The identification of the multi-drug resistant organisms, vancomycin resistant enterococci (VRE) and extended spectrum β-lactamase producing Enterobacteriaceae (ESBL-E), in the ICU: examining the interplay between patient colonisation and environmental contamination.

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A thesis submitted to the School of Postgraduate Studies, Faculty of Medicine and Health Sciences, Royal College of Surgeons in Ireland, in fulfilment of the degree of Doctor of Medicine

Supervisors: Dr Deirdre Fitzgerald-Hughes Prof Hillary Humphreys

March 2016
I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree Doctor of Medicine is my own personal effort. Except where explicitly noted in the text, all environmental sampling and laboratory work was entirely my own work. Where any of the content presented is the result of input or data from a related collaborative research programme, this is duly acknowledged in the text. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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Student Number _____________________________________________

Date _______________________________________________________

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<tr>
<td>Agg</td>
<td>Aggregation substance</td>
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<tr>
<td>APACHE</td>
<td>Acute physiology and chronic health evaluation</td>
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<td>ASC</td>
<td>Active surveillance cultures</td>
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<tr>
<td>BC</td>
<td>Bed control panel</td>
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<td>BDU</td>
<td>Bed days used</td>
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<td>BH</td>
<td>Beaumont Hospital</td>
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<td>BHI</td>
<td>Brain heart infusion</td>
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<tr>
<td>bla</td>
<td>β-lactamase</td>
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<td>blaNDM</td>
<td>New Delhi metallo-β-lactamase</td>
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<tr>
<td>BSI</td>
<td>Blood stream infection</td>
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<td>CBA</td>
<td>Columbia Blood Agar</td>
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<td>CC</td>
<td>Clonal clusters</td>
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<tr>
<td>CDC</td>
<td>Centre for Disease Control and Prevention</td>
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<tr>
<td>CH</td>
<td>Chart holder</td>
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<tr>
<td>CLB</td>
<td>Cell lysis buffer</td>
</tr>
<tr>
<td>CML</td>
<td>Clinical Microbiology Laboratory</td>
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<tr>
<td>CPE</td>
<td>Carbapenemase-producing Enterobacteriaceae</td>
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<tr>
<td>CRE</td>
<td>Carbapenem resistant Enterobacteriaceae</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palendronic repeats</td>
</tr>
<tr>
<td>CSB</td>
<td>Cell suspension buffer</td>
</tr>
<tr>
<td>CTX-M</td>
<td>Cefotaxime hydrolyzing capabilities</td>
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<tr>
<td>CVC</td>
<td>Central venous catheter</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>DS</td>
<td>Drip stand</td>
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<tr>
<td>EARS-Net</td>
<td>European Antimicrobial Resistance Network</td>
</tr>
<tr>
<td>ECDC</td>
<td>European Centre for Disease Control</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ESBL</td>
<td>Extended spectrum beta lactamases</td>
</tr>
<tr>
<td>Esp</td>
<td>Extracellular surface protein</td>
</tr>
<tr>
<td>EUCAST</td>
<td>European Committee on Antimicrobial Susceptability Testing</td>
</tr>
<tr>
<td>GeIE</td>
<td>Gelatinase</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
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<tr>
<td>HCAI</td>
<td>Health care associated infections</td>
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<tr>
<td>HDU</td>
<td>High dependency units</td>
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<tr>
<td>HPSC</td>
<td>Health Protection and Surveillance Centre</td>
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<tr>
<td>ICU</td>
<td>Intensive care units</td>
</tr>
<tr>
<td>IDSA</td>
<td>Infectious Diseases Society of America</td>
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<tr>
<td>IPCT</td>
<td>Infection prevention and control team</td>
</tr>
<tr>
<td>LTCF</td>
<td>Long term care facility</td>
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<tr>
<td>MALDI-TOF MS</td>
<td>Matrix assisted laser desorption &amp; ionisation time of flight mass spectrometry</td>
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<tr>
<td>Term</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
</tr>
<tr>
<td>SHV</td>
<td>Sulfhydryl variable</td>
</tr>
<tr>
<td>ST</td>
<td>Sequence types</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA</td>
</tr>
<tr>
<td>TEM</td>
<td>Temoneira</td>
</tr>
<tr>
<td>TEN</td>
<td>Tris-EDTA-NaCl</td>
</tr>
<tr>
<td>UC</td>
<td>Urinary catheter</td>
</tr>
<tr>
<td>UPGMA</td>
<td>Unweighted pair group average method</td>
</tr>
<tr>
<td>VIM</td>
<td>Verona integron-encoded metallo-βlactamase</td>
</tr>
<tr>
<td>VRE</td>
<td>Vancomycin resistant enterococci</td>
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<td>WGS</td>
<td>Whole genome sequencing</td>
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<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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We also quantified the burden of ESBL-E and VRE carriage among patients admitted to the ICU over this time period and examined the interplay between environmental and patient isolates. Of 157 patients who were included in the study, 9.5% were colonised with ESBL-E and 19.1% were colonised with VRE. There were no epidemiological links between the patient and environmental isolates for the ESBL-E isolates. There were a number of epidemiologically linked isolates among the VRE from both the environmental and patient samples.

Genetic characterisation of a selection of vancomycin resistant Enterococcus faecium (VREfm) isolates, by pulsed-field gel (PFGE) and next generation sequencing (NGS), showed that there was genetic diversity among the patient isolates, but that the environmental isolates displayed more clonal relationships. NGS demonstrated that despite the clonality of the environmental isolates, there were a variety of strain matches identified, and indeed the reverse was true for the patient isolates, highlighting the complexity of enterococcal populations and transmission in the hospital milieu.
Acknowledgements

The fact that I am writing the acknowledgements to this thesis is nothing short of a minor miracle. It could not have been achieved without the help of a lot of people along the way.

First and foremost, my enormous thanks to my supervisor Deirdre. Your patience, enthusiasm and clarity was inspirational. You have an air of calm and sense of perspective and humour that was very helpful in my many moments of uncertainty. I couldn’t have had a more understanding supervisor. As I am your first true MD submission, you may well stick to the PhDs after this!

To Hilary thank you for giving me the opportunity to do this research and for the departmental support in funding it over the couple of years. Thank you for your advice over coffee, your encouragement, for understanding my last minute tendencies and for being a mentor to me both in this and my clinical career over the years

Thank you to all in the clinical microbiology department, both past and present. To Tania and Marta, my office compatriots, it has been a pleasure and privilege to work alongside such talented scientists. Thank you also for our bartering arrangements and for ordering my consumables for me. Thank you to Liam and Tania, for navigating me around the lab and the equipment, the art of plate pouring, and for keeping me company as we each talked to ourselves. A sign of genius I’m sure. To Siobhan, may you always hold onto your positive attitude and sunny outlook. Good luck with your PhD, and Post Doc. To Niall, thank for your stories, your sense of humour and drama and for the choice advice, in restaurant and holidays, as well as thesis writing. Congratulations on the house, and may all the associated drama be in the past. I also thank Ann, the anchor of the department, for being so welcoming and for all the chats, as you calmly and firmly keep us all on the straight and narrow. And to Paolo, Tony, Anna-Rose and Hannah for the stories, the rose, lunchtime chats and words of encouragement. Good luck to all of you in your endeavours. To Dr. Eanna, for my wild cooking book, I use it a lot. Congratulations on graduating. Good luck in the new job.

I also extend my enormous thanks to Mairéad for all the help with organizing my data, for your support and listening to me. I couldn’t have got through it without you. Who knew counting could be so difficult.
To the clinical microbiology laboratory of Beaumont hospital, my heartfelt thanks to you all. Especially to Ann, who always had my samples separated for me, to Mary who was always there to answer any queries, teach me how to use the Maldi, and generally being wonderful.

John, Sheila, Fionnula and Caoimhe of the infection prevention and control team for sharing your hospital data, and putting mine into context and also for your help in navigating the BHIS patient system.

I wish to acknowledge the patients and staff in the ICU in Beaumont Hospital.

To Caroline and all the nursing staff who were always very accommodating and welcoming to me as they saw me arrive with my black box of swabs. Also to Joanne Mulvihill, the data analyser for ICU for answering all my queries and to Dr. James O’Rourke, whose enthusiasm is infectious and who supported me doing the project in the ICU.

I also thank my microbiology colleagues, Kirsten, Cara and Eileen from St.Vincent’s University Hospital and Jonathan Collins from Tallaght Hospital for being so generous with both your methods and your experience in PFGE.

I acknowledge and thank Graeme and Alex from Pathogenica,Inc for collaborating with us on this project and for doing the sequencing on some of the samples.

Thank you also to my friends, both in and out of microbiology. To ‘les Filles’ for your friendship and support in all aspects of my life. Welcome to the two new arrivals. This incubation period was that of an elephant! To Lillian, Lorraine, Joanne, Sinead for being lovely colleagues as well as friends, for listening to my woes and being so supportive.

To Suzanne thank you very much for your support and accommodating the time away from the BonS to write this, it would have been impossible otherwise.

To my Mum and Dad, what can I say, thank you for always supporting me, for being generous to a fault and always having my back. Mom thank you for all the help in formatting this, and to you and Augusta for the inspirational words, the reiki, candles and any other hocus pocus available.

I dedicate this to my darling husband, Matt for all the love, help, patience, pedantry, understanding in the face of meltdowns over the years and who means more to me than I can put into words and to my darling little Róisín who helps me to remember what is truly important.
Chapter 1

General Introduction
1.0 General Introduction

Modern medicine - from organ transplantation to chemotherapies for malignancies, from abdominal surgery to immunotherapy for autoimmune conditions - relies on the treatment and prevention of infection. The rise of antibiotic resistance in healthcare systems and the community makes infection treatment and prevention more challenging. The World Health Organisation (WHO) and World Economic Forum have stated that antimicrobial resistance is the greatest risk to human health and a public health emergency [1].

Bacteria have evolved for billions of years alongside natural antimicrobial agents and indeed, many of our antibiotics were originally isolated from microorganisms. They have therefore developed mechanisms of survival to defend themselves and their ecological niches. Evidence of past battles are present in the genomes of many remote bacteria carrying genes encoding for resistance mechanisms to antibiotics with which they have never come in contact [2]. Multi-drug resistant organisms are the scourge of modern healthcare. In the US alone, it is estimated that antibiotic resistant organisms will cause at least 2 million illnesses and contribute to 23,000 deaths [3]. The Infectious Diseases Society of America (IDSA) has highlighted a number of significant antibiotic resistant organisms, Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa and Enterobacter spp, the so called ESKAPE organisms to be of particular concern due to their ability to avoid the biocidal activity of antibiotics.[4]

1.1 Health care associated infections.

Health care associated infections (HCAI), defined as an infection acquired in a hospital or health care facility 48-72h after admission, affect millions of hospitalised patients worldwide. The European Centre for Disease Control (ECDC) estimates that approximately 4 million people acquire these infections every year in the EU with an estimated prevalence of 7%. A recent Centre for Disease Control and Prevention (CDC) study, examining the prevalence of HCAI in acute US hospitals, reported that of the 11,282 patients surveyed, 452 (4%) had at least one HCAI [5]. The numbers of
HCAI reported are proportionally higher in patients admitted to intensive care units (ICUs). The WHO HCAI fact sheet reports that, in developed countries, the rate of HCAI in patients admitted to ICU patients is in the region of 30%. In developing countries, the rate is 2-3 fold higher, with patients in neonatal ICUs being at the highest risk. In a recent US Point prevalence study (PPS), 15% of patients surveyed were in critical care, and of these, 9% had HCAI representing 34% of all patients with HCAI [5]. A recent European PPS showed that in most hospitals surveyed the highest rate of HCAI were in ICUs which had an overall rate of 19.5% [6] The rate of HCAI reported for Ireland in augmented care units (which included high dependency units, HDU, ICUs and paediatric ICUs and NICUs) was 16.5%, three fold higher than the baseline rate of 5.2% [6, 7]. In the English PPS survey conducted in 2011, the prevalence was 23.4% almost four times that of the baseline prevalence rate of 6.4% [8].

The factors which contribute to the higher rate of HCAI in critical care units are multifactorial, and include, increased numbers of patients with co-morbidities, increased prevalence of invasive devices (central venous catheter (CVC), urinary catheter (UC)) and increased use of antibiotics. In the Irish PPS mentioned earlier, patients surveyed in the ICUs had the highest prevalence of medical devices with 67% of those surveyed having at least one invasive medical device in situ and 50.4% of patients in augmented care units were receiving antibiotics [7, 8].

Internationally, a similar situation in ICUs is reported in a large multicentre study (EPIC II) of prevalence and outcomes of infections in patients from 1265 ICUs in 75 different countries including 13,700 adult patients. On the day of the study, 9084 patients (71%) were receiving antibiotics and 7087 patients were considered infected (51%) [9].

The cost of infections is multi faceted with increased morbidity and mortality for patients, and increased risk of developing infections with multi-drug resistant organisms (MDRO) resulting in, prolonged hospital stay and increased therapeutic costs. In Europe HCAI are estimated to cost in the region of 7 billion euro and 16
million extra days in hospital [10]. Since 2005, 88 of 147 developing countries and 36 of 46 developed countries who are UN member states have pledged to reduce HCAIs by signing the pledge of WHO’s First Global Patient Safety Challenge. The 2011 PPS of HCAI and antibiotic use in the UK found that there was, in England, an overall reduction in HCAI from the previous survey carried out in 2006 from 8.2-6.4%. [8] In Ireland, a similar survey carried out in 2012 found that the rate of HCAI had marginally increased from 4.9% in 2006 to 5.2% in 2012 [7], but, however, there were differences in the methodology used in both studies, making direct comparison difficult.

1.2 Microbiology of HCAIs

In the Irish 2012 PPS, which recorded a HCAI prevalence rate of 5.2% [7], 251 of the infections recorded (52%) had at least one causative microorganism identified representing 310 microorganisms. Of these the highest proportion of infections (35%) was due to Enterobacteriaceae. *E.coli* accounted for 56% of infecting isolates and *Klebsiella pneumoniae* and *K.oxytoca* accounted for 19%. Most of the isolates (60%) retained susceptibility to 3rd generation cephalosporins. However 26 patients (25%) had infections with Enterobacteriaceae that were resistant to 3rd generation cephalosporins. *Staphylococcus aureus* was the next most commonly isolated pathogen, with 37% of the isolates resistant to flucloxacillin. Enterococci were the third most isolated pathogen with vancomycin or glycopeptides resistance being identified in 26% of isolates.

This distribution of micro-organisms isolated from patients with HCAI, reported in the Irish PPS study is echoed in the findings of other European countries, with Enterobacteriaceae accounting for the highest number of HCAI followed by *S.aureus*, *Pseudomonas* spp. and *Enterococcus* spp. Interestingly this differs from US data where in a recent PPS of HCAI 4% of patients surveyed were found to have a HCAI. Where a pathogen was identified, (372 of 504 infections), *Clostridium difficile* was the commonest pathogen identified in patients, representing 12% of the HCAI
identified. \textit{S.aureus} was the next most commonly isolated organism accounting for 10.7\% of infections and the Enterobacteriaceae were next with \textit{Klebsiella pneumoniae/K.oxytoca} and \textit{E.coli} being reported in 9.9\% and 9.3\% of cases, respectively. \textit{Enterococcus} spp accounted for 8.7\% of infections [5].

1.3 Enterobacteriaceae

The Enterobacteriaceae, a large family of Gram negative rods are present in significant numbers as part of the normal gut flora of humans and animals. As a group, they commonly cause infections of the urinary tract, respiratory tract and intra-abdominal infections, both in the community and in hospital settings. \textit{E.coli} is by far the most commonly reported pathogen in these infections and furthermore accounts for 50\% of blood stream infections (BSI) reported to the European Antimicrobial Resistance Network (EARS-Net) from Ireland in 2014.[11]

1.4 Mechanisms of Gram negative resistance to beta-lactam antibiotics.

Gram-negative bacteria have numerous mechanisms by which they develop antimicrobial resistance including target site alteration (eg altered penicillin binding proteins), porin loss/alterations and production of inactivating enzymes. [12, 13] The Gram-negative cell envelope with its additional outer membrane is a barrier to the entry of some penicillins to their cell wall target, peptidoglycan. In addition, the activation of membrane efflux pumps may also scavenge and remove antibiotics including the \textit{β}-lactam classes. However the main mechanism of resistance among Enterobacteriaceae is the production of \textit{β}-lactamases enzymes that inactivate \textit{β} -lactam antibiotics [14]. Beta-lactam antibiotics are a family of antibiotics comprising of the penicillins, penicillins with beta-lactamase inhibitor combinations, cephalosporins, monobactams and carbepenems. The common structural feature among them is the four membered \textit{β}-lactam ring. They exert their antibacterial activity by inhibiting the cross linking of peptidoglycan chains in the bacterial cell wall by binding to transpeptidases (penicillin-binding proteins) in the periplasmic
space of Gram-negative bacteria. Enterobacteriaceae (and indeed other bacteria)
may carry diverse \textit{bla} genes that encode β-lactamase enzymes capable of
destroying the β-lactam ring of a wide range of penicillins, cephalosporins and more
recently carbapenem antibiotics.

\textbf{1.5 Classification of beta-lactamases}

As mentioned earlier, β-lactamase production is the commonest mechanism of
Gram negative resistance to β-lactam antibiotics. The widespread use, mis-use and
overuse of these types of antibiotics has resulted in the continued evolution of
these enzymes towards greater diversity of substrate specificity i.e. the extended-
spectrum β-lactamases, including even last resort carbapenem-inactivating
enzymes (the carbapenemases). By 2009, more than 890 different beta-lactamases
had been reported, \url{http://www.lahey.org/Studies/}. [13].

The β- enzymes have multiple classification systems which have been described
over the last four decades. The two most commonly used are the structural Ambler
class classification and the functional classification by Bush, Jacoby and Medieros.
[14, 15]. The Ambler class, based on conserved and distinguished amino acid motifs
divides the enzymes into Classes A-D. Class A, C and D utilise serine for beta-lactam
hydrolysis and Class B requires zinc at the active site for hydrolysis - the so called
metallo β-lactamases. The functional classification system of Bush, Jacoby and
Medieros, which is continually growing and evolving with increasingly refined
molecular methods, incorporates substrate and inhibitor profiles to group the
enzymes to reflect their phenotype in clinical isolates. They are divided into 3 major
groups which generally correlate with the more broadly based molecular
classification and are further subdivided into subgroups. Group 1 are
cephalosporinases, (Ambler class C), which are usually encoded on chromosomes of
many Enterobacteriaceae. These are chromosomal AmpC β-lactamases. They have
a high affinity for cephalosporins and monobactams, (aztreonam), and are not
inhibited by the β-lactamase inhibitor clavulanate. Many species, such as
\textit{Citrobacter freundii}, \textit{Serratia marcescens} and \textit{Enterobacter cloacae}, have low levels
of these enzymes, but expression can be induced in the presence of β-lactam
antibiotics. Also included in this group are the plasmid mediated AmpC enzymes. Group 2 are penicillinases and cephalosporinases, (Ambler class A and D), and are the most numerous group. They include the penicillinases of Gram positive organisms such as $S.\text{aureus}$ and the plasmid-mediated TEM1 and 2 and SHV in Gram negative organisms. Group 3 are the metallo- $\beta$ -lactamases, (Ambler class B), and include chromosomal and plasmid encoded enzymes including the carbepenmases, VIM, IMP and $\text{blaNDM-1}$. [16]

1.6. The extended spectrum beta lactamases (ESBLs)

Bacteria evolved to produce enzymes capable of hydrolyzing the second and third generation cephalosporins and the $\beta$-lactam with $\beta$ -lactamase inhibitor combinations, which had been developed in the 70’s and 80’s to overcome TEM and SHV1 $\beta$-lactamases. Extended spectrum $\beta$-lactamases were first isolated in Europe in 1982 and $\text{Klebsiella pneumoniae}$ was the organism most commonly associated with outbreaks, predominantly in the health care setting [17]. The extended spectrum beta-lactamases are in phylogenetic group 2be and are Ambler class A. They include numerous mutants of TEM and SHV and CTX-M enzymes. The TEM and SHV ESBLs evolved from point mutations in their parent TEM and SHV enzymes, CTX-M enzymes, which are now internationally disseminated were derived from the chromosomes of the $\text{Kluyvera}$ spp, mobilized onto plasmids by insertion sequences .[13] The CTX-M enzymes hydrolyse cefotaxime in preference to ceftazidime. They confer resistance to penicillins, broad spectrum cephalosporins including cefotaxime, ceftriaxone, ceftazidime and monobactams. They are inhibited by clavulanate, tazobactam and sulfabactam, and have little activity against cephemycins and carbepenems. [18]

1.7. Dissemination of ESBL Enterobacteriaceae

Until the late 1990’s the predominant ESBL types in Europe, and indeed throughout the world, were the TEM and SHV enzyme variants.[19] CTX M type ESBLs have become the dominant type of ESBL with over 150 variants identified[20]. The CTX M type ESBLs proliferated in human gut Enterobacteriaceae increasingly being identified in $E.\text{coli}$ causing infections in the community and $\text{Klebsiella}$ spp associated
with hospital infections. Prior to 2000, no CTX-M enzymes were identified in isolates in the UK. In 2001 there was an outbreak, involving 30 patients, of *Klebsiella pneumoniae* with a CTX-M 26 β-lactamase, and a survey of 900 *E. coli* isolates from hospitals in UK and Ireland found four isolates with CTX-M 15. Just four years later, a survey of cephalosporin resistant isolates from 16 laboratories in the UK found 1127 isolates of which 51% were *E. coli* and the largest group of these had CTX-M enzymes. This was also found to be the predominant ESBL type found in the *Klebsiella* spp submitted [19]. CTX-M type ESBLs now account for 10% of invasive *E. coli* isolates in Ireland, and gut carriage of ESBL *E. coli* was identified in 40% of nursing home residents in Belfast and 55% nursing home residents in Galway but their prevalence is reported as 1-2% in other outpatient populations [21, 22]. There is geographic variation in the ESBL rates among population groups around the world. The highest rates of ESBL in *E. coli* and *Klebsiella pneumoniae* occur in India, at 80%, China 60%, Eastern and Southern Asia, Latin America and Southern Europe around 30%, whereas Northern Europe, North America and Australasia have a rate of 7-10% [13]. The distribution of cephalosporin resistance, a marker of potential ESBL rates, among invasive *E. coli* and *Klebsiella pneumoniae* throughout Europe is illustrated in the EARS-net map Figure 1.0. ESBL positive isolates are commonly also resistant to the aminoglycosides, fluoroquinolones and trimethoprim, but remain susceptible to the carbapenems thus leading to problems with empiric antimicrobial choice as the rates increase. The increasing widespread use of carbapenems has fueled the growing resistance to this family of antibiotics, which up until 10 years ago retained almost universal activity against the Enterobacteriaceae.[19].
1.8. Animal reservoirs of ESBL-E.

As well as increasing in the human population, numerous studies have been published over the last decade on the recovery of ESBL-E from domesticated animals both pets and animals for food production. The widespread use of antibiotics in farming practice is most likely responsible for this large reservoir of antimicrobial resistance in farming and subsequently in the food chain, but humans could also be the source of animal infections. Antibiotic use is linked to resistant isolates in both farm and farmers. A study looking at resistant isolates from pigsties, pigs and farmers in pig farms in Denmark compared 19 pig herds with high use of third and fourth generation cephalosporins, with 20 pig farms with no cephalosporin use. ESBL-producing *E. coli* was detected in pig stool samples from 79% of the farms with high use of cephalosporins compared with samples from pigs testing positive from 20% of the farms with no consumption. ESBL-producing *E. coli* was also detected in 19 of the 195 farmer participants and all but one had contact with pigs[23]. Studies of broiler chickens in the Netherlands, found clonally related ESBL producing *E. coli* in broiler meat and humans, suggesting broiler meat as a source of ESBL producing *E. coli* causing infection in humans.[24] Fish farms in China, where there is high use of antibiotics in aquaculture, have detected ESBL producing
E. coli from 17% of fish tested. Investigators also found 25% of fish tested had plasmid mediated quinolone resistance genes [25]. A study carried out on healthy volunteers attending an infection control conference showed that previous contact with pets increased by almost seven-fold the chance to be colonized with ESBL positive bacteria [26].

1.9. Risk factors for acquisition of ESBL-E
Risk factors associated with ESBL-E acquisition include age >65y, prior antibiotic therapy, including third generation cephalosporins and fluoroquinolones, prior hospitalization, travel to countries of high endemicity, such as India, China, [13] [27] and contact with pets [27].

1.10. Treatment options for ESBL-E
The widespread global dissemination of ESBL-E and their associated acquisition of multiple drug resistant plasmids has complicated the issue of empiric therapy in the setting of presumed Gram negative sepsis or severe infection. Antibiotics which in general retain activity against ESBL-Es include carbepenems, aminoglycosides, the fluoroquinolones, ciprofloxacin, trimethoprim, fosfomycin, nitrofurantoin, tigecycline and cefipime, a 4th generation cephalosporin. However resistance to these antibiotics can be co-acquired making empiric choices difficult. Inadequate initial antimicrobial therapy has been shown to increase the risk of death in patients with sepsis [28], and this is more likely to occur in patients with bloodstream infections (BSI) due to ESBL producing Enterobacteriaceae. Carbapenems are the mainstay of treatment for severe infections with ESBL-E, occasionally in conjunction with an aminoglycoside. As mentioned above, the increasing use of carbapenems, the last line of defence in β-lactam antibiotics, have given rise to the growing number of isolates with plasmid medicated carbapenemases. Isolates with these resistance determinants, are susceptible to few antibiotics, such as the polymyxins (colomycin), some aminoglycosides and tigecycline.
1.11 Enterococci.

Enterococci are Gram positive facultative anaerobic bacteria. Most strains in the Enterococcus genus possess the characteristics summarized by Sherman in 1937 such as the ability to grow in 6.5% NaCl and to grow at 10 and 45°C at pH 9.6, and, for the most part, to survive at 60°C for 30 min. They were also noted to hydrolyse aesculin in the presence of bile, a biochemical means of identification [29]. They are the most commonly identified streptococci in the intestine of humans and other vertebrate animals, and therefore were initially called *Streptococcus faecalis* in 1906. In 1984 they were recognized as a separate genus [30]. The term ‘Enterocoque’ was first described in a French article in the 1899 describing commensal streptococci in the gastrointestinal tract, with pathogenic potential [31].

In the same year, a case of endocarditis caused by enterococci was reported in the literature, ‘A case of acute endocarditis caused by *Micrococcus zymogenes*’ with a description of the pathogen [32]. This organism was subsequently identified as an Enterococcus. There are a number of Enterococcal species associated with human infection. They are generally opportunistic organisms which normally have low virulence in the human host. The two species most commonly associated with human infections are *Enterococcus faecium* and *Enterococcus faecalis*. Sites of infection include the urinary tract, often associated with urinary catheters, intra-abdominal infections, bloodstream infections (BSI), including catheter related BSI, health care associated endocarditis, and surgical wounds or chronic ulcers.

Enterococci have adapted to their environment in the human gut, where they are clearly outnumbered by anaerobic commensals. In a normal host, they establish symbiotic relationships with the commensal flora and the immune system. One of the effects antibiotics have on the human gut is one of altering the balance of the commensal flora, favouring the enterococci which have inherent resistance to a number of antibiotics. Broad spectrum antibiotics have the additional effect of down regulating the intestinal expression of an intestinal antimicrobial peptide, Reg III gamma. This antimicrobial peptide is produced by intestinal epithelial cells through their activation by competing anaerobic Gram negative organisms.
Colonisation with enterococci is also facilitated by increased gastrointestinal pH from the use of proton pump inhibitors which are widely prescribed, both in the ICUs and indeed in the community at large. [33]

Enterococci have become an important cause of HCAI. Most invasive enterococcal infections occur within hospitals in patients with underlying medical conditions whose normal flora has been affected by prior antibiotics therapy. Resistant strains of *E. faecium* has been identified by the CDC and IDSA as a triple threat pathogen due to its capacity to colonise the human intestine and the human skin and to persist within the environment [4]. *E. faecalis* and *E. faecium* rank among the top five most prevalent hospital acquired pathogens. In 2013, cases of vancomycin resistant enterococci (VRE), in hospitalised patients, numbered 20,000 cases and were associated with 1300 deaths. Of these VRE cases, 77% were *E. faecium*. [3]

### 1.12. Antibiotic resistance in Enterococci

Enterococci are inherently resistant to a number of antibiotics, including cephalosporins and macrolides, and acquired resistance to amoxicillin, aminoglycosides and glycopeptides is increasing. Much of enterococcal resistance stems from acquired mobile genetic elements. *E. faecalis* strains, which are usually amoxicillin susceptible, are responsible for the majority of infections, but the for minority of resistant strains, whereas *E. faecium* has become the reservoir for vancomycin resistance in humans. [34] Vancomycin is the standard treatment for enterococcal infection, however glycopeptide (vancomycin and teicoplanin) resistance has become widespread since it was first identified in the 1980s. Resistance to these antibiotics is encoded by *van* genes which encode for enzymes which modify precursors for the vancomycin binding site, reducing vancomycin affinity for the target. There are 8 known *van* genes, and vancomycin resistance among enterococci can be acquired or intrinsic. [35] Acquired resistance, with acquisition of plasmids encoding for *vanA* and *vanB* genes, account for the majority of resistant genotypes and are responsible for acquired resistance among *E. faecalis* and *E. faecium*. [36] These resistance determinant are encoded on small mobile genetic elements-transposons. *VanA* is located on Tn1546, which is transferred only
after integration into plasmids or conjugative transposons. VanB consists of three subtypes, vanB1-3 and is located on transposon Tn1547. This integrates into bacterial chromosome. Subtype 2 is the most common worldwide and is associated with a conjugative transposon Tn1549 which is present in Gram positive commensal anaerobic bacteria. These plasmids and conjugative transposons facilitate the horizontal transfer of resistant genes between bacterial strains and species [37]. Enterococcus gallinarum and Enterococcus durans harbor vanC genes which are chromosomal, encode for intrinsic vancomycin resistance, and are therefore not counted among surveillance numbers for vancomycin resistant enterococci (VRE) nor are they subject to infection control measures. Strains which acquire vanA genes express high level resistance to both vancomycin and teicoplanin, whereas strains with vanB resistance have variable resistance to vancomycin, with typically lower mean inhibitory concentrations (MICs), but retain susceptibility to teicoplanin [33].

Vancomycin resistant enterococci were first identified by Uttley et al in 1988. The first cases were an outbreak of vancomycin resistant E. faecium and E. faecalis in an English hospital [38]. There quickly followed a report from France and the first US cases were described from an outbreak of E. faecalis in a hospital in Missouri in 1989. The increase in isolation of enterococci with acquired resistance coincided with the increased use of vancomycin to treat the growing numbers meticillin resistant S. aureus (MRSA) infections globally, infections due to coagulase negative staphylococci and Clostridium difficile. In the mid 1990’s doctors in the United States were using 12 times the amount of vancomycin in hospitals that was being prescribed by their colleagues in the Netherlands [39]. In Europe and Australia, the widespread use of the glycopeptide, avoparocin in animals, resulted in genetically diverse strains carrying vancomycin resistance determinants in the community. Widespread banning of these agents in farming in the 1990, initially slowed the rate of VRE, but it has continued to increase throughout Europe, though with widely varying rates throughout the 28 member states as illustrated by the EARS-net Map for vancomycin resistance in enterococci Fig 1.1[34]. In the United States,
vancomycin resistance was 77% among *E. faecium* isolates from health care associated infection in 2013. [3] Ireland has the highest rate of vancomycin resistance among invasive VRE isolates in Europe at 46% 2014 [11] as illustrated in figure 1.1.

![Figure 1.1 EARS-Net map of the European distribution of vancomycin resistance among *E. faecium* and *E. faecalis* invasive isolates in participating European countries.](image)

### 1.13. Virulence factors in VRE nosocomial lineages

The majority of multi-drug resistant enterococcal infections are caused by specific lineages of *E. faecalis* and *E. faecium*. In the case of *E. faecalis*, hospital adapted drug resistant lineages include strains from multi-locus sequence typing clonal clusters (CCs) CC2, CC9, CC28, CC40. The clonal complex CC17 is widely disseminated and responsible for most HCAI involving *E. faecium*. [40, 41] These CCs have adapted to facilitate gastro-intestinal (GI) tract colonisation, person-to-person transmission and survival in the hospital environment. These adaptations include antibiotic resistance, and also virulence factors that promote aggregation and biofilm formation such as extracellular surface protein (Esp), and aggregation substance (Agg) and gelatinase (GelE) that may play a role in facilitating persistance of VRE in healthcare settings. [42] Esp is a surface protein that promotes biofilm formation in enterococci. Aag is a pheromone-inducible surface protein that promotes
aggregation during bacterial conjugation in \textit{E. faecalis}\cite{43}. A role for GelE (a metalloprotein that cleaves collagen) in biofilm development has also been described\cite{44}. These lineages also have modifications of cell wall glycoproteins enabling particularly \textit{E. faecalis} to be resistant to phagocytosis and enhances general environmental persistence \cite{40}.

1.14. Molecular typing of VRE

With their increasing contribution to HCAI and hospital outbreaks, the characterization and differentiation of strains has become an important infection control tool. Different epidemiological questions require different typing techniques providing different distinguishing power.\cite{45}

1.14.1. Polymerase chain reaction (PCR) based typing

PCR based typing became prominent in the 1990s, but has limitations in relation to reproducibility and discriminatory power. This in part overcome by a very standardized approach using a commercial kit from Diversilab, Biomerieux, Marcy-l’Etoile, France. Although the use of this method of typing in outbreak settings has been published, its discriminatory power has not been proven on reference strain sample sets and its application requires specialised equipment and significant cost \cite{45}. Plasmid based PCR is used to determine the presence of resistance genes and virulence genes.

1.14.2. Pulsed field gel electrophoresis (PFGE)

Macro-restriction analysis in pulsed field gel electrophoresis is the ‘Gold standard’ for typing enterococcal strains and has been used extensively for molecular characterization of VRE outbreaks, or establishing clonal patterns in an institution. The method, however, involves a time consuming procedure and is technically laborious, demanding experienced personnel for both laboratory work and subsequent data analysis. This method is DNA fragment based, using band pattern identification, and gives no additional information. It is also less portable and inter-laboratory comparisons of images and fragment patterns are challenging, as the
banding patterns may be prone to DNA polymorphisms within strains [46]. A harmonized protocol is also lacking for VRE, reinforcing the difficulty with inter-laboratory comparisons.[45, 47]

1.14.3 Multi-locus sequence typing (MLST)

Multi-locus sequence typing, is a method based on identifying differences in amino acid sequences of housekeeping genes. Mutational changes in housekeeping genes occur infrequently and are not exposed to any selective pressure as may be the case for cell surface and cell wall components or determinants associated with antimicrobial resistance. In this method, a combination of seven loci distributed across the genome and associated with a suitable discriminatory power are sequenced and analysed for similarity. MLST compares populations of bacteria and can designate them into sequence types (ST)s. For example the classical hospital associated *E. faecium* clonal complex CC 17 includes ST17 and ST 18. However, in addition to the core genome which determines ST, the accessory genome content of specific clones may be associated with specialisation in the hospital milieu [45]. MLST typing is internationally comparable for enterococci.[48]

1.14.4 Whole genome sequencing/ next generation sequencing

Whole genome sequencing (WGS) may provide a new horizon in outbreak investigation, finding out transmission routes of pathogens and tracing emergence and spread of resistant bacteria. In the future they will probably play a role in rapid diagnostics also. Targeted gene sequencing/next generation sequencing (NGS) has the potential to provide typing results and detect resistance genes in a single assay, guiding both timely treatment and infection control decisions and facilitating rapid tracking of resistant clones. In relation to VRE, a commercial sequenced-based assay which targets a selection of short DNA regions for the detection of *vanA* and *vanB* from a single sample is now available (Hospital Acquired Infection HAI Biodetection™, Pathogenica, Boston). The software can also be used to type the strains and provide phylogenetic comparisons for sets of isolates [49]
1.15 Clinical manifestations of VRE.

The most common clinical manifestation of VRE is colonization of the GI tract. This colonization is without symptoms but serves as a reservoir for transmission to other patients. An unpublished survey by Wrenn et al carried out in a tertiary referral hospital in Dublin found that 40% of the screened stool samples were positive for VRE [50]. An American study found that 14% of stool samples submitted for *C. difficile* testing were also VRE positive [51]. On average, 8-10% of colonised patients go on to develop infection, although the ratio of infected to colonized patients depends on the population. It is substantially lower in healthy immune-competent patients, but is associated with a poor outcome in significantly immune-suppressed patients, for example patients undergoing allogeneic bone marrow transplantation [52]. Vancomycin resistance has been shown to be an independent predictor of death in enterococcal BSI. A study comparing vancomycin resistant to vancomycin susceptible enterococcal BSI found that clinical failure was higher with VRE BSI and vancomycin resistance to be an independent predictor of crude mortality. All cause mortality was higher - 52% versus 27% - in patient with VRE BSI [53]. VRE BSI has also been associated with longer hospital stays and increased hospital costs [54]. It has been suggested that the high mortality rates may be related to the underlying health of the patients who have developed VRE infections, and that VRE colonisation or infection is related to a complicated medical course or prolonged hospital stay rather than due to organism related factors.

1.16. Risk Factors for VRE acquisition

Risk factors for VRE are widely reported in the literature. A recent review, divided these into both intrinsic and extrinsic risk factors for VRE acquisition. Among the intrinsic, or patient related factors were underlying immunosuppresion (including history of malignancy, haematological malignancies and neutropenia), chronic underlying conditions, (which may reflect health care contact and previous antibiotic exposure), previous or current antibiotic therapy with cephalosporins, anti-anaerobic agents including β-lactam/β-lactamase combinations and vancomycin. The extrinsic factors include previous hospitalization- including
transfers from other hospitals, previous ICU stay, presence of medical devices e.g. urinary catheters or intravascular catheters, and also accommodation in a room where the previous occupant was VRE positive.[55]. A retrospective study carried out in a Brazilian hospital investigating risk factors for acquisition and infection with VRE in the context of an outbreak, did not find an association with ICU admission or vancomycin use. However, they found that the environment had a significant role in VRE acquisition in patients and was, in that case, associated with problems with the hospital sewerage systems[56].

1.17. Screening practice for MDROs in critical care units in Ireland

1.17.1 ICUs

ICUs, or augmented care units are specially staffed and equipped areas of a hospital dedicated to the treatment and monitoring of patients with life-threatening conditions. They provide specialist medical, nursing and other allied health professional care with specialist skills in dealing with these conditions. They include all levels of critical care defined as follows- level 2 care (high dependency- to actively treat and support critically ill patients with primarily single organ failure; level 3 care (intensive care- to actively manage and treat critically ill patients with two or more organ failures)[57].

ICUs were first set up to care for the sickest of patients on the battle field during the Crimean war, and were pioneered by Ibsen, a Danish anaesthetist to include mechanical ventilation during a large polio outbreak in Denmark in the 1950s to care for patients with respiratory failure[58]. They subsequently evolved to include post operative recovery and further to be the specialist areas they are now.

An Irish national survey of the current practices and infrastructure in place in critical care units across the country for infection control and hygiene purposes was undertaken in 2012 [59]. This survey found screening practices for MDRO to vary widely between hospitals and was organism dependent. Active surveillance for MDRO in 14 Level 3 ICUs in the country included the following:- admission screening for meticillin resistant Staphylococcus aureus (MRSA) carriage and weekly
screening thereafter was conducted in 100% of units surveyed; admission screening for rectal carriage of VRE or ESBL-E or carbapenem resistant Enterobacteriaceae (CRE) was carried out in 9 (64%), 3 (21%) and 5 (33%), respectively.

A similar survey was carried out in the United States in 2008 and found a wide variety in the screening practices of National Healthcare Safety Network hospital ICUs. Of those units that responded, 57%, 59% routinely screened for MRSA, 22% screened for VRE, and 12% screened for multi-drug resistant Gram negative bacteria [60]. The CDC recommends use of barrier precautions for confirmed cases but do not recommend routine surveillance cultures in low prevalence settings [61], however the Society for Healthcare Epidemiologist of America recommends surveillance cultures for all high risk admissions and the use of preemptive contact precautions for patients pending culture results [62].

Recent Health Protection and Surveillance Centre (HPSC) guidelines on control of MDRO, recommend screening for all these organisms in high risk areas, such as ICUs [50] These guidelines were updated in 2014 to include ESBL-Es in response to the findings that 55% of residents of long term care facilities (LTCF) have positive rectal screens when tested [22, 50]

1.17.2. Screening

Screening is defined in the Oxford English Dictionary as “a system of checking for the presence or absence of something, typically a disease”. In infection terms, it forms part of a series of infection prevention and control measures to reduce the transmission of infections.

1.17.2.1. Passive screening

Passive screening for MDRO is done by detection of these organism in clinical cultures sent to the microbiology laboratory for investigation of a possible infection, be it a urine sample for investigation of a urinary tract infection, or a blood culture from a septic patient. Only a small proportion of patients carrying VRE have a
clinical infection, therefore this method misses a considerable reservoir of patients who may potentially transmit to others.

1.17.2.2. Active surveillance cultures (ASC) for VRE

Active surveillance cultures, or active screening involves regularly or episodically culturing VRE from patients’ stool samples or rectal swabs. The frequency of sampling may vary as may laboratory methods. The goal of screening is to rapidly identify carriers to reduce risk of cross transmission to other patients. The benefit of this is dependent on the rapidity and sensitivity of the laboratory methods used[55]. Chromogenic agars have improved standard culture methods in reducing the time to detection, and PCR methods further reduce this time.

The impact of active screening is often measured in conjunction with other control measures. An outbreak of VRE BSIs in a haematology unit in Texas, USA, experienced an eight fold reduction in the density of VRE BSI after active screening of all stool samples for VRE. Another study involving an oncology unit found a reduction in VRE BSI from 2.1 patients per 1000 patients days to 0.45 as well as a reduction in attributed cost after implementation of admission and subsequent weekly screening for VRE [63]. Another prospective study on the effect of admission screening, versus active screening of stool samples sent for *C. difficile* versus passive screening showed that greater than 91% of VRE were detected through active screening versus 8% from clinical samples. There was an estimated cost reduction based on a reduction of BSI and reduced transmission [64]. Hayden et al found increased cleaning resulted in a reduction of VRE acquisition, from 33 per 1000 patient days at risk to 16.84 per 1000 patient days at risk during enhanced cleaning. Patients were screened within 48 hours of admission and daily thereafter but the patients were only isolated with contact precautions if clinical samples were positive [65]. In this study ACS did not contribute to the reduction in acquisition.

Active screening for VRE is currently recommended in Ireland for high risk groups, for example patients admitted to ICUs, haematology/oncology units, renal dialysis units and patients admitted from another hospital, patients with a previous history
of VRE colonization and where appropriate ‘high risk’ contacts of known VRE positive patients in the setting of an outbreak.[50]

1.17.2.3. ASC for ESBL-E

Despite the large problem with ESBL-E both in the community and in hospital settings, guidelines for infection prevention and control interventions for ESBL-E in hospitals are lacking and screening is not routine practice. Despite updated national guidelines here in Ireland recommending screening for high risk patients, [66], there are limited data as to the impact of routine screening for these isolates [67]. Gardam et al in 2002 investigated the effectiveness of screening for multi-drug resistant Enterobacteriaceae (MDRE) in a multi-organ transplant unit. Of 287 patients admitted 24% were colonized with MDRE. Of those, 9% developed clinical infections. Of those colonized, the majority of patients had unique strains and none of the infections were found to be due to patient to patient transmission. The authors concluded that continuing the ASC was not cost effective in their setting [68]. Interestingly, none of the colonizing isolates were CTX-M ESBL producing E.coli, which has since become a dominant worldwide resistance gene associated with ESBL production in E.coli. A recent survey of screening practice in Canadian hospitals has also sought to address this question. They found that 50% of the hospitals in Toronto used admission screening as a means to detect and to control the spread of ESBL-E. They compared the hospitals that undertook active admission screening to those that didn’t and looked at the effect this had on the incidence of healthcare acquisition of ESBL-E. ESBL-E were identified by phenotypic methods only. The researchers found a 49% reduction in overall healthcare associated acquisition of ESBL-E in the screening hospitals compared to the non screening hospitals, and a similar reduction in incidence of HCA ESBL-E BSI [69].

1.18. Chlorhexidine washes in patients

Chlorhexidine is a topical antiseptic which has been used throughout the world since the 1950s. It has a safely record in both adults and children and has been used
in a variety of concentrations and formulations for handwashing, treating gingivitis and in pre-operative skin preparation. Chlorhexidine gluconate is a water soluble biguanide which binds to the negatively charged bacterial cell wall. At low concentrations, chlorhexidine alters the osmotic balance within the cell affecting cell membrane integrity, and at higher concentrations results in the precipitation of cellular structures, resulting in cell death. It has broad-spectrum activity against both Gram positive and Gram negative bacteria, yeasts and some viruses. It has no activity against chlorhexidine as a measure to prevent the transmission of and reduce infections with MDRO [71, 72]. MDRO frequently colonise the skin of patients in ICU, and reducing the bioburden on body surfaces may prevent the development of infection and reduced cross-transmission. Vernon et al in 2006, found that daily bathing of patients in ICU with chlorhexidine washcloths instead of soap based washcloths, reduced the VRE acquisition rates in their patients, and also resulted in a reduction of environmental contamination with VRE [73]. Climo et al the following year, in a single centre found that this measure reduced the acquisition of VRE in ICU patients by 30% and decreased the rate of BSI in ICUs by a remarkable 66%[72]. A more recent multi-centre cluster-randomised cross over trial looked at the effect of chlorhexidine bathing of ICU patients on acquisition of MDROs and hospital acquired BSI. The MDRO included were MRSA and VRE. The study found that there was a 23% reduction in the acquisition of both MRSA and VRE during the periods of bathing with chlorhexidine and that it was especially effective at reducing the overall risk of BSI in ICU patients, the most significant reduction was seen in coagulase-negative staphylococci. There was also a beneficial effect on catheter related BSIs and fungal BSIs, but the previously dramatic reduction in VRE BSI was not seen [72]. A caution to these favourable results, is the potential for emerging resistance. Already a number of pseudomonas strains have high level resistance to chlorhexidine [70]. Enterococci were also found to have increased MICs when compared with staphylococcci tested [70]. There is also the risk of allergic reactions which though rare, have been documented [74]. Further independent studies are
needed to assess the specific impact on MDR Gram negative organisms, which are lacking.

1.19 The contribution of environmental contamination to acquisition and dispersal of HCAIs.

It has been estimated that the majority of pathogens causing HCAI in intensive care units ICUs come from the patient themselves (40-60% of cases), followed by the hands of healthcare workers in 20-40% cases, the alteration of flora from antibiotic use in 20% and other sources, including the hospital environment, being responsible for another 20%. [75] Hospital surfaces have been implicated in the cross transmission of many pathogens like MRSA, VRE and resistant *Acinetobacter* and *Pseudomonas* spp.[76]

There are many infection prevention and control strategies to reduce HCAI. They include reducing person to person transmission of infections by means of implementing good hand hygiene practice, standard precautions in the care of all patients, active surveillance cultures for targeted MDROs, with isolation of individuals colonised or infected with them and the use of care bundles for invasive device insertion [77]. There are also a host of environmental controls, from controlled ventilation in high risk area, such as operating theatres, ICUs and oncology units to, active monitoring of water for legionella, monitoring endoscopy rinse waters and cleaning of patient environment. With all these interventions, there has been an improvement in a number of HCAI, including the steady decline of invasive infections with MRSA. The percentage of *S.aureus* BSI due to MRSA in Ireland has gone from 45% in 2008 to 18% in 2014[11]. Despite these interventions, the overall numbers of *S.aureus* BSIs have stayed constant and the rates of other resistant organisms like VRE and ESBL-E continue to increase. Overall, HCAI remain a significant problem in health care settings, especially in ICUs as outlined above.

Up until a decade ago, the role the environment played in transmission of resistant organisms was considered negligible[78]. Since then, many studies have shown that
micro-organisms can survive for long periods of time on hospital surfaces. The degree of contamination and duration of survival differs between species. This has been shown to be anything from 7 days to 46 months for MRSA and VRE, to a number of hours to months for Enterobacteriaceae, *Acinetobacter* spp and *Pseudomonas* spp[76, 79, 80]. The survival times vary under different conditions including humidity, surface type, and the presence of organic material, with increased humidity and the presence of protein or serum resulting in the longer survival of Gram negative bacteria [80].

Many studies have investigated the presence of patient commensal or pathogenic flora on the surfaces around them in hospital. The proportion of surfaces contaminated vary widely, from 1-27% for MRSA on general hospital wards, up to 64 % of surfaces in a burns unit with MRSA patients. The degree of environmental contamination is dependent on the degree to which patients are colonised, with heavily colonised patients shedding more in their environment[81]. This is particularly true of patients with diarrhea and with resistant organisms isolated from multiple sites. Studies, looking at both gut colonization of MSRA and VRE have shown that patients who have diarrhea and gut colonization with MRSA are more likely to contaminate their environment. Boyce et al found that 59% of environmental surfaces were positive in patients’ rooms, when the patients had diarrhea and colonization of their gut with MRSA, versus 23% surface contamination in the rooms of patients who were MRSA positive, with no diarrhea [82]. This is also true of VRE. In an earlier study Boyce et al found in an outbreak of VRE, the rooms of patients who had diarrhea had environmental contamination in 46% of sites tested, versus 15% in the rooms of patients who were VRE positive with no diarrhea [83]. A study conducted in an ICU, Bonten et al, showed that overall 12% of environmental samples were positive for VRE, but the degree of contamination increased to more than 60 % in rooms of patients colonised with VRE at three or more body sites [84].

Contamination of rooms of patients colonised by ESBL has also been investigated. In a study looking at environment contamination of rooms of ESBL positive children in
France, 4% of environmental samples were contaminated [79]. Being colonised or infected with ESBL \textit{Klebsiella} spp was more likely to result in environmental contamination than being colonised or infected with ESBL \textit{E.coli} [79]. Judge et al also found ESBL E in the environment of a patient with ESBL \textit{Klebsiella pneumoniae} in a urine sample [85]. They found the survival time of these isolates on surfaces to be only a few hours.

The persistence of multi drug resistant organisms in the environment occurs. Whether this is due to poor cleaning or the formation of biofilm on the surfaces has been subject to research.

It is routine practice to have a ‘terminal clean’ of a room after the discharge of any patient.[86]. The rooms of patients with resistant organisms should be cleaned with 1 in 1000ppm chlorine or other disinfectant after the patient has been discharged. Carling et al showed in a multicentre study that the cleaning of ICUs after discharge of patients to be sub-optimal. A baseline audit of room cleaning after patient discharge, showed that only 49% surfaces had been cleaned. This improved to 82% after a number of educational sessions and feedback cycles to cleaning staff. The improvement also required the recruitment of additional housekeeping staff, education of environmental supervisors and commitment from hospital management[87]. Hayden et al had similar baseline results from another study carried out in a ICU, which showed that increased cleaning resulted in reduction of VRE acquisition, from 33 per 1000 patient days at risk to 16.84 per 1000 patient days at risk during enhanced cleaning, even when there was a lapse in hand hygiene compliance rates[65]. Another group took the opportunity of a decommissioned ICU to take more definitive environmental samples with cutting implements. Researchers took sections from different materials in the ICU after the ICU had two so called ‘terminal cleans’ with 500 ppm free chlorine solution. MDRO, including MSRA, VRE and ESBLs were cultured from 52% of 44 samples. Forty -one of 44 samples, 93% were visually confirmed to have biofilm infecting their surface. Eighteen of these surfaces were stored for 12 months, and confocal laser imaging with live dead staining confirmed viable organisms present in all samples after 12
months. This shows that bacteria can persist in dry biofilm in the hospital environment [88]. The role this plays in onward transmission remains to be assessed.

Hospital environments are not sterile places. Hospitals are places where people, with varying degrees of underlying illness and immunosuppression, are placed in an enclosed, somewhat controlled environment for the care of diverse medical and surgical problems. As typical mammals, humans shed the equivalent of their entire intestinal microbiome every 2-3 weeks [40]. In hospitalised patients, a proportion goes into the hospital environment. The role that the hospital environment plays in the transmission, acquisition and subsequent infection with multi-drug resistant organisms has been debated over the last decade. It has gone from being regarded as having a generally negligible effect on infection transmission [89] to being implicated in the acquisition of MRSA, VRE, resistant Gram negative organisms and other organisms in both general hospital wards and general ICUs [79, 90, 91].
1.20. Hypothesis and aims of this research

Patients are shedding MDRO into the ICU environment and this environment is a reservoir for the dissemination and cross transmission of MDROs.

In this thesis we focus on two MDROs, VRE and extended spectrum β-lactamase producing Enterobacteriaceae ESBL-E in the context of environmental contamination in an ICU and patient colonisation. Of the many MDRO that present a risk to vulnerable patients, we focused on these organisms in the context of their increasing frequency in Ireland. The aims were therefore

1. To describe and quantify the contamination of the ICU environment with VRE and ESBL-E. Particular reservoirs for the target MDRO within the ICU environment were sought and an analysis of the effect of quality improvement measures which occurred during the course of the study on environmental contamination is outlined.

2. To address the burden of patient colonisation with the target MDROs, particularly in relation to ESBL-Es. We assess the patient demographics and risk factors for ESBL-E and VRE carriage or infection.

3. To correlate links between the patients and the environment from both an epidemiological and a molecular perspective.
Chapter 2

Materials and Methods
2.1. Setting
Beaumont Hospital, Dublin is an 820 bed tertiary referral teaching hospital. It is a national referral centre for neurosurgery, cochlear implantation, neurology, and renal transplantation and a regional referral centre for many specialties for the North East of Ireland. The hospital has a 12 bedded ICU and a 10 bedded specialist neurosurgical ICU. The general ICU has six beds in an open plan area and six isolation rooms. A schematic of the floor plan of the general ICU is shown in Appendix 1. This study took place in the general ICU only.

2.2. Environmental sampling
The environment of all occupied bed spaces in the general ICU was sampled twice weekly over seven, three-week time periods, from October 2012-June 2014. Six ‘high touch’ areas were sampled around each bed. These were; (1) the patient monitor above the bed, (2) the control panel of the drip stand adjacent to the bed, (3) the bed control panel on the right hand side of the bed, (4) the bed mattress (5) the unit sinks and (6) the chart-holder holding the patient’s clinical notes and nursing care flow sheets, later the computer keyboard. Figure 2.1 shows one of the newer isolation rooms with five of the six high touch sites (excluding the unit sink) indicated by a red star. The unit introduced computerised clinical charts in March 2014. At this time, the chart holder was replaced with ceiling mounted and mobile computer terminals with keyboards as shown in Figure 2.2.

Each area was swabbed using Copan eSwabs™ (liquid Amies elution swabs collection and transport system, Copan Diagnostics, Italy). An area of approximately 5cm² was swabbed on the flat surfaces (e.g. mattress, chart-holder). On high-touch items (e.g. patient monitors, drip stand), the areas deemed to be most frequently touched i.e. the control buttons, were sampled. The same area (approximately) was swabbed from each piece of equipment on each sampling occasion.
Figure 2.1. The equipment in one of the isolation rooms in the ICU. Areas marked by a star are examples of the “High touch” surfaces sampled around each bed space. Arrows indicate close-up views of the control panel of the drip stand and the bed control panel. Photos by Ray Lohan, Media Services Department, RCSI
Figure 2.2 Chart holders, monitors and keyboards. Illustrations of the chart holders (A) compared with the monitors and keyboards (B) which replaced them. In the main ward area the terminals are ceiling mounted. In the isolation rooms they are on mobile units. Photos by Ray Lohan, Media Services Department, RCSI.

2.2. Hospital hygiene measures in place in the ICU.
There is a dedicated member of the cleaning staff allocated to the ICU every day. They are rostered on from 7am to 7pm. There is also an on call service if required after hours. There is no specific checklist for cleaning. Bed spaces are cleaned one at a time and all bed spaces are cleaned with 1000 ppm sodium dichloroisocyanurate (Precept, Advanced Sterilization products, Ontario, Canada)

Staff hand hygiene audits in ICU are carried out monthly and reported back to the hospital hygiene and infection prevention and control committee quarterly and audits of environmental hygiene standards are carried out twice yearly as per national hygiene standards [92].

2.3. Patient samples
As part of their clinical care, patients in the ICU are screened on admission and weekly thereafter, by a rectal swab, for the presence of resistant flora for infection
prevention and control purposes. The target organisms for this screening are VRE and CRE.

For the purposes of this study, the VRE isolates from patients were provided by the Clinical Microbiology Laboratory (CML), Beaumont Hospital. Once processed for routine screening, rectal swabs, from all patients present in the ICU at the time of environmental sampling, were collected from the CML and were cultured to screen for the presence of ESBL producing Enterobacteriaceae. The use of patients’ VRE screening swabs for the purpose of recovering ESBL-E if present, received ethical approval from the Beaumont Hospital Ethics Committee (approval number 12/74, Appendix 2)

Patient demographics and details of ICU stay were recorded including bed number, diagnosis, presence of devices and antibiotic treatment. These data were collected on a form at each visit (appendix 3), inputted and stored on an Access database and subsequently in MS Excel spreadsheets (Microsoft OfficeTM).

Additional surveillance data on all VRE screens carried out in BH over the 20 months duration of the study, was obtained from the laboratory information system. This data was received by a personal communication from Mairead Skally, Surveillance scientist, Beaumont Hospital. In addition to this, a data trawl of the laboratory information system was carried out to quantify the numbers of ESBL clinical isolates identified from inpatients in Beaumont hospital over the time of the study. These constituted the first ESBL isolate per patient identified from any clinical isolate sent during the course of routine care. This additional data was obtained to place the ICU data in the wider context of the hospital data.

2.4 Culture media

Pre-poured plates of MacConkey agar, Columbia Blood Agar (CBA) and Brilliance UTI Clarity agar were obtained from Fannin LIP, Galway, Ireland. Brilliance UTI clarity, a chromogenic agar, was used for presumptive identification of enterococci and
Enterobacteriaceae. Appearance of pink colonies on this agar was presumptive for \textit{E. coli}, dark blue presumptive for \textit{Klebsiella spp.}, and small turquoise colonies presumptive for enterococci. Representative plates showing each of these organisms are shown in Figure 2.3. For isolation of VRE, pre-poured plates of VRE Select (BioRad) or VRE Chrome ID (Biomerieux) were used. For the isolation of ESBL producing \textit{Enterobacteriaceae}, pre-poured plates of ESBL Brilliance agar (Oxoid) were used. Representative plates showing growth of VRE and ESBL-E are shown in Figure 2.4. quid culture media were prepared in-house in Milli-Q water (Millipore Ireland, Cork, Ireland) using dehydrated culture media (Oxoid, UK or Sigma Aldrich, UK). For solid media, 1-2% agar was added (Sigma Aldrich, UK). Media was autoclaved at 121°C for 15 min and allowed to cool. Solid media was poured into petri dishes aseptically and cooled to solidify. Plates were stored inverted at 4°C for up to two weeks.

\textbf{Figure 2.3 Presumptive identification of target organisms.} The appearance of enterococci (A), \textit{E.coli} (pink) and \textit{K.pneumoniae} (dark blue) (B) and \textit{Enterobacteriaceae} spp (C) on Brilliance UTI chrome agar.
Figure 2.4 Appearance of resistant target organisms on selective chromogenic agars. The typical appearance of vancomycin resistant *E. faecium* is represented by purple colonies on VRE Select® agar. A susceptible clinical strain was inoculated onto the lower half of the plate and showed no growth (A). The typical appearance of *Klebsiella pneumoniae* ATCC 700603 (ESBL-E positive control) which grows as sea green colonies on ESBL Brilliance agar. The ESBL-negative control (*E. coli* ATCC 25922) is also shown on the lower half of the plate but did not produce colonies on this agar (B).

2.5 Bacterial reference strains

In addition to patient and environmental isolates, reference strains were used in this study. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as negative and positive controls for phenotypic identification of ESBL-E. *E. faecalis* ATCC 29212 and *E. faecalis* ATCC 51299 were used as negative and positive controls for phenotypic identification and susceptibility testing of VRE and as reference strains for PFGE. *Salmonella enterica subsp. enterica serovar Braendurup* H9812 was used as an internal reference for PFGE.

2.6 Bacterial growth and storage.

Isolates obtained on agar slopes were sub-cultured onto agar plates (CBA or Mueller Hinton) to obtain isolated colonies and all isolates recovered from swabs were streaked for purity onto UTI Brilliance agar (LIP, Galway). Plates were inverted and grown overnight (16-18 h) at 37°C in a static incubator and were stored inverted at 4°C. Colonies from these plates were sub-cultured as required for up to two weeks after which time, fresh plates were prepared from cryopreserved stocks.
For liquid culture, single colonies were inoculated into 2 ml liquid medium and grown overnight at 37°C in a shaking incubator at 150-200 rpm (Gallenkamp, Environmental). Cryopreserved stocks of bacterial strains and isolates were stored at -20°C on Protect Bacterial Preserver beads (Technical Service Consultants, Heywood, Lancashire, UK) by emulsifying isolated colonies from overnight culture in the cryopreservation fluid surrounding the beads. For recovery from storage, single beads were used to inoculate agar plates which were incubated overnight at 37°C.

2.7 Buffers, Solutions and Enzymes
All buffers and solutions were prepared using de-ionised water (Millipore, Cork, Ireland) or sterile water (Baxter, UK). The pH of buffers was adjusted using a pH meter (Denver Instrument, model 215) which was calibrated before measurement. Enzymes were prepared as stock solutions in buffers recommended by the suppliers and were stored in aliquots at -20°C.

2.8 Phenotypic identification and susceptibility testing

2.8.1 ESBL double-disc synergy test
Confirmatory phenotypic tests for ESBL production were carried out on isolates of *E. coli*, *Klebsiella spp* and *Enterobacter* spp from both patient and environmental samples. Tests were carried out and interpreted according to European committee for antimicrobial susceptibility testing (EUCAST) guidelines using MASTDISCS™ID cefotaxime and cefepime ESBL Detection Discs (Mast Diagnostics, Merseyside, UK). Briefly, isolated colonies from overnight cultures were suspended in sterile saline to a turbidity equivalent to a 0.5 Macfarland standard. A sterile swab was used to spread this suspension evenly onto MH agar plates and dried. Discs were placed onto the agar plates and incubated overnight at 37°C for 18-24h. Zone diameters were measured and a zone difference of greater or equal to 5mm was regarded as positive for ESBL production [93]. The appropriate ESBL-positive and negative reference strains were used as controls.
2.8.2 Vancomycin E-test

MIC values for vancomycin confirmed VRE and were determined using E-test strips (Biomerieux). Briefly, a bacterial suspension was prepared in saline to a turbidity equivalent to a 0.5 Macfarland standard. This suspension was spread uniformly on MH agar using a cotton swab and allowed to dry. An E-test strip was placed on the centre of the plate using a sterile forceps. Plates were incubated overnight at 37°C for 24h. The MIC value was indicated at the point of intersection of the zone of inhibition, with the E-test strip. Appropriate positive and negative reference strains were used as controls [93].

2.9 Identification of isolates using matrix assisted laser desorption & ionisation time of flight mass spectrometry (MALDI-TOF MS.)

A MALDI Biotyper (Brüker Daltonik MALDI Biotyper) was used for MALDI-TOF MS to identify bacteria. Isolated colonies from samples grown overnight on an agar plate were evenly and thinly spread with a wooden applicator onto the stainless steel target plate supplied with the MALDI Biotyper. Matrix solution (1 µl) was added to the sample and was allowed to dry. Each isolate was analysed in duplicate. The target plate was loaded into the MALDI Biotyper for analysis. The lid was closed and the vacuum was allowed to reach a level of 3^{-6} pa or less. Measured peaks were then compared to the Brüker isolate database and a score value of greater than 2 represented a good identification.

The MALDI Biotyper measures highly abundant 16S rRNA proteins found in all microorganisms. The characteristic patterns of these abundant proteins are used to reliably and accurately identify particular microorganisms. Identification is performed by matching the measured protein fingerprint against the Brüker MALDI biotype database.
The acceleration parameters are set by Bruker to match the isolate database which contains greater than 4000 strains of over 2000 well characterised microbial species. For species identification the size range generally used is between 2 and 20 kilodaltons. The instrument is calibrated weekly using a Bruker bacterial test standard which is supplied by the manufacturer. This contains *E. coli* DH5α and 2 additional proteins (RNase and myoglobin) in the upper mass range. This enables calibration over a mass range of 4-17kDa containing some additional proteins which can be used for instrument mass calibration as well as a performance verification standard [94].

2.10 Identification of VRE from patient swabs.

Rectal swabs, taken from patients on admission to the general ICU as part of their clinical care, were processed for identification of VRE by the Clinical Microbiology Laboratory of Beaumont Hospital. The swabs were plated onto selective Chrome ID VRE and incubated for 24h at 37°C. The target organisms grow as either purple (*E. faecium*) or blue (*E. faecalis*) colonies at 24h on this medium. The identification of the organisms was confirmed by MALDI-TOF MS as described above. The MIC for vancomycin was determined using E-test strips (Biomerieux) as described in Section 2.8.2.

2.11 Identification of ESBL-E from patient swabs

For identification of ESBL-E from patients, patient rectal swabs were inoculated first onto Brilliance ESBL selective agar (Oxoid, UK) and incubated at 37°C for 18-24h. Colonies that were blue or pink (*E. coli*) or dark blue or sea green (*Klebsiella* spp., *Enterobacter* spp., *Serratia* spp., *Citrobacter* spp.) were sub-cultured onto UTI Brilliance agar for purity. Presumptive *E. coli* (pink colonies) or other Enterobacteriaceae e.g. *Klebsiella* spp and *Enterobacter cloacae* (dark blue/purple colonies) were confirmed using MALDI-TOF MS as described in Section 2.8. Confirmation of the ESBL phenotype was carried out by double disk synergy test as per EUCAST guidance as described in Section 2.8.1.
2.12 Recovery of target organisms from environmental samples

For the recovery of ESBL-E and VRE from the ICU environment, each environmental swab (Copan eSwabs) was transferred into 2 ml of brain heart infusion (BHI) broth for enrichment and incubated overnight (16-18h) at 37°C in a shaking incubator (Gallenkamp, Leicester, UK) set at 150-200 rpm. A 10 μl loop of the enriched suspension was streaked onto UTI Brilliance agar (Oxoid, UK) to isolate single colonies. Enterococci and Enterobacteriaceae (colonies appearing blue, blue green, turquoise or pink) were sub-cultured onto VRE-Select (Biorad)/VRE ChromeID (Biomerieux) or Brilliance. Presumptive positive VRE and ESBL-E, were speciated based on their growth on selective chromogenic agars and were confirmed using MALDI-TOF MS as described in Section 2.8. Phenotypic confirmation of ESBL-E was done using the MAST discs described in Section 2.8.1 and vancomycin MICs for VRE were determined using E-tests as described in Section 2.8.2.

2.12 Genetic analysis and molecular typing

2.12.1 Typing using Pathogenica HAI Biodetection kit

The HAI BioDetection™ system (Pathogenica Inc. Boston, USA) is a next generation sequencing system developed for surveillance and monitoring of HCAI in healthcare facilities. The kit uses molecular inversion probe technology and targeted sequencing for identification and subtyping of bacteria. This system was used to both identify resistance genes and type a selection of patient and environmental VRE isolates from this study. Typing and analysis was carried out by Pathogenica Inc in Boston.

Charcoal-containing cotton swabs (ProbactTM, UK) were inoculated with overnight growth of VRE and sent to Pathogenica Inc in Boston, USA for analysis using the HAI Biodetection™ kit.
2.12.2. Pulsed field gel electrophoresis (PFGE) for VRE

A modification of the German Enterococcal Reference Laboratory PFGE method (obtained by personal communication from Dr. Kirsten Schaffer, Microbiology Department, St Vincent's University Hospital, Dublin) (Appendix 4) was used with further changes based on the modifications of methods described by B. Murray and by D Turabelidze and B Saeedi [47, 95, 96] and (Dr Jonathan Collins, Microbiology Department, Tallaght Hospital, Dublin Personal communication).

Preparation of DNA plugs from clinical isolates and reference strains

Overnight bacterial cultures on blood agar were suspended in 2ml TEN (buffer to an optical density (OD) at 600 nm of 1.0. A 1ml aliquot of each suspension was centrifuged at 14000rpm for 10s. The supernatant was removed and the pellet was washed with 500μl TEN and re-centrifuged before re-suspension in 200μl TEN. The suspension was pre-incubated at 55°C. An equal volume of molten 2% SeaKem® agarose in TE buffer (10 mM Tris, 1mM EDTA, pH 8.0) was mixed with the bacterial suspension. The agarose suspension was dispensed into plug moulds supplied by Bio-Rad and allowed to solidify.

Plug lysis

Plugs were lysed overnight in 1 ml EC buffer (6 mM containing 100μl lysozyme (4mg/ml) at 37°C in a shaking incubator (Gallenkamp, Leicester, UK) set at 200 rpm. The buffer was replaced with 2 ml cell suspension buffer (CSB) (100mM Tris, 100mM EDTA, pH 7.5) and incubated at 55°C for 45 min. CSB was replaced with fresh CBS buffer containing Proteinase K (100 μg/ml) and was incubated at 55°C for 1h. The plug was washed 4 times with fresh CSB at 20 min intervals and stored at 4°C overnight.
Restriction

Slices of the gel plug (2.5 mm) were incubated in 200 μl TE buffer at 25°C for 15 min. The remainder of the plug was stored in CSB at 4°C. TE Buffer was removed and the plug slice was pre-incubated with 150 μl of CutSmart® buffer (New England Biolabs) at 25°C for 30 min followed by restriction with Sma1 enzyme (30U) (New England Biolabs) for 4 h at 25°C. The enzyme-buffer combination was replaced with fresh 0.5M TBE.

Preparation of control plugs

In addition to the use of a Lambda ladder and *E. faecalis* reference strain, ATCC 29212 as PFGE marker and references, the reference strain H9812, *Salmonella enteric subsp. enterica* serovar *Braendurup* was used as a size reference. Agarose plugs of this strain were prepared according to the PulseNet standardised protocol for *E. coli* [97]. Briefly, overnight cultures of *S. braenderup* H9812 were resuspended at an OD$_{600}$ of 1.4 in CSB. An aliquot of this suspension (400 μl) was mixed with 1 mg/ml proteinase K and an equal volume of 1% Seakam Gold agarose containing 1% sodium dodecyl sulphate (SDS) at 55°C was added. The suspension was dispensed into plug moulds and allowed to solidify for 30 min. Plugs were lysed in 5ml cell lysis buffer (CLB) (50mM Tris, 50mM EDTA, 1% sarcosyl, pH 8.0 containing 100μg/ml proteinase K) at 55°C in a shaking incubator for 1.5-2h or overnight. The plugs were washed 4 times at 50°C with preheated TE buffer and sterile water, alternating at 15 min intervals over 1 h. Plugs were either stored in TE buffer at 4°C or were restricted. Plug slices (2.5mm) were restricted with 50U Xba1 in 1X H buffer (Roche) at 37°C for 1.5-2h or overnight. The restriction reaction was stopped by replacing the enzyme mix with 0.5X TBE (Tris-Borate-EDTA).

Electrophoresis running condition, gel staining and analysis

PFGE was carried out in a 1% Seakem agarose gel prepared in 0.5X TBE buffer using a CHEF-DR™II electrophoresis system (Bio-Rad Laboratories, CA). PFGE was performed over 20 h, using an initial switch time of 3.5 s, a final switch time of 25s for 12 h at 200 V, followed by a second cycle of initial switch time 1s and final
switch time 5s, for 8h at 200V. Both cycles were carried out at 14°C with an inclusion angle of 45°. Gels were stained with ethidium bromide (10mg/ml) for 30min and then rinsed for 30min with distilled water. The gels were viewed and photographed using GeneSys gel documentation system and a G:BOX transilluminator (Syngene, Cambridge, UK) and saved as a file tagged TIFF image. Banding patterns were compared and analysed using GelCompar®II software (Ver.6.5, Applied Maths). The extent of variability was determined by the Dice coefficient using a tolerance of 1% and strains were clustered according to the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Clonal groups were assigned based on a similarity of ≥80%.
Chapter 3

Bacterial contamination of surfaces in the general ICU, an observational study
3.1. Introduction

The hospital environment is increasingly recognised as a reservoir for bacteria, and the inanimate environment is often identified a source of hospital acquired infections [80]. The rise in HCAI, especially among the critically ill, and continued emergence of multidrug resistant bacteria over the last decade, has prompted surveillance of the environment surrounding vulnerable patients [98]. An international study representing data from 1265 ICUs in 75 countries, of the prevalence and outcomes of infection in ICUs, EPIC II, reported that infection was present in 51% of the 13796 patients included in the study. Of the patients that had an organism identified, 44% were due to MDRO [9]. The most commonly isolated resistant bacteria were *Pseudomonas aeruginosa*, MRSA and *Acinetobacter baumanii*. VRE accounted for 3.8% and ESBL-E accounted for 1.9%[9]. In the most recent point prevalence survey of HCAI and antimicrobial use in Ireland, published in November 2012, augmented care units, which includes ICUs, had a HCAI rate of 16% compared with the overall average of 5.2%[7].

The rate of VRE among enterococcal-related BSI and ESBL-E among Enterobacteriaceae related BSI infections has increased dramatically in Ireland over the last decade [50]. According to the ECDC figures from EARS-net, Ireland has the highest rates in Europe of vancomycin resistance among bloodstream isolates of *E. faecium* at 46.3% in 2014 up from 37.1% in 2006 [11]. The rate of ESBL-E production among *E.coli* and *K. pneumoniae* in 2014 was 10.1% and 11.3% up from 2.5 and 8.6% respectively. *E.coli* accounts for the majority of invasive isolates notified to the EARS-Net by a factor of two. With these emerging patterns, increasing resistance in these organisms is a matter of grave concern both nationally and internationally [3, 10].

Hospital surfaces have been implicated in the cross transmission of many pathogens like MRSA, VRE, resistant *Acinetobacter spp.* and *Pseudomonas spp.*[76]. Hospital surface investigation is usually carried out in response to an outbreak, and in conjunction with other infection prevention and control interventions[86]. This is largely due to the constraints on resources, both on infection prevention and
control personnel to carry out the screening and the laboratory resources. It may also be due to the transient nature of environmental contamination, and low yields from environmental screening. Pathogens associated with HCAI do however persist in the environment, but to a varying degree [99]. Most Gram positive bacteria, such as enterococci, can survive on dry surfaces for months. For Gram negative bacteria, reported viability is variable and dependent on species. *Acinetobacter* species can survive for months, like the Gram positive organisms. Enterobacteriaceae have been reported to survive from hours to months [80, 85, 99]. The environmental conditions, including temperature and humidity can increase the persistence of some Gram negative organism including *Pseudomonas* spp and also the presence of organic material such as protein or serum for Enterobacteriaceae assists survival [80, 99]. Many recent studies have found that environmental contamination with MDRO has contributed to patient acquisition of these organisms. For example, Marci Drees et al found that prior room contamination was highly predictive of VRE acquisition in an ICU [91], and earlier Bonten et al investigated the colonisation of patients with VRE and environmental contamination in a medical ICU and found of 13 patients whose rooms were colonised with VRE, three (13%) acquired VRE[84]. A study looking at the effect of enhanced cleaning on acquisition of VRE in an ICU found that after feedback and education the rate of surfaces cleaned went from a baseline of 48% to 84 % and that the rate of patient VRE acquisition in the unit went from 33 per 100- patient days at risk, to 16.84 per 1000 during period of enhanced cleaning [65].

Another study investigating the acquisition of resistant Gram negative organisms among patients in a general ICU from prior room occupants found that a positive carrier status of previous room occupant was a risk factor for acquisition of MDR *P. aeruginosa* and *A. baumanii* in subsequent room occupants. However the authors pointed out that the environment was not sampled in this study, so its role in transmission is speculative [100]. Although infection prevention and control strategies are largely based on preventing person-to-person transmission, to reliably inform policy, surveillance outside of outbreaks should also be investigated.
to better inform evidence-based recommendations [86]. While infection prevention and control interventions, contribute to the reduced transmission of antimicrobial-resistant bacteria, routine, strategic and enhanced surveillance of the environment is necessary to inform local and national policy underpinning infection prevention.

In this study, environmental contamination of the ICU environment with VRE and ESBL-E bacteria was investigated. We chose these organisms because these organisms have been increasing over the last number of years as highlighted above. Furthermore, in relation to VRE, Ireland is the only European country where vancomycin resistance among invasive *E. faecium* isolates exceeds 25% [11]. A recent typing study of invasive VRE isolates from an Irish tertiary referral hospital, compared the major STs in Ireland to those of other countries and additionally aimed to characterise virulence genes among Irish isolates [48]. The study reported four predominant STs among Irish VRE isolates, ST17, ST18, ST78 and ST203. While, ST17 and 18 represent classical hospital strain types, ST78 and ST203 are comparatively new STs, but all four STs have been reported previously among isolates from Europe, Asia and Australia [101, 102].

In this Chapter, we describe an observational study carried out in a general ICU, in which VRE and ESBL-E on ‘high touch surfaces’ in the environment of patient bed spaces were targeted specifically as potential reservoirs of these resistant organisms. Enhanced surveillance of these sampling sites took place twice weekly over seven three week periods spanning two years from October 2012 to June 2014. This sampling approach provided a snapshot of basal environmental contamination with these MDROs outside of outbreaks in the protean environment of an ICU.

### 3.2 Results

**3.2.1. Overview of the total bacterial contamination of ICU surfaces.**

A total of 1722 swabs were taken from the environment of 157 patients during seven three week intervals from October 2012 to June 2014. For some patients, their environment was sampled on more than one occasion, as their length of stay in ICU exceeded 48 h. This included patients who moved beds, and therefore
environment, within the ICU. The majority of the environmental swabs, 1206/1722 (70 %) were positive for bacterial growth. Of the swabs that were positive for bacterial growth, the majority of isolates recovered represented microorganisms of the skin flora (68%), gut flora (21%) and other Gram positive and Gram negative environmental bacteria (9%). The variety of microorganisms recovered is illustrated in Figure 3.1. Of these, only those belonging to Enterobacteriaceae (n= 60) and Enterococcus spp. (n=186) were investigated further to identify sub-populations of ESBL-E and VRE among them (highlighted as extended segments in Figure 3.1). A subsection of the isolates recovered from environmental swabs over each time period of the study is illustrated in figure 3.2. The Enterobacteriaceae isolates from ‘high-touch’ sites included E.coli, Klebsiella spp., Enterobacter spp., Pantoea spp. and Proteus spp.

![Figure 3.1 The variety of microorganisms isolated from the ICU environmental swabs.](image)

The extended segments represent the proportion of VRE and ESBL-E recovered.
The variety of microorganisms with potential infection risk to patients isolated from the ICU environment over time

Table 3.1. Culture results from environmental swabs including the number and proportion which were positive for VRE and ESBL-E.

<table>
<thead>
<tr>
<th>Contamination detected at environmental sites (n=1206/1722)</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sites positive for bacterial growth</td>
<td>1206 (70)</td>
</tr>
<tr>
<td><em>Bacteria detected</em></td>
<td></td>
</tr>
<tr>
<td>Sites positive for VRE</td>
<td>108 (6.3)</td>
</tr>
<tr>
<td>Sites positive for ESBL-E</td>
<td>10 (0.6)</td>
</tr>
<tr>
<td>Sites positive for other microorganisms</td>
<td>1088 (63)</td>
</tr>
<tr>
<td>Sites negative for bacterial growth</td>
<td>516 (30)</td>
</tr>
</tbody>
</table>

3.2.2. VRE and ESBL-E contamination of the ICU environment

Of the ‘high touch’ sites investigated, 108/1722 (6.3%) were positive for VRE and 10/1722 (0.6%) were positive for ESBL-E (Table 3.1). VRE accounted for 108/186 (37%) of the enterococci isolated from the environmental sites and ESBL-E accounted for 10/60 (17%) of the Enterobacteriaceae identified from environmental sites (Figures 3.3).
Figure 3.3 VRE/ESBL-E among environmental Enterococci/Enterobacteriaceae. The proportion of Enterococci recovered from the ICU environment which were VRE (a) and ESBL-E as a proportion of Enterobacteriaceae recovered from environmental swabs (b).

3.2.3. Prevalance of VRE/ESBL-E environmental contamination over seven sampling periods

The recovery of VRE and ESBL-E from ‘high-touch’ surfaces was investigated for seven sampling periods of three week duration spanning two years. VRE was recovered from the ICU environmental sites in all of the seven screening periods, and ESBL-Es were recovered in 5/7 (71.4%) of the sampling periods (Figure 3.4). Contamination levels with VRE/ESBL-E varied over each of the sampling periods with periods 2 and 5 having the highest percentage of samples positive for the MDRO at 13% and 12.7% of samples respectively as illustrated in Figure 3.5. A biphasic pattern was observed for the detection of VRE/ESBL-E in the ICU, with relatively high detection levels in the January/February periods compared to other sampling periods.
Figure 3.4 The number of VRE and E SBL-E recovered from ICU environmental sites in each of the study time periods. The time periods investigated included seven periods spanning October 2012-June 2014. Each time period represents 3 weeks of sampling where sampling was conducted on two days each week.
The quantity of environmental contamination with MDRO (VRE/ESBL-E) in each sampling period with respect to the total number of environmental samples taken at the time. Each time period represents 3 weeks of sampling where sampling was conducted on two days each week. The total number of environmental samples taken during each sampling period is represented by the blue bars, red bars represent combined VRE/ESBL-E numbers recovered in each time period.

### 3.2.4. Contamination levels with VRE/ESBL-E in each bed space of the ICU

VRE was recovered throughout the ICU environment from samples taken from high touch surfaces in each bed space, (Figure 3.6). Of the total number of environmental swabs taken from each bed space, 5% were positive for VRE or ESBL-E from beds 1-6, 10% were positive from beds 7-10 and 4% from beds 11 and 12. The ICU has six isolation rooms, namely beds 7-12, as shown in the plan of the unit (Figure 3.6b). The most commonly used beds for patients colonised with resistant flora are beds 7 to 10. Sixty nine of the total 157 patients (44 %) included in the study occupied beds 7-10 at least once over the course of the study. Three of them were in two isolation rooms and 23/69 (33.3%) were colonised with a VRE or ESBL-E. Of the sites found to be positive for VRE, a greater proportion 67/108,(61%) were from samples from these rooms compared to other bed spaces of the ICU (Figure 3.7). Of the 10 ESBL-E environmental isolates, 4 (40%) were recovered from these rooms. Comparing the combined level of contamination of the environment with
MDRO in beds 1-6 (open area) to beds 7-10 (single isolation rooms), there was more contamination in the isolation rooms compared to the open ward and this difference was statistically significant (p<0.0005).

**Figure 3.6 Distribution of VRE/ESBL-E among ICU beds.** (a) The distribution of ESBL-E and VRE among the beds of the general ICU. The box illustrates the isolation rooms. As seen in the plan of the ICU layout (b), isolation rooms 11 and 12 are negative pressure rooms and are separate from the other 4 isolation rooms.
3.2.5. Potential reservoirs of VRE/ESBL-E among the high touch surfaces of the ICU.

The target organisms were recovered from all high touch surfaces sampled (Figure 3.8). The sites most frequently contaminated by VRE and or ESBL-E were the bed control panel (BC), drip stand (DS) and chart holder (CH), together accounting for 64% of contaminated sites. Where VRE was recovered from a patients environment, it was distributed throughout all surfaces sampled, with the greatest numbers recovered from dripstands, bed control panels and chart holders. Although relatively low in numbers compared to VRE, where ESBL-E were recovered, they were found in equal numbers on bed controls, chartholders, dripstands and sinks. No ESBL-E were recovered from mattresses, monitors or keyboards.
A number of improvement strategies were undertaken in ICU during the course of, but independent of the study. The first was the installation of a clinical information system (electronic patient record and ICU monitoring system) which replaced the paper flow sheets and also the mobile chart holders. The second was the introduction of chlorhexidine wipes to clean patients.

The installation of the clinical information system took place over two stages. The first of these involved the temporary relocation of the unit, including the equipment, to a general ward in the hospital for a period of three weeks (two of which were in sampling period 3, June/July 2013). This was to facilitate the required building works for the installation of the ceiling mounts. The temporary unit was a general ward, but maintained its bed numbers. Comparing the sampling results for June in 2013 and 2014, 202 and 200 swabs were taken respectively from the environment. Of those, 15/202 (7.4%) and 10/200 (5%) were positive for a target organism.
was no statistically significant difference in the recovery of MDROs from the environment between the two time points, i.e. time period three, when the unit was at its temporary location and time period seven, a year later, when the ICU was back at its usual location (p=0.3).

The second stage occurred nine months later in April 2014, sampling period 6, and involved removing all the chart holders and replacing them with the ceiling mounted and mobile computer terminals illustrated in Figure 2.2 (Chapter 2). A comparison of positive swabs (positive for bacterial growth) and swabs positive for the targeted organisms, VRE/ESBL-E is shown in Figure 3.9. There were 211 swabs taken from the chart holders over the course of the study. Of these 184 (87%) were positive for bacterial growth, with 23 (12.5%) positive for VRE/ESBL-E. There were 69 samples taken from the keyboards installed once the chart holders were removed. Of these samples, 31 (45%) were positive for bacterial growth, of which 9% were target organisms (VRE/ESBL-E). As shown in figure 3.9, a statistically significant higher level of overall contamination was found on chart holders compared to keyboards (p<0.005). However, no statistically significant difference was noted when comparing the level of recovery of the targeted MDROs from these two pieces of equipment. The keyboards were a flat, silicone model Medigenic® Medical Keyboard by Esterline technologies which are equipped with a reminder to clean, with both audio and visual alerts.
Figure 3.9 The comparison of the recovery of VRE and ESBL-E between chart holders and keyboards in the ICU. The keyboards replaced the chart holders during the course of the study. *** = p<0.005, NS = not significant.

Chlorhexidine wipes were introduced as part of the ICU patient bathing protocol in the ICU and started in the autumn of 2013, prior to the sampling time period 4. This was in an effort to reduce the number of MDRO acquisitions in the unit. The overall contamination of the environment was compared before and after introduction of the wipes for patient bathing. Pre chlorhexidine the percentage of sites sampled that was positive for bacterial growth was 75% of sites sampled and of those 12% were positive for a MDRO. After the introduction of chlorhexidine for patients this rate went down to 70% of all samples, of which 8% were positive as illustrated in figure 3.10.
Figure 3.10 Before and after chlorhexidine introduction: Percentage of samples with growth and with MDRO recovered. The proportion of environmental sites positive for both general bacterial growth and the study target organisms, VRE and ESBL-E, before and after the introduction of chlorhexidine wipes for patient bathing. Chlorhexidine wipes were introduced in the unit at the end of 2013. ** = p<0.05, NS = not significant.

3.3. Discussion
The microbiome of the ICU is variable and subject to change due to many factors including the patient cohort, changes in staff, cleaning regimes, infection prevention and control policies and compliance with these policies. The seven sampling periods investigated here can be regarded as ‘snapshots’ in time over 20 months based on environmental sampling of ‘high-touch’ surfaces of the ICU. The target MDROs, VRE and ESBL-E were recovered from the ICU environment, in low numbers (overall rate 6.3% VRE, 0.7% ESBL-E). Given the many variables that contribute to contamination rates it is unsurprising that the literature is conflicting regarding the rates of environmental contamination, with some studies reporting
similar rates to that of the present study but others reporting higher or lower rates. For example, a Singaporean study investigating environmental contamination on a general hospital ward found, of pooled samples of 82 environmental sites, 2% were positive for VRE and 1% were positive for ESBL Klebsiella pneumoniae [103].

Another study looking at the role of the ICU environment and health care workers hands on transmission of MDROs, found that of 605 environmental samples, 1.3% were positive for enterococci and of these 25% were VRE[104]. Notably, we did not limit our study to the bed spaces of patients with these specific MDRO infections, an approach taken by others, which may yield greater numbers over longer sampling periods and serve as a baseline for interventional studies [105, 106]. One such study, reported a similar baseline rate of 6.0% surface contamination with VRE based on six high-touch surfaces in 37 ICU rooms [106]. Another found a high rate of environmental contamination with another MDRO, CRE, in an ICU with a positive environmental samples being found in the environment of 30/34 (88%) patients representing 24% of the samples taken, the majority being taken from sites on the bed (sheet by the pillow, and adjacent to the patient legs)[107].

In this present study, no specific reservoirs for these organisms were identified in the ICU environment. Our study confirms, albeit in low numbers, previous reports however, that surfaces close to the patient and frequently touched by staff, namely the bed controls, drip stands and chart holders, harbour the majority of these organisms [86]. It was previously shown from laboratory studies that VRE persists on gloved and un-gloved fingertips of healthcare workers for up to 60 min and survived on telephone hand-pieces, bedrails and tabletops for 60 min, 24 h and up to 7 days respectively[108]. While the survival of Gram-negative bacteria have not been as comprehensively studied as Gram-positives such as MRSA, laboratory studies also support the survival of Enterobacteriaceae including ESBL-E from 2-3 hours to a number of months [80, 85].

The temporary relocation of the unit for 3 weeks had no impact on the contamination of the environment, despite the smaller space and the change in the layout. Over this time, environment contamination with VRE/ESBL-E was 7.1%,
which was comparable to the time period of the following year (May/June 2014), where 5% of sites were positive. The Electronic chart system went online in the unit at the beginning of March 2014 and at that time chart holders, were replaced by ceiling mounted and mobile computer terminals with keyboards. The associated keyboards have easy to clean surfaces which flash and sound an alarm when cleaning is recommended and have an antibacterial cover (Fig 2.2 B). With the increasing replacement of paper based medical notes with electronic records and laboratory information systems by healthcare institutions, there are many more keyboards now in clinical areas which are a potential environmental reservoir of transmissible bacteria. Early studies looking at the organisms present on computer keyboards found many were contaminated including studies showing 8-42% contamination with MRSA [109]. The cleaning of keyboards is also problematic with some studies showing significant bacterial load when tested [110]. Medical keyboards or antibacterial keyboards have been developed to reduce the risk of transmission of bacteria. A validation study performed in the Institute for Medical Microbiology and Hygiene at the Philipps University of Marburg Germany on the Medigenic Medical keyboards found a 6 log reduction in bacterial contamination with a variety of bacterial samples tested in vitro [111]. Another study carried out in University College Hospitals, London found that the Medigenic Keyboard™ with flat silicone surface and warning light improved hand hygiene practice and increased cleaning episodes when compared to other easy to clean models [112]. The same model of keyboard was introduced in the ICU in BH during the course of the present study. Our study found low rates of bacterial contamination recovered from the keyboards when compared with the chart holders which was statistically significant p<0.005. This highlights a positive impact of technology on the overall bacterial burden of the ICU environment.

A number of studies have found up to a 30% reduction in patient colonisation with MDRO after introduction of daily bathing of ICU patients with chlorhexidine impregnated wipes [70]. The introduction of chlorhexidine wipes into this ICU appeared to have a positive impact on environmental contamination rates when
the proportion of positive samples were compared before and after this intervention (Figure 3.10). This positive effect of chlorhexidine wipes on the inanimate environment is not widely described but has been reported elsewhere [73]. Furthermore, the impact of chlorhexidine bathing on this patient cohort with regard to reduction of MDROs is not yet apparent, an area that will be discussed in Chapter 4.

As demonstrated in other studies, the environment adjacent to colonised patients is usually the most contaminated [78, 86]. This was also shown to be the case in this study. The majority of positive samples were clustered around the isolation rooms 7-10. These are where colonised patients are most often accommodated. The difference between the proportion of MDROs collectively recovered from these rooms was statistically higher than for the open plan area of the ward, beds 1-6 (p<0.0005). This is an important consideration for the risk of patient acquisition of MDRO from rooms previously accommodating patient colonised with an MDRO as reported in other studies [91, 100]. This could inform cleaning protocols for different parts of the unit concentrating on the most contaminated areas.

Interestingly the other isolation rooms on the other side of the unit, are new, larger rooms, which comply with size standards for ICU rooms [59], had few representative samples but are rarely used due to staffing issues.

Low numbers of VRE and ESBL-E were recovered in the general ICU environment, but the rates found were comparable to reported studies. There appeared to be a seasonal element to the burden of MDRO on surfaces, but the rate found between each sampling period is variable. The most contaminated sites were concentrated in the isolation rooms where colonised patients are accommodated in keeping with other studies [79, 91, 113, 114].

The modernisation of the patient record to a computerised patient information system facilitated the replacement of cumbersome, contaminated equipment with a cleaner alternative. Whether this small improvement continues into the future may depend on how the maintenance of the keyboards is delegated.
Chlorhexidine wipes have been shown to reduce acquisition of MDRO[72]. However, there is little in the literature on the effect this has on the patient environment. This study shows that there is a reduction in the overall contamination of the environment with micro-organisms which may contribute to the reported reductions in acquisitions of MDRO. Further studies, with larger numbers would be needed to confirm this.
Chapter 4

An investigation of the dynamics of patient carriage/infection with VRE/ESBL-E in an ICU.
4.1 Introduction

The patient population in the ICU is amongst the sickest population in the hospital. They are the patient group who are most at risk of HCAI. Multicentre studies have shown that this population of patients have not only increased prevalence of infections in general, but 44% of the infections in ICU are due to MDRO [9]. A recently published Irish PPS of HCAI and antimicrobial use report a prevalence of HCAI in the intensive care setting of 16.5%, which is echoed in a similar PPS in England where the rate of HCAI in ICU is 23.5%, three times higher than the overall prevalence of HCAI in the general hospital population [7, 8]. The rate of invasive infections by VRE notified by Ireland to EARS-net showed an increase in the rate of vancomycin resistance in *E. faecium* isolates from 37.1% in 2006 to 46.3% in 2014. Ireland has the highest rate of VRE among invasive enterococcal isolates reported to EARS-net and is the only country where the rate of VRE exceeds 25% [11].

According to surveillance data from EARS-net, ESBL production in *E. coli* increased from 2.5% in 2006 to 10.1% in 2014 and in *K. pneumoniae* from 8.6% to 11.3% over the same period [11]. *E. coli* are the most common invasive isolates reported to EARS-net by a factor of two. Increasing resistance among *E. coli*, causing infections in both the hospital and community is therefore concerning.

The prevalence of ESBL-E and VRE carriage in healthcare environments including ICU is less well documented than the prevalence of infection. However, in many instances, infection is preceded by colonisation and therefore, in addition to the adjacent physical environment, patient carriage with ESBL-E/VRE represents an often overlooked infection risk [52, 79, 85, 113]. An epidemiology study conducted recently on isolates from patients in this study hospital (Beaumont Hospital, Dublin) and its catchment area, identified patients admitted from nursing homes as a potential reservoir for ESBL producing *E. coli* [115]. Furthermore, a recent study investigating the rate of colonisation with a variety of MDRO in LTCFs in the west of
Ireland found that 56% of residents were colonised with ESBL producing *E. coli*, 8% with ESBL-producing *K. pneumoniae* and 3% with VRE.[22]

Comparison of two prevalence studies of ESBL-E colonisation among healthy adults attending a community health clinic in France showed that there had been a 10-fold increase in the faecal carriage of ESBL-E from 0.6% in 2006 to 6% in 2011. The studied subjects had none of the previously identified risk factors for ESBL-E and the isolates recovered belonged to diverse STs[116]. A similar study conducted in Spain a decade earlier found that the rate of ESBL-E carriage among inpatient and outpatient populations had increased from 0.3% and 0.7% in 1991 to 11.8% and 5.5% respectively, in 2003 and faecal samples from a group of 108 healthy volunteers also tested in 2003 were positive for ESBL *E.coli* in 3.7% of the samples [117]. Further afield, a study from a paediatric unit in Madagascar found that 21% of 244 infants admitted to the paediatric unit had ESBL-E in their faecal samples, which increased to 57% carriage on discharge [118]. A recent Irish study found ESBL-E carriage rates of 14.3 % among patients in ICU [119]. Overall, these studies highlight the increasing prevalence of these organisms over the last decade in the community as well as in hospitalised patients outside of outbreaks. Particularly in vulnerable patients, such as those in ICUs, the early detection of ESBL-E carriage may help to identify possible cross infection risks.

For VRE, current national guidelines on screening for MDROs other than MRSA recommend passive screening and active surveillance culture (ASC). Passive screening involves testing all enterococci isolated from clinical samples for glycopeptide resistance where susceptibilities are indicated. ASC is recommended for all admissions to high risk areas which include admission to ICUs, HDUs, haematology/oncology units, transplantation units. As previously discussed, this occurs to varying degrees in critical care units in Ireland, unlike for MRSA screening, where there is 100% reported ASC undertaken in all critical care units [59]. In additions, ASC for VRE is recommended for patients with a history of VRE colonisation or infection on re-admission to hospital, patients transferred from another hospital in Ireland or from a hospital abroad. Also any “at risk” patients
who have been contacts of known VRE patients during an outbreak of VRE are also screened [50]. For ESBL-Es, current recommendations recommend testing all Enterobacteriaceae isolated from clinical samples for cephalosporin resistance as per EUCAST guidance with additional phenotypic testing for ESBL-Es as appropriate on samples, where susceptibility testing is indicated [93]. Active surveillance testing for ESBL-E has recently been recommended, as for VRE, for patients in ICUs, HDUs, haematology/oncology units, transplantation units and transfers from hospitals abroad and at home (where possible and in consultation with referring infection prevention and control team (IPCT), and also from LTCFs [66]. This is in response to growing reports of multi-drug resistant *Klebsiella pneumoniae* being notified and the results of the recently published study on nursing home colonisation with MDRO [11, 22]. The benefit of screening for ESBL-E has yet to be determined [120].

In the general wards of Beaumont Hospital, VRE and ESBL-E are identified from patient clinical specimens (e.g. wound swabs, urine, blood culture) taken on suspicion of infection. However active surveillance cultures are carried out in ‘high risk’ areas only, as described above. In the general ICU in BH, patients are routinely screened on admission for carriage of MRSA (nose and groin swab), VRE and CRE (rectal swab) and weekly thereafter as per national guidelines. If positive, the patients are isolated in a single room, where possible, and contact precautions are put in place. Where screening reveals that a patient is VRE-positive but previously unknown to be positive, this is referred to as a new case, for infection prevention and control purposes. Screening for ESBL-E by rectal swab or stool sampling is not currently part of the routine admission screening policy in the ICU in BH. ESBL-E positive patients are identified based on the recovery of these isolates from clinical specimens taken for clinical suspicion of infection with new identifications referred to as new cases. The numbers of new cases of VRE and ESBL-E are discussed at the ICPT’s weekly meeting. Hospital-wide surveillance data is also available for VRE and ESBL-E. In the case of VRE, surveillance includes carriage and infecting isolates (e.g. from rectal screens and clinical specimens such as wound swabs, blood cultures,
catheter and mid stream urines) whereas for ESBL-E, surveillance data includes only isolates from clinical specimens taken on clinical grounds.

In this Chapter, an investigation of VRE/ESBL-E colonisation of patients in the ICU, conducted over the seven periods in which environmental screening took place as outlined in Chapter 2 Section 2.2 is described. In addition, wider hospital surveillance data in relation to VRE/ESBL is included to determine the overall burden of these MDROs in the hospital for the years in which the ICU study was conducted and for the year prior to commencement of the study. The contribution of targeted screening or ASC to the identification of potential patient reservoirs for VRE/ESBL-E in this environment was also investigated.

4.2. Results

4.2.1. ICU patient characteristics and demographics

Throughout the screening periods of this study, 157 patients were available from whom screening specimens were taken and environmental samples were taken from their surroundings, while they were in ICU (as discussed in Chapter 3). Demographic and relevant clinical information collected for all ICU patients participating in the study during the seven study periods is summarised in Table 4.1. Of the 157 patients, 95 (60.5%), were male. The mean age was 61 years (range 16-87 years). Eighty-three (53%) were surgical patients. The majority were mechanically ventilated, 104/157 (66.2%) and 121/157 (77%) had a central venous catheter (CVC) or urethral catheter (UC) or both. The mean acute physiology and chronic health evaluation (APACHE) II score was 21 (range 0-40). The APACHE II score is a “severity of disease” classification system, scored from 0-71, for patients admitted to ICU and it is applied within 24 hours of ICU admission[121]. The majority, 134 patients (85.3%), had received antibiotics during their stay in the ICU. The most commonly used antibiotic was piperacillin-tazobactam accounting for 46% of prescriptions. Meropenem accounted for 17% of prescriptions. The majority of patients (89/134, 67%) were treated with two or more agents and 34% received vancomycin or a third generation cephalosporin. Vancomycin was prescribed to
21% (28/134) patients. Antibiotic exposure history of the patients while in ICU is shown in Figure 4.1.

Table 4.1 Characteristics and demographics of the patients in the ICU included in the study

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Patient population n=157</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender, n(%)</td>
<td>95 (60.5)</td>
</tr>
<tr>
<td>Mean age in years (range)</td>
<td>61 (16-87)</td>
</tr>
<tr>
<td>Mean length of stay in days (range)</td>
<td>8 (1-48)</td>
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<tr>
<td>Mean APACHE II score(^a) (range)</td>
<td>21 (0-40)</td>
</tr>
<tr>
<td>Exposure to antibiotics in ICU(^b), n (%)</td>
<td>134 (85)</td>
</tr>
<tr>
<td>MDRO(^c) colonised, n (%)</td>
<td>39 (24.8)</td>
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<td>Specialty, n (%)</td>
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<td>Medicine</td>
<td>74 (47)</td>
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<tr>
<td>Surgery</td>
<td>83 (53)</td>
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<td>Co-morbidities n (%)</td>
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<td>Immunosuppression</td>
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</tr>
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<td>Abdominal surgery</td>
<td>16 (18)</td>
</tr>
<tr>
<td>Devices, n (%)</td>
<td></td>
</tr>
<tr>
<td>Central venous catheter</td>
<td>121 (77)</td>
</tr>
<tr>
<td>Urinary catheter</td>
<td>121 (77)</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>104 (66)</td>
</tr>
<tr>
<td>Surgical drain</td>
<td>33 (21)</td>
</tr>
</tbody>
</table>

\(^a\)APACHE II Acute Physiology and Chronic health score- and ICU disease severity score
\(^b\) the antibiotics received by patients in the study are shown in Figure 4.2 and included piperacillin/tazobactam, co-amoxiclav, vancomycin, clarithromycin etc.
\(^c\)MDRO = multi drug resistant organisms
Figure 4.1 Antibiotics received by patients in ICU. The types of antibiotics received by patients in the ICU during their ICU stay were recorded. For each antibiotic/antibiotic group, data are expressed as a percentage of patients in ICU during the study periods that received at least one dose of the antibiotic (n=134). The ‘other’ category included co-trimoxazole, and antiviral and antifungal agents. CXM=cefuroxime, CTX=cefotaxime, CAZ=ceftazidime

4.2.2. Characteristics of ICU Patients colonised with VRE/ESBL-E

The total number of patient colonised with the target MDROs over the course of the study was 39(24.8%). As six patients were co-colonised with VRE and ESBL-E, the numbers colonised with each organism totalled 45. A summary of the patient demographics and other relevant characteristics by MDRO type (VRE and ESBL-E) is shown in Table 4.2. The correlation between the recovery of a targeted MDRO and age-group among the patient cohort is illustrated in Figure 4.2.

Thirty patients were colonised with VRE. The mean age of the patients colonised with VRE was 64 (range 34-85). Mean APACHEII score was 24(range 12-38). The division between medical and surgical patients was equal at 15 each. Eighteen patients (60%) were admitted from another ward in the hospital, 25/30 (83%) had a
CVC and/or urinary catheter (UC) or both in situ and 12/30 (40%) had abdominal surgery. All patient colonised with VRE had been prescribed an antibiotic. Of these, six (20%) received vancomycin as part of their antibiotic treatment in ICU. Of the patients included, two patients were treated for invasive VRE infection, one for a catheter related BSI and the other for a VRE surgical site infection. They both had initially been treated with vancomycin.

Fifteen patients in the ICU had ESBL-E isolated either from the screening samples, or from previous clinical samples, prior to admission to ICU. The ESBL-E identified from patients screens included predominantly \textit{E.coli} 9/15 (60\%), \textit{Enterobacter cloacae} complex 4/15 (27\%), \textit{Klebsiella} spp. 2/15 (13\%) (\textit{Klebsiella pneumoniae} (1) \textit{oxytoca} (1)).

The mean age of the patients colonised with ESBL-E was 66y (range 21-83). The mean APACHEII score was 18 (range 7-38), nine were on mechanical ventilation (60\%), 11/15 (73\%) had a CVC, 12/15 (80\%) had a UC in situ and three had surgical drains present one of which was a chest drain. One of the patients was on immunosuppressant therapy post-transplant and one had a history of diabetes mellitus. Thirteen of the 15 (87\%) received antibiotics while in ICU. All but one of those receiving antibiotics were on an agent which had activity against the ESBL-E identified. This patient received cefuroxime and metronidazole prophylaxis for a renal transplant, both of which have no activity against ESBL-E. There were no patients with an ESBL-E BSI over the course of the study. Six of the 15 patients, (37.5\%) were admitted directly to the ICU from the community.
Table 4.2. Characteristics and demographics of the patients colonised with the target MDRO in the ICU included in the study

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>VRE positive n=30</th>
<th>ESBL-E positive n=15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender, n(%)</td>
<td>16 (53)</td>
<td>11 (73)</td>
</tr>
<tr>
<td>Mean age in years (range)</td>
<td>64 (34-85)</td>
<td>66 (21-83)</td>
</tr>
<tr>
<td>Mean length of stay in days (range)</td>
<td>11 (1-42)</td>
<td>8 (1-26)</td>
</tr>
<tr>
<td>Mean APACHE II score (range)</td>
<td>24 (12-38)</td>
<td>18 (7-38)</td>
</tr>
<tr>
<td>Exposure to antibiotics in ICU, n (%)</td>
<td>30 (100)</td>
<td>13 (87%)</td>
</tr>
<tr>
<td>Vancomycin exposure</td>
<td>6 (20)</td>
<td></td>
</tr>
<tr>
<td>Specialty, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medicine</td>
<td>15 (50)</td>
<td>8 (53)</td>
</tr>
<tr>
<td>Surgery</td>
<td>15 (50)</td>
<td>7 (47)</td>
</tr>
<tr>
<td>Co-morbidities n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>11 (37)</td>
<td>3 (20)</td>
</tr>
<tr>
<td>Malignancy</td>
<td>6 (20)</td>
<td>0</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>4 (13)</td>
<td>0</td>
</tr>
<tr>
<td>Steroids</td>
<td>7 (23)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>12 (40)</td>
<td>3 (20)</td>
</tr>
<tr>
<td>Abdominal surgery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Devices, n (%)</td>
<td>25 (83)</td>
<td>11 (73)</td>
</tr>
<tr>
<td>Central venous catheter</td>
<td>25 (83)</td>
<td>12 (80)</td>
</tr>
<tr>
<td>Urinary catheter</td>
<td>18 (60)</td>
<td>9 (60)</td>
</tr>
<tr>
<td>Mechanical ventilation</td>
<td>7 (23)</td>
<td>3 (20)</td>
</tr>
<tr>
<td>Surgical drain</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.2. Correlation between age ranges of ICU patients and recovery of MDRO. The dark blue bar represents the age distribution of the patients who were colonised with the target MDRO, and the light blue bar, the percentage of patients in each range who were colonised with the target MDROs. The percentage of each age group colonised with MDRO are represented by the line plot.

4.2.3. The impact of targeted screening on VRE/ESBL-E recovery from ICU patients

The positive effect of targeted screening or ASC in identifying previously unknown MDRO positive patients is summarised in Figure 4.3. The number of patients in the ICU with VRE over the course of the seven time periods of the study was 30/157, (19.1%). Examination of VRE screening results on patient admission and during their hospital stay revealed 19/30 (63.3 %) were VRE-colonised but previously unknown and were recorded by the IPCT as new cases of VRE. Therefore, based on this study alone, VRE-ASC resulted in the identification of an additional 19/157 (11.6 %) of ICU patients as VRE-positive compared to only 11/157 (7%) in the absence of ACS. Of these 19 new cases, 14 were VRE positive on admission screening,i.e within 48h of admission to ICU, and five patients acquired VRE in the ICU (admission screen negative, subsequent screen positive).
Screening of rectal swabs over the seven sampling periods revealed that 15/157 (9.6%) ICU patients were ESBL-E-positive. Six of 157 patients (3.8%) in ICU were co-colonised with both VRE and ESBL-E. Nine of the 15 patients (60%) identified as ESBL-E positive during their ICU stay, had no prior history of isolation of ESBL-E. Therefore targeted screening for ESBL-E identified an additional 9/157 (5.7%) patients as positive compared to 6/157 (3.8%) in the absence of targeted screening. Based on the timing of swabs taken for screening, 2/9 of previously unknown ESBL-positive patients (22%) acquired ESBL-E in the ICU. The numbers of patients in the ICU, previously unknown to carry VRE or ESBL-E are shown in Figure 4.3. These patients were identified from rectal screening swabs (taken initially for the purpose of VRE screening but used also for ESBL-E recovery in this study) taken either on ICU admission or weekly thereafter.

![Figure 4.3 VRE and ESBL-E positive rates in the ICU over seven study periods.](image)

Data shown represents the % of patients in ICU over seven study periods that were VRE or ESBL positive and include those that were known positives based on investigation of clinical specimens and those that were identified based on targeted screening (previously unknown).
4.2.4. Patterns of VRE/ESBL-E detection among ICU patients over time

The burden of VRE/ESBL-E in the ICU, based on isolates recovered from ICU patients over the time-periods of the study is illustrated in Figure 4.4. The prevalence rate of the targeted MDROs varied from 13 % to 33 % among the seven time periods investigated but no specific trends or patterns in rates recovered over the study periods, for example, due to seasonal factors, were apparent.

Figure 4.4. The burden of targeted MDROs (VRE and ESBL-E) in the ICU by time period. The number of patients in the ICU included in the study is represented in blue and the number with MDRO detected from screening samples over each sampling period is represented in red. The percentage of patients positive for MDRO in the ICU is marked by the percentage for each time period. The total number of patients across sampling periods (160) includes three patients admitted in more than one time period. The total number of MDRO positive patients (46) includes those co-colonised with both organism (6) and one VRE positive patient that was counted in 2 time periods, as they were re-admitted a year later.

4.2.5. Hospital-wide surveillance of VRE/ESBL-E in patients in Beaumont Hospital.

For the years 2012 to 2014, the number of VRE screens taken in the hospital (two ICUs, HDU haematology and oncology patients and known VRE positive patients) increased from 1508 to 1989 but, as shown in Fig 4.5., the rate of VRE positive isolates remained at approximately 9% from 137/1508 (9.1%) to 179/1989 (9.0%).
While no ASC took place for ESBL-E, based on positive clinical specimens, there was a statistically significant increase in the annual incidence of ESBL-E hospital-wide, from 0.46 per 1000 bed days used (BDU) in 2011 to 0.67 per 1000 BDU in 2014 (p<0.05), representing an increase of 44% over the 4 years. The number of ESBL-E positive isolates (where only the first isolate of ESBL-E per patient was included) increased from 102 to 151 during this time (Personal communication from Mairead Skally) (Figure 4.6). The numbers of positive (VRE and ESBL-E) isolates identified from clinical specimens (VRE and ESBL-E) and rectal swabs (VRE only) within the seven sampling periods only, which would include those from ICU patients, along with the numbers in ICU alone are illustrated in Figure 4.7. This shows the burden of these organisms in the hospital compared to the ICU in particular. For VRE, no obvious relationship between the two populations was apparent. However, in relation to the number of ESBL-E recovered from ICU patients, similar trends in ESBL-E numbers recovered hospital-wide during the study periods were observed but these trends are based on identification of ESBL-E from clinical specimens alone and not from active surveillance.

Figure 4.5. The total number of VRE screens taken in Beaumont Hospital from 2012-2014 and the number of VRE-positive screens. The total number of screens taken in BH (including those in ICU) with the positive screens highlighted in the darker shade. The % of screens positive for VRE in each year was 9%, 8.5% and 9% for years 2012, 2013, 2014, respectively. The total number of positive VRE screens over the three years was 483. Data from BH surveillance by personal communication from Mairead Skally.
Figure 4.6. The number of ESBL-E isolated from clinical specimens from patients in Beaumont Hospital over 2011-2014 inclusive. The number of patients positive for ESBL-E over the years 2011 to 2014 showing an increase in the numbers identified in the hospital patients from the year preceding the study and including the years in which the study took place. Data from infection prevention and control surveillance, personal communication Mairead Skally.

Figure 4.7. Comparison of the numbers of VRE and ESBL positive patients in the ICU to the hospital data during each of the 7 three-week sampling periods of the study. The solid lines represent the number of patients in the ICU from whom ESBL-E (red line) and VRE (blue line) were isolated over each sampling period. The red dotted line represents the number of patients where ESBL-E was isolated from clinical samples across the hospital (including ICU). The blue dotted line represents the number of patients where VRE was isolated from screening and clinical samples from across the hospital (including ICU).
4.2.6. The location of the patients with MDRO within the ICU.

As shown in Figure 4.8., the patients colonised with MDRO were distributed throughout the beds in the ICU. Of those colonised with VRE, 75% were in an isolation room at the time of sampling, and remained in an isolation room. Of those found to be ESBL-E colonised, 53% were in one of isolation rooms 7-11. Six were co-colonised with VRE and two were isolated for other reasons, namely diarrhoea. Of the ESBL-E positive patients, 47% were distributed throughout the open plan area of the ICU.
Figure 4.8. The distribution of patients with MDRO in each bed in the ICU. The total number of patients colonised with VRE or ESBL-E in the ICU per bed space (a). One VRE patient was moved from bed 4 to bed 7 so is counted twice in the numbers. The schematic plan of the ICU is included for reference (b) of location of the isolation rooms highlighted by the black boxes.
4.2.7. The effect of chlorhexidine on the number of patients colonised with MDRO.

Chlorhexidine wipes, containing 2% chlorhexidine gluconate in a non-alcohol and non-alkaline base, were introduced into ICU for washing patients in October 2013. The numbers of colonised patients in the unit before chlorhexidine introduction was 14/64 (22%) of the patients studied and increased to 26/96 (27%) of the patients included in the study as shown in figure 4.9. Therefore, in this study, the practice of bathing patients using chlorhexidine wipes did not appear to significantly change the patient colonisation rates with the target MDROs. p=0.45 with 95%CI.

![Figure 4.9. The effect of daily chlorhexidine bathing on the prevalence of patients colonised with MDRO in the ICU.](image)

Comparing the number of patients colonised with an MDRO in the unit during the sampling periods before and after the introduction of chlorhexidine wipes for bathing patients.
4.3 Discussion.

There are a wide range of approaches to the infection prevention and control interventions in relation to VRE and ESBL-E both within countries and internationally [60, 61, 69]. In Ireland, national guidelines recommend the screening of at risk patients, which would include patients in ICU, for VRE and now recently ESBL-E [50, 66]. A recent survey of infection prevention and control practices, including screening practices, in Irish critical care units show that 61% of units screened for VRE and 21% screened for ESBL-E [59]. The prevalence of ESBL-E is increasing throughout the world and yet there is no clear guidance as to the best approach to screening high risk patients for enteric MDROs like ESBL-E [120].

There were 157 patients included in the study, and 25.7% were colonised with one or more of the target MDROs. Of those with VRE, 11/30 (37%) were known to be colonised on admission and were isolated. The majority of patients with resistant flora identified while in the ICU were admitted with the resistant isolate. On average, 75% of patients, (7/9 for ESBL-E and 14/19 for VRE), were positive for MDRO on admission screens, i.e. within 48h of admission. Interestingly, the overall rate of colonisation of patients with VRE of 19.6% is higher than the overall rate of 9% positivity in all patients screened in the hospital. This is keeping with previous studies that the rate of resistant organisms is higher in ICUs [6, 100]. Vancomycin was prescribed to 6/30, (20%) of the patients colonised with VRE, and accounted for 21% (28/134) of the antibiotic prescriptions in the ICU patients included in the study. Although from reports of antimicrobial consumption in Ireland, this centre trends around the national mean and is on the lower side for the hospital type range, it is difficult to ascertain whether this is comparable to other centres or indeed countries in relation to glycopeptide prescribing. The carriage of and infection with VRE is impacted on by antibiotic consumption [39]. However, in Ireland, antimicrobial consumption data report vancomycin consumption, along with metronidazole and nitrofurantoin. They all accounted for 10% of hospital prescriptions in 2013[122]. For this group, European antimicrobial consumption data catagorise vancomycin/glycopeptides as ‘other antibiotics J01x’ grouping them
together with a number of other antibiotics, therefore comparisons on consumption data for particular antibiotics are not possible at present [123]. Whether this has a bearing on the high rates of VRE in Ireland, compared to our European neighbours requires better breakdown of antimicrobial classes in the surveillance data.

In this study 9.8% of the ICU patients included were colonised with ESBL-E. The majority of these (53%) were *E.coli*. This is lower than the rate reported in a recently published Irish study which reported the rate of colonisation in their ICU patients to be 14.3% [119]. Other studies from Europe report variable results of ESBL-E carriage rates from 5% to 15% among admissions to intensive care settings [124, 125]. The rates of ESBL-E in both the community and hospitals are increasing, as illustrated locally by the increasing number of ESBL-E isolates from patients in this hospital from 2011 to 2014 (Personal communication from Mairead Skally, IPCT, BH) and reports of increasing carriage among people in community settings, returning travellers and nursing home residents, where the prevalence reported varies from 6%-55% [21, 22, 27, 116].

The importance of early detection lies in the appropriate selection of empiric antimicrobials in the setting of sepsis. The increasing prevalence of ESBL-E, consequently drives the use of carbapenems as first line therapy against these organisms, thus leading to the emergence of carbapenem resistance [19]. A French study published recently, found 15% of their ICU admissions were colonised with an ESBL-E, and 12% of the patients were admitted from the community. Despite the high carriage rate, 1.4% of infections on admission to the ICU were due to ESBL-E. This increased to 10% among the ICU acquired infections [116]. Recent EARS-net data show increasing ESBL production in invasive isolates of *E.coli* and *K. pneumoniae* from Ireland increasing from 2.5% and 8.6% in 2006 to 10.5% and 11.3%, respectively in 2014 [11]. The HPSC started a surveillance programme in 2014 in light of the increased reporting of multi-drug resistant *K. pneumoniae* being reported from hospitals throughout the country [66].
Only six of the 15 patients who were positive for ESBL-E, had a prior history of ESBL-E, and if ASC were in place routinely this would have detected an additional nine patients, (5.6%). All patients identified with ESBL-E colonisation, who required antibiotics, were on appropriate therapy. One patient, who had hospital acquired pneumonia, colonised with ESBL-E, had been in ICU 9 days prior to ESBL screen and may have benefited from an earlier switch to meropenem. There is little cephalosporin use in the ICU in BH, representing only 14% of prescriptions, mostly for surgical prophylaxis. Piperacillin-tazobactam is the most commonly used antibiotic in this unit, accounting for 43% of prescriptions, and most ESBL-E isolates from this hospital are susceptible to piperacillin-tazobactam (personal communication from Mary O’Connor, Clinical Microbiology Laboratory, BH). However, this requires ongoing surveillance, but the current empiric choice of antimicrobials in this setting is usually appropriate.

The burden of ESBL-E among the patients in the ICU was relatively low, however the patients were placed throughout the ICU as illustrated in figure 4.8. This may facilitate the spread of these organisms to the environment and present a risk of cross transmission to other patients [100]. Chlorhexidine wipes were introduced in the ICU over the course of this study as a measure to reduce the rate of MDRO colonisation in the ICU. Observations of MDRO colonisation among the patients studied, however showed a small increase, rather than a decrease in the percentage of patients colonised. This is at odds with the published literature, which in many instances shows a reduction of MDRO acquisition and subsequent reduction of BSI [72]. However, the numbers in this study are likely too low to show benefit with regard to acquisition of MDRO, but hospital ICU data collected on all patients since the introduction of the measure failed to show a benefit as of yet (personal communication IPCT BH).

This study identified 9.8% patients admitted to the ICU are colonised with ESBL-E, and the distribution over time, matches the number of these infections in the hospital. The benefit of ASC for ESBL-E is unclear. The question arises as to whether
such a policy may increase the carbepenem prescriptions, and subsequently drive carbepenem resistance. The proportion of colonised patients who develop infections is yet to be determined and the infrastructure to act on the results is a challenge [120]. The ICU had an average occupancy rate of 110% (range 108-138%) over the 3 years in which this study took place (personal communication, Johanna Mulvihill, ICU audit team BH). Our colleagues elsewhere have started screening based on the findings of an ESBL-E positive rate of 14.3% in their ICU [119]. Surveillance data shows that for the moment, the prevalence of these organisms is increasing and ASC has been shown to reduce BSI due to MDRO over time [69]. Therefore starting ASC before BSI with ESBL-E becomes a larger problem, may be the way forward, though further studies are needed to assess associated laboratory impact and patient benefit.
Chapter 5

Analysis of clinical and molecular relationships between vancomycin resistant *E. faecium* isolates recovered from ICU patients and their environment and investigation of the clinical relationships between ESBL-E isolates.
5.1. Introduction

Epidemiological studies are important in tracking the transmission of microorganisms, particularly those associated with HCAI, between hospitalized patients and the physical environment in which they are placed. Hospital surveillance maps infections within a ward or unit and may record and track colonization of patients with specific microorganisms. The surveillance data collected may then be used as a basis for the instigation of actions aimed at reducing the burden of colonization or infection. Audits may also be undertaken to further investigate possible clinical transmission events by using molecular techniques to type bacteria. This allows them to be grouped based on their genetic characteristics and allows more reliable inferences to be made regarding the spread of particular clones in the healthcare setting. The molecular typing of bacteria has become common practice in the surveillance and monitoring of pathogens especially in the setting of an outbreak [126].

In the busy environment of hospital wards, the linking of patient acquisition of MDRO to the hospital environment is challenging. The use of clinical epidemiology observations alone has many limitations and confounding factors. These include frequency and quality of cleaning, staff compliance with hand hygiene, the bacterial survival on surfaces and also the possibility of MDRO like VRE arising de novo from a patient’s flora, due to antibiotic exposure or acquisition of resistant genes from commensal anaerobes [86, 127]. Molecular typing of isolates, by establishing the extent of genetic relatedness between recovered isolates, can provide additional information to either support possible clinical links or to discount them, giving a more accurate picture of the patterns of transmission that may occur.

5.1.1. Pulsed-field gel electrophoresis (PFGE)

In terms of available molecular typing methods, macro-restriction analysis by pulsed-field gel electrophoresis (PFGE) has been shown to be particularly useful in the differentiation and clustering of bacteria based on their genetic relatedness. It is
still considered the ‘gold standard’ for enterococcal strain typing, especially in the setting of outbreaks [45]. The standard for enterococcal typing analysis accepts the following parameters: three band differences indicate related strains, six or less band difference between strains suggests that the strains may be related, and greater than six band differences represent unrelated strains [128]. The enterococcal genome, like the genomes of many other pathogens, has been described as flexible, and prone to a significant level of genetic recombinations [45, 127]. There appears to be inconsistency in relation to the stability of DNA banding patterns seen on the macro-restriction of enterococci. For example Bonten et al reported little genetic variation within individual patients, however, Morrison et al found that there were DNA polymorphisms within a single strain of vancomycin resistant \textit{E. faecium}, with up to seven band difference found in isolates belonging to a single strain [46, 129]. PFGE for enterococci is also technically difficult, time consuming and relatively expensive. It is also without internationally agreed standardized running times or band pattern difference analysis, making inter laboratory comparisons challenging [45].

\textbf{5.1.2 Multi locus sequence typing (MLST)}

Multi locus sequence typing (MLST) typing is less discriminating, but more suitable to population-based analysis of isolates over longer time periods. It was developed in the late 1990s and is based on the sequencing of seven housekeeping genes. The sequences can be queried on publicly available online databases to determine the genetic relatedness of strains. Epidemiological studies of \textit{E. faecium} outbreaks using MLST analysis have indicated that its population structure involves a number of predominant ST types (e.g. ST17, ST18, and ST78) associated with hospital infections which are designated as clonal complex (CC)17 and have adapted to the hospital environment [37, 41, 130]. However Bayesian analysis of Population Structure (BAPS) of the MLST data on \textit{E. faecium} has found that further subdivisions exist within the established hospital STs, thus questioning the single descendant clonal lineages of hospital strains. Therefore BAPS analysis suggests that the majority of clinical isolates currently circulating belong to three different lineages.
These clonal lineages have helped guide the selection of strains for WGS projects [131].

5.1.3 Whole Genome Sequencing (WGS)

WGS could be transformative for the practice of clinical microbiology by encompassing the steps of identification, resistance determinants characterisation and strain typing all in one method [132]. This has tremendous potential for real time investigation of hospital outbreaks. Enterococcal genomes have evolved as a streamlined genome from the highly competitive environment of the GI tract [133]. The genome of the initial hospital strain of *E. faecalis* V 583 was first sequenced in 2003. It was the first vancomycin-resistant *E. faecalis* strain seen in the USA. Analysis identified that 26% of the genome was comprised of mobile elements, including acquired pathogenicity islands, transposons, phages and plasmids [134]. When it was compared to a susceptible, commensal strain from oral cavities OG1FR, the genomes had clustered regularly interspaced short palindromic repeat (CRISPR) loci which were lacking in strain V583. CRISPR loci are derived from extrachromosomal DNA and provide bacteria with a sequence specific acquired defense against plasmids and phages. Many studies, as mentioned above have shown that most hospital infections are caused by hospital endemic, clonal lineages that lack CRISPR loci. There is a strong correlation between the absence of a CRISPR *cas* locus and the presence of acquired antibiotic resistance in both *E. faecalis* and *E. faecium* [133]

The first fully sequenced genome of *E. faecium* was published in 2012, from an Australian group, characterising Aus004, a vancomycin resistant strain from a patient with a BSI [135]. Shortly afterwards, another genome sequence was completed, TX16, isolated from a patient who had endocarditis in 1992 [136]. There are now 31 publicly available *E. faecium* genome sequences and a number of draft genomes sequenced by Sanger and Illumina sequencing. The draft assemblies are a consequence of the large number of repeats within protein coding sequences and
other repetitive elements, which represent a challenging bioinformatics problem [131]. These are published online from publicly available databases, such as the US-based NCBI Genomes Database and the European Nucleotide Archive (ENA), where there are currently 163 draft genome sequences of \textit{E. faecium}. WGS of 16 \textit{E. faecalis} strains and eight \textit{E. faecium} strains, in combination with MLST typing have divided the \textit{E. faecium} strains into two distinct Clades (defined as a group that contains a common ancestor and all its descendants). Clade A consists of infection derived strains and Clade B consists of human commensal strains. However, there are as yet only limited data, and the majority of strains sequenced so far are from either European or American sources.

\textbf{5.1.4 Next Generation Sequencing (NGS)}

NGS, or targeted gene sequencing, is a high throughput method of generating millions of reads at a low cost base, by targeting specific genes sequences, to detect species, resistance determinants and virulence genes simultaneously, rather than sequencing the whole genome. There are a number of methods including pyrosequencing, Dye termination synthesis, semiconductor and direct detection [137].

The Pathogenica HAI BioDetection ™ system discussed elsewhere uses semiconductor chemistry to translate chemical information onto a semiconductor chip. This commercial assay is based on 300 probes that capture and amplify short DNA sequences, selected by the company based on WGS analysis. The technology simultaneously sequences all of the probe targets from any organism in a sample, reducing the hands-on time needed to identify and type bacterial species, while detecting resistance genes at the same time. The assay can detect numerous organisms implicated in HCAI including \textit{Acinetobacter baumannii}, \textit{A. pittii}, \textit{A. calcoaceticus}, \textit{Clostridium difficile}, \textit{E. coli}, \textit{E. faecalis}, \textit{E. faecium}, \textit{Enterobacter aerogenes}, \textit{E. cloacae}, \textit{K. oxytoca}, \textit{K. pneumoniae}, \textit{Proteus mirabilis}, \textit{Pseudomonas aeruginosa}, \textit{Staphylococcus aureus}, \textit{S.epidermidis}, \textit{S.saprophyticus}, and \textit{S. haemolyticus} [138]. This technology could be a potentially powerful epidemiological
tool combining diagnostic with typing data to track pathogens in patients and the environment in real time.

In this chapter, possible transmission events in an ICU, outside of outbreaks, were investigated based on clinical epidemiology (e.g. temporal and spatial links between patients and their environment) and the use of molecular typing. A selection of VRE isolates (*E. faecium*) recovered from patients in the ICU and their environment (as described in Chapters 3 and 4) were subjected to PFGE analysis and NGS analysis to investigate these tentative clinical epidemiological links between isolates.

5.2. Results

5.2.1. Study setting and purpose

As described in Chapters 3 and 4, VRE and ESBL-E were recovered from patients in the ICU and from the high touch surfaces in the patients’ environment. The numbers of ESBL-E recovered were too small to warrant epidemiological analysis supported by molecular typing and therefore only limited clinical epidemiological associations, both temporal and spatial were investigated for ESBL-E. However, molecular typing (using PFGE analysis and targeted NGS) was carried out on a selection of patient and environmental VRE isolates (*E. faecium* only) to determine the extent of their genetic relatedness. VRE*fm* isolates were selected as they were the predominant species isolated in the initial sampling period and among the isolates characterised by NGS. These data were investigated in association with clinical epidemiological links between isolates in relation to time and location of recovery of the isolates in an attempt to track the spread of VRE over discrete time periods and outside of outbreak situations.

5.2.2. Clinical epidemiological associations between patient and environmental MDRO in an ICU

From the 157 patients included in the study, a total of 189 unique patient and bed number associations were identified and investigated. On the majority of sampling
occasions (56%) no targeted bacteria were identified from the patient or from their adjacent environment. There were 15/189 (8%) sampling occasions where both the patient and environment were positive for VRE or ESBL-E. A number of possible MDRO transmission events were identified on investigation of the patterns of recovery of the targeted MDROs with respect to date and location of the MDRO-positive patient/environmental site. For example, during the early part of the first sampling period, there was only one patient in the ICU colonised with VRE. Their environment was also contaminated with VRE, as was the environment of a number of bed spaces in the ICU at that time, illustrating potential shedding and dispersal into the environment. At least four examples of possible transmission of VRE from a patient to their environment were identified based on the recovery of VRE from an environmental site which was previously negative but became positive after a VRE-positive patient occupied the bed space. Two examples of possible transmission of VRE from the environment to a patient in the ICU were observed over the course of the study based on sequential recovery of VRE from the ICU bed-space accompanied by a change in the occupying patient status from VRE-negative to VRE-positive. In the case of one of these ‘possible environment to patient transmission’ events, the patient became VRE-positive nine days after admission and placement in an environment positive for VRE. The other, tenuous transmission event involved a patient acquiring VRE having spent 48 hours in a room in which the environment had sampled VRE-positive. However the acquisition arose after the patient left the ICU. Interestingly, there was a relatively high number of cases 58/189 (31%) where either the patient or their environment was positive for VRE but with no evidence of transmission in either direction (i.e. the environment remained positive and the patient remained negative or vice versa). Of the patients who were positive for ESBL-E, only 2 patients had an ESBL-E isolated from an environmental sample at the same time. In one of these however, the patient was colonised with an ESBL *E.coli* and the environment was positive with an ESBL *K.pneumoniae*. Where the patient was colonised with both ESBL-E and VRE, of 105 environmental sites sampled, two sites (1.9%) were positive for an ESBL-E and 16 (15%) were positive for VRE.
5.2.3. Analysis of the genetic relatedness of VRE isolates recovered from patients and their environment.

Of the 138 VRE isolates recovered in total over the seven sampling periods of this study, comprising *E. faecium*, *E. faecalis*, *E. gallinarum* and an isolate of *Paenibacillus* spp (identified as non-VRE by Sanger sequencing by Pathogenica inc), 71 VRE (*E. faecium*) isolates were typed and analysed for genetic relatedness using PFGE. Of these 71 isolates, 49 were environmental samples and 22 were patient samples, from 16 different patients. Three of the patient samples were from a single patient, representing one rectal screen and two blood culture isolates. The other patient samples comprised of one other blood culture isolate and samples from screening rectal swabs.

Visual inspections of the PFGE pattern of each isolate identified 32 different PFGE types (pulse types). These were manually assigned to PFGE types ‘I to XXXII’ Figure 5.1 summarises the diversity of PFGE types found, based on this visual analysis. Of these isolates, there were 23 unique pulse types, and clusters of 11, 10, 8, 5 and 4. The rest of the isolates showed a genetic relationship with one or two other isolates. Of the unique pulse types, six were from patient samples and the rest were from environmental samples.
Figure 5.1 Diversity of pulse types among the VRE isolates - each colour represents a different pulse type generated by PFGE numbers 1 to 32 in roman numerals. The size of the pie chart slice represents the number of isolates in each pulse type.

Analysis of the PFGE patterns using GelCompare software (GelCompare®II software version 6.5, Applied Maths) revealed a similar pattern of diversity among recovered isolates. There were 32 different PFGE types and among these there were three PFGE clusters. One comprising of eight isolates another comprising of nine isolates and a third comprising of five closely related isolates (cluster analysis based on a band tolerance of 1%) (Figure 5.2). Two of the three PFGE clusters contained environmental isolates only, whereas the third cluster contained patient and environmental isolates. The overview of the isolates also illustrated that the same patient could have different pulse types, showing diversity of strains within individuals as well as between individuals. There were also three instances where one environmental and one patient isolate were linked together. In each instance the isolates were epidemiologically independent from each other in both time, and location within the ICU.
Figure 5.2. Dendogram of a selection of vancomycin resistant isolates. Analysis of 71 E. faecium isolates by PFGE using GelCompare®II software (version 6.5, Applied Maths). The extent of variability was determined by the Dice coefficient using a 1% tolerance. The isolates include 49 environmental (green) and 22 patient (red) isolates from ICU in Beaumont Hospital, Dublin, over seven sampling periods during the 18 months from Oct 2012-June 2014. Three large clusters are identified.
PFGE cluster I

As shown in Figure 5.2 and 5.3, this cluster contains eight isolates recovered from the environment of the ICU. All were recovered within two days of each other (all during sampling period 1) and from five different bed spaces (beds spaces 2,6,7,8,9). These isolates were recovered from widely distributed locations within the environment of the ICU, and encompassed the open plan area and isolation rooms. This may suggest widespread dissemination of VRE in the ICU from patient or other sources with inadequate cleaning.

![PFGE Cluster I](image)

**Figure 5.3. Cluster I from PFGE analysis.** This cluster represents eight environmental isolates, all related in time, taken within 2 days of each other, and distributed throughout the ICU as illustrated by the bed numbers. The different colours represent different beds.

PFGE cluster II

The second cluster is illustrated in Figure 5.4 and consists of nine isolates recovered from the ICU environment. Eight isolates in this cluster were recovered on a single day during sampling period 5, from three different bed spaces (beds 4, 8, 9). The other isolate in this cluster was recovered two days later from the same bed space as three of the others (bed space 8), illustrating a degree of strain persistence in the environment of the ICU. Interestingly, all of these beds were occupied by patients colonised with VRE but of the two whose isolates were included in PFGE, their isolates did not cluster with the environmental isolates.
Figure 5.4. Cluster II from PFGE analysis. This cluster includes environmental isolates taken within three days from three bed spaces in the ICU, illustrating dispersal and persistence in the environment. There were no patient isolates associated with this cluster. The colours indicate different beds.

PFGE cluster III

The third cluster contains four patient isolates and one environmental isolate. The patient isolates include a blood culture isolate and a screen sample from a single patient. This patient was readmitted to ICU and having been initially colonised with VRE, developed a BSI 20 days following readmission. Another of the patient samples in this cluster was also a blood culture isolate from a patient who was in the ICU at the same time as the first patient, during sampling period 2, and developed a VRE BSI four weeks later. This patient’s rectal swab had a vancomycin-susceptible *E. faecium*. The third patient in this group overlapped with all the others in the ICU.

The clustering of these patient isolates in PFGE cluster III could represent cross transmission within the unit over this time period. In terms of the geographical relationships in ICU between the patients associated with cluster III, there did not appear to be a direct geographical link between patients, with isolates being recovered from distant locations in the ICU (e.g. in isolation rooms). This may illustrate indirect cross-transmission, perhaps aided by the hands of health care workers or inadequate cleaning, rather than direct patient-to-patient transmission.

There was one environmental isolate in Cluster III. This was independent in time and place from the other isolates in this cluster, having been isolated a year after the other isolates.
**Figure 5.5. Cluster III from PFGE analysis.** This represents a cluster of VRE isolates from both patient and environmental sources related in time in the ICU with the exception of isolate H57. The red colour represents patient isolates. The green colour represents the environment. Patient BC represents a blood culture isolate as opposed to a rectal swab for the screens.

### 5.2.4 Targeted gene sequencing analysis of isolates.

Pathogenica, Ltd., as mentioned in chapter 2, section 2.12.1 and above have developed a NGS technique that targets a selection of short DNA regions and is able to detect the presence of several different pathogens and resistance genes simultaneously, while only sequencing a select number of kilobases of genome. They also have developed software which types the strains, providing phylogenetic trees for sets of isolates. The target pathogens detected by their technology are among the common causes of HCAI including enterococci, *E.coli*, *S.aureus*, *A.baumanii* and a number of others.

In March 2013, 48 enterococcal isolates recovered during this study, including 35 environmental and 13 patient isolates, were sent to Pathogenica Ltd., Boston, USA, for characterisation. These isolates were recovered in sampling periods one and two. A selection of both VRE and VSE samples covering environment and patient isolates were chosen, in order to challenge the technology in a number of ways – firstly to verify the isolate identification capability of the technology, secondly to assess the ability to distinguish between susceptible and resistant isolates, and finally as a method to type the isolates, characterising the relationships between them.

Their initial analysis confirmed a mixture of *E. faecium* and *E. faecalis* among the isolates, 38/48 (80%) of which were *E. faecium* and 8/48, (16.6%) were *E. faecalis*. 

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Source</th>
<th>Date</th>
<th>Bed number</th>
</tr>
</thead>
<tbody>
<tr>
<td>H71</td>
<td>Patient BC</td>
<td>30 Jan 2013</td>
<td>6</td>
</tr>
<tr>
<td>H72</td>
<td>Patient screen</td>
<td>9 Dec 2012</td>
<td>11</td>
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<tr>
<td>H66</td>
<td>Patient screen</td>
<td>7 Jan 2013</td>
<td>4</td>
</tr>
<tr>
<td>H57</td>
<td>Chart Holder</td>
<td>30 Jan 2013</td>
<td>8</td>
</tr>
<tr>
<td>H65</td>
<td>Patient BC</td>
<td>25 Jan 2013</td>
<td>11</td>
</tr>
</tbody>
</table>
One isolate of *E. gallinarum* and one isolate of *Paenibacillus* spp were also identified. The technology identified a resistance mechanism in 23/48 (48%) of the samples, all of which were identified as having the *vanA* gene. Pathogenica confirmed the presence of the *vanA* gene with PCR, which they found to be 100% concordant with the NGS findings. However, this finding was at odds with the MIC data of our isolates, where an additional seven isolates, four *E. faecium* and three *E. faecalis*, should also have been identified as VRE having MICs between eight and 64 µg/ml for vancomycin, (the breakpoint for resistance being a MIC of 4 µg/ml) indicating resistance to vancomycin. These were subsequently characterised as having the *vanB* gene by PCR and Sanger sequencing, which identified the presence of both *vanA* and *vanB* in nine of the *E. faecium* isolates.

Subsequently, the 24 VRE *E. faecium* (VREfm) isolates identified were further characterised and typed. Eleven were patient isolates and 13 environmental isolates. The relationships between these isolates are represented in Figure 5.6.

Although only carried out on a subset of the study isolates, the relationships between the isolates as detected by NGS, matched to a degree the PFGE analysis. Two main clusters were identified, divided by time and containing both environmental and patients isolates in each. Group 1 comprised mainly environmental isolates and contained isolates very similar to Cluster I by PFGE. Within this group, NGS also included two isolates from patients who were in the ICU at the time these samples were taken from the environment, however, the strain matches vary, complicating the interpretation. Group 2 comprised mainly patient isolates and included all of the patient isolates in cluster III identified by PFGE. This included three isolates, one rectal swab, and two later blood culture isolates, and other patients who were in the unit at the same time as each other, suggesting person-to-person spread in the ICU.
Figure 5.6. Dendogram of VRE isolates using HAI BioDetection™, Pathogenica Ltd.

Dendogram generated by targeted Next Generation Sequencing of 24 isolates characterised by targeted gene sequencing using HAI BioDetection™, Pathogenica Ltd. The arrow indicates the possible cluster division of genetic relatedness. Strain match of the isolates represent the closest match to the available sequences published in the gene databases. Patient BC = Blood culture isolate.

NGS carried out in Boston by Pathogenica Ltd

Strain typing involves matching the strain identified to the closest strain among bacterial strains whose draft genome has been sequenced and banked. These sequences are available for comparison in gene banks, like NCBI Genomes Database,
in the US and the European Nucleotide Archive (ENA), and where there are currently 163 draft genome sequences of *E. faecium*. There were six different strains identified among our isolates. Of these, there were two predominant strains, C1904 12/24 (50%), of which 9 (75%), were patient isolates. Strain S447 was confirmed in 9/24 (37.5%), of which seven (77.7%) were environmental isolates. There was one each of four other strain types.

### 5.3. Discussion

Establishing accurate epidemiological relationships between isolates in the clinical environment is often challenging. VRE is found more frequently on surfaces than Gram-negative pathogens and this was supported by our findings here. It is possible that this pattern of recovery of VRE may be because it is shed in larger numbers or because it persists for longer on surfaces or because it is easier to detect in the environment. There were more occasions where VRE was isolated in the environment of a patient colonised with VRE than was the case for ESBLs. However the numbers of ESBL-E positive patients were relatively low compared to the VRE positive patients. Only two patients, positive for ESBL-E, had an ESBL-E isolated from an environmental sample at the same time. The first was a patient colonised with *E. cloacae*, and *E. cloacae* was also isolated from their surrounding environment. This patient had intra-abdominal sepsis after a cholecystectomy and had a previous history of ESBL colonization. This patient also had an intra-abdominal drain in situ at the time of sampling, possibly increasing the likelihood of environmental contamination. In case 2, however, the patient and environmental ESBL-E samples did not match. The patient was colonised with an ESBL positive *E. coli* and the environment was positive for an ESBL *K. pneumoniae*.

Due to the small numbers of isolates recovered and the limited clinical epidemiological links between them, ESBL-E isolates were not typed.

Visual inspection of PFGE patterns of the VRE isolates showed 32 different pulse types indicating a relatively diverse population structure. However, three pulse
types appeared to predominate in the collection, at least on visual analysis. For two of these, exclusively environmental isolates were included whereas the third pulse type was found amongst both patient and environmental isolates.

Gelcompare II software clusters pulse types based on the extent of variability as determined by the Dice coefficient and is therefore more discriminatory than visual comparisons. The Gelcompare II cluster analysis also identified three clusters, two containing environmental isolates only and one mix of patient and environmental isolates. The environmental clusters contained isolates which were linked in time, with evidence of environmental persistence, or recontamination with the same strain. In the cluster that contained both patient and environmental isolates, the one environmental isolate was independent of the isolates by one year, and none of those patients was readmitted to the ICU over that time. Also, the PFGE pulse types illustrated that the same patient could have different pulse types, showing diversity of strains within individuals as well between individuals. The pairs of isolates, one of environmental and one of patient origin, showed again that the relationships are not straightforward.

Ireland has the highest rates of VRE in Europe with 46% of the enterococcal isolates submitted to the EARS-Net being resistant to vancomycin [19]. A recently published study from another Irish tertiary hospital investigated the clonal relationships between a collection of bloodstream isolates, spanning 42 months, from both their hospital and two other hospitals. They found five predominant pulse types, one consisting of a large number of isolates involved in an outbreak. Eighteen of their isolates were unique, 11 of which were isolates from BSI from their institution, indicating a polyclonal population of VRE among patients[48]. However, that study predominantly focused on BSI isolates. Our study compared environmental isolates and isolates recovered as part of screening for VRE. Our study also found genetic diversity among the patient isolates, which in the non-outbreak setting is somewhat reassuring. A Malaysian study examined collections of clinical VRE isolates and also report genetic diversity among their isolates, with a predominance of MLST CC17 among a small number analysed [139]. In our study, there was evidence of person
to person transmission within the unit. For example, two patients who were in the unit at the same time, but in separate isolation rooms at either end of the unit, showed closely related VRE strains, suggesting the transmission was facilitated by poor hand hygiene. Genetic characterization of the environmental samples in this study shows that there is clonal spread of VRE among environmental isolates from our ICU, which differ from the patient isolates. A US study from an ICU showing a link between the environment and increased risk of patient acquisition of VRE did not genetically characterize the isolates [91]. Our study suggests that the presence of VRE in a bed space may or may not be linked to subsequent acquisition of VRE in another patient in that environment. Moore et al. investigated contamination in different wards, including ICU and did not find VRE contamination in isolation room housing VRE colonised patients. [114]. The Ryan et al. study referred to earlier included PFGE analysis of five environmental isolates in addition to the patients isolates [48]. Each environmental isolate in their setting clustered with the corresponding patient isolates in five separate clusters. Temporal relationships between environmental and patient isolates were not clearly apparent. However, for two of the clusters, the environmental and patient isolates were recovered from the same ward, one during the outbreak and the other linking patient isolates to environmental contamination in the haematology ward. These results and those described in the present study, suggest further complexities to the relationship between environment and patient VRE isolates in the non-outbreak setting.

Another complicating issue is that researchers have shown that VRE acquisition is not only linked to the circulating VRE strains in other patients and the environment in a hospital, but also to colonising and circulating strains of hospital adapted susceptible enterococcal strains. Howden et al. applied WGS and comparative analysis to a collection of E. faecium isolates, both vancomycin-susceptible and -resistant, and to five vanB positive anaerobic commensal bacteria collected from human faeces samples [127]. They were investigating the hypothesis that VRE may arise de novo from acquisition of vancomycin resistance determinants arising from transposon Tn1549-like elements found in commensal anaerobes. These Tn1549-
like elements have been found to be present in up to 50% of healthy VRE negative adults in Australia [140]. They found that in 18 of 36 VRE isolates, the transposon Tn1549 had been acquired by independent insertion events, rather than cross transmission. Phylogenetic comparison of their isolates to international isolates found that this may be an international phenomenon. These findings suggest that acquisition of antibiotic resistance amongst enterococci within a patient may be common, occurring in the bowel whilst under antibiotic pressure, and may offer an explanation as to why infection control measures do not control VRE [127].

In this study, NGS did not give significant additional information with regard to the clustering of the isolates. It did, however, confirm that different strains predominated in the environmental isolates and the patient isolates. This technology may need to be used in conjunction with phenotypic or other genotypic confirmatory methods, such as PCR for resistance genes. In this study, we noted that the selected DNA fragments missed the required target on many occasions resulting in failure to identify resistance mechanisms among the isolates as occurred in our vanB isolates. It is also possible that DNA mutations, occurring naturally in collections such as ours, may alter the selected targets resulting in poor binding to the probes. Furthermore, with this technology, difference between strains are determined based on a number of single nucleotide polymorphism (SNIP) differences, therefore both WGS and NGS rely on the existence of sufficiently large reference sequence databases [137]. Recently, the HAI Biodetection™ system was compared to amplified fragment length polymorphisms (AFLP) to track an outbreak of ESBL-E in nursing homes in The Netherlands. Investigators found that the kit compared favourably in terms of usability and performance. However, similar to our findings here for VREfm, it failed to detect a number of ESBL genes, and its phylogenetic tree, though comparable required further work.[138]

This technology is in its infancy, and although it failed to correctly identify a number of our VRE isolates, the technology is evolving rapidly and the cost associated with it is decreasing.[137]. We were among the first groups to apply this technology to patient isolates and also to VRE specifically. Its strength lies in its ability to combine
the recovery of multiple organisms, potentially directly from a sample, and a number of the surveillance processes including resistance determinants, bacterial characterization and typing in one method.

In summary, genetic profiling of our patient isolates showed genetic diversity whereas the environmental isolates showed more clonal relationships. The clonality of the environmental isolates offers an opportunity to address this aspect of infection prevention and control by more careful cleaning of the environment, as the clones were present independently during particular times during the study. This would require investment in education, feedback for cleaning staff and possibly the recruitment of additional staff to make a real impact. Some of these interventions have shown positive outcomes as described in other studies [65, 87].

An additional route of patient acquisition of VRE is the possibility that VRE may occur de novo in patients through the acquisition of resistant determinants by susceptible colonizing, hospital adapted clones from host-associated normal anaerobic flora. Expanding screening to susceptible enterococci, although labour and resource intensive, may be needed to control the rise of VRE [127]. This would be particularly important in the Irish setting where rates are among the highest in Europe. New rapid methods, such as NGS based screening may make this more feasible.
Chapter 6

General discussion, conclusions and further work.
6.1. General Discussion

In this project we investigated the contribution that patients and the physical environment in which they are placed and cared for, make to the acquisition of infection and transmission of MDRO, with ESBL-E and VRE as the target organisms. The study was conducted outside of an outbreak setting in a general ICU, to assess the baseline contamination of the environment over a number of sampling periods and to correlate contamination to ICU activities where possible. We also investigated high touch surfaces in the ICU as potential reservoirs of VRE and ESBL-E in the environment of the ICU, focusing on surfaces in the immediate patient environment. This was in the context of increasing VRE and ESBL-E being recovered from bloodstream isolates in Ireland[11, 50].

We found VRE in 9% of our environmental isolates. This baseline rate is higher than recently reported in the literature for similar studies in both general wards (2 %) and ICU rooms (5 %) although the VRE rates reported in the literature vary considerably [103, 106]. ESBL-E contamination of the environment was low in our study at 0.6%, which is at odds with some studies and in agreement with others investigating specifically Gram-negative contamination of the environment [79, 100, 107]. The limited numbers of ESBL-E recovered in this study were dispersed throughout the ICU. We identified a group of older isolation rooms in our ICU as particular reservoirs of VRE and there were significantly more of these contaminated than for both the open plan area and the newer isolation rooms.

Standard size recommendations exist for ICU isolation rooms [57, 59]. While the newer rooms in our ICU conform to the standard size (beds 11 and 12), this is not the case for the older rooms (beds 7 to 10). The smaller size may result in more cramped conditions or may hamper proper cleaning of the environment, which may contribute to the persistence of VRE in the environment. The general ICU operates at an average of 110% capacity. The recommended operating capacity for ICUs is 85%, to allow for timely access when needed [57]. Reduction in capacity would also give flexibility to the system to allow for improved cleaning. Practice improvement
initiatives (e.g. installation and roll out of an electronic patient record and introduction of chlorhexidine wipes to wash patients) implemented by the ICU team during the course of this study had a positive impact in reducing the contamination of the environment. However, further investigation of changes in practice over additional or longer periods would be important to confirm that such small but significant improvements are sustained.

We also investigated the burden of VRE and ESBL-E colonization in the patients in our ICU. Practice varies both nationally and internationally in relation to screening for these organisms and there is no clear guidance as to the best strategy [59, 120]. In addition, policies are often implemented in the absence of a solid evidence base. We found that 19% of patients were colonised with VRE and 9.8% colonised with ESBL-E. Patients with VRE were generally more ill than ESBL-E colonised patients, and had longer lengths of stay than ESBL-E colonised patients. Both groups had prior antibiotic therapy in >80% of cases. Of the patients with VRE, 36% (11/30) were known to be positive on admission. Of those with unknown status that were screened on admission to ICU, 73% (14/19) were VRE positive within 48 hours of admission.

The status of ESBL-E among patients is unknown in ICU, unless they have a previously positive clinical isolate. Of the patients admitted to ICU during this study who were found to be ESBL-E positive following targeted screening, 40% (6/15) were already known to have ESBL-E in their flora, whereas the other 9 were previously unknown. Therefore, ASC could have picked up an additional 5.7% (9 out of 157 admissions) of patients colonized with ESBL-E. Active surveillance for ESBL-E colonization for ICU admissions would have an impact on antimicrobial choices, should they require treatment. Currently, there is very little cephalosporin use in this ICU, and there are few ESBL-E infections in the ICU as exemplified by these snapshots of time, so a more longitudinal investigation may be warranted to justify screening all admissions. The rate of 9.8% is in agreement with other studies where recent Irish data reported 14.3% carriage in ICU patients and European data show variable ESBL-E carriage of between 5-15% of admission to ICU [119, 125]. Data
from LTCF in Ireland and Northern Ireland show that carriage among residents is high at 40-56% [21, 22]. This has prompted a national recommendation to screen for ESBL-E in this population, which has not been implemented across the board. The debate on whether or not to screen continues in the literature [120]. The data presented in this thesis shows that, at least for these snapshots of time, there was little clinical impact in knowing the ESBL status of patients. There is a well functioning antimicrobial stewardship culture in the ICU and empiric treatment for infections are appropriate for the majority of patients, given current antimicrobial susceptibility data. The lack of ACS for ESBL-E did result in patients being cared for in the open area of the ICU. The dispersal of EBSL-E in the unit was broader than VRE, which is a reflection of this. Again the numbers were small, but epidemiological investigation did not support links between patients and environmental contamination with these organisms.

Interestingly, chlorhexidine wipes for cleaning patients, introduced to reduce colonization with MDRO, had little impact on acquisition of either VRE or ESBL-E in patients, which differs from the literature [72]. It did, however, have the unexpected finding of a significant reduction in the overall bacterial burden in the ICU environment. Further analysis, in time with larger number of patients will inform us as to the true effect.

Lastly we carried out a detailed characterization of a selection of the VRE isolates from both environmental and patient isolates, using PFGE and NGS. These investigations showed differences in the molecular epidemiology of the environmental and patient VREfm isolates that may contribute to their transmission patterns. The environment isolates were clonal in time and space, whereas the patient isolates, showed more marked genetic diversity. Genetic diversity among the patient isolates as shown by mainly sporadic PFGE types in the non-outbreak setting is somewhat reassuring, in that silent outbreaks were not apparent. VRE is endemic in our institution and MLST analysis would help to put these strains in a more population based context, which would form part of future work. Further analysis of CCs among VRE have shown that the population structure is more
diverse than previously thought, unlike other problematic Gram-positive HCAI such as MRSA which is limited to only five clonal lineages [131, 141].

In this study, there was evidence of limited person-to-person transmission within the unit. A recent Irish study also showed polyclonal pattern of VRE isolates among mostly bloodstream isolates, with molecular epidemiological but not always clinical epidemiological links to a small number of environmental isolates [48]. Linking the environment to the patients isolates, shows that though there may be genetic relationships between isolates, in our study they did not always support the assertion that the environment is the source of infection where VRE were recovered in the non outbreak setting [78]. We did not include staff hand carriage of resistant organism in this study, which may have contributed to the patterns of transmission of VRE. NGS confirmed the relationships established by PFGE patterns, but interestingly showed a diversity of strains among the environmental isolates. In the small number of isolates that were sequenced by NGS, all the patient isolates, except one, matched to the same strain type. This shows the complex dynamics that underpin the interface between patients and the environment.

WGS of VRE and Gram positive anaerobes of the commensal gut flora has suggested that commensal flora may also contribute to the acquisition of VRE specifically through acquisition of vanB resistance determinants by susceptible enterococci from anaerobes. Based on their findings, the authors suggest screening for hospital adapted susceptible strains of E. faecium [127]. This discovery is interesting in the context of the present study given that 45% of our strains were confirmed to contain the vanB gene, which is associated with the transposon Tn1549 present in commensal Gram positive anaerobic bacteria [142]. Further investigation of this potential source of VRE would be best achieved using sequencing methods such as NGS. NGS has the capacity to rapidly identify target pathogens, detect resistance determinants and characterize relationships between them. In this era of antibiotic resistance it is vital to understand and prevent transmission in our crowded hospitals. This is particularly true in ICU, where patients are among the sickest in the hospital and at the highest risk of acquiring a HCAI in particular MDRO [6, 9].
6.2 Conclusion and future work.

There is a case for screening patients admitted from nursing homes for ESBL-E based on the higher burden of carriage among these patients. In the present study, ESBL-E carriage was detected at a relatively low rate of 9.8% of our ICU admissions. Nonetheless, screening all ICU patients may be prudent as it may prevent further increases in resistant isolates causing BSI in ICUs as is the case in other European jurisdictions [6].

Screening for VRE in our hospital showed that, of those that were admitted to ICU and screened, 73% were positive on admission screening, within 48h. Investigating the ‘source ward’ of these patients may provide an opportunity to identify VRE carriers in target groups in the hospital, expanding ACS to these patients, thus limiting transmission if transferred to ICU. These target groups will differ in different hospitals depending on the specialty populations within each hospital. In addition, the tracking of other organisms associated with antibiotic resistance and HCAI from hospital wards to ICU could be targeted to particular patient populations in the same way.

Furthermore a more detailed analysis of antibiotic history, over patients’ entire hospital stay, and indeed in the previous 3 months, could indicate the true exposure of their flora to antibiotics. This would contribute to a deeper understanding of the role antibiotics have on the emergence of resistance organisms from the human microbial flora.

In the present study, molecular typing of VRE isolates, confirmed some instances of patient-to-patient transmission in the ICU. Clearly an operating capacity of >100% is not sustainable, nor is it safe for patients or staff and furthermore it may contribute to the patient burden of MDRO through cross transmission events. In this regard, our study highlighted distant isolation rooms within the unit housing patients colonized with VREfm of the same PFGE type. The finding of close clonal associations among environmental isolates recovered from the ICU highlight the challenges of cleaning the ICU. This requires better supports and training for
cleaners, which is often low on priority lists when resources are limited. From an infrastructure point of view, reconfiguring the older isolation rooms would be required for a sustained reduction in contamination of the environment. A follow-up study might confirm the benefit of this in terms of reduced environmental contamination.

The complexity of enterococcal transmission is evident from our study. Further investigations that facilitate comparisons of patterns of transmission, that include, environmental and clinical isolates (both colonising and invasive strains) are needed. This approach would contribute to a clearer understanding of the epidemiology of VRE persistence and transmission. In an Irish context, where rates of VRE invasive infection are the highest in Europe, we urgently need to identify modifiable factors that contribute to the success of VRE in our healthcare facilities [11]. Ultimately, this level of investigation would be best achieved by the establishment of a VRE reference laboratory in Ireland.

Epilogue

Dr. William H Stuart, US Surgeon General (1965-1969) has often been misquoted as saying that ‘it is time to close the book on infectious diseases and declare the war against pestilence won’. The records show that he in fact said the opposite[143]. During the golden years of antibiotic development, when control of infections was at hand, the dramatic reduction in mortality due to infectious diseases had tapered off and the public health focus had shifted to tackle chronic diseases such as cardiovascular and respiratory disease. This change of focus was not to be at the exclusion of infectious diseases, but rather to signal a shift of emphasis.

For several decades, the medical world acted in line with the misquote, turned its back on infectious diseases, and became complacent with antibiotic use. Bacteria, who have been here long before us, and will persist and flourish long after us, took advantage. Clearly the book is not closed on infectious diseases – it continues to be
written. It is however time to close the book on this contribution to a fraction of this ongoing work.
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57. NICE, *Core Standards for ICU.* 2013.


93. EUCAST, *EUCAST Guidelines on the detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance*. 2013, European Society for Clinical Microbiology and Infectious Diseases.


Appendices
Appendix 1. Schematic of general ICU
Appendix 2. Ethics approval
Ethics (Medical Research) Committee - Beaumont Hospital
Notification of ERC/IRB Approval

Principal Investigator: Prof. Hilary Humphreys
REC reference: 12/74
Protocol Title: The identification and characterisation of antimicrobial resistant bacteria in the ICU environment and the factors contributing to the frequency of their isolation.

Ethics Committee Meeting Date: 28th September 2012
Final Approval Date:

From: Ethics (Medical Research) Committee - Beaumont Hospital, Beaumont, Dublin 9

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Prof. Alice Stanton
ERC/IRB Convenor’s Signature
Approval # 1, dated 18th October 2012

24 OCT 2012
Appendix 3 Data collection forms
**ENVIRONMENT**

- **Bed no.**
- **Date of sampling**
- **Day cleaned**
  - [ ] Today
  - [ ] Yesterday

**Antibiotic history**
- AMC
- CSM
- MTZ IV
- AET
- DAP
- MTZ PO
- CIP
- GEN
- PTZ
- CLA
- LSE
- VAN IV
- CTX
- MBR
- VAN PO
- Other AM

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<th>Site</th>
<th>BHI</th>
<th>UTI / CTX</th>
<th>VRE</th>
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<td>POS / NEG</td>
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**Resistant flora present**
- [ ] MRSA
- [ ] VRE
- [ ] CRE
- [ ] ESBL

**Episode no.**

**DOB**

**167**
Appendix 4. German Enterococcal reference laboratory PFGE protocol
<table>
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Datum: 19.10.2004

**Analylt mit Abkürzungen:**

DNA-Fragmente verschiedener Bakterien (z.B. *Staphylococcus aureus*, *Enterokokken*, *E.coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*).

**Verantwortliche:**

Frau Stresemann  
Frau Fröse  
Frau Stapel

**Methode:**

1. Kultivierung  
2. DNA-Präparation:  
   - Einbettung der Bakterienzellchen in Agaroseblöckchen  
   - Lyse der Zellmembran und Verdau störender Nukleasen  
   - Enzymverdau der Bakterien-DNA  
   - Einbettung in Agarosegel  
3. Pulsfeldelektrophorese

**Testprinzip:**

Bakterienzellen werden in ein Agaroseblöckchen eingebettet und die Zellwände mit Detergentien und Proteasen (zusätzlich Lysostaphin / Lysozym bei grampositiven Bakterien) lysiert.  
Anschließend wird das Bakterienchromosom, das durch die Gelmatrix vor Scherbruch geschützt ist, mit selten schneidenden Restriktionsenzymen in Fragmente getrennt.  
Durch diesen Restriktionsverdau werden wenige aber große Fragmente erzeugt, die durch spezielle Elektrophoresegeräte mit Spannungserscheinung unter Kühlung ca. 24h (entsprechend der jeweils zu untersuchenden Spezies) aufgetrennt werden und charakteristische Bandenmuster ergeben, die miteinander verglichen werden.  
Ein Marker dient der Größenbestimmung der Fragmente.

**Referenzbereich:**

---

**Meßbereich:**

---

**Einheiten:**

---

**Umrundungsfaktoren oder Formeln:**

---

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</table>

<table>
<thead>
<tr>
<th>Stoff/Hersteller</th>
<th>Beschreibung</th>
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</thead>
<tbody>
<tr>
<td>N-Lauroylsarcosine</td>
<td>L-5125 / 50g</td>
</tr>
<tr>
<td>Polyoxyethylene 20 Cetyl Ether</td>
<td>P-5884 / 100g</td>
</tr>
<tr>
<td>Deoxycholic Acid</td>
<td>D-6752 / 100g</td>
</tr>
<tr>
<td>Fa. Merck: Proteinase K</td>
<td>124568 / 100mg oder 500mg</td>
</tr>
<tr>
<td>Tris-Kleber III</td>
<td>1.08421 / 1kg</td>
</tr>
<tr>
<td>Na triumhydroxid Plätzchen</td>
<td>1.06495.0250 / 250g</td>
</tr>
<tr>
<td>Borsäure</td>
<td>165 / 500g</td>
</tr>
<tr>
<td>Fa. Riedel de Haen: Na triumchlorid</td>
<td>31434 / 5kg</td>
</tr>
<tr>
<td>Fa. Serve: Tris (=Tris(hydroxymethyl)aminomethane) research grade</td>
<td>37190 / 5kg</td>
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<tr>
<td>Fa. Bio Rad: Ethidium Bromide</td>
<td>161-0433 / 10ml</td>
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<tr>
<td>Fa. Biolabs (Restriktionenzyme): Dra I</td>
<td>RO 129L</td>
</tr>
<tr>
<td>Not I</td>
<td>RO 189L</td>
</tr>
<tr>
<td>Sma I</td>
<td>RO 141L</td>
</tr>
<tr>
<td>Spe I</td>
<td>RO 133L</td>
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<tr>
<td>Xba I</td>
<td>RO 145L</td>
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</table>

Selbsthergestellte Lösungen:

SeaKorn Gold Agarose (Schmelzpunkt: ≥90°C)

Für gramnegative Bakterien:


Dateiname: AM-MI-511B_PFGE.doc
<table>
<thead>
<tr>
<th>CSB (Cell Suspension Buffer)</th>
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</thead>
<tbody>
<tr>
<td>Tris / HCl (1M Stammlösung pH 8,0)</td>
</tr>
<tr>
<td>EDTA (0,5M Stammlösung pH 8,0)</td>
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<tr>
<td>mit steriлем Aqua dest ad 100ml auffüllen.</td>
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<table>
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<td>EDTA (0,5M Stammlösung pH 8,0)</td>
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<tr>
<td>10 % Sarkosyl (N-Lauroylsarcosine)</td>
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<tr>
<td>mit steriлем Aqua dest ad 500ml auffüllen.</td>
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<table>
<thead>
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<th>TE-Puffer</th>
</tr>
</thead>
<tbody>
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<td>Tris / HCl (1M Stammlösung pH 7,6)</td>
</tr>
<tr>
<td>EDTA (0,5M Stammlösung pH 8,0)</td>
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<tr>
<td>10ml Tris / HCl Stammlösung und 2ml EDTA Stammlösung mit Aqua dest ad 1000ml auffüllen.</td>
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<table>
<thead>
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<th>EC-Puffer</th>
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<tbody>
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<td>Tris / HCl (1M Stammlösung pH 7,4)</td>
</tr>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>EDTA (0,5M Stammlösung pH 8,0)</td>
</tr>
<tr>
<td>Brij 68 (Polyoxylethylene 20 Cetyl Ether)</td>
</tr>
<tr>
<td>Sarkosyl (N-Lauroylsarcosine)</td>
</tr>
<tr>
<td>Desoxycholat (Deoxycholic Acid)</td>
</tr>
<tr>
<td>mit steriлем Aqua dest ad 500ml auffüllen, pH 7,5 – 8,0 (einstellen mit 10N NaOH)</td>
</tr>
<tr>
<td>Lösung auf Magnetrührer ansetzen, nicht autoklavieren (Detergenzien werden zerstört).</td>
</tr>
</tbody>
</table>

EDTA 0,5 M pH 8,0 (Stammlösung)
EDTA (Tritex III, Molare Masse = 372,24 g/l) 18,12g
<table>
<thead>
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<th>Arbeitsanweisung zur Methodendurchführung</th>
<th>Seite 6 von 21</th>
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<td>Version: B</td>
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NaOH (Natriumhydroxid, Molare Masse = 40,0 g/l) 15g mit Aqua dest. ad 1000ml auffüllen.

**Ansatz:**
EDTA und NaOH und ca. 400ml Aqua dest. in einen Meßkolben geben und mit NaOH auf genau pH 8,0 einstellen (nur dann löst sich EDTA). Erst wenn EDTA vollständig gelöst ist (braucht Zeit) mit Aqua dest. auffüllen. Lösung in kleine Flaschen verteilen und autoklaven.
(EDTA fängt Magnesium ab und verhindert dadurch einen Verlust von DNA.)

**Ethidium Bromide (EtBr – Stammlösung)**
c = 10 mg/ml Lagerung im Kühlschrank (Mikrobiologie Raum 052090)
Ethidium Bromide ist cancerogen / Handschuhe tragen!

**Färbelösung:**
60μl Stammlösung in ca. 600ml steriles Aqua dest. pipettieren, geeignet für mehrere Färbungen. Alle Färbelösungen nur im dafür vorgesehenen Sonderabfallbehälter (Mikrobiologie Raum 052291) entsorgen.

**Lysostaphin**
Ansatz: c = 1mg/ml
Lösen in 1 ml 0,001-0,005 M Essigsäure (pH 4,0 – 4,8)
(6μl Essigsäure (100%ig) plus 50ml Aqua dest. ergibt ca. 0,002 M)
à 300μl aliquotieren, bei −20°C lagern (mindestens 6 Monate haltbar).

**Lysozym**
Ansatz: c = 20mg/ml
Lösen in Aqua dest.
Ca. 4-5ml ansetzen, d.h. 100mg in 5ml Aqua dest. lösen.
à 300μl aliquotieren, bei −20°C lagern.
nach Gebrauch verwerfen, niemals erneut einfrieren.

**NaCl 5M (Stammlösung)**
292,7g Natrumchlorid (NaCl M = 58,44g/l) in 1000ml Aqua dest. (Meßkolben) auf dem Magnetrührer lösen. Lösung in Flasche auffüllen und autoklaven.

**Proteasase K**
Ansatz: c = 20mg/ml

**Dateiname: AM-Mi-511B_PFGE.doc**
100mg in 5ml sterillem Aqua dest. lösen
à 1ml aliquotieren, bei −20°C lagern.

**TBE-Puffer**
Ansatz einer 10x TBE Stammlösung:
- Tris (v. Serva): 108g
- Borsäure: 55g
- EDTA (0,5 M): 20ml
mit Aqua dest. ad 1000ml auffüllen und auf dem Magnetrührer lösen (pH 8,0). Lösung autoklavieren.

0,5x TBE Elektrophorese Gebrauchspuffer: 10x TBE 1 : 20 verdünnen.

**TEN-Puffer**
- Tris (v. Serva): 12g
- EDTA: 29g
- NaCl: 8,7g
mit Aqua dest. ad 1000ml auffüllen (Meßkolben) pH 7,5 (mit 10N NaOH einstellen), auf kleine Flaschen verteilen und autoklavieren.

Tris / HCl 1 M pH 8,0 (Stammlösung)
121,1g Trizma (Tris M = 121,1g/l) in 1000ml Aqua dest. lösen. Zunächst nur wenig Aqua dest. hinzufügen und mit 1M oder 4M HCl den pH einstellen. (Man braucht viel Volumen HCl).
Niemals mit NaOH ausgleichen!

### Methodendurchführung:

**Gramnegative Bakterien**

**Vorbereitung:**
- Keime frisch überimpfen (Überwachtkultur auf Blutagar anfertigen).
- Protokolloben vorschreiben (Kurzdaten: 1, 2, 3, ..., ausführliche Daten: Befundnummer, Name, Keimart, Enzym).
- 10ml Glas-Röhren (im Glaslager vorrätig) vorschreiben (Kurzdaten) und 2ml CSB (=Cell Suspension Buffer) vorlegen.
- Eppendorffläschchen vorschreiben (Kurzdaten).
- Küvetten (Fa. Sarstedt, No. /REF 67.742 im Lager vorrätig) mit Kurzdaten beschriften.
- 24-Well-Zellkulturplatte (Fa. Greiner, No. 662160) mit Kurzdaten beschriften.
- Soakern Gold Agarose (1% ig in TE-Puffer gelöst) aufkochen und bei 55°C im Wasserbad halten.
<table>
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<tr>
<th>Keim</th>
<th>Enzym</th>
<th>Zeitprogramm</th>
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</thead>
<tbody>
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<td>Staphylococcus sp.</td>
<td>Sma I</td>
<td>5 / 15 / 10 Std.</td>
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<tr>
<td></td>
<td></td>
<td>15 / 60 / 13 Std.</td>
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Ist die Elektrophorese beendet, wird das Gel vorsichtig aus der Kammer entnommen (Handschuhe) und in einem Ethidium-Bromid-Bad für 30 – 60min gefärbt (Ethidium Bromid ist cancerogen und befindet sich in einem separaten Raum (Mikrobiologie Raum 052261), der nur mit vorher angezogenen Nitrile-Handschuhen betreten werden darf).

Nach der Färbung wird das Gel 30 – 60min in Aqua dest. gewaschen, um die Hintergrundfärbung zu reduzieren, anschließend wird es photographiert.

Genaue Angaben zum Herolab Gerät befinden sich in der Geräte-SOP.

**Enterokokken**

**Vorbereitung:**

- Keime frisch überimpfen (Übernachtkultur auf Biotagar anfertigen).
- Protokollbogen vorschreiben (Kurzdaten: 1, 2, 3,..., ausführliche Daten: Befundnummer, Name, Keimart, Enzym).
10ml Glasröhren (im Glaslager vorrätig) vorschreiben (Kurzdaten) und 2ml TEN-Puffer vorlegen.

Eppendorfröhrchen vorschreiben (Kurzdaten).

Küvetten (Fa. Sarstedt, No./REF 67.742: im Lager vorrätig) mit Kurzdaten beschriften.

24-Well-Zellkulturplatte (Fa. Greiner, No. 662160) mit Kurzdaten beschriften.

Seakern Gold Agarose (2%-ig in EC-Puffer gelöst) aufkochen und bei 55°C im Wasserbad halten.

Gießform vorbereiten (mit warmem Wasser spülen, trocknen und mit 70%igem Alkohol desinfizieren).

1.Tag

Gesamtes Keimmaterial mit Tüpfel von Blutplatte abnehmen und in 2ml TEN-Puffer einreiben, kurz vortexen.


Trübungsmessung bei 610nm Wellenlänge. Geforderte Extinktion: 0,7 – 1,0 (evtl. andere Verdünnungen wählen). Der Leerwert besteht aus 1ml TEN-Puffer.

1ml dieser Küvetten-Keimsuspension in vorgeschriebene Eppendorfröhrchen pipettieren.

Bei 14000 U für 10 Sek. zentrifugieren.

Überstand mit Eppendorfpipette absaugen und 500µl TEN-Puffer pipettieren, vortexen.

Bei 14000 U für 10 Sek. zentrifugieren.

Überstand absaugen, 200µl EC-Puffer pipettieren, vortexen.

Proben bei 55°C im Thermomixer halten.

200µl 2%-ige Seakern Gold Agarose dazugeben und ca. 10mal mit Pipette mischen.

Blockchen gießen.

Mit allen folgenden Proben genauso verfahren.

Blockchen 10 Min. bei Zimmertemperatur erstarrn lassen.

Greiner Zellkulturplatte mit 1ml EC-Puffer befüllen.

Blockchen vierteln und ein Viertel in Zellkulturplatte geben.

100µl Lysozym (Konzentration: 20mg/ml) pro Viertel hinzufügen, Zellkulturplatte bei 36±1°C für 1,5 Std. inkubieren.

EC-Puffer absaugen.

2ml CS-Puffer pipettieren (CS = Cell Suspension)

Zellkulturplatte im Thermomixer 45Min. bei 55°C und 500rpm schüttern.

CS-Puffer absaugen.

2ml frischen CS-Puffer pipettieren, 10µl Proteinase K pro Cup dazugeben.

Platte im Thermomixer 1 Std. bei 55°C und 500rpm schüttern.
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1. CS-Puffer absaugen.
2. 2ml frischen CS-Puffer pipettieren.
3. Platte im Thermomixer 45 Min. bei 55°C und 500rpm schütteln.
4. CS-Puffer absaugen.
5. Letzten Waschgang noch 1mal wiederholen.
6. 2ml frischen CS-Puffer pipettieren und Blöckchen bei 4°C im Kühlschrank aufbewahren.

-> Laufgel herstellen (1% Agarose in 0,5 x TBE-Puffer)
   d.h.: 1g + 100ml bei normaler Gel-Gießkammer,
   1,4g + 140ml bei größerer Gel-Gießkammer.
   Agarose-Gemisch aufkochen und anschließend bei 55°C halten (Wasserbad).

2. Tag
Enzymverdau und PFGE-Start

1. 96-Well Mikrotiterplatte (Flachboden) mit Tessa vollständig abkleben (Schutz vor einfallenden Enzymen).
2. den Deckel der Mikrotiterplatte dem Bedarf entsprechend beschreiben und Position der Blöckchen auf Arbeitsliste eintragen.
3. benötigte Anzahl Cups öffnen (nach Benützung auf Unterseite mit Edding markieren).
4. 300µl CS-Puffer in Mikrotiterplatte vorlegen.
5. Gelblöckchen mit desinfiziertem Spatel aus Zellkulturplatte entnehmen, auf dem Spatel mit Skalpell ein Viertel abteilen und dieses Viertel in das entsprechende Närpfchen der Mikrotiterplatte geben.
   Hilfsmittel: schwarze Unterlage als Kontrast u. diese mit Frischhaltefolie abgedeckt.
   Restliches Blöckchen in Zellkulturplatte zurückgeben.
6. Enzympuffer herstellen (pro Cup 50µl, 2-3 Cups zusätzlich berechnen).

Das Restriktionsenzym soll in einer Aktivität von 10 U pro Cup vorliegen.

Ansatz von 50µl Enzympuffer:

| ca. 10U Enzym |
| 0,5µl DTT |
| 0,5µl BSA |
| 5µl 10 x Puffer |
| ≤44,5µl Aqua dest. (steril) |

=> liegt 100-fach konz. vor  
=> s.o.  
=> liegt 10-fach konz. vor  

Sollte die PFGE nicht sofort laufen, kann das Gel in frischem 0,5 x TBE-Puffer bei Kühlschranktemperatur gelagert werden.

Pulsfeldgelelektrophorese

→ Elektrophoresekommer mit 2000ml 0,5x TBE Laufpuffer befüllen.
→ Gel vorsichtig in Kammer positionieren (Handschuhe!).
→ CHEF III Steuergert, Pumpe und Kühler (Reihenfolge beachten) anschalten.
→ Temperatur des Kühlerätes auf 14°C einstellen und abwarten bis die gewünschte Temperatur erreicht ist.
→ CHEF III Steuergert programmieren
  Block: I, Initial Time: variabel, Final Time: variabel, Run Time: variable Volts: 6, Included Angle: 120,
  Start
  (die gewählten Zeitprogramme sind abhängig vom Bakterienstamm und vom Enzym)

Enzyme und Zeitprogramme für Analyse folgender Spezies:

<table>
<thead>
<tr>
<th>Keim</th>
<th>Enzym</th>
<th>Zeitprogramm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterococcus sp.</td>
<td>Sma I</td>
<td>1 / 25 / 23 Std.</td>
</tr>
</tbody>
</table>

→ Lauf starfen.

Ist die Elektrophorese beendet, wird das Gel vorsichtig aus der Kammer entnommen (Handschuhe) und in einem Ethidiun-Bromid-Bad für 30 – 60min gefärbt. (Ethidium Bromid ist cancerogen und befindet sich in einem separaten Raum (Mikrobiologie Raum 052261), der nur mit vorher angezogenen Nitrile-Handschuhen betreten werden darf).

Nach der Färbung wird das Gel 30 – 60min in Aqua dest. gewaschen, um die Hintergrundfärbung zu reduzieren, anschließend wird es photographiert.

Genaue Angaben zum Herolab Gerät befinden sich in der Geräte-SOP.
Färbelösung zu alt.
Degeneriertes Lysoenzym.
Gel unzureichend gewaschen.

**Indikation:**

Bei gehäuftem Auftreten desselben Bakteriums in einem Bereich (Station) liegt der Verdacht einer Übertragung auf einen Patienten nahe, z.B. durch das Personal. In diesem Fall gibt eine PFGE Aufschluß darüber, ob die untersuchten Stämme identisch sind oder nicht. Die Indikation zu einer PFGE wird von dem verantwortlichen Hygienearzt gestellt.

**Kriterien für die analytische Freigabe der Ergebnisse:**

Abweichung der Kontrollbänder zueinander sollte ≤ 10% betragen. Pearson coefficient.

**Auswertung und Dokumentation:**

Die vergleichende Analyse der Bandenmuster für die einzelnen Stämme kann in folgende Kategorien eingeordnet werden (nach Tenover):

- **Klasse I – identisch:**
  Die Bandenmuster der Proben sind identisch.

- **Klasse II – eng verwandt:**
  Bei einem genetischen Unterschied können 1-2 Banden differieren, die Bandenmuster können als Subtypen bezeichnet werden.

- **Klasse III – vielleicht verwandt:**
  Bei zwei genetischen Unterschieden können 2-3 Banden differieren, die Bandenmuster können als Subtypen bezeichnet werden.

- **Klasse IV – verschieden:**
  Bei drei und mehr genetischen Unterschieden werden mehr als 4 Banden differieren, die Bandenmuster gehören verschiedenen Stämmen an.


**Probenlagerung nach der Untersuchung:**

Die überzähligen Agaroseblöckchen werden in Eppendorfbehältnissen mit 1 ml CHEF-TE überführt und im Kühlschrank gelagert.

**Angaben zur Methodenvalidierung:**

Unterlagen sind im Ordner zur Methodenvalidierung.

Dateiname: AM-MI-511B_PFGE.doc
Qualitätskontrolle:

Auf jedem Agarosegel wird ein enzymverdauter Kontrollstamm von Staphylococcus aureus NCTC 8325 mitgeführt, der als Marker zum Größenvergleich und als interne Qualitätskontrolle dient. 
Je nach Größe des Gels variiert auch die Anzahl der Kontrollen. 
Bei einem Gel mit 20 Spuren werden 3 Kontrollstämme mitgeführt, die sich an Position 1,10 und 20 befinden, bei 30 Spuren sind es 4 Kontrollstämme an Position 1, 10, 20 und 30. 
Die Größe der DNA-Fragmente dieses Kontrollstammes liegt im Bereich 16 – 673 kb.

Literatur: