into two groups clinical frailty score (1-4=fit, 5-9=frail), frailer patients were older ($p=0.005$), had a higher Charlson comorbidity index ($p=0.003$), were prescribed more drugs at admission ($p<0.001$) and were less mobile ($p<0.001$). There was slight evidence that frailer patients had fewer teeth ($p=0.058$).

4.3.3 Operative and anaesthetic data

The majority of patients (86/93) had sustained fractures to the neck of femur, of which 43/87 were intracapsular, and 43/86 were intertrochanteric. The remaining six patients had fractured the femoral shaft (n=2) or an ankle (n=5). The operations performed for each type of fracture are shown in Table 20. One patient was discharged for three days but readmitted for operation when conservative treatment failed, and this was treated as one admission as described above. One patient was operated upon but the fracture was found to

be old and healed and therefore was closed without procedure, and two other patients were managed without operation due to alignment of fracture.

In terms of anaesthetic type, 53% of patients (n=49) received a general anaesthetic with laryngeal mask airway and femoral nerve block, and 29 (31%) received a general anaesthetic, endotracheal tube and femoral nerve block. Five patients (5%) received general anaesthetic and endotracheal intubation only, and seven (8%) received spinal anaesthesia. The type of anaesthetic was unknown for one patient.

Table 20. Type of fracture and operations performed on study patients (n=90)

Fracture type	Number of patients	Operation	Number of patients (n=91)*
Intracapsular neck of femur	43	Hemiarthoplasty	37
		AO** screws	4
		Exeter bipolar hemiarthoplasty	2
Intertrochanteric fracture of neck of femur	42	Dynamic hip screw	34
		Intramedullary hip screw	8
Femoral shaft fracture	2	Open reduction and internal fixation of femur	2
Ankle fracture	5	Open reduction and internal fixation of ankle	5

*2 patients had stable ankle/femoral fractures which were subsequently not operated upon and 1 patient was operated upon but found to have a healed hip fracture, and no treatment was undertaken.

** AO = Algemeinshaft fur osteosynthesefragen

4.3.4 Findings from oral health examinations

Fifty patients possessed teeth, with a range of 0-28, median of 5, and lower and upper quartiles of 0 and 20 respectively. Of these, 24 also wore removable dentures. Forty-three patients had no teeth and all but one wore removable dentures. In total, 66 patients wore removable dentures. The numbers of plaque scores recorded by dental status are shown in Table 21.

No statistically significant variation was seen between dental nor denture plaques score over the three examinations (Figure 6), nor between fitter and frailer patients over time. Dental findings are summarised in Table 83, Appendix G.

Table 21. Frequency of plaque scores undertaken per examination and dental status

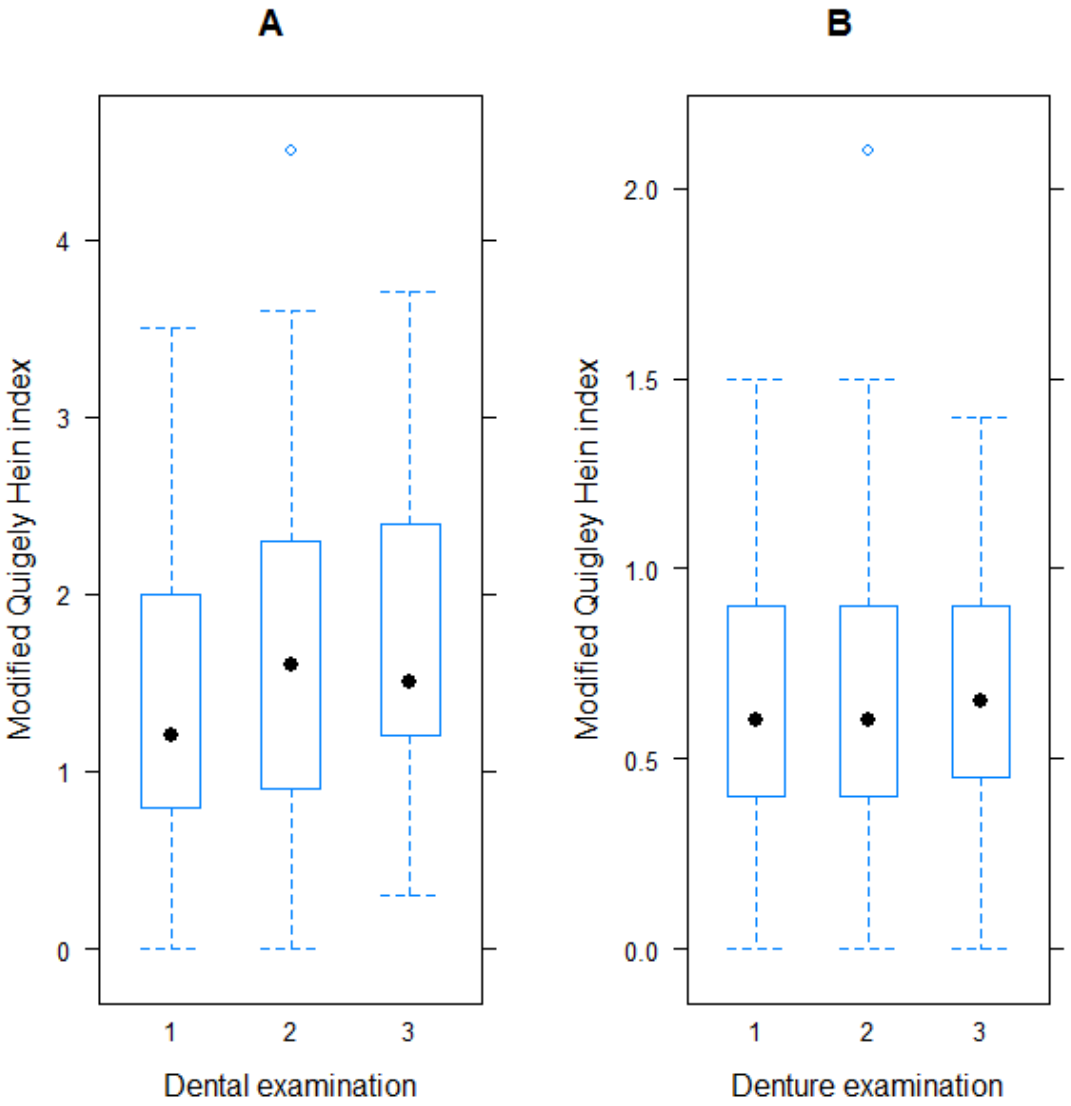
Examination day	*Total (n=92*)*	*Dentate (n=50)*	*Denture wearing (n=67)*
1	92	49	60
7	78	44	54
14	61	33	44

*One patient had neither teeth nor dentures

The distribution of xerostomia inventory (XI) scores (n=58) taken on day 14 ranged from 0-46, with median 16.5, and lower and upper quartiles 10 and 26 respectively. The data were non-parametric, even after logarithmic transformation. Therefore a binomial value based on low XI score (0-10) and high score (>10) was calculated. A score of 0-10 was chosen to represent those who either had responded "very often" to only one or two questions or who responded less strongly to several questions (or indeed those with a score of 0, n=3). High XI score (n=40) was not significantly associated with age, clinical frailty scale, Charlson comorbidity index, number of drugs prescribed at admission, nor prescription of cognitively active drugs at admission. There was

a slight association between high XI score and number of comorbidities (p=0.055, Fisher's exact test).

Figure 6. Plots of modified Quigley Hein indices from dental and denture examinations of study patients on day 1, day 7 and day 14 (A= Dental examinations only, B=Denture examinations only)



4.3.5 Results from real-time PCR assays of oral samples

A total of 816 samples of extracted nucleic acid (408=tongue swabs, 408=throat swabs) from 93 patients were successfully tested using the novel multiplex real-time PCR assays. Human DNA was found in all samples, as evidenced by positive GAPDH assay results. Thresholds for positive identification of target pathogens are described in 3.4.3. Distributions of Ct values for positive results from PCR assays are shown in Figure 1, Appendix G. Ct values from the GAPDH assay were normally distributed, but the distributions of Ct values from the other assays were negatively skewed and zero-inflated.

Frequencies of patients with different numbers of time points sampled are shown in Table 22. Two sets of samples were taken late due to an oversight in recording when follow-up should occur for each patient. Eleven patients missed one sample for logistical reasons or memory error; seven missed sample two, two missed sample three and two missed sample four. The first sample was taken preoperatively in 50 patients and post-operatively in 43 patients.

Table 22. Frequencies of patients with different numbers of time points sampled

Number of time points sampled	*Frequency (n=93)*
5	55
4	24
3	5
2	4
1	2

Mean CT values from the GAPDH assay were compared over the 5 sampling points using generalized linear modeling with Penalised Quasi-Likelihood (

Table 23), and no significant difference was found. This suggests differences seen in target organism assays over time were likely to be real.

Table 23. Mixed effect model fitted using penalized quasi-likelihood relating CT values for the GAPDH assay over time in hospital

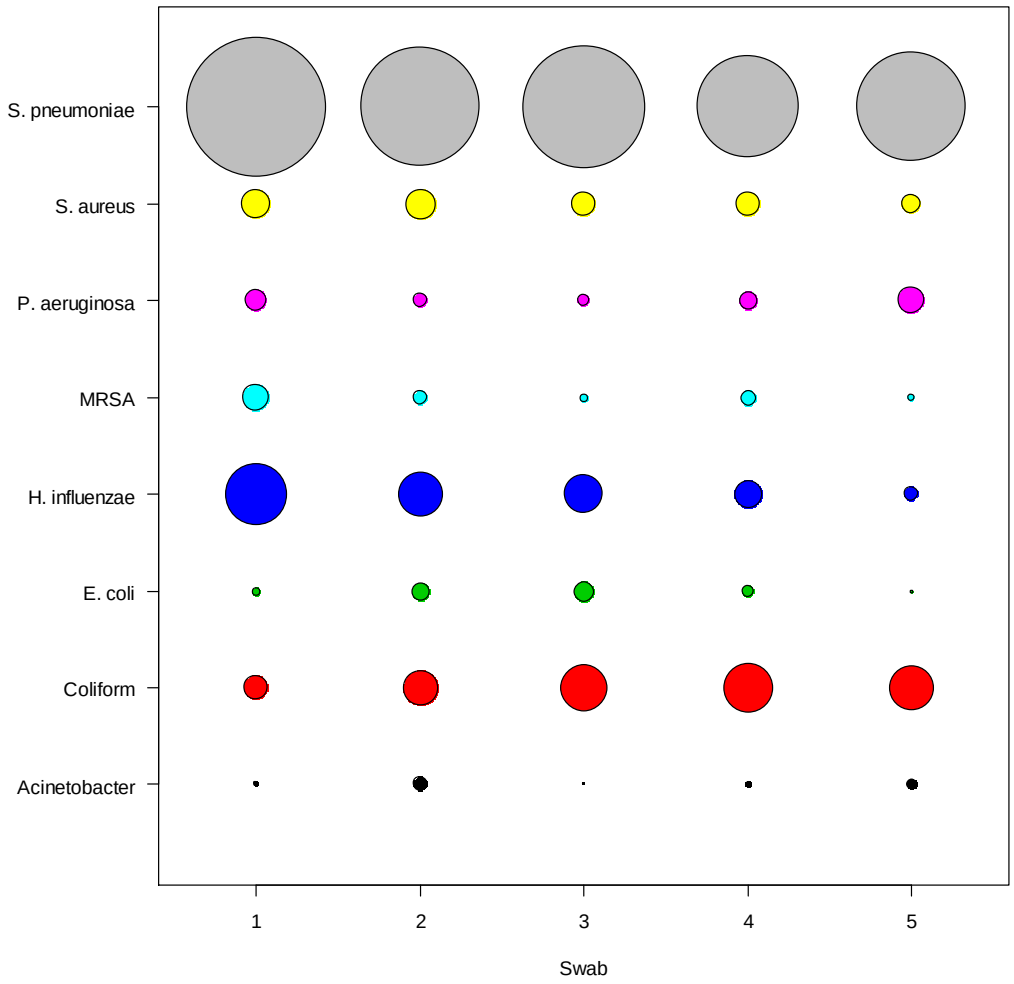
Target	*Value*	*Standard error*	*DF*	*T value*	*P value*
GAPDH	-0.023	0.024	722	-0.951	0.342

(Intercept) Residual, StdDev: 2.257 2.815

Individual patient results are shown in Appendix F. Twenty patients had all negative swabs. Of the remaining 73, 17 had transient acquisition of a single pathogen on one day only, and the other 56 had single (n=26) or mixed pathogen colonisation (n=30). Of single pathogen colonisation, *S. pneumoniae* was most common (n=20), followed by *P. aeruginosa* (n=6), *H. influenzae* (n=4) and *E. coli*/*Acinetobacter* spp (both n=2). *S. aureus* colonisation alone was seen in one patient. Certain combinations of pathogens were seen more frequently. Disregarding the persistence of the organisms, the commonest combinations of organisms were permutations containing *S. pneumoniae* and *H. influenzae* (n=11), *S. aureus* and MRSA (n=10), *E. coli* and *H. influenzae* (n=5), and *S. pneumoniae* and *P. aeruginosa* (n=5).

To examine the effect of time in hospital on prevalence of target pathogens, abundance of each of the target organisms at the five sampling time points was plotted in Figure 7 (raw numbers are shown in Table 84, Appendix G). *S. pneumoniae* was most abundant throughout the sampling period, and appeared to decline over time. *H. influenzae* was next common and also appeared to decline significantly over time in hospital. MRSA, *S. aureus*, *P. aeruginosa*, *E. coli* and *Acinetobacter* spp were comparatively rare. Fewer than 5% of samples overall were positive for "hospital" pathogens (MRSA, *E. coli*, *P. aeruginosa* and *Acinetobacter* spp.), and coliforms were seen in around 8% of samples. The results from the coliform assay are included here for interest but will not be shown in later analyses.

Figure 7. Abundance of target organisms in all samples over time



Note: The number of patients with samples positive for each organism at each sampling point is represented by the area of the shaded circles. Several organisms may arise from a single patient sample. *S. pneumoniae, H. influenzae* and coliforms were detected most commonly in all patients.

However this plot does not take the persistence of colonisation of the organisms into account. For example, patients with a positive *E. coli* result on sample 1 or sample 2 may both have positive results on sample 3. More important in terms of clinical relevance is when organisms were first seen. Results from PCR assays from individual patients (Appendix F) were used to define when acquisition and colonisation first occurred. Acquisition was defined to have occurred if samples were positive at one or more time points (either tongue, or throat or both samples positive). Colonisation was defined as samples positive at two or more time points, which need not have been consecutive (tongue or

throat or both samples positive). Figure 8 shows the first day of acquisition or colonisation of target organisms, and Figure 9 shows the first day of colonisation only. Acquisition was more common than colonisation in all organisms apart from *S. pneumoniae*. Colonisation was rare in *E. coli* (2%), MRSA (2%), *P. aeruginosa* (4%), *Acinetobacter* spp. (2%), moderately common in *S. aureus* (8%) and *H. influenzae* (9%), and common in the case of *S. pneumoniae* (29%). The majority of both acquisition and colonisation began early in admission (day 0-3). When considering colonisation only (Figure 9), it is even clearer that the first day of colonisation was within the first three days of admission for the majority of organisms. Only MRSA and *P. aeruginosa* began to colonise at day 7, albeit rarely. It is unclear from these analyses whether colonisation occurred as a result of events surrounding admission or whether these results reflected earlier colonisation in the community. There were no marked differences between the incidence of positive results between patients whose first sample had been taken pre- versus post-operatively.

Figure 8. First day patients acquired each of the seven target organisms (area of circles represents number of patients who first acquired each organism by swab number taken)

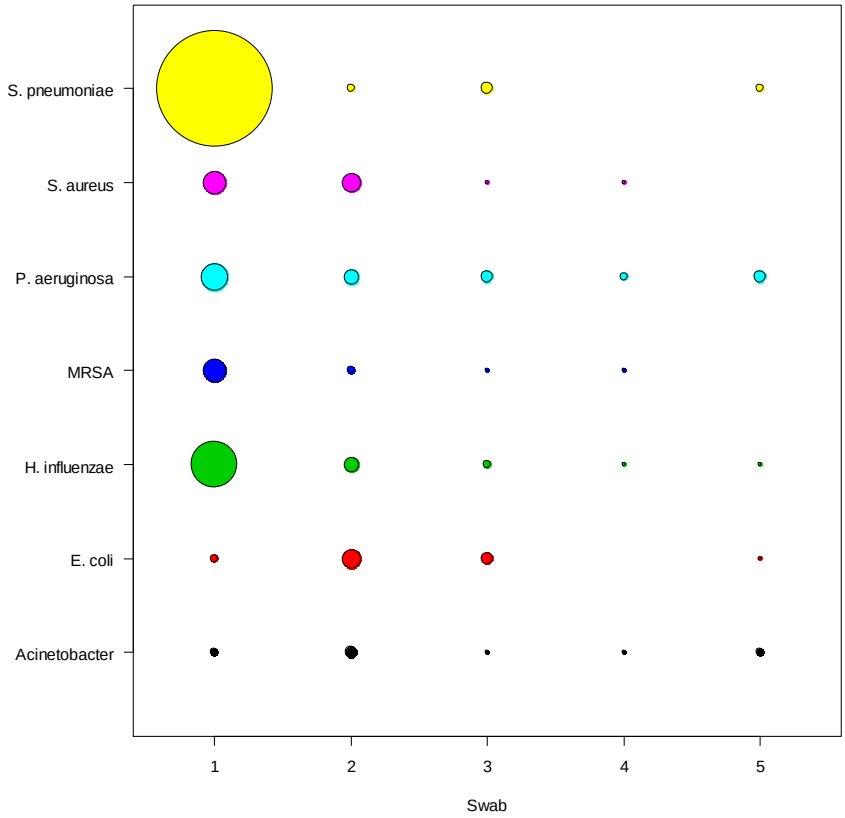
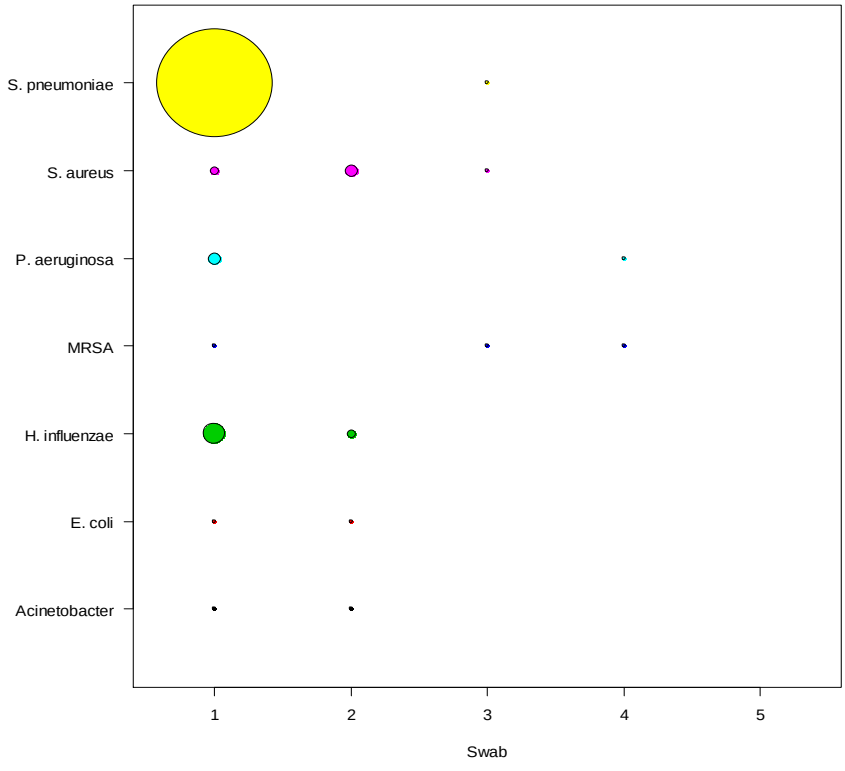


Figure 9. First day patients became colonised with target organisms



A generalised linear model with Penalised Quasi-Likelihood (GLMMPQL) was used to investigate changes in presence/absence of individual organisms over time using Ct value as a binary variable by patient (Table 24). Tongue and throat swabs were treated equally.

Table 24. GLMMPQL model relating presence/absence of organism over time in individual patients to investigate whether changes over time in hospital occurred.

Organism	*Coefficient*	*Standard error*	*DF*	*T*	*P value*
H. influenzae	-0.222	0.043	315	-5.123	0
S. pneumoniae	-0.053	0.030	315	-1.759	0.080
Coliform	0.003	0.203	315	0.017	0.986
E. coli	-0.082	0.044	315	-1.879	0.061
P. aeruginosa	-0.016	0.030	315	-0.541	0.589
S. aureus	0	0	315	-13.984	0
MRSA	-0.039	0.208	315	-0.187	0.852
Acinetobacter spp	0.024	0.027	315	0.861	0.390

DF =degrees of freedom

Only *H. influenzae* was shown to decline significantly over time, with an odds ratio of 0.44. Occurrence of *H. influenzae* declined by 20% at each sampling point. There was slight evidence that *S. pneumoniae* and *E. coli* decreased with time. GlmmPQL modelling of *S. aureus* above produced wide standard error and a large negative coefficient and therefore a simple generalised linear model was also undertaken, the results of which were non-significant (Table 25).

Table 25. Generalised linear model relating presence of *S. aureus* over time in individual patients to investigate whether changes over time in hospital occurred.

Organism	*Estimate*	*Std. Error*	*z value*	*P value*
S. aureus	-0.027	0.049	-0.552	0.581

Null deviance: 198.94 on 408 degrees of freedom, Residual deviance: 198.63 on 407 degrees of freedom, AIC: 202.63

4.3.6 Complications during admission and length of stay

Complications were common among the study patients (n=90), with a median of eight complication events per patient (range 0-50). Aspiration events were observed in four patients by the study investigator (VE) at study visits. Cerebrovascular disease had been documented in 16 patients (18%), of which one of these had a witnessed aspiration event. Post-operative cough occurred in 41 patients (46%). Eight patients had one or more falls in hospital. Despite receiving prophylaxis against deep vein thrombosis (in the form of once daily subcutaneous low molecular weight heparin), five patients (6%) developed a deep vein thrombosis (DVT) in hospital. Twenty patients (22%) developed one or more urinary tract infections. Ten (11%) developed either a hyper- or hypoactive delirium. Eight patients (9%) developed an infection, excluding urinary tract infection and hospital acquired pneumonia (e.g. wound infection). Sixteen patients (18%) developed non-infectious acute illnesses (e.g. vomiting, digoxin toxicity). Other complications included new drugs being started (all patients were prescribed new drugs which usually included calcium and a bisphosphonate to reduce fractures) and complications specific to a particular patient. Four patients were transferred to intensive care for escalation of treatment, of whom two patients developed HAP (not related to ventilation). All patients transferred to intensive care died.

Length of hospital stay ranged from 4-265 days, with a median of 25 days and lower and upper quartiles of 14 and 53 days respectively. Increased length of stay was significantly associated with the combination of increased number of

complications, increased clinical frailty score and presence of urinary tract infection during admission (Table 26).

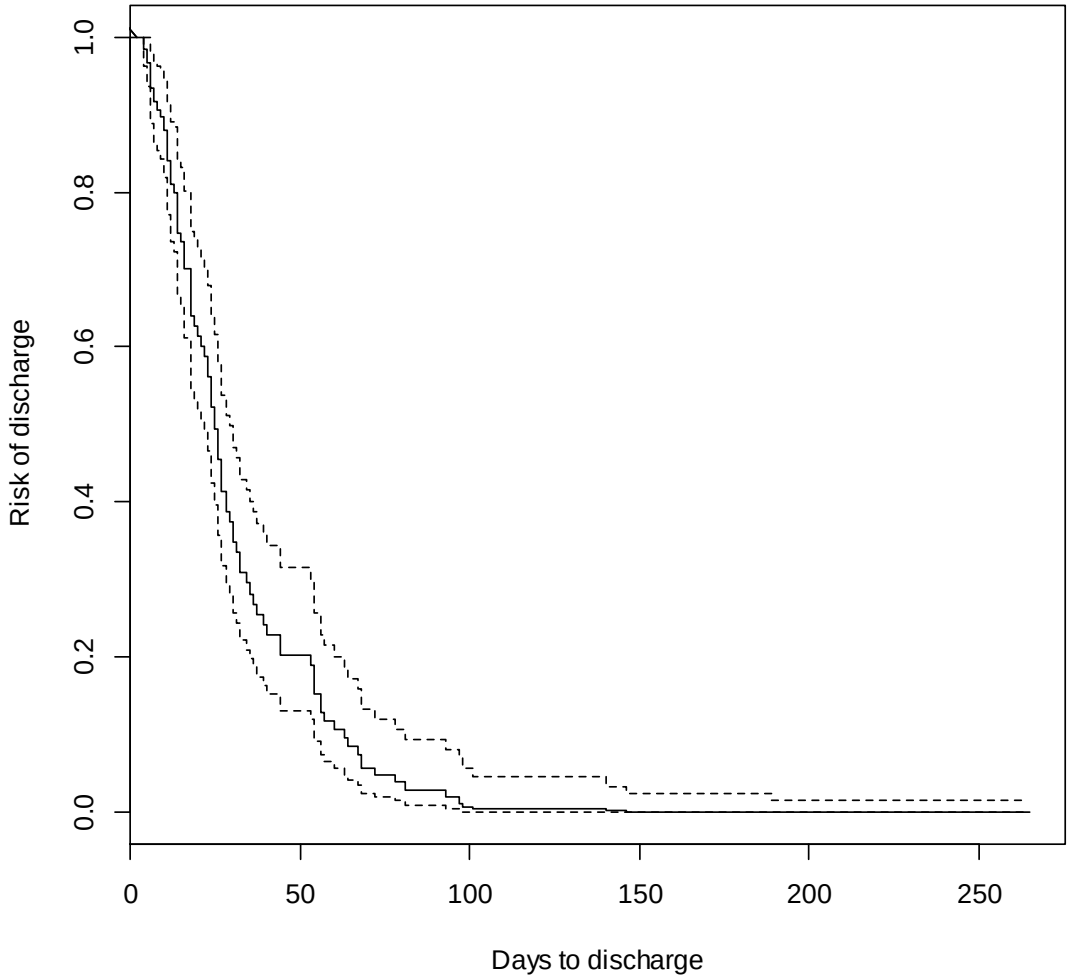
Table 26. Relating length of hospital stay with patient variables using Cox's proportional hazards.

Variable	*Coefficient*	*Exponential (coefficient)*	*Standard error (coefficient)*	*Z value*	*P value*
Number of complications	-0.066	0.936	0.016	-4.188	<0.001***
Clinical frailty score	-0.208	0.812	0.090	-2.310	0.021 *
Urinary tract infection	-0.508	0.602	0.276	-1.838	0.066 .

Table 27. Chi squared results from variables include in the survival analysis

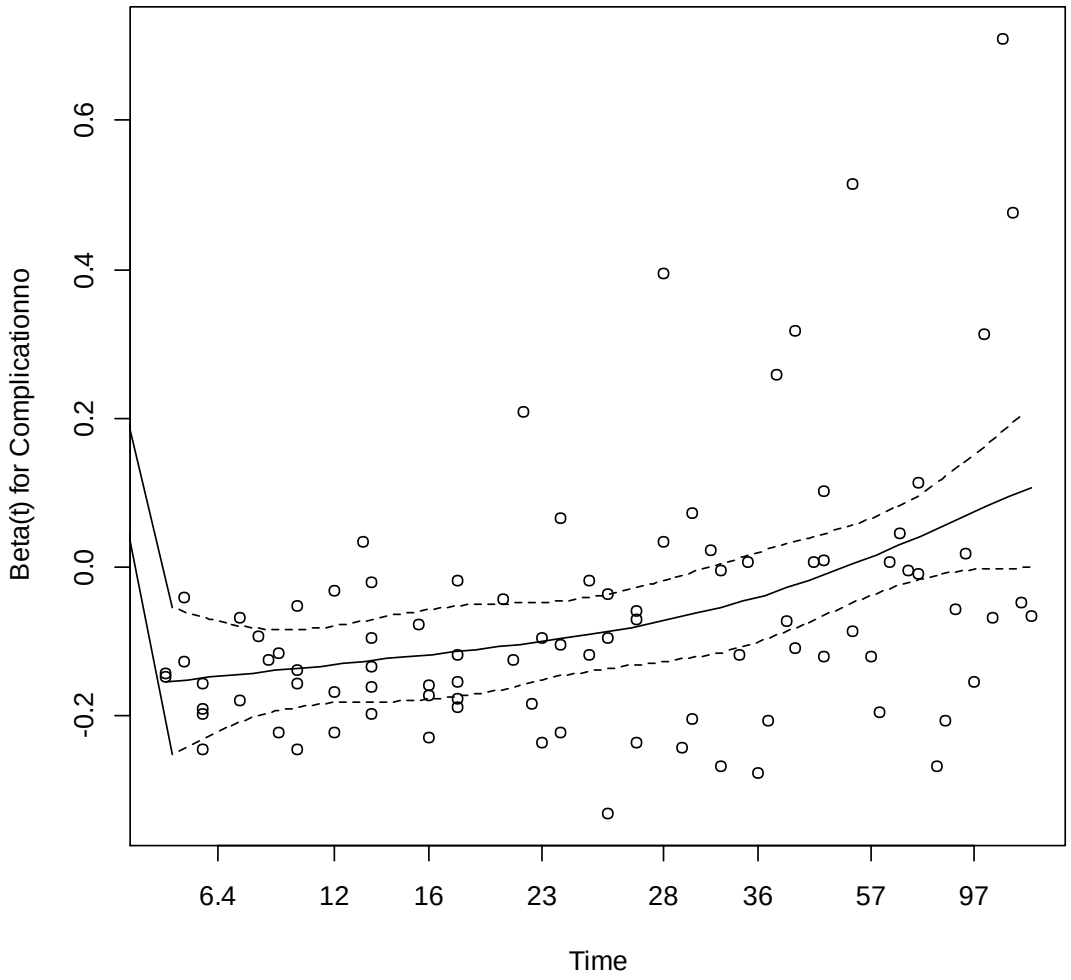
Variable	*Rho*	*Chi squared*	*P value*
Number of complications	0.405	20.057	<0.001
Clinical frailty score	-0.099	1.522	0.022
Urinary tract infection	0.054	0.244	0.062
Global	NA	23.625	<0.001

Figure 10. Survival curves of length of stay in hospital of study patients (Confidence intervals plotted with dashed line, solid line represents survival curve. The risk of discharge declines sharply until day 50, less sharply until day 100 and then flattens as the vast majority of patients have no been discharged.)



The proportional hazards assumption of the Cox regression were tested and the chi squared values associated are shown in Table 27. The risk of discharge in relation to significant covariates was not proportional and increased with time spent in hospital (Figure 11).

Figure 11. Risk of discharge plotted against time in hospital and number of complications (Each circle represents a patient and the solid line represents the risk of discharge, with dotted lines representing confidence intervals around this)



4.3.7 HAP and death during admission

In total 10/90 (11%) patients developed HAP during the hospital admission, and 18/90 (20%) of patients developed HAP or LRTI at any point during the study period (of whom three patients developed LRTI during admission.). Microbiological results from patients who developed HAP are shown in Table 85, Appendix G. Three patients who developed HAP were found to have active cancer during the hospital admission (metastatic lung cancer, metastatic prostate cancer and high grade glioma of brain), and these patients all died during follow up. Two of these patients received radiotherapy during follow up

(but after the oral sampling period) and subsequently developed HAP. The median number of days to HAP was 27 (range 2-88). Diagnosis of HAP was further characterised using two definitions (British Society for Antimicrobial Chemotherapy, BSAC and American Thoracic Society, ATS) which were concordant in all 10 patients. Seven patients were characterised as "probable HAP" and three more as "possible HAP". Two of the "possible HAP" patients were diagnosed from death certificate, but did not have new changes on chest radiograph (or did not have a chest radiograph due to severity of illness) and therefore did not fulfil BSAC or ATS criteria. However these patients were sufficiently unwell that they were treated for HAP based on clinical findings. Initial antibiotics prescribed included cefuroxime and clarithromycin (n=2), coamoxiclav (n= 4), Piperacillin-tazobactam (n=2), doxycycline (n=1), and chloramphenicol (n=1, patient with *S. aureus* bacteraemic pneumonia). Two patients were prescribed additional metronidazole to cover anaerobic causes of pneumonia, due to suspicion of aspiration. Of the ten patients with HAP, five had two courses of antibiotics for HAP due to recurrence of symptoms after treatment had stopped, and one was prescribed three courses of antibiotics in total (patient 97). Patients who were prescribed two or more courses of antibiotics were no more likely to die than those prescribed only one course of antibiotics. Case vignettes of those who developed HAP are described in Table 86 in Appendix G.

In total, ten patients died in hospital (10.8%); six of those died from HAP, one from pressure sores and probable wound infection, one from acute interstitial pneumonitis (extensive invasive respiratory cultures negative), one from cardio-respiratory failure and pulmonary embolus and one died from gram-negative bacterial sepsis secondary to catheter insertion. HAP was therefore the leading cause of in-hospital death. Length of stay before death in hospital ranged from 5-146 days with a median of 32.5 days. Death after admission for lower limb fracture was highly significantly associated with residence in either institution or hospital prior to admission.

Table 28. Generalised linear model relating death from all causes during the entire study with residence in either institution or hospital prior to admission

	Estimate	*Std. Error*	*z value*	*P value*	*OR (95% confidence intervals)*
Residence in hospital or institution	2.036	0.638	3.190	0.001 **	7.66 (2.19-26.74)

Null deviance: 82.175 on 92 degrees of freedom, Residual deviance: 72.314 on 91 degrees of freedom, AIC: 76.314

4.3.8 Discharge and follow up

Ten patients died in hospital (10.8%) and the remaining 83 patients survived to discharge (Table 29). The majority of these patients returned home, with eight patients changing from home to institution: five to residential care, and three to nursing care.

Table 29. Hospital discharge destinations of study patients

Discharge destination	*Number of patients (n=93)*
Died in hospital	10
Own home	67
Residential home	11
Nursing home	5

A further five patients died during the 90 days following discharge from non-HAP causes, leaving 78/93 (84%) alive at 90 days after admission, or a crude mortality rate of 16% at 90 days. Eight (10%) patients received antibiotics for lower respiratory tract infection during the 90 day follow up period. Owing to lack of access to chest x-ray in the community it was not possible to determine

whether these patients in fact had pneumonia. Ultimately 8/10 patients who had HAP during the study died (six from HAP, one possibly form HAP and the other from high-grade glioma), while none of the patients with HAP/LRTI diagnosed after discharge died. HAP was responsible for 40% of the deaths during the entire study period, and 80% of patients who developed HAP died.

4.4 Discussion

It has been assumed that increased length of stay in hospital was associated with increased rates of colonisation with "hospital" type commensal pathogens, such as *E. coli*, *P. aeruginosa*, and *Acinetobacter*. However results above suggest that both transient acquisition and colonisation occur for the first time within the first three days of admission in the majority of organisms. Only MRSA and *P. aeruginosa* began to colonise at day 7, and these events were rare. It is unclear whether detection of these organisms early in admission represented the oral flora acquired from the community, or whether organisms were acquired as a result of a combination of events surrounding the fracture, including the use of peri-operative antibiotics. Studies on the oral flora of subjects receiving antibiotics (amoxicillin, ceftriaxone, penicillin) have reported a rapid but transient reduction in numbers of commensal organisms (e.g. viridans Streptococci) [251-253] and commensal pathogens such as *S. pneumoniae*, Branhamella (*Moraxella cattarhalis*) and *H. influenzae* [254], with restoration of previous flora either during therapy or after discontinuation of antibiotic treatment. One study reported cefpirome had no effects on pharyngeal flora [255]. In our study 20 people were colonised with *S. pneumoniae* throughout admission despite peri-operative antibiotics (which included antibiotics active against *S. pneumoniae*), which suggests that the antibiotics had little effect on this organism, possibly due to the brevity of the course (three doses only). There were no great differences between the incidence of positive results between patients whose first sample had ben taken pre- versus post-operatively, therefore it is likely that events proximal to the operation influenced the occurrence of colonisation. It must also be noted that, given the small number of samples taken, the risk of two samples being colonised starting

on sample 3 or later must be lower as there are fewer opportunities for the sample to be positive.

In general, these findings suggest that the hospital environment is not a major source of colonisation of target pathogens in this cohort of patients. Even transient acquisition (swab positive at only one time-point) was more common in the first 3 days of admission. These findings cannot be applied to patients in other settings such as those on general medical wards or intensive care units. In practical terms, this means that the window for intervening to prevent putative sequelae of oral colonisation with respiratory pathogens is within 72 hours of admission to hospital in this cohort.

It is difficult to compare levels of colonisation of the target pathogens with those found in other studies due to differences in definitions, particularly given this was a relatively small study. However colonisation with respiratory commensal pathogens was generally relatively infrequent, apart from with *S. pneumoniae* (29%), which was high when compared with estimates of 1-13% in nasopharygeal samples from young adults [256]. *H. influenzae* has previously been detected from 3-19% of young adults [256]. Previous studies have suggested prevalence of *S. aureus* colonisation as ranging from 23-48% [29, 257], so estimates here are low, probably again due to differences in definition.

The tongue and throat, as opposed to dental plaque, were sampled because it was assumed that the risk of HAP would be higher if colonising organisms were present anywhere in the mouth rather than specifically in dental plaque. A previous study identified that coliform bacteria, *S. aureus* and *P. aeruginosa* were more commonly identified in the oral rinse, tongue or throat samples than from dental or dental plaque [245]. Dental or denture plaque often only became positive when all other sites were also positive. However, a study by Furrier et al. demonstrated that coliform positive dental plaque was likely to give rise to nosocomial infection whereas *S. aureus* infected dental plaque was not [42]. These assumptions need to be tested further to clarify the optimal sampling sites for future studies.

It was felt to be important to include older adults with cognitive impairment in the study because poor cognition has been associated with higher risk of HAP [258], and also to aid translation of study findings to "real" populations of patients. Therefore a combination of tongue and throat swabs was used to sample the oral microbiota, which was shown to be the minimally inclusive sampling combination in those with cognitive impairment in an earlier study [245]. The gold standard method for detecting oral microorganisms such as *S. aureus*, coliform bacteria and Pseudomonads was shown to be the oral rinse sample [245]. Thus results from the final assays may have missed some organisms which would have been detected by an oral rinse. Nasal sampling was not undertaken but also may have increased yield of target micro-organisms, especially for *S. pneumoniae* [259]. There were few data available regarding the prevalence of hospital respiratory pathogens (e.g. *P. aeruginosa*, *E. coli*) from nasal samples compared with oral sampling.

The patient cohort in general was representative of a population of "well" persons with lower limb fracture, but some biases occurred at recruitment. In particular, 20/190 patients (11%) who refused to participate cited that taking part would be "too much" for them suggesting a degree or strain or anxiety contributing to refusal. In addition, 35/190 (18%) were moribund or deemed too aggressive to take part in the study. Aggression was associated with dementia, and patients were often bed-bound and could or would not open their mouths to have swabs taken, which was taken as refusal of assent to participate. The withdrawal rate was low (3/93, 3%). Recruitment took place on Monday to Friday only, and patients who were admitted on either Friday evening or Saturday were not included. This is unlikely to have introduced systematic bias in the type of patient recruited as there are presumably no differences between people who fall on a Thursday or a Friday. However missing these patients could have introduced a difference in outcome in terms of the ward care received, due to lower weekend staffing levels as compared to weekday. Of all the patients who potentially could have been recruited, 74% were female. However, 54% of females declined, compared with 42% of males. The proportion of women recruited was 69%, less than the 80% expected

(around 80% of hip fracture patients are said to be female) but may reflect that proportionally more men were willing to participate.

The rate of HAP in this study (10%) was similar to the 9% found in a previous UK study of hip fracture patients [23]. The cohort in this study was a "well" population, and patients with acute medical illness, patients who were moribund and patients who were aggressive or uncooperative as a result of moderate-severe cognitive impairment were excluded at recruitment. In addition, seven patients had fractures of either ankle or femoral shaft, and were likely to be less frail and therefore less at risk of HAP than were the patients with fractured neck of femur.

It could be argued that by ensuring complete follow up of patients in hospital, ascertainment bias occurred. However the diagnosis of HAP was made by an orthogeriatrician independent of this study, and either a chest radiograph plus other signs or a death certificate diagnosis plus other signs were needed to verify a diagnosis. Diagnosis of HAP is difficult because there is no gold standard diagnostic test. The prospective nature of follow up is likely to have increased accuracy of diagnosis as compared with retrospective follow up. While it could be argued that including the use of death certificate data is less objective than a positive chest radiograph, two patients who were clinically suspected to have died from HAP but were too unwell to undergo chest radiography would have been otherwise missed. The above criteria represent the best diagnosis that can be made without invasive respiratory sampling or computed tomography scanning.

Two patients underwent radiotherapy after active cancer was discovered during hospital admission, and subsequently developed HAP. While chemo- or radio-therapy were exclusion criteria for the study, pathological fracture was not, and therefore these patients were included. However their risk of HAP may have been greater due to both cancer and radiotherapy, and there was an argument for excluding these two patients from analysis. Central to this work has been the attempt to make the findings as relevant to a "real" population of

patients as possible, in order to aid translation into everyday clinical work, and therefore, it was decided to include these patients in the analysis.

Previous publications have suggested that nursing home residents with HAP were more likely to "present atypically"- that is with a delirium [14, 15], not getting out of bed ("off legs"), falls or decreased oral intake compared to younger patients. In this study, 7/10 patients fulfilled both ATS and BSAC criteria for pneumonia, one only had one minor criterion but had a positive chest radiograph and two patients were too unwell for chest radiography and were diagnosed by death certificate. The criteria for ATS and BSAC diagnoses are slightly different in terms of temperature and white cell count thresholds, and also in that the BSAC guidelines include decreased oxygen saturations. Anecdotally, sometimes one would have to look harder to detect relevant clinical findings (e.g. green phlegm on nightgown in a patient not apparently coughing), but the typical clinical signs of pneumonia were still present in this older age group, and other modes of presentation were additional to, rather than instead of, the traditional clinical presentation of pneumonia.

The death rate in this cohort was higher than previously suggested (60% compared with 18-43%), despite exclusion of the sickest patients at recruitment. It is unclear why the mortality rate was higher in this study than others and may relate to complete follow up (i.e. greater ascertainment of outcomes) or may be related to there being an over representation of males within the cohort, who are more likely to develop HAP. Regardless, HAP was the leading cause of death in hospital in this cohort, and was associated with death in 40% of those who died during the study. Therefore efforts to prevent HAP are warranted, given the scale of the problem of lower limb fractures in older people.

Patients admitted from a location other than their own homes were approximately seven times more likely to die in hospital. This has been attributed to patients in institutional care having a poorer prognosis from pneumonia than community dwelling persons [260]. Given the strength of this effect, however it may be prudent to class these patients as high risk when

counselling patients and families about outcomes after hip fracture. In a retrospective study of 195 nursing home residents who sustained a hip fracture, 76.9% died in the 1.4 year median follow up, and the risk of death was 70% greater if there had been an episode of pneumonia within six months of hip fracture [240]. There is no single strategy to prevent deaths in patients admitted from other locations, but patients transferred from other hospitals may need greater attention placed on their care if a full recovery is anticipated, with proactive management of existing conditions and shared care between other specialties where appropriate. In addition, the information may help to plan escalation of therapy and to prepare the patient and family about possible outcome, if they wish for this information.

Few patients underwent spinal anaesthesia in this study, which is commonly used when operating on patients with hip fracture in other centres (in Scotland around 60% undergo spinal anaesthesia)[261]. No airway is inserted when patients undergo spinal anaesthesia, although sedative drugs are given which may increase the risk of aspiration or respiratory depression. While spinal anaesthesia is recommended in patients with hip fracture, there is no clear evidence of clinical benefit, though one study suggested that respiratory morbidity was higher after general anaesthetic in octogenarian patients [262]. It is possible that increased instrumentation of the airway in patients in this study could have increased the rate of HAP but would not be expected to account for increased mortality.

Dental plaque scores were relatively high, indicating unmet oral health needs in older patients with lower limb fracture in hospital, but there were no significant differences in scores over time, or between fitter and frailer patients. Patients anecdotally reported struggling to clean their teeth for a few days after operation because they couldn't walk to a sink, however this observation did not correlate with statistically significant increase in plaque at examination on day 7. However this study may have been underpowered to detect small differences in plaque scores (see 5.5). The finding that there was no difference to plaque scores between scoring time points is in contrast with those from

another study of ICU patients [42]. However, plaque scoring on day seven may have been too late to detect transient changes, or small changes may not have been detected by the modified Quigley Hein index.

The modified Quigley Hein index was used in preference to other plaque scores because it was a visual score (and therefore did not necessitate the removal of plaque and was repeatable for inter-examiner alignment) and because of its ability to discriminate between larger and smaller plaque loads. Previous studies indicated that some older patients have extensive denture or dental plaque, and it was hypothesised that these patients would be more likely to be colonised by respiratory commensal pathogens, if plaque was the stable reservoir of infection. Other possible plaque scoring systems (e.g. Silness and Loe or a dichotomous scoring system) require the use of a dental probe or do not distinguish between heavier loads of plaque. Disclosing tablets were not used, as brushing to remove the colouration could have interfered with the outcomes being studied, but this could have produced a greater degree of intra- and inter-rater variation, and potentially led to more conservative scoring. Indeed, neither of the two medical research nurses associated with this project achieved sufficiently high agreement with the PI (VE) to undertake plaque scoring as part of the study. Ideally, intra-rater calibration ought to have occurred at several time points during the study and included more surfaces. Because examinations took place at the bedside, lighting was suboptimal and air-drying of teeth was not undertaken, which could have contributed to poorer detection of smaller amounts of plaque. In retrospect, full mouth scoring may not have been needed [263], which would have decreased time taken for examinations and potentially left more time for calibration. Three forms of bias have been identified when undertaking plaque scoring [263]- leniency, central tendency and the halo effect. Leniency, the tendency to score all teeth similarly, and central tendency bias combined could have led to increased scoring of middle values. The halo effect, where the general oral appearance, discrepant with the actual state of the teeth leads an examiner to score more or less leniently was more likely to have led to generally increased scores in older hospitalised patients. If these biases occurred they could have contributed to a

lack of difference between examinations. However because scoring was undertaken by a single examiner, the effects from these biases are likely to have been minimised, as these biases are likely to be more pronounced when aligning several examiners.

The same scoring system was used for teeth and dentures, but dentures were removed, (and some drying will have therefore taken place) and examined in better light, and smaller deposits of plaque may have been seen on dentures because of this. It was difficult to know how to score debris between but not on teeth, and it may have been better in terms of subsequent analysis to have scored this debris according to the height of plaque, as per the Quigley Hein index, rather than simply assigning a score of 3, as this would have created a central tendency bias, and again could have contributed to not finding a significant difference between scores.

A composite plaque score was created so that all the patients could be included in analysis relating to plaque, and the higher of the dental or denture quartile score was used in analysis. Previous studies had not shown any association between amount of plaque and presence of respiratory pathogens [42, 153], so using the higher score added strength to accepting the null hypothesis. Overall, small differences between scores may have been minimised, but larger differences between very little plaque and large amounts of plaque were adequately characterised.

Several areas highlighted in this study warrant further investigation. Most clinically relevant would be to validate the risk factors for increased length of stay in hospital, as this could be used to plan discharge and escalation of care. Specifically, preventing urinary tract infections via reducing urinary catheter use (the commonest cause of UTI in hospital patients [264]) has the potential to save money by reducing patient stay. It would also be interesting to compare oral colonisation dynamics of respiratory commensal pathogens with patients in other setting such as medical admissions wards and other surgical wards, and to sample for longer into the admission. Once more bacterial genomic

sequencing data for other coliform bacteria becomes available, it will be possible to investigate the role these bacteria have in the development of HAP.

4.4.1 Conclusions

Oral colonisation with respiratory commensal pathogens begins predominantly within the first three days of admission in older patients with lower limb fracture, and not as a result of time spent in hospital. If oral hygiene interventions are implemented with a view to modifying the oral microbiota, these should be undertaken at the start of hospital admission in this group of patients. Patients admitted to hospital with lower limb fracture from locations other than home should be treated as having a high risk of death in hospital, and care planned accordingly. Oral health in older patients in hospital with lower limb fracture is poor in some cases, with patients frequently reporting sensation of dry mouth. HAP occurred in 11% of patients, with a crude mortality of 60%, and was the leading cause of death in hospital after lower limb fracture. Efforts to understand the pathogenesis of HAP and identify modifiable risk factors are necessary.

Chapter 5

Investigating risk factors for the development of hospital acquired pneumonia in older patients with lower limb fracture

5.1 Introduction

In order to better understand the pathogenesis of HAP in patients with lower limb fracture in relation to the different groups of putative risk factors, a cohort of patients were followed prospectively to detect oral colonization with seven common respiratory commensal pathogens, levels of dental plaque, subjective sensation of oral dryness, and incidence of HAP during hospital admission. To identify the most important risk factors for HAP, data on the majority of previously reported risk factors were collected in these patients, and correlation between these variables was assessed using a correlation matrix. The cohort of patients in this study were a "real", prospective population, and the study was approached with a clinical viewpoint, with variables utilised that are used and recorded in everyday clinical practice in the UK. Univariate generalised linear modelling and Fisher's exact test were used to investigate associations between cases of HAP and patient risk factors, which included complications occurring during admission. Risk factors likely to impact on colonisation events with individual pathogens were then selected for inclusion in two conceptual models. These models were then challenged using multivariate generalised linear modelling. Infection events were investigated using individual variables from the models.

5.1.1 Aims of this Chapter

The individual aims of the Chapter are described below.

1. Describe the extent to which patient variables interact using a correlation matrix

2. Investigate whether HAP or HAP/LRTI was associated with recorded patient variables

3. Investigate whether HAP or HAP/LRTI was associated with acquisition or colonisation with target pathogens

4. Investigate whether colonisation with individual target pathogens was associated with variables contained in the medical or dental models

5. Investigate whether certain combinations of pathogens or absence of pathogens related to either HAP or HAP/LRTI up to 3 months or other patient variables

5.2 Methods

Data collection, study variables follow up and ethical and funding are described in 4.2. Three patients who withdrew were excluded from analyses in this Chapter because they were not followed up for HAP or LRTI after withdrawal (therefore n=90).

5.2.1 Definitions of acquisition and colonisation of commensal pathogens

Acquisition of organisms was defined as the presence of an organism at any point over the five sampling time points. Seven binary variables, corresponding to each of the target organisms, were calculated per patient. In previous studies investigating repeated microbiological samples, colonization by an organism was defined as two or more consecutive swabs positive for the same organism [265, 266]. Colonisation was thought to be important clinically, and was distinguished from transient carriage of that organism (single sample positive) which was felt to be clinically less important [35]. However it could be argued that non-consecutive positive swabs still constitute a risk to the lungs, as any simultaneous episode of aspiration could result in an increased risk of HAP. Non-consecutive positive samples may represent repeated transient acquisition via the nose or mouth, or the waxing and waning of numbers of that organism, with smaller numbers not easily detected, particularly by culture-based methods. Any sequelae related to carriage of these organisms may be proportional to number of days colonized (i.e. five days colonized may carry a greater risk than two days colonized). Therefore analysis was also conducted using a colonization index. The total number of

positive samples divided by the number of samples taken was calculated for each bacterium (e.g. 6/10=0.6 for *S. aureus*) per patient, and this value was known as the colonization index. Tongue and throat samples were therefore considered equally. This meant that colonisation was seen as a continuum, rather than a binary state, and the effect of persistent or intermittent colonisation could be determined. A previous group developed a colonization index to assist in determining when to treat intensive care patients with candidal colonization at risk of invasive infection [267]. However this index was calculated by taking the sum of the log10 of concentrations of candida isolated from 1ml of oropharngeal washing sample and dividing by the number of washings taken (maximum two). No other colonization indices have been published to the best of this author's knowledge. Colonisation status was also analysed as a binomial variable, with "uncolonised" referring to the 20 patients with negative samples for all target organisms over all time points.

5.2.2 Definitions of HAP and HAP/LRTI

Two outcome variables were used in analyses in this chapter: HAP and HAP/LRTI. Cases of HAP were identified when the responsible clinician started antibiotics for HAP, and further characterised by both British Society for Antimicrobial Chemotherapy (BSAC) and American Thoracic Society (ATS) guidelines to ensure diagnoses were robust and comparable (see 4.2.6). Pneumonia is distinguished from lower respiratory tract infection (a less serious illness) by the presence of infiltrates on chest radiograph. In order to fulfil BSAC/ATS guidelines for HAP, new infiltrates were required on a chest radiograph reported by a radiologist. In addition, "Pneumonia" as primary cause of death on death certificate was acceptable where patients were too unwell to tolerate chest radiography. HAP was diagnosed during hospital stay only. However, patients were followed until three months after discharge for episodes of pneumonia, which are now referred to as "healthcare associated pneumonia" [11]. Routinely UK General Practitioners (GP) do not request chest radiography when treating respiratory infection, and therefore it was not possible to determine whether these patients had pneumonia. Therefore episodes of lower respiratory tract infection (LRTI) were also recorded

throughout admission and up to three months after discharge. Cases of LRTI were identified by hospital clinician or GP starting antibiotics for LRTI at any point during the study period; new infiltrates on chest radiograph were not required for diagnosis. HAP/LRTI therefore referred to any cases of HAP during hospital admission, plus any cases of LRTI at any point during the study period.

5.2.3 Conceptual models of the roles of patient demographics and oral health variables in oral colonization with respiratory commensal pathogens

It was hypothesised that the same risk factors which caused HAP would also influence clinically important colonisation events with respiratory commensal pathogens, given that infection follows adherence and colonisation of an infecting organism. In order to generate clinically relevant models to explain colonisation with respiratory commensal pathogens, two sets of variables were considered: the first from previously published studies considering HAP, and the second from overriding general health variables which are likely to impact on other variables (including two frailty scores, one mobility score and a comorbidity score). These variables were later formed into two models to explain colonisation with respiratory commensal pathogens. The first considered risk factors proximal to the site of colonisation, specifically involving factors of the mouth, dentition and plaque (dental model, Figure 12). The second involved assessing the extent to which patient demographics and comorbidity were risk factors for colonisation by each colonising pathogen (medical model, Figure 13). These models only included variables present at admission, as other events could have happened at any time throughout the admission and not necessarily within the 14 day sampling period. The dental model was smaller to allow any effects from the dental variables to be seen, as fewer patients could be included in this analysis (e.g. only a proportion wore dentures), and only pertinent non-dental demographic variables were included.

Overriding health variables such as age, gender, functional indices and comorbidity index were included in both models. The effects of tobacco smoke on colonisation with respiratory commensal pathogens is unknown, but is

known to be associated with combined obstructive pulmonary disease (COPD, see below) and to influence oral conditions such as periodontitis, and it seemed prudent to include this variable in both models. All dental variables other than xerostomia index were included in the dental model, and plaque scores at day 7 and day 14 were substituted for admission plaque score when testing models. The relationship between colonisation with individual respiratory commensal pathogens and xerostomia score was analysed separately, due to number of missing values.

Deprivation has not been tested in relation to HAP or colonisation with respiratory pathogens, and was included in both models to generate further hypotheses. Cerebrovascular disease, a common cause of dysphagia, is included in the medical model, as pneumonia is a common complication after stroke. COPD was included in the model because again pneumonia is common in patients with COPD, antibiotic use is often frequent (which in turn may be a risk factor for colonisation with non-oral bacteria) and some of the drugs prescribed commonly for COPD such as oral steroids or inhaled steroids may also increase the risk of pneumonia. Dementia, ability to self consent and place of residence were included in the medical model, as pneumonia is common in nursing home residents, who in turn have a high prevalence of dementia and cognitive impairment. Diagnoses of dementia were sometimes made during admission, usually where cognitive impairment was milder, and inability to self consent was a simple method of detecting all persons with significant cognitive impairment. Aspiration was not included in the models because there was no biological explanation for an effect on oral colonisation. Nasogastric tubes were inserted in two patients after the sampling period had finished and therefore were not included in the models.

Figure 12. Patient variables used in dental model
Age + Gender+ Clinical frailty score +Barthel index + Charlson index + Residence + Number of Teeth + Dentures + Admission plaque score + Deprivation score*+Smoking

*UK indices of multiple deprivation

Figure 13. Patient variables used in medical model
Age+Gender+COPD+Antibiotics pre admission + Ability to self-consent + Residence + Barthel index + Weight + Number of teeth + Admission plaque score + Clinical frailty score +HABAM score + Smoking + Charlson index + Cerebrovascular disease + Dementia

5.2.4 Data analysis

The list of putative risk factors contained variables that were potentially related. Therefore the extent of the association between variables was investigated using correlation matrices. Correlation matrices were constructed in R using the polycor package, with Pearson correlation coefficients calculated for continuous variables. Where data types were categorical or ordinal, polycoric correlation was used to assess associations. Correlations of either greater that 0.2 or lower than -0.2 were considered noteworthy in terms of explaining clinically relevant colinearity between variables (rather than significant associations per se). While correlation coefficients of >0.8 are usually considered significant, here understanding weaker colinearity between variables potentially helped to explain significant results obtained in multivariate analyses relating carriage of organisms with patient characterstics.

Generalised linear modeling with a binomial error structure was undertaken to investigate HAP/LRTI and colonisation with individual organisms in relation to uni- and multi-variate explanatory variables respectively. During multivariate analysis, stepwise removal of non-significant variables in the two models was undertaken until only significant variables remained. Fisher's exact test was also used to investigate the relationships between HAP and explanatory variables where there were proportionally few cases compared to controls. Although covariates such as Barthel index and Clinical Frailty Score

were categorical, given the number of categories (minimum 9), they were considered as continuous variables.

Canonical correspondence analysis (CCA) was used to investigate relationships between colonisation patterns amongst patients and patient demographics. CCA is based on correspondence analysis (CA). CA is an iterative matrix approach which, in this case, seeks to investigate and separate the main trends in variation in an individual by a measured characteristics data matrix. To achieve this the correlations between individual patients and their colonisation status are maximised, such that patients with similar statuses have similar 'scores' on what are analogous to principle component axes. Scores for an individual patient on one axis are the weighted average of a set of complementary scores for the individual colonising agents for the same axis (as in a principal components analysis). The scores for patients are therefore derivable from the scores of the colonization variable present for the patient and vice versa. CCA was undertaken using the Vegan package in R. Patient variables which had been found to be significantly associated with colonization with individual organisms in generalized linear models were included as covariates, along with HAP as an outcome variable. An organism by patient matrix was used to assess likely dependence between patient co-variates and colonization with target organisms. The raw data matrix was subjected to CCA with patient demographic information used as covariates to explain the patterns of colonization amongst patients. Two patients were excluded from the CCA as one had no plaque score (patient with no teeth or dentures) and the other had no weight recorded.

5.3 Results

5.3.1 Investigating the extent of correlation between key variables used in the study

Results from the correlation analyses are shown in Table 30, and standard errors for the analyses are shown in Table 87, Appendix H. Correlations of either greater that 0.2 or lower than -0.2 were considered noteworthy (coefficients of >0.205 or <0.205, significant at 5% level with 90 degrees of

freedom), and are typed in boldface. Deprivation was associated with increased weight, fewer teeth, increased tendency to wear complete dentures, and being a current smoker. There were slight (0.19) correlations between deprivation and increased clinical frailty score and increased plaque score at admission. Smoking was associated with increased age, increased number of teeth, increased likelihood of dementia, and decreased likelihood of wearing complete dentures. Dementia was associated with increased age, decreased Barthel index, increased Clinical Frailty Score, Decreased HABAM (mobility) score and increased Charlson index. Increased Charlson index (increased number and severity of illnesses) was in turn associated with increased age, being male, a decreased Barthel index, increased Clinical Frailty score and decreased HABAM score. Increased HABAM score (better mobility) was associated with lower age, being female, home-living, increased Barthel index (more independent) and decreased Clinical frailty score. Increased Clinical frailty score was associated with increased age, being male, residence in institution or hospital prior to admission, and lower Barthel index (less independent). An increased Barthel index (more independent) was associated with lower age, being female, and home-living. Residence in a location other than home was associated with increased age. Increased weight was associated with home-living.

Inability to sign the consent form oneself was associated with increased age, residence somewhere other than home, decreased Barthel index (less independent), increased clinical frailty score, decreased HABAM (mobility) score, increased likelihood of dementia, and decreased likelihood of combined obstructive respiratory disease (COPD). COPD was associated with lower age, being female, not having dementia and current smoking. Having been prescribed antibiotics within three months prior to admission was associated with decreased Barthel index, increased likelihood of having dementia, ex- or never smoking and inability to sign the consent form oneself.

Presence of complete dentures was associated with increased age, decreased number of teeth, increased clinical frailty score and decreased HABAM score.

Increased tooth number was associated with lower age. Increased plaque at admission was associated with decreased Barthel score (less independent), decreased number of teeth, increased likelihood of having dementia and increased likelihood of wearing complete dentures.

Given the degree of interaction between covariates, outcome variables were subjected to multivariate analysis where possible.

Table 30. Correlation matrix of patient variables used in the study

	Age	Female	Residence	Barthel	Weight	Teeth	Frailty	HABAM	Charlson	Dementia	Dentures	Smoking	Plaque1	IMD	COPD	Consent	Antibiotics
Age		Polyserial	Pearson	Pearson	Pearson	Pearson	Pearson	Pearson	Pearson	Polyserial	Pearson	Pearson	Pearson	Pearson	Polyserial	Pearson	Polyserial
Female	**-0.21**		Polyserial	Polyserial	Polyserial	Polyserial	Polyserial	Polyserial	Polyserial	Polychoric	Polyserial	Polyserial	Polyserial	Polyserial	Polychoric	Polyserial	Polychoric
Residence	**0.30**	-0.17		Pearson	Pearson	Pearson	Pearson	Pearson	Pearson	Polyserial	Pearson	Pearson	Pearson	Pearson	Polyserial	Pearson	Polyserial
Barthel	**-0.31**	**0.20**	**-0.38**		Pearson	Pearson	Pearson	Pearson	Pearson	Polyserial	Pearson	Pearson	Pearson	Pearson	Polyserial	Pearson	Polyserial
Weight	-0.14	-0.19	**-0.29**	-0.02		Pearson	Pearson	Pearson	Pearson	Polyserial	Pearson	Pearson	Pearson	Pearson	Polyserial	Pearson	Polyserial
Teeth	**-0.24**	0.07	0.03	0.13	0.00		Pearson	Pearson	Pearson	Polyserial	Pearson	Pearson	Pearson	Pearson	Polyserial	Pearson	Polyserial
Frailty	**0.33**	**-0.42**	**0.38**	**-0.54**	-0.08	**-0.23**		Pearson	Pearson	Polyserial	Pearson	Pearson	Pearson	Pearson	Polyserial	Pearson	Polyserial
HABAM	**-0.39**	**0.24**	**-0.39**	**0.52**	0.05	**0.22**	**-0.74**		Pearson	Polyserial	Pearson	Pearson	Pearson	Pearson	Polyserial	Pearson	Polyserial
Charlson	**0.43**	**-0.35**	0.15	**-0.40**	0.18	-0.11	**0.35**	**-0.38**		Polyserial	Pearson	Pearson	Pearson	Pearson	Polyserial	Pearson	Polyserial
Dementia	**0.23**	-0.15	0.17	**-0.41**	-0.11	0.06	**0.38**	**-0.28**	0.20		Polyserial	Polyserial	Polyserial	Polyserial	Polychoric	Polyserial	Polychoric
Dentures	**0.23**	-0.20	0.02	-0.19	0.06	**-0.87**	**0.20**	**-0.22**	0.05	-0.03		Pearson	Pearson	Pearson	Polyserial	Pearson	Polyserial
Smoking	0.20	0.19	-0.03	-0.02	0.06	**0.22**	-0.13	0.04	0.08	**0.31**	**-0.22**		Pearson	Pearson	Polyserial	Pearson	Polyserial
Plaque1	-0.03	0.15	-0.10	**-0.20**	-0.02	**-0.32**	0.11	-0.03	0.07	**0.58**	**0.30**	-0.10		Pearson	Polyserial	Pearson	Polyserial
IMD	-0.02	-0.18	-0.09	-0.19	0.18	**-0.28**	**0.20**	-0.18	0.11	0.13	**0.27**	**-0.31**	**0.22**		Polyserial	Pearson	Polyserial
COPD	**-0.23**	**0.37**	-0.11	0.07	-0.06	-0.11	-0.06	0.05	-0.04	**-0.67**	0.03	**-0.31**	-0.12	0.13		Polyserial	Polychoric
consent	**0.38**	0.08	**0.39**	**-0.49**	-0.20	-0.15	**0.31**	**-0.38**	0.17	**0.43**	0.14	0.03	0.08	0.12	**-0.21**		Polyserial
Antibiotics	0.14	0.03	0.11	**-0.24**	-0.06	0.17	**0.20**	-0.10	0.19	**0.29**	-0.13	**0.28**	0.02	-0.16	0.18	**0.21**	

Barthel=Barthel index, Teeth=number of teeth, Frailty=Clinical frailty score, Charlson=Charlson index, Plaque1=Admission plaque quartile score, IMD= Indices of multiple deprivation score, COPD=Combined obstructive respiratory disease, Consent= ability to sign consent for oneself, Antibiotics=Prescribed antibiotics within three months prior to hospital admission

5.3.2 Investigating associations between HAP or HAP/LRTI and patient demographic factors

Univariate generalised linear models were used to investigate associations between HAP/LRTI and patient factors during the whole study period (cases n=18). Where number of cases was low (HAP n=10), associations between HAP and explanatory variables were analysed using Fisher's exact test.

HAP was not significantly associated with any patient demographic variables tested, however there was slight evidence of associations with age (p=0.06), Charlson index (p=0.07) and increased deprivation score (p= 0.06). Full results are shown in Table 88, Appendix H. HAP/LRTI up to 3 months after discharge were significantly associated with increased Charlson comorbidity index, increased clinical frailty score, and decreased HABAM (mobility) score (Table 31). Interestingly HAP/LRTI was not associated with increased age.

Table 31. Relating HAP/LRTI and patient factors using univariate generalised linear modelling (significant variables shown only)

Variable	*Estimate*	*Standard error*	*Z value*	*P value*	*Residual deviance*	*Odds ratio (95% confidence intervals)*
Clinical frailty scale	0.527	0.196	2.68	<0.01**	81.648 on 88	1.69 (1.15-2.49)
Mobility (HABAM)	-0.047	0.023	-2.064	0.04 *	85.788 on 88	0.95 (0.91-1.00)
Charlson index	0.389	0.119	3.267	<0.01**	78.302 on 88	1.48 (1.17-1.86)

Null deviance =90.072 on 89

5.3.3 Investigating associations between HAP or HAP/LRTI and oral colonisation with respiratory commensal pathogens

HAP was significantly associated with persistent colonisation with *E. coli* both using Fisher's exact test (p=0.036) and using generalised linear modelling (Table 32). The model was somewhat underdispersed and the lower confidence interval for the odds ratio was zero.

Table 32. Univariate generalised linear model relating HAP and oral colonisation index with *E. coli*

Variable	*Estimate*	*Standard error*	*Z value*	*P value*	*Odds ratio (95% confidence intervals)*
E. coli	0.503	0.2561	1.963	0.050	1.65 (1.00-2.73)

Null deviance: 62.79 on 89 degrees of freedom

Residual deviance: 57.80 on 87 degrees of freedom

AIC: 63.8

HAP was not associated with being either colonised with any or uncolonised by all pathogens. HAP was not associated with acquisition of any individual pathogen. HAP was not associated with a positive PCR assay (coliform assay not included) with any organism at admission only. In addition, neither death due to any cause nor cough were associated with a positive PCR assay result at admission.

HAP/LRTI was significantly associated with persistent *S. aureus* colonisation (Table 33). There was slight evidence that persistent colonisation with MRSA was also associated with HAP/LRTI (Table 34). Analyses of HAP with *S. aureus* and HAP/LRTI with *E. coli* are included from completeness in Tables 94-96, Appendix H. However, these results need to be interpreted in light of the power of the study (see 5.4).

Table 33. Univariate generalised linear model relating HAP/LRTI and oral colonisation index with *S. aureus*

Variable	*Estimate*	*Standard error*	*Z value*	*P value*	*Odds ratio (95% confidence intervals)*
S. aureus	0.426	0.181	2.357	0.018 *	1.53 (1.07-2.18)

Null deviance: 90.072 on 89 degrees of freedom

Residual deviance: 82.835 on 87 degrees of freedom

AIC: 88.835

Table 34. Univariate generalised linear model relating HAP/LRTI and oral colonisation index with MRSA

HAP/LRTI	*Estimate*	*Standard error*	*Z value*	*P value*
MRSA	0.546	0.305	1.793	0.073 .

Null deviance: 90.072 on 89 degrees of freedom

Residual deviance: 84.483 on 87 degrees of freedom

AIC: 90.483

Absence of cough was significantly associated with absence of detection of respiratory commensal pathogens on any sample (p= 0.002, Odds ratio= 5.75 95% confidence intervals 1.67- 25.75). However cough was not associated with positive PCR assays at admission only.

5.3.4 Investigating associations between HAP or HAP/LRTI and oral health variables

Neither HAP nor HAP/LRTI were more common in dentate patients, those who wore dentures, those with increased number of teeth, or with higher plaque quartile scores at admission, day 7 or day 14. HAP and HAP/LRTI were not associated with increased denture plaque indices at days 1, 7 or 14. In dentate patients only, HAP and HAP/LRTI were not associated with increased

modified Quigley Hein scores. HAP and HAP/LRTI were not commoner in those with a high xerostomia score.

5.3.5 Investigating associations between HAP or HAP/LRTI and in-hospital events and outcomes

Table 35 and Table 36 show postoperative delirium, witnessed aspiration episodes, and falls during hospital admission were all significantly more common in patients who developed HAP or HAP/LRTI. Two of the three aspiration episodes in patients who subsequently developed HAP occurred prior to the episode of HAP, and one was simultaneous. HAP was also associated with cough. HAP was not associated with length of operation, time to operation, or type of anaesthetic. There was no association between HAP or HAP/LRTI and pre-operative albumin levels. Full results are shown in Tables 89-90, Appendix H.

Table 35. Associations between HAP and significant in hospital events using Fisher's exact test

Variable	*HAP (N=10)*	*No HAP (n=80)*	*P value*	*Odds ratio (95% confidence intervals)*
Aspiration	3/10	1/80	<0.01	30.88 (2.18 -1773.08)
Delirium	4/10	6/80	0.01	7.89 (1.28-46.31)
Fall	3/10	5/80	0.04	6.21 (0.80-41.00)
Cough	9/10	32/80	<0.01	13.17 (1.69 -601.85)

Table 36. Univariate generalised linear models relating HAP/LRTI at any point and significant in-hospital events

Variable	*Estimate*	*Standard error*	*Z value*	*P value*	*Residual deviance (df)*	*Odds ratio (95% confidence intervals)*
Aspiration	2.653	1.189	2.231	0.026	84.104 on 88	14.20 (1.38-146.06)
Delirium	1.640	0.701	2.338	0.019	84.870 on 88	5.15 (1.30-20.37)
Fall	2.180	0.790	2.758	0.006	82.292 on 88	8.85 (1.88-41.65)

Null deviance= 90.072 on 89 degrees of freedom

Both HAP ($p< 0.001$, Fisher's exact test) and HAP/LRTI were associated with increased number of complications during hospital stay (Table 37), though the effect size was small.

Table 37. Univariate generalised linear model relating HAP/LRTI with number of complications during hospital admission

Variable	*Estimate*	*Standard error*	*Z value*	*P value*	*Odds ratio (95% confidence intervals)*
Number of complications	0.100	0.030	3.291	<0.001 ***	1.10 (1.04-1.17)

Null deviance: 90.072 on 89 degrees of freedom

Residual deviance: 76.628 on 88 degrees of freedom

AIC: 80.628

Death from any cause was strongly associated with both HAP and HAP/LRTI, but more so with HAP. Death was still associated with prior HAP when patients with pneumonia and active cancer were not included (P= 0.001 , odds ratio= 17.23, 95% confidence intervals= 2.44-203.26, Fishers exact test), as shown in Table 40

.

Table 38. Univariate generalised linear model relating death from all causes and HAP

Variable	*Estimate*	*Standard error*	*Z value*	*P value*	*Odds ratio (95% confidence intervals)*
HAP	3.731	0.884	4.220	<0.001 ***	41.71 (7.37-235.95)

Null deviance: 81.101 on 89 degrees of freedom, Residual deviance: 57.482 on 88 degrees of freedom, AIC: 61.482

Table 39. Univariate generalised linear model relating death from all causes and HAP/LRTI at any point during the study

Variable	*Estimate*	*Standard error*	*Z value*	*P value*	*Odds ratio (95% confidence intervals)*
HAP/LRTI	2.005	0.619	3.239	0.001	7.43 (2.21- 24.99)

Null deviance: 81.101 on 89 degrees of freedom, Residual deviance: 70.657 on 88 degrees of freedom, AIC: 74.657

Table 40. Univariate generalised linear model relating death from any cause and HAP in persons without active cancer

Variable	*Estimate*	*Standard error*	*Z value*	*P value*	*Odds ratio (95% confidence intervals)*
HAP	2.904	0.902	3.220	0.001 **	18.25 (3.11- 106.95)

Null deviance: 81.101 on 89 degrees of freedom, Residual deviance: 69.445 on 88 degrees of freedom, AIC: 73.445

5.3.6 Oral colonisation with respiratory commensal pathogens and patient factors

Acquisition of any organism was associated with decreased Barthel index, prescription of a cognitively active drug at admission and post-operative cough (Tables 98-99, Appendix H). Multivariate generalised linear modelling was used to investigate the associations between colonisation with individual organisms and patient factors using both the medical model (Table 41) and the dental model (Table 42). Multivariate models were generated for all seven target organisms. However the final models for *E. coli* and *Acinetobacter* using the medical model were underdispersed, which was likely due to the paucity of positive results from these two assays (21/816 or fewer) and are not shown in Table 41. Full results are shown in Tables 117-125, Appendix H.

Univariate analysis was also undertaken and results are shown in Tables 108-116, Appendix H (models for *S. pneumoniae* and *H. influenzae* were somewhat overdispersed, while models of *Acinetobacter* were underdispersed). Colonisation rather than acquisition was used to investigate associations with patient factors, as HAP or HAP/LRTI were associated only with increased oral colonisation index of *S. aureus* and *E. coli*. However significant results from relating acquisition of target organisms with patient factors using multivariate analysis are shown in Tables 100-107, Appendix H for completeness.

Models of *S. aureus* indicated that being male, being prescribed antibiotics in the three months prior to sampling, increased Charlson index, being fitter

(increased Barthel or decreased Clinical frailty score), decreased weight and current smoking were associatied with increased colonisation indices. In addition, colonisation with *S. aureus* was associated with both dementia and inability to self consent in the medical model. Factors that were included in both models but only appeared significantly in one model included low deprivation score (dental model) and number of teeth (medical model) .

The models explaining MRSA colonisation resulted in broadly concordant results for both models and included recent use of antibiotics, current or history of smoking, higher Barthel index (more independent), higher Charlson index (more serious illnesses). Number of teeth was included in both models as a variable but was only significant in the medical model.

Colonisation with *E. coli* was explained using the dental model only, and significant variables included being male, possessing few teeth but not wearing dentures, current smoking and lower Barthel index (less independent).

Neither model contained significant risk factors for *P. aeruginosa* colonisation. There were 16 patients with positive samples for *P. aeruginosa*, but only nine with more than one sample positive and the highest index was 0.4 (in only 1 patient). Being female, increased Charlson index and increased clinical frailty score were significant risk factors using the medical model initially, but became insignificant during stepwise removal of non-significant variables.

Colonisation with *S. pneumoniae* was explained by being female, possessing increased number of teeth, current or history of smoking, lower Charlson index (fewer serious illnesses) and increased HABAM (mobility) score. Variables included in both models but significant in only one model were decreased clinical frailty score, and lower deprivation score (dental model), and decreased plaque score at admission and no use of recent antibiotics (medical model).

H. influenzae colonisation was explained by being female, having combined obstructive pulmonary disease (COPD) and decreased Barthel score (less independent). Of variables included in both models but only significantly

associated with *H. influenzae* colonisation in one were increased deprivation score and lower age (dental model), and current smoking, presence of dementia and decreased number of teeth (medical model).

Colonisation with *Acinetobacter* spp was not associated with any variable tested in either model. In the above models, there was discordance between the significance of some variables included in both models. However the correlation analysis above demonstrated extensive correlation between risk factors, and this is considered further in the discussion.

Colonisation with *S. pneumoniae* was not associated with a significant decrease the colonisation indices of *S. aureus* or *E. coli* (univariate generalised linear modelling).

Table 41. Multivariate generalised linear models relating oral colonisation index of target organisms with patient factors using the medical model

Organism	*Positive risks*	*Negative risks*
S. aureus	Male* Recent antibiotics*** Charlson index*** Dementia*** Unable to self-consent*	Weight* Teeth number* Frailty score* Ex** or never smoker***
MRSA	Recent antibiotics*** Barthel** Charlson index** Ever smoked***	Teeth number**
P. aeruginosa	Nil significant	Nil significant
S. pneumoniae	Female** Teeth number*** HABAM*** Can't self-consent * Current/Ex smoker*	Plaque score quartile at admission* Recent antibiotics** Charlson index**
H. influenzae	Female* COPD** Current smoker* Dementia***	Barthel* Number of teeth**

*p<0.05, **p<0.01, ***p<0.001

Table 42. Multivariate generalised linear models relating oral colonisation with target organisms against patient and dental factors using the dental model

Organism	*Positive risks*	*Negative risks*
S. aureus	Charlson index** Admission plaque* Barthel index* Male** Current smoking**	Low deprivation score
MRSA	Age* Charlson index* Current or ex smoker* Barthel index (less frail)*	N/A
E. coli	No dentures* Male*** Current smoking***	Few teeth** Barthel index (more frail)***
P. aeruginosa	Nil significant	Nil significant
S. pneumoniae	Female* More teeth*** Current smoking***	Clinical frailty score (less frail)* Charlson index (fewer serious illnesses)* Deprivation score (less deprived)
H. influenzae	Dentures (complete) ** Female** Deprivation score (more deprived)**	Age (younger)** Barthel score (more frail)***
Acinetobacter spp	Nil significant	Nil significant

*p<0.05, **p<0.01, ***p<0.001

5.3.7 Oral colonisation with S. aureus and xerostomia

The binomial xerostomia score was not entered into either the full medical or simple dental model due to number of missing values, and is therefore considered separately. Increased colonisation with *S. aureus* was related to a high xerostomia score (Table 43).

Table 43. Generalised linear model relating *S. aureus* colonisation with xerostomia (binary variable)

Variable	*Estimate*	*Standard error*	*Z value*	*P value*	*Odds ratio (95% Confidence intervals)*
S. aureus	2.488	1.023	2.432	0.015*	12.04 (1.62- 89.40)

Null deviance: 99.919 on 57 degrees of freedom

Residual deviance: 86.836 on 56 degrees of freedom

(32 observations deleted due to missingness)

AIC: 115.26

5.3.8 Multivariate analysis: Canonical correspondence plots of pathogens, patients and patient variables

Patient variables entered into the CCA included age, Barthel score, clinical frailty score, Charlson index, dementia, number of teeth, plaque quartile score at admission, weight, presence of HAP and presence of HAP/LRTI, along with colonisation indices per organisms and per patient. In the CCA plot (Figure 14) the first axis appears to represent a trend from (positive scores) frail individuals to those with teeth (negative scores), which probably represents a spectrum of frailty, with fitter patients possessing more teeth. The second (y) axis was less clear, although the Barthel and Charlson indices were closest to being parallel to this axis. This implies that high Charlson scores were associated with low (negative) second axis scores.

S. pneumoniae colonisation was associated with increased number of teeth and increased independence, as found previously in this Chapter. *S. pneumoniae* and *S. aureus* co-colonisation were associated with higher Barthel score (meaning more independent persons). Increased frailty (measured by the clinical frailty score) and increased Charlson comorbidity index were associated with co-colonisation with MRSA, Acineobacter spp and *P. aeruginosa*. Interestingly, *H. influenzae* and *E. coli* co-colonisation were seen in a number of patients. The results from multivariate GLMs earlier in the chapter showed that both *E. coli* and *H. influenzae* were associated with increased frailty. *H. influenzae* was geographically distant from MRSA. Patients colonised with MRSA were typically community dwelling and less frail, whereas the reverse was true of patients colonised with *H. influenzae*, which may account for the plotted distance between these variables. In summary, the CCA reiterates what has already been found using multivariate GLMs, but adds that certain bacteria co-colonise recurrently, and in association with defined patient characteristics.

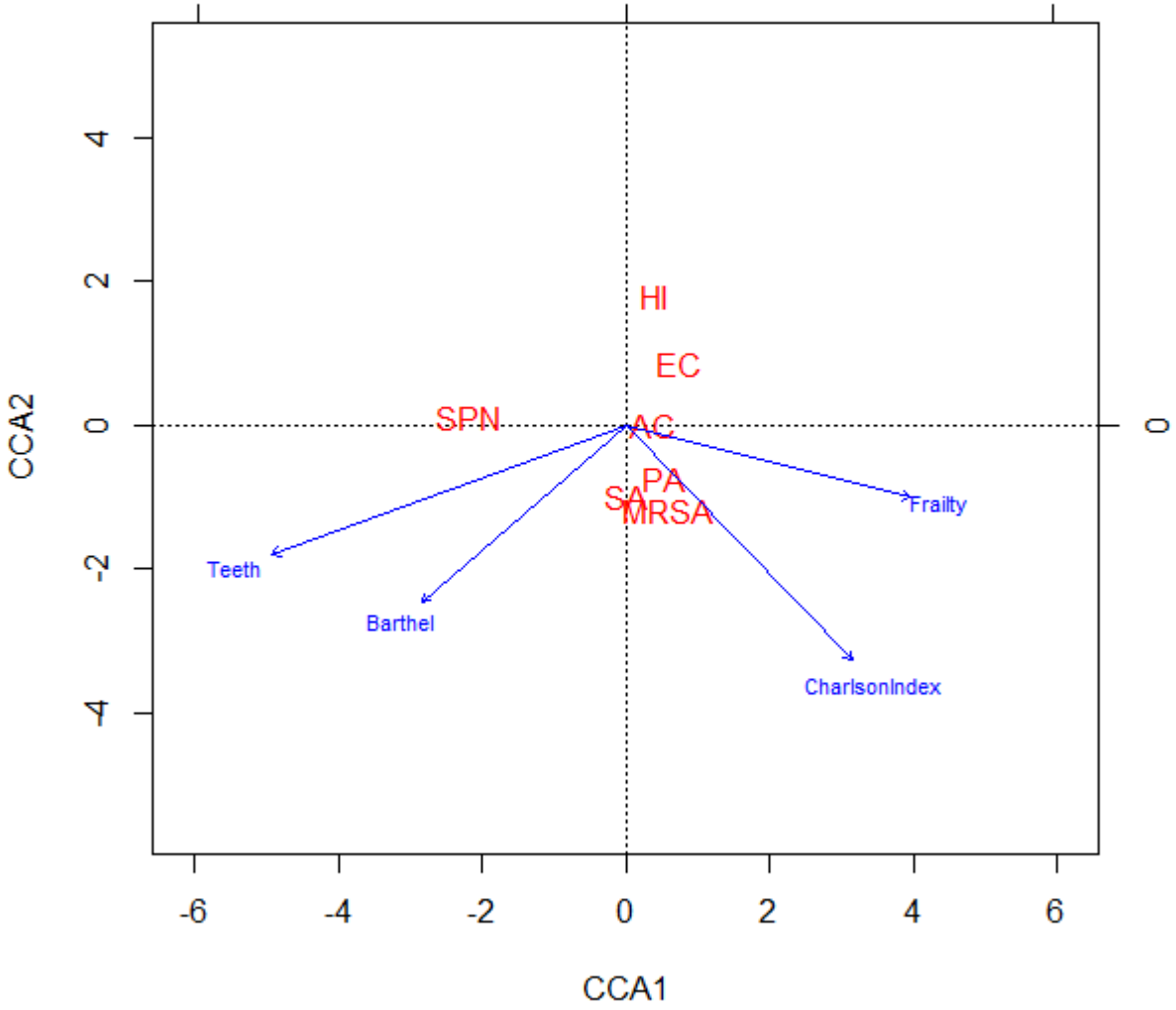


Figure 14. Canonical correspondence analysis demonstrating relationships between colonisation indices of target bacteria and demographic variables in study patients

5.4 Discussion

The covariates with the greatest effect size on HAP were in-hospital events such as aspiration, falls, post-operative cough, presence of delirium. Falls and delirium either preceded or occurred simultaneously with HAP, and may have been associated with the presentation of HAP itself rather than risk factors per se. It should be noted that apart from falls, the other three variables were observed and noted by the primary investigator at times of study visits, and were not measured objectively. The effect sizes of significantly associated covariates in this study, such as Charlson comorbidity index or Clinical frailty score were small.

Aspiration and function of swallow were not objectively tested as the aim of the study was not to record aspiration pneumonia. Rather aspiration episodes were observed and recorded by the study investigator as part of routine follow up. Thus the strength of effect of aspiration on HAP is likely to be overestimated in this study, and needs further research in this population. However assessment of swallowing function by speech and language therapists or other trained personnel may identify patients at higher risk of HAP. Similarly the primary reason for noting both cough and delirium was as evidence that HAP was absent, rather than the reverse. These results may suggest that the simple (and free) bedside test of simply asking the patient if they have a cough might be a predictor of subsequent HAP, in keeping with a previous study [56], and warrants further investigation.

The association between HAP and witnessed aspiration episodes begs the question whether the cases of HAP were in fact aspiration pneumonia. The other important question is whether there is any value dividing the two conditions. Aspiration pneumonia is diagnosed by clinical suspicion of dysphagia or impaired cough reflex in a patient with pneumonia [105], but does not have an associated objective diagnostic criteria, and the episodes of aspiration are usually unwitnessed. The three patients who developed HAP at the same time as, or after, a witnessed aspiration episode would therefore be defined as having aspiration pneumonia. Marik argues that pneumonia

secondary to aspiration of oropharyngeal contents should be considered separately to both community and hospital acquired pneumonia [105], despite acknowledging that all respiratory pathogens must be aspirated into the lungs to cause pneumonia of any type, and that aspiration occurs during sleep in healthy subjects [132]. Aspiration of oropharyngeal contents can lead to CAP as well as HAP in older persons [268], and the underlying insult , clinical syndrome and treatment are the same whether "aspiration" has taken place or not. Careful studies of underlying aetiology in patients with aspiration pneumonia revealed that the most frequent bacterial causes were *S. pneumoniae*, *S. aureus*, *H. influenzae* and Enterobactericae [104, 269], and anaerobic bacteria (Streptococci) were isolated in only two patients (though antibiotics had already been given). Aspiration of orophayngeal contents may be better treated as a risk factor for pneumonia, rather than considering aspiration pneumonia as a separate entity. Risks for aspiration include decreased conscious level, dysphagia and intestinal obstruction [269]. However, presence of nasogastric tube or endotracheal tube ought also to be included as risk factors for aspiration, in that biofilms develop on both [118, 135], facilitating the transmission of bacteria to the lungs. However aspiration of acid, oils, vomitus, food or other foreign body are more likely to cause a chemical pneumonia or aspiration pneumonitis, and these conditions would be better served by being defined as a separate condition, because infection is less likely to be present. The presence of a nasogastric tube is an important risk factor in this regard, but data on nasogastric tube insertion was incomplete in this study and were therefore not analysed. However both patients who had nasogastric tubes inserted developed HAP, one before and one after nasogastric tube insertion. Nasogastric tube insertion indicates a person at risk of aspiration (the indication for a tube is either due to dysphagia or bowel obstruction) and also provides a reservoir of oropharyngeal bacteria to the tracheal entrance. Therefore even nasogastric tube insertion after HAP has occurred identifies aspiration as a risk factor for HAP.

HAP was associated with more persistent colonization of *E. coli*, in keeping with a previous study conducted in intensive care patients which found *E. coli*

in dental plaque to be associated with nosocomial infections [42]. HAP/LRTI was associated with increased colonization with *S. aureus*, and there was slight evidence of an association with persistent colonisation with MRSA. A previous culture-based study identified salivary colonisation with *S. aureus* as a risk factor for aspiration pneumonia [101]. Terpenning et al. found that aspiration pneumonia was associated with increased number of teeth, S. sobrinus or *S. aureus* in saliva and P. gingivalis in dental plaque [29]. However these results need to be interpreted in light of the sample size, as is discussed below.

Neither HAP nor HAP/LRTI was associated with acquisition of respiratory commensal pathogens. Colonisation is thought to be more significant than acquisition as adherence suggests evasion of host immune defences (although this assumes that the body recognises the bacterium as a pathogen rather than a commensal). The main disadvantage of using the colonization index was that geographical repetition (tongue and throat positive) was considered equally with temporal repetition (sample 1 tongue and sample 2 tongue). However, wider geographical distribution of the organism may constitute greater load and it was decided that tongue and throat samples should be considered separately rather than combining the two sets of results. A similar approach was also used in the Candida colonization index [267].

In contrast with two previous studies which found a relationship between plaque score and respiratory infection in dentate institutionalised older persons [44, 45], in this study HAP was not associated with any measured dental variables. Abe et al. only included 62/81 patients within the analysis, for reasons which were unclear [45]. Mojon et al. used only clinical findings to diagnose pneumonia, with follow up time of 1 year [44], which may have increased the proportion of patients diagnosed as pneumonia.

Factors associated with oral colonization with respiratory commensal pathogens appeared to be pathogen specific. *S. pneumoniae* colonization occurred in generally fitter individuals, whereas *H. influenzae* colonization appeared commoner in frailer patients. *H. influenzae* colonization was associated with COPD which would be expected given that *H. influenzae*

infections are so common in patients with COPD. COPD in turn was associated with lower age (in the correlation matrix) which explains why *H. influenzae* was simultaneously associated with lower age and increased Barthel index. Despite *S. pneumoniae* being one of the commonest causes of pneumonia, increased colonization was neither associated with HAP nor with frailty factors. One study suggested that biofilm formation necessary for establishing colonization is enhanced by the lack of the capsule, a major determinant of virulence [270]. Where there is a strong antibody response to a commensal organism, it is usually cleared, but *S. pneumoniae* is known to persist for 2-4 weeks in healthy adults [256]. Antibody response to an organism may be generated by colonization without infection [271]. However it is unclear whether there was a benefit to patients being colonised with *S. pneumoniae*, and it is more likely that colonization was related to epidemiological contacts.

It is unclear why *S. pneumoniae* was associated with increased numbers of teeth (and correspondingly why *H. influenzae* was associated with fewer teeth) and it seems likely that number of teeth is a confounder for health status, rather than being causally associated. There is no other literature suggesting an association between these organisms and tooth number. The correlation matrix showed that having fewer teeth was associated with increased age, but not the individual functional scores. These observations require further investigation. Data on previous vaccination against *S. pneumoniae* were not collected, and this might be an important variable to investigate in future to explain some of the variation seen.

Colonisation with *P. aeruginosa* and *Acinetobacter* spp were not explained by any of the variables in either model. This is unlikely to be due to these organisms being infrequent colonizers (4% and 2% of study patients respectively), as *E. coli* only colonized 2% of study patients, and was found to be associated with several study variables. VAP due to *P. aeruginosa* has been associated with increased length of mechanical ventilation, increased age, use of antibiotics at admission and transfer form a medical unit or ICU [272]. In one study of intensive care patients, *Acinetobacter* colonization only occurred after spending

two weeks on intensive care [266]. Previously identified risk factors for nosocomial *Acinetobacter* infection include increased length of stay, increased bed transfers, increased duration and number of antibiotics, prior colonization with *A. baumannii*, use of central venous catheters, mechanical ventilation and cardiovascular or respiratory failure [266, 273]. It may be that patients in this study did not frequently exhibit these findings during the sampling period, or that variables important in determining colonization with these organisms were not collected in this study, perhaps such as changes in the structure of the oral microbiota or changes at a cellular immunity level. In addition, in the case of *Acinetobacter* spp., the assay may have detected non-pathogenic species, which could have contributed to non-significant analysis with patient factors. In the CCA, co-colonisation with *Acinetobacter* spp, *P. aeruginosa* and MRSA appeared to be associated with increased frailty, suggesting a common underlying set of risk factors.

Colonisation with *S. aureus* and MRSA was explained by similar risk factors, as would be expected given their genetic similarities, which included current smoking, male sex, increased Charlson score and recent antibiotics use. The latter two risk factors indicate likely prior contact with healthcare settings. Interestingly though, both the CCA and the multivariate GLM (dental model) indicated that both MRSA and *S. aureus* were more frequently seen in home dwelling patients rather than those from institutions or transferred from hospital. It is unclear why males would be more susceptible to *S. aureus* colonization than females. Colonisation with *S. aureus* was associated with fewer teeth (in the medical model), and increased dental plaque (in the dental model), though both variables were included in both models. In addition MRSA was also associated with fewer teeth in the medical model only. There appeared to be conflict between explaining *S. aureus* colonization using the medical and dental models for variables relating to frailty. In the medical model, dementia and inability to self-consent were significant, but in the dental model, higher Barthel index (more independent) was significantly associated. The reasons for this are unclear, and it may be that several groups of persons are at risk of *S. aureus* colonization (given that it is a commensal even in healthy

adults). Structural equation modeling might help understand better the relationships between *S. aureus*/MRSA colonization and these variables.

Colonisation with *S. pneumoniae* did not inhibit colonization with other organisms such as *S. aureus* and *E. coli*. In previous studies of colonization in children, *S. pneumoniae* has been shown to decrease the colonization rate of *S. aureus* [274], and in turn, increased pneumococcal vaccine uptake has been associated with an increase in otitis media caused by *S. aureus* infection in children, with co-colonisation with non-vaccine type *S. pneumoniae* [275]. Mouse models suggested that antibodies to *S. pneumoniae* cross-reacted with *S. aureus* and inhibited its ability to colonise [274]. However, given that antibody production and function appears to decline with age [276], these effects may be less pronounced in older patients.

Colonisation with *S. aureus*, MRSA, *S. pneumoniae*, *H. influenzae* and *E. coli* was associated with current and/or previous smoking, in keeping with previous studies [43, 277-279], and suggests a common underlying mechanism. However one study suggested that cigarette smoke inhibited growth of *S. pneumoniae* and *S. aureus*, rather promoting growth of Klebsiella, Enterobacter and Pseudomonas [280]. Underlying mechanisms contributing to altered bacterial community structure are likely to be multifactorial. One study found an initial pro-inflammatory host response to colonization with respiratory commensal pathogens specifically in smokers [277]. Other mechanisms may include decreased mucociliary clearance by respiratory epithelial cells and increased adherence to respiratory epithelial cells in smokers [281, 282]. Both bacterial and viral infections are also commoner in smokers or those exposed to tobacco smoke, which may relate to impaired cellular and humoral immunity, and changes to epithelial cells in the respiratory tract [281, 282]. Pulmonary defences against *H. influenzae* specifically may be impaired in smokers [283]. Smokers also appear to exhibit altered oral bacterial communities in general, with changes found including fewer alpha-haemolytic Streptococci [284], a more diverse and anaerobic flora [285] and increased prevalence of bacteria belonging to Socransky's "red" and "orange" group bacteria [286]. Another

interesting finding has been that all the pathogens tested for in this study have been identified from cigarettes themselves using 16s PCR [287].

It was interesting to note that *S. pneumoniae* and *S. aureus* appeared to be associated with less deprived persons and *H. influenzae* with more deprived persons. Previously colonization with both *S. pneumoniae* and *H. influenzae* has been associated with deprivation in children [256]. Again this may be a confounding effect, given that *H. influenzae* was associated with COPD which is in turn associated with smoking, a factor correlated with deprivation. *H. influenzae* was also associated with denture wearing, and denture wearing was in turn correlated with increased deprivation. Respiratory pathogens have previously been shown to colonise dentures [288], but denture wearing in general was not strongly associated with colonisation with other pathogens. It is unclear whether denture wearing was a confounder for deprivation or whether there was a direct association between denture wearing and *H. influenzae* colonisation.

In the dental model, only oral colonisation with *S. aureus* was associated with higher admission plaque scores. Given that the pathogens were identified from tongue and throat rather than dental plaque, it was not possible to determine whether *S. aureus* was associated with mature plaque or whether both are confounders for poor general health, and this is an area for future research. This work attempted to stratify patient risk of HAP by the extent of oral colonization with respiratory commensal pathogens, and sampling tongue and throat yielded more target pathogens than did sampling dental or denture plaque [245]. While respiratory pathogens have previously been identified in dental plaque [140, 153, 289], two studies found no link between dental plaque index and colonisation of dental plaque with respiratory pathogens [42, 153]. There are several possible reasons for this. First, dental plaque may not influence the development of pneumonia by being the stable source of respiratory pathogens but may indirectly affect the risk of HAP by other means. Second, it may be that adherence to dental plaque is pathogen dependent. Fourrier et al. found that *S. aureus* in dental plaque was not linked to

subsequent VAP, while colonisation with gram negative rods was "highly predictive" of VAP [42]. HAP has been found more commonly in dentate persons [44, 101], and if these findings can be believed (studies have not been powered by numbers of edentate persons), the underlying reasons remain unclear.

One of the major limitations of this study is that caries was not included as a covariate. Aspiration pneumonia has been significantly associated with number of decayed teeth in two studies [30, 101]. Nor did we test for caries related organisms which have previously been associated with aspiration pneumonia [101]. Future research investigating the role of plaque in determining colonisation of *S. aureus* would need to examine these variables. Data were collected on blood transfusion, but this data was not analysed because it was discovered during follow up that the recording of transfusion varied in location (between notes, fluid charts, anaesthetic charts, and nursing documentation), and it could not be determined whether patients already included in the study actually had received blood products. Poor filing of loose charts and nursing documentation precluded an effective retrospective search for these data.

It should be noted that the colonization index data were zero inflated and therefore there is a possibility of over-predicting the significance of patient factors where colonization index was larger than zero. A zero could either represent genuine absence of colonization, or that a particular organism could colonise that patient but other factors (measured or unmeasured) in the environment preclude this. Nasopharyngeal sampling was not undertaken in this study, which would have been likely to increase the yield of *S. pneumoniae* in particular and possibly *H. influenzae* also [259]. However another study showed that oropharyngeal sampling of *S. pneumoniae* and *H. influenzae* in adults was comparable to nasopharyngeal sampling [290]. In addition, an organism could have been present on days not sampled. A zero could also mean a bacterial species which has a single nucleotide polymorphism (SNP) in either primer or probe locations was not detected. During validation of the

PCR assays, it was noted that a small number of bacteria previously identified in a microbiology laboratory by biochemical means were not detected by the PCR assays (see 3.4.2). A zero result depends on the sensitivity and efficiency of the PCR assays. While PCR assays can technically detect a single copy of an organism, this is not always the case if PCR efficiency is less than 100%. However PCR is more sensitive than culture.

An assumption has been made that the organisms detected by real time PCR are potentially pathogenic. It is known that both *H. influenzae* and *S. aureus* have a clonal population structure and only certain clones cause disease, while others may be non-pathogenic [235]. Assays for organisms tested in this study were designed using all sequence data available rather than only sequence data from known pathogenic clones which may mean that non-pathogenic organisms have been detected. However, the assays were tested against clinical isolates (i.e bacteria which had been isolated from an infected site) and performed well. However non-pathogenic species could have been identified by the assays.

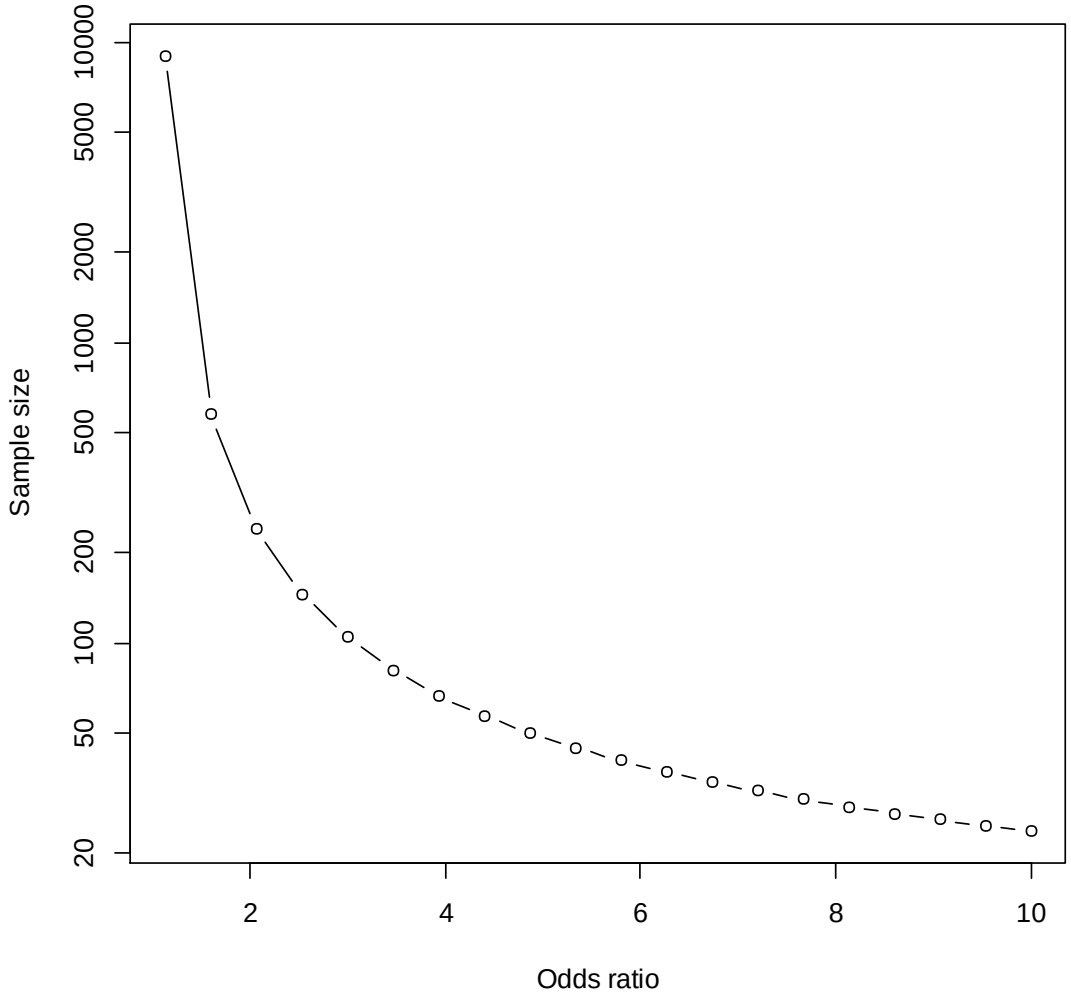
Some cross-reactivity could have occurred in the *S. aureus* and *E. coli* assays (which also therefore affected results of the MRSA assay), as described in 3.4.2, which could have produced false positive results. The *S. aureus* assay detected two organisms from the specificity panel (S. constellatus and a group C β-haemolytic Streptococcus), and the *E. coli* assay detected S. constellatus, E. facecium and a haemolytic *Streptococcus* group B. These issues occured despite in silico testing using BLAST (Basic Local Alignment Search Tool), and this demonstrates the difficulties in designing molecular assays for bacteria-rich environments. Only 14/93 (15%) patients in this study were colonised by *S. aureus*, which is lower than prevalence estimates from previous studies (23-48%) [257]. In addition, the prevalence of group C streptococci in a study of 1796 hospital in-patients was only 0.3% [291] and S. constellatus was detected in 1/65 persons in another study [292], suggesting that the majority of *S. aureus* detected in this study were likely to be true positives. Sequencing the false-positive strains detected is the only way of knowing whether genuine cross-

reactivity occurred or whether transformation of genes form other bacteria has occurred.

This was a relatively small study, and is insufficiently powered to detect some results found. Recruitment rate of the study was limited mainly by the number of patients refusing to participate or who could not be consented for logistic reasons (see 4.3.1). However given the number of variables collected, the prolonged and intensive nature of follow up, the time needed for DNA extraction of samples, and the concomitant development of the multiplex PCR assays, it would have been difficult to accommodate more study patients. While the sample size was relatively small, the quality of study design and implementation was high.

Figure 15 shows the sample sizes needed to detect a series of odds ratio in binomial logistic regression with 80% power and a 5% significance level [293]. This study was adequately powered to distinguish between variables where the odds ratio was just under 4, but insufficiently powered to determine smaller differences than this. This includes the findings relating HAP and HAP/LRTI with *E. coli* and *S. aureus* colonization (whose odds ratios were 1.65 and 1.53 respectively). Therefore these findings need to be corroborated in a larger study.

Figure 15. Range of sample sizes required to detect given effect sizes (odds ratios) with 80% power and at 5% significance level



Spinal anaesthesia was only used in 8% of study patients, as is the practice in Newcastle upon Tyne Foundation Hospitals, while in other hospitals spinal anaesthesia would be the norm when operating on hip fractures in particular. No airway manipulation is undertaken with spinal anaesthesia which may reduce the risk of HAP. Medically unwell patients were excluded so results are not generalisable to this group. This study only sampled patients for the first 14 days after admission, and oral bacterial communities may have changed after this time, affecting the risk of HAP at a later point in the patient's admission. Data on time to ambulation after surgery were not recorded. One study suggested that increased time to ambulation was associated with post operative pneumonia and delirium [294].

The findings from this study suggest that future studies should focus on establishing if dentate persons are at higher risk of HAP and identifying underlying mechanisms, in order to inform intervention studies. Future oral hygiene intervention studies to prevent HAP should not be targeted only towards those people with heavier plaque. In addition, next generation identification methods could be used to identify changes in microbial communities and identify as yet unknown pathogens, given that the aetiological agent is found in less than 60% of cases of HAP in older people [17].

5.4.1 Conclusions

This is a relatively small study but is one of the few which combines medical, dental and microbiological findings with HAP defined with chest radiograph as an outcome. Colonisation with respiratory commensal pathogens is likely to be both a confounder (between frailty/illness and HAP) and the source of infecting organism(s). The risk of HAP is therefore likely to be determined by the balance between overarching patient factors (which affect the oral flora and increase the risk of HAP) and the risk of aspiration. The relationships between OCRP, oral hygiene and HAP are clearly complex. Given that *S. aureus* was associated with dental/denture plaque and also with HAP, further research into the relationships between these variables, and into oral hygiene as an intervention in pneumonia appear warranted.

Chapter 6 Discussion

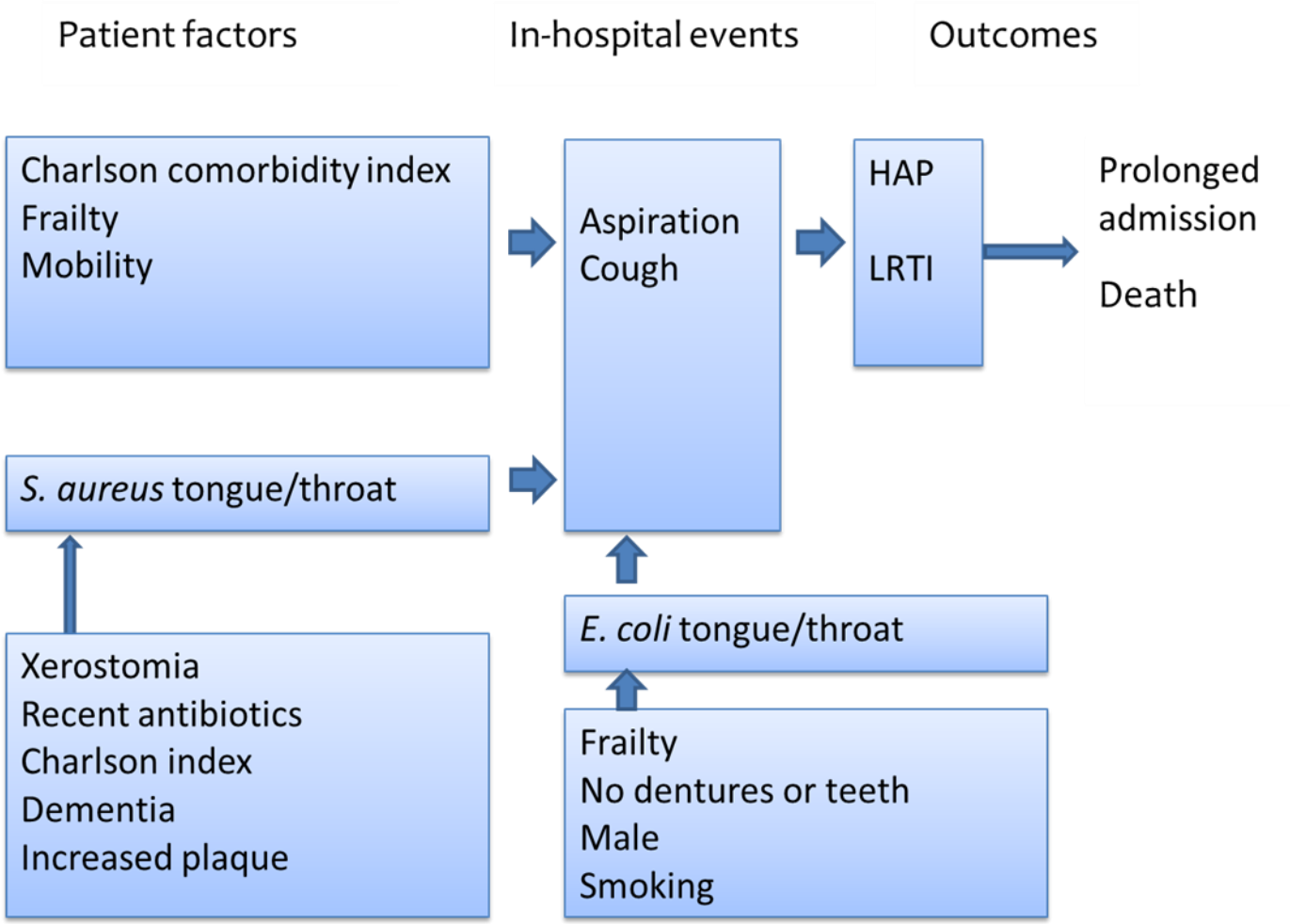
Hospital acquired pneumonia (HAP) is a common and frequently fatal complication after lower limb fracture in UK patients, and currently no strategies for prevention are routinely employed in UK hospitals in patients on orthopaedic or other general hospital wards. In this study, the incidence of HAP and HAP/LRTI were 11% and 20% respectively, and 60% of deaths in hospital and 40% of deaths over the whole study period were preceeded by HAP. Of the 70-75,000 patients who fracture a hip annually then [51], approximately 7,500 will develop HAP and 15,000 will develop HAP/LRTI. Taking a more conservative estimate of 40% mortality, this would translate to 3,000 deaths from HAP after hip fracture, which would account for 12% of the 25,696 pneumonia deaths in England and Wales in 2011.

The most striking finding in this study is that oral carriage of "hospital" type bacteria begins, in the most part, within 72 hours of hospital admission, challenging the idea that these organisms are hospital-acquired. While results from this study cannot discern whether the organisms were simply acquired early in admission or were present in the community, these findings suggest that further study of oral carriage of potential respiratory pathogens is needed, perhaps in elective surgical patients. Results from this study suggest that certain patient characteristics seem to encourage carriage with particular organisms or combinations of organisms, and this is upheld by patients from settings such as nursing homes or with underlying disease tending to be infected by "hospital" type bacteria. It is possible that while the environment remains important, the host is the key determinant of the nature of colonizing organisms. This concept challenges current thinking about the origin of hospital acquired infection. In order to reduce the risk of infection in hospitalized patients in the future, we need to better understand the composition and dynamics of colonization at "entry sites" and how these are affected by illness, immune function and other external factors. One could

hypothesise that manipulation of organisms or immunity in the mouth could lower the risk of a variety of infections, both in the hospital and in the community.

In this study, HAP was associated with host frailty and prior oral colonisation with respiratory commensal pathogens, but the strongest determinants for the development of HAP were witnessed aspiration episodes and post-operative cough. These findings imply that interventions to prevent HAP should be directed toward patients with the above risk factors. Overall the results suggest that colonising respiratory bacteria only act as pathogens when permitted to do so by breaches in host defences. However, colonisation with respiratory bacteria was also associated with pathogen specific host factors, inferring that adverse health of the host promoted both colonisation and subsequent respiratory infection with respiratory pathogens. Figure 1 shows a diagram demonstrating these relationships. Thus colonising respiratory commensal pathogens were simultaneously both confounders for frailty associated host factors and risk factors for HAP.

Figure 16. Relationships between patient factors, in-hospital events and respiratory tract infection in older patients with lower limb fracture



HAP was associated only with colonisation (and not acquisition) of respiratory commensal pathogens, in keeping with established dogma. Colonisation occurred early within the sampling period and is likely to represent flora acquired in the community rather than in the hospital. However, given that the sampling period was only 14 days, it is not possible to comment on late acquisition of respiratory commensal pathogens. The median onset of HAP was later than quoted in other studies (27 days compared with 7-11 days in other studies [21, 22, 26]) and a longer sampling period may have revealed late colonisation with respiratory commensal pathogens in some patients.

Events that facilitate the delivery of potential pathogens to the lungs appears to be key risk factors for the development of HAP, and are common to the groups of patients at particular risk of HAP (e.g. patients on intensive care, stroke patients). However, given that all pneumonias involve aspiration to some degree [105], the concept of aspiration pneumonia perhaps ought to be discarded, and aspiration considered simply as a risk factor for pneumonia. Few previous studies have undertaken objective measures of aspiration, and further research is needed to correlate the risk of HAP with the degree of aspiration in order to stratify patients without clinically obvious aspiration risk. Delirium was also associated with HAP, and changes in conscious level are central to the diagnosis of delirium. It was not possible to determine whether delirium contributed to an increased risk of aspiration, leading to HAP, or whether aspiration occurred independently which gave rise to HAP which then led to delirium. The truth may be a combination of both of these, with delirium resulting in further aspiration, and increased and continual inoculum of oral bacteria transmitted to the lungs, however the sample size was too small to disaggregate these risk factors.

HAP was the most prevalent healthcare associated infection in the 2011 English National Point Prevalence Survey of Healthcare Associated Infection [20], and nationally coordinated efforts to prevent the occurrence of HAP are needed. Given the mortality associated with HAP in this and other studies, investigating the efficacy of putative interventions such as disinfection of oral surfaces,

management of xerostomia and removal of denture and dental plaque appear warranted in non-ventilated patients. The MRSA decolonisation protocols already in place in many NHS hospitals may offer a starting place for such interventions, given the importance of S.aureus in the development of subsequent HAP/LRTI. While such interventions are primarily directed towards *S. aureus*, it is likely that they would be equally effective in preventing colonisation of *E. coli* and other respiratory pathogens in those at risk of HAP, due to the inclusion of chlorhexidine 0.12% mouthwash. Interventions to prevent HAP may also be usefully investigated in other settings such as nursing homes, stroke units and colorectal and upper abdominal surgical wards, all of whom have relatively high rates of HAP. Managing xerostomia would need to be addressed separately, and a further study is needed to verify the association with *S. aureus*, and to trial therapies such as rehydration, avoidance of certain drugs, and use of lubricants such as oxygenated glycerol triesters [295], with *S. aureus* colonisation and subjective assessment of xerostomia as endpoints.

Other approaches which have not been adequately trialled in non-ventilated patients include the use of probiotics [296], or the introduction of specific bacteria which can inhibit the colonisation of others, known as bacterial interference [297]. Iwase et al. found that the introduction of Staphylococcus epidermidis which possessed the serine protease "Esp" was able to inhibit colonisation with *S. aureus* and destroy previous biofilm formation [298]. Antibodies to *S. pneumoniae* have been shown to cross-react with *S. aureus*, decreasing nasal colonisation with the latter [274]. Introduction of non-pathogenic *E. coli* into the intestines of patients with ulcerative colitis have been shown to prolong the maintenance phase, when combined with mesalazine treatment [299]. It is likely that as we begin to understand how bacterial communities interact using next generation sequencing techniques, our ability to manipulate the resident flora on body surfaces will no longer be limited simply to eradication.

HAP was not directly associated with dental factors in this group, though colonisation with individual respiratory pathogens were significantly

associated with number of teeth, amount of dental and denture plaque at admission and sensation of xerostomia during hospital admission. Thus HAP may be indirectly related to the presence of certain dental factors via colonisation of putative aetiological pathogens. Given that HAP has also been previously associated with number of decayed teeth and presence of P. gingivalis [29, 30], further research is needed to understand these relationships better. The exclusion of caries and P. gingivalis identification, which are potentially important risk factors, is one of the important limitations of this study. P. gingivalis has been found to modify both commensal oral flora and the local host immune function to promote periodontal disease[143], and may also prove to be important in the development of HAP. Further work is needed to understand the relationships between dentition, dental and denture plaque, caries and the oral microbiota in more detail.

Perhaps the major limitation to this study is its size, in part due to fewer patients than expected presenting with hip fracture during the study period, and the study was not powered to make some of the associations drawn from the results. However the detail involved in follow up of this study would perhaps be too great for a larger or multi-centre trial. Now that several studies have made similar conclusions regarding significant patient risk factors, follow up in future studies could be simplified to include only important risk factors.

Yield of respiratory commensal pathogens from sampling the oral microbiota could have been increased by either taking an oral rinse sample or by the addition of nasopharyngeal samples [245, 259, 300]. However the former would have necessitated the exclusion of patients with moderate to severe dementia and the latter may have been less acceptable to patients (nasopharyngeal samples are uncomfortable). A compromise would be to add a simple nasal swab, accepting that the yield of some bacteria would probably be lower.

It is known that influenza and other viruses such as respiratory syncytial virus may facilitate infection by *S. pneumoniae* [75, 76, 301, 302] and that HAP may be caused by viruses [303]. By not testing samples for viruses, important factors in the development of colonisation with respiratory commensal pathogens and

HAP may have been overlooked. However both cost and time played a factor in the decision not to analyse samples for viruses, and this is an area which could be addressed in the future using duplicate samples taken during the study. In addition, the local oral immune response, was not assessed in relation to colonising pathogens. It is likely that immunosenescence plays a prominent part in determining whether both colonisation and infection are successful in older patients, and much work is needed in this area. Another important risk factor which was not assessed was alcohol intake. Pneumonia is known to be more common in those with higher alcohol intake [304]. There was insufficient information in patient admission documentation to ensure complete ascertainment of this risk factor, and future studies of colonisation with respiratory commensal pathogens should include this risk factor.

Though the real time multiplex PCR assays designed for this study coped well with bacterially rich samples, manufacturer contamination of reagents meant that only strongly positive results were accepted, which may have led to the exclusion of true weak positive results in some patients. In addition, there is a risk that cross-reactivity occurred between both the *S. aureus* and *E. coli* assays and certain Streptococcal samples. Without sequencing the relevant samples, it is not possible to determine whether any cross-reacitvity may have occurred during testing of study samples, but given the relatively small numbers of patients who had positive tests with these assays, any effects from cross-reactivity are likely to be small. A number of patients appeared to be colonised by a non-*E. coli* coliform (Appendix F), however these results could not be analysed in relation to patient factors due to significant contamination of reagents seen in water and non-template samples. In addition, lack of whole genome sequence data precluded design of assays for *Klebsiella pneumoniae*, *Serratia marcescens* and Enterobacter *cloacae*. While the latter two organisms are not common causes of HAP, *K. pneumoniae* is, and its exclusion may have meant clinically important results were not seen. As better software facilitating the comparison of entire genomes becomes available, it is likely that assays will be designed to identify specific Enterobactericeae, and similar studies as this will

be needed to assess the dynamics of colonisation with these organisms, with particular emphasis on frailty.

This study is among the first to investigate HAP in relation to the risk of aspiration episodes, *S. aureus* colonisation and increased frailty and comorbidity simultaneously and prospectively. While several groups have investigated HAP in surgical patients or nursing home residents, few have investigated HAP in patients with lower limb fracture, and risk factors common to both the former groups were identified in the latter. Studies investigating factors associated with colonisation rather than infection are also rare in non-ventilated patients, and the combination of respiratory pathogens investigated in this study is novel in this group. Important risk factors for colonisation with *S. aureus* have been identified in this work, which may lead to the design of more robust and effective intervention protocols. In addition, this study is unique in identifying colonisation with *E. coli* as important in non-ventilated patients.

Another strength of this study was that a single geriatric medicine trained clinician conducted the majority of patient follow up, and the observation of aspiration episodes in particular was opportunistic and probably related to the time spent on each study visit. Importantly, diagnosis of HAP was robust and independent of the study. The investigator was blinded to oral sample results due to anonymisation and analysis of samples after patient follow up was complete. Adequate sampling was ensured by the inclusion of the GAPDH assay, meaning that all samples negative for respiratory pathogens had a positive test for human DNA .

While patient factors were more important than the components of the oral microbiota in determining whether HAP occurred, understanding how the oral microbiota changes in response to the presence of respiratory commensal pathogens, or indeed the reverse, may be crucial in understanding how to manipulate the oral flora without the use of disinfection. Recognition of changes in the oral microbiota could potentially be used as biomarkers for HAP, and the duplicate samples taken during this study will be analysed to this

end. While there do not appear to be adverse effects from disinfectants such as chlorhexidine, there may be advantages in the longer term in artificially manipulating the oral microbiota using other commensal bacteria to promote health. Next generation sequencing technology is now able to determine the composition of bacterially rich samples rapidly and accurately, to genus level, and investigation of oral samples is currently being undertaken as part of the Human Microbiome Project [305]. This technology has allowed researchers to redefine the "core" oral flora, and is likely to lead to the identification of novel organisms, some of which may be implicated in disease. Further studies of bronchoalveolar lavage samples using this technology are also likely to explain the aetiology of pneumonia in those patients in whom no causative organism is found (estimated at 40% [17]).

Further research is also needed to better understand the pathogenesis of the clinical syndrome of pneumonia at a tissue and molecular level, to enable accurate gold standard diagnosis of pneumonia clinically. Currently, invasive respiratory sampling in conjunction with chest radiography is needed to make the best diagnosis [10, 19]. However invasive sampling is rarely undertaken in older people on general hospital wards, and may be considered inappropriate in the UK in these patients due to frailty. Meanwhile, a consensus statement on the diagnostic criteria which should be used in research trials in non-ventilated patients is needed. More standardised terminology or subject headings would also improve retrieval rates when searching for articles on nosocomial pneumonia in older people and potentially improve design of future trials by allowing researchers to assess more of the relevant literature.

Perhaps another important next step is to raise awareness among clinicians of risk factors, the importance of prompt treatment and likely outcomes in HAP. It may also be useful to translate published risk factors into a clinical tool to facilitate teaching about and assessing those with this condition. In patients in locations at high risk of HAP, assessing their risk of HAP at admission may be useful in planning care. Cluster randomised intervention trials are needed to assess the efficacy of interventions discussed above.

In studies of older people in hospital, it is standard practice to assess variables at the start of admission and observe how these variables relate to an event later in admission. However this method may be flawed because it assumes that the baseline characteristics and health state of the patient remain the same, when in fact sequential events over the course of admission may modulate the risk profile of the patient either positively or negatively. Complications such as infection, pain, falls with or without head injury, dehydration, delirium, drug toxicity, urinary retention, acute renal failure, prescription of new drugs, blood transfusions and other events occur commonly in these patients during admissions to hospital. In addition, the psychological state of the patient may change, and anxiety and depression are commonly experienced by hospital patients [306, 307], and both of these, along with alcohol withdrawal and bereavement reactions were present in the patients in this study. Psychological stress has been shown to increase the rate of upper respiratory tract infections [308], and presumably also impacts on other medical conditions. In this group of patients, of whom a third are likely to die within one year, psychological factors may be just as important as the occurrence of physical illnesses in determining outcome (for example when facing necessary transition to a residential or nursing home from previously dwelling at home). Equally, patients may recover, and risk factors noted at admission may have improved with time. Structural equation modelling may be one method of analysing complex and dynamic longitudinal data in future work.

5.5 Conclusions

HAP was a significant clinical problem in the group studied, and was associated with host frailty, oral colonisation with specific respiratory commensal pathogens, witnessed aspiration episodes and post-operative cough. In turn, colonisation with respiratory commensal pathogens was associated with oral and dental risk factors, in addition to host frailty factors. These risk factors identify patients at high risk of HAP and suggest *E. coli*, *S. aureus*, dental plaque and dry mouth as possible targets for future intervention studies. Further studies are needed to determine whether organisms causing HAP are hospital or in fact community acquired commensal pathogens.

Chapter 7 Appendices

7.1 Appendix A.

7.1.1 Columbia blood agar

Columbia agar base (Oxoid Ltd, UK) containing horse blood.

7.1.2 Skimmed milk, tryptone, glucose glycerol (STGG)

Made at Freeman Hospital Microbiology laboratory. STGG was made by dissolving 10ml glycerol, 0.5g glucose, 3.0g oxoid tryptone soy broth and 2.0g of skimmed milk powder into 100ml distilled water. Aliquots of 1ml of STGG were added to sterile 2ml microtubes.

7.1.3 Chocolate agar Made at Freeman Hospital Microbiology laboratory.

7.1.4 Amplitaq Gold DNA Polymerase

(containing 250U of 5U/µl Amplitaq Gold) with GeneAmp 10x PCR Gold buffer (containing 150mM Tris-HCL, pH8.0, 500mM KCl) and 25 mM magnesium chloride solution (Applied Biosystems).

7.1.5 Tris Borate EDTA (TBE) Buffer

100ml TBE buffer 10x solution (Severn Biotech Ltd, UK) was added to 900ml deionised water and 100µl of ethidium bromide added to make 1 litre of TBE buffer with ethidium bromide concentration 0.5µl/ml.

7.1.6 Agarose gel electrophoresis loading buffer

BlueJuice™ 10x Gel loading buffer which contained 65% (w/v) sucrose, 10mM Tris-HCl (pH 7.5), 10mM EDTA, and 0.3% (w/v) Bromophenol blue. The recommended concentration was 2x (one part buffer with 4 parts sample).

(Invitrogen, Life Technologies Ltd, Paisley , UK)

7.1.7 Agarose

Microsieve (3:1) for DNA 500-1500bp was used to create agarose gels for electrophoresis (Flowgen Bioscience Ltd, UK).

7.1.8 DNA ladder

Superladder low 100bp ladder 100µg/ml (Thermo Fisher Scientific, Surrey, UK).

7.1.9 PCR water

Sigma-Aldrich Company Ltd, UK

7.1.10 Deoxynucleotide triphosphate bases

For PCR 10mM mix with dTTP (Applied Biosystems, Warrington, UK) containing 2.5mM of each dATP, dCTP,dGDP and dTTP.

7.1.11 Ethanol

7.1.12 DNeasy blood and tissue kit

For purification of DNA from bacteria was obtained from Qiagen UK Ltd.

7.1.13 Ethidium Bromide

10mg/ml, Invitrogen, Life Technologies Ltd, Paisley , UK

7.1.14 Tris-EDTA buffer

Contains 1.0M Tris HCl and 0.1M EDTA (Sigma-Aldrich Company Ltd, UK)

7.1.15 Triton X

Made by Sigma-Aldrich Company Ltd, UK 100x.

7.1.16 Lysozyme

From chicken egg white 100mg/ml, made by Sigma-Aldrich Company Ltd, UK

7.1.17 Taqman® Universal PCR Master mix

Applied Biosystems (Warrington, UK)

7.1.18 Probes

5'FAM, 3'Deep dark quencher II and 5'CY5-3'Black hole quencher 1 probes from Eurogentec Ltd (Southampton, UK)

Taqman VIC-TAMRA probe from Applied Biosystems (Warrington, UK)

7.1.19 Oligonucleotide primers

Oligold oligonucleotide primers were manufactured by Eurogentec Ltd, Southampton, UK

7.1.20 Salmon sperm DNA 10mg/ml

Invitrogen, Life Technologies Ltd, Paisley , UK

7.1.21 Moltaq 16s DNA Polymerase

Molzym, VH-bio Ltd, Gateshead, UK

7.1.22 Exonuclease III

Promega UK, Southampton, UK

7.2 Appendix B. PCR assays targeted towards organisms causing HAP retrieved from Medline search (October 2008)

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
P. aeruginosa	16s RNA	Forward 480 GTT ACC AAC AGA ATA AGC Reverse 1240 TTG TAC CGA CCA TTG TAG Probe 640 AGC TCA GTA GCT TTT GGA		[309]
P. aeruginosa	16S RNA	Forward GTG CCT GCA GCC GCG GTA AT Reverse TGC GCC ACT AAG ATC TCA AG		[200]
P. aeruginosa	oprL	PAL1 ATG GAA ATG CTG AAA TTC GGC PAL2 CTT CTT CAG CTC GAC GCG ACG		[219]
P. aeruginosa	OprL	PAL1 ATG GAA ATG CTG AAA TTC GGC PAL2 CTT CTT CAG CTC GAC GCG ACG OprL-FL TGC GAT CAC CAC CTT CTA CTT CGA GT OprL-LC CGA CAG CTC CGA CCT GAA G		[310]
P. aeruginosa	16S rRNA /oprL	oprL PAL1 ATG GAA ATG CTG AAA TTC GGC PAL2 CTT CTT CAG CTC GAC GCG ACG 16s rRNA Forward GAG GAA GGT GGG GAT GAC GT Reverse AGC CCC GGG AAC GTA TTC AC		[311]
P. aeruginosa	exoS, exoT, exoU, exoY	Exo S MP5 GCG AGG TCA GCA GAG TAT CG MP3 TTC GGC GTC ACT GTG GAT GC exoT		[312]

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
		MP5 AAT CGC CGT CCA ACT GCA TGC G MP5 TGT TCG CCG AGG TAC TGC TC exoU MP5 CCG TTG TGG TGC CGT TGA AG MP3 CCA GAT GTT CAC CGA CTC GC exoY MP5 CGG ATT CTA TGG CAG GGA GG MP3 GCC CTT GAT GCA CTC GAC CA		
P. aeruginosa	ALGd GDP Mannose dehydrogenase	VIC1 TTC CCT CGC AGA GAA AAC ATC VIC2 CCT GGT TGA TCA GGT CGA TCT		[218]
Pseudomonas sp	16s rDNA	PA-GS-F GAC GGG TGA GTA ATG CCT A PA-GS-R CAC TGG TGT TCC TTC CTA TA		[313]
P. aeruginosa	16s rDNA	PA-SS-F GGG GGA TCT TCG GAC CTC A PA-SS-R TCC TTA GAG TGC CCA CCC G		[313]
P. aeruginosa	23s rRNA	PaFP TCC AAG TTT AAG GTG GTA GGC TG PARP CTT TTC TTG GAA GCA TGG CAT C Probe- PA23FAM AGG TAA ATC CGG GGT TTC AAG GCC		[314]
P. aeruginosa	gyrB	gyrB-398 CCT GAC CAT CCG TCG CCA CAA C gyrB-620 CGC AGC AGG ATG CCG ACG CC Probe: SYBR green		[315]
P. aeruginosa	ecfx	ECF1 ATG GAT GAG CGC TTC CGT G ECF2 TCA TCC TTC GCC TCC CTG		[228]
P. aeruginosa	ETA	ETA1 GAC AAC GCC CTC AGC ATC ACC AGC		[316]

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
		ETA2 CGC TGG CCC ATT CGC TCC AGC GCT Probe ETA3 AGC CAC ATG TCG CCG ATC TAC ACC		
P. aeruginosa	algD, nested	Initially PAL1 GAC AGG TTG AGC TTG TGG PAL3 GAA TTC CTC CGC GAG AGC Then PAL 2 CGA ACT GGA CAA GCA GAC PAL4 GCA GAT CAC GTC CAT CAC		[317]
Pseudomonas sp	groES	Forward ATG AAG CTT CGT CCT CTG CAT Reverse GTC TTT CAG CTC GAT	Heat shock protein	
P. aeruginosa		16s rDNA F CGG YCC AGA CTC CTA CGG G R TTA CCG CGG CTG CTG GCA C GyrB F CCT GAC CAT CCG TCG CCA CAA C R CGC AGC AGG ATG CCG ACG CC ETA F GAC AAC GCC CTC AGC ATC ACC AGC R CGC TGG CCC ATT CGC TCC AGC GCT AlgD F TTC CCT CGC AGA GAA AAC ATC R CCT GGT TGA TCA GGT CGA TCT Oprl F GCT CTG GCT CTG GCT GCT R AGG GCA CGC TCG TTA GCC	Compared a variety of assays with gold standard	[214]
P. aeruginosa	Exotoxin A	ETA1 GACAAC GCC CTC AGC ATC ACC AGC		[318]

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
		ETA2 CGC TGG CCC ATT CGC TCC AGC GCT		
Pseudomonas sp	16S RNA	F GGT CTG AGA GGA TGA TCA GT R TTA GCT CCA CCT CGC GGC		[319]
P. aeruginosa	oprI	F ATG AAC AAC GTT CTG AAA TTC TCT GCT R CTT GCG GCT GGC TTT TTC CAG	PCR –Elisa	[320]
P. aeruginosa	FliC	F GCC TGC AGA TCG CCA ACC R GGC AGC TGG TTG GCC TG		[321]
P. aeruginosa	16s r RNA	F1 GGCAGT AAG TTA ATA CCT TGC T R1 CCT TAG AGT GCC CAC CCG AG F2 GCG CGC GTA AGT GGT TCA GC	In ovine fleece washings. 2nd primer for nested PCR with R1	[322]
Enterobacter sp.			No papers retrieved- only ones found related to Enterbacter sakazakii or ESBL genes or typing- but see below	
Serratia marcescens	16s rRNA	F GGT GAC CTT AAT ACG TTC ATC AAT TG (435-460) R GCA GTT CCC AGG TTG AGC C (595-613) P TGC GCT TTA CGC CCA GTA ATT CCG A (534-558)	Real time- also included resistance genes	[323]
Serratia marcescens, Klebsiella, Enterobacter	16s RNA in .	Universal forward primer GGC GGC AGG CCT AAC Universal reverse primer CAG GCA GTT TCC CAG ACA TTA CT	Taqman assay Probes but not primers specific to sequences	[324]

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
Acinetobacter baumannii	16s-23s ITS	Probe FAM- AGC AAG CTC TCT GTG CTA CCG CTC GA-TAMRA F CAT TAT CAC GGT AAT TAG TG R AGA GCA CTG TGC ACT TAA G		[325]
Acinetobacter baumannii	16s-23s	recA gene F CCT GAA TCT TCT GGT AAA AC R GTT TCT GGG CTG CCA AAC ATT AC FGAG TTT GAT CCT GGC TCA R CCG GTC CTC TCG TAC	PCR plus restriction analysis	[326]
Acinetobacter baumannii	16s-23s ITS	AGT GTG ATC TGA CGA AGA CAC ATT AAC T	Oligonucleotide probe for hybridisation not primers	[327]
Klebsiella pneumoniae	16srRNA	EMBL accession no X93214 126bp target sequence 44-170 K16SF 15-mer position 44-58 K16SR 16-mer position 155-170	Real time From blood culture bottles	[328]
Klebsiella pneumoniae	Haemolysin gene	EMBL accession no AF293352	No primers described- "PCR performed as described elsewhere…	[329]

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
			unpublished work"	
Klebsiella pneumoniae	gyrA	Genbank accession AJ292307 Sense primer 219 CTT TTC TGT TCA GAC CTG CG 238 Antisense primer 182 TAG CCC CTA ACG TCG ATC AT 163 Sense probe 273 AAT ACG GTT TAT TCA TGG CG 254 Antisense probe 141 GCT TCG AAT TGG CGA TTT CA 160		[330]
Escherichia coli	16s rRNA, ITS, 23s r RNA	UP CAA TTT TCG TGT CCC CTT CG DN GTT AAT GAT AGT GTG TCG AAA C	Used SYBR green	[331]
Escherichia coli		Various see paper	Cdt genes- Primers generated non-specific products and were not suitable for typing	[332]
Escherichia coli (and group B *Streptococcus* and Listeria monocytogenes)	16s rDNA	F AAC TGG AGG AAG GTG GGG AT R AGG AGG TGA TCC AAC CGC A	Not species specific	[333]
Escherichia coli	SfmD	F ACT GGA ATA CTT CGG ATT CAG ATA CGT R ATC CCT ACA GAT TCA TTC CAC GAA A 6-FAM CAG CAG CTG GGT TGG CAT CAG TTA TTC	Real time arm Good sensitivity/specificity	[334]

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
		G-TAMRA In Opticon 2		
Escherichia coli	16s, B-galactosidase	16S F TCT CCA GAA CAT CAT CCT G R GAG CTT GAC AAA GTG GTC GT B-Galactosidase F CTT GCC TGG TTT CCG GCA CCA GAA R AAC CAC CGC ACG ATA GAG ATT CGG G	Not specific enough	[335]
Escherichia coli	lamb	F GGA TAT TTC TGG TCC TGG TGC CG R ACT TGG TGC CGT TGT CGT TAT CC Biotin labelled probe TGC GTG ATA ACT ATC GTC TGG	PCR-ELISA Primers from Bej et al. 1990	[336]
Escherichia coli	16s rRNA	F ACA CGG TCC AGA ACT CCT ACG R GCC GGT GCT TCT TCT GCG GGT AAC GTC A SYBR Green used to quantify		[337]
Escherichia coli	16s rRNA	F CAT GCA AGT CGA ACG GTA ACA G R GCG ACG TTA TGC GGT ATT AGC PROBE TGC TTT GCT GAC GAG TGG CGG A		[338]
Escherichia coli	lacZ	No primers described	From Kane et al.	[339]
Escherichia coli	5s rRNA	F TGC CTG GCG GCC GTA GCG CG R ATG CCT GGC AGT TCC CTA CT		[340]
Escherichia coli	gfp	F CAG TGG AGA GGG TGA AGG TG R AAA GGG CAG ATT GTG TGG AC		[341]
Escherichia coli	B-	F CTT TGC CTG GTT TCC GGC ACC AGA A		[342]

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
	galacatosidase	R ACC CAC CGC ACG ATA GAG ATT CGG G		
Escherichia coli	16s	F GCT AAT ACC GCA TAA CGT TCG (168-187)		[343]
		R AAC GCT TGC ACC CTC CGT A (552-527)		
Escherichia coli	Uid, lacZ	lacZ	Lindow man	[344]
		F ATG AAA GCT GGC TAC AGG AAG GCC	From Fricker 1994	
		R GGT TTA TGC AGC AAC GAG ACG TCA		
		Uid		
		F TGT TAC GTC CTG TAG AAA GCC C		
		R AAA ACT GCC TGG CAC AGC AAT T		
Escherichia coli	cnf	F CGC TTG GAC TGG GGA TAA TT		[345]
		R CTT CAT AGT AGA TGC CGC TC		
Escherichia coli	malB	F TCG CCA CAC GCT GAC GCT GAC CA	Also detects Shigella	[346]
		R TTA CAT GAC CTC GGT TTA GTT CAC AGA		
Escherichia coli	uidA or 16s	F AAA ACG GCA AGA AAA AGC AG		[347]
	rRNA	R ACG CGT GGT TAC AGT CTT GCG		
*Escherichia coli**	lacZ and uidA	lacZ		[203]
		F ATG AAA GCT GGC TAC AGG AAG GCC		
		R GGT TTA TGC AGC AAC GAG ACG TCA		
		uidA		
		F AAA ACG GCA AGA AAA AGC AG		
		R ACG CGT GGT TAC AGT CTT GCG		
Escherichia coli	GroEL	F TGA AAC GYG GTA TCG ACA AA	Reverse transcript	[215]
		R CTG CAT ACC TTC AAC MAC GTC C	real time PCR	
		Probe		
		6-FAM CCT TCT TTA CCG ACT TTI TCC ATC GCT T		
		TAMRA		

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
Escherichia coli	uidA	Inititally F ATC ACC GTG GTG ACG CAT GTC GC R CAC CAC GAT GCC ATG TTC ATC TGC Then F TAT GAA CTG TGC GTC ACA GCC R CAT CAG CAC GTT ATC GAA TCC	Nested	[348]
Escherichia coli	UidA		Based on Jefferson et al., another by Blanco et al. Used oligonucleotide gene probes	[203]
Escherichia coli	lacZ, lamb			[203]
Escherichia coli	uidA		Used Bej et al. 1991 primers	[349]
Escherichia coli	malB	Not listed	As per Furrer et al. and Candrian et al. Were seeking toxigenic strains.	meyer
Escherichia coli	16s rRNA			[205]
Escherichia coli	lacZ	F- CTT TGC CTG GTT TCC GGG ACC AGA A R- ACC CAC CGC ACG ATA GAG ATT CGG G		[350]
Escherichia coli	B-galactosidase	F- CTT TGC CTG GTT TCC GGC ACC AGA A R- AAC CAC CGC ACG ATA GAG ATT CGG G	Same primers as above.	Kane

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
S. pneumoniae	lytA	GGA AAG ACC CAG AAT TAG GT GTC TGA GTG GTT GTT TGG TT 308BP	Comparing different swab types	[351]
S. pneumoniae	lytA,	LytA-F ACG CAA TCT AGC AGA TGA AGC LytA-R TGT TTG GTT GGT TAT TCG TGC Probe FAM- TTT GCC GAA AAC GCT TGA TAC AGG G- TAMRA	Real time	[352]
S. pneumoniae	lytA	No primers listed in article.	Real time multiplex PCR from Sheppard et al.	[353]
S. pneumoniae	lytA versus Ply.	Ply F ACG TGA CAA TGT AGT T R CCG GTA CAC TCT CTA AT FL GGA AGT CTT GAC TCC TAG G-FLU 640 TGG GAC AGA AAT GGG C LytA F CAG CGG TTG AAC TGA TTG A R TGG TTG GTT ATT CGT GCA A FL GAA AAC GCT TGA TAC AGG GAG TT 640 AGC TGG AAT TAA AAC GCA CGA G	Real time, lytA was more sensitive	[354]
S. pneumoniae		No primers listed in paper	Real time, clinical study, from sputum, primers from Kais et al.	[210]
S. pneumoniae	ply	GenBank accession m17717	Multiplex reverse	[330]

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
		Antisense primer 552 CCA CTT GGA GAA AGC TAT CGC T 531 Sense primer 558 CCC AGC AAT TCA AGT GTT CG 557 Sense probe 465 CCC ACT CTT CTT GCG GTT GA 484 Antisense probe 673 TGA GCC GTT ATT TTT TCA TAC TG 651	line blot hybridisation of bacterial respiratory pathogens- S Pneumoniae, S. pyogenes, *S. aureus*, *H. influenzae*, Bordetella, Klebsiella, Legionella and others.	
S.pneumoniae	lytA versus pneumolysin	Pneumolysin M17717 F CTA CCA ACG ACA GTC GCC TCT A (397-418) R ATT ATT CTC TAA CAA GGT CTC ATC CAC TAC (464-435) Probe FAM CCT GGA GCA CTT CT MGB (420-433) LytA F ACG CAA TCT AGC AGA TGA AGC (306-326) R TGT TTG GTT GGT TAT TCG TGC (386-406) Probe FAM TTT GCC GAA AAC GCT TGA TAC AGG G TAMRA (330-354)	Real time	[355]
S. pneumoniae	ply gene	No primers listed in this paper.	Real time, from Corless et al.	[356]
S. pneumoniae	Compared lytA ply, psaS	LytA AE005672 nuc pos F1841014, R 1840961 P 1840985 F ACG CAA TCT AGC AGA TGA AGC A	Real time	[357]

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
		R TCG TGC GTT TTA ATT CCA GCT FAM GCC GAA AAC GCT TGA TAC AGG GAG- BHQ1 PsaA U53509 F166, R 279 P 219 F GCC CTA ATA AAT TGG AGG ATC TAA TGA R GAC CAG AAG TTG TAT CTT TTT TCC G HEX CTA GCA CAT GCT ACA AGA ATG ATT GCA GAA AGA AA PHOS Ply AE008539 F 721 R 798 P 742 F GCT TAT GGG CGC CAA GTC TA R CAA AGC TTC AAA AGC AGC CTC TA FAM-CTC AAG TTG GAA ACC ACG AGT AAG AGT GAT GAA PHOS		
S. pneumoniae	16s	Not useful to this study		[358]
S. pneumoniae		No primers listed in this paper.	Used PCR technique from Pai et al.	[359]
S. pneumoniae	ply	F AGC GAT AGC TTT CTC CAA GTG G (531-552) R CTT AGC CAA CAA ATC GTT TAC CG (603-583) P ACC CCA GCA ATT CAA GTG TTC GCG (556-580)	From Walker et al., real time, direct from sputum.	[211]
S. pneumoniae	ply	No primers listed in this paper.	From Saukkoriipi et al.	[360]
S. pneumoniae	ply	F TGC AGA GCG TCC TTT GGT CTA T R CTC TTA CTC GTG GTT TCC AAC TTG A Amplicon 80 bp	From Corless et al. which was based on Walker et al. 1987	[361]
S. pneumoniae	ply	LytA 429 bp	From Corless et al.	[362]

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
		F AAC CAT ATA GGC AAG TAC AC R ATC ATG CTT AAA CTG CTC AC Ply 81 bp F TGC AGA GCG TCC TTT GGT CTA T R CTC TTA CTC GTG GTT TCC AAC TTG A		
S. pneumoniae	Compared ply lytA	lytA 308 bp from Nagai et al. 2001 sense CAA CCG TAC AGA ATG AAG CGC anti-sense TTA TTC GTG CAA TAC TCG TGC G ply 329 bp from Salo et al. 1995 sense ATT TCT GTA ACA GCT ACC AAC GA antisense GAA TTC CCT GTC TTT TCA AAG TC spn9802 162 bp Suzuki et al. 2005 143F CAA GTC GTT CCA AGG TAA CAA GTC T 304R CTA AAC CAA CTC GAC CAC CTC TTT Spn9828 227bp Suzuki et al. 2005 19F GGC ATT GTG AAT GGA TTG ATT G 245R TCA TGT GCA TCC CAA GCT ACA	With primers used to be highly specific- spn9802 and spn9828	[363]
S. pneumoniae	lytA	La5_Ext and La3_Ext from Obregon et al .	Used primers from Salo et al., nested PCR	[364]
S. pneumoniae		ply 208bp OUTER		[365]

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
		F ATT TCT GTA ACA GCT ACC AAC GA R GAA TTC CCT GTC TTT TCA AGT C INNER F CCC ACT TCT TCT TGC GGT TGA R TGA GCC GTT ATT TTT TCA TAC TG		
S. pneumoniae	lytA,	No primers listed in this paper	Multiplex PCR from Stralin et al. 2005	[191]
S. pneumoniae	ply	From Saukkoriipi et al. 2002 Ply F ACT CTT CTT GCG GTT GAT CG R TGA GCC GTT ATT TTT TCA TAC TG P TCT CCA AGT GGA AGA CCC CAG CAA 640 CAA GTG TTC GCG GAG CGG TAA AC	Compares Real time PCR for ply, conventional PCR and other tests.	[366]
S. pneumoniae	lytA	No primers listed in this paper.	Serotyping outer primers only from Messmer et al	[221]
S. pneumoniae	Unclear	No primers listed in this paper.	PCR as per McAvin et al. 2001	[367]
S. pneumoniae	ply	No primers listed in this paper.	Real time, direct on sputum, based on Greiner et al. 2001	[368]
S. pneumoniae	ply	F TGC AGA GCG TCC TTT GGT CTA T R CTC TTA CTC GTG GTT TCC AAC TTG A	Multiplex based on Corless 2001	[369]
S. pneumoniae	Compared ply, lytA	No primers listed in this paper.	(outer primers only) and psaA for specificity from Messmer 1997,	[370]

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
S. pneumoniae	ply	F ACT CTT CTT GCG GTT GAT CG R TGA GCC GTT ATT TTT TCA TAC TG 640 CAA TTG TTC GCG GAG CGG TAA AC P TCT CCA AGT GGA AGA CCC CAG CAA	Morrison et al 2000 Real time, compared with culture in children. Based on pneumolysin sequence from Walker et al., using modified version of primers from Salo et al.	[188]
S. pneumoniae	lytA	Genbank AF345846 173 bp F CAG CGG TTG AAC TGA TTG A R TGG TTG GTT ATT CGT GCA A Donor probe GAA AAC GCT TGA TAC AGG GAG TT Acceptor probe AGC TGG AAT TAA AAC GCA CGA G		[371]
S. pneumoniae	16s	Not relevant to this study.		[372]
S. pneumoniae	lytA	Prototype sequence 94813 191-454 F GGA GTA GAA TAT GGA AAT TAA TGT R GCT GCA TAG GTC TCA GCA TTC CAA		[373]
S. pneumoniae	16s	Not relevant to this study.	Simultaneous detection of meningitis pathogens	[374]
S. pneumoniae	ply	Sense TTT CTG TAA CAG CTA CCA ACG A Anti sense GAA TTC CCT GTC TTT TCA AAG TC	From Salo/ Toikka et al.	[375]
S. pneumoniae	ply	Primers not relevant to this study or previously	Used broad range	[376]

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
		described.	and more specific primers then confirmed using ply by Corless et al	
S. pneumoniae	lytA	Primers above.	From Gillespie et al.	[377]
S. pneumoniae	unclear	SP1 ATC GAA ATT AAT GTG AGT A SP2 AGC TCT CAG CAT TCC A	From Hassan-King et al.	[378]
S. pneumoniae	ply	Sp sense GAC AAT ACA GAA GTG AAG GCG G Sp antisense ATA GGC ACC ACT ATG ATC CAG C Sp d1 TCG GCA AGC CTG GAT GAT CTG CTG T FLUOR SPA1 640- AGC GAC TGC CTT CTT GAA TCA AGT CCT CTA -P		[379]
S. pneumoniae	PBP2	No primers listed in this paper.	From Dowson et al.	[380]
S. pneumoniae	ply, lytA,	Ply , outer 1006-1032 AAT GCA CTG TTA CAT CAA CGC TGG AAA 1047-1073 GAT ACA ACT CTG ATT CCA ATG TCG AAT Inner 424-450 AAT AAT GTC CCA GAT AGA ATG CAG TAT 473-499 TGG AAC AAC TCA AGG TCA AGT TTG GTT LytA Outer	Nested PCR	[381]

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
		599-627 AAA ATC AAT GGC ACT TGG TAC TAC TTT 634-660 TAT ATG CTT GCA GAC CGC TGG AGG AAG Inner 107-133 AGA ATG AAG CGG ATT ATC ACT GGC GGA 172-198 AAC GGT TGC ATC ATG CAG GTA GGA CCT		
S. pneumoniae	lytA	LYTA F CGG ACT ACC GCC TTT ATA TCG R GTT TCA ATC GTC AAG CCG TT		[382]
S. pneumoniae	ply and atpA	ply F AGC GAT AGC TTT CTC CAA GTG G (531-552) R CTT AGC CAA CAA ATC GTT TAC CG (583-605) AptA F CGC TAA TTT ACA GTA TGA C (783-801) R TAA ATC CAC GAC GAC GAA C (847-865)		[209]
S. pneumoniae	lytA	No primers listed	Based on Garcia et al. 1986	[383]
S. pneumoniae	Unclear	No primers listed in this paper.	Based on Garcia et al. 1986	[384]
S. pneumoniae	16S	F AGT CGG TGA GGT AAC CGT AAG R AGG AGG TGA TCC AAC CGC A		[385]
S. pneumoniae	ply	F TGC AGA GCG TCC TTT GGT CTA T (894-915)	Based on Walker et	[216]

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
		R CTC TTA CTC GTG GTT TCC AAC TTG A (974-950) P VIC- TGG CGC CCA TAA GCA ACA CTC GAA (941-918)	al. 1987.	
S. pneumoniae	16s	Lower primer (r) Genbank X58312 CTA CGC ATT TCA CCG CTA CAC (567-587) Upper primer AAG GTG CAC TTG CAT CAC TAC C (106-127)		[386]
H. influenzae	gyrB	GenBank accession U32738 Sense primer 5974 GAA TAT CCA CAG GAA TCC CG 5993 Antisense primer 6028 GAT GAT AAT TCT GTA TCG GTG CAA 6005 Sense probe 5926 GAA GCA CAG TCA TAA TAA CTT CTG CT 5951 Antisense probe 6159 GTC CTG GTA TGT ATA TCG G 6138	Multiplex reverse line blot hybridisation of bacterial respiratory pathogens	[330]
H. influenzae	bex	Not relevant to this study as only type b included.	Multiplex from Corless et al.	[369]
H. influenzae	bexA	No primers listed in this paper.	Multiplex from Corless et al.	[387, 2007 #992]
H. influenzae	16s	16s rRNA -538bp F TCC TAA GAA GAG CTC AGA GAT R TGA TCC AAC CGC AGG TTC C	Multiplex with *H. influenzae*	[382]

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
H. influenzae	frdB	F ATC GAA AGT TTA GAG GCA A (328-348) R TTC TTT CGA TGG ATG TGG TT (392-412)		[209]
H. influenzae	bexA	No primers listed in this paper.	As per Falla et al. 1994	[383]
H. influenzae	16s rRNA then P6	No primers listed in this paper.	As per Stralin et al. 2005	[191]
H. influenzae	16a	No primers listed in this paper.	Nested PCR based on Radstrom et al.	[388]
H. influenzae	16s rRNA then P6	16s rRNA (538 bp) F TCC TAA GAA GAG CTC AGA GAT (4800-4820) R TGA TCC AAC CGC AGG TTC C (5337-5319) P6 (296 bp) F TTG GCG GWT ACT CTG TTG CT (174-193) R TGC AGG TTT TTC TTC ACC GT (469-449)		[201]
H. influenzae	23s then specific primers	23s F GCG ATT TCY GAA YGG GGR AAC CC R TTC GCC TTT CCC TCA CGG TAC T (where Y is C or T and R is A or G) Specific F GTG AGG AGA ATG TGT TGG GAA G R GGT TGT AGG ACT GCA ATG TGG ACT C	Real time, used SYBR green some primers from Anthony et al. 2000	[389]
H. influenzae	Unclear	No primers listed in this paper.	Based on Falla et al.	[384]

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
H. influenzae	Hel encoding P4 outer membrane protein.	F GAT CCG AAT TCC TTA AAA GGA AT R ATT AAA TAT TGG ATC CAG TAA AAA CTG AAC Internal probe from Sambrook et al. deduced from previously published sequence of hel (Green et al 1991). Gcg acg tca atc tcg tgc ggt tcg ggg tgc gg	Based on Reidl and Mekalaanos 1996.	[390]
H. influenzae	16s rRNA then specific primers	F GG AGT GGG TTG TAC CAG AAG TAG R AGG AGG TGA TCC AAC CGC A		[385]
H. influenzae	bexA	F GGC GAA ATG GTG CTG GTA A (142-160) R GGC CAA GAG ATA CTC ATA GAA CGT T (241-217) P TET-CAC CAC TCA TCA AAC GAA TGA GCG TGG (189-163)	Based on Kroll et al. 1988	[216]
H. influenzae	16s	F CGT ATT ATC GGA AGA TGA AAG TGC (177-200) R CTA CGC ATT TCA CCG CTA CAC (682-702)	Modification of prev PCR designed by Hendolin et al.	[220]
H. influenzae	16s	Oligonucleotide probe H inf GGA GTG GGT TGT ACC AGA ATA GAT	Universal primers, amplicon cloned into *E. coli* then hybridised and sequenced	[391]
H. influenzae	16s then species specific	F CCT AAG AAG AGC TCA GAG R AAG GAG GTG ATC CAY CCG CAM MTT C Y=G OR A		[392]

Target organism	*Gene target*	*Primers/Probe*	*Comments*	**Reference**
H. influenzae	16s	M=G OR C Lower primer (R) GENBANK accession no M35019 CTA CGC ATT TCA CCG CTA CAC (679-699) Upper primer (F) CGT ATT ATC GGA AGA TGA AAG TGC (177-200)		[386]
H. influenzae		Not relevant to this study (type b only)		{Hassan-King, 1996 #1019
H. influenzae	P6	F AAC TTT TGG CGG TTA CTC TG R CTA ACA CTG CAC GAC GGT TT P TAA ATA TGA CAT TCA TGG TG	Based on Hotomi et al. and specific internal probe	[393]
H. influenzae	16s	Not relevant to this study.		[374]
H. influenzae-		Not relevant to this study (type b only)		[394]

7.3 Appendix C Optimising primers and probes

Table 44. Primer optimisation for *P. aeruginosa* real-time PCR assay

Primer concentrations (µM)	RN Rep 1	RN Rep 2	RN Rep 3	RN Rep 4	Mean value RN
1. 0.05/0.05	2.00	2.06	2.10	2.10	2.07
2. 0.05/0.3	n/a	3.51	3.55	3.57	3.54
3. 0.05/0.9	n/a	3.56	3.62	3.64	3.61
4. 0.3/0.05	1.49	1.48	1.46	1.47	1.48
5. 0.3/0.3	3.21	3.22	3.19	3.19	3.20
6. 0.3/0.9	3.54	3.54	3.52	3.49	3.52
7. 0.9/0.05	1.50	1.50	1.52	1.52	1.51
8. 0.9/0.3	3.07	3.01	3.03	3.02	3.03
9. 0.9/0.9	3.46	3.44	3.40	3.38	3.42

RN= level of fluorescence

Table 45. Mean RN values by primer concentration for *P. aeruginosa* real-time PCR assay

		Forward Primer (µM)		
		0.05	0.3	0.9
Reverse Primer (µM)	0.05	2.07	1.48	1.51
	0.3	3.54	3.20	3.03
	0.9	3.61*	3.52	3.42

*Number in bold indicates highest RN (level of fluorescence).

Table 46. Primer optimisation assay for *Acinetobacter* spp. real-time PCR assay

Primer concentrations (µM)	RN Rep 1	RN Rep 2	RN Rep 3	RN Rep 4	Mean RN
1. 0.05/0.05	0.36	0.36	0.36	0.36	0.36
2. 0.05/0.30	0.97	0.96	1.00	1.05	1.00
3. 0.05/0.9	1.14	1.17	1.21	1.28	1.20
4. 0.3/0.05	0.49	0.49	n/a	n/a	0.49
5. 0.3/0.3	1.11	1.11	1.17	1.16	1.14
6. 0.3/0.9	1.31	1.34	1.33	1.33	1.33
7. 0.9/0.05	0.51	0.49	0.49	0.49	0.50
8. 0.9/0.3	1.16	1.14	n/a	1.10	1.13
9. 0.9/0.9	1.42	1.37	1.36	1.32	1.37

RN= level of fluorescence

Table 47. Mean RN values by primer concentration for *Acinetobacter* spp. real-time PCR assay

		Forward Primer (µM)		
		0.05	0.3	0.9
Reverse Primer (µM)	0.05	0.36	0.49	0.50
	0.3	1.00	1.14	1.13
	0.9	1.20	1.33	1.37*

*Number in bold indicates highest RN (level of fluorescence).

Table 48. Primer optimisation assays for *E. coli* real-time PCR assay

Primer concentrations (µM)	RN Rep 1	RN Rep 2	RN Rep 3	RN Rep 4	Mean RN
1. 0.05/0.05	0.07	0.07	0.07	0.07	0.07
2. 0.05/0.30	0.13	0.12	0.13	0.12	0.13
3. 0.05/0.9	0.18	0.15	0.17	0.17	0.17
4. 0.3/0.05	0.13	0.13	0.13	0.14	0.13
5. 0.3/0.3	0.22	0.22	0.22	0.23	0.22
6. 0.3/0.9	0.27	0.25	0.25	0.23	0.25
7. 0.9/0.05	0.15	0.17	0.17	0.18	0.17
8. 0.9/0.3	0.27	0.28	0.30	0.31	0.29
9. 0.9/0.9	0.31	0.30	0.32	0.34	0.32

RN= level of fluorescence

Table 49. Mean RN values by primer concentration for E coli real-time PCR assay

		Forward Primer (µM)		
		0.05	0.3	0.9
Reverse Primer (µM)	0.05	0.07	0.13	0.17
	0.3	0.13	0.22	0.29
	0.9	0.17	0.25	0.32*

*Number in bold indicates highest RN (level of fluorescence).

Table 50. Primer optimisation assays for *H. influenzae* real-time PCR assay

Primer concentrations (µM)	RN Rep 1	RN Rep 2	RN Rep 3	RN Rep 4	Mean RN
1. 0.05/0.05	0.46	0.46	0.45	0.45	0.46
2. 0.05/0.30	0.78	0.79	0.80	0.78	0.79
3. 0.05/0.9	0.75	0.76	0.77	0.76	0.76
4. 0.3/0.05	0.84	0.82	0.82	0.83	0.83
5. 0.3/0.3	1.30	1.30	1.30	1.31	1.30
6. 0.3/0.9	1.98	1.94	1.93	1.93	1.95
7. 0.9/0.05	3.34	3.23	3.31	n/a	3.29
8. 0.9/0.3	2.31	2.29	2.33	2.36	2.32
9. 0.9/0.9	2.80	2.81	2.80	2.78	2.80

RN= level of fluorescence

Table 51. Mean RN values by primer concentration for *H. influenzae* real-time PCR assay

		Forward Primer (µM)		
		0.05	0.3	0.9
Reverse Primer (µM)	0.05	0.46	0.83	3.29*
	0.3	0.79	1.30	2.32
	0.9	0.76	1.95	2.80

*Number in bold indicates highest RN (level of fluorescence).

Table 52. Primer optimisation assays for *S. pneumoniae* real-time PCR assay

Primer concentrations (µM)	RN Rep 1	RN Rep 2	RN Rep 3	Mean RN
1. 0.05/0.05	0.26	0.28	0.29	0.27
2. 0.05/0.30	0.56	0.56	0.57	0.56
3. 0.05/0.9	0.58	0.58	0.58	0.58
4. 0.3/0.05	0.77	0.76	0.75	0.76
5. 0.3/0.3	1.31	1.33	1.30	1.31
6. 0.3/0.9	0.58	0.70	0.73	0.67
7. 0.9/0.05	0.74	0.74	0.77	0.75
8. 0.9/0.3	1.35	1.27	1.20	1.27
9. 0.9/0.9	1.28	1.16	1.06	1.17

RN= level of fluorescence

Table 53. Mean RN values by primer concentration for *S. pneumoniae* real-time PCR assay

		Forward Primer (µM)		
		0.05	0.3	0.9
Reverse Primer (µM)	0.05	0.27	0.76	0.75
	0.3	0.56	1.31*	1.27
	0.9	0.58	0.67	1.17

*Number in bold indicates highest RN (level of fluorescence).

Table 54. Primer optimisation assays for *S. aureus* real-time PCR assay

Primer concentrations (µM)	RN Rep 1	RN Rep 2	RN Rep 3	Mean RN
1. 0.05/0.05	0.01	0.02	0.00	0.01
2. 0.05/0.30	0.35	0.37	0.37	0.36
3. 0.05/0.9	0.55	0.58	0.57	0.56
4. 0.3/0.05	0.06	0.04	0.05	0.05
5. 0.3/0.3	0.53	0.54	0.54	0.54
6. 0.3/0.9	1.04	1.03	1.03	1.03
7. 0.9/0.05	0.06	0.06	0.04	0.05
8. 0.9/0.3	0.54	0.51	0.54	0.53
9. 0.9/0.9	1.22	1.24	1.21	1.22

RN= level of fluorescence

Table 55. Mean RN values by primer concentration for *S. aureus* real-time PCR assay

		Forward Primer (µM)		
		0.05	0.3	0.9
Reverse Primer (µM)	0.05	0.01	0.05	0.05
	0.3	0.36	0.54	0.53
	0.9	0.56	1.03	1.22*

*Number in bold indicates highest RN (level of fluorescence).

Table 56. Primer optimisation assays for MRSA real-time PCR assay

Primer concentrations (µM)	RN Rep 1	RN Rep 2	RN Rep 3	Mean RN
1. 0.05/0.05	0.29	0.23	0.22	0.25
2. 0.05/0.30	0.50	0.52	0.51	0.51
3. 0.05/0.9	0.56	0.59	0.50	0.55
4. 0.3/0.05	0.18	0.17	0.17	0.17
5. 0.3/0.3	0.54	0.60	n/a	0.57
6. 0.3/0.9	0.66	0.65	0.66	0.66
7. 0.9/0.05	0.18	0.20	0.17	0.18
8. 0.9/0.3	0.51	0.52	0.54	0.52
9. 0.9/0.9	0.68	0.67	0.72	0.69

RN= level of fluorescence

Table 57. Mean RN values by primer concentration for MRSA real-time PCR assay

		Forward Primer (µM)		
		0.05	0.3	0.9
Reverse Primer (µM)	0.05	0.25	0.17	0.18
	0.3	0.51	0.57	0.52
	0.9	0.55	0.66	0.69*

*Number in bold indicates highest RN (level of fluorescence).

7.4 Appendix E Multiplex experiments

Table 58. Comparing Ct results between single and multiplex assays for detection of *S. aureus* (in triplicate)

Mastermix	*SA*	*SA/MRSA*	**SA/MRSA**
DNA	SA	SA	SA/MRSA*
1	26.5852	26.3493	Not tested
2	26.6317	26.5689	Not tested
3	26.4555	26.5343	Not tested
Mean Ct	26.557	26.484	n/a

Table 59. Comparing Ct results between single and multiplex assays for detection of MRSA (in triplicate)

Mastermix	*MRSA*	*SA/MRSA*	**SA/MRSA**
DNA	MRSA	MRSA	SA/MRSA*
1	24.4962	27.1405	Not tested
2	24. 5869	27.7436	Not tested
3	24. 6082	24.6371	Not tested
Mean Ct	24.564	27.507	n/a

*Note that MRSA samples should generate a positive *S. aureus* assay, therefore a mix of MRSA and *S. aureus* was not tested

Table 60. Comparing Ct values between single, duplex and triplex assays for the detection of *H. influenzae* (in triplicate)

Mastermix	*HI*	*HI/PA*	*HI/PA*	*HI/EC*	*HI/EC*	*HI/EC/PA*	*HI/EC/PA*	*HI/EC/PA*	**HI/EC/PA**
DNA	HI	HI	HI/PA	HI	HI/EC	HI	HI/PA	HI/EC	HI/EC/PA
1	36.487	38.6307	30.6829	35.2364	32.5103	37.0809	31.3183	35.3651	undetected
2	37.2918	37.1985	30.6882	35.7509	33.1181	36.8125	31.3995	35.7525	Undetected
3	36.5912	37.6238	30.6249	36.1353	33.3209	35.4145	31.8521	35.589	Undetected
Mean Ct	36.79	37.818	30.665	35.708	32.983	36.436	31.523	35.569	n/a

Table 61. Comparing Ct values between single, duplex and triplex assays for the detection of *E. coli* (in triplicate)

Mastermix	*EC*	*PA/EC*	*PA/EC*	*HI/EC*	*HI/EC*	*HI/EC/PA*	*HI/EC/PA*	*HI/EC/PA*	**HI/EC/PA**
DNA	EC	EC	PA/EC	EC	HI/EC	EC	PA/EC	HI/EC	HI/EC/PA
1	30.1247	30.6819	28.6078	29.6558	26.1065	30.6125	28.9384	27.1936	29.0775
2	31.0637	30.2209	28.3917	28.5439	26.4113	28.4829	29.2493	28.1229	28.9725
3	31.3854	30.1572	27.6238	28.3862	26.4236	27.8717	29.6509	28.1686	28.1986
Mean Ct	30.858	30.353	28.208	28.862	26.314	28.989	29.280	27.828	28.750

Table 62. Comparing Ct values between single, duplex and triplex assays for the detection of *P. aeruginosa* (in triplicate)

Mastermix	*PA*	*PA/EC*	*PA/EC*	*HI/PA*	*HI/PA*	*HI/EC/PA*	*HI/EC/PA*	*HI/EC/PA*	**HI/EC/PA**
DNA	PA	PA	PA/EC	PA	HI/PA	PA	PA/EC	HI/PA	HI/EC/PA
1	30.5769	31.2838	24.6422	31.7001	24.3825	32.7515	25.4427	24.8215	24.7829
2	31.4181	31.0338	24.799	32	24.4466	33.0158	25.4372	24.7689	24.6309
3	31.2252	31.2442	24.6588	31.4659	24.5328	32.3955	25.4817	24.8649	25.0388
Mean Ct	31.073	31.187	24.7	31.722	24.454	32.721	25.454	24.818	24.818

Table 63. Comparing Ct values between single, duplex and triplex assays for the detection of *S. pneumoniae* (in triplicate, unsuccessful)

Mastermix	*SPN*	*SPN/AC*	*SPN/AC*	*SpN/KP*	*SpN/KP*	*SpN/AC/KP*	*SpN/AC/KP*	*SP/AC/KP*	**SP/AC/KP**
DNA	SPN	SPN	SPN/AC	SPN	SPN/KP	SPN	SPN/AC	SPN/KP	SP/AC/KP
1	26.1805	25.5667	26.5304	26.1961	26.5942	26.1439	26.8811	27.2794	27.2641
2	25.6963	25.732	26.5491	26.1679	26.6463	26.1959	27.0135	27.2067	27.6847
3	25.7144	25.7508	26.4554	26.1389	26.7246	26.1749	27.1426	27.2423	28.4039
Mean Ct	25.864	25.683	26.512	26.168	26.655	26.172	27.012	27.243	27.784

Table 64. Comparing Ct values between single, duplex and triplex assays for the detection of *Acinetobacter* spp. (in triplicate, unsuccessful)

Mastermix	*AC*	*SPN/AC*	*SPN/AC*	*AC/KP*	*AC/KP*	*SpN/AC/KP*	*SpN/AC/KP*	*SP/AC/KP*	**SP/AC/KP**
DNA	AC	AC	SPN/AC	AC	AC/KP	AC	SPN/AC	AC/KP	SP/AC/KP
1	24.3764	27.8862	32.3168	24.8689	24.4955	30.6591	-	-	-
2	24.449	28.1899	30.2157	24.8053	24.7093	30.2648	-	-	-
3	24.4683	28.0207	29.8046	24.9128	24.6608	29.7077	-	-	-
Mean Ct	24.431	28.032	30.779	24.862	24.622	30.211			

Table 65. Comparing Ct values between single, duplex and triplex assays for the detection of and *K. pneumoniae* (in triplicate, unsuccessful)

Mastermix	*KP*	*AC/KP*	*AC/KP*	*SpN/KP*	*SpN/KP*	*SpN/AC/KP*	*SpN/AC/KP*	*SP/AC/KP*	**SP/AC/KP**
DNA	KP	KP	AC/KP	KP	SPN/KP	KP	AC/KP	SPN/KP	SP/AC/KP
1	23.0732	22.8747	22.6586	23.3086	23.4017	23.336	23.217	23.3877	23.2491
2	23.1504	22.841	22.6435	23.2129	23.3701	23.3115	23.1483	23.3631	23.4117
3	23.2129	23.6007	22.6454	23.4278	23.3219	23.2583	23.144	23.2812	23.5127
Mean Ct	23.146	23.105	22.649	23.316	23.365	23.302	23.170	23.344	23.391

Table 66. Comparing Ct values between single and duplex assays for the detection of human DNA (in triplicate, unsuccessful)

Mastermix	*GAPDH*	*GAPDH/AC*	**GAPDH/AC**
DNA	GAPDH	GAPDH	GAPDH/AC
1	33.6427	33.6085	Undetected
2	34.0986	33.5802	Undetected
3	33.6439	34.1632	Undetected
Mean Ct	33.795	33.784	-

Table 67. Comparing Ct values between single and duplex assays for the detection of *Acinetobacter* spp. (in triplicate, unsuccessful)

Mastermix	*AC*	*GAPDH/AC*	**GAPDH/AC**
DNA	AC	AC	GAPDH/AC
1	24.8246	24.5818	23.0684
2	24.8249	24.603	23.175
3	24.393	24.5648	23.1462
Mean Ct	24.681	24.583	23.130

Table 68. Comparing Ct values between single and duplex assays for the detection of MRSA (in triplicate, unsuccessful)

Mastermix	*MRSA*	*MRSA/CPS*	**MRSA/CPS**
DNA	MRSA	MRSA	MRSA/CPS
1	24.9438	35.668	Undetected
2	25.7939	36.535	Undetected
3	25.5241	37.8119	Undetected
Mean Ct	25.4206	36.67163	-

Table 69. Comparing Ct values between single and duplex assays for the detection of *S. pneumoniae* (in triplicate, unsuccessful)

Mastermix	CPS	MRSA/CPS	MRSA/CPS
DNA	CPS	CPS	MRSA/CPS
1	27.518	28.0828	27.0539
2	27.7417	28.177	26.6535
3	27.5532	28.1275	26.6862
Mean Ct	27.6043	28.1291	26.79787

Table 70. Comparing Ct values between single and duplex assays for the detection of *E. coli* (in triplicate, unsuccessful).

Mastermix	EC	EC/CPS	EC/CPS	EC/KP	EC/KP	EC/CPS/KP	EC/CPS/KP	EC/CPS/KP	EC/CPS/KP
DNA	EC	EC	EC/CPS	EC	EC/KP	EC	EC/CPS	EC/KP	EC/CPS/KP
1	25.1294	28.7024	32.9775	29.0949	30.6719	-	-	-	-
2	24.8923	28.9511	34.2371	29.9062	29.8771	-	-	-	-
3	24.7652	29.2655	32.2764	29.716	30.707	-	-	-	-
Mean Ct	24.929	28.973	33.164	29.572	30.419	-	-	-	-

Table 71. Comparing Ct values between single and duplex assays for the detection of *S. pneumoniae* (in triplicate, unsuccessful).

Mastermix	CPS	EC/CPS	EC/CPS	CPS/KP	CPS/KP	EC/CPS/KP	EC/CPS/KP	EC/CPS/KP	EC/CPS/KP
DNA	CPS	CPS	EC/CPS	CPS	CPS/KP	CPS	EC/CPS	CPS/KP	EC/CPS/KP
1	26.6306	26.1481	26.2846	26.4499	26.1913	26.4099	32.9567	26.1636	37.1052
2	26.6465	26.1992	26.0974	26.4767	26.1955	25.8513	32.6769	26.1496	38.4368
3	26.6428	26.1286	26.1092	26.483	26.2324	26.3295	34.0337	26.0725	-
Mean Ct	26.640	26.158	26.164	26.470	26.206	26.197	33.222	26.129	37.771

Table 72. Comparing Ct values between single and duplex assays for the detection of *K. pneumoniae* (in triplicate, unsuccessful).

Mastermix	KP	EC/KP	EC/KP	CPS/KP	CPS/KP	EC/CPS/KP	EC/CPS/KP	EC/CPS/KP	EC/CPS/KP
DNA	KP	KP	EC/KP	KP	CPS/KP	KP	EC/KP	CPS/KP	EC/CPS/KP
1	23.7908	23.3899	18.1262	24.0414	23.4762	24.0313	18.4347	23.2428	18.3136
2	23.7054	23.386	18.1056	24.4812	23.5007	24.0307	18.6057	23.1998	18.4107
3	23.692	23.4714	17.9184	24.3407	23.3383	23.9806	18.3493	23.108	18.4531
Mean Ct	23.729	23.416	18.050	24.288	23.438	24.014	18.463	23.184	18.392

7.5 Appendix D Inclusivity and specificity testing

Table 73. Species tested against *Acinetobacter* spp. assay for inclusivity

Organism	Organism code	CT value
Acinetobacter junii	B139	34.9
Acinetobacter haemolyticus	B105	39.3
Acinetobacter lwoffii	B106	36.3
Acinetobacter lwoffii	B114	37.0
Acinetobacter baumannii	BC56	35.3
Acinetobacter lwoffii	BC57	36.4
Acinetobacter junii	BC59	34.0
Acinetobacter lwoffii	BC69	36.8
Acinetobacter lwoffii	BC70	34.7
Acinetobacter baumannii	BC68	20.9
Acinetobacter baumannii	BC99	20.5
Acinetobacter junii	M031	33.6
Acinetobacter sp.	M029	20.4

Table 74. Species tested against *S. pneumoniae* assay for inclusivity

Organism	Organism code	CT value
Streptococcus pneumoniae	B116	22.00
Streptococcus pneumoniae	BC20	22.75
Streptococcus pneumoniae	BC132	20.82
Streptococcus pneumoniae	BC21	21.85
Streptococcus pneumoniae	BC22	22.24
Streptococcus pneumoniae	E003	23.23
Streptococcus pneumoniae	E004	24.09
Streptococcus pneumoniae	E006	22.68
Streptococcus pneumoniae	E007	23.14
Streptococcus pneumoniae	E008	23.28
Streptococcus pneumoniae	SPN clin 1	24.41
Streptococcus pneumoniae	SPN clin 2	24.52
Streptococcus pneumoniae	E001	22.1
Streptococcus pneumoniae	E002	22.6

Table 75. Species tested for inclusivity against *P. aeruginosa* assay

Organism n=28	Organism code	CT value
Pseudomonas aeruginosa	C056	17.2
Pseudomonas aeruginosa	C057	17.4
Pseudomonas aeruginosa	C058	16.6
Pseudomonas aeruginosa	C060	18.3
Pseudomonas aeruginosa	C062	18.0
Pseudomonas aeruginosa	C063	17.0
Pseudomonas aeruginosa	C080	18.1
Pseudomonas aeruginosa	C092	17.8
Pseudomonas aeruginosa	C096	17.3
Pseudomonas aeruginosa	C094	17.4
Pseudomonas aeruginosa	B130	Not detected
Pseudomonas aeruginosa	B036	17.7
Pseudomonas aeruginosa	B037	17.2
Pseudomonas aeruginosa	B047	Not detected
Pseudomonas aeruginosa	B049	Not detected
Pseudomonas aeruginosa	B071	17.1
Pseudomonas aeruginosa	BC15	17.8
Pseudomonas aeruginosa	BC16	17.2
Pseudomonas aeruginosa	BC17	17.1

Pseudomonas aeruginosa	B076	18.3
Pseudomonas aeruginosa	BC18	17.6
Pseudomonas aeruginosa	BC19	17.3
Pseudomonas aeruginosa	BC48	18.7
Pseudomonas aeruginosa	BC49	17.3
Pseudomonas aeruginosa	BC65	16.9
Pseudomonas aeruginosa	B013	14.6
Pseudomonas aeruginosa	B121	15.2
Pseudomonas aeruginosa	B024	15.4

Table 76. Species tested against *H. influenzae* assay for inclusivity

Organism n=17	Organism code	CT value
Haemophilus influenzae	JP1	19.5
Haemophilus influenzae	JP2	19.2
Haemophilus influenzae	JP3	20.3
Haemophilus influenzae	JP4	19.9
Haemophilus influenzae	JP5	19.2
Haemophilus influenzae	JP6	19.2
Haemophilus influenzae	JP7	20.2
Haemophilus influenzae	JP8	21.3
Haemophilus influenzae	JP9	Not detected
Haemophilus influenzae	JP10	20.1
Haemophilus influenzae	JP11	20.2
Haemophilus influenzae	JP12	20.5
Haemophilus influenzae	JP13	19.4
Haemophilus influenzae	JP14	19.7
Haemophilus influenzae	JP15	19.7
Haemophilus influenzae	JP16	21.6
Haemophilus influenzae	C081	20.3

Table 77. Species tested against *S. aureus* assay for inclusivity

Organism n=16	Organism code	CT value
Staphylococcus aureus	C001	28.62
Staphylococcus aureus	C002	27.68
Staphylococcus aureus	C003	27.60
Staphylococcus aureus	C004	26.59
Staphylococcus aureus	C006	26.87
Staphylococcus aureus	C007	26.37
Staphylococcus aureus	C008	25.14
Staphylococcus aureus	C009	26.64
Staphylococcus aureus	C012	31.31
Staphylococcus aureus	B021	24.85
Staphylococcus aureus	B025	29.54
Staphylococcus aureus	B039	29.89
Staphylococcus aureus	B049	28.51
Staphylococcus aureus	B063	27.50
Staphylococcus aureus	B073	Not detected

Table 78. Species tested against MRSA assay for inclusivity

Organism n=17	Organism code	CT value
MRSA	C010	27.70
MRSA	C011	26.63
MRSA	C021	24.59
MRSA	C043	26.32
MRSA	C049	23.92
MRSA	C050	26.23
MRSA	C051	23.58
MRSA	C052	24.78
MRSA	C053	26.60
MRSA	C054	25.21
MRSA	B006	24.87
MRSA	B017	23.52
MRSA	M023	24.37
MRSA	M024	25.33
MRSA	M020	25.60
MRSA	M022	23.17
MRSA	BC71	26.20

Table 79. Species tested against *E. coli* assay for inclusivity

Organism n=30	Organism code	CT value
Escherichia coli	C065	24.4
Escherichia coli	C066	24.6
Escherichia coli	C067	24.2
Escherichia coli	C068	24.2
Escherichia coli	C069	24.3
Escherichia coli	C070	24.2
Escherichia coli	C073	23.5
Escherichia coli	C074	24.4
Escherichia coli	C075	24.2
Escherichia coli	C076	24.1
Escherichia coli	C093	23.6
Escherichia coli	B001	24.8
Escherichia coli	B023	24.1
Escherichia coli	B031	23.7
Escherichia coli	B032	23.7
Escherichia coli	B038	23.8
Escherichia coli	B040	26.1
Escherichia coli	B045	25.1
Escherichia coli	B061	Not detected

Escherichia coli	B066	Not detected
Escherichia coli	B070	24.3
Escherichia coli	B072	Not detected
Escherichia coli	B075	Not detected
Escherichia coli	NCTC 9001	23.0
Escherichia coli	NCTC 12900	23.6
Escherichia coli	NCTC10418	24.1
Escherichia coli	NCTC 12923	23.1
Escherichia coli	NCTC 13167	24.0
Escherichia coli	NCTC 13216	24.4

Table 80. Panel of bacteria tested against final multiplexed assays for specificity, showing CT values from testing

Organism	*Code*	*S. aureus*	*MRSA*	*E. coli*	*P. aeruginosa*	*S. pneumoniae*	*H. influenzae*	*Acinetobacter spp*	*Coliform*
Streptococcus oralis	11427								37.75
Streptococcus salivarius	AS214								37.53
Streptococcus mutans	10449								35.53
Streptococcus mitis	AS148								37.17
Streptococcus oralis	A23								38.17
Streptococcus sanguis	AS218								36.63

Organism	*Code*	*S. aureus*	*MRSA*	*E. coli*	*P. aeruginosa*	*S. pneumoniae*	*H. influenzae*	*Acinetobacter spp*	*Coliform*
Streptococcus mitis	12261								
Streptococcus salivarius	8606								36.81
Streptococcus gordonii	10231								37.17
Streptococcus sanguis	7865								36.71
Streptococcus oralis	11427								37.24
Actinomyces israelii	01/30								36.50
Streptococcus constellatus	11325								37.14

Organism	*Code*	*S. aureus*	*MRSA*	*E. coli*	*P. aeruginosa*	*S. pneumoniae*	*H. influenzae*	*Acinetobacter spp*	*Coliform*
Streptococcus salivarius	8618								37.11
Corynebacteria	01/0509								36.99
Streptococcus sanguis	7863								36.47
Streptococcus mutans	R471								35.98
Streptococcus mitis	BC30								36.80
Streptococcus agalactiae	B107								36.90
Streptococcus constellatus	BC38								38.11

Organism	*Code*	*S. aureus*	*MRSA*	*E. coli*	*P. aeruginosa*	*S. pneumoniae*	*H. influenzae*	*Acinetobacter spp*	*Coliform*
Alpha-haemolytic streptococcus	BC51								39.12
Streptococcus oralis	B095								35.92
Alpha-haemolytic *streptococcus*	B123								37.33
Haemolytic *Streptococcus* group B	B065			28.96					16.65
Lactobacillus	B147								35.54
Streptococcus anginosus	B145								34.91

Organism	*Code*	*S. aureus*	*MRSA*	*E. coli*	*P. aeruginosa*	*S. pneumoniae*	*H. influenzae*	*Acinetobacter spp*	*Coliform*
Streptococcus constellatus	B079			23.72					15.96
Streptococcus constellatus	B086	25.68							34.34
Alpha-haemolytic *streptococcus*	B027								33.48
Streptococcus agalactiae	B010								34.85
Beta-haemolytic *Streptococcus* group C	B037	26.60							37.80
Haemolytic *Streptococcus*	B002								35.49

Organism	*Code*	*S. aureus*	*MRSA*	*E. coli*	*P. aeruginosa*	*S. pneumoniae*	*H. influenzae*	*Acinetobacter spp*	*Coliform*
group B									
Streptococcus sanguis x	B012								36.23
Enterococcus faecalis	B034								35.86
Enterococcus faecium	B060			21.83					15.78
Streptococcus species	B011								36.03
Enterococcus faecium	B059								34.83
Staphylococcus epidermidis	B051								36.78

Organism	*Code*	*S. aureus*	*MRSA*	*E. coli*	*P. aeruginosa*	*S. pneumoniae*	*H. influenzae*	*Acinetobacter spp*	*Coliform*
Staphylococcus epidermidis	B056								14.12
Staphylococcus epidermidis	B046								13.66
Alpha-haemolytic *Streptococcus*	B022								33.10
Streptococcus hominis	B053								33.28
Staphylococcus aureus	C001	28.40							37.35
MRSA	C010		22.41						37.15
Acinetobacter baumannii	BC56							30.00	37.10

Organism	*Code*	*S. aureus*	*MRSA*	*E. coli*	*P. aeruginosa*	*S. pneumoniae*	*H. influenzae*	*Acinetobacter spp*	*Coliform*
Acinetobacter haemolyticus	B105							34.77	37.10
Acinetobacter lwoffii	B106							30.27	37.56
Klebsiella pneumoniae	B043								13.99
Klebsiella oxytoca	B111								13.99
Escherichia coli	C095			21.89					14.30
Pseudomonas aeruginosa	BC16				16.58				
Enterobacter *clocae*	B141								13.14

Organism	*Code*	*S. aureus*	*MRSA*	*E. coli*	*P. aeruginosa*	*S. pneumoniae*	*H. influenzae*	*Acinetobacter spp*	*Coliform*
Serratia marcescens	B118								13.34
Bacillus fragilis	ATCC 25285								23.87
Lactococcus	No code								36.38
Streptococcus pneumoniae	E003					15.06			
Streptococcus pneumoniae	E006					14.89			37.69
Haemophilus influenzae	918386						ND		37.72
Haemophilus influenzae	918366c						14.00		38.02
Haemophilus	NCTC						14.44		37.13

Organism	*Code*	*S. aureus*	*MRSA*	*E. coli*	*P. aeruginosa*	*S. pneumoniae*	*H. influenzae*	*Acinetobacter spp*	*Coliform*
influenzae	11931								

ND= Not detected. GAPDH assay negative for all bacteria tested.

7.6 Appendix F Multiplex real-time PCR results by patient

Note: Patients who developed HAP are highlighted in green text

ID:1	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S.aureus	•	•								
	MRSA	•	•								
	E. coli										
	P. aeruginosa										
	H. influenzae	•	•								
	S. pneumoniae	•	•			•	•				
	Acinetobacter										
	Coliform										

ID:2	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•	•	•	•	•	•	•	•	•	•
	Acinetobacter										
	Coliform										

ID:3	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli					•					
	P. aeruginosa										
	H. influenzae	•	•								
	S. pneumoniae		•								
	Acinetobacter										
	Coliform						•				

ID:4	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli	•	•			•	•	•	•		
	P. aeruginosa										
	H. influenzae	•	•								
	S. pneumoniae										
	Acinetobacter										
	Coliform	•	•			•	•	•	•	•	•

ID:5	Day	1		3		5		7		14	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S.aureus										
	MRSA										
	E. coli					•	•				
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae		•			•	•	•	•	•	•
	Acinetobacter										
	Coliform					•	•				

ID:6	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli				•						
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae										
	Acinetobacter			•	•						
	Coliform	•		•	•						

ID:7	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•	•	•	•	•	•	•	•	•	•
	Acinetobacter										
	Coliform										

ID:8	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae	•									
	S. pneumoniae										
	Acinetobacter										
	Coliform				•						•

ID:9	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform			•							

ID:10	Day	0-2		3		4-6		7-9			
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae		•								
	S. pneumoniae					•					
	Acinetobacter										
	Coliform							•			

ID:11	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa			•							
	H. influenzae										
	S. pneumoniae	•	•	•	•	•	•			•	•
	Acinetobacter										
	Coliform	•		•		•	•			•	•

ID:12	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa									•	•
	H. influenzae		•								
	S. pneumoniae										•
	Acinetobacter										
	Coliform					•	•	•	•	•	•

ID:13	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform	•	•			•	•	•	•	•	•

ID:14	Day	0-2		3		4-6		7-9			
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa	•									
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform										

ID:16	Day	0-2		3		4-6		7-9			
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus	•	•								
	MRSA	•									
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform										

ID:18	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli						•				
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform						•				

ID:21	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•	•	•		•		•	•	•	•
	Acinetobacter										
	Coliform										

ID:25	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•		•							
	Acinetobacter										
	Coliform										

ID:28	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli									•	
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform									•	•

ID:29	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae										
	Acinetobacter									•	
	Coliform									•	•

ID:30	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa				•						
	H. influenzae	•	•	•	•	•	•	•		•	•
	S. pneumoniae										
	Acinetobacter						•				
	Coliform										

ID:32	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus			•	•						
	MRSA			•	•						
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform									•	•

ID:33	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•	•								
	Acinetobacter										
	Coliform										

ID:34	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform			•							

ID:35	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•	•	•	•	•	•	•	•		
	Acinetobacter										
	Coliform										

ID:37	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa		•								
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform										

ID:38	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa					•					
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform										

ID:39	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa					•					
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform										

ID:40	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa		•								
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform										

ID:43	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus					•	•	•	•	•	•
	MRSA							•	•		•
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae										
	Acinetobacter	•									
	Coliform										

ID:44	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus			•							
	MRSA			•							
	E. coli			•							
	P. aeruginosa	•				•					
	H. influenzae										
	S. pneumoniae										
	Acinetobacter			•						•	
	Coliform										

ID:45	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae										
	Acinetobacter	•							•		
	Coliform										

ID:46	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										•
	S. pneumoniae									•	•
	Acinetobacter			•							
	Coliform										

ID:47	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa				•						
	H. influenzae					•	•				
	S. pneumoniae										
	Acinetobacter										
	Coliform					•	•			•	

ID:48	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•	•	•	•	•	•	•	•		
	Acinetobacter										
	Coliform										

ID:49	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•	•	•	•	•	•	•	•	•	•
	Acinetobacter										
	Coliform										

ID:50	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae		•		•	•	•	•	•	•	•
	S. pneumoniae										
	Acinetobacter										
	Coliform										

ID:51	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•									
	Acinetobacter										
	Coliform										

ID:52	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•	•	•		•		•		•	
	Acinetobacter										
	Coliform										

ID:53	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										•
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform										

ID:54	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa								•		•
	H. influenzae	•									
	S. pneumoniae	•			•						
	Acinetobacter										
	Coliform										

ID:57	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•	•	•	•	•	•	•	•		
	Acinetobacter										
	Coliform										

ID:58	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae					•		•			
	Acinetobacter										
	Coliform										

ID:59	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae	•	•			•					
	S. pneumoniae	•	•			•				•	•
	Acinetobacter									•	
	Coliform										

ID:61	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli				•						
	P. aeruginosa										
	H. influenzae							•	•		
	S. pneumoniae										
	Acinetobacter										
	Coliform										

ID:63	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•	•	•	•	•					
	Acinetobacter										
	Coliform										

ID:64	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus	•	•								
	MRSA	•	•								
	E. coli										
	P. aeruginosa	•			•	•	•			•	
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform										

ID:65	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•	•			•		•	•	•	•
	Acinetobacter										
	Coliform										

ID:70	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•	•	•		•	•	•	•	•	
	Acinetobacter										
	Coliform										

ID:71	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus	•									
	MRSA	•									
	E. coli	•									
	P. aeruginosa										
	H. influenzae	•	•	•		•		•	•		
	S. pneumoniae										
	Acinetobacter							•			
	Coliform	•									

ID:72	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae	•		•							
	S. pneumoniae	•	•	•		•	•			•	•
	Acinetobacter										
	Coliform										

ID:73	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa									•	•
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform										

ID:74	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa			•							
	H. influenzae										
	S. pneumoniae	•									
	Acinetobacter										
	Coliform										

ID:76	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus	•				•		•			
	MRSA	•									
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae						•				
	Acinetobacter										
	Coliform										

ID:77	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•	•	•	•	•	•				
	Acinetobacter										
	Coliform										

ID:78	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus			•	•	•					
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae					•					
	S. pneumoniae		•	•	•	•	•	•	•	•	
	Acinetobacter										
	Coliform										

ID:79	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae			•	•						
	S. pneumoniae			•	•						
	Acinetobacter										
	Coliform										

ID:80	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli			•							
	P. aeruginosa										
	H. influenzae			•		•	•				
	S. pneumoniae										
	Acinetobacter										
	Coliform			•							

ID:81	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa							•			
	H. influenzae										
	S. pneumoniae	•	•	•	•	•		•		•	•
	Acinetobacter										
	Coliform										

ID:82	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae				•						
	S. pneumoniae	•	•	•		•	•	•	•		
	Acinetobacter										
	Coliform										

ID:83	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus				•		•			•	•
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform										

ID:85	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus			•	•	•		•	•		
	MRSA					•		•			
	E. coli			•		•	•	•	•		
	P. aeruginosa		•								
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform					•	•	•	•		

ID:86	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus	•	•	•	•	•	•	•	•		
	MRSA	•	•	•	•	•	•	•	•		
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform										

ID:87	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae			•							
	Acinetobacter										
	Coliform										

ID:88	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae		•								
	S. pneumoniae										
	Acinetobacter										
	Coliform										

ID:90	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae			•	•					•	
	S. pneumoniae										
	Acinetobacter										
	Coliform										

ID:93	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•	•	•	•	•	•	•	•		
	Acinetobacter										
	Coliform										

ID:96	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa						•				
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform										

ID:97	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa	•	•					•	•		
	H. influenzae										
	S. pneumoniae										
	Acinetobacter										
	Coliform										

ID:98	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•	•	•	•	•		•	•		
	Acinetobacter										
	Coliform										

ID:99	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus										
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•	•	•	•	•	•	•		•	•
	Acinetobacter										
	Coliform										

ID:101	Day	0-2		3		4-6		7-9		13-15	
		Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat	Tongue	Throat
	S. aureus							•			
	MRSA										
	E. coli										
	P. aeruginosa										
	H. influenzae										
	S. pneumoniae	•		•		•		•			
	Acinetobacter										
	Coliform										

7.7 Appendix G Additional tables relating to Chapter 4

Table 81. Demography of study participants

Factor	Median (range) n=93
Age	82 (65-101)
Weight (in kilograms)	62 (36-102)*
Barthel score	20 (4-20)
HABAM score	53 (18-67)
Clinical frailty score	4 (1-9)
Number of teeth	4 (0-28)
Number of prescribed drugs at admission	6 (0-19)
Number of comorbidities	6 (0-15)**
Number of complications	7 (0-50)
COPD (%)	17 (18.28% 95%CI 10.26-26.3)

*n=92 **One patient did not have hearing or vision recorded.

Table 82. Numbers of patients prescribed drugs previously associated with pneumonia

Drug	Number of patients prescribed drug
Statin	50
Sedating/cognitively active drugs*	33
Proton pump inhibitor	26
Angiotensin converting enzyme inhibitor	21
Either inhaled or oral steroid	17
Antibiotic in 3 months prior to admission	15
Steroid inhaler	14
Prednisolone 5mg	6

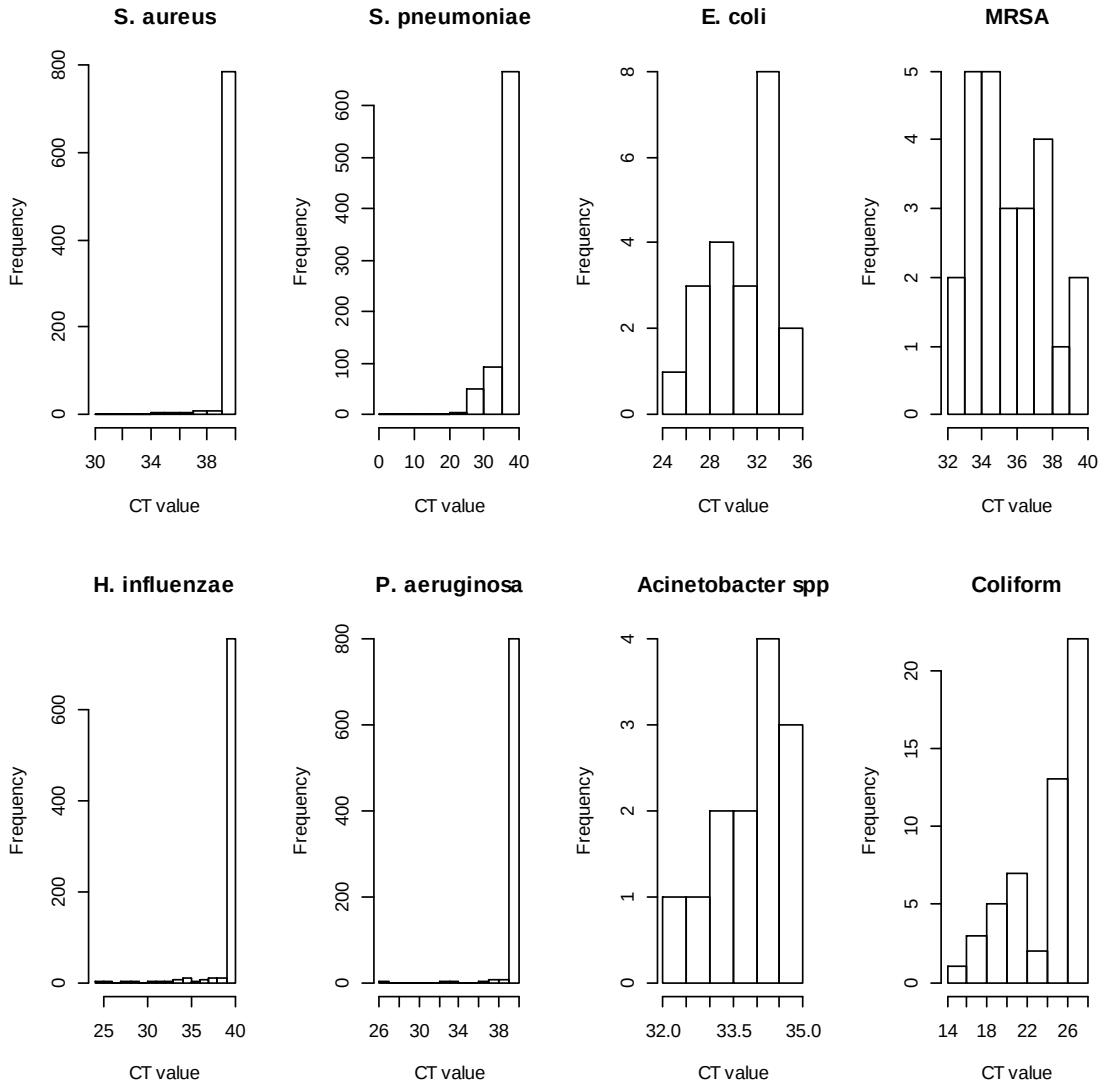
*Sedating or cognitively active drugs include opiates, moderate opiates, selective serotonin reuptake inhibitors, tricyclic antidepressants, anti-epileptic drugs, cholinesterase inhibitors (for dementia), hypnotics, major tranquilisers, gabapentin and pregabalin.

Table 83. Oral examination results by dentate status

	Dentate with or without dentures (n=50)	Dentures only (n=42)	P value**
Median Number of teeth	5	n/a	n/a
Median Xerostomia inventory score	17 (n=31)	15 (n=27)	0.132
Median Dental plaque index	1.2 (n=49)	n/a	n/a
Median Denture plaque index	0.55 (n=20)	0.7 (n=17)	3.544e-06

Note: One patient had neither teeth nor dentures ** Wilcoxon test

Figure 17. Histograms of Ct values from positive real-time PCR assays for each target organism (note varying scales on both axes)



Note: Ct values<35 only are shown for *E. coli* and *Acinetobacter* spp., and <27 for the coliform assay

Table 84. Comparing number of samples positive for target organisms by PCR over time

Organism	Swab 1 (%)*	Swab 2 (%)	Swab 3 (%)	Swab 4 (%)	Swab 5 (%)	Total positive (%)	Patients positive* (%)
S. pneumoniae	52/186 (27.96)	40/168 (23.81)	41/168 (24.40)	33/162 (20.37)	29/132 (21.96)	195/816 (22.89)	38/93 (40.86)
H. influenzae	23/186 (12.36)	15/168 (8.93)	13/168 (7.74)	9/162 (5.56)	4/132 (3.03)	64/816 (7.84)	23/93 (24.73)
S. aureus	11/186 (5.91)	10/168 (5.95)	8/168 (4.76)	8/162 (4.94)	5/132 (3.79)	42/816 (5.15)	14/93 (15.05)
MRSA	10/186 (5.38)	5/168 (2.98)	3/168 (1.79)	5/162 (3.09)	2/132 (1.52)	25/816 (3.06)	11/93 (11.83)
E. coli	3/186 (1.61)	6/168 (3.57)	7/168 (4.17)	4/162 (2.47)	1/132 (0.76)	21/816 (2.57)	11/93 (11.83)
P. aeruginosa	8/186 (4.30)	5/168 (2.98)	4/168 (2.38)	6/162 (3.70)	7/132 (5.31)	30/816 (3.68)	19/93 (20.43)
Acinetobacter spp	2/186 (1.08)	5/168 (2.98)	1/168 (0.59)	2/162 (1.19)	3/132 (2.27)	16/816 (1.96)	8/93 (8.60)
Coliform	9/186 (4.84)	12/168 (7.14)	16/168 (9.52)	16/162 (9.87)	12/132 (9.09)	65/816 (7.97)	19/93 (20.43%)

*Includes any patient with one or more positive samples (tongue or throat)

Table 85. Respiratory commensal pathogens identified in mouths of patients who subsequently developed HAP

Study ID	Organisms seen	Days to HAP
4	Persistent *E. coli* plus *H. influenzae* on day 1 only	2
5	Persistent *S. pneumoniae* and *E. coli* on day 5 only	16
22	No pathogens detected	40
25	*S. pneumoniae* swab 1 and 2 only	38
28	*E. coli* on swab 5 only	33
32	MRSA swab 2 only	8
43	*Acinetobacter* spp on day 1, mixed *S. aureus* and MRSA d5,7,14	88
47	*P. aeruginosa* swab 2 only, *H. influenzae* swab 3 only	27
83	Persistent *S. aureus* in throat from swab 2	88
97	*P. aeruginosa* on swab 1 and 4 (tongue and throat)	30

Table 86. Case vignettes of patients who developed HAP

Patient	Background	Events during admission
4	81 year old male. Nursing home resident Vascular dementia, non- insulin dependent diabetes mellitus, bronchiectasis, recurrent lower respiratory tract infections	48 hours after admission developed fever, green sputum, fever 38.8, CXR shows left basal consolidation. IV antibiotics for 3 days then oral antibiotics. Speech and language review which suggested needed thickened fluids. Developed hyperactive delirium (saying "I'm going to die and no one will help me") then further episode fever, green sputum, upper abdominal pain. Witnessed aspiration following day. Discharged back to nursing home and died of likely pneumonia after course of cefalexin antibiotics around 1 month after discharge
5	85 year old female COPD/asthma, pulmonary hypertension, pulmonary eosinophilia, macular degeneration, cataracts, recent neurological event thought to be stroke	5 days after admission sudden weakness in arm and witnessed aspiration (by VE) plus chest pain. MRI scan showed high grade glioma accounting for neurological change, and troponin was positive indicating non-st elevation myocardial infarction. Produced green sputum but chest x-ray clear on 29/04/09. 01/05/09 Computed tomography pulmonary angiogram (CTPA) done for ?pulmonary embolus but found patchy consolidation. Sputum culture from 29/04/09 showed *S. pneumoniae*. Commenced intravenous antibiotics. Urinary tract infections diagnosed on 12/05 and 19/05. Further chest x ray changes plus raised white cell count, treated as HAP with oral

Patient	Background	Events during admission
		antibiotics. Discharged to continuing care home given terminal illness. Died within 3 months in continuing care.
22	81 year old male Atrial fibrillation, heart failure, anaemia, leg ulcers, cellulitis, benign prostatic hypertrophy	Admitted after fall in hospital while being treated for cellulitis. Decision not to operate because fracture well aligned. Treated with antibiotics for cellulitis, physiotherapy. Discharged "non-weight bearing" after 3 weeks. Readmitted 3 days later after started weight-bearing at home, fracture became unstable and painful. Decision to operate but delays pending investigations (echocardiogram). Developed severe Clostridium difficile infection 24 hours after operation. MRSA positive (nose) so eradication therapy commenced. 48 hours later still sick so antibiotic for C. difficile changed to vancomycin. Transferred to intensive care for fluid balance monitoring after further 24 hours. Given ionotropic support and nasogastric tube inserted for feeding. Developed hyperactive delirium. Oxygen level dropped, white cell count raised and commenced antibiotics for HAP. Died on intensive care after 6 days. Death certificate stated 1A pneumonia, 1b hip fracture II C. difficile colitis

Patient	Background	Events during admission
25	79 year old female Osteoarthritis, non-insulin dependent diabetes, hypertension, breast cancer, previous tuberculosis, fractured clavicle, cholecystectomy, hysterectomy.	Problems with pain control, increased doses of morphine sulphate. Queries about alcohol intake as empty bottles found at home. Depression but didn't want antidepressants. ECG showed indications for pacemaker but patient didn't want one inserted. Slow rehabilitation. One month after admission, cough, grey sputum (grew *S. pneumoniae*), chest x ray showed consolidation. Treated with oral antibiotics. Discharged home.
28	93 year old male Osteoarthritis	Cough 48 hours after admission but chest x- ray clear. 3 days later oxygen levels, dropped to 92%, blood pressure dropped, temperature 37.5, auscultatory findings at left lung base. Treated with intravenous antibiotics for 10 days. Constipation after but otherwise well. 14 days later profound fluid resistant hypotension, tachycardia, hypoxia, decreased responsiveness. CXR showed right lower lobe consolidation. Intravenous antibiotics started. Died 3 days later, after antibiotic change.
32	101 year old female	7 days after admission developed high respiratory rate, high white cell count and C reactive protein. New right basal consolidation on chest x ray. Treated with oral

Patient	Background	Events during admission
	Atrial fibrillation, ischaemic heart disease, heart failure, falls, peripheral vascular disease, fem-pop bypass, throat cancer, skin cancer (Squamous cell), previous hip replacement	antibiotics. Day 14 antibiotics for C. difficile started.Day 21 died, death certificate 1a bronchopneumonia, 1b old age, II fractured neck of femur
35	79 year old female Ischaemic heart disease with coronary stent insertion, total hip replacement, previous fractures, tinnitus, gallstones, COPD/asthma, osteoarthritis, hiatus hernia, mastoidectomy	Day 3 post-operative anaemia, transfusion and subsequent transfusion reaction treated with prednisolone and salbutamol. Day 4 still anaemic so further transfusion and further transfusion reaction. Day 7 post op had green phlegm, *H. influenzae* isolated from sputum but chest x-ray bibasal atelectasis. Treated for LRTI with oral antibiotics. Day 8 oral thrush treated with nystatin. Day 36 further operation as implant was cutting through bone and then three UTIs before discharge.
43	83 year old male	Operation delayed as needed treatment for heart failure, then postoperative atrial fibrillation treated with metoprolol. Day 34, coliform UTI. Day 44 clot retention and

Patient	Background	Events during admission
	Heart failure, prostate cancer, anaemia, hypertension	haematuria from active prostate cancer. Transferred to Freeman hospital for radiotherapy and Proteus UTI treated with oral antibiotics. Following day developed bleeding per rectum and transferred to Royal Victoria Infirmary for treatment. Difficulty making decision about going into institutional care, said would consider suicide but for religious beliefs.Day 57 developed rattly but unproductive cough, hypoxia, fever, tachycardia and hypotension but with no chest x ray change. He was given IV Tazocin (antibiotic) for HAP. Died 5 days later and death certificate recorded as 1A Pneumonia II Metastatic prostate carcinoma.
47	85 year old female Ischaemic heart disease, heart failure, non-insulin dependent diabetes, lymphoma	Pressure sores on sacrum and heels developed and debrided. Day 5 dysphagia therefore not eaten and blood sugar low. Also cough productive of green sputum but chest x ray clear. Treated with intravenous antibiotics for aspiration pneumonia though no witnessed aspiration plus oral prednisolone in case lymphoma contributing (though recent CT suggested no change). Day 7 green sputum on nightgown and hypoactive delirium- no verbal interaction. Day 28 transferred to Freeman hospital but noted to have HAP (consolidation on chest x ray) on arrival and treated with intravenous antibiotics (tazocin). Limited improvement over following month then deteriorated.

Patient	Background	Events during admission
		Died on day 69, death certificate 1a Pneumonia II Hip fracture, lymphoma
83	77 year old female Non-insulin dependent diabetes, hypertension, hypercholesterolaemia	Day 4 hyperactive delirium from UTI treated with oral antibiotics. Day 11 fall off bed. Day 12 DVT treated with warfarin. Day 29 further hyperactive delirium and given regular haloperidol. Day 57 regular quetiapine for ongoing confusion. Day 67 three further falls and two CT head scans. Warfarin stopped due to risk of falls. Day 84 lorazepam added. Day 86 became unwell and given oxygen and tinzaparin for ?pulmonary embolus. Grew *S. aureus* in blood cultures and clinically evident pneumonia on auscultation. Chest x ray showed atelectasis and effusion. 4 days IV antibiotics given. Further clinical signs of HAP 3 days later so further 14 days IV antibiotics given. Day 104 nasogastric tube inserted for feeding but removed after kept being pulled out . Several further UTIs. Discharged to nursing home
97	70 year old female Depression and recent suicide attempt, breast cancer, lung cancer, hypertension,	Day 5 suicidal thoughts, UTI treated with oral antibiotics. Day 6 seen by psychiatry and deemed moderate to high suicide risk and placed under constant observation. Day 10 mood better but cough with green phlegm, treated with oral antibiotics. Day 15 bone biopsy showed pathological fracture and oncology review said "incurable metastatic

Patient	Background	Events during admission
	hypercholesterolaemia,	cancer". Day 17 radiotherapy commenced. Day 21 cough with green phlegm and chest x ray showed consolidation so treated with oral antibiotics. Day 29 acute respiratory failure, transferred to high dependency unit, chest drain inserted and non-invasive respiratory support commenced. Antibiotics changed to intravenous tazocin. Died day 30, death certificate 1a pneumonia, 1b metastatic squamous lung cancer, II pathological fracture of right neck of femur, breast cancer.

7.8 Appendix H Additional data relating to Chapter 5

Table 87. Standard errors for correlation matrix of patient variables

	Age	Female	Residence	Barthel	Weight	Teeth	Frailty	HABAM	Charlson	Dementia	Dentures	Smoking	Plaque1	IMD	COPD	Consent
Age																
Female	0.14															
Residence	0.10	0.13														
Barthel	0.10	0.13	0.09													
Weight	0.10	0.13	0.10	0.11												
Teeth	0.10	0.14	0.11	0.10	0.11											
Frailty	0.09	0.11	0.09	0.07	0.11	0.10										
HABAM	0.09	0.13	0.09	0.08	0.11	0.10	0.05									
Charlson	0.09	0.12	0.10	0.09	0.10	0.10	0.09	0.09								
Dementia	0.23	0.27	0.17	0.13	0.23	0.21	0.18	0.17	0.19							
Dentures	0.10	0.13	0.11	0.10	0.11	0.03	0.10	0.10	0.11	0.22						
Smoking	0.10	0.14	0.11	0.11	0.11	0.10	0.10	0.11	0.11	0.21	0.10					
Plaque1	0.11	0.14	0.10	0.10	0.11	0.09	0.11	0.11	0.11	0.17	0.09	0.10				
IMD	0.11	0.13	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.19	0.10	0.10	0.10			
COPD	0.14	0.19	0.18	0.17	0.15	0.16	0.16	0.16	0.16	5.92	0.15	0.14	0.15	0.15		
Consent	0.09	0.15	0.09	0.08	0.10	0.10	0.10	0.09	0.10	0.12	0.10	0.11	0.11	0.10	0.19	
Antibiotics	0.15	0.21	0.14	0.13	0.17	0.15	0.15	0.15	0.15	0.26	0.16	0.16	0.16	0.17	0.22	0.13

Barthel=Barthel index, Teeth=number of teeth, Frailty=Clinical frailty score, Charlson=Charlson index, Plaque1=Admission plaque score, IMD= Indices of multiple deprivation score, COPD=Combined obstructive respiratory disease, Consent= ability to sign consent for oneself, Antibiotics=Prescribed antibiotics within three months prior to hospital admission

Table 88. Associations between HAP and patient factors using Fisher's exact test

Variable	HAP (n=10) median (min-max)	No HAP (n=80) median (min-max)	P value
Age	82 (70-101)	82 (65-96)	0.061
Female	N=5	N=56	0.281
Clinical Frailty Scale	5 (3-7)	4 (1-9)	0.570
Charlson index	6 (4-11)	5 (2-11)	0.067
HABAM (mobility score)	50.5 (18-61)	53 (20-67)	0.194
Barthel score	19 (4-20)	20 (4-20)	0.143
Number of teeth	7 (0-27)	3.5 (0-28)	0.353
Admission plaque quartile score	3 (1-4)	2 (1-4)	0.265
Plaque quartile score at 7 days	3.5 (1-4)	3 (1-4)	0.403
Plaque quartile score at 14 days	3 (1-4)	2 (1-4)	0.451
Deprivation score	14.02 (3.71-49.48)	22.40 (2.32-71.74)	0.058
Sedating drug at admission	N=6	26	0.157

Table 89. Relating HAP/LRTI and patient factors using univariate generalised linear modelling

Variable	Estimate	Standard error	Z value	P value	Null deviance	Residual deviance
Age	0.06208	0.03937	1.577	0.115	90.072 on 89	87.378 on 88
Clinical frailty scale	0.5266	0.1964	2.682	0.007 **	90.072 on 89	81.648 on 88
Decreased mobility	-0.04710	0.02282	-2.064	0.039*	90.072 on 89	85.788 on 88
Barthel index	-0.07789	0.06217	-1.253	0.210	90.072 on 89	88.605 on 88
Charlson index	0.3890	0.1191	3.267	0.001 **	90.072 on 89	78.302 on 88
Number of teeth	0.01721	0.02649	0.650	0.516	90.072 on 89	89.656 on 88
Admission plaque quartile score	0.3267	0.2359	1.385	0.166	89.623 on 88	87.645 on 87
Day 7 plaque quartile score	0.3673	0.2684	1.368	0.171	79.159 on 77	77.171 on 76$
Deprivation score (IMD)	-0.004427	0.013755	-0.322	0.748	89.623 on 88	89.518 on 87
Female	-0.9555	0.5399	-1.770	0.077.	90.072 on 89	86.971 on 88
Sedating drug	1.0441	0.5389	1.937	0.053 .	90.072 on 89	86.288 on 88

Table 90. Associations between HAP and in-hospital events using Fisher's exact test

Variable	HAP (N=10)	No HAP (n=80)	P value	Odds ratio (95% confidence intervals)
Length of operation (mins)	90 (45-165)	90 (24-150)*	0.1754	
Delirium	4/10	6/80	0.01	7.89 (1.28-46.31)
Aspiration	3/10	1/80	<0.01	30.88 (2.18 -1773.08)
UTI	3/10	17/80	0.6865	
Fall	3/10	5/80	0.04	6.21 (0.80-41.00)
Cough	9/10	32/80	<0.01	13.17 (1.69 -601.85)
Infectious illness	2/10	6/80	0.2162	
Increase in analgesia	3/10	13/80	0.3747	
Transfusion	1/10	15/80	0.6839	

*Three patients had no operation

Table 91. Univariate generalised linear models relating HAP/LRTI and in-hospital events

Variable	Estimate	Standard error	Z value	P value	Null deviance (df)	Residual deviance (df)
Aspiration	2.6532	1.1892	2.231	0.026 *	90.072 on 89	84.104 on 88
Delirium	1.6397	0.7013	2.338	0.019 *	90.072 on 89	84.870 on 88
UTI	0.7282	0.5820	1.251	0.211	90.072 on 89	88.575 on 88
Transfusion	-0.09685	0.70276	-0.138	0.89	90.072 on 89	90.053 on 88
Operation length	0.015566	0.009781	1.591	0.112	88.708 on 86	86.086 on 85
Fall	2.1800	0.7904	2.758	0.006 **	90.072 on 89	82.292 on 88
Analgesia increase	0.3567	0.6492	0.549	0.583	90.072 on 89	89.781 on 88
Infection (not HAP or LRTI)	0.9858	0.7842	1.257	0.209	90.072 on 89	88.617 on 88
Non-infectious illness	0.3567	0.6492	0.549	0.583	90.072 on 89	89.781 on 88

df= degrees of freedom

Table 92. Comparing patient characteristics between patients with and without HAP/LRTI

Variable	HAP/LRTI (n=18) median (min-max)	No HAP/LRTI (n=72) median (min-max)	P value
Age	82.5 (70-101)	82 (65-96)	0.304
Female	9/18	52/72	0.087
Frailty	5 (3-9)	4 (1-7)	0.006**
Charlson index	6 (4-11)	4 (2-11)	<0.001**
HABAM	50 (18-61)	53 (20-67)	0.010**
Barthel	19 (4-20)	20 (4-20)	0.013*
Number of teeth	6 (0-27)	3.5 (0-28)	0.406
Admission plaque quartile score	3 (1-4)	2 (1-4) (1 NA)	0.24
Plaque quartile score at 7 days	3 (1-4) (2 NAs)	3 (1-4) (10 NAs)	0.1626
Deprivation score	16.39 (3.71-65.94)	22.4 (2.32- 71.74)	0.9764
Sedating drug at admission	10/18	22/72	0.05844

Table 93. Comparing in-hospital events and patient outcomes between patients with and without HAP/LRTI

Variable	HAP/LRTI (n=18) median (min-max)	No HAP/LRTI (n=72) median (min-max)	P value	Odds ratio (95% CI)
Delirium	5/18	5/72	0.021	5.25 (1.05- 26.54)
Aspiration	3/18	1/72	0.022*	14.17 (1.06- 782.77)
Cough	13/18	28/72	0.009	4.29 (1.27- 17.08)
Increase in analgesia	4/18	12/72	0.504	
Length of operation	95 (45-165)	22 (4-265)	0.267	
Length of stay	37.5 (13-140)	22 (4-265)	0.002**	
Died during study	8/18	7/72	0.001**	7.52 (1.93-30.84)

Table 94. Generalised linear model relating HAP and *S. aureus* colonisation index

Variable	*Estimate*	*Std. Error*	*z value*	*P value*
S. aureus	0.2889	0.1737	1.663	0.0963

Null deviance: 62.79 on 89 degrees of freedom, Residual deviance: 60.05 on 87 degrees of freedom, AIC: 66.05

Table 95. Generalised linear model relating HAP/LRTI and MRSA colonisation index

Variable	*Estimate*	*Std. Error*	*z value*	*p value*	*OR*	*95%CI*
MRSA	0.5463	0.3047	1.793	0.073 .	1.74	0.96-3.17

Null deviance: 90.072 on 89 degrees of freedom, Residual deviance: 84.483 on 87 degrees of freedom, AIC: 90.483

Table 96. Generalised linear model relating HAP/LRTI with *E. coli* colonisation index

Variable	*Estimate*	*Std. Error*	*z value*	*P value*
E. coli	0.3297	0.2266	1.455	0.1456

Null deviance: 90.072 on 89 degrees of freedom, Residual deviance: 86.972 on 87 degrees of freedom, AIC: 92.972

Table 97. Comparing acquisition of pathogens between patients with and without HAP/LRTI

Variable	*HAP/LRTI (N=18)*	*No HAP/LRTI (n=72)*	*P value*
H. influenzae	5/18	18/72	0.765
S. aureus	5/18	9/72	0.136
S. pneumoniae	6/18	31/72	0.596
MRSA	4/18	7/72	0.214
P. aeruginosa	3/18	15/72	1
E. coli	3/18	8/72	0.44
Acinetobacter spp.	1/18	7/72	1

Table 98. Comparing patient characteristics between uncolonised and colonised persons

Variable	Uncolonised (N=20)	Colonised (n=73)	P value	Odds ratio (95% confidence intervals)
Age (median)	82	82 (65-101)	0.90	
Female	13/20	51/73	0.79	
Clinical frailty score	4 (2-6)	5 (1-9)	0.25	
Barthel score (median)	20 (17-20)	20 (4-20)	0.01**	N/A
HABAM score (median)	52 (20-67)	53 (18-67)	0.90	
Charlson index (median)	4 (2-10)	5 (2-11)	0.18	
Number of teeth (median)	2 (0-27)	6(0-28)	0.53	
COPD	2/20	16/73	0.34	
Dementia	0/20	6/73	0.33	
Diabetes mellitus	0/20	9/73	0.20	
Cerebrovascular disease	5/20	11/73	0.32	
Cognitively active drug	3/20	30/73	0.04*	3.90 (1.00- 22.61)
Proton pump inhibitor	4/20	22/73	0.57	
Statin	10/20	40/73	0.80	
ACE inhibitor	6/20	15/73	0.38	
Inhaled steroid	4/20	10/73	0.49	
Oral steroids	1/20	5/73	1	
Either oral/inhaled steroids	4/20	13/73	0.76	
Recent antibiotics	1/20	14/73	0.18	

HABAM= Hierarchical assessment of Balance and Mobility score, COPD= combined obstructive pulmonary disease, ACE= Angiotensin converting enzyme

Table 99. Comparing infection-related outcomes between colonised and uncolonised persons

Variable	Uncolonised (N=20)	Colonised (n=73)	P value	Odds ratio (95% confidence intervals)
HAP	1/20	9/73	0.446	
HAP/LRTI	2/20	16/73	0.343	
Cough	3/20	38/73	0.004	6.04 (1.56-34.91)
UTI	5/20	15/73	0.760	
Other post-operative infection	2/20	6 /73	0.68	
Length of stay	25.5(11-265)	25 (4-189)	0.347	
Death during study	2/20	13/73	0.511	

UTI= Urinary tract infection

Table 100. Summary of multivariate analysis using generalized linear modeling relating acquisition of individual organisms and significant associations with patient characteristics

Organism	Significant patient characteristics	Odds ratio
S. aureus	Recent antibiotics	5.83
MRSA	Recent antibiotics	5.40
	Higher Charlson index	1.27
E. coli	Lower Barthel score	0.80
P. aeruginosa	Increased weight	1.04
	Higher clinical frailty score	1.49
S. pneumoniae	More teeth	1.09
	Lower Charlson index	0.76
H. influenzae	Fewer teeth	0.95
	Dementia	8.26
Acinetobacter spp	Lower Barthel score	0.86

Table 101. Multivariate generalized linear model relating acquisition of *S. pneumoniae* (binomial variable) with medical model of patient characteristics

Variable	Estimate	Standard Error	z value	P value
Number of teeth	0.08542	0.02503	3.413	0.001 ***
Charlson index	-0.27569	0.11936	-2.310	0.021 *

Null deviance: 125.80 on 92 degrees of freedom, Residual deviance: 105.35 on 90 degrees of freedom, AIC: 111.35

Table 102. Multivariate generalized linear model relating acquisition of *H. influenzae* (binomial variable) with medical model of patient characteristics

Variable	Estimate	Standard Error	z value	P value
Number of teeth	-0.05226	0.03024	-1.728	0.084.
Dementia	2.11181	0.93669	2.255	0.024 *

Null deviance: 104.041 on 92 degrees of freedom, Residual deviance: 95.567 on 90 degrees of freedom, AIC: 101.57

Table 103. Multivariate generalized linear model relating acquisition of *S. aureus* (binomial variable) with medical model of patient characteristics

Variable	Estimate	Standard Error	z value	P value
Recent antibiotics	1.7636	0.6458	2.731	0.006**

Null deviance: 78.797 on 92 degrees of freedom, Residual deviance: 71.777 on 91 degrees of freedom, AIC: 75.777

Table 104. Multivariate generalized linear model relating acquisition of MRSA (binomial variable) with medical model of patient characteristics

Variable	Estimate	Standard Error	z value	P value
Recent antibiotics	1.6858	0.7151	2.357	0.018 *
Charlson index	0.2394	0.1359	1.762	0.078 .

Null deviance: 67.608 on 92 degrees of freedom, Residual deviance: 58.333 on 90 degrees of freedom, AIC: 64.333

Table 105. Multivariate generalized linear model relating acquisition of *P. aeruginosa* (binomial variable) with medical model of patient characteristics

Variable	Estimate	Standard Error	z value	P value
Weight	0.03890	0.01937	2.008	0.044*
Clinical frailty score	0.39928	0.19347	2.064	0.0390 *

Null deviance: 93.713 on 91 degrees of freedom , Residual deviance: 85.920 on 89 degrees of freedom (1 observation deleted due to missingness), AIC: 91.92,

Table 106. Multivariate generalized linear model relating acquisition of *E. coli* (binomial variable) with medical model of patient characteristics

Variable	Estimate	Standard Error	z value	P value
Barthel	-0.22796	0.06984	-3.264	0.001**

Null deviance: 67.608 on 92 degrees of freedom, Residual deviance: 56.531 on 91 degrees of freedom, AIC: 60.531

Table 107. Multivariate generalized linear model relating acquisition of *Acinetobacter* spp (binomial variable) with medical model of patient characteristics

Variable	Estimate	Standard Error	z value	P value
Barthel	-0.15503	0.07161	-2.165	0.030 *

Null deviance: 54.542 on 92 degrees of freedom, Residual deviance: 50.446 on 91 degrees of freedom, AIC: 54.446

Table 108. Summary of significant results from univariate analysis using generalised linear models relating colonisation index of individual organisms and patient factors (*S. aureus*, MRSA, *E. coli* and *P. aeruginosa*)

Organism	Positive risk factors (odds ratio)	Negative risk factors (odds ratio)
S. aureus	COPD (2.87)	Female (0.38)
	Recent Antibiotics (4.61)	Never smoked (0.26)
	Charlson index (1.17)	
MRSA	Age (1.08)	Female (0.26)
	COPD (3.89)	Never smoked (0.11)
	Recent antibiotics (6.46)	Decreased weight (0.97)
	Charlson index (1.24)	
E. coli	Unable to self consent (3.33)	Female (0.13)
	Residence in institution (6.86)	Never smoked (0.16)
	Clinical frailty score (1.84)	Decreased weight (0.96)
	Dementia (9.42)	Number of teeth (0.91)
	Plaque quartile score on admission (1.66)	Mobility score (0.92)
		Barthel score (0.81)
P. aeruginosa	Weight (1.03)	
Acinetobacter spp	Residence in institution (6.23)	Low barthel score (0.85)
	High clinical frailty score (1.66)	Low mobility score (0.95)

Table 109. Summary of significant results from univariate analysis using generalised linear models relating colonisation index of individual organisms and patient characteristics (*S. pneumoniae*, *H. influenzae* and *Acinetobacter* spp.)

Organism	Positive risk factors (odds ratio)	Negative risk factors (odds ratio)
S. pneumoniae	Female (2.08)	Age (0.96)
	Barthel score (1.18)	No recent antibiotics (0.61)
	Number of teeth (1.05)	Community living (0.12)
	HABAM score (1.06)	Low clinical frailty score (0.75)
		Ex smoker (0.37)
		Never smoked (0.68)
		Low charlson index (0.78)
		No cerebrovascular disease (0.56)
H. influenzae	Female (2.24)	Age (0.96)
	COPD (3.08)	Low Barthel score (0.90)
	Unable to self consent (1.84)	Fewer teeth (0.92)
	Residence in institution (2.29)	Low mobility score (0.96)
	High clinical frailty score (1.27)	Ex smoker (0.45)
	Dementia (4.10)	Never smoker (0.36)
	Plaque quartile admission score (1.29)	

Table 110. Univariate generalised linear models relating colonisation index (*S. aureus*) and baseline patient variables

Factor	Estimate	Standard Error	Z value	P value	Odds ratio	95% confidence intervals	Residual deviance
Age	0.02337	0.02219	1.053	0.292			161.76 on 91 df
Female	-0.9589	0.3190	-3.006	0.003 **	0.38	0.21-0.72	153.90 on 91 df
COPD	1.0537	0.3267	3.225	0.001 **	2.87	1.51-5.44	153.37 on 91 df
Recent antibiotics	1.5289	0.3231	4.732	<0.001 ***	4.61	2.45-8.69	142.36 on 91 df
Self-consent	-0.7869	0.5831	-1.349	0.177			160.52 on 91 df
Residence institution	-17.1101	1361.8872	-0.013	0.990			153.08 on 90 df
Residence hospital	0.0117	0.7456	0.016	0.987			
Barthel score	0.09586	0.06854	1.399	0.162			160.29 on 91 df
Weight	-0.02538	0.01295	-1.960	0.050			157.96 on 90 df
Number of teeth	-0.005428	0.016606	-0.327	0.744			162.78 on 91 df
Clincial Frailty Score	-0.03751	0.10503	-0.357	0.721			162.76 on 91 df
HABAM score	0.008352	0.015600	0.535	0.592			162.59 on 91 df
Ex-smoker	-0.2566	0.3861	-0.665	0.5063			153.95 on 90 df
Never smoker	-1.3487	0.5276	-2.556	0.011 *	0.26	0.09-0.73	
Charlson index	0.15844	0.06236	2.541	0.011 *	1.17	1.04-1.32	156.91 on 91 df
CVD	-0.4470	0.4853	-0.921	0.357			161.95 on 91 df
Dementia	0.6832	0.4976	1.373	0.17			161.26 on 91 df
Plaque quartile score admission	0.2637	0.1424	1.852	0.064 .			158.75 on 90 df

COPD=Combined obstructive pulmonary disease, HABAM= Hierarchical balance and mobility score, CVD=cerebrovascular disease

Null deviance 162.89 on 92 df , df=degrees of freedom, *p<0.05,**p<0.01,and ***p<0.001

Table 111. Univariate generalised linear models relating colonisation index (MRSA) and baseline patient variables

Factor	Estimate	Standard Error	Z value	P value	Odds ratio	95% confidence intervals	Null Deviance	Residual deviance
Age	0.07505	0.02950	2.544	0.011 *	1.08	1.02-1.14	113.45 on 92 df	106.75 on 91 df
Female	-1.3432	0.4234	-3.172	0.002 **	0.26	0.11-0.60	113.45 on 92 df	102.84 on 91 df
COPD	1.3593	0.4101	3.315	0.001 ***	3.89	1.74-8.70	113.45 on 92 df	103.28 on 91 df
Recent antibiotics	1.8654	0.4138	4.508	<0.001 ***	6.46	2.87-14.53	113.45 on 92 df	94.10 on 91 df
Self-consent	-0.2706	0.5838	-0.463	0.643			113.45 on 92 df	113.21 on 91 df
Residence in institution	-16.5567	1361.8872	-0.012	0.990			113.45 on 92 df	107.06 on 90 df
Residence in hospital	0.5651	0.7579	0.746	0.456				
Barthel score	0.02511	0.06506	0.386	0.700			113.45 on 92 df	113.29 on 91 df
Weight	-0.03505	0.01724	-2.033	0.042 *	0.97	0.93-1.00	112.96 on 91 df	108.40 on 90 df
Number of teeth	-0.04927	0.02635	-1.87	0.062 .			113.45 on 92 df	109.26 on 91 df
Clinical Frailty Score	0.2144	0.1317	1.628	0.104			113.45 on 92 df	110.83 on 91 df
HABAM score	-0.01803	0.01733	-1.040	0.298			113.45 on 92 df	112.43 on 91 df
Ex-smoker	0.3973	0.5573	0.713	0.476			113.45 on 92 df	99.067 on 90 df
Never smoker	-2.2242	1.1233	-1.980	0.048 *	0.11	0.01-0.98		
Charlson index	0.21243	0.07756	2.739	0.006**	1.24	1.06-1.44	113.45 on 92 df	106.58 on 91 df
CVD	-0.4380	0.6223	-0.704	0.482			113.45 on 92 df	112.90 on 91 df
Dementia	-0.4934	1.0306	-0.479	0.632			113.45 on 92 df	113.18 on 91 df
Plaque quartile score on admission	0.1925	0.1802	1.068	0.285			113.08 on 91 df	111.92 on 90 df

COPD=Combined obstructive pulmonary disease, HABAM= Hierarchical balance and mobility score, CVD=cerebrovascular disease, df=degrees of freedom *p<0.05,**p<0.01,and ***p<0.001,

Table 112. Univariate generalised linear models relating colonisation index (*E. coli*) and baseline patient variables

Factor	Estimate	Standard Error	Z value	P value	Odds ratio	95% confidence intervals	Null Deviance	Residual deviance
Age	0.04904	0.03071	1.597	0.110			108.68 on 92 df	106.06 on 91 df
Female	-2.0487	0.5111	-4.008	<0.001 ***	0.13	0.05-0.35	108.68 on 92 df	88.22 on 91 df
COPD	-0.1188	0.5573	-0.213	0.831			108.68 on 92 df	108.64 on 91 df
Recent antibiotics	-0.7271	0.7459	-0.975	0.33			108.68 on 92 df	107.53 on 91 df
Self-consent	1.2026	0.3452	3.484	<0.001***	3.33	1.69-6.55	108.68 on 92 df	98.86 on 91 df
Residence institution	1.9250	0.4319	4.457	<0.001***	6.86	2.94-15.98	108.68 on 92 df	89.354 on 90 df
Residence hospital	-14.9045	1357.6515	-0.011	0.991				
Barthel score	-0.20937	0.03423	-6.117	<0.001 ***	0.81	0.76-0.87	108.68 on 92 df	76.025 on 91 df
Weight	-0.04435	0.02305	-1.924	0.054 .	0.96	0.91-1.00	69.960 on 91 df	65.771 on 90 df
Number of teeth	-0.09109	0.03620	-2.516	0.012 *	0.91	0.85-0.98	108.68 on 92 df	99.052 on 91 df
Clinical Frailty Score	0.6100	0.1365	4.468	<0.001***	1.84	1.41-2.40	108.68 on 92 df	88.334 on 91 df
HABAM score	-0.078338	0.014761	-5.307	<0.001***	0.92	0.90-0.95	108.68 on 92 df	82.257 on 91 df
Ex smoker	-0.8067	0.4679	-1.724	0.085			108.68 on 92 df	100.48 on 90 df
Never smoker	-1.8187	0.6856	-2.653	0.008 **	0.16	0.04-0.62		
Charlson index	-0.007187	0.099062	-0.073	0.942			108.68 on 92 df	108.68 on 91 df
CVD	-0.3427	0.6260	-0.547	0.584			108.68 on 92 df	108.36 on 91 df
Dementia	2.2429	0.4506	4.977	<0.001***	9.42	3.90-22.78	108.68 on 92 df	89.197 on 91 df
Plaque quartile score on admission	0.5048	0.2097	2.408	0.016 *	1.66	1.10-2.50	106.87 on 91 df	100.40 on 90 df

COPD=Combined obstructive pulmonary disease, HABAM= Hierarchical balance and mobility score, CVD=cerebrovascular disease, df=degrees of freedom, *p<0.05,**p<0.01,and ***p<0.00

Table 113. Univariate generalised linear models relating colonisation index (*P. aeruginosa*) and baseline patient variables

Factor	Estimate	Standard Error	Z value	P value	Odds ratio	95% confidence intervals	Null Deviance	Residual deviance
Age	-0.005945	0.025532	-0.233	0.816			97.063 on 92 df	97.009 on 91 df
Female	0.4218	0.4382	0.963	0.336			97.063 on 92 df	96.073 on 91 df
COPD	0.5921	0.4082	1.45	0.147			97.063 on 92 df	95.122 on 91 df
Recent antibiotics	-0.5730	0.6159	-0.93	0.352			97.063 on 92 df	96.063 on 91 df
Self-consent	-1.544	1.002	-1.540	0.124			97.063 on 92 df	92.857 on 91 df
Residence institution	-1.3553	1.0231	-1.325	0.185			97.063 on 92 df	91.842 on 90 df
Residence hospital	-15.7869	1357.6515	-0.012	0.991				
Barthel score	0.01607	0.05759	0.279	0.780			97.063 on 92 df	96.982 on 91 df
Weight	0.03239	0.01271	2.548	0.011 *	1.03	0.91-1.14	97.063 on 92 df	90.253 on 90 df
Number of teeth	0.01135	0.01861	0.61	0.542			97.063 on 92 df	96.697 on 91 df
Clincial Frailty Score	0.1000	0.1209	0.827	0.408			97.063 on 92 df	96.385 on 91 df
HABAM score	0.004375	0.017880	0.245	0.807			97.063 on 92 df	97.002 on 91 df
Ex smoker	0.4618	0.6375	0.724	0.469			97.063 on 92 df	96.433 on 90 df
Never smoker	0.4614	0.6603	0.699	0.485				
Charlson index	0.1269	0.0757	1.677	0.0936			97.063 on 92 df	94.444 on 91 df
CVD	0.5428	0.4231	1.283	0.199			97.063 on 92 df	95.548 on 91 df
Dementia	-16.7507	1839.2432	-0.009	0.993			97.063 on 92 df	93.176 on 91 df
Plaque quartile score on admission	0.2293	0.1660	1.381	0.167			96.627 on 91 df	94.676 on 90 df

COPD=Combined obstructive pulmonary disease, HABAM= Hierarchical balance and mobility score, CVD=cerebrovascular disease, df=degrees of freedom, *p<0.05,**p<0.01,and ***p<0.001

Table 114. Univariate generalised linear models relating colonisation index (*S. pneumoniae*) and baseline patient variables

Factor	Estimate	Standard Error	Z value	P value	Odds ratio	95% confidence intervals	Null Deviance	Residual deviance
Age	-0.03924	0.01076	-3.646	<0.001 ***	0.96	0.94-0.98	359.72 on 92 df	346.39 on 91 df
Female	0.7301	0.1992	3.666	<0.001 ***	2.08	1.40-3.07	359.72 on 92 df	344.85 on 91 df
COPD	0.25071	0.19050	1.316	0.188			359.72 on 92 df	358.03 on 91 df
Recent antibiotics	-0.50171	0.24947	-2.011	0.044 *	0.61	0.37-0.99	359.72 on 92 df	355.29 on 91 df
Self-consent	0.02973	0.18914	0.157	0.875			359.72 on 92 df	359.70 on 91 df
Residence institution	-2.10432	0.59253	-3.551	<0.001 ***	0.12	0.04-0.39	359.72 on 92 df	334.96 on 90 df
Residence hospital	0.09508	0.36919	0.258	0.797				
Barthel score	0.16253	0.04272	3.805	<0.001 ***	1.18	1.08-1.28	359.72 on 92 df	337.51 on 91 df
Weight	-0.001848	0.005838	-0.317	0.752			359.26 on 91 df	359.16 on 90 df
Number of teeth	0.050890	0.007926	6.421	<0.001 ***	1.05	1.04-1.07	359.72 on 92 df	317.71 on 91 df
Clinical Frailty Score	-0.28837	0.05499	-5.244	<0.001 ***	0.75	0.67-0.83	359.72 on 92 df	330.79 on 91 df
HABAM score	0.054108	0.009557	5.661	<0.001 ***	1.06	1.04-1.08	359.72 on 92 df	321.48 on 91 df
Ex smoker	-1.0045	0.2133	-4.710	<0.001 ***	0.37	0.24-0.56	359.72 on 92 df	335.37 on 90 df
Never smoker	-0.3947	0.2081	-1.896	0.058 .	0.68	1.45-1.01		
Charlson index	-0.25026	0.04831	-5.18	<0.001 ***	0.78	0.71-0.86	359.72 on 92 df	327.38 on 91 df
CVD	-0.57153	0.24866	-2.298	0.022 *	0.56	0.35-0.92	359.72 on 92 df	353.86 on 91 df
Dementia	-0.14315	0.34180	-0.419	0.675			359.72 on 92 df	359.54 on 91 df
Plaque quartile score on admission	-0.29922	0.07242	-4.132	<0.001 ***	0.74	0.64-0.85	357.11 on 91 df	339.40 on 90 df

COPD=Combined obstructive pulmonary disease, HABAM= Hierarchical balance and mobility score, CVD=cerebrovascular disease, df=degrees of freedom, numbers to 2 d.p., *p<0.05,**p<0.01,and ***p<0.001

Table 115. Univariate generalised linear models relating colonisation index (*H. influenzae*) and baseline patient variables

Factor	Estimate	Standard Error	Z value	P value	Odds ratio	95% CI	Null deviance	Residual deviance
Age	-0.04393	0.01722	-2.551	0.011 *	0.96	0.93-0.99	189.49 on 92 df	183.04 on 91 df
Female	0.8046	0.3398	2.368	0.018*	2.24	1.15-4.35	189.49 on 92 df	182.99 on 91 df
COPD	1.1243	0.2683	4.19	<0.001 ***	3.08	1.82-5.21	189.49 on 92 df	173.25 on 91 df
Recent antibiotics	0.3513	0.3168	1.109	0.267			189.49 on 92 df	188.33 on 91 df
Self-consent	0.6107	0.2619	2.332	0.020*	1.84	1.10-3.08	189.49 on 92 df	184.68 on 91 df
Residence institution	0.8282	0.3153	2.627	0.009 **	2.29	1.23-4.25	189.49 on 92 df	178.13 on 90 df
Residence hospital	-16.3114	1357.6515	-0.012	0.990				
Barthel score	-0.10073	0.02754	-3.657	<0.001 ***	0.90	0.86-0.95	189.49 on 92 df	178.07 on 91 df
Weight	-0.006119	0.010017	-0.611	0.541			187.78 on 91 df	187.40 on 90 df
Number of teeth	-0.07959	0.02029	-3.923	<0.001 ***	0.92	0.89-0.96	189.49 on 92 df	167.90 on 91 df
Clincial Frailty Score	0.23538	0.08551	2.753	0.006 **	1.27	1.07-1.50	189.49 on 92 df	181.88 on 91 df
HABAM score	-0.03654	0.01050	-3.480	0.001***	0.96	0.94-0.98	189.49 on 92 df	178.25 on 91 df
Ex smoker	-0.7893	0.3127	-2.524	0.012 *	0.45	0.25-0.84	189.49 on 92 df	180.90 on 90 df
Never smoker	-1.0097	0.3562	-2.835	0.005 **	0.36	0.18-0.73		
Charlson index	-0.08676	0.06644	-1.306	0.192			189.49 on 92 df	187.67 on 91 df
CVD	0.08809	0.33331	0.264	0.792			189.49 on 92 df	189.42 on 91 df
Dementia	1.4117	0.3346	4.219	<0.001 ***	4.10	2.13-7.91	189.49 on 92 df	174.88 on 91 df
Plaque quartile score on admission	0.2516	0.1161	2.167	0.030*	1.29	1.02-1.61	188.58 on 91 df	183.75 on 90 df

COPD=Combined obstructive pulmonary disease, HABAM= Hierarchical balance and mobility score, CVD=cerebrovascular disease, df=degrees of freedom, numbers to 2 d.p., *p<0.05,**p<0.01,and ***p<0.001

Table 116. Univariate generalised linear models relating colonisation index (*Acinetobacter* spp) and baseline patient variables

Factor	Estimate	Standard Error	Z value	P value	Odds ratio	95% CI	Null deviance	Residual deviance
Age	0.007832	0.042237	0.185	0.853			52.703 on 92 df	52.669 on 91 df
Female	-0.5855	0.6102	-0.960	0.337			52.703 on 92 df	51.810 on 91 df
COPD	0.8797	0.6331	1.39	0.165			52.703 on 92 df	50.956 on 91 df
Recent antibiotics	0.6434	0.6836	0.941	0.347			52.703 on 92 df	51.904 on 91 df
Self-consent	0.5611	0.5952	0.943	0.346			52.703 on 92 df	51.942 on 91 df
Residence institution	1.8297	0.6156	2.972	0.003 **	6.23	1.86-20.83	52.703 on 92 df	44.213 on 90 df
Residence hospital	-15.2114	2238.3889	-0.007	0.995				
Barthel score	-0.16780	0.05036	-3.332	0.001***	0.85	0.67-0.93	52.703 on 92 df	43.946 on 91 df
Weight	-0.03499	0.02576	-1.358	0.174			50.462 on 90 df	50.462 on 90 df
Number of teeth	-0.06468	0.04354	-1.486	0.137			52.703 on 92 df	49.817 on 91 df
Clincial Frailty Score	0.5078	0.1885	2.693	0.007**	1.66	1.15-2.40	52.703 on 92 df	45.703 on 91 df
HABAM score	-0.04762	0.02249	-2.118	0.034 *	0.95	0.91-1.00	52.703 on 92 df	48.757 on 91 df
Ex smoker	0.5796	1.0850	0.534	0.593			52.703 on 92 df	52.363 on 90 df
Never smoker	0.5484	1.1233	0.488	0.625				
Charlson index	0.03372	0.13482	0.250	0.803			52.703 on 92 df	52.642 on 91 df
CVD	0.5736	0.6833	0.839	0.401			52.703 on 92 df	52.060 on 91 df
Dementia	0.3821	1.0586	0.361	0.718			52.703 on 92 df	52.585 on 91 df
Plaque score quartile on admission	0.1491	0.2939	0.507	0.612			43.915 on 91 df	43.655 on 90 df

CI= confidence intervals, COPD=Combined obstructive pulmonary disease, HABAM= Hierarchical balance and mobility score, CVD=cerebrovascular disease, df=degrees of freedom, numbers to 2 d.p., *p<0.05,**p<0.01,and ***p<0.0

Table 117. Multivariate generalised linear model relating colonisation index (*S. aureus*) and baseline patient characteristics (medical model)

Patient variable	Estimate	Standard error	Z value	P value
Female	-1.19617	0.41121	-2.909	0.004 **
Recent antibiotics	2.60964	0.48437	5.388	<0.001 ***
Self-consent	-2.11681	0.81878	-2.585	0.010 **
Weight	-0.04233	0.01624	-2.606	0.010**
Number of teeth	-0.05705	0.02403	-2.374	0.018 *
Frailty score	-0.57686	0.19224	-3.001	0.003 **
Not smoking currently	-2.08445	0.57667	-3.615	<0.001***
Charlson index	0.46496	0.11340	4.100	<0.001 ***
Dementia	2.17319	0.69347	3.134	0.002 **

Null deviance: 162.081 on 91 degrees of freedom, , Residual deviance: 85.298 on 82 degrees of freedom (1 observation deleted due to missingness) AIC: 140.31

Table 118. Multivariate generalised linear model relating colonisation index (*S. aureus*) using the dental model

Patient variable	Estimate	Standard error	Z value	P value
Charlson index	0.22338	0.07684	2.907	0.004 **
Plaque quartile score day 1	0.40577	0.16075	2.524	0.012 *
Female	-1.04207	0.35366	-2.947	0.003 **
Deprivation score (IMD)	-0.01747	0.01003	-1.742	0.082 .
Current smoking	-1.18256	0.45728	-2.586	0.010 **
Barthel index	0.16317	0.07445	2.192	0.028 *

Null deviance: 161.26 on 90 degrees of freedom, Residual deviance: 128.57 on 84 degrees of freedom, (2 observations deleted due to missingness), AIC: 177.58

Table 119. Multivariate generalised linear model relating colonisation index (MRSA) and baseline patient variables (medical model)

Patient variable	Estimate	Standard error	Z value	P value
Recent antibiotics	2.73471	0.48441	5.645	<0.001 ***
Barthel index	0.26737	0.08777	3.046	0.002 **
Number of teeth	-0.07573	0.02776	-2.728	0.006 **
Ever smoked	-3.15218	1.06884	-2.949	0.003 **
Charlson index	0.34315	0.10948	3.134	0.002 **

Null deviance: 113.445 on 92 degrees of freedom, Residual deviance: 57.507 on 87 degrees of freedom, AIC: 94.774

Table 120. Multivariate generalised linear model relating colonisation index (MRSA) using the dental model

Patient variable	Estimate	Standard error	Z value	P value
Age	0.09624	0.03858	2.494	0.013*
Charlson index	0.21556	0.09634	2.238	0.025 *
Ever smoked	-2.44064	1.02793	-2.374	0.018 *
Barthel index	0.17428	0.08653	2.014	0.044 *

Null deviance: 113.445 on 92 degrees of freedom, Residual deviance: 85.862 on 88 degrees of freedom, AIC: 121.13

Table 121. Multivariate generalised linear model relating colonisation index (*S. pneumoniae*) and baseline patient variables (medical model)

Patient variable	Estimate	Standard error	Z value	P value
Plaque quartile score day1	-0.23552	0.09256	-2.545	0.011 *
Female	0.65446	0.24166	2.708	0.007 **
Recent antibiotics	-0.89130	0.29322	-3.040	0.002 **
Self-consent	0.43530	0.21823	1.995	0.046 *
Number of teeth	0.04441	0.01044	4.253	<0.001 ***
HABAM	0.04803	0.01113	4.315	<0.001 ***
Ever smoked	-0.53310	0.21077	-2.529	0.011*
Charlson index	-0.14629	0.05318	-2.751	0.006 **
Dementia	1.17022	0.45638	2.564	0.010 *

Null deviance: 357.11 on 91 degrees of freedom, Residual deviance: 247.91 on 82 degrees of freedom (1 observation deleted due to missingness) AIC: 374.3

Table 122. Multivariate generalised linear model relating colonisation index (*S. pneumoniae*) using the dental model

Patient variable	Estimate	Standard error	Z value	P value
Clinical frailty score	-0.126637	0.061986	-2.043	0.041*
Charlson index	-0.117752	0.052891	-2.226	0.026 *
Teeth number	0.043151	0.009431	4.576	<0.001 ***
FemaleTRUE	0.534771	0.236574	2.260	0.024 *
Current smoking	-0.892464	0.228295	-3.909	<0.001***
Deprivation score (IMD)	-0.010290	0.005465	-1.883	0.060.

Null deviance: 353.82 on 91 degrees of freedom, Residual deviance: 260.40 on 85 degrees of freedom, (1 observation deleted due to missingness), AIC: 377.49

Table 123. Multivariate generalised linear model relating colonisation index (*H. influenzae*) and baseline patient variables (medical model)

Patient variable	Estimate	Standard error	Z value	P value
Female	0.93102	0.39793	2.340	0.019*
COPD	0.96067	0.34225	2.807	0.005**
Barthel index	-0.07338	0.03587	-2.046	0.041 *
Number of teeth	-0.07072	0.02155	-3.282	0.001 **
Not currently smoking	-0.83846	0.36119	-2.321	0.020 *
Dementia	1.86245	0.40935	4.550	<0.001 ***

Null deviance: 189.49 on 92 degrees of freedom, Residual deviance: 118.88 on 86 degrees of freedom, AIC: 188.72

Table 124. Multivariate generalised linear model relating colonisation index (*H. influenzae*) using the dental model

Patient variable	Estimate	Standard error	Z value	P value
Age	-0.054508	0.020021	-2.723	0.006 **
Dentures	0.498582	0.160148	3.113	0.002 **
Female	1.293585	0.411110	3.147	0.002 **
IMD	0.021399	0.007844	2.728	0.006 **
Barthel index	-0.168932	0.036667	-4.607	<0.001 ***

Null deviance: 188.29 on 91 degrees of freedom, Residual deviance: 114.28 on 86 degrees of freedom, (1 observation deleted due to missingness), AIC: 179.68

Table 125. Multivariate generalised linear model relating colonisation index (*E. coli*) using the dental model

Patient variable	Estimate	Standard error	Z value	P value
Teeth number	-0.23707	0.07618	-3.112	0.002 **
Dentures	-0.65410	0.30161	-2.169	0.030 *
Female	-2.30236	0.62617	-3.677	<0.001 ***
Current smoking	-2.40923	0.67333	-3.578	<0.001 ***
Barthel index	-0.22273	0.05207	-4.278	<0.001 ***

Null deviance: 108.682 on 92 degrees of freedom, Residual deviance: 34.083 on 87 degrees of freedom, AIC: 69.894

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