Wound infection following Hepatopancreatobiliary (HPB) surgery – a measure of predictive surgical and transmission factors and patient outcomes.

L E CHAMBERS

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Wound infection following Hepatopancreatobiliary (HPB) surgery – a measure of predictive surgical and transmission factors and patient outcomes.

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List of abbreviations

AMR - Antimicrobial resistant

CoNS – Coagulase negative staphylococci

COPD - Chronic obstructive pulmonary disease

CPE - Carbapenemase producing Enterobacteriaceae

CRP – C reactive protein

CVA – Crystal violet assay

ECC – *Enterobacter cloacae* complex

ESBL - Extended spectrum beta-lactamase

FBC – Full blood count

IBD - Irritable bowel disease

IBS - Irritable bowel syndrome

IL - Interleukin

HB - Haemoglobin

HCT - Haematocrit

HPB - Hepatopancreatobiliary

MBASO - Mean basophils

MCH - Mean corpuscular haemoglobin

MCHC - Mean corpuscular haemoglobin concentration

MCV - Mean corpuscular volume

MDR – Multi-drug resistant

MEOS - Mean eosinophils

MLYMPH - Mean lymphocytes

MMONO - Mean monocytes

MNEUT - Mean neutrophils

MRSA - Methicillin resistant *Staphylococcus aureus*

MSSA – Methicillin sensitive *Staphylococcus aureus*

NYHA - New York Heart Association

PLT - Platelets

PPE – Personal protective equipment

RBC - Red blood cell

SAP – Surgical antimicrobial prophylaxis

SIRS - Systemic inflammatory response syndrome

SSI - Surgical site infection

VRE - Vancomycin resistant Enterococci

WBC - White blood cell

XDR – Extensively drug resistant

ZOI – Zone of inhibition

Abstract

Incidence of surgical site infections (SSIs) following hepatopancreatobiliary (HPB) surgery can be as high as 20 – 40 %. SSIs, particularly those caused by antimicrobial resistant (AMR) organisms, are a significant burden for both patients and the NHS. The aim of this study was to determine risk factors, incidence and the source of these infections and to measure how bacteria that can cause SSIs can form biofilms. Patients' surgical sites were swabbed before and after surgery as well as different surfaces on the HPB ward. The bacteria were identified and their AMR was determined. Patient demographics, comorbidities and full blood counts were analysed to determine risk factors associated with SSIs. Biofilm assays (crystal violet, XTT and bacterial percentage coverage), using three of the isolates found on patients (*Enterobacter cloacae*, *Enterococcus faecium* and *Staphylococcus haemolyticus*) were conducted. The incidence of SSIs was 23.1 % and risk factors identified included bile leak, use of drains, pancreatic surgery, open surgery, long surgery and long hospital stay. Statistical analysis showed poor post-operative nutrition, post- operative pneumonia and return to the operating theatre as being significant risk factors for SSI. The bacteria found to cause SSIs were all gut commensals that were isolated from the drain fluid and not from the wound swabs, suggesting transmission occurred during surgery. High levels of multi-drug resistant (MDR) and extensively drug resistant (XDR) species were isolated, particularly XDR coagulase negative staphylococci. The surfaces with the most MDR and XDR species included most of the bathroom surfaces, the nurses' phone and computer keyboard, bedside cabinet and the soap dispenser. *In vitro* biofilm assays showed that AMR could develop among bacteria in a polymicrobial biofilm and this could therefore occur within a polymicrobial SSI and hospital setting, making treatment more difficult. It is clear that more needs to be done to prevent SSIs following HPB surgery and that the hospital can still act as a reservoir for MDR and XDR bacteria.

Chapter 1. Introduction

1.1 Introduction to surgical site infections

Surgical site infections (SSI) are defined as an infection that occurs at the site of surgery within 30 days after surgery (Mangram *et al.*, 1999). SSIs are divided into three categories: superficial incisional SSIs that infect the skin and subcutaneous tissue; deep incisional SSIs that effect the deep soft tissue and organ/space SSIs where the infection involves any other part of the anatomy including organs and excluding the incision (Mangram *et al.*, 1999).

Figure 1. Different categories of surgical site infections.
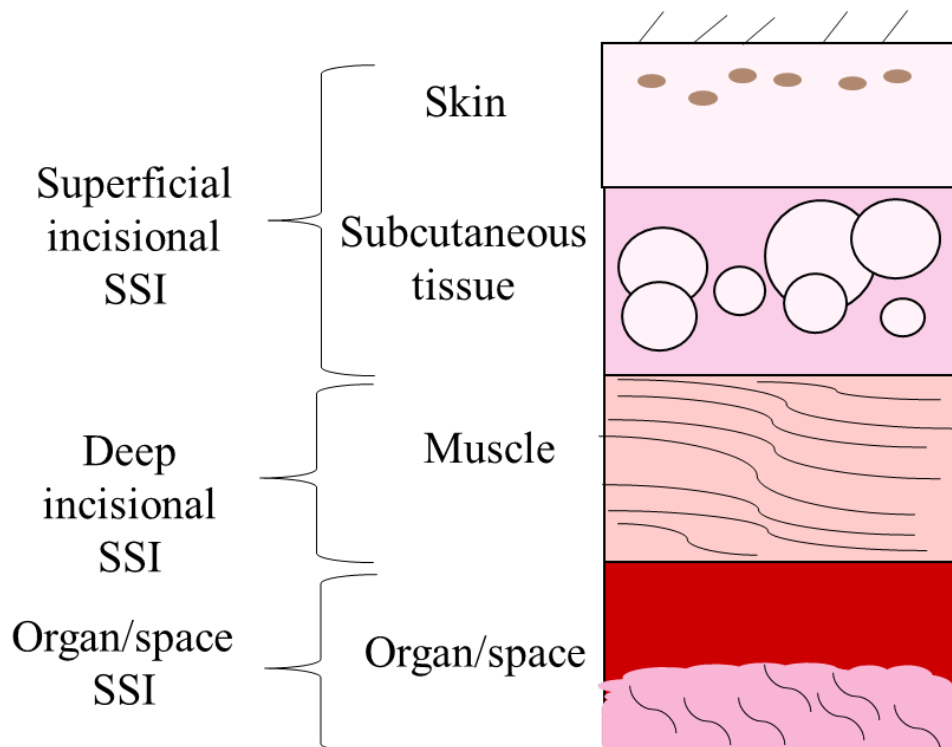
Surgical site infections are the most common type of healthcare associated infection (Zinn *et al.*, 2013). Incidences of SSIs can be as high as 20 %, depending on the procedure and methods of data collection (Leaper *et al.*, 2004). Surgical site infections increase the length of hospital stay (Coello *et al.*, 1993; Plowman *et al.*, 2001; Kirkland *et al.*, 1999) and

this results in an increased financial burden, when considering bed stay, treatment, nursing care and diagnostics (Leaper *et al.*, 2004). The estimated median cost of an SSI to the NHS was £5,239 per patient in 2006 (Urban, 2006). More recent estimates of the cost of SSIs to the NHS are as high as £10,000 per person, with deep-incisional SSIs costing as much as £100,000 per patient (Rothwell, 2020). SSIs also increase mortality rates, and it has been suggested that patients with a SSI are 2 - 11 times more at risk of death compared to patients without a SSI (Olson and Lee, 1990; Engemann *et al.*, 2003; Kirkland *et al.*, 1999). Furthermore, when antimicrobial resistant organisms cause SSIs, this can result in an even higher financial burden and prolonged hospital stay (Bassetti *et al.*, 2013). The number of deaths per year due to antimicrobial resistant infections is currently estimated to be 700,000. However, by 2050 it has been estimated that 10 million deaths per year will occur due to antimicrobial resistant infections. This outweighs the number of yearly deaths caused by cancers (O'neill, 2014). Moreover, it is believed that up to 60 % of SSIs are preventable (Anderson *et al.*, 2014).

In 1990 - 1996, the most common bacteria isolated from SSIs included *Staphylococcus aureus,* Coagulase-negative *Staphylococci* spp., *Enterococcus* spp. and *Escherichia coli* (Mangram *et al.*, 1999)*.* The findings of a public health surveillance report (2006 - 2017) of causative agents of a variety of different types of SSIs in 10,874 inpatients is shown (Figure 2). These results demonstrated that SSIs caused by Methicillin- Resistant *Staphylococcus aureus* (MRSA) had significantly decreased from 25 % in 2006 to 3 % in 2017. The incidence of SSIs caused by Methicillin-Sensitive *Staphylococcus aureus* (MSSA) has also decreased, albeit less significantly, from 14 % in 2006 to 9 % in 2017. However, the rates of SSIs caused by *Enterobacteriaceae* has increased significantly from

14 % in 2006 to 29 % in 2017 (Public Health England, 2017). As well as an increase in SSIs

caused by *Enterobacteriaceae,* there has also been an increase in antimicrobial resistant

strains of *Enterobacteriaceae*, particularly carbapenemase producing *Enterobacteriaceae*

(CPE), which creates even more problematic issues (Elgohari *et al*., 2017).
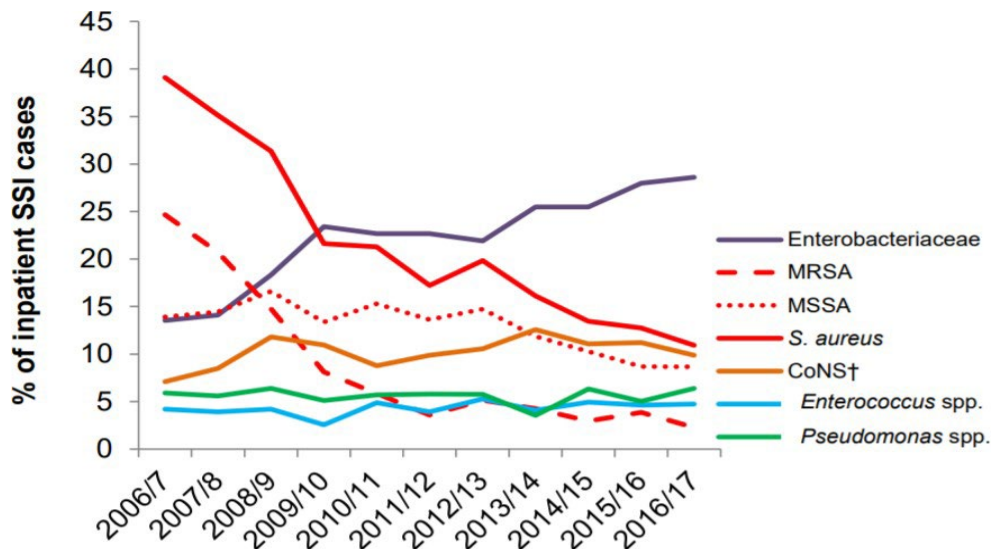
Figure 2. Trends in microorganisms reported as causing inpatient SSIs, all surgical categories in NHS hospitals, UK. From: Public Health England. Surveillance of surgical site infections in NHS hospitals in England, 2016 to 2017. †Coagulase-negative Staphylococcus

Over the past 30 years, postoperative hospital stay has steadily decreased (CDC, 1992).

This underlines the need for post discharge surveillance, because many patients may not

follow preventative measures for the development of SSIs in their own home, and in

addition they are exposed to organisms found in the community (Manniën *et al*., 2006).

Recently some healthcare institutes have started using a web-based application that

outpatients can use to monitor their wounds and thus detect an SSI promptly (Sanger *et*

al., 2017). This involves the patients sending photographs of their surgical wounds via text

or email to the hospital, this results in early diagnosis and fewer unnecessary hospital

visits (Sanger *et al*., 2016).

Approximately 16 % of patients that undergo HPB surgery are readmitted (Lucas *et al.*, 2013) with pancreaticoduodenectomy having the highest readmission rates of all surgery (15 % - 20 %) (Martin *et al.*, 2011). Furthermore, the incidence of SSIs after hepatectomy has been reported to be 3.1 % – 14 % (Harimoto *et al.*, 2011; Moreno Elola-Olaso *et al.*, 2012; Virani *et al.*, 2007). A surveillance report by the ECDC found that between 2014 and 2017, there was a statistically significant increasing trend for both the percentage of SSIs and the incidence density of SSIs following laparoscopic cholecystectomy in Europe (European Centre for Disease Prevention and Control, 2019).

1.2 Physiology of organs

1.2.1 Physiology of liver and liver Cancer

The liver is an important organ that has multiple functions which include fat-soluble vitamin storage and/or metabolism, bile production, bilirubin metabolism, thyroid hormone function and drug metabolism (Kalra *et al.*, 2022). Hepatocytes are divided into three zones. These include zone I which is the periportal region and plays a large role in oxidative metabolisms such as beta-oxidation, gluconeogenesis, bile formation, cholesterol formation, and amino acid catabolism. As this zone is closest to oxygenated blood and nutrients it is the first to regenerate. Zone II sits between zone I and zone III and is defined as the pericentral region. Zone III helps with detoxification, biotransformation of drugs, ketogenesis, glycolysis, lipogenesis, glycogen synthesis, and glutamine formation. It has the lowest perfusion due to its distance from the portal triad (Kalra *et al.*, 2022).

The immune response of a healthy liver is divided into physical, chemical and immunological defences. Physical defences include the biliary sphincter and hepatic

junctions, and these act as a barrier to microorganisms. Bile salts protect the liver against infection by providing a chemical barrier. Kupffer cells are a type of macrophage that are found in large quantities in the liver. These cells stop toxins and enteric organisms from passing into the blood stream via the hepatic portal vein. Patients undergoing liver resections have fewer Kupffer cells and are therefore at a greater risk of developing sepsis (Jarnagin, 2016). Other immunological defences include immunoglobulin A (Emmrich *et al.*, 1998; Scott-Conner and Grogan, 1994), fibronectin (Wilton *et al.*, 1987) and complement factors (Sumiyoshi *et al.*, 1997). Patients with cancer are immunosuppressed due to upregulation of T regulatory cells and myeloid-derived suppressor cells, unresponsive and/or decreased T cells, inflammatory cytokine release and immunosuppressive cell signalling receptors and/or ligands and are therefore more prone to developing infections (Hotchkiss *et al.*, 2013). This lack of immunity among liver cancer patients is likely to be a contributing factor to the high infection rates (20 % - 40 %) following HPB surgery (Ceppa *et al.*, 2013).

Different types of primary liver cancer include hepatocellular carcinoma, liver angiosarcoma and hepatoblastoma (Figure 3). Hepatocellular carcinoma is the most common primary hepatic malignancy and develops in the hepatocytes. Hepatic angiosarcoma is rare and only accounts for 2 % of primary liver tumours (Mani and Van Thiel, 2001). Hepatic angiosarcoma originates from endothelial cells (Selby *et al.*, 1992). Hepatoblastoma is extremely rare and usually found in children as it originates from the primitive hepatic stem cells during embryogenesis of the liver (Wu *et al.*, 2017). Hepatectomy or liver resection is the surgical procedure performed to remove a tumour from the liver (Tsim *et al.*, 2010).

1.2.2 Physiology of the pancreas and pancreatic cancer

The pancreas is responsible for producing bile and various hormones. These hormones include insulin, amylin, glucagon, somatostatin, ghrelin and pancreatic polypeptide (El-Sayed and Mukherjee, 2019). The pancreas is divided into three sections; the head is the large, rounded section next to the duodenum, the body of the pancreas is the middle section and the tail of the pancreas is the narrow section. Pancreatic malignancies are divided into endocrine and exocrine subtypes. Pancreatic ductal adenocarcinomas account for 85 % of pancreatic cancers and are therefore the most common type of pancreatic malignancy (Dindyal and Spalding, 2019). Pancreatic ductal adenocarcinomas are exocrine neoplasms and effect the epithelial cells of the pancreatic ducts. Other exocrine tumours include mucinous tumours, intraductal papillary mucinous neoplasms and solid pseudo papillary tumours. Endocrine pancreatic cancers only account for 5 % of pancreatic cancer and most of these are non-functioning neuro- endocrine tumours (Dindyal and Spalding, 2019). The most commonly used surgical procedure for the removal of pancreatic cancer at the head of the pancreas is

Whipple pancreatoduodenectomy; this involves resection of the distal stomach or a variant that preserves the pylorus (Dindyal and Spalding, 2019). A pancreatoduodenectomy can be carried out using a laparoscopic or an open procedure.

1.2.3 Physiology of biliary duct and biliary duct cancer

The bile ducts are channels that connect the liver and gallbladder to the small bowel. The bile ducts carry bile, which is the fluid responsible for breaking down fats in food. The bile ducts consist of the left and right hepatic ducts, which come from the liver, and together these form the hepatic ducts. The cystic duct comes from the gallbladder, the hepatic

duct and cystic duct both form the common bile duct. Cancer of the bile duct is referred

to as cholangiocarcinoma. There are two main types of bile duct cancer; intrahepatic

cholangiocarcinoma, which is found in bile ducts within the liver and extrahepatic bile

duct cancer, which may form in hilum region or the distal region. Surgical interventions

for biliary duct malignancies include hemihepatectomy, which is used for hepatic duct

cancers, and pancreatoduodenectomy, which is used for distal and middle bile duct
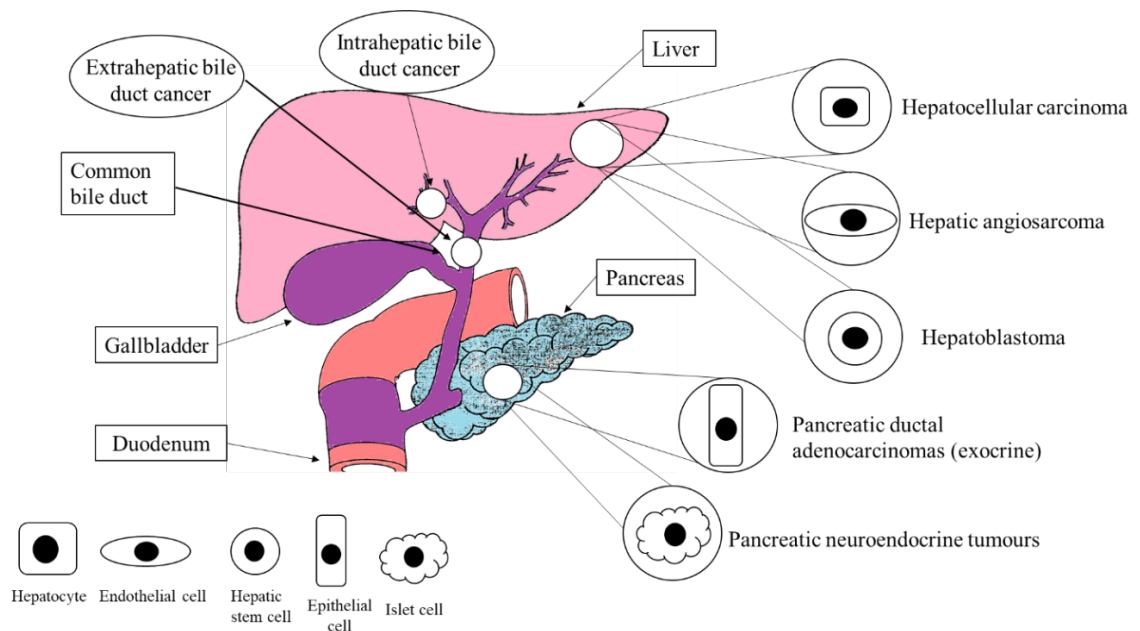
cancers (Seyama and Makuuchi, 2007).

Figure 3. Physiology of the hepatopancreatobiliary tract and locations of different types of cancer.

1.3 Risk factors of surgical site infections

Predisposing risk factors (such as comorbidities), intraoperative surgical risk factors and

post-operative risk factors of SSIs following HPB surgery have been studied and these are

discussed.

1.3.1 Predisposing risk factors of surgical site infections

Diabetes

Research on the development of SSIs in patients with diabetes is conflicting. One study found that patients who had a pancreatoduodenectomy who had diabetes mellitus had a significantly lower (p = 0.014) incidence of SSIs (Barreto *et al.*, 2015). However, other studies have found that when patients with diabetes undergo surgery, they are at a greater risk of developing an SSI (Martin *et al.*, 2016; Yang *et al.*, 2014; Finney *et al.*, 2003; Kneuertz *et al.*, 2012). For example, a meta-analysis of 14 studies found that patients with diabetes were almost twice as likely to develop an SSI when compared to non-diabetic patients (Zhang *et al.*, 2015). A number of reasons can explain the higher rates of SSIs in diabetic patients; firstly, diabetic patients often suffer from small vessel disease where there is a decrease of nutrients and oxygen flow to peripheral tissues and thus a reduced systemic ability to fight infections (Turina *et al.*, 2005). Secondly, high blood glucose levels impair the function of monocytes and leukocytes (Mowat and Baum, 1971; Bagdade *et al.*, 1978; Delamaire *et al.*, 1997). Finally, diabetic patients often experience peripheral neuropathy and this decreases the release of neuropeptides, disrupting the healing response (Twigg *et al.*, 2001). Another meta-analysis looking into SSIs and diabetes concluded that both pre- and post-operative hyperglycaemia were associated with an increased incidence of SSI. However, diabetes remained a significant risk factor for SSI even when hyperglycaemia was controlled. The reason for this is unknown but it could be because diabetes often causes vascular problems and white blood cell dysfunction (Martin *et al.*, 2016).

Poor glucose control has been associated with an increase of SSIs following HPB surgery. Ambiru *et al*., (2008) conducted a study involving 265 patients undergoing HPB surgery for cancer in a hospital in Japan. The findings showed that when the glucose levels were not maintained at a level of <200 mg/dL, 52 % of patients developed an SSI in comparison to 20 % when glucose levels were adequately controlled (Ambiru *et al*., 2008).

Smoking

Nicotine use is known to delay primary wound healing (Mangram *et al*., 1999; Nagachinta *et al*., 1987; Nolan *et al*., 2017; Jones and Triplett, 1992; Daly, 2009) and thus the longer a wound takes to heal, the more likely it is to become infected. Carbon monoxide binds to haemoglobin and shifts the oxyhaemoglobin dissociation curve to the left, which ultimately reduces the oxygen supply and could contribute to the development of an SSI (Rietbrock *et al*., 1992; Sørensen, 2012; Hopf *et al*., 1997). Nicotine can also cause vasoconstriction and therefore reduce blood circulation (Rejali *et al*., 2005). Another factor to consider is that smoking is known to cause respiratory and cardiovascular disease and thus it might be these clinical manifestations that increase the risk of developing an SSI and not primarily smoking alone (Messner and Bernhard, 2014).

Research has shown that CO levels can reduce significantly in only 12 hours after smoking cessation (Woehlck *et al*., 1999; Shannon-Cain *et al*., 2002). However, a study found that smoking cessation prior to surgery does not necessarily reduce the incidence of SSIs following gastrointestinal surgery (Kuri *et al*., 2011), therefore implying that the long-term effects of smoking make individuals more susceptible to SSIs, possibly due to such factors as exposure to toxins in the cigarettes.

Obesity

Obesity is a known risk factor for many types of SSI, although many obese patients may also have other comorbidities such as cardiac and respiratory problems, so it is difficult to know if obesity alone is a causative risk factor of SSIs (Pantalone *et al.*, 2017). From April 2016 to March 2017, a surveillance study of SSIs was conducted across 201 NHS hospitals and 8 independent sector NHS treatment centres in the UK and included 139,691 patients of which 1,635 developed SSIs, 495 of the operations included in this study were HPB. Of the patients undergoing HPB surgery, between April 2015 and March 2016, 43.7 % were classified as obese (BMI ≥ 30 kg/m^2) in comparison to 39 % from April 2016 to March 2017, with HPB surgery and cholecystectomy patients having the highest rates of obesity (Public Health England, 2017). A study in Shanghai, China aimed to identify risk factors for SSIs following hepatic resection in 7,388 patients, between 2010 and 2011; of these participants, 27.3 % were obese. Results showed that obesity significantly predicted incisional SSIs but not other forms of SSIs (Yang *et al.*, 2014). High infection rates in obese patients are likely due to tissue oxygen pressure and Kabon *et al.*, (2004) concluded that wound and tissue hypoxia commonly occurred in obese patients perioperatively (Kabon *et al.*, 2004). SSIs may also occur due to reduced blood circulation in fat tissues and subsequently a reduced circulation of immune cells throughout the tissues in obese patients (Nyström *et al.*, 1987).

Weight loss/anorexia

Weight loss is a common problem in patients undergoing surgery, in particular those with malignancies and those receiving treatment for cancers; for example, weight loss is often a side effect of chemotherapy (Sánchez-Lara *et al.*, 2013). Depending on treatment,

location and type of tumour 40 % - 80 % of cancer patients suffer some form of malnutrition (Sánchez-Lara *et al.*, 2013). Malnutrition is considered a risk factor for the development of SSIs and the European Society for Parenteral and Enteral Nutrition (ESPEN) guidelines state that surgery should be postponed in patients of a high nutritional risk status until their nutritional status improves (Weimann *et al.*, 2006). An American study found that pre-operative weight loss of > 4.5 kg was more common ($p = 0.03$) in the 1744 participants who were undergoing colorectal, liver or pancreatic resections (Ejaz *et al.*, 2017). However, Kneuertz *et al.*, (2012) found that recent weight loss ($p = 0.69$) and being underweight ($p = 0.55$) were not associated with the development of SSIs following HPB surgery (Kneuertz *et al.*, 2012).

1.3.2 Factors leading to infection prior to surgery

Skin preparation

If surgical wounds are infected from the patient's own skin flora, then disinfection of the patient prior to surgery may result in a decreased incidence of SSIs. There are different methods that can be used to disinfect the skin prior to surgery, for example an antiseptic solution may be applied to the surgical site prior to surgery, or the patient may shower and wash using an antiseptic solution.

The National Institute for Health and Care Excellence (NICE) guidelines state that an alcohol-based solution of chlorhexidine is the first choice of antiseptic and must be used immediately before skin incision (National Institute for Health and Care Excellence, 2019). A randomised control trial of patients undergoing general surgical procedures found that operative skin preparation using chlorhexidine-alcohol (9.5 %) significantly reduced the SSI rates by 41 % compared to povidone-iodine (Darouiche *et al.*, 2010). Pre-operative

antiseptic showering is not commonplace in the NHS, although it has been shown that rates of infection were 2.3 % in those who did not shower at all, 2.1 % in patients who showered with ordinary soap and infection rates were reduced to 1.3 % among patients who showered with disinfecting soap containing hexachlorophene (Schwartz *et al.*, 1999).

The NICE guidelines suggest that hair should not be removed from the surgical site prior to surgery unless completely necessary and should be performed using a clipper with a single-use head (National Institute for Health and Care Excellence, 2008). Razor use can increase the chance of SSI as they cause epidermal injury, therefore, NICE suggests avoiding the use of razors. One study found that the incidence of SSIs in patients who had hair removed with clippers was 11.2 %, which was significantly less than that the group who had hair removed with a razor (20 %) (Kurien *et al.*, 2018).

1.3.3 Factors leading to infection during surgery

Operating times

The literature shows that operating times of over 2 hours increased the risk of SSIs (Schwartz *et al.*, 1999). One study, using 4817 participants who had undergone a pancreatoduodenectomy found that an increased operative time was associated with increased mortality (p = 0.001) and morbidity (p = 0.0001) (Ball *et al.*, 2010). Another study found that operating time was significantly longer in patients that developed SSIs following liver resection (p = 0.002) (Fukami *et al.*, 2019). Razavi *et al.* (2005) study found that duration of surgical operation was a significant risk factor when operations lasted 30 minutes or less, 3 % of patients developed an SSI whilst when operations lasted 6 hours or over the risk of SSI increased to 18 % (Razavi *et al.*, 2005).

Type of surgery

Laparoscopic (or keyhole) surgery is becoming more of a standard procedure and there is much evidence to suggest that infection rates are lower in laparoscopic procedures than open surgery. For example, one study found that laparoscopic cholecystectomies had a 1.1 % SSI rate whilst open cholecystectomies had a 4 % infection rate (Boni *et al*., 2006). In a cased-matched control study of 50 patients, López-Ben *et al*., (2014) found that laparoscopic hepatectomies in comparison to open hepatectomies resulted in an increased incidence of SSI. Rates of SSI in laparoscopic surgery patients were 2 % whilst 18 % of open surgery patients developed an SSI (López-Ben *et al*., 2014). However, this study also found that the mean operating time for laparoscopic surgery was 95 minutes longer than for open surgery and this finding contradicts what was previously discussed as longer operating times are associated with higher SSI rates (Fukami *et al*., 2019). One reason that laparoscopic surgery may reduce the risk of developing an SSI is because the surgical site is smaller and therefore there is a smaller surface area to become contaminated. A meta-analysis found that laparoscopic abdominal surgery compared to open surgery reduced SSI incidence by 70 % - 80 % in obese patients (Shabanzadeh, 2012).

Use of drains during surgery

Drains are often used at the end of surgery to allow the drainage of liquids such as blood away from the surgical dead space to improve wound healing and prevent infections (Scevola *et al*., 2002). In HPB surgery, drains may be used to remove bile as it is toxic to surrounding tissues (Petrowsky *et al*., 2004). For example, the retention of bile may result in liver injury because it induces apoptosis or necrosis of hepatocytes (Attili *et al*., 1986).

Results from randomized control trials have shown that in hepatic surgery the use of drains may not reduce infection rates and may in fact increase the risk of infections in some patients undergoing hepatectomy (Petrowsky *et al*., 2004). However, one meta-analysis found that prophylactic drains did not reduce the occurrence of bile collections and this contradicts the objective of this technique (Petrowsky *et al*., 2004). Furthermore, drains may act as a channel for bacteria to spread to the wound thus increasing the risk of SSIs (Willett *et al*., 1988; Dougherty and Simmons, 1992). Late removal of surgical drains can increase the risk of infections including wound infections (Bassi *et al*., 2010). It has been found that retrograde drain infections increase when drain placement is prolonged for more than four postoperative days (Shirata *et al*., 2017).

Blood Transfusion

The literature is contradictory in regard to blood transfusion as a risk factor for SSIs. Ball *et al*., (2010) reported that transfusion of red blood cells after pancreatoduodenectomy was associated with increased morbidity within 30 days after surgery (Ball *et al*., 2010). Furthermore, Zhang *et al*. (2016) found that patients who had a blood transfusion were 3.2 times more likely to develop an infection (Zhang *et al*., 2016). However, other studies have found that blood transfusion was not associated with increased infection rates (Sutton *et al*., 2014; Ecker *et al*., 2016). Although blood transfusion is often necessary in complex HPB procedures with high blood loss, it has been reported that many blood transfusions during pancreatoduodenectomies do not meet predetermined criteria and are therefore not necessary (Ross *et al*., 2013). Thus, it is important blood transfusions are only used when patients fit the predetermined criteria.

1.3.4 Factors leading to infection post-surgery

Bile leakage

Bile leakage is common complication following liver resection (Fukami *et al*., 2019). Bile leakage can result in the release of pancreatic enzymes, which can digest tissues and therefore result in bacterial infections and inflammation (Nagai *et al*., 1989; Naruse *et al*., 2000; Zhang *et al*., 2003). In one study 10.5 % of 458 patients undergoing hepatic resection developed a bile leakage, and of these 7 % (p = 0.003) developed an SSI (Braunworth *et al*., 2019). It has been shown that repeat hepatectomy was a risk factor for both SSIs and bile leakage and therefore re-operation was a major risk factor for SSIs after HPB surgery (Sadamori *et al*., 2013).

1.4 Bacteria involved in hepatopancreatobiliary surgical site infections

One study investigated risk factors, clinical impact and preventative methods of SSI in patients undergoing hepatectomies for hepatocellular carcinoma. The causative microorganisms of incisional SSIs were found to be methicillin-resistant *Staphylococcus aureus* (MRSA) (29 %), coagulase-negative *Staphylococci* (CoNS) (21 %), *Enterobacter cloacae* (12.5 %), methicillin-sensitive *Staphylococcus aureus* (MSSA) (8 %), *Klebsiella* spp. (4 %) and *Enterococcus faecalis* (4 %). The causative microorganisms of organ and space SSIs were CoNS (33 %), *Enterococcus faecalis* (14 %), MRSA (12 %), *Enterococcus faecium* (10 %), MSSA (8 %), *Enterobacter cloace* (5 %), *Streptococcus* spp., *Bacteroides* spp., *Escherichia coli*, *Klebsiella* spp., *Candida* spp. (3 %), *Serratia* spp., *Pseudomonas* spp. and other *Enterococcus* spp. (1 %) (Shirata *et al*., 2017). Another study found that *Enterococcus* spp. (*n* = 59) were the leading cause of SSIs following HPB surgery followed

by *S. aureus* (*n* = 23 MSSA, *n* = 14 MRSA), *Klebsiella* spp. (*n* = 18), *Pseudomonas aeruginosa* (*n* = 13) and *Enterobacter* spp. (*n* = 10) (Takahashi *et al.*, 2018). It can therefore be concluded that Gram-positive surgical site infections are usually caused by *Staphylococcus* spp. including MRSA, CoNS and *Enterococcus* spp., whilst Gram-negative surgical site infections are not as common but are often caused by *Enterobacter* spp., *Klebsiella* spp. and *Pseudomonas* spp.

1.5 Key resistant bacteria

The emergence of antimicrobial resistance is due to a number of factors. Firstly, a major factor is the overuse and misuse of antibiotics, for example prescribing antibiotics in the absence of a bacterial infection e.g. when a patient has a viral infection. Resistance may also occur when a patient is given a sublethal dose or does not finish their full course of antibiotics (Ayukekbong *et al.*, 2017). The genes which encode antimicrobial resistance mechanisms can be found on the chromosome or on plasmids. Plasmids are transposable and thus can be spread among bacteria via horizontal gene transfer (Summers, 2006).

1.5.1 Methicillin resistant *Staphylococcus aureus* (MRSA)

Staphylococcus aureus is a commensal of the nasal passage in approximately 30 % of the population (Wertheim *et al.*, 2005). As well being a coloniser, *S. aureus* can be pathogenic, causing a variety of skin infections and SSIs (Lowy, 1998). Methicillin resistant *Staphylococcus aureus* has been described as a great threat in both the community and hospitals (Andersson *et al.*, 2011). However, the incidence of MRSA has decreased significantly in the past 10 years (Figure 2). In 2001, the UK Government introduced mandatory reporting of MRSA bacteraemia in NHS hospitals (Edgeworth, 2011). This was introduced because of an increase in epidemic MRSA (EMRSA) (EMRSA-15 and EMRSA-16)

in UK hospitals with > 40 % of *S. aureus* bacteraemia isolates being found to be resistant to methicillin (Pearson *et al.*, 2009). Alongside reporting of cases, another reason for the reduction in MRSA SSIs in the UK, is likely the introduction of the screening of inpatients for MRSA. Where patients were swabbed before surgery and then subsequently decontaminated if MRSA was isolated.

Penicillin binding protein (PBP) are proteins involved in the formation of peptidoglycan, which is a building block of the bacterial cell wall. Methicillin inhibits PBPs and thus interferes with the construction of the bacterial cell wall (Stapleton and Taylor, 2002). There are two main mechanisms of methicillin resistance in *S. aureus*. Firstly, *mecA* is a gene found in bacteria, which encodes a low affinity PBP 2a and results in methicillin not being able to bind to the PBP as it normally would, resulting in methicillin resistance (Beck *et al.*, 1986). *mecA* is located on the Staphylococcal cassette chromosome (SCC) (Hiramatsu *et al.*, 1999; Katayama *et al.*, 2000). Secondly, methicillin resistance in *S. aureus* can occur through enzymatic inactivation of the antibiotic (Pantosti *et al.*, 2007). *bla*Z is one of the genes responsible for the production of β-lactamase, which is an enzyme that hydrolyses β-lactams, this means that the β-lactam cannot target the cell wall and kill the bacteria via cell lysis (Lowy, 2003).

1.5.2 Enterobacteriaceae

Before 2000, carbapenemase producing Enterobacteriaceae (CPE) were rare and most resistance was attributed to AmpC beta-lactamases, extended spectrum beta-lactamases (ESBL) or porin deficiency (Doi and Paterson, 2015). Due to the more recent dissemination of CPE, the World Health Organisation (WHO) has classified carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and carbapenem-resistant and third

generation cephalosporin-resistant Enterobacteriaceae as a critical priority (Tacconelli *et al*., 2018). The hospitalized and critically ill are most at risk of invasive CPE infections and these infections can result in mortality in up to 40 % of cases (Doi and Paterson, 2015). The most prevalent types of carbapenemases across the globe are KPC, VIM, IMP, NDM, and OXA-48 are routinely reported as the cause of infections (Hansen, 2021).

CPE and their resistance plasmids can spread rapidly, resulting in outbreaks in healthcare facilities. One notable outbreak occurred in the northwest of England and involved *Klebsiella pneumoniae* carbapenemase (KPC) producing Enterobacteriaceae (Cantón *et al*., 2012). Another outbreak of Enterobacteriaceae, capable of producing the carbapenemase OXA-48, occurred in a renal unit in London (Thomas *et al*., 2013).

1.5.3 Vancomycin resistant Enterococci (VRE)

Vancomycin resistant Enterococci (VRE) do not usually cause infections in the community, however, they often cause nosocomial infections (Gastmeier *et al*., 2014). For example, in Europe, *Enterococcus* spp. are responsible for 9.6 % of all nosocomial infections (Gastmeier *et al*., 2014). VRE bloodstream infections result in a significantly higher mortality rate when compared to vancomycin susceptible enterococci blood stream infections (Song *et al*., 2003; Salgado and Farr, 2003). A retrospective matched case-control study, conducted in Germany, compared hospital costs of 42 individuals with nosocomial VRE infections and 42 individuals with nosocomial vancomycin sensitive Enterococcus (VSE) infections. It was found that in those with VRE the median overall hospital costs were higher than those with VSE infections (EUR 37,971 vs. EUR 23,025; p = 0.049) (Puchter *et al*., 2018).

1.5.4 Resistant bacteria in the Northwest England

In hospitals in Manchester, there has been an observed higher number of cases of

carbapenemase producing Enterobacteriaceae, with one report stating that between 2009 and 2017, 60 people had died from CPE blood-stream infections in a hospital in Manchester. It is thought that the main source of these infections are poorly designed sinks which allow contaminated water to splash back onto patients (Davies, 2017). These alarming rates of CPE in Manchester hospitals made national news and resulted in an increased surveillance effort as well as improved sink facilitates to stop harbouring these resistant organisms (Trepanier *et al*., 2017; Davies, 2017).

1.6 Transmission of bacteria to surgical site infections

1.6.1 Transmission from endogenous sources

The source of microbial contamination of surgical sites is thought to be usually from the endogenous microbes on a patient's skin (Ayliffe, 1991) and these bacteria are usually Gram-positive cocci, such as *Staphylococcus* spp. If the surgical site is near the perineum or groin, then faecal microbes such as anaerobic bacteria and Gram-negative aerobes may cause SSIs (Sganga *et al*., 2016).

The skin is the largest organ in the body and is home to many different species of colonizing bacteria. It is estimated that human commensal bacteria are equally as abundant as human cells (Sender *et al*., 2016). The type of bacteria found on the body depends on the area of the body and the type of skin. *Propionibacteria* spp. are more likely to colonise sebaceous sites whilst *Staphylococcus* spp. and *Corynebacterium* spp. are more likely to colonise warm and moist areas such as the feet, armpits and bends of the elbows (Byrd *et al*., 2018). Commensal bacteria play an important role in preventing pathogenic bacteria colonizing the skin as they take up space and nutrients, thus outcompeting pathogenic bacteria and this phenomenon is termed 'colonization

resistance' (Buffie and Pamer, 2013). Bacteria can also produce antimicrobial bacterial peptides (AMPs) which can protect the host from infection by killing other bacteria (Hassan *et al.*, 2012). Some species of bacteria are skin commensals that may turn pathogenic in the right circumstances, for example, *S. aureus* is a skin colonizer but when *S. aureus* enters the bloodstream it can result in an inflammatory response which could be fatal (Kwiecinski and Horswill, 2020). Dysbiosis is defined as when changes in the microbiome results in an increase of disease-causing microbes and thus may predispose an individual to disease. Factors that may cause dysbiosis include taking antibiotics or a weakened immune system (DeGruttola *et al.*, 2016).

In terms of SSIs, skin commensals may contaminate the surgical wound during or after surgery and result in an SSI, therefore transforming the commensal skin bacteria from a colonizer to a pathogen. To avoid contamination of a patients' surgical site from their own microflora, it is important that the area of surgery is cleaned adequately with an antiseptic solution.

Many skin commensals such as *S. aureus* and *S. epidermidis* may be spread into the environment by skin scales and approximately 10^6 bacteria are shed from an individual each day (Davies and Noble, 1962). An early study looked at the bacterial composition of wound washing and found that 50 % of the strains isolated were from the patients' own skin and 20 % of strains were identical to those of surgical staff (Burke, 1963
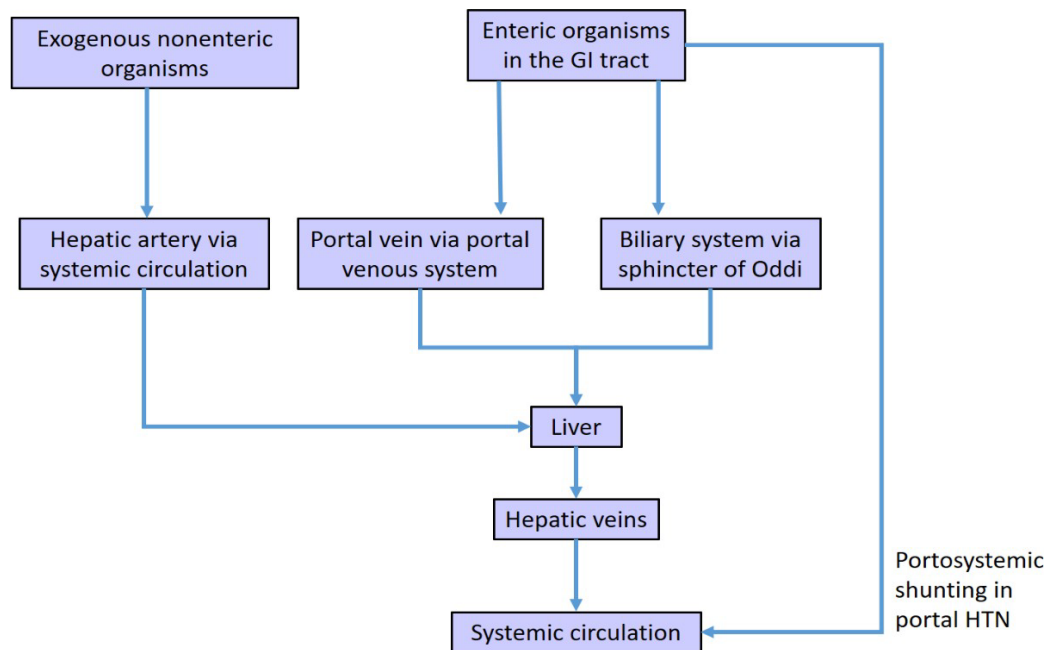
Figure 4. Exogenous organisms and organisms from the GI tract may enter the blood stream. Adapted from Jarnagin, 2016.

Gastrointestinal surgery may result in gastric microbes causing the infection such as Gram-negative bacilli (e.g. *E. coli*) and Gram-positive organisms (e.g. *Enterococcus* spp.) (Altemeier *et al*., 1968). During surgery, pathogens from the gut may enter the liver via the portal venous system, which carries blood to the liver from the gastrointestinal tract (Figure 4). Since the gut contains many bacteria, this may result in liver infection if the immune response is not functioning properly (Jarnagin, 2016). The sphincter of Oddi in the biliary system is another way in which enteric organisms may enter the liver and eventually the blood stream. The hepatic artery can be used by exogenous organisms to invade the liver and the blood stream via systemic circulation. Endocarditis therefore increases the risk of both liver and blood stream infections (Jarnagin, 2016).

1.6.2 Transmission from exogenous sources

Hospital surfaces act as reservoir for many different microbial pathogens and this has been shown in papers published as early as the 1960s (Sanborn, 1963). Evidence of the role in contaminated hospital surfaces in the development of hospital-acquired infections can be shown by modelling transmission routes. For example, Lawley *et al*., (2010) used a murine model to show how *Clostridium difficile* was spread through contaminated cages (Lawley *et al*., 2010). In another study, researchers placed cauliflower mosaic virus on a telephone in a neonatal intensive care unit. Various sites on the wards were tested for viral DNA to determine how contamination may occur and it was shown that the virus spread rapidly to different areas. The sites that were most likely to contain the viral DNA were blood gas analysers, computer mice, telephone handles, medical charts, ventilator knobs, door handles, radiant warmer control buttons, patient monitors, personnel hands, the nurse's station, resident physician charting area, changing room and staff break room (Oelberg *et al*., 2000). This highlights the need for regular cleaning of touch points as these act as fomites and pathogens may be passed between healthcare workers hands, the hospital environment and patients. The way in which bacteria are spread among a hospital ward may be different to viral DNA although this study does provide a helpful insight into how nosocomial pathogens can be transmitted.

Disinfection of hospital surfaces results in a decrease of hospital acquired infections (Dancer, 2014). However, Manian *et al*. (2011) found that MRSA and *Acinetobacter baumannii* could still be found in 27 % of rooms that had been bleached four times (Manian *et al*., 2011). A diverse selection of organisms can be found on the floor of hospitals due to high numbers of people walking between wards and the environment

outside the hospital and carrying organisms on their shoes. The use of disinfectant on hospital floors has been found to reduce the number of organisms by 90 % - 95 %, however, within only 1 - 2 hours the number of organisms reaches the peak it was originally, and prior to cleaning (Ayliffe, 1991).

Vancomycin resistant Enterococci (VRE) have been shown to be able to survive on surfaces for up to 4 years (Wagenvoort *et al.*, 2011). As Enterococci are one of the most common species isolated from HPB surgical sites (Jarnagin, 2016) the fact that this species may survive for extended periods on surfaces may be the reason for high infection rates. Furthermore, it has been estimated that VRE hand contamination is acquired from 10 % of contacts from either the patient or the immediate area surrounding the patient (Hayden *et al.*, 2008).

A systematic review found that many species of Gram-positive and Gram-negative organisms are able to persist for months at a time, the duration of persistence on fomites for different pathogens which are likely to be found in HPB surgical sites are summarised (Table 1). Gram-negative organisms are generally able to survive on inanimate objects for longer periods of time with *Klebsiella* spp. persisting for up to 30 months and *E. coli* and *P. aeruginosa* surviving on surfaces for up to 16 months (Dickgiesser, 1978).

Table 1 .The duration of persistence of pathogens on dry inanimate surfaces. Adapted from (Kramer *et al.*, 2006)

Species of bacteria	Duration of persistence (range)
Escherichia coli	1.5 hours – 16 months
Enterococcus spp. including VRE and VSE	5 days – 4 months
Klebsiella spp.	2 hours to > 30 months
Pseudomonas aeruginosa	6 hours – 16 months; on dry floor: 5 weeks
Serratia marcescens	3 days – 2 months; on dry floor: 5 weeks
Shigella spp.	2 days – 5 months
Staphylococcus aureus, including MRSA	7 days – 7 months
Streptococcus pneumonia	1 – 20 days
Streptococcus pyogenes	2 days – 6.5 months

1.7 Systemic Inflammatory Response Syndrome and Sepsis

Sepsis is a complication of SSIs. The systemic inflammatory response (SIRS) was first

defined at the American College of Chest Physicians/Society of Critical Care Medicine

Consensus Conference in Chicago in 1991 where the aims of this conference were to

further categorise sepsis (Bone *et al.*, 1992). The conclusions of this conference were that

SIRS is defined as the body's reaction to trauma, burns, infections and pancreatitis and

other insults. SIRS is characterised by two or more of the following clinical manifestations:

body temperature above 38°C or below 36°C; heart rate above 90 beats per minute;

respiratory rate greater than 20 breaths per minute or hyperventilation, manifested by a

$PaCO_2$ of less than 32 mm Hg and a white blood cell count greater than 12,000/cu mm or

fewer than 4,000/cu mm or more than 10 % immature neutrophils, without a known

cause of abnormal white blood cell count such as chemotherapy, neutropenia and

leukopenia (Bone *et al.*, 1992). Sepsis is defined as the systemic response to an infection, although the clinical manifestations are identical to SIRS, this makes it difficult to determine if the inflammatory response is due to an infection or another means such as trauma if there are no visible signs of trauma or infection (Balk and Bone, 1989). Multiple organ dysfunction is when organ function cannot maintain homeostasis, this may be absolute or relative. For example, relative organ dysfunction could be when a patient has normal cardiac output and systemic oxygen delivery, yet tissues are not oxygenated adequately (Bone *et al.*, 1992). Severe sepsis is defined as sepsis with one of the following: hypoperfusion abnormality, sepsis-induced hypotension or organ dysfunction (Bone *et al.*, 1992).

In 2001, a group of experts met to revisit the 1992 sepsis guidelines and concluded that there was not enough evidence to suggest a change in the definitions of sepsis, although the list of signs and symptoms should be increased to reflect clinical bedside experience and the diagnostic criterion for SIRS is oversensitive and nonspecific (Table 2) (Levy *et al.*, 2003). Thus, a new classification scheme for sepsis was formed and named The predisposition, infection (or insult), response and organ dysfunction (PIRO).
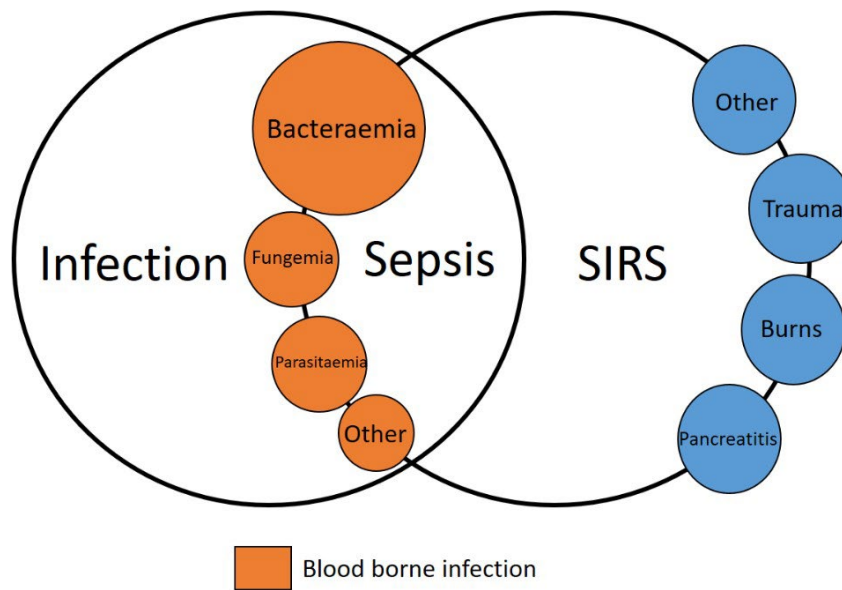
Figure 5. Diagram showing the relationships between infection, sepsis and SIRS. Other non-infectious causes of SIRS include surgery, ischemia and autoimmune disorders. Adapted from: (Bone *et al.*, 1992).

Table 2. The predisposition, infection (or insult), response and organ dysfunction (PIRO) system for staging sepsis.

Predisposition	Premorbid illness. Cultural/religious beliefs, age and sex. Genetic polymorphisms of inflammatory response components.
Insult infection	The culture and sensitivity of infecting pathogens. Can use assays to determine microbial products (e.g. LPS) and gene transcript profiles.
Response	SIRS, septic shock and CRP. Can be measured by non-specific inflammatory markers (e.g. IL-6) or impaired host-response.
Organ dysfunction	A number of failing organs or score (e.g. SOFA).

The incidence of sepsis after surgery is increasing and in the USA approximately one third

of all sepsis cases occur following surgery (Vogel *et al.*, 2009; Bateman *et al.*, 2010).

Likewise, in an observational study in the US from 1979 – 2000, the incidence of sepsis

increased by 8.7 % per year, although sepsis-associated mortality decreased from 27.8 %

to 17.9 % (Martin *et al.*, 2003). Martin *et al.* (2003) discussed that the reason behind this

increase in incidence and suggested that it was due to more invasive procedures,

including those that were immunosuppressive, combined with an increase in AMR

infections (Martin *et al.*, 2003). The reason behind a decrease in sepsis-associated

mortality could be due to improved intensive care facilities (Lichtenstern *et al.*, 2007).

One study looked at nosocomial infection in intensive care units (ICUs) in Europe which

included 3,147 patients and found that 37 % of ICU patients had an infection and 24 % of

these infections were acquired in the ICU (Vincent *et al.*, 2006). AMR pathogens are

extremely prevalent in ICUs posing even more of a threat (Archibald *et al.*, 1997). The

most common type of infection found in these patients were respiratory (68 %) followed

by abdominal infections (22 %) (Vincent *et al.*, 2006). However, following general surgery

the source of sepsis in 85 % of patients was found to be intra-abdominal (Barie *et al.*,

2004).

It was originally thought that sepsis was mainly caused by Gram-negative organisms,

however, it has been shown that Gram-positive bacteria are most commonly the cause of

sepsis (Martin *et al.*, 2003; Solomkin *et al.*, 2004). Bacterial toxins play a major role in the

inflammation process during sepsis and different types of bacteria may produce different

toxins. For example endotoxins or lipopolysaccharide (LPS) found in the outer membrane

of Gram-negative bacteria bind to immune cells and promote the secretion of pro-

inflammatory cytokines (Ramachandran, 2014). Type I toxins disrupt host cells without

entering them and these include superantigens, such as those produced by *S. aureus.*

These activate large quantities of T cells and result in a cytokine storm such as the process

that occurs during toxic shock syndrome (Ramachandran, 2014). Type II toxins invade host cell membranes and disrupt host cell defence mechanisms from within the cell and examples of type II toxins include haemolysins and phospholipases (van der Poll and Opal, 2008). Examples of bacteria that produce type II toxins include *Clostridium perfringens* (Freedman *et al*., 2016), and *Streptococcus pyogenes* (Barnett *et al*., 2015). Type III toxins have an A and B subunit; the B subunit binds to the host cell and the A subunit release enzymes that damage the host cell (van der Poll and Opal, 2008). Examples of type III toxins are Shiga, anthrax and the cholera toxin.

LPS causes immune cells to express IL-8, IL-6, IL-1β, IL-1, IL-12, TNFα and IFNγ, although TNFα is a key component in endotoxic shock and causes tissue damage and evidence from clinical trials and animal sepsis models has shown that anti-TNF antibodies may help in treating septic shock (van der Poll and Opal, 2008).

The elderly are at a greater risk of developing sepsis and also have an increased risk of mortality associated with sepsis (Martin *et al*., 2006). One study looked into the incidence of sepsis among elderly patients (<65) who had undergone surgery between 2006 and 2011. This study found that sepsis was most commonly associated with abdominal surgery and mortality due to sepsis was significantly associated with higher age, women, development of organ dysfunction, respiratory or abdominal infection and a failure to identify the causative microorganism (Bouza *et al*., 2015). This highlights the need for a rapid diagnostic method to detect microbes in the blood.

1.8 Biofilm formation in surgical wounds

It has been reported that at least 80 % of SSIs are associated with biofilms (Mangram *et al.*, 1999). Wounds are likely to have bacteria (either endogenous or exogenous) in them as they do not have a protective covering of skin. Initially, the hosts' immune system kills or prevents overgrowth of these bacteria in the wound. However, if these bacteria attach to the wound surface and proliferate then a biofilm will form and this can evade eradication by the hosts' immune system and antimicrobials. The wound is then in a biofilm infected state (Percival *et al.*, 2015). Biofilms are communities of microorganisms adhered to each other or surfaces and surrounded by extracellular polymeric substance (EPS) (Flemming and Wingender, 2010).

Biofilms can form under static conditions, in liquid media, where there is no replenishment of nutrients. This is the case for the biofilms studied in the *in vitro* biofilm assays in this work. These biofilms often grow at the bottom of the well and also at the liquid-air interface (Hung *et al.*, 2013; Vlamakis *et al.*, 2013). Biofilms can also develop under flow conditions, where nutrients are constantly replenished (Sternberg *et al.*, 1999; Teal *et al.*, 2006). This is the case for *in vivo* biofilms, including the formation of biofilms in wounds. There are six stages in biofilm formation in wounds. The first stage is the formation of the conditioning film. This is where the adsorption of macromolecules on the surface occurs and this changes the physiochemical properties of the surface, enabling bacteria to adhere to it (Lorite *et al.*, 2011). Stage two is microbial adhesion and co-adhesion which facilitated by various attachment appendages, such as fimbriae and pili (Percival *et al.*, 2015). Stage three is when the microbes on the wound surface divide

and the formation of distinct microcolonies occurs. The primary colonizing bacteria alter the microenvironment, aiding secondary and tertiary microorganisms to colonize the biofilm (Percival *et al.*, 2015:2017). At stage four, EPS is produced and this helps the bacteria further adhere to the surface. EPS is composed of proteins, polysaccharides, glycolipids, extracellular enzymes, metal ions and extracellular DNA (Branda *et al.*, 2005; Flemming and Wingender, 2010). At stage five microbial homeostasis happens and the biofilm is a complex system of microcolonies with water channels that act as a circulatory system for nutrients and waste (Percival *et al.*, 2015:2017). Stage six involves detachment and reattachment of microbes and thus the dispersal of microbes to colonize new surfaces. Clumps containing thousands of microbes can re-enter the exudate in the wound bed (Percival *et al.*, 2015:2017).
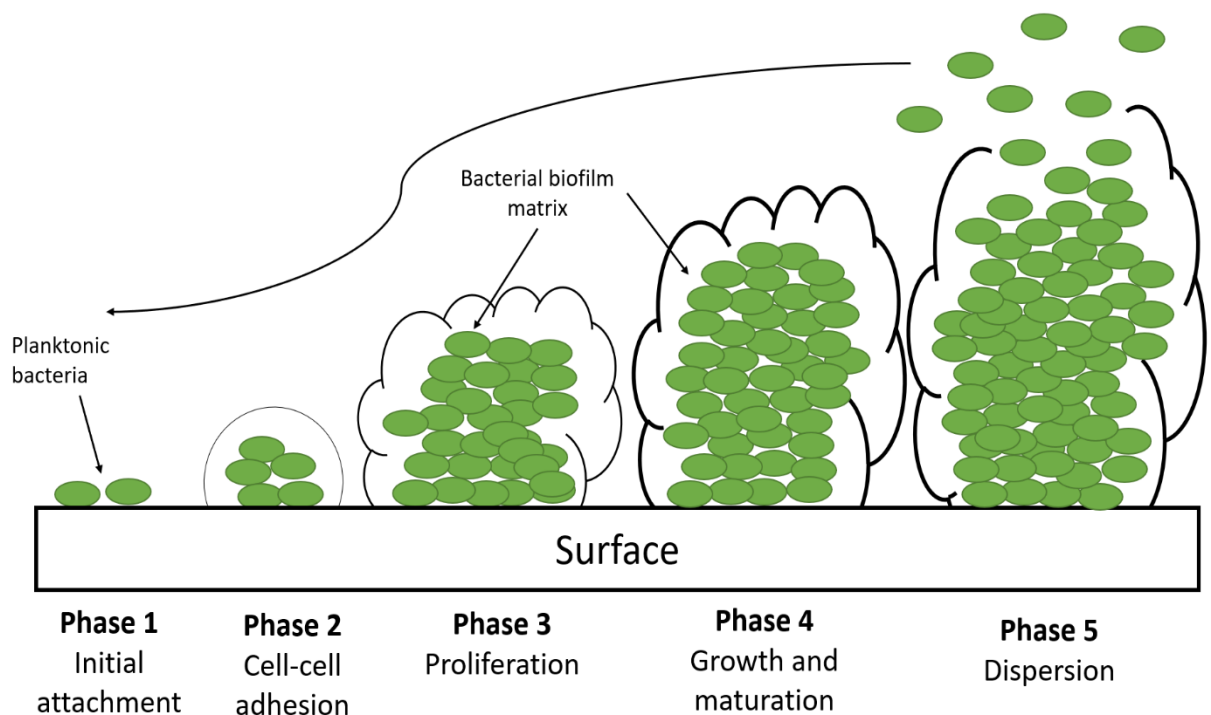

Figure 6. Simplified diagram of flow biofilm formation. Adapted from Aiyer *et al.*, (2018).

Bacteria can form polymicrobial biofilms in wounds and it has been shown that biofilms delay wound healing and increase the risk of chronic infection (European Wound

Management Association, 2004; Kirker *et al.*, 2009; Kathju *et al.*, 2009; Zhao *et al.*, 2010; Kanno *et al.*, 2010; Gurjala *et al.*, 2011; Fazli *et al.*, 2011; Kirker *et al.*, 2012; Roy *et al.*, 2014; Percival *et al.*, 2015). Furthermore, mixed communities of microorganisms may also prevent wound healing via the production of destructive enzymes and toxins (Wolcott *et al.*, 2010).

The 5 step model as shown (Fig. 6) does not however describe the complexity of biofilm formation in real life clinical settings. This model is based on *in vitro* surface based studies using *P. aeruginosa* (Sauer *et al.*, 2002; Klausen *et al.*, 2003; Pamp *et al.*, 2009). The model does not account for non-surface attached aggregates (Sauer *et al.*, 2022). For example, bacteria could bind to molecules in host fluid through surface adhesion interactions. Sauer *et al.* (2022) proposed a new biofilm formation model where the three basic events observed in all biofilm formation, aggregation, growth, disaggregation replace the 5 step model (Sauer *et al.*, 2022). These events occur whether *in vitro*, *in situ* or *in vivo* and therefore this 3 step model may be more representative of biofilm formation.

Biofilm formation can result in decreased susceptibility to antibiotics and therefore make SSIs caused by biofilms more difficult to treat. It has been shown that microbial cells within biofilms are 10 – 1000 times more resistant to antimicrobials than planktonic cells (Mah, 2012). Persister cells are one way in which biofilms can evade antimicrobial treatment and are often tolerant to antibiotics. Persister cells were first discovered in *Staphylococcus* spp. in 1944 (Bigger, 1944). These are cells that are in a dormant state and thus antibiotics are not effective because antimicrobials are generally only active against growing cells (Miyaue *et al.*, 2018). It is believed that persister cells enter the dormant state due to toxin-antitoxin systems (Schuster and Bertram, 2013; Wood *et al.*, 2013;

Harms *et al.*, 2016). Persister cells exhibit only temporary resistant phenotypes and this makes them distinguishable from the permanent antimicrobial resistance found in bacteria as a result of horizontal gene transfer and genetic mutations (Miyaue *et al.*, 2018).

A diagnostic guideline has been suggested for the presence of biofilms infections after surgery. The following are factors that may indicate a post-surgical biofilm infection 1) microbial evidence of an infection post-surgery 2) microscopic evidence of microbial aggregation 3) records of a biofilm pre-disposing condition e.g. implanted medical device, infective endocarditis 4) recurrent infection at the same site with organism that are clonally identical 5) evidence of failure of antimicrobial treatment 6) local or systemic signs of infection which may get better with antibiotics but then return once the course of antibiotics is finished (Hall-Stoodley *et al.*, 2012; Hall *et al.*, 2014).

1.9 Treatment of surgical site infections

For maximum survival rates following HPB surgery, SSI early diagnosis, competent source control and prompt and adequate antimicrobial therapy is vital. For septic patients who have recently had HPB surgery a broad-spectrum antimicrobial is necessary (Lichtenstern *et al.*, 2007). Treatment of SSIs should include an empirical antibiotic, which has activity against anaerobic bacteria such as metronidazole, co-amoxiclav, piperacillin-tazobactam or meropenem. In patients who are known MRSA carriers or those who are at risk of MRSA carriage, an antibiotic that includes activity against locally prevalent MRSA strains should be used. Topical antibiotics for the use of surgical wound healing are not advised due to risks of unknown absorption, toxicity, allergies and antimicrobial resistance (National Institute for Health and Clinical Excellence, 2008). A randomised control trial

found that applying a chloramphenicol ointment to the wound at the end of surgery and 3 days after surgery did not prevent an SSI compared to a control group, which were not given any treatment (Kamath *et al*., 2005). Therefore, it may be suggested that in certain circumstances there are no benefits in applying a topical antimicrobial after surgery.

1.9 Measures to prevent surgical site infections

1.9.1 Surgical antibiotic prophylaxis

The use of surgical antibiotic prophylaxis (SAP) to prevent SSIs was established in the 1960s and has been repeatedly shown to be effective (Miles *et al.*, 1957; Burke, 1961; Polk and Lopez-Mayor, 1969; DiPiro *et al.*, 1985; Classen *et al.*, 1992). SAP is routinely given to HPB surgery patients. SAP guidelines (antibiotic given, time and length of prophylaxis) differ between countries and even between healthcare facilities within the same country. One reason for this may be geographical differences in the organisms causing SSIs, the fact that the prevalence of AMR bacteria differs between countries and also varying costs of antibiotics. The World Health Organisation (WHO) recommends that pre-operative prophylaxis should be administered within 120 minutes prior to the surgical incision, while considering the half-life of the antibiotic (World Health Organization, 2018).

More recent studies have shown that SAP may not reduce the risk of SSIs. Ren *et al.* (2013) found that the use of surgical antibiotic prophylaxis both pre-operatively and post-operatively among HPB surgery patients did not significantly reduce the incidence of SSI, when compared to a group that had one antibiotic dose pre-operatively (Ren *et al.*, 2013). Furthermore, prolongation of prophylaxis is a major determinant of AMR and also *C. difficile* infection, as antibiotics alter the normal gut microbiota resulting in an increase of *C. difficile* (World Health Organization, 2018; Hopkins *et al.*, 2002). It has been reported that a single dose of SAP can result in an increase of AMR in the bacteria colonizing the patient and therefore could cause AMR SSIs (Roberts *et al.*, 1978; Bratzler *et al.*, 2013; Khalil *et al.*, 2016).

1.9.2 Environment cleaning, disinfection and sterilisation

To prevent contamination of surgical sites from the environment cleaning, disinfection and sterilisation of the hospital ward and equipment is required. Any items that do not come into contact with skin are considered low risk items and are cleaned (physically remove microorganisms using detergent). Items that come into contact with mucous membranes, are considered medium risk items, and are disinfected a process that reduces the number of microorganisms to a level at which they are not harmful. Spores will not usually be destroyed. Items that penetrate skin/mucous membranes and are considered high-risk items are sterilised (a process that removes or destroys all microorganisms, including spores) (Manchester University NHS Foundation Trust, 2018).

1.9.3 Hand hygiene

Good hand hygiene procedures are one of the most important infection control methods for preventing SSIs. Guidelines state hands should be sanitized with either an alcohol hand rub or washed with a liquid soap and water before and after patient contact. If hands are visibly soiled then liquid soap and water should be used (Manchester University NHS Foundation Trust, 2018).

1.9.4 Personal protective equipment (PPE)

Personal protective equipment (PPE) such as gloves, aprons, gowns, goggles and fluid-repellent surgical masks primarily protect the healthcare worker from contaminated bodily fluids. PPE also indirectly protects a patients surgical wound from infection as correct use can prevent transmission of pathogens in a hospital ward or surgical theatre (NICE, 2008; WHO, 2009). However, one study highlighted that the PPE must be sterile in order to prevent SSIs (Anjum *et al*., 2022).

1.9.5 Screening of methicillin resistant *Staphylococcus aureus* (MRSA), carbapenemase producing Enterobacteriaceae (CPE) and vancomycin resistant Enterococcus (VRE)

Another infection control method used in hospitals is routine screening for MRSA, CPE and VRE in patients undergoing surgery. Nasal/groin/faecal swabs should be taken prior to surgery and during their hospital stay. Any patient found to be colonised by these resistant organisms will be given suppression therapy prior to surgery to prevent contamination of their surgical site/sites. Any patient who develops a new MRSA, CPE, VRE infection or colonisation must be isolated in a side room and healthcare workers should take extra precaution when handling these patients, such as wearing a disposable plastic apron and disposable gloves on patient contact and on contact with the patient's immediate environment, attention to hand hygiene must also be given. Extra effort should be made to decontaminate the isolation rooms, for example, once a CPE infected/colonised patient is discharged the room must be cleaned and then fogged using hydrogen peroxide vapour (Cawthorne and Manchester University NHS Foundation Trust, 2016; Pagett *et al*., 2019).

Table 3. Key bacteria that cause SSIs and infection control methods.

Skin commensals	Infection control methods	Gastrointestinal organisms	Infection control Methods	Environmental organisms	Infection control methods
Staphylococcus aureus	• Skin preparation using Chlorohexidine prior to surgery (If Chlorohexidine allergy use 70% isopropyl alcohol wipe). • Good hand hygiene practice. • PPE equipment	*Enterobacter cloacae*	• Antimicrobial prophylaxis. • Good hand hygiene practice. • PPE equipment.	*Pseudomonas* spp.	• Clean, disinfect or sterilise hospital wards and equipment using Chlor-clean solution. • Single use equipment where appropriate. • Good hand hygiene practice. • PPE equipment.
Coagulase negative Staphylococci		*Bacteroides* spp.		*Serratia* spp.	
Streptococcus spp.		*Escherichia coli*			
		Candida spp.			
		Klebsiella spp.			
		Enterococcus faecalis			
		Enterococcus faecium			

1.9.6 Predicative care plans

A sustainable action plan for the reduction of hospital acquired infections (HAIs) caused by antimicrobial resistant species is desperately needed. To prevent AMR SSIs, a predicative care plan may be a suitable route. Many patients are routinely given the same prophylactic antibiotics prior to surgery. A more personal and tailored method may be a better approach. For example, patients could be given an antibiotic based on bacterial growth from a swab and the antibacterial sensitivities of these species. This may not always be possible for treatment of an SSI as microbiology cultures can take over 24 hours to grow and identify. However, in terms of prophylaxis, in elective surgery the surgical sites could be swabbed beforehand, and sensitivities performed thus enabling the clinician to select potentially the most effective antibiotic.

1.10 Impact of COVID-19

Covid-19 has changed the way healthcare is provided. For example, PPE such as masks and gloves are more commonly worn and antimicrobial hand gel is more commonly used. The cleaning of surfaces has become more commonplace. Face-to-face meetings have been avoided where possible, for example pre-surgery clinics may be held virtually instead. All the measures used to prevent the spread of Covid-19 are likely to also impact the incidence of other infections such as SSIs. Previously, antibacterial hand gel used in hospitals has resulted in a decrease of MRSA infections. Therefore the heightened use of antibacterial hand gel and other PPE methods could result in a decrease in other bacteria such as Gram-negative species that can cause SSIs.

Covid-19 also impacted this research specifically. The university laboratory was out of access due to nationwide lockdowns for almost a year and therefore some bacterial samples

were unusable on return and laboratory work could not be conducted for a significant amount of time during the research degree. Access to the hospital to collect patient samples was not permitted following the Covid-19 outbreak and therefore the collection and analysis of patients samples was heavily impacted and this resulted in a smaller sample size than anticipated. The research therefore had to change course and the biofilm assays were conducted as a result of the limitations due to Covid-19.

The aims of this work was to determine the incidence and risk factors of SSIs in HPB surgery patients by collating laboratory findings with patient information. Secondary aims were to investigate which bacteria were found on the hospital surfaces. Another aim was to measure how bacteria isolated from patients form biofilms over time by performing bacterial percentage coverage assays, crystal violet assays and tetrazolium salt reduction assays.

Chapter 2. Methods

2.1 Participants, collection of patient information and statistical analysis for risk factors

2.1.1 Ethical approval

Ethical approval for the collection of patient samples and patient data was issued by the Central Bristol Research Ethics Committee on 21/09/2018.

2.1.2 Participants

Participants were patients undergoing hepatopancreatobiliary (HPB) surgery at the Manchester Royal Infirmary (Manchester University NHS Foundation Trust) between 2018 and 2019. The majority of the patients were having surgery to investigate and/or remove malignancies from the HPB tract. Patients were given a participant information sheet to read and informed consent for inclusion was obtained. Those patients who lacked mental capacity, those who could not read or communicate in English, those with a known brain abscess and patients with a known class 3 infection were excluded from this study. Surgeries were either laparoscopic or open surgeries and included liver resection, pancreaticoduodenectomy, cholecystectomy and pancreatectomy and combinations of the above. All patients were admitted to the high dependency unit (HDU), for at least one night, following their surgery. Prophylaxis was continued for 48 hours post- operatively.

2.1.3 Patient information

Patient information such as demographics, comorbidities, surgical factors and post-operative information was collected following the patients' surgical procedures (Table 4). The information was collected from the patient data system used at the hospital and

stored according to the Data protection Act 2018. An SSI was defined as an infection that

occurred where the surgery was performed, within 30 days. This was confirmed by

positive wound swab cultures, positive drain fluid cultures or SSI reported in the patient's

hospital records.

Table 4. The factors investigated.

Preoperative medical factors	Intraoperative surgical factors	Postoperative factors
Sex	Type of surgery	Return to theatre
Age, years	Laparoscopic surgery	Post-operative sepsis
Weight	Operating times	Post-operative sustained bile leak and further intervention
Chronic lung disease	Bile leakage	Urine infection
COPD	Drain used	Prolonged stay in hospital (days)
Current smoker	Total blood loss (mL)	Weight loss
Diabetes mellitus (type 1)	Excessive haemorrhage	Pneumonia
Diabetes mellitus (type 2)	Synchronous surgery – liver and bowel	Wound infection
Ischemic heart disease		Fistula
NYHA heart failure grade		Diarrhoea
Long term steroid use		
Hypertension		
Crohn's disease		
Ulcerative colitis		
MRSA screen		
CPE screen		
VRE screen		

2.1.4 Collection of full blood count and C reactive protein results

Full blood counts (FBC) and C reactive protein (CRP) tests are routinely performed

throughout the patient's hospital stay. The pre-surgical blood tests were taken the day

before the surgery and the post-operative blood tests were taken within two weeks

following the surgery, depending on time of discharge from hospital. The full blood

counts included the following blood cells: white blood cell, red blood cell, haemoglobin,

haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean

corpuscular haemoglobin concentration, mean platelets, mean neutrophils, mean

lymphocytes, mean monocytes, mean eosinophils and mean basophils. The total average

and ranges of the FBC and CRP were calculated for the SSI group and non-SSI group.

2.1.5 Statistical analysis for risk factors of surgical site infection

Fisher's exact tests were performed for variables where there were more than five patients with the risk factor. Cross tabulation was also performed. Wilcoxon rank sum test with continuity correction was performed on continuous variables. Due to the small sample size the results were interpreted with caution.

2.2 Patient and hospital environment sample collection

2.2.1 Collection of patient samples

Microbial swabs were taken from the site of surgery prior to surgery and on day 1, 7, 14 and 28 following surgery, where possible, unless they were discharged before this time period, in which case a final swab was obtained on the day of discharge. Sterile charcoal swabs (Transystem ™ COPAN, Italy) were dipped into 2 mL of sterile saline before being wiped across the patients' skin. Wound dressings were removed, and the swab was gently wiped as close to the surgical wound as possible without inflicting pain on the patient. The swabs were then transported to the laboratory in a diagnostic specimen container which was compliant with Packaging Instruction 650, and therefore suitable for diagnostic specimens assigned to UN3373.

2.2.2 Collection of environmental samples

Selected surfaces of the wards the HPB surgery patients were admitted to before and after surgery were swabbed to recover the bacteria. These surfaces were on the

hepatopancreatobiliary (HPB) ward, surgical admissions ward and high dependency unit (HDU). These areas were divided into the patient bedside, the nurses' workstation, ward surfaces and the patient/visitor bathroom. The swab was streaked across a 10 cm by 10 cm area, 3 times, in a criss-cross direction (Whitehead *et al.*, 2008). Once the samples had been collected from the patient and the hospital ward, they were transported to Manchester Metropolitan University in line with Health and Safety Executive (HSE) regulations.

2.3 Culture, identification and antibiotic susceptibility of bacteria from patients and the hospital ward

2.3.1 Microbial culture of patient and ward samples

The collected environmental and clinical swabs were streaked onto Columbia horse blood agar (Oxoid, UK), MacConkey agar (Oxoid, UK), chocolate agar with 0.05 g/500 mL of thiamine (Acros organics, USA) and sabouraud agar (Oxoid, UK) within 3 hours of collection. They were incubated aerobically and anaerobically using specific growth parameters (Table 5).

Table 5. Microbiological agar used to recover the bacterial species from the swabs.

Media	Time	Temperature	Aerobic/anaerobic
Columbia horse blood agar	Up to 7 days	37°C	Aerobic
MacConkey agar	18 - 24 h	37°C	Aerobic
Sabouraud agar	14 days	30°C	Aerobic
Columbia horse blood agar	Up to 7 days	37°C	Anaerobic
Chocolate agar with thiamine	Up to 7 days	37°C	Aerobic

2.3.2 Biochemical bacterial identification

A Gram-stain, catalase and oxidase test was performed on each pure culture and a

coagulase test was carried out on Gram-positive, catalase positive cocci. Initially, the appropriate API tests (bioMérieux, France) were used to further identify the organisms. However, API tests were not used for later samples and species were identified by 16s sequencing. Once the swab had been streaked onto the above media the swab was vortexed in 2 mL of 15 % glycerol and stored at -80°C for future genetic analysis. The charcoal swabs were also frozen at -80 °C for future analysis.

2.3.3   Polymerase chain reaction

16s sequencing was used to identify the organisms. The 16s ribosomal RNA is the RNA component of the 30s small subunit of the prokaryotic ribosome, the genes that code this component are referred to as the 16s rRNA gene and due to slow rates of evolution in this region they are often sequenced and used for identification of bacteria. 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') are commonly used primers for amplifying the DNA between positions 27 and 1492 of bacterial 16S rRNA genes which is nearly the full length of the 16s rRNA gene.

For each PCR reaction 0.5 µL of forward primer (27f), 0.5 µL of reverse primer (1492R), 12 µL of 2x MyTaq red and 12 µL of distilled H_2O were added to a PCR Eppendorf tube to give a volume of 25 µL. A 0.2 µm filter (Sarstedt AG and Co. KG, Germany) was used to filter sterilise the distilled H_2O. A single pure colony of the bacteria to be identified was touched with a sterile pipette tip and mixed into the PCR mix. The cycles for the PCR reaction are shown (Table 6).

Table 6. Parameters of PCR cycles.

Temperature	Time	Number of cycles
95 °C	1 min	X 1
95 °C	30 s	X30
50 °C – 68 °C	30 s	
72 °C	1 min / kb	
72 °C	5 mins	X1
10 °C	Hold	

2.3.4 Gel electrophoresis

Gel electrophoresis was used to determine if bacterial DNA was present in the samples.

To make a 1 % agarose gel, 3 g of agarose was added to 300 mL of 1x TBE buffer and

dissolved on full power in an 800 W microwave. The 1 % agarose was poured into the gel

box and 3 µL of Midori Green Advance Stain (Nippon Genetics EuropeGmbH, Germany)

was added and mixed. A gel comb was inserted to create wells and the gel was left for 30

mins to set. 1x TBE buffer was poured to the maximum line of the gel electrophoresis box

and a 1kb hyperladder l (Bioline, UK) was added to the first well. All samples were spun in

a PCR centrifuge for 2 min at 7,000 rpm and only the supernatant was loaded into the

wells to avoid the sample sticking to the wells during gel electrophoresis. Three

microliters of sample were loaded into the wells. The gel was run at 100 V for 40 min. The

bands were visualised under UV (Syngene, UK).

2.3.5 PCR purification

A QIAquick® PCR Purification kit (50) (Qiagen, UK) was used to purify the PCR samples

that expressed clear bands in the gel electrophoresis. The QIAquick® PCR Purification kit

purifies DNA by a fast bind-was-elute procedure and PCR products are passed through a

silica-membrane to yield >100 bp of DNA up to 10 kb. A Nanodrop spectrophotometer

(Thermofisher Scientific, USA) was used to determine the DNA concentration in each

sample. The Nanodrop spectrophotometer was blanked using 1 µL of elution buffer (Qiagen, UK) and 1 µL of the sample was loaded onto the sample stage and a reading taken and recorded. This was repeated for all the samples and the sample stage and arm cleaned using a 2-ply medwipe between each reading. Samples were only used if DNA concentration was between 20 µg/µL - 80 µg/µL and the A260/A280 was between 1.8 – 2 thus demonstrating an adequate amount of DNA for 16s analysis.

2.3.6 16s rRNA sequencing

The final volume of the samples that were sent off for 16s sequencing were 10 µL with 1 µL of forward primer (27f) at 5 pmol. The remaining 9 µL was the amount of purified DNA product needed to make the concentration of DNA 20 µg/µL - 80 µg/µL and this was diluted with filter sterilised H_2O. A GATC barcode was attached to the Eppendorf and the samples were sent off for sequencing at EUROFINS genomics, Germany. The returned sequences were then compared with published sequences in the GenBank database by using the BLASTN algorithm (http://www.ncbi.nlm.nih.gov/blast).

2.3.7 Antimicrobial resistance testing

Antimicrobial resistance (AMR) of clinical pathogens was determined by using the Kirby Bauer disk diffusion method (Bauer *et al*., 1959). Antibiotic discs (Oxoid, UK) on Mueller Hinton agar (Oxoid, UK) were used (Table 7). Zones of inhibition were measured and compared to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) Clinical Breakpoints Tables (V. 12.0) to determine if the isolate was sensitive, intermediate or resistant (Tables 8 - 14). Initially only zones of inhibition (ZOI) of pathogenic species were conducted and some strains were unable to grow on Mueller Hinton agar and therefore some AMR data is missing. Currently there are no EUCAST breakpoints for *Micrococcus* spp., *Aerococcus* spp., *Dermacoccus* spp., *Kocuria rhizophila*

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and consequently AMR could also not be determined for these species.

Table 7. Concentrations of antibiotics used for disc diffusion method.

Antibiotic	Concentration (µg)
Cefoxitin	30
Ciprofloxacin	5
Gentamicin	10
Norfloxacin	10
Erythromycin	15
Fusidic acid	10
Tetracycline	30
Vancomycin	5
Meropenem	10
Ampicillin (for *Enterococcus* spp.)	2
Ampicillin (For Enterobacterales)	10
Piperacillin-tazobactam	36

Table 8. EUCAST breakpoints (v12.0) for *Staphylococcus* spp.

Antibiotic	Zone diameter breakpoints (mm)	
	S≥	R<
Cefoxitin (*S. aureus* and CoNS except *S. epidermidis* and *S. lugdunensis*)	22	22
Cefoxitin (*S. epidermidis* and *S. lugdunensis*)	27	27
Ciprofloxacin (*S. aureus*)	50	21
Ciprofloxacin (CoNS)	50	24
Erythromycin	21	21
Fusidic acid	24	24
Gentamicin (*S. aureus*)	18	18
Gentamicin (CoNS)	22	22
Norfloxacin	17	17
Tetracycline	22	22

Table 9. EUCAST breakpoints (v12.0) for *Enterococcus* spp.

Antibiotic	Zone diameter breakpoints (mm)	
	S≥	R<
Norfloxacin	12	12
Vancomycin	12	12
Ampicillin	10	8

Table 10. EUCAST breakpoints (v12.0) for *Acinetobacter* spp.

Antibiotic	Zone diameter breakpoints (mm)	
	S≥	R<
Ciprofloxacin	50	21
Gentamicin	17	17
Meropenem	21	15

Table 11. EUCAST breakpoints (v12.0) for Enterobacteriales.

Antibiotic	Zone diameter breakpoints (mm)	
	S≥	R<
Cefoxitin	19	19
Ciprofloxacin	25	22
Gentamicin	17	17
Meropenem	22	16
Ampicillin	14	14
Piperacillin-tazobactam	20	20

Table 12. EUCAST breakpoints (v12.0) for *Pseudomonas* spp.

Antibiotic	Zone diameter breakpoints (mm)	
	S≥	R<
Ciprofloxacin	50	26
Piperacillin-tazobactam	50	18
Meropenem (*P. aeruginosa*)	20	14
Meropenem (Pseudomonas other than *P. aeruginosa*)	24	18

Table 13. EUCAST breakpoints (v12.0) for *Bacillus* spp.

Antibiotic	Zone diameter breakpoints (mm)	
	S≥	R<
Erythromycin	24	24
Norfloxacin	21	21
Vancomycin	10	10
Meropenem	25	25

Table 14. EUCAST breakpoints (v12.0) for *Corynebacterium* spp.

Antibiotic	Zone diameter breakpoints (mm)	
	S≥	R<
Ciprofloxacin	50	25
Tetracycline	23	24
Vancomycin	17	17

2.4 Biofilm assay methods

2.4.1 Bacterial suspensions

The strains used were *E. faecium*, *E. cloacae* and a *S. haemolyticus* that were all isolated from patients having HPB surgery at a hospital in Manchester, UK. The *E. cloacae* was extensively drug resistant, *S. haemolyticus* was multi-drug resistant. A single colony was inoculated into 10 mL tryptone soy broth (TSB) (Oxoid, UK) and vortexed for 10 s. The inoculated broth was incubated at 37°C in an orbital shaker at 200 rpm. After 24 h, the cultures were centrifuged at 1271 *g* for 10 min and the supernatant was removed. The bacterial pellet was washed in 10 mL of 0.85 % saline by vortexing for 10 s and centrifugated again at 1271 *g* for 10 min. The supernatant was removed and 10 mL of TSB was added and vortexed for 10 s. The optical density (OD) was adjusted to 1.0 ± 0.1. Miles and Misra were performed to determine colony forming units (CFU/mL). This was carried out using serial dilution and 20 µL of each dilution dropped onto tryptone soy agar (Oxoid, UK) in triplicate. The plates were incubated at 37°C overnight and the colonies were counted. The CFU per mL was calculated with the following formula;

$$Average\ no.\ of\ colonies\ in\ each\ dilution\ \times 50\ \times dilution\ factor$$

These were 1.25×10^9 CFU/mL for *E. cloacae*, 3.98×10^8 CFU/mL for *S. haemolyticus* and 3.0×10^6 CFU/mL for *E. faecium*.

2.4.2 Bacterial coverage methods on synthetic skin

The bacterial % coverage measurements were taken at 24 h, 48 h and 7 days. Initially the bacteria (*E. faecium*, *E. cloacae* and *S. haemolyticus*) were grown overnight in 30 mL of tryptone soy broth (TSB) (Oxoid, UK) at 37°C. Synthetic skin (Amazon, UK) was cut into 1 cm by 1 cm squares and soaked in 100 % ethanol for 10 min, washed with 2.5 mL of sterile H_2O and air dried for 1 h in a Class II Microbiological safety cabinet. Nine of the sterile synthetic skin squares were stuck into a Petri dish with double sided sticky tape and 30 mL of bacterial suspension was added. These were incubated at 37°C for a total of 7 days. The following day, three of the synthetic skin squares were removed and 3 mL of TSB was added to the Petri dish and the remaining squares were incubated in media again. The three squares were washed with 2.5 mL of distilled H_2O and air dried for 1 h. The squares were then moved to 12 well plates and 2 mL of 0.03 % acridine orange was added for 2 mins. The squares were again washed with 2.5 mL of d H_2O and air dried in the dark for 1 h. The biofilm was visualised and bacterial coverage determined using Cell F software (Olympus). This was repeated after 48 h and 7 days.

2.4.3 Steel coupon preparation

Polished 304 grade stainless steel coupons were cut into 1 cm x 1 cm squares. They were soaked in 70 % ethanol for 10 min and washed with 2.5 mL of sterile water. They were air dried for 1 h.

2.4.4 Biofilm assay preparation

Steel coupons were soaked in undiluted acetone, methanol and ethanol for 10 min for each solvent and washed with 1 mL of water after each step. They were air dried for 1 h. Nine coupons were placed into 12 well culture plates and TSB was added to the empty

wells as a negative control. For each variation of species one 12 well culture plate was used. The combinations of bacteria species were

- *E. cloacae*

- *S. haemolyticus*

- *E. faecium*

- *E. cloacae* and *S. haemolyticus*

- *E. cloacae* and *E. faecium*

- *E. faecium* and *S. haemolyticus*

- *E. cloacae*, *E. faecium* and *S. haemolyticus*

One mL of the single species (OD 1.0) was added to nine of the wells for the single species assay. For the two species assays 50 µL of each species (OD 1.0) were added to the wells. For the culture plate with all three species, 33.3 µL of the inoculated broth (OD 1.0) for each species was added to the wells. The culture plates were sealed with parafilm and incubated at 37°C.

2.4.5 Crystal Violet Assay

Crystal violet assays were performed using a modified protocol used by Amin *et al*. (Amin *et al*., 2020). After the culture plates had been incubated for 24 h, 48 h and 7 days the assays were performed. Three coupons for each timeframe were removed and the 12 well plates were sealed and returned to the incubator if necessary. The coupons were washed with 1 mL of distilled H_2O and airdried for 2 h in a Class II Microbiological safety cabinet. The coupons were then placed into another 12 well culture plate and 1 mL of 0.1 % crystal violet was added to each well and left for 30 min. The coupons were washed three times with distilled H_2O and dried at room temperature for 1 h. After drying, 1 mL of 33 %

glacial acetic acid (Fisher Scientific, UK) in H_2O was added to the wells, mixed and transferred to a cuvette. The absorbance was measured at 590 nm and 33 % glacial acetic acid was used to bank the spectrophotometer. These were all carried out in triplicate, averaged and the standard deviation was calculated.

2.4.6 Tetrazolium salt reduction assay (XTT)

XTT assays were performed using an adapted protocol used by Karaky *et al*. (Karaky *et al*., 2020). A stock solution of XTT was made by dissolving 4 mg of XTT in 10 mL of sterile PBS at 37 °C. This was stored at -20 °C. A menadione (0.4 mM) solution was made and supplemented with D-glutamine (50 mM). The working concentration of XTT and menadione was a ratio of 5:1. Following the growth of biofilms the coupons were removed for each timeframe (24 h, 48 h and 7 days). The coupons were carefully washed three times with 1 mL sterile H_2O and the coupons were placed in wells of a new 12 well culture plate. One mL of XTT-menadione solution was added to each well and these were incubated in the dark for 3 h at 37°C. After incubation the supernatant was removed from the wells and the absorbance was read at 490 nm. The XTT-menadione solution was used to blank the spectrophotometer. The averages and standard deviation were calculated.

2.4.7 Antimicrobial resistance testing

The inoculated broth was removed from each well at the same time the coupons were removed to perform the assays (24 h, 48 h and 7 days). The inoculated broth was grown on selective media to separate the different species, or TSA for the single species assays. For *E. faecium* bile esculin agar (Sigma Aldrich, Germany) was used, for *E. cloacae* MacConkey agar (Oxoid, UK) and for *S. haemolyticus* mannitol salt agar (Oxoid, UK) was used. Disc diffusion was then performed on each isolate using the Kirby Bauer method (Bauer *et al*., 1959), as explained previously.

Chapter 3. Risk factors of surgical site infections and full blood count analysis

3.1 Introduction

Pre-operative risk factors of surgical site infections (SSI) following hepatopancreatobiliary

(HPB) surgery include patient comorbid conditions (obesity, cardiovascular disease,

bleeding disorders), malnutrition and hepatopancreatobiliary (HPB) pathology (bactibilia,

malignancy, biliary obstruction) (Ceppa *et al.*, 2013). Intraoperative risk factors include

long operating times, use of drains and significant blood loss. Post-operative risk factors

of SSIs include pancreatic and biliary fistulae (Ceppa *et al.*, 2013). Bacteria that

contaminate surgical sites often originate from the GI tract and for example one study

found the dominant microorganisms in HPB surgical wounds were *Enterococcus* spp. (55

%), *S. aureus* (45 %), alpha Streptococci (36 %), *Klebsiella* spp. (27 %) and *Enterobacter*

spp. (18 %) (Howard *et al.*, 2006). The patient records of twenty-six patients undergoing

HPB surgery were analysed. The aims were to determine any pre-operative, intra-

operative and post-operative risk factors of SSI following HPB surgery and also to see if

full blood count results showed markers of infection. Another objective was to identify

which organisms were causing these infections and this could imply how the bacteria

may have contaminated the surgical site. It was found that gut bacteria isolated from

drain fluid were the cause of SSIs in all (23.1 %) of the patients who developed an SSI.

Risk factors for SSIs following HPB surgery included bile leak, use of drains, pancreatic

surgery, open surgery, long surgery, long hospital stay, poor post- operative nutrition,

post-operative pneumonia and return to the operating theatre.

Analysis of the average full blood count and C reactive protein (CRP) results found that

following surgery patients in the SSI group had WBC, PLT, MNEUT and CRP results that

were out of range.

3.2 Results

3.2.1 Patient demographics, comorbidities and preoperative factors

Twenty-six participants were included in this study and the demographics and comorbidities are shown (Table 15). Of these patients 65 % were male and 35 % were female. The majority of the participants were over 50. The BMI of the patients was heathy (n = 10), overweight (n = 9) and obese (n = 7). Three patients had chronic lung disease and three other patients had chronic obstructive pulmonary disease (COPD). Six of the 26 patients were current smokers. Two patients had type 1 diabetes and 3 patients had type 2 diabetes. Two of the patients had ischemic heart disease. Half of the patients had hypertension. One patient had Crohn's disease and another patient had ulcerative colitis. The patients were routinely screened by the hospital for Vancomycin resistant enterococcus (VRE), carbapenemase producing Enterobacteriaceae (CPE) and methicillin resistant *Staphylococcus aureus* (MRSA) during their hospital stay. One patient was found to be colonised by CPE and three by VRE. No patients were colonised by MRSA.

Table 15. Preoperative medical factors of patients.

Preoperative medical factors		N
Sex	Female	9
	Male	17
Age, years	<29	1
	30 – 49	2
	50 – 69	11
	70 +	9
Weight	Healthy	10
	Overweight	9
	Obese	7
Chronic lung disease	Yes	3
	No	23
COPD	Yes	3
	No	23
Current smoker	Yes	6
	No	20
Diabetes mellitus (type 1)	Yes	2
	No	24
Diabetes mellitus (type 2)	Yes	3
	No	23
Ischemic heart disease	Yes	2
	No	24
NYHA heart failure grade	Grade 1	4
	Grade 2	3
Long term steroid use	Yes	1
	No	25
Hypertension	Yes	13
	No	13
Crohn's disease	Yes	1
	No	25
Ulcerative colitis	Yes	1
	No	25
MRSA screen	Positive	0
	Negative	26
CPE screen	Positive	1
	Negative	25
VRE screen	Positive	3
	Negative	23

3.2.2 Surgical factors

The most common surgical procedure was liver resection (n = 7) (Table 16). A higher

number of open surgical procedures were carried out (n = 18) than laparoscopic (n = 8).

The majority (n = 20) of the operations took 2 h – 6 h to complete. A bile leakage occurred

in six of the 26 patients. A drain was used in 17 of the operations. One patient had an

excessive haemorrhage. Three of the patients had synchronous surgery of the liver and

bowel.

Table 16. Intraoperative surgical factors of patients.

Intraoperative surgical factors		*N*
Type of surgery	Liver resection	7
	Pancreaticoduodenectomy	4
	Liver resection and cholecystectomy	4
	Pancreatectomy	3
	Cholecystectomy	3
	Pancreaticoduodenectomy and resection of antrum of stomach	3
	Spleen preserving distal pancreatectomy	2
Laparoscopic surgery	Yes	8
	No	18
Operating times	< 2 hours	3
	2 - 6 hours	20
	> 6 hours	3
Bile leakage	Yes	6
	No	20
Drain used	Yes	17
	No	9
Total blood loss (mL)	100 – 500	4
	500-1000	2
	1000+	2
Excessive haemorrhage	Yes	1
	No	25
Synchronous surgery – liver and bowel	Yes	3
	No	23

3.2.3 Postoperative factors

Three of the patients were returned to theatre for further surgical intervention and another three had postoperative sepsis. Seven of the 26 patients had a bile leak during or after surgery which then required further intervention. Two patients had a urine infection following surgery. Most patients (n = 17) were in hospital for >10 days. Seven patients experienced post-operative weight loss. Four patients suffered from post-operative pneumonia. A total of six patients had wound infections (SSIs). A further two patients had a fistula. Four patients experienced post-operative diarrhoea (Table 17).

Table 17. Postoperative factors of patients.

Postoperative factors		N
Return to theatre	Yes	3
	No	23
Post-operative sepsis	Yes	3
	No	23
Post-operative sustained bile leak and further intervention	Yes	7
	No	19
Urine infection	Yes	2
	No	24
Prolonged stay in hospital (days)	<10	17
	>10	6
	>20	3
Weight loss	Yes	7
	No	19
Pneumonia	Yes	4
	No	22
Wound infection	Yes	6
	No	20
Fistula	Yes	2
	No	24
Diarrhoea	Yes	4
	No	22

3.2.1 Surgical site infections

Of the 26 patients, 6 (23.1 %) [95 % binomial confidence intervals 9.0 - 43.6 %], developed

SSIs. All of the patients with an SSI had open surgery as opposed to laparoscopic surgery

and drains were used in all cases where infection occurred (Figure 7). The surgeries all

involved the pancreas and these included pancreaticoduodenectomy, pancreatectomy

and pancreaticoduodenectomy with resection of antrum of the stomach. All of the

operations took over 2 h. All of the 6 patients except one were in hospital for over 10

days. Four of the 6 patients had a bile leak which needed further intervention. Four of the

6 patients had poor nutrition and calorific intake following surgery. Half of the 6 patients

were returned to theatre. Half of the patients experienced post-operative pneumonia.
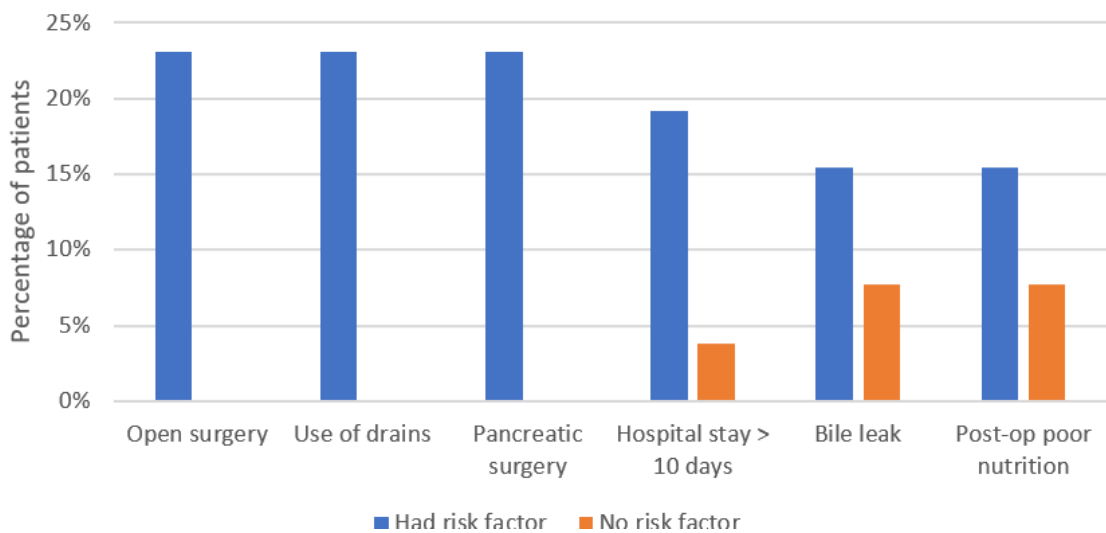
Fifty percent of the patients were smokers.

Figure 7. Percentage of patients with SSIs and potential contributing factors.

The incidence of SSIs along with risk factors were statistically analysed and the key

findings were as follows. The patients with hypertension were less likely (p = 0.015) to

have an SSI. Crosstab showed that patients who were returned to theatre after the initial

surgery were more likely to develop an infection, however, due to small sample size this

finding should be treated with caution. Patients who did not experience a post-operative

sustained bile leak and further intervention were less likely to experience an infection (p =

0.028). With increasing length of stay the likelihood of an infection appeared to increase.

Those who had infections had longer stays in hospital (p = 0.008). Patients with

pneumonia appeared to be more likely to get an infection. Those patients with better

nutrition and calorie intake were less likely to get an infection (p = 0.028). Those who had

infections had a broader spread of weight loss, thus suggesting those with infections lost

more weight.

3.2.2 Species that caused SSIs

The bacteria found to cause SSIs were identified from drain fluid cultures that were

conducted by the staff at the hospital, apart from *Enterobacter cloacae* which was also

identified from a wound swab (Table 18). The species that caused SSIs in the six patients

were *Citrobacter freundii*, *Enterobacter kobei*, *E. cloacae* and *Clostridium perfringens*,

Enterobacter spp. and in one patient it was not known which species caused the

infection. All of the identified *Citrobacter* spp. and *Enterobacter* spp. were ESBL and AmpC

producing strains.

Table 18. Bacteria cultured from drain fluid or wound swabs taken by the hospital in patients who had SSIs.

Species 1	Species 2
Citrobacter freundii	
Citrobacter freundii	*Streptococcus milleri*
Enterobacter cloacae	*Clostridium perfringens*

Unknown	
Enterobacter spp.	
Enterobacter kobei	

3.2.3 Blood analysis

The range and average blood test results both pre-surgery and post-surgery for all patients with SSI and without SSI were calculated (Tables 19 and 20). The were no differences in the average blood test results when considering the normal reference range between pre-surgery and post-surgery in the no SSI group apart from MNEUT being higher after surgery (8.14 x 10ˆ9/L). Average RBC, HCT and CRP were out of range both before and after surgery in the no SSI group.

RBC (before 4.17 x10ˆ12/L, after 3.42 x10ˆ12/L), HB (before 126.50 g/L, average 105.17 g/L) HCT (before 0.37, after 0.30) CRP (before 18.33 mg/L, after 129.67 mg/L) were all out of the reference range before and after surgery in the patient group that got an SSI. In the positive SSI group, additional blood tests were out of the reference range after surgery. These were WBC (11.45 x 10ˆ9/L), PLT (422.17 x 10ˆ9/L) and MNEUT (9.16 x 10ˆ9/L). The average level of CRP was particularly high after surgery in the SSI group (124.5 mg/L) when compared to the non-SSI group (12.9 mg/L). Interestingly, the CRP average was also

significantly higher before surgery in the SSI group (18.33 mg/L) when compared to the non-SSI group (6.42 mg/L).

One patient who had a SSI demonstrated RBC, HB, HCT PLT, and CRP were out of range following surgery. In another patient WBC, RBC, HB, HCT, PLT, MNUET, MMONO, MBASO and CRP were out of range following surgery. In another patient, RBC, HB, HCT, PLT and CRP were out of range after surgery. One patient demonstrated WBC, RBC, HB, HCT, PLT, MNUET, MLYMPH and CRP out of the reference range following their surgery. The blood results for another patient showed that WBC, RBC, HB, HCT, PLT, MNEUT, MMONO and CRP were out of range after surgery. In another patient, the following blood test results that were out of the refence range included RBC, HB HCT and CRP.

Table 19. Full blood cell count and CRP averages and ranges, before and after surgery in non-SSI patients.

| Blood test | No surgical site infection | | | | UoM | Ref. Range |
| | Pre-op | | Post-op | | | |
	Range	Average	Range	Average		
WBC	9.0	7.00	12.7	10.58	x 10ˆ9/L	4.0-11.0
RBC	1.7	4.30	2.4	3.74	x10ˆ12/L	4.50-6.00
HB	57.0	130.35	57.0	113.85	g/L	130-180
HCT	0.2	0.38	0.2	0.33	Ratio	0.400-0.520
MCV	17.0	88.45	17.0	89.65	Fl	80-98
MCH	5.9	30.40	6.2	30.58	Pg	27.0-33.0
MCHC	37.0	344.15	33.0	341.15	g/L	320-365
PLT	257.0	240.45	351.0	255.05	x 10ˆ9/L	150-400
MNEUT	7.3	4.31	11.8	8.14	x 10ˆ9/L	1.80-7.50
MLYMPH	2.0	1.85	2.3	1.36	x 10ˆ9/L	1.00-4.00
MMONO	0.8	0.63	1.3	0.86	x 10ˆ9/L	0.20-1.00
MEOS	0.5	0.15	1.0	0.20	x 10ˆ9/L	0.00-0.40
MBASO	0.1	0.04	0.1	0.04	x 10ˆ9/L	0.00 - 0.10
CRP	20.1	6.42	213.0	76.75	mg/L	0 - 5.0

Table 20. Full blood cell count and CRP averages and ranges, before and after surgery in SSI patients.

| Blood test | Surgical site infection | | | | UoM | Ref. Range |
| | Pre-op | | Post-op | | | |
	Range	Average	Range	Average		
WBC	2.50	7.03	9.2	11.45	x 10ˆ9/L	4.0-11.0
RBC	2.67	4.17	1.01	3.42	x10ˆ12/L	4.50-6.00
HB	78.00	126.50	16	105.17	g/L	130-180
HCT	0.24	0.37	0.044	0.30	Ratio	0.400-0.520
MCV	12.00	88.50	16	89.33	Fl	80-98
MCH	5.90	30.50	4.9	30.92	Pg	27.0-33.0
MCHC	21.00	345.17	27	346.83	g/L	320-365
PLT	133.00	237.17	628	422.17	x 10ˆ9/L	150-400
MNEUT	2.19	4.34	7.21	9.16	x 10ˆ9/L	1.80-7.50
MLYMPH	0.99	1.87	1.3	1.37	x 10ˆ9/L	1.00-4.00
MMONO	0.37	0.59	0.87	0.71	x 10ˆ9/L	0.20-1.00
MEOS	0.37	0.20	0.22	0.14	x 10ˆ9/L	0.00-0.40
MBASO	0.07	0.05	0.14	0.07	x 10ˆ9/L	0.00 - 0.10
CRP	8.00	18.33	204	129.67	mg/L	0 - 5.0

3.3 Discussion

3.3.1 Bacteria responsible for SSIs

Five of the SSIs were determined to have come from the drain cultures, although one patient also had a positive wound swab with a different species to the drain culture. The bacteria causing the infection in another patient was not determined and infection was diagnosed only by the patients' symptoms and blood analysis. Since the SSIs were not discovered by wound swabs but drain cultures, in most cases, this may suggest that the bacteria that caused the SSIs originated from the organ or space surrounding the drain and not just infections of the upper layers of skin surrounding the incision. Despite staphylococci including MDR strains being the most frequently identified species from the surgical sites, *Citrobacter* spp., *Enterobacter* spp., *Clostridium perfringens* and *Streptococcus milleri* were found to be the cause of SSIs in the patients included in this study. *Enterobacter* spp. and *Citrobacter* spp. are Enterobacteriaceae and those isolated were ESBL/AmpC producing, this therefore supports the observation that the rates of SSIs caused by antimicrobial resistant Enterobacteriaceae is increasing (Public Health England, 2017).

Citrobacter freundii caused SSIs in two of the six cases. *C. freundii* is Gram-negative species in the family Enterobacteriaceae. Citrobacter infections are most commonly seen in hospitalized patients with multiple comorbidities and these infections are often acquired in the hospital environment (Lipsky *et al.*, 1980; Samonis *et al.*, 1991; Lavigne *et al.*, 2007; Mohanty *et al.*, 2007). Samonis *et al.*, (2009) investigated the different species of *Citrobacter* spp. infections in 78 hospital patients and *C. freundii* was found to be the most common (71.8 %) causative species of HAI (Samonis *et al.*, 2009). Thapa *et al.*,

(2010) highlighted the fact that MDR *Citrobacter* spp. are an important cause of SSIs and were the most common species isolated from surgical sites (23/29) and 20 of the 23 *Citrobacter* spp. recovered in the Thapa study were also found to be MDR (Thapa *et al.*, 2010).

Enterobacter **spp**. caused 50 % of the SSIs in this study. Enterobacter are in the Enterobacteriaceae family. Enterobacter are normal inhabitants of the human and animal digestive system. Enterobacter species are nosocomial pathogens that are capable of causing variety of infections including SSIs, urinary tract infections (UTI), osteomyelitis, respiratory infections, soft tissue infections, and endocarditis among many others (Ramirez, 2021). *Enterobacter* spp. have been reported to cause up to 44 % of SSIs following spinal surgery (Dubée *et al.*, 2012). The World Health Organisation (WHO) released a list of AMR bacteria in 2017 and carbapenem-resistant Enterobacteriaceae CRE were in the critical priority group, meaning new antibiotics are desperately needed to combat these infections (Ramirez, 2021). Jang and Yoon (2019) found an association between SSI caused by *Enterobacter* spp., higher BMI (p = 0.036) and longer hospital stay (p = 0.01) when compared to a non-Enterobacter SSI group in patients undergoing spinal surgery (Jang and Yoon, 2019). However, in this work, no correlation was determined. Although, like the work of Jang and Yoon *et al*. (2019) the patients who had an SSI caused by *Enterobacter* spp. had long hospital stays (over 2 weeks).

E. cloacae are perhaps one of the most clinically relevant Enterobacter species and part of the Enterobacter Cloacae Complex (ECC). *E. cloacae* caused an SSI in one patient in this study. This species is part of the normal gut microflora and may cause infection in

immunocompromised hosts (Keller *et al*., 1998). Enterobacter were the second most common genus (38.5 %), and *E. cloacae* was the second most common species identified in the ascites of 30.8 % of patients on day 3 following pancreatoduodenectomy (Itoyama *et al*., 2020).

E. kobei was found to cause an SSI in one patient. *E. kobei* is very similar to *E. cloacae* and is part of the ECC. *E. kobei* was distinguished from other *E. cloacae* because it gave a negative Voges-Proskauer reaction. *E. kobei* has been identified from blood, sputum, throat and urine samples. However, the clinical significance of this species is unknown, and this may be as a result of this species being wrongly identified as *E. cloacae* (Kosako *et al*., 1996).

Streptococcus milleri group are microaerophilic commensals, often found in the oral cavity, gastro-intestinal tract and genitourinary tract (Piscitelli *et al*., 1992). This group is part of the intestinal flora of 20 – 50 % of the population. The *S. milleri* group are associated with abdominal, thoracic and hepatic sepsis. Hardwick *et al*., (2000) stated that *S. milleri* should be considered as the causative pathogen of abdominal sepsis, particularly in patients who have had surgery or multiple drainage procedures (Hardwick *et al*., 2000). Furthermore, *Streptococcus anginosus* (part of the *S. milleri* group) have been found at higher levels in patients with IBS and IBD (Janket *et al*., 2015). In patients with *S. milleri* infections, including SSI, mortality has been associated with polymicrobial infections, malignancies and an age of 65 or more (Al Majid *et al*., 2020). Furthermore, Majid *et al*. (2020) found that *S. milleri* infection (46%) was often associated with Enterobacteriaceae polymicrobial infections.

Clostridium perfringens is found in the human gastrointestinal tract among other places.

It is associated with acute gastrointestinal infection (Yao, 2021). *C. perfringens* is the most common cause of gas gangrene. This species commonly causes surgical wound infections, particularly in biliary or intestinal surgeries due to contamination of the surgical wound from gut bacteria (Stevens *et al*., 2012; Takehara, 2018). It has also been found that *C. perfringens* can colonise the biliary tree (Leal *et al*., 2008). One patient had surgery involving both the pancreas and the stomach and therefore this may explain why *C. perfringens* and *E. cloacae* (another gut bacterium) were found in the drain fluid extracted from this patient. In agreement with our findings, a case study found that a 65-year-old woman, who had a pancreatectomy to remove a malignancy, had a polymicrobial SSI and abscess which included both *E. cloacae* and *C. perfringens* (Tabarelli *et al*., 2009). There was also an earlier case study that reported sudden death as result of *C. perfringens* infection following a pancreaticoduodenectomy (Königsrainer *et al*., 2007). Furthermore, Tabarelli *et al*. (2009) concluded that *C. perfringens* must be considered a source of life-threatening infection after pancreatic resection (Tabarelli *et al*., 2009).

3.3.2 Risk factors of SSIs

Bile leak

Four of the six patients who had SSIs had a bile leak during surgery. Under normal circumstances the bile is normally sterile (Isla *et al*., 2007). However, bile contamination is considered a risk factor for infectious complications following pancreaticoduodenectomy (PD) (Okano *et al*., 2015). It has been reported that 20 % of patients with HPB diseases

without any previous biliary tract intervention had contaminated bile juice at surgery

(Itoyama *et al.*, 2020). Itoyama *et al.* (2021) found that bacterial cultures collected from

bile during PD surgery and those collected from ascites on day 3 post-surgery showed

94.9 % similarity (p < 0.0001) (Itoyama *et al.*, 2021). Moreover, the Itoyama study found

that 4 of these patients had positive drain cultures on day 21 post-surgery and 30 of these

bacterial cultures were the same species with the same antibiotic resistance profiles as on

day 3.

Drains
In all of the six cases of SSI a drain was used. Drains are routinely used following

pancreatectomy to reduce post-operative complications, such as infection, by draining

fluid (Menghua *et al.*, 2020). Counterintuitively, pathogens may enter the abdominal

cavity via the drainage tubes, contaminating aseptic fluid collections (Menghua *et al.*,

2020). Late removal of surgical drains can increase the risk of infections including wound

infections (Bassi *et al.*, 2010). It has been found that retrograde drain infections increase

when drain placement is prolonged for more than four postoperative days (Shirata *et al.*,

2017). These findings are in agreement with those presented in this work as all of the

patients with an SSI had a drain fitted.

Type of surgery
All patients that developed SSIs had surgery involving the pancreas. Septic complications

following pancreas surgery are common (35 %) and SSIs are the most common septic

complication following pancreatic surgery (Okano *et al.*, 2015). Joliat *et al.*, (2018) found

that in 549 patients that had pancreatic surgery, 26 % had an SSI, of these incisional SSIs

were in 70 patients, 50 patients had an organ/space SSI and 24 had incisional and

organ/space SSI (Joliat *et al.*, 2018). In the results reported here, only three of the 26

patients had a pancreaticoduodenectomy and resection of antrum of stomach and all of these patients developed an SSI, perhaps indicating the involvement of the stomach during the surgical procedure a cause of infection. However, in all patients (apart from one where the cause of infection was not known) species that are found in the gastrointestinal tract were cultured from drain fluid. This could be due to the close proximity of the pancreas to the duodenum and stomach.

Open surgery
All of the patients that developed SSIs had open surgery as opposed to laparoscopic surgery. A systematic review and meta-analysis compared patient outcomes of laparoscopic PD and open PD (Feng *et al*., 2021). Five studies found a significantly lower rate of wound infection in the open surgery group (OR: 0.35; 95 % CI: 0.22–0.56; *p* < 0.0001) (Asbun and Stauffer, 2012; Delitto *et al*., 2016; Stauffer *et al*., 2017; van Hilst *et al*., 2019; Huang *et al*., 2020).

Operation length
All of the patients who had SSIs were operated on for over 2 h and in two cases the surgery lasted over 6 h. There was only one other patient that was operated on for over 6 h, who did not develop a SSI. A systematic review on studies investigating operative times and incidence of SSIs across a variety of different surgery types found that the likelihood of SSI increased with extended operating times (Cheng *et al*., 2017). The mean operating time was 30 min longer in patients who had SSIs than those who did not (Cheng *et al*., 2017). Cheng *et al*., (2017) also found that the chances of developing a post-operative complication (such as SSI) increased with increasing operative time increments. For example, it increased by 14 % for every 30 min and 21 % for every 60 min (Cheng *et al*.,

2017). So, in agreement with the results presented here, it may be that increased

operating times resulted in increased incidence of SSI.

Long hospital stay
A large percentage (83.3 %) of the patients that had an SSI were in hospital for over 10

days. There is a large body of observational evidence supporting the association between

SSI and total/postoperative stay (Ortona *et al*., 1987; Vegas *et al*., 1993; Kirkland *et al*.,

1999; Hollenbeak *et al*., 2000; Jenney *et al*., 2001; Whitehouse *et al*., 2002; McGarry *et

al*., 2004; Coskun *et al*., 2005; Coello *et al*., 2005; Weber *et al*., 2008). This could be a risk

factor because patients could be more likely to pick up AMR bacteria when in hospital

(Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic

Infectious Diseases (NCEZID), Division of Healthcare Quality Promotion (DHQP), 2019).

However, it is more likely that these patients were in hospital for a longer period of time

post-surgery because they had an SSI that required a prolonged hospital stay to treat.

Indeed, Mujagic *et al*., (2018) found an association between postoperative length of

hospital stay and SSI but not pre-operative hospital stay and SSI (Mujagic *et al*., 2018).

However, whether prolonged hospital stay is a causative factor of SSIs or SSIs result in a

prolonged hospital stay, both the treatment of SSIs and prolonged hospital stay will be

costly to the NHS and result in longer waiting times for a hospital bed.

Poor post-operative nutrition
Those patients with better post-operative nutrition and calorie intake were found to be

less likely to get an infection (p = 0.028). There is currently little literature regarding post-

operative weight loss as a risk factor for SSI, although there are various studies reporting

pre-operative poor nutrition as a risk factor. For example, Skeie *et al*, (2018) studied

patients undergoing aortocoronary bypass, caesarean, inserting prosthesis in hip joint,

colon surgery and cholecystectomies (open and laparoscopic) and found a significantly

higher incidence of SSI in those with nutritional risk (11.8 %), when compared to those

without (7.0 %) (p = 0.047) (Skeie et al., 2018). Additionally, Shinkawa et al. (2012) found

that preoperative nutritional risk index was associated (p < 0.05) with SSI in patients

undergoing pancreaticoduodenectomy (Shinkawa et al., 2013). One study looking post-

operative weight loss among patients undergoing posterior spinal fusion found that

clinically severe postoperative weight loss was associated with a significantly increased

superficial wound infection incidence (13.6 vs. 2 %, p = 0.047) (Tarrant et al., 2015).

However, post-operative wight loss as a risk factor of SSI may differ among different

surgeries and more research is needed in regard to post-operative weight loss as a risk

factor of infection among HPB surgery patients specifically. Moreover, weight loss usually

occurs due to reduced nutritional intake, but it could also be due to a catabolic state

caused by an increased stress response following surgery (Skeie et al., 2018). This stress

response might result in decreased immune response, poor wound healing and infection.

Pneumonia
Like SSIs, hospital-acquired pneumonia has been suggested to be another infective

complication found in hospital in patients. It has been shown that elective surgery

increased the cause-specific hazard ratios for nosocomial pneumonia (Wolkewitz et al.,

2008). In contrast, Elliot et al. (2017) found no significant association between pneumonia

incidence and SSI in pancreatic surgery patients (Elliott et al., 2017). It may be that if a

patient has one infection following surgery, they may be prone to developing other

infections due to an increased stress response that alters the immune defence. Gundel et

al. (2018) investigated pulmonary complications and SSIs in 1400 patients undergoing

laparotomy (Gundel et al., 2018). It was found 3.5 % of patients developed pneumonia

and 19.6 % of patients developed an SSI. However, like much of the current literature, the link between these two types of infection was not investigated further. To the authors knowledge, there is currently little literature on post-operative pneumonia as a risk factor of SSI following HPB surgery and more research is needed in this area.

Return to theatre

Three of the six patients who had an SSI were either returned to theatre for a further surgical procedure or had a previous HPB surgery. The Dutch surveillance network for healthcare-associated infections PREZIES includes 'prior surgery,' 'multiple surgical procedures,' and 'repetitive surgeries' as risk factors for SSI, however, more evidence is required. Verberk *et al*. (2017) found no association between prior surgery and the incidence of SSIs, but multiple surgeries (OR 1.27) and repetitive surgeries (OR 2.31) have been shown to increase the odds of developing an SSI (Verberk *et al*., 2017). However, none of the surgeries investigated in those studies were classified as HPB. One reason that a return to surgery might result in an increased risk of SSI, is that more deep tissue is handled, thus increasing the risk of a deeper SSI (Surgical site infection surveillance service (SSISS), 2021). Another reason could be that longer surgery is associated with increased risk of SSI and repeat surgery would mean more time spent on the operating table when compared to a single surgical procedure (Pessaux *et al*., 2003).

3.3.3 Blood analysis

In the SSI patients the average WBC count was higher than in the non-SSI group. Following surgery, the inflammatory process begins when WBCs travel to the surgical site and this is mediated by cytokines and acute phase proteins (Germolec *et al*., 2018). This results in an increase of neutrophils in the blood and a decreased lymphocyte count, this is referred to as neutrophil to lymphocyte ratio (NLR) and is often used as biomarker of

inflammation (Bhat *et al.*, 2013). Predictably, the average neutrophil count was above the reference range following surgery in the non-SSI group and was even higher in the SSI group. However, the lymphocyte count was within range when looking at averages in SSI and non-SSI groups. Only one SSI patient demonstrated that the lymphocyte count was below reference range post-surgery.

The CRP levels were above range in all of the average pre-surgery, post-surgery, SSI and non-SSI groups. However, CRP levels were even higher in the post-operative SSI group and in all of the SSI patients individually. CRP is a biomarker instigated by tissue damage, infection, inflammation and malignancy and is the most widely used for diagnosing infections following surgery (Daryapeyma *et al.*, 2014; Lowsby *et al.*, 2015). As these patients have malignancies it would be expected that their CRP would be higher than normal prior to surgery. Hart *et al*. (2020) conducted a review and found that CRP was 10 – 50 µg/mL in patients with liver cancer (Carr *et al.*, 2018) and 3 - 50 µg/mL in patients with pancreatic cancer (Chen *et al.*, 2018; Hart *et al.*, 2020). The tissue damage and inflammation from surgery would then result in a higher CRP level and then the patients who developed an infection would have a further increase in CRP levels in their blood.

The average platelet (PLT) count was higher than normal after surgery in the SSI group but was within range before surgery and both before and after surgery in the non-SSI group. Liu *et al*. (2022) also found that PLT count increased post-surgery in patients with an SSI following repair of a femoral neck fracture surgery (Liu *et al.*, 2022). PLTs were involved in blood clotting and thus preventing bleeding (Jackson, 2011). It also known that bacteria can cause PLT aggregation which may suggest why PLTs are high in SSI

patients (Clawson, 1971). Other studies have also found that increased PLTs are associated with an increase of infection rates (Nurden *et al.*, 2012; Li and Emsley, 2013).

3.4 Conclusions

This research highlights the role of gut bacteria in SSIs following HPB surgery, since all the patients who had pancreaticoduodenectomy and resection of antrum of stomach had an SSI. Furthermore, all of the patients who had a SSI had organisms that were found in the GI tract isolated from their drain fluid following surgery. This is further supported since all of the patients who had an SSI had surgery involving the pancreas and the pancreas in close proximity of the GI tract. All of the patients, who got an SSI, had open surgery as opposed to laparoscopic and thus this may be an important risk factor for SSI following HPB surgery. Post-operative risk factors identified were poor post operative nutrition and longer hospital stay (> 10 days), although it was difficult to determine if the infection itself was causing these factors.

Chapter 4. Bacterial colonisation of bacteria found on the hospital ward

4.1 Introduction

Hospitals act as reservoirs for multi-drug resistant (MDR) bacteria and extensively drug resistant (XDR) bacteria due to high antibiotic use in patients and hands-on care from healthcare workers. MDR bacteria are defined as non-susceptibility to at least one agent in three or more antimicrobial categories and XDR are defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (Magiorakos *et al*., 2012). Work of others has shown that that the microbiome of a hospital is incredibly similar to that of the patients (Lax *et al*., 2017), to the authors knowledge, it has not been shown whether this also applies to patient's surgical sites. The aims of this chapter were to determine the types of bacteria and the levels of AMR in bacteria found on hepatopancreatobiliary (HPB) surgery patients and on surfaces of a HPB hospital ward. Patients were swabbed on their surgical site before and after surgery and the bacteria isolated were identified and tested for antibiotic resistance. Areas of the HPB ward were also swabbed and the isolates were identified and tested for antibiotic resistance. From the patients, 11 XDR and 22 MDR species were identified. All of these were *Staphylococcus* spp. apart from the XDR *E. cloacae*. From the hospital ward 6 MDR species and 13 XDR species were identified, these were all *Staphylococcus* spp. except an XDR *Citrobacter koseri* found in the bathroom sink and a MDR *Pseudomonas stutzeri* isolated from the nurses' computer keyboard. *Acinetobacter lwoffii* which was found on the floor and had intermediate resistance to ciprofloxacin. *S. epidermidis* found on the shower outlet and was resistant to cefoxitin, ciprofloxacin, erythromycin, fusidic acid, gentamicin and norfloxacin. *S. epidermidis* which was found on the shower control was

resistant to cefoxitin and had intermediate resistance to ciprofloxacin. *S. epidermidis* was isolated from the soap dispenser, which was resistant to cefoxitin, ciprofloxacin, gentamicin and norfloxacin. *S. epidermidis* found on the sink tap and nurses' computer keyboard which was resistant to all of the antibiotics tested. A methicillin resistant *S. hominis* was found on the touch-screen patient TV.

4.2 Results

4.2.1 Bacteria colonizing the surgical wounds

A variety of bacterial species were isolated from the skin or surgical sites of patients although only six patients had SSIs. Gram-positive organisms were isolated more frequently than Gram-negative organisms and *Staphylococcus* spp. were by far the most commonly identified. *S. epidermidis* was most frequently isolated and was colonizing the surgical sites of 14 patients (Table 21). Generally, the number of organisms isolated from patients on day 1 following surgery decreased from the number found on the pre-operative swabs. For patients who stayed in hospital longer than a week the number of organisms colonizing their surgical wounds generally then increased. Gram-positive rods such as *Corynebacterium* spp., *Brevibacterium* spp. and *Paenibacillus* spp. were only isolated prior to surgery. One patient had a particularly high number of Gram-positive organisms on their skin prior to surgery, with four different species identified. The Gram-

negative bacilli found on patients' surgical sites included *Acinetobacter lwoffii*, *Enterobacter cloacae*, *Enterobacter ludwigii* and *Pseudomonas aeruginosa*.

The AMR profiles of some of the bacteria isolated were determined. There were isolates that AMR could not be determined due to EUCAST breakpoints for those species not existing or unforeseen circumstances. Many of the CoNS were methicillin resistant, MDR or XDR. *S. epidermidis* and *S. haemolyticus* showed particularly high levels of resistance to antibiotics with some strains being resistant to all antibiotics tested (Table 23).

Table 21. Numbers of different bacteria species found on patients.

Species	Total	Pre-op	Day 1	Day 7	Day 14	Day 28	No. of patients
Gram positive cocci							
Staphylococcus epidermidis	24	7	8	5	3	1	14
Staphylococcus haemolyticus	15	7	2	4	1	1	12
Staphylococcus capitis	10	6	2	2	0	0	8
Staphylococcus hominis	20	9	5	5	1	0	13
Staphylococcus aureus	7	5	2	0	0	0	5
Staphylococcus warneri	3	1	1	1	0	0	3
Micrococcus yunnanensis	2	2	0	0	0	0	2
Micrococcus luteus	5	3	2	0	0	0	4
Micrococcus spp.	4	1	3	0	0	0	3
Enterococcus faecium	1	1	0	0	0	0	1
Dermacoccus nishinomiyaensis	3	2	0	1	0	0	3
Staphylococcus saprophyticus	1	0	0	1	0	0	1
Staphylococcus sciuri	2	1	0	0	1	0	2
Staphylococcus auricularis	1	0	0	1	0	0	1
Staphylococcus simulans	2	1	0	1	0	0	2
Staphylococcus pasteuri	1	1	0	0	0	0	1
Staphylococcus lugdunensis	2	1	1	0	0	0	2
Kocuria kristinae	1	0	1	0	0	0	1
Kocuria rhizophila	2	2	0	0	0	0	2
Rothia terrae	1	1	0	0	0	0	1
Gram negative cocci							
Neisseria subflava	2	1	0	0	1	0	2
Moraxella osloensis	1	0	1	0	0	0	1
Gram negative rods							
Acinetobacter lwoffii	3	2	1	0	0	0	3
Enterobacter cloacae	1	0	0	0	1	0	1
Enterobacter ludwigii	1	1	0	0	0	0	1
Pseudomonas aeruginosa	1	0	0	1	0	0	1
Gram positive rods							
Gordonia otitidis	1	1	0	0	0	0	1
Paenibacillus glucanolyticus	1	1	0	0	0	0	1
Paenibacillus amylolyticus	1	1	0	0	0	0	1
Corynebacterium aurimucosum	1	1	0	0	0	0	1
Corynebacterium imitans	1	1	0	0	0	0	1
Corynebacterium sundsvallense	1	1	0	0	0	0	1
Corynebacterium singulare	1	1	0	0	0	0	1
Brevibacterium paucivorans	1	1	0	0	0	0	1
Brevibacterium casei	1	1	0	0	0	0	1
Brevibacterium spp.	1	0	0	1	0	0	1
Helcobacillus massiliensis	1	1	0	0	0	0	1

Table 22. Species isolated from wound swabs at different stages.

Pre-operation	Day 1 post-operation	Day 7 post-operation	Day 14 post-operation	Day 28 post-operation	Discharge from hospital
Paenibacillus glucanolyticus *S. aureus*	*Micrococcus spp.* *Moraxella osloensis*				
Brevibacterium casei *Micrococcus spp.* *S. aureus* *S. haemolyticus*	*Acinetobacter lwoffii* *Micrococcus spp.* *S. aureus*				
S. aureus *S. epidermidis*	*S. hominis* *S. warneri*	*S. capitis* *S. epidermidis* *S. saprophyticus*			
A. lwoffii *S. epidermidis*	*S. epidermidis*	*Brevibacterium frigoritolerans*	*Enterobacter cloacae* *S. epidermidis*		

S. haemolyticus S. hominis		S. haemolyticus			
			S. sciuri		
S. aureus S. capitis	S. aureus S. capitis S. haemolyticus				
S. aureus S. capitis S. epidermidis	Micrococcus spp. S. epidermidis S. hominis.				
Helcobacillus massiliensis Neisseria subflava S. epidermidis S. haemolyticus S. hominis S. sciuri	Kocuria kristinae S. epidermidis	S. epidermidis S. haemolyticus S. warneri			
Brevibacterium paucivorans Corynebacterium aurimucosum Corynebacterium imitans Corynebacterium sundsvallense S. capitis		Pseudomonas aeruginosa S. epidermidis			

S. hominis *S. lugdunensis* *S. simulans*	*S. haemolyticus*	Unidentified yeast		-	-
A. lwoffii *Enterobacter ludwigii* *Micrococcus yunnanensis* *S. capitis* *S. haemolyticus*	*Dermacoccus spp.* *Micrococcus luteus*				
Gordonia otitidis *M. luteus* *Rothia terrae* *S. hominis* *S. pasteuri*	*Micrococcus spp.*	*S. haemolyticus*			
Dermacoccus nishinomiyaensis *S. hominis*	No growth	*S. epidermidis* *S. hominis*			*Corynebacterium amycolatum* *S. epidermidis*
S. epidermidis *S. haemolyticus* *S. hominis*	No growth	*S. hominis*	*S. haemolyticus*		

Enterococcus faecium M. luteus S. capitis Unidentified yeast	M. luteus S. epidermidis	S. capitis S. haemolyticus S. hominis	S. hominis		
M. luteus S. haemolyticus	S. haemolyticus			S. epidermidis S. haemolyticus	
Corynebacterium singulare M. luteus Micrococcus yunnanensis	No growth				
S. hominis	S. capitis S. hominis				
S. epidermidis S. hominis S. saprophyticus	S. hominis				
D. nishinomiyaensis Kocuria rhizophila S. hominis S. lugdunensis	S. hominis	S. haemolyticus S. hominis S. simulans			
S. epidermidis	No growth				
Paenibacillus amylolyticus					

S. hominis	S. epidermidis S. hominis				
S. epidermidis	S. epidermidis S. lugdunensis				
Staphylococcus spp.		D. nishinomiyaensis			
S. epidermidis S. hominis	No growth				
K. rhizophila S. haemolyticus	No growth				
S. warneri	S. epidermidis				
S. capitis	S. epidermidis	S. epidermidis S. hominis	Neisseria subflava S. epidermidis		

Table 23. Antimicrobial resistance profiles of bacteria isolated from patient surgical wound swabs before and after surgery (S = sensitive, I = Intermediate, R = Resistant).

Bacteria species	Time of swab	Cefoxitin	Ciprofloxacin	Erythromycin	Fusidic acid	Gentamicin	Norfloxacin	Tetracycline	Vancomycin	Meropenem	Ampicillin	Piperacillin-tazobactam
S. aureus	Pre-op	S	S	S	S	S	S	S				
S. aureus	Day 1	S	S	S	S	S	S	S				
Acinetobacter lwoffii			I			S				S		
S. aureus	Pre-op	S	S	S	S	S	S	S				
S. warneri		S	S	S	S	S	S	S				
S. capitis	Day 7	S	S	S	S	S	S	S				
S. epidermidis		R	R	R	R	I	R	S				
S. saprophyticus		S	S	R	S	S	S	S				
Acinetobacter lwoffii			I			S				S		
S. epidermidis	Pre-op	I	S	S	S	S	S	S				
S. haemolyticus		R	I	S	S	R	R	S				
S. epidermidis	Day 1	R	R	R	R	R	R	S				
S. haemolyticus	Day 7	R	R	S	S	R	R					
S. epidermidis		R	S	R	S	S	S	I				
S. sciuri	Day 14	S	S	S	R	S	S	S				
Enterobacter cloacae		R	S			S				R	R	R
S. aureus	Pre-op	S	S	S	R	S	S	I				

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S. capitis		S	S	S	S	S	S	S				
S. aureus		S	S	S	R	S	S	S				
S. capitis	Day 1	R	S	S	S	S	S	S				
S. haemolyticus		S	S	S	S	S	S	R				
S. aureus		S	S	S	S	S	I	S				
S. capitis	Pre-op	S	S	S	S	I	S	S				
S. epidermidis		S	S	S	S	S	S	S				
S. epidermidis	Day 1	S	S	I	S	S	S	S				
S. hominis		S	I	R	S	S	R	S				
S. sciuri		S	S	R	R	S	S	S				
S. hominis		S	S	R	R	S	S	S				
S. epidermidis	Pre-op	S	I	S	S	R	R	R				
S. haemolyticus		R	S	R	S	S	S	S				
S. epidermidis		R	R	R	R	R	R	S				
S. haemolyticus	Day 7	R	R	R	R	R	R	I				
S. epidermidis		I	S	R	R	S	S	R				
S. auricularis	Day 7	S	S	R	S	S	S	S				
S. capitis		S	S	R	R	S	S	R				
S. simulans		S	S	S	R	S	S	S				
S. hominis	Pre-op	S	S	S	S	S	S	S				
Corynebacterium imitans			I					S				
S. lugdunensis		S	S	S	S	S	S	S				
S. haemolyticus	Day 1	R	R	S	S	R	R	S				
Pseudomonas aeruginosa			I							S		I
S. haemolyticus		S	S	S	R	S	S	S				
S. capitis	Pre-op	S	S	S	S	S	S	S				
Acinetobacter lwoffii			S		S					S		
S. hominis	Pre-op	S	S	I	S	S	S	S				

Organism	Time											
S. pasteuri		S	S	S	R	S	S	I				
S. hominis		R	I	S	S	S	S	S				
S. hominis	Day 7	R	I	R	S	S	S	S				
S. epidermidis	Day 7	R	S	R	S	R	I	S				
S. epidermidis	Discharge	R	S	R	R	R	S	S				
Corynebacterium amycolatum	Discharge		R				R	I				
S. epidermidis	Pre-op	R	I	S	S	S	S	S				
S. hominis	Pre-op	R	I	R	R	R	S	R				
S. haemolyticus	Pre-op	R	I	S	R	S	S	R				
S. hominis	Day 7	R	S	R	R	R	S	S				
S. haemolyticus	Day 14	R	R	R	S	R	R	S				
S. epidermidis	Day 1	R	R	S	S	R	R					
S. haemolyticus	Day 7	R	R	S	S	R	R	S				
S. capitis	Day 7	R	R	S	S	R	R	S				
Enterococcus faecium	Pre-op						R		S		R		
S. haemolyticus	Day 1	S	S	S	S	S	S	S				
S. epidermidis	Day 28	R	R	R	R	R	R	R				
S. haemolyticus	Day 28	R	R	S	S	R	R	S				
Corynebacterium singulare	Pre-op		S					S	S				
S. hominis	Pre-op	S	S	S	S	S	S	S				
S. capitis	Day 1	R	S	S	S	S	R	S				
S. hominis	Day 1	S	S	S	S	S	S	S				
S. epidermidis	Pre-op	S	S	S	S	S	S	S				
S. hominis	Pre-op	R	I	R	R	R	S	R				
S. saprophyticus	Pre-op	S	S	R	R	S	S	S				
S. hominis	Day 1	I	R	S	R	R	S	S				
S. hominis	Pre-op	R	S	S	S	S	S	S				
S. lugdunensis	Pre-op	R	S	S	S	S	S	S				

Species	Timepoint											
S. hominis	Day 1	R	S	S	S	S	S	S				
S. simulans	Day 7	R	S	S	S	S	S	S				
S. haemolyticus		R	R	S	S	R	R	S				
S. hominis		R	R	S	S	S	S	S				
S. epidermidis	Pre-op	S	S	R	R	S	S	R				
S. hominis	Pre-op	R	S	S	R	S	S	S				
S. epidermidis	Day 1	R	S	S	R	S	S	S				
S. hominis		R	S	S	R	S	S	S				
S. epidermidis	Pre-op	R	S	S	S	S	S	S				
S. lugdunensis	Day 1	R	S	S	S	I	S	S				
S. epidermidis		S	S	S	S	S	S	S				
S. epidermidis	Pre-op	S	I	S	S	S	S	S				
S. hominis		S	I	R	S	S	S	S				
S. haemolyticus	Pre-op	R	I	R	R	S	S	S				
S. warneri	Pre-op	S	I	S	R	S	S	S				
S. epidermidis	Day 1	R	S	R	S	S	R	S				
S. capitis	Pre-op	S	I	S	R	S	S	S				
S. epidermidis	Day 1	S	S	S	R	I	S	S				
S. epidermidis	Day 7	R	S	R	R	I	S	S				
S. hominis		S	R	R	R	R	S	S				
S. epidermidis	Day 14	R	R	R	R	R	R	S				

4.2.2 Bacteria recovered from the hospital ward

Samples were taken from areas immediately surrounding the patients and across the ward to determine the bacterial species that could be recovered from the surfaces. The bacterial species recovered were also tested for their susceptibility to antibiotics. Gram positive cocci were the most frequently recovered type of bacteria (81 %) followed by Gram-positive Bacillus (7.9 %) and Gram-negative Bacillus (9.5 %) (Table 24).

Staphylococcus epidermidis was isolated from hospital surfaces more frequently than any other species (*n* = 15), followed by *Staphylococcus haemolyticus* (*n* = 7), *Staphylococcus capitis* (*n* = 6), *Staphylococcus hominis* (*n* = 5) and *Micrococcus* spp. (*n* = 5). *Pseudomonas stutzeri* was the most commonly isolated Gram-negative species (*n* = 2). The computer keyboard at the nurses' workstation had the largest number of different species (*n* = 7), followed by the floor on the HPB ward (*n* = 5), the patient bedside chair (*n* = 4), the soap dispenser on the corridor of the HPB ward (*n* = 4) and nurses' phone (*n* = 4).

Nurses Area

The nurses' workstation located in the high dependency unit (HDU) was sampled to recover bacterial species. The nurses' computer keyboard had the largest number of species, these included *S. epidermidis*, *Dermacoccus nishinomiyaensis*, *S. haemolyticus*, *Rothia dentocariosa*, *Roseomonas mucosa*, *Kocuria marina* and *Pseudomonas stutzeri. P. stutzeri* was MDR and resistant to meropenem and tested intermediate resistance to piperacillin-tazobactam and ciprofloxacin. The *S. epidermidis* and *S. haemolyticus* found on the computer keyboard were XDR. The PC screen on the nurses' workstation was swabbed and no species were isolated*. S. epidermidis*, *S. capitis*, *Micrococcus* spp. and *Brevibacterium luteolum* were identified from the swab taken from the nurses' phone. The *S. capitis* isolated from the phone was XDR.

Table 24. Total number of different species found on the hospital surfaces.

Morphology	Species	N	Total %
	Staphylococcus epidermidis	15	81 %
	Staphylococcus haemolyticus	7	
	Staphylococcus capitis	6	
	Staphylococcus hominis	5	
	Micrococcus spp.	5	
	Staphylococcus saprophyticus	2	
	Dermacoccus nishinomiyaensis	2	
Gram positive cocci	*Enterococcus faecalis*	1	
	Micrococcus luteus	1	
	Staphylococcus sciuri	1	
	Aerococcus viridians 2	1	
	Kocuria marina	1	
	Staphylococcus succinus	1	
	Staphylococcus xylosus	1	
	Dietzia cinnamea	1	
	Staphylococcus pettenkoferi	1	
	Bacillus cereus	1	7.9 %
	Microbacterium phyllosphaerae	1	
Gram-positive bacillus	*Paenibacillus lautus*	1	
	Rothia dentocariosa	1	
	Brevibacterium luteolum	1	
	Pseudomonas stutzeri	2	9.5 %
	Citrobacter koseri	1	
Gram-negative bacillus	*Acinetobacter lwoffii*	1	
	Raoultella planticola	1	
	Roseomonas mucosa	1	
Fungus	Yeast	1	1.6 %
		63	

Figure 8. Number of different species found on each surface.

Patient bedside surfaces

MDR and XDR CoNS were identified on the cabinet, chair, pillow, TV and mattress with some of these isolates expressing resistance to all of the antibiotics tested. The pathogenic bacteria *Enterococcus faecalis* was identified from the pillow used by a patient and was resistant to norfloxacin and ampicillin but sensitive to vancomycin. The potentially pathogenic Gram-positive rod, *Bacillus cereus* was identified on the bed rail and resistant to meropenem and expressed intermediate resistance to vancomycin. *Raoultella planticola* is a Gram-negative bacillus that was isolated from the cabin*et* although, it was sensitive to all antibiotics tested. No bacteria were isolated from the swab taken from the clipboard at the end of the patients bed.

Surfaces that were further away from the patient vicinity were sampled. From these, those that were XDR included *S. xylosus* and *S. epidermidis* isolated from the soap dispenser and a MDR *S. capitis* isolated from the glove dispenser. The floor was contaminated with two potentially pathogenic Gram-negative rods, *Acinetobacter lwoffii* and *P. stutzeri.* The *P. stutzeri* was intermediately resistant to ciprofloxacin and resistant to piperacillin-tazobactam. No bacteria were identified from the swab taken from the wall on the hospital ward.

Patient bathroom surfaces

Samples were taken from surfaces in the patient bathroom. Apart from two CoNS, all of the bacterial species found in samples from the bathroom were XDR organisms. An unidentified yeast was also extracted from the sink plug. A potentially pathogenic Gram-negative bacillus, *Citrobacter koseri* was isolated from the sink plug. This isolate was XDR and expressed intermediate resistance to cefoxitin, gentamicin and meropenem. Furthermore, it was resistant to ampicillin and piperacillin-tazobactam, which was the antibiotic used as prophylaxis for HPB surgery in this specific hospital.

Table 25. Antimicrobial resistance profiles of bacteria isolated from a HPB ward.

Ward area	Ward surface	Species	Cefoxitin	Ciprofloxacin	Gentamicin	Norfloxacin	Erythromycin	Fusidic acid	Tetracycline	Vancomycin	Meropenem	Ampicillin	Piperacillin-tazobactam
Nurses' workstation	Nurse desk	*Dermacoccus nishinomiyaensis*	/	/	/	/	/	/	/	/	/	/	/
		S. epidermidis	S	I	S	S	S	S	R				
	Nurse desk	*S. capitis*	R	I	S	S	S	S	S				
	Nurse phone	*S. epidermidis*	R	I	S	S	R	S	S				
		Micrococcus spp.	/	/	/	/	/	/	/	/	/	/	/
		S. capitis	S	R	R	R	R	R	R				
		Brevibacterium luteolum	/	/	/	/	/	/	/	/	/	/	/
	Nurse keyboard	*S. epidermidis*	R	R	R	R	R	R	R				
		Dermacoccus nishinomiyaensis	/	/	/	/	/	/	/	/	/	/	/
		S. haemolyticus	R	R	R	R	R	R	R				
		Rothia dentocariosa	/	/	/	/	/	/	/	/	/	/	/
		Roseomonas mucosa	/	/	/	/	/	/	/	/	/	/	/
		Kocuria marina	/	/	/	/	/	/	/	/	/	/	/
		Pseudomonas stutzeri		I							R		I
Patient bathroom	Sink tap	*S. haemolyticus*	R	R	R	R	R	S	S				
		S. epidermidis	R	R	R	R	R	R	R				
	Plug	*Citrobacter koseri*	I	S	I						I	R	R
		Yeast	/	/	/	/	/	/	/	/	/	/	/
		S. haemolyticus	R	I	R	S	R	I	R				
	Shower outlet	*S. epidermidis*	R	R	R	R	R	R	S				
		S. hominis	S	S	S	S	R	R	S				
	Shower control	*S. epidermidis*	R	I	S	S	S	S	S				
Ward surfaces	Floor	*Micrococcus spp.*	/	/	/	/	/	/	/	/	/	/	/
		S. saprophyticus	S	I	S	S	R	S	S				
		S. epidermidis	S	S	S	S	S	S	R				
		Acinetobacter lwoffii		I	S	S					S		
		Pseudomonas stutzeri		I							S		R
	Glove dispenser	*S. hominis*	S	S	S	S	R	S	R				
		S. capitis	S	S	S	S	S	S	S				
		S. succinus	S	S	S	S	S	S	S				
	Soap dispenser	*S. haemolyticus*	R	S	S	S	S	R	S				
		Paenibacillus lautus	/	/	/	/	/	/	/	/	/	/	/

Group	Surface	Bacteria											
		S. xylosus	R	R	R	R	R	R	S				
		S. epidermidis	R	R	R	R	S	S	S				
	Floor	*S. capitis*	S	I	S	S	S	S	S				
		S. epidermidis	S	S	R	S	S	S	S				
		Dietzia cinnamea	/	/	/	/	/	/	/	/	/	/	/
		S. pettenkoferi	R	I	S	S	S	S	S				
	Glove dispenser	*S. capitis*	R	I	S	S	R	R	S				
Patient bedside	Cabinet	*Micrococcus spp.*	/	/	/	/	/	/	/	/	/	/	/
		S. haemolyticus	R	R	R	R	R	R	R	/	/	/	/
	Chair	*S. haemolyticus*	S	I	S	R	R	S	S				
		S. epidermidis	S	R	S	R	S	S	S				
		Micrococcus spp.	/	/	/	/	/	/	/	/	/	/	/
		S. hominis	R	R	R	R	R	S	S				
	Table	*S. epidermidis*	S	S	S	S	R	S	S				
		Aerococcus viridians 2	/	/	/	/	/	/	/	/	/	/	/
		S. capitis	S	R	S	S	S	R	S				
	Pillow	*Enterococcus faecalis*				R				S		R	
		Micrococcus spp.	/	/	/	/	/	/	/	/	/	/	/
		S. hominis	S	S	S	R	R	R	S				
	Bed rail	*S. epidermidis*	R	R	S	S	S	S	S				
		Bacillus cereus				S	S			I	R		
	Curtain rail	*S. epidermidis*	S	S	S	S	S	S	S				
	Cabinet	*Raoultella planticola*	S	S	S						S	S	S
		Micrococcus luteus	/	/	/	/	/	/	/	/	/	/	/
		S. haemolyticus	R	R	R	R	R	R	R				
	TV	*S. saprophyticus*	R	R	S	S	R	R	S				
		S. hominis	R	S	S	S	S	S	S				
		S. sciuri	S	R	S	S	S	R	S				
	Chair	*S. epidermidis*	S	S	S	S	S	R	S				
	Mattress	*Microbacterium phyllosphaerae*	/	/	/	/	/	/	/	/	/	/	/
		S. epidermidis	R	R	S	R	R	S	S				

4.2.3 Transmission of bacteria between patients and surfaces

Some of the bacteria recovered had the same antimicrobial resistance profiles and these included *S. aureus, Acinetobacter lwoffii, S. capitis, S. haemolyticus, S. epidermidis* and *S. hominis*.

4.3 Discussion

An XDR *E. cloacae* was resistant to piperacillin-tazobactam, cefoxitin, meropenem and ampicillin. Piperacillin-tazobactam is the antibiotic given as prophylaxis to surgical patients, thus it is of concern that some bacteria were found to be resistant to this antibiotic. *Pseudomonas stutzeri* was found on the floor that was resistant to piperacillin-tazobactam and on the nurses' computer keyboard with intermediate resistance to piperacillin/tazobactam. *C. koseri* which was XDR, including, resistant to piperacillin/tazobactam was isolated from the plug in the sink in patient bathroom. Studies have shown that most *C. koseri* isolates are susceptible to piperacillin/tazobactam and one study found that *Citrobacter* spp. resistance to piperacillin/tazobactam did not exceed 10 % (Deveci and Coban, 2014; Hrbacek *et al*., 2021). *Citrobacter* spp. have previously been susceptible to almost all antibiotics, however, resistance genes can spread easily in this species via plasmid and chromosomal mediated genes (Deveci and Coban, 2014).

4.3.1 Most frequently identified bacteria

S. epidermidis was identified from surfaces in higher numbers than other species and was identified on 15 surfaces. *S. epidermidis* is part of the normal skin and mucosa microbiome (Otto, 2009). Wojtyczka *et al*., (2014) also found that *S. epidermidis* was the most frequently recovered CoNS (26.2 %) from hospital surfaces (Wojtyczka *et al*., 2014). *S. epidermidis* was once considered a harmless contaminant of clinical samples although it is now apparent that it has an important role in nosocomial infections and that nosocomial genotypes of *S. epidermidis*

colonize patients and healthcare workers, causing a large number of healthcare associated infections (Becker *et al.*, 2007; Miragaia *et al.*, 2007; Hira *et al.*, 2010; Conlan *et al.*, 2012; Widerström *et al.*, 2012; Rolo *et al.*, 2012; Mendes *et al.*, 2012; Saffari *et al.*, 2016). Multi-drug resistant *S. epidermidis* phenotypes are often the cause of such infections and this species often forms biofilms on indwelling medical devices, thus negatively impacting antimicrobial treatment (Becker *et al.*, 2014). The most commonly isolated bacterium from prosthetic joint infections is *S. epidermidis* and this species accounts for approximately 30 % - 50 % of such infections (Zimmerli *et al.*, 2004; Arciola *et al.*, 2005; 2006; Campoccia *et al.*, 2009; Peel *et al.*, 2012).

S. haemolyticus was the second most frequently isolated species from the hospital ward and was found on seven surfaces. *S. haemolyticus* is a CoNS and commonly found in the armpit of humans. This species is the second most frequently identified species from blood cultures (Takeuchi *et al.*, 2005). In terms of prevalence on hospital wards, multi-drug resistant *S. haemolyticus* that produces biofilms and has been identified from neonatal ICUs and hospital wards (Monsen *et al.*, 2005; Widerström *et al.*, 2006; Liakopoulos *et al.*, 2008). Again, Wojtyczka *et al.*, (2014) also found that *S. haemolyticus* was the second most frequently isolated CoNS species from the hospital environment (Wojtyczka *et al.*, 2014) and may cause infection in immunocompromised hosts such as those having chemotherapy. *S. haemolyticus* is considered the most antibiotic resistant of the CoNS species (Cavanagh *et al.*, 2014). The SCC*mec* type V gene, responsible for methicillin resistance, has been found in *S. haemolyticus* (Froggatt *et al.*, 1989; Chiew *et al.*, 2007). High levels of methicillin resistance were found in the *S. haemolyticus* strains isolated from patients and the hospital wards and 81 % of the strains tested were methicillin resistant, MDR or XDR.

S. capitis was found on six different surfaces. This species is a CoNS, a human skin commensal and normally found surrounding the sebaceous glands on the face, scalp, and neck (Schleifer, 1975; Froggatt *et al*., 1989). *S. capitis* often causes biofilm-related infections such as endocarditis (Nalmas *et al*., 2008), catheter related bacteraemia (Tristan *et al*., 2006) and urinary tract infections (Oren and Merzbach, 1990). Dissemination of *S. capitis* in healthcare environments have been reported, including a tertiary care facility in China and an ICU in Greece (Papadimitriou-Olivgeris *et al*., 2013; Zhou *et al*., 2015). Moreover, during prosthetic joint surgery, *S. capitis* has been reported to be the predominant CoNS in laminar air flow (Månsson *et al*., 2015). One study found that of CoNS identified from hospital surfaces (air, walls, floors and medical equipment) 17.2 % of these *were S. capitis* (Wojtyczka *et al*., 2014).

S. hominis was identified on five different hospital surfaces. *S. hominis* is a CoNS and human skin commensal and often isolated from axillae and pubic areas high in apocrine glands (Schleifer, 1975; Kloos and Musselwhite, 1975). Like other CoNS may cause disease in immunocompromised hosts such as those with predisposed illness (Choi *et al*., 2008).

Micrococcus spp. were also found on five different surfaces. This genus of bacteria are Gram-positive cocci that are human skin commensals. These species rarely cause disease, but *Micrococcus folliculitis* may in immunocompromised humans such as those with HIV (Smith *et al*., 1999). *Micrococcus* spp.

are known to shed from human skin and be released indoor air potentially aiding transmission between people (Kooken *et al*., 2012).

4.3.2 Bacteria colonizing surgical sites

The number of species isolated on day 1, generally decreased from the pre-surgery swab and then increased thereafter. This is likely because of decontamination of the surgical site just before the incision and then the wound dressings protecting the wound from contaminants. Then during their post-operative hospital stay, the skin surrounding the wound is recolonised from the environment or their own microbiome. A variety of other different pathogenic and non- pathogenic species were isolated and summaries of these species are discussed below.

Enterococcus faecium is part of the normal GI flora but known to cause UTIs, bacteraemia, endocarditis and infect wounds (Agudelo Higuita and Huycke, 2014). Healthcare associated strains of *E. faecium* are often vancomycin resistant although this strain was sensitive to vancomycin (Agudelo Higuita and Huycke, 2014).

Dermacoccus nishinomiyaensis is not normally considered a human pathogen and is commonly found on exposed skin of the face, hands and legs of humans. Although it has been reported to cause paediatric catheter-related bacteraemia (Joron *et al*., 2019). This species was also found on the nurses' computer keyboard.

Rothia terrae is a Gram-positive coccus, which was originally isolated from soil in Taiwan and there are no reports of infections caused by this organism.

However, its relative *R. dentocariosa* has been identified to be an opportunistic pathogen causing septicaemia, endocarditis and other serious infections (Chou *et al.*, 2008).

Moraxella osloensis is a Gram-negative bacillus that is a mutualistic symbiont of a slug-parasitic nematode. There are reports of isolation from anaesthetic agents (Bennett *et al.*, 1995) and sink traps in hospitals (Rosenthal and Gilardi, 1978). There are also rare reports of infection in humans and has been isolated from the nasopharynx of healthy adults (Berger and Felsen, 1976). There have been 12 documented cases of invasive infection by *M. osloensis* and these include endocarditis, meningitis, osteomyelitis, septic arthritis, vaginitis and bacteraemia (Samir *et al.*, 2000).

Neisseria subflava is commonly found in the nasopharynx and urogenital tract of humans. A rare cause of invasive diseases such as meningitis, endocarditis, ocular infections, arthritis and bacteraemia (Baraldès *et al.*, 2000).

A. lwoffii is a Gram-negative aerobic bacillus that is found on the skin and oropharynx of 25% of healthy people (Regalado *et al.*, 2009). It is often prevalent in healthcare settings and may cause a variety of infections including SSIs in immunocompromised hosts. It may cause bacteraemia, particularly in catheterised patients, those with cancer and the immunosuppressed. In the UK, in 2020, *A. lwoffii* was reported to be the most common cause of *Acinetobacter* spp. bacteraemia causing 30 % of Acinetobacter bacteraemia cases (UK Health Security agency, 2021). Furthermore, *Acinetobacter* spp. bacteraemia rates per 100,000 were found to be highest (2.05) in the Northwest of England compared to other areas of the England and the sharpest increase in rates between 2018 – 2020 (UK Health Security agency, 2021). A*. lwoffii* often expresses high levels of antibiotic resistance. Strains isolated expressed intermediate resistance to ciprofloxacin. Conversely, Musyoki *et al.*, (2019) found that *A. lwoffii* had relatively high susceptibility

to ciprofloxacin (80 – 85 %) (Musyoki *et al*., 2019).

Pseudomonas aeruginosa is a Gram-negative bacillus that can cause infections in the urinary tract, respiratory tract, burns and surgical sites, usually in an immunocompromised host (Goldman and Schafer, 2011). *P. aeruginosa* expressed intermediate resistance to piperacillin/tazobactam and ciprofloxacin. Piperacillin-tazobactam resistance in *P. aeruginosa* is becoming more common. In a study that recovered *P. aeruginosa* isolates in 127 intensive care units, it was found that 14.4 % were resistant to piperacillin-tazobactam (Harris *et al*., 2002). Harris *et al*., 2002 found that patients who were exposed to piperacillin-tazobactam had an increased risk (odds ratio [OR] = 6.82; 95% confidence interval [CI], 4.56 to 10.21), of carrying piperacillin-tazobactam resistant *P. aeruginosa* strains (Harris *et al*., 2002).

Gordonia otitidis are Gram-positive bacilli that are environmental isolates and only described as human pathogens in a small number of case reports (Riegel *et al*., 1996; Drancourt *et al*., 1997; Lesens *et al*., 2000). *Paenibacillus glucanolyticus* is a ubiquitous, Gram-positive bacillus species found in soil, air, water and food. Spores are resistant to heat, cold and disinfectants so can survive on surfaces for prolonged periods (Celandroni *et al*., 2016). There is a rare report of this species causing endocarditis in elderly diabetic

man (Ferrand *et al.*, 2013).

Corynebacterium aurimucosum are Gram-positive bacilli that are considered human commensals. There are various case reports of identification of this species in human clinical samples such as urine, wounds and cerebrospinal fluid samples (Leal *et al.*, 2016). However, it is uncertain as of yet if they are the cause of infection. *Corynebacterium imitans* are rarely recovered from clinical samples with the exception being five reports of isolation from blood samples (Bernard *et al.*, 2002) and two isolates from urine samples (Hirokawa *et al.*, 2013). *Corynebacterium sundsvallense* has previously been isolated from a sinus culture and blood culture (Bernard *et al.*, 2002). *Corynebacterium amycolatum* is a Gram-positive bacillus that is part of the normal skin and mucous membrane flora (Knox and Holmes, 2002). *C. amycolatum* can cause a variety of infections and there are reports of isolation from pus, urine, catheter tips, blood, sputum, prostatic secretion, ear infections and cerebrospinal fluid (Sengupta *et al.*, 2015).

Brevibacterium paucivorans is an aerobic Gram- positive bacillus that are associated with dairy products but also may be found on human skin. There have been rare reports of Brevibacterium causing catheter-related

bacteraemia mainly in immunocompromised hosts. There is one reported case of

bacteraemia caused by *B. paucivorans* (Asai *et al.*, 2019). *Brevibacterium casei* is Gram-positive bacilli often found in soil but also on human skin. *B. casei* is an emerging opportunistic pathogen of the *Brevibacterium* spp*.* and it is the most common species isolated from human clinical samples.

4.3.3 Ward surfaces

The surfaces which were regularly touched by patients were shown to have a range of bacteria and these included the floor, shower outlet, shower control, sink tap, soap dispenser and the patient touch-screen TV. These are all surfaces that are regularly touched by patients and healthcare workers and are considered touchpoints.

4.3.4 Bacteria identified on hospital ward surfaces

S. epidermidis was isolated from hospital surfaces more frequently than any other species, followed by *S. haemolyticus*, *S. capitis*, *S. hominis* and Micrococcus spp. *Pseudomonas stutzeri* was the most commonly isolated Gram-negative species. In agreement to the findings of Lax *et al.*, (2017), *Staphylococcus* spp. were the most frequently identified genera on the hospital ward in this study (43). Other dominant genera identified from various surfaces in the hospital were *Staphylococcus* spp. (116), *Streptococcus* spp. (83), *Corynebacterium* spp. (77) and *Acinetobacter* spp. (27).

4.3.5 Bedside surfaces

A variety of different microorganisms were identified from the clinical environment. *Bacillus mycoides* was found on the touch-screen TV system. *B. mycoides* is a spore forming

Gram-positive bacillus that is found in soil (Stratford *et al*., 2013). Although *B. mycoides* is very closely related to *B. anthracis, B. cereus* and *B. thuringiensis* and the ribosomal DNA sequence similarity is >99.4 %, DNA-DNA re-association cannot differentiate these species (Von Wintzingerode *et al*., 1997). Therefore, the identification of this bacterium is uncertain. Studies have shown that Gram-positive bacilli are often isolated from touch-screen technology, for example, one study found that Bacillus was the second most commonly isolated organisms from nurses' phones (Dorost *et al*., 2018). There are no current reports of *B. mycoides* causing infections although its relative *B. cereus* is known to cause toxin mediated gastrointestinal infections, skin infections and there is a case study of a patient with a catheter related blood-stream infection caused by *B. cereus* (Wu *et al*., 2019).

Micrococcus luteus (formerly *Micrococcus lysodeikticus*)*, S. haemolyticus and Raoultella planticola* were found on the bedside cabinet. *R. planticola* is a non-motile, aerobic, encapsulated Gram-negative rod that is similar in appearance to *Klebsiella pneumonia. R. planticola* is commonly found in water, soil and fish (Drancourt *et al*., 2001; Teo *et al*., 2012), and has been cited as causing pneumonia, bacteraemia, necrotizing fasciitis, cystitis, cholecystitis, pancreatitis, hepatic disease and soft tissue infections (O' Connell *et al*., 2010; Wolcott and Dowd, 2010; Kim *et al*., 2012; Olson *et al*., 2013; Xu *et al*., 2015; Demiray *et al*., 2016). *Micrococcus luteus* primarily inhabits human skin although it has also been detected in mucous membranes and soil (Kocur *et al*., 2006). *M. luteus* is an opportunistic pathogen and has been reported as a cause of bacteraemia (von Eiff *et al*., 1996; Peces *et al*., 1997), septic shock (Albertson *et al*., 1978), septic arthritis (Wharton *et al*., 1986), endocarditis (Glupczynski *et al*., 1986; Dürst *et al*., 1991; Seifert *et al*., 1995), meningitis (Fosse *et al*., 1985) and pneumonia (Souhami *et al*., 1979). Despite this, to the authors' knowledge there are no reports of *M. luteus* being recovered from hospital surfaces.

Following swabbing of the bedside table, *Aerococcus viridians* 2, *S. epidermidis* and *S. capitis* were identified from the bedside table. *Aerococcus* viridians 2 is a Gram-positive coccus and often isolated from food and the environment and is a common pathogen of crustaceans and animal and an opportunistic pathogen of immunocompromised humans. *Aerococcus viridians* 2 may cause UTIs, meningitis, wound infections, osteomyelitis, septic arthritis but most commonly causes endocarditis and bacteraemia (Chen *et al.*, 2012). *A. viridians* has often been reported to be found in occupied hospital rooms and there are two case reports of patients that had developed a nosocomial UTI caused by *A. viridians* (Mohan *et al.*, 2017).

Fabrics are a surface commonly found in hospitals and include mattresses, bedding, medical gowns, laundry bags, privacy curtains and surgical drapes. Contaminated fabrics in hospitals can contain high numbers of microorganisms from bodily substances such as blood, urine, vomit, faeces and skin. Microorganisms commonly found on hospital textiles include skin flora, CoNS, *Bacillus* spp. and Gram-negative species (Blaser *et al.*, 1984). *Enterococcus faecalis, Micrococcus* spp. and *S. hominis* were found on a used patient pillow cover. *E. faecalis* is a Gram-positive coccus that is part of the normal gut micro-flora and it can cause a wide range of infections including urinary tract infections, endocarditis, bacteraemia, and wound infections (Kau *et al.*, 2005). Literature on the length of survival of microorganisms on textiles after laundering is contradictory with a variety of different laundering temperatures suggested. For example, Orr *et al*. (2002) found that Enterococci can survive laundering temperatures as high as 71°C (Orr *et al.*, 2002). In the UK the Department of Health guidelines are to launder linen at 60°C for 10 min, however, Wilcox

and Jones (1995) found that *Enterococcus faecium* could survive these conditions (Wilcox and Jones, 1995).

Another high touch area is the bed rails in hospitals which are touched by staff, visitors and patients and this area is also likely to come into contact with bodily fluids. The bodily fluids can adsorb to the bed rail surface and aid the survival of microorganisms by protecting against desiccation and reacting with antimicrobial agents (Hirai, 1991; Lambert and Johnston, 2001). In this study, *B. cereus* and *S. epidermidis* were found on the bed rail. *B. cereus* is a Gram-positive bacillus and can cause gastro-intestinal infections, anthrax-like progressive pneumonia, sepsis and central nervous system infections, especially in immunocompromised people (Bottone, 2010). Ali *et al.*, (2014) found that *B. cereus* was the second most commonly isolated species from healthcare workers and hospital surfaces in an intensive care unit and operation theatre in a hospital in Elkhomes, Libya (Ali *et al.*, 2014). There are reports of nosocomial outbreaks in ICUs due to *B. cereus* due to inadequate disinfection procedures and contaminated medical equipment (Bryce *et al.*, 1993; Gray *et al.*, 1999; Van Der Zwet *et al.*, 2000). Ali *et al.*, (2014) also found that in the absence of contaminating soil, bacterial transfer from fingertips to bed rail ranged from 38 % to 64 %, whilst transfer from rail to fingertip ranged from 22 % to 38 % (Ali *et al.*, 2012).

4.3.6 Patient and visitor bathroom

Sinks, drains and faucets in patient bathrooms are known to act as vehicles in the transmission of bacteria such as *Acinetobacter baumannii* and *P. aeruginosa* (Smismans *et al.*, 2019). Bacteria can contaminate sinks when healthcare workers and patients wash contaminated hands. It has been suggested that sink drains in hospitals contain $10^6–10^{10}$ colony-forming units (CFU)/mL of bacteria, of which approximately $10^3–10^5$ CFU/mL are Gram-negative rods, especially with waterborne bacteria (Döring *et al.*, 1991). Sinks have been identified as a source of Gram-negative outbreaks in hospitals and Kramer *et al.*

(2005) found that 100 % of sinks in a neonatal ICU were contaminated with Gram-negative bacilli (Kramer *et al.*, 2005). *Staphylococcus* spp. were the predominant species identified from areas of the patient bathroom, although *Citrobacter koseri* and a yeast were found on the sink plug. *C. koseri* is a motile Gram-negative bacillus and an opportunistic pathogen. Infections that this species can cause include endocarditis, brains abscess, urinary tract infections, wound infections, respiratory, meningitis, and sepsis (Wanger, 2017). One study found that *Citrobacter freundii* was commonly isolated from sinks although there are no reports of *C. koseri* contamination of hospital sinks (De Geyter *et al.*, 2017). Gram-negative bacilli are often found in large numbers in sinks in healthcare facilities and in a neonatal ICU 100 % of the sinks were colonised by Gram-negative rods (Kramer *et al.*, 2005). All of the bacteria identified from the bathroom apart from two CoNS were XDR. Weinstein *et al.*, (1991) found that in an ICU sink drain, a range of *P. aeruginosa* isolates which varied over time were identified with more than half of these having high-level resistance to gentamicin and tobramycin. Furthermore, chlorhexidine resistance in these strains correlated with chlorhexidine use at the sinks (Weinstein, 1991).

An XDR *S. epidermidis* (resistant to cefoxitin, ciprofloxacin, gentamicin and norfloxacin) was found on the shower control in the patient bathroom. Species that have been identified from hospital shower faucets include *Legionella* spp. and other Gram-negative species such as *Pseudomonas* spp. (U.S. Department of Health and Human Services Centers for Disease Control and Prevention (CDC), 2003). Inhalation of the aerosols generated from faucets may expose patients to these pathogens (U.S. Department of Health and Human Services Centers for Disease Control and Prevention (CDC), 2003) and have been reported to result in cases of infection of the respiratory tract (Gonzalez-Martin, 2019).

4.3.7 Surfaces on the ward

XDR *S. epidermidis* and *S. xylosus* were identified from the swab taken from the soap dispenser. Contaminated hand soap dispensers are associated with the risk of hand contamination after use (Sartor *et al*., 2000). However, if used correctly, once the soap has been applied this should decontaminate the hands. Brooks *et al*., (2002) reported chlorhexidine resistance in Gram-negative species identified from a contaminated soap dispenser which could result in survival of bacteria on the hands (Brooks *et al*., 2002). In this study, the *S. haemolyticus* was methicillin resistant and the *S. xylosus* and *S. epidermidis* were resistant to cefoxitin, ciprofloxacin, gentamicin and norfloxacin. The findings in this work were in agreement with Brooks *et al*., (2002) who swabbed 28 soap dispensers and of these 68 %, were contaminated, and CoNS were isolated from 25 % of the swabs (Brooks *et al*., 2002). Furthermore, many of the species isolated had high levels of antibiotic resistance. One of these species was XDR MRSA, which was also resistant to ciprofloxacin, clindamycin, erythromycin, gentamicin, methicillin, penicillin, tetracycline, trimethoprim–sulfamethoxazole and cefazolin, and was only susceptible to vancomycin (Brooks *et al*., 2002). *S. xylosus* was found on the soap dispenser and has previously been isolated from a clinical environment, for example Dziri *et al*. (2016) swabbed various surfaces in a hospital and of the CoNS recovered 4.8 % of these were *S. xylosus* (Dziri *et al*., 2016).

Paenibacillus lautus was also cultured from the soap dispenser on the hospital ward. This species is a Gram-positive bacillus that is an opportunistic human pathogen and has been found to cause blood-stream infections (Grady *et al*., 2016). It has been isolated from a tick (*Ixodes granulatus* Supino) found on a *Sundamys muelleri* rat and it could potentially be transmitted to humans via a tick bite (Loong *et al*., 2018). *P. lautus* is able to survive on surfaces for long periods and is resistant to heat, cold and common disinfectants. Soap

dispensers on the hospital ward are cleaned regularly and this resistance to cleaning products may explain why it was still isolated (Celandroni *et al.*, 2016).

A diverse selection of organisms can be found on the floor of hospitals due to high footfall between wards and the environment outside the hospital and transmitting organisms via footwear. The use of disinfectant on hospital floors reduces the number of organisms by 90 % - 95 %, although it has been shown that within only 1 - 2 hours, bacterial colonisation returned to pre-cleaning levels (Ayliffe, 1991). *A. lwoffii* was found on the floor. *A. lwoffii* is resistant to conventional detergent and alcohol disinfectants, irradiation, and desiccation and this may explain why it was isolated from the floor (Strassle *et al.*, 2012). Its relative *A. baumannii* is able to persist from 5 days to 5 months on healthcare equipment (Kramer *et al.*, 2005). Oberauner *et al.*, (2013) swabbed surfaces on an ICU in Austria and found the most prevalent genus was *Acinetobacter* spp*.* (24 %) (Oberauner *et al.*, 2013).

S. saprophyticus was also found on the floor and is part of the normal human flora and is commonly found in the perineum, rectum, urethra, cervix, and gastrointestinal tract. *S. saprophyticus* is known to cause urinary-tract infections, particularly in young sexually active females (Ehlers, 2020). Dziri *et al*. (2016) swabbed various surfaces in a Tunisian hospital and found of all CoNS isolated, *S. saprophyticus* was the second most commonly isolated species (36.1 %), after *S. haemolyticus* (45.8 %) (Dziri *et al.*, 2016).

Pseudomonas stutzeri was isolated from the side of the floor on the hospital ward and also the nurses' computer keyboard*. P. stutzeri* is widely distributed in the environment and an opportunistic pathogen; infections this species is associated with include bacteraemia (Goetz *et al.*, 1983; Keys *et al.*, 1983), endocarditis, (Rosenberg *et al.*, 1987), pneumonia (Carratala *et al.*, 1992; Campos-Herrero *et al.*, 1997) and skin infections (Puzenat *et al.*, 2004).

4.3.8 Nurses' area

A variety of different species were found on the nurses' computer keyboard. Computers are often used on the hospital wards although they are not usually waterproof or designed for regular disinfectant and thus may serve as reservoirs for pathogens (Lu *et al*., 2009). Factors that were found to influence the level of contamination of computer keyboards included the texture of the surface, the proximity to patients and how frequently the keyboard was used (Rutala *et al*., 2006). The species that were clinically significant included *Pseudomonas stutzeri*. *P. stutzeri* has been found on computer components on a hospital ward by others. For example, *P stutzeri* was identified on 1.4 % of computer interfaces (4/282) in a study that looked at contaminants of computers on hospital wards (Lu *et al*., 2009). The *P. stutzeri* identified on the keyboard was resistant to all of the antibiotics tested. *Pseudomonas* spp. often have limited susceptibility to disinfectants and antimicrobials due to their intrinsic resistance often caused by multiple mechanisms. These include efflux pumps, low outer membrane permeability and β-lactamases synthesis. It has also been reported that *Pseudomonas* spp. can acquire almost all known resistance mechanisms (Pang *et al*., 2019).

Contamination of computer surfaces (monitor, mouse and keyboards) is common and one study swabbed 25 computer keyboards in various healthcare facilities found that more than 50 % of keyboards were contaminated with potentially pathogenic microorganisms. These pathogens included CoNS (100 %), diphtheroids (80 %), *Micrococcus* spp. (72 %), *Bacillus* spp. (64 %), non-fermentative Gram-negative rods (36 %), *Enterococcus* spp. (12 %), oxacillin resistant *S. aureus* (4 %) and oxacillin sensitive *S. aureus* (4 %) (Rutala *et al*., 2006). Hartmann *et al*., (2004) found that ICU station computers were contaminated with pathogens at a higher rate (6.3 %) than other surfaces (Hartmann *et al*., 2004). Furthermore, keyboard contamination has been associated with mouse contamination (Lu

et al., 2009). Oberauner *et al*., (2013) identified the computer keyboard in a central nurse's station as having the highest number of CFUs (512), in comparison to other surfaces, and had 6 different species (Oberauner *et al*., 2013). To prevent computer surface contamination a disposable plastic barrier is recommended by the CDC.

As people have begun to use mobile phones and computers with increased frequency these have also become hot spots for the transmission of HAIs (Brady *et al*., 2006:2009:2011; Rutala *et al*., 2006; Tekerekoğlu *et al*., 2011; La Fauci *et al*., 2016). Unsurprisingly, in a study by Lax *et al*., (2015) the strongest correlations of bacteria were between hospital staff and their mobile phones (Lax *et al*., 2015). *Brevibacterium luteolum* was isolated from the nurse's phone, and these are Gram-positive rods found in soil. There are no reports of this species being of clinical importance. However, there are reports of other *Brevibacterium* spp. being isolated from clinical environments such as blood samples (Wauters *et al*., 2000). *Rothia dentocariosa* is a bacterium of low virulence that is part of the normal oral flora. *R. dentocariosa* was isolated from the nurses' keyboard. *Roseomonas mucosa* was also found on the nurse's computer keyboard. It is a Gram-negative coccobacillus that is part of the skin microbiota and an opportunistic pathogen, which may cause catheter-related infections, infections during dialysis and surgical wound infections (Romano-Bertrand *et al*., 2016). *R. mucosa* has previously been found on a bandage trolley in an ICU (Oberauner *et al*., 2013).

A systematic review on the contamination of computer peripheral device in healthcare settings by Ide *et al*., (2019) who found that the most frequent contaminants of computer equipment were skin commensals. However, pathogenic organisms were also found, and this included *S. aureus*, MRSA, *C. difficile* and VRE (Ide *et al*., 2019). However, the researchers struggled to find evidence to suggest a link between contaminated computer equipment and infection and/or colonisation of patients. It has been shown that

decontamination of other fomites results in reduced HAIs (Otter *et al.*, 2011:2013; Falagas *et al.*, 2011; Murphy *et al.*, 2012; Donskey, 2013; Ejemot-Nwadiaro *et al.*, 2015). Although keyboards may harbour pathogenic species, they can be difficult to decontaminate due to irregular surfaces, incorrect use of cleaning products and potential damage from cleaning products (Dettenkofer and Block, 2005).

4.4 Conclusions

Overall, none of the organisms isolated from the patient or ward swabs were found to cause SSIs. On day 1 following surgery, the number of species colonizing the wound generally decreased from the pre-operative swab, suggesting sufficient decontamination of the surgical site before surgery. The wounds were then re-colonized throughout the duration of the patient's hospital stay. No bacteria identified from hospital surfaces caused SSIs, however, there were species with matching antibiotic resistance profiles found on patients and the hospital ward. These surfaces were high-touch points including the floor, shower outlet, shower control, soap dispenser, sink tap, nurses' computer keyboard and the patient touch-screen TV. Many of the bacteria found on hospital surfaces were MDR and XDR, suggesting more needs to be done to eradicate antimicrobial-resistant bacteria in healthcare settings.

Chapter 5. In vitro bacterial biofilm assays

5.1 Introduction

Biofilms are reported to cause at least 80 % of surgical site infections (SSIs) (Mangram *et al.*, 1999). Multi-species SSIs account for a significant proportion of SSIs and one study found that 42 % of SSIs, in patients who had undergone tumour removal surgery, were polymicrobial (Rolston *et al.*, 2014). The aims of this chapter were to measure how bacteria, isolated from surgical patients, formed single species and multi-species biofilms over time including the surface area the biofilm covered, overall mass of the biofilms and the respiring cells within the biofilms. A secondary aim was to determine if antimicrobial resistance changed over time and whether this was influenced when the bacteria were grown in a multi-species biofilm. Three potentially pathogenic strains of bacteria isolated were used in the experiments, which included *Staphylococcus haemolyticus* which was multi-drug resistant (MDR), *Enterobacter cloacae* which was extensively drug resistant (XDR) and *Enterococcus faecium* which is a pathogen known to cause SSIs. Crystal violet assay (CVA), tetrazolium salt reduction assay (XTT) and percentage coverage were performed using these three species and with all the different co-culture and multi-culture biofilms. Biofilm growth was observed at 24 h, 48 h and one week and disc diffusion method was used to see if the antimicrobial resistance profiles changed over time.

5.2 Results

5.2.1 Bacterial coverage on synthetic skin

Biofilms of *S. haemolyticus*, *E. faecium* and *E. cloacae* were grown on synthetic skin and bacterial coverage was observed after 24 h, 48 h and 7 days (Figure 9 -11). Experiments were run in triplicate and averages and standard deviation were calculated. After 24 h, *S.*

haemolyticus covered 5.2 % of the synthetic skin and after 48 h the bacteria covered 11.92 % whilst after 7 days it covered 24.22 % of the synthetic skin (Figure 9). *E. faecium* covered 1.66 % of the synthetic skin after 24 h, 11.97 % after 48 h and 19.91 % after 7 days (Figure 10). *E. cloacae* covered 17.12 % of the synthetic skin after 24 h, 17.72 % after 48 h and 35.9 % after 7 days. *E. cloacae* covered more of the synthetic skin in a significantly shorter length of time than any of the other species as it had covered 17.12 % of the skin after just 24 h (Figure 11) compared to *S. haemolyticus* (5.2 %) and *E. faecium* (1.66 %). After one week *E. cloacae* covered a significantly larger percentage (35.9 %) of the synthetic skin than *S. haemolyticus* (24.22 %) and *E. faecium* (19.91 %). *E. faecium* covered less of the surface than any of the other species and also took more time to grow (Figure 10).
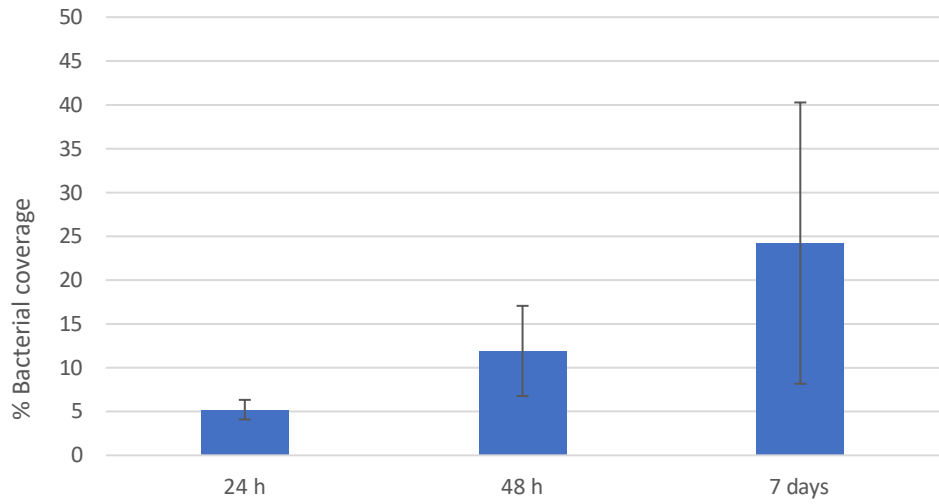
Figure 9. Percentage of *S. haemolyticus* biofilm coverage on synthetic skin.
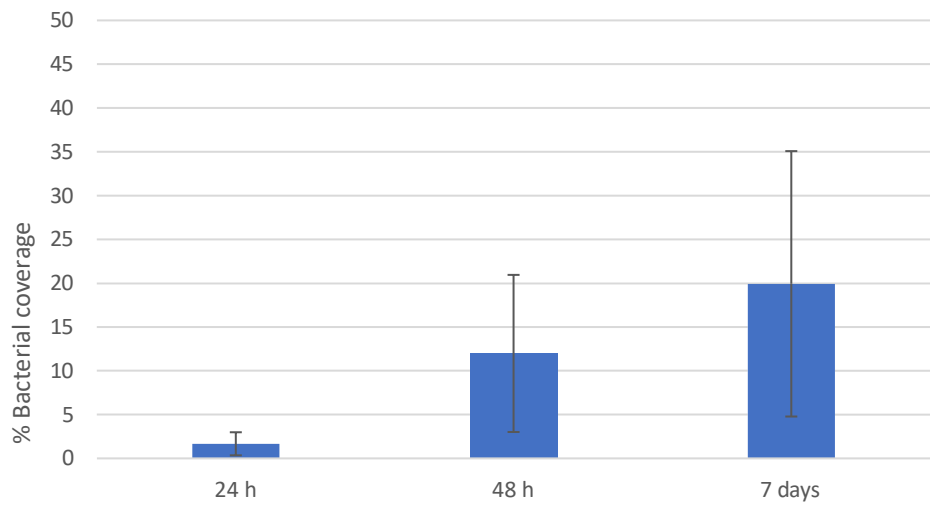
Figure 10. Percentage of *E. faecium* biofilm coverage on synthetic skin.
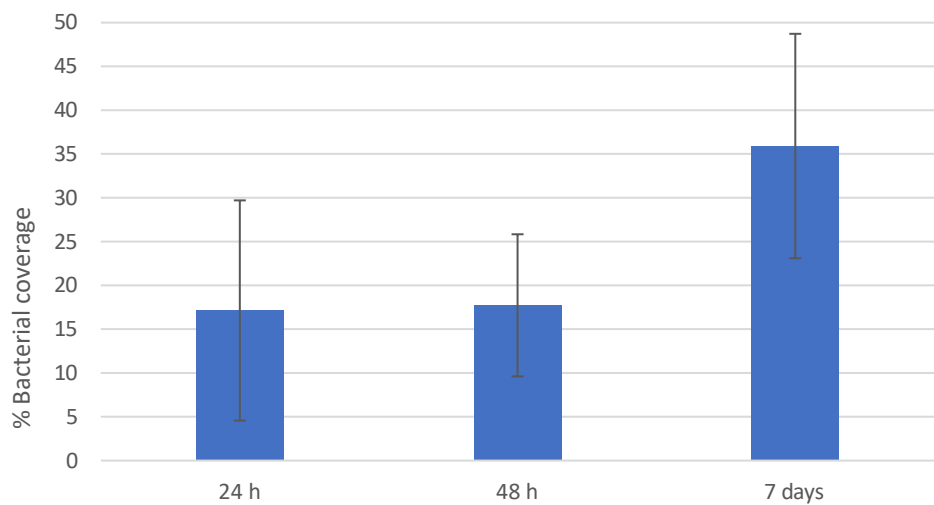
Figure 11. Percentage of *E. cloacae* biofilm coverage on synthetic skin.

5.2.2 Crystal violet and XTT biofilm assays

Single species biofilms
Enterobacter cloacae

The results from the crystal violet assay for *E. cloacae* in a single species culture showed that

the biofilm grew over time (Figure 12). After 24 h the average optical density (OD) was 0.16,

0.18 at 48 h and 0.84 after 7 days. The XTT reduction assay also showed an increase in *E.*

cloacae biofilm with increased time. At 24 h the OD was 0.0075, at 48 h it was

0.03 and on day 7 it was 0.706. The OD readings for the CVA was significantly higher than

the XTT at all of the different timepoints.

Staphylococcus haemolyticus

Both the crystal violet assay and the XTT reduction assay showed that the number of

bacteria reduced at 48 h (CVA 0.13, XTT 0.026) from the result at 24 h (CVA 0.25, XTT

0.0.032). After 7 days the numbers then increased significantly (CVA 1.28, XTT 0.71)

(Figure 13). Of the three species used in the assays, the OD at one week, for both CVA and

XTT for *S. haemolyticus* was significantly higher than the other species.

Enterococcus faecium

The density of the *E. faecium* biofilm increased over time for both the crystal violet

biofilm assay and the XXT reduction assay (Figure 14). At 24 h the OD was 0.29 for the

crystal violet assay and 0.03 for the XTT assay. After 2 days the OD for the crystal violet

assay was 0.294 and 0.06 for the XTT assay. After one week the OD for the crystal violet

assay was 0.26 and for the XTT assay was 0.38. The CVA on day 7 was particularly high for

all of the species and nearly double the XTT OD.
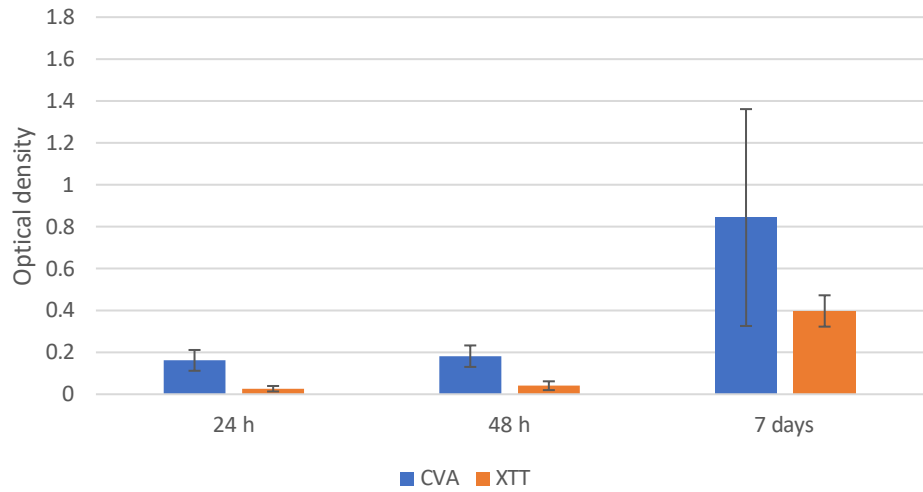
Figure 12. Crystal violet and XTT biofilm assay for *E. cloacae*.
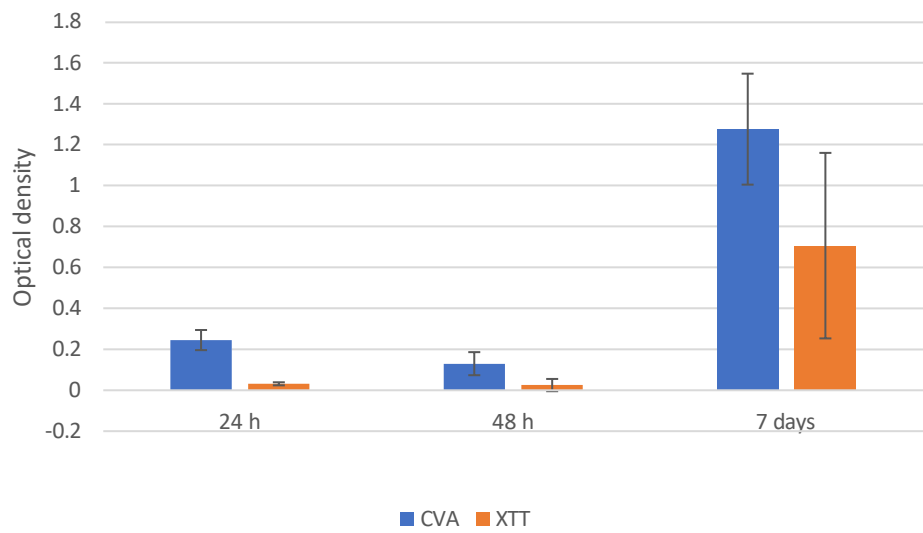
Figure 13. Crystal violet and XTT biofilm assay for *S. haemolyticus.*
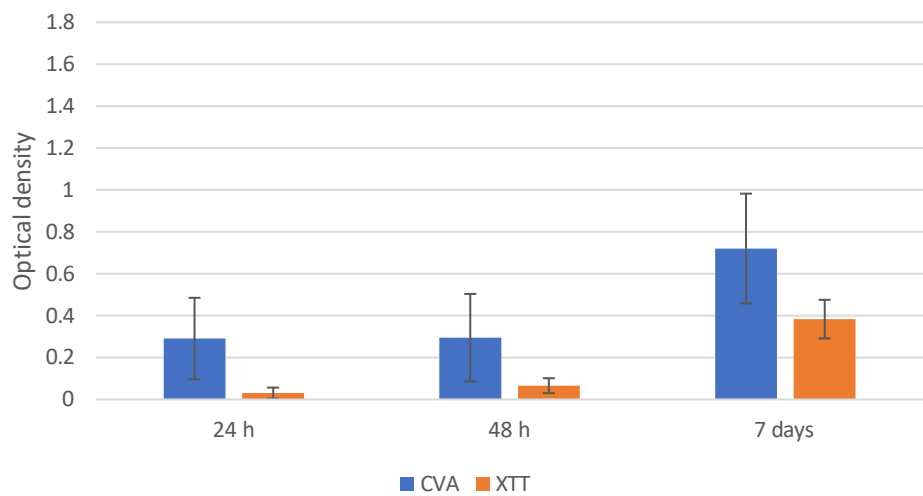
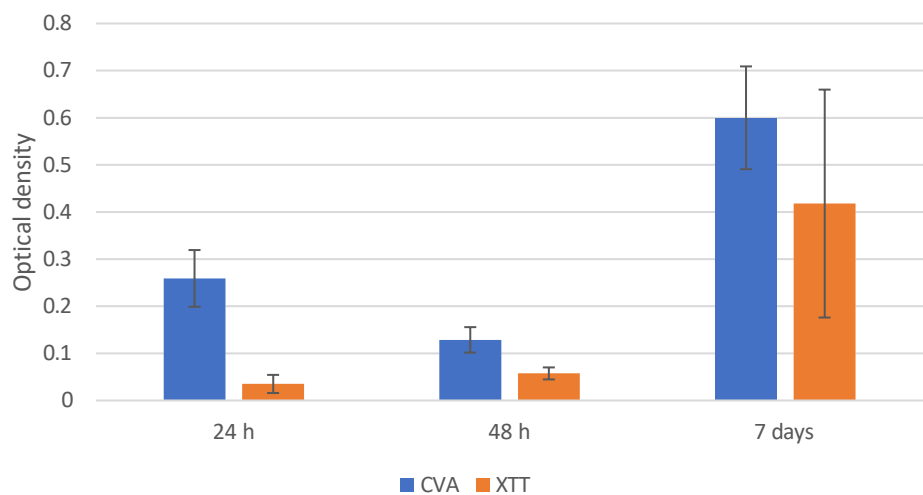Figure 14. Crystal violet and XTT biofilm assay for *E. faecium.*

Co-culture biofilms

Enterobacter cloacae and Staphylococcus haemolyticus

The crystal violet assay results were different and showed that the biofilm decreased at 48 h from 24 h and then increased at the reading taken on day 7. At 24 h the OD was 0.22, at 48 h it was 0.2 and at 7 days it was 0.17 (Figure 15). The average OD of the *E. cloacae* and *S. haemolyticus* biofilm increased over time when the XTT reduction assay was performed. The OD reading at 24 h was 0.024, 48 h was 0.067 and at 7 days it was 0.42. After 7 days, the OD for both CVA and XTT was significantly lower than any of the other co-culture biofilms.

Enterobacter cloacae and Enterococcus faecium

The *E. cloacae* and *E. faecium* biofilm increased as time went on for both the crystal violet biofilm assay and the XTT assay. At 24 h the OD was 0.24 for the crystal violet assay and 0.033 for the XTT assay. Forty-eight hours after the assays were set up, the OD for the crystal violet biofilm assay was 0.25 and for the XTT assay it was 0.036. After one week the OD for the crystal biofilm assay was 0.609 and for the XTT assay it was 0.47 (Figure 16).

Enterococcus faecium and Staphylococcus haemolyticus

The results from the crystal violet assay and the XTT assay for *E. faecium* and *S. haemolyticus* showed that the OD decreased from 24 h at 48h and the increased at the 7-day OD reading. The OD results for the crystal violet biofilm assay were 0.259 (24 h), 0.13 (48 h) and 0.6 (7 days). For the XTT assay the average OD readings were 0.035 (24 h), 0.057 (48 h) and 0.42 (7 days) (Figure 17).
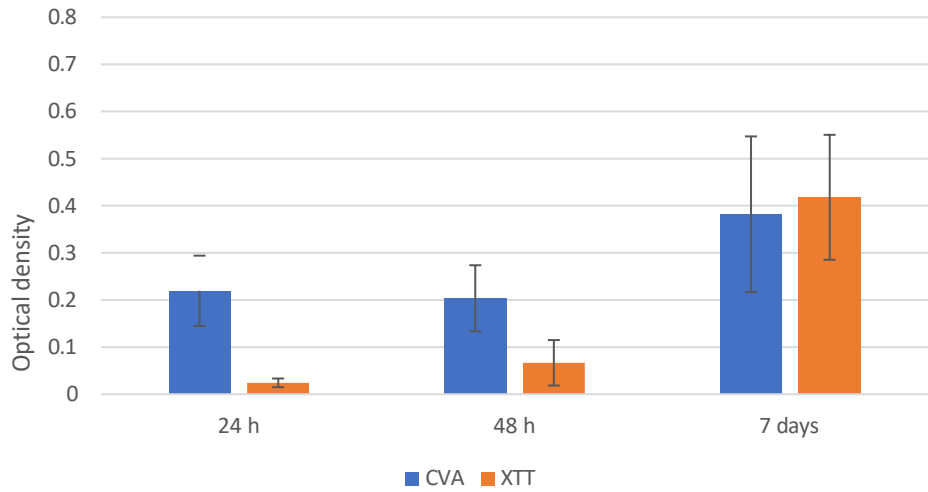
Figure 15. Crystal violet and XTT biofilm assay for *E. cloacae* and *S. haemolyticus.*
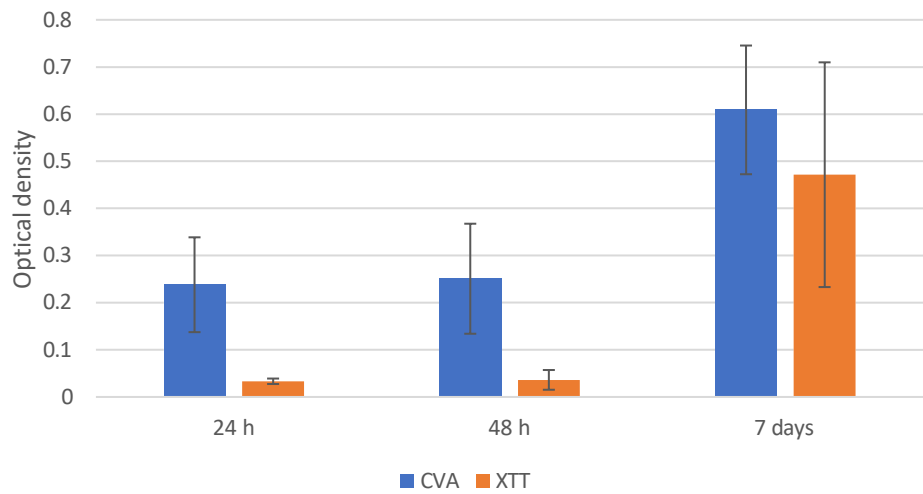
Figure 16. Crystal violet and XTT biofilm assay for *E. cloacae* and *E. faecium.*

Figure 17. XTT and crystal violet biofilm assay for *E. faecium* and *S. haemolyticus.*

Multiple species biofilm
Enterobacter cloacae, Enterococcus faecium and Staphylococcus haemolyticus

The average OD taken for the crystal violet assay and XTT assay increased as time went on

for the biofilm which included all three species used in this study. The crystal violet

biofilm OD readings were 0.16 (24 h), 0.19 (48 h) and 0.48 (7 days). The XTT reduction

assay readings were 0.037 (24 h), 0.055 (48 h) and 0.45 (7 days) (Figure 18).
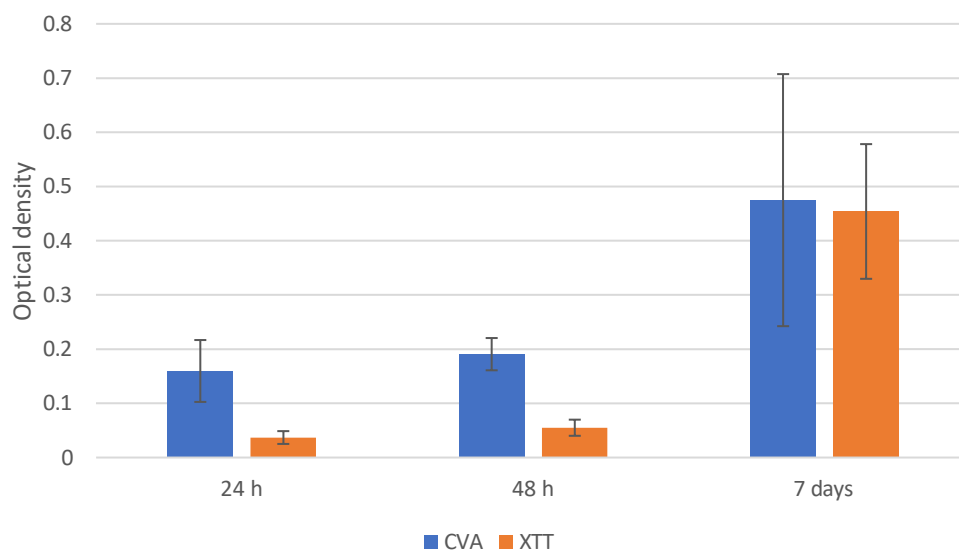
Figure 18. Crystal violet and XTT biofilm assay for *E. faecium*, *S. haemolyticus* and *E. cloacae.*

5.2.3 Antimicrobial sensitivities of the different combinations of bacteria at 24 h, 48 h and 7 days
Enterococcus cloacae

The *E. cloacae* strain used in this study was XDR and resistant to cefoxitin, meropenem,

ampicillin and piperacillin-tazobactam. Gentamicin resistance was observed after 48 h

when *E. cloacae* as grown singularly.

Gentamicin resistance was also seen at 7 days when *E. cloacae* was grown with *E. faecium*

(Table 26).

Table 26 Antimicrobial sensitivities of *E. cloacae* grown with different combinations of bacteria at 24 h, 48 h and 7 days

Enterobacter cloacae				
Before	EC	EC and SH	EC and EF	EC, EF and SH
Cefoxitin	R	R	R	R
Ciprofloxacin	S	S	S	S
Gentamicin	S	S	S	S
Meropenem	R	R	R	R
Ampicillin	R	R	R	R
Piperacillin-tazobactam	R	R	R	R
24 h				
Cefoxitin	R	R	R	R
Ciprofloxacin	S	S	S	S
Gentamicin	S	S	S	S
Meropenem	R	R	R	R
Ampicillin	R	R	R	R
Piperacillin-tazobactam	R	R	R	R
48 h				
Cefoxitin	R	R	R	R
Ciprofloxacin	S	S	S	S
Gentamicin	R	S	S	S
Meropenem	R	R	R	R
Ampicillin	R	R	R	R
Piperacillin-tazobactam	R	R	R	R
7 days				
Cefoxitin	R	R	R	R
Ciprofloxacin	S	S	S	S
Gentamicin	S	S	R	S
Meropenem	R	R	R	R
Ampicillin	R	R	R	R
Piperacillin-tazobactam	R	R	R	R

EF = *Enterococcus faecium*, EC = *Enterobacter cloacae*, SH = *Staphylococcus haemolyticus*

Staphylococcus haemolyticus

The *S. haemolyticus* was isolated from a patient was initially resistant to cefoxitin, ciprofloxacin, gentamicin and norfloxacin. No changes in resistance were observed when *S. haemolyticus* was grown on its own. From 24 h erythromycin resistance developed in all of the co-cultures and the multi-species biofilm (Table 27).

Intermediate resistance to fusidic acid was also seen in the multispecies biofilm.

Table 27. Antimicrobial sensitivities of *S. haemolyticus* grown with different combinations of bacteria at 24 h, 48 h and 7 days.

Staphylococcus haemolyticus				
Before	SH	EC and SH	EF and SH	EC, EF and SH
Cefoxitin	R	R	R	R
Ciprofloxacin	R	R	R	R
Erythromycin	S	S	S	S
Fusidic acid	S	S	S	S
Gentamicin	R	R	R	R
Norfloxacin	R	R	R	R
Tetracycline	S	S	S	S
24 h				
Cefoxitin	R	R	R	R
Ciprofloxacin	R	R	R	R
Erythromycin	S	R	R	R
Fusidic acid	S	S	S	S
Gentamicin	R	R	R	R
Norfloxacin	R	R	R	R
Tetracycline	S	S	S	S
48 h				
Cefoxitin	R	R	R	R
Ciprofloxacin	R	R	R	R
Erythromycin	S	R	R	R
Fusidic acid	S	S	S	I
Gentamicin	R	R	R	R
Norfloxacin	R	R	R	R
Tetracycline	S	S	S	S
7 days				
Cefoxitin	R	R	R	R
Ciprofloxacin	R	R	R	R
Erythromycin	S	R	R	R
Fusidic acid	S	S	S	S
Gentamicin	R	R	R	R
Norfloxacin	R	R	R	R
Tetracycline	S	S	S	S

EF = *Enterococcus faecium*, EC = *Enterobacter cloacae*, SH = *Staphylococcus haemolyticus*

Enterococcus faecium

The *E. faecium* used in this study was isolated from a patient and was resistant to norfloxacin and ampicillin but sensitive to vancomycin. No changes in antimicrobial resistance were observed in any of the different combinations of bacteria and at any of the different times of extraction (Table 28).

Table 28. Antimicrobial sensitivities of *E. faecium* grown with different combinations of bacteria at 24 h, 48 h and 7 days.

Enterococcus faecium				
Before	EF	EC and EF	EF and SH	EC, EF and SH
Norfloxacin	R	R	R	R
Vancomycin	S	S	S	S
Ampicillin	R	R	R	R
24 h				
Norfloxacin	R	R	R	R
Vancomycin	S	S	S	S
Ampicillin	R	R	R	R
48 h				
Norfloxacin	R	R	R	R
Vancomycin	S	S	S	S
Ampicillin	R	R	R	R
7 days				
Norfloxacin	R	R	R	R
Vancomycin	S	S	S	S
Ampicillin	R	R	R	R

EF = *Enterococcus faecium*, EC = *Enterobacter cloacae*, SH = *Staphylococcus haemolyticus*

5.3 Discussion

SSIs are frequently caused by biofilms, including multi-species biofilms (Mangram *et al*., 1999). Biofilms are more difficult to treat with antimicrobials and biofilm wounds are often chronic in nature (Sharma *et al*., 2019). Biofilm assays, using bacteria isolated from HPB surgery patients, were used to measure biofilm growth over time and also determine if antimicrobial resistance can develop in these biofilms.

In SSIs following HPB surgery, *Enterococcus* spp. can form biofilms on biliary stents (Lee, 2017). *Enterococcus* spp. have several virulence proteins that aid biofilm formation. These are aggregation substance (Agg), *Enterococcus faecalis* endocarditis-associated antigen A (EfaA), adhesion of collagen of *E. faecalis* (Ace) and biofilm on plastic operon (Bop) (Nallapareddy and Murray, 2006). *Enterococcus* spp. also express pili which facilitate adhesion, initiating the start of biofilm formation (Mandlik *et al*., 2008). Furthermore, *E.*

faecalis also uses the quorum sensing system, fsr (faecal streptococci regulator) locus, to form biofilms (Hancock and Perego, 2004).

One study that looked at biofilm formation in CoNS found that, following *S. epidermidis*, *S. haemolyticus* was the second most common CoNS that formed biofilms (Shrestha *et al*., 2017). Fredheim *et al*. (2009) also found that biofilm formation was a common phenotype in clinical *S. haemolyticu*s strains. Through various *in vitro* biofilm assays, Fredheim *et al*. (2009) found low levels of the *ica* operon in *S. haemolyticus* showing that they may form polysaccharide intercellular adhesin independent biofilms. Furthermore, they found that extracellular DNA may help form the mature biofilm matrix of *S. haemolyticus* and this is not found in other CoNS (Fredheim *et al*., 2009).

The majority of biofilm associated infections are caused by Enterobacteriaceae (Sommer *et al*., 2013). Misra *et al*., (2022) used field emission-scanning electron micrography and demonstrated nanotube formation between *E. cloacae* cells thus suggesting a means of communication (Misra *et al*., 2022). Ramos-vivas *et al*. (2019) found that the rate of biofilm formation in *Enterobacter* spp*.* was low (4 %). However, it was also shown that an *E. cloacae* isolated from hepatic transplant infection samples formed moderate to strong biofilms (Ramos-Vivas *et al*., 2019), thus demonstrating the variability between strains.

5.3.1 Bacterial percentage coverage

The percentage coverage of bacteria on a surface could indicate the potential for biofilm growth. The bacterial percentage coverage of *E. cloacae* on the synthetic skin did increase at 48 h but not significantly (0.6 %), when compared to the other species. Although, of all the single species, *E. cloacae* had the highest % coverage at one week but this did not correlate with the XTT or CV assay results. This suggests that the *E. cloacae* biofilms

covered a larger surface area than the other species but did not have more of a biological or respiring mass overall.

After one week, the *E. faecium* biofilm covered the least percentage of the surface area, when compared to the other species. The bacterial % coverage results for *E. faecium* did correlate with the CV and XTT results as the OD readings at day 7 were lower than the other species. Furthermore, the CFU for *E. faecium* (3.0×10^6 CFU/mL) was lower than the other species, suggesting this strain does not grow particularly quickly or form biofilms as quickly as the other species used in this research.

5.3.2 Crystal violet and XTT assays

Crystal violet is a basic protein stain that can dye viable cells, dead cells and the extracellular matrix. This makes it a useful tool for determining the amount of biofilm mass (Pitts *et al*., 2003). 2,3-bis (2-methoxy-4- nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) is a tetrazolium salt. The XTT can be deacidized by enzymes in the cytoplasm of the respiratory chain to a water-soluble formazan in viable cells and therefore can be used as a way to measure respiring cells (Xu *et al*., 2016).

5.3.3 Single culture biofilms

Overall on day 7, for all of the single species biofilms, in both the crystal violet assay and XTT reduction assay the biofilm had increased in density since the 24 timepoint. However, the XTT results were significantly less than the CVA results. The XTT results are likely to be a more accurate representation of the living biofilm because crystal violet will stain dead bacteria and the extracellular matrix, whilst XTT can measure living bacteria as XTT measures metabolic activity (Doğan *et al*., 2021). However, the biofilm mass of *S. haemolyticus* did decrease at 48 h, when grown individually and this could also be

explained as the biofilm progressed to the dispersion phase of formation (Misra *et al*., 2022).

Visually in the current study the *S. haemolyticus* biofilm did look more dense than any of the other species biofilms and this was shown by the CV and XTT OD readings at one week, as they were higher than the other species. However, *S. haemolyticus* did not have the largest % coverage, indicating that the biofilm did not cover a larger surface area but had a larger mass. Grzebyk *et al*. (2013) also conducted crystal violet biofilm assays using *S. haemolyticus* and *S. epidermidis* and found that *S. haemolyticus* was more likely (97 %) to produce biofilms than *S. epidermidis* (Grzebyk *et al*., 2013). Furthermore it was found that *S. haemolyticus* had a stronger ability to produce mucus (Grzebyk *et al*., 2013).

5.3.4 Co-culture biofilms

The OD readings for the CVA and XTT on co-culture and multi-culture biofilms were all significantly less than the results from the single species biofilms. This could suggest that the bacteria were inhibiting the growth of the other species. This has been shown with *in vitro* models using *S. aureus* and *P. aeruginosa.* For example, Gomes-Fernandes *et al*. (2022) looked at growth competition in co-cultures (*P. aeruginosa* and *S. aureus*) and found that *P. aeruginosa* inhibited the growth of *S. aureus* (Gomes-Fernandes *et al*., 2022). Sycz *et al*. (2021) performed single, co-culture and triple species biofilm assays using *E. cloacae*, *E. coli* and *P. aeruginosa*. In agreement with our findings, in the dual and triple species biofilms, at all stages of biofilm growth, there was a significant reduction ($p \leq 0.05$) in *E. cloacae* cells when compared to the number of cells in the single *E. cloacae* biofilm (Sycz *et al*., 2021). This was attributed to the antagonistic interactions between the species of bacteria. To the authors knowledge the biofilm combinations of the three species used in the current research have not been studied.

Like the single species biofilms, in some instances the OD decreased at the 48 h reading. Misra *et al*. (2022) performed crystal violet biofilm assays with *E. cloacae* isolates and found that at 96 h – 120 h the biofilm decreased, and they accounted this as correlating with the shift from maturation to the dispersion phase of biofilm formation (Misra *et al*., 2022). This is in agreement with this work whereby the OD reading of *E. cloacae* in the crystal violet assay with *E. cloacae* and *S. haemolyticus* reduced at 48 h from 24 h. Although this was not found in just the *E. cloacae* biofilm or any other bacteria combinations.

It has been shown that some bacteria can enter a viable but nonculturable (VBNC) state. This is when the cells are living but unable to grow on media as they normally would (Oliver *et al*., 2005). VBNC cells are still metabolically active and carry out respiration, although they have a lower metabolic rate (Shleeva *et al*., 2004). This could be an explanation of why the CV readings are significantly higher than the XTT OD measurements in all instances. Bacteria may enter the VBNC state when they are exposed to stressful situations, such as starvation (Du *et al*., 2007). In the static biofilms assays presented here, there was likely limited nutrients in the medium and consequently this could have led the bacteria to go into the VBNC state. It has been demonstrated that *E. cloacae* is capable of entering the VBNC state (Oliver *et al*., 2010). *E. faecium* is reported to enter the VBNC state at low temperatures and due to starvation (Lleò *et al*., 2001). To the authors knowledge the VBNC state has not been reported in *S. haemolyticus*. However, VBNC *S. aureus* and *S. epidermidis* strains have been isolated from biofilms growing in catheters (Zandri *et al*., 2012).

5.3.5 Antimicrobial resistance

It has been shown that biofilms reduce susceptibility to antimicrobials. In this study, the AMR of the bacteria cultured from a polymicrobial biofilm was used to determine if it was

changed over time. Erythromycin resistance developed in *S. haemolyticus* from 48 h in all the different combinations of bacteria except where it was grown with no other species. The incidence of clinical Staphylococcus isolates with resistance to macrolides has increased in recent years and resistance can be spread between Staphylococcal species (Lim *et al.*, 2002).

Macrolide resistance is these species is normally due to modification of ribosomal RNA, mediated by erythromycin resistance methylase (*erm*) genes (Schmitz *et al.*, 1999). Erythromycin resistance in Enterococci is also associated with *erm* genes, *erm*(A), *erm*(B) and *erm*(C), although, the predominate erythromycin resistance gene in Enterococcus is the *erm*(B) gene (Celik *et al.*, 2014). The mobile genetic element Tn*2009,* which contains erm(B) has been found in *Acinetobacter junii* and there is evidence it can be present in *E. cloacae* as well (Ojo *et al.*, 2006). Furthermore, the dissemination of mobile genetic elements with antibiotic resistance cassettes can occur between species (Schroeder and Stephens, 2016).

Gentamicin resistance in *E. cloacae* developed when *E. cloacae* was grown on its own at 48 h but interestingly it was susceptible at 7 days. When *E. cloacae* was grown with *E. faecium*, gentamicin resistance was observed at 7 days. A study found that only 3.6 % of Enterobacter species were gentamicin resistant and differences in gentamicin resistance were seen between nosocomial (12.5 %) and community-acquired isolates (1.19 %) (Al-Tawfiq *et al.*, 2009). *Enterococcus* spp. are known to express low levels of gentamicin resistance which is thought to be due to the intrinsic reduced permeability to these antibiotics (Hollenbeck and Rice, 2012). In *Enterococcus* spp., high level resistance to aminoglycosides occurs through the acquisition of genes. These genes are most frequently the *aac(6')-Ie-aph(2'')-Ia* gene which encodes AAC(6')Ie-APH(2'')Ia (Chow, 2000; Hollenbeck and Rice, 2012; Miller *et al.*, 2014). Chow *et al*. (2001) found that a

similar gene*, aac(6')-Im* and *aph(2")-Ib* was found in both *E. faecium* and *E. coli* and could be transmitted via horizontal gene transfer (Chow *et al*., 2001)*. If the genes encoding aminoglycoside resistance mechanisms can be transmitted between Gram-positive and Gram-negative bacteria via horizontal gene transfer this may suggest how the gentamicin resistance occurred in *E. cloacae*. However, this does not explain how resistance to gentamicin occurred when *E. cloacae* was grown without any other bacteria present. This could have been because of the presence of persister cells. An *in vivo* study on polymicrobial wound infection in mice, found that when four species were used instead of one, wound healing was impaired and there was increased tolerance to antimicrobials (Dalton *et al*., 2011).

5.4 Conclusions

It was found that the OD reading increased significantly from 24 h to 7 days in both the crystal violet assay and the XTT assay. The OD readings from the XTT assays were significantly less than the crystal violet, showing that the levels of respiring bacteria were low. In some instances the OD readings reduced at 48 h from 24 h and this was attributed to the biofilm being in the dispersion phase of formation. The XTT and CV measurements of the co-culture and multispecies biofilms were significantly lower than the single species biofilms, suggesting antagonistic interactions occurred between the bacteria. Development of erythromycin resistance was observed after 24 h when *S. haemolyticus* was grown in dual and multi-species biofilms. Intermediate resistance to fusidic acid was also seen at 48 h when *S. haemolyticus* was grown with the other two species. Gentamicin resistance was seen in *E. cloacae* when it was grown with *E. faecium* at 48 h. This could be due to horizontal transmission of resistance genes within the biofilm. Although, in one instance gentamicin resistance was observed at 48 h in an *E. cloacae* biofilm without any other species present and this could have been due to the presence

of persister cells.

Chapter 6. Conclusions and future work

Overall, no bacteria that were colonising the surgical sites were found to cause surgical site infections. In most instances, SSI was detected by positive drain fluid cultures. The species that did cause SSIs were all gut bacteria, suggesting that the bacteria were endogenous bacteria from the patients' own gastrointestinal tract. This indicated that the bacteria were transferred to the surgical site during surgery. Another factor that further supports this, was that, in the six incidences of SSIs, all of the surgeries were pancreatic and in all of the patients where surgery also involved the stomach, they developed a SSI. Future work could include swabbing of the operating theatre and also the surgeons as the findings indicate bacterial transmission to the wound occurred during surgery.

One limitation of this study was the fact that many bacteria are non-culturable and these may play a role in infection. Furthermore, swabbing of the surgical site might not pick up all of the bacteria and whole genome sequencing might be a more accurate (although costly) way to identify the bacteria. The small patient sample size is another limitation, in which the Covid-19 epidemic played a significant role. Future work could include the recruitment of more participants and this would further validate the findings. However, despite the small sample size the incidence of SSIs (23.1 %) was similar to that reported by others (20 % - 40 %) (Ceppa *et al*., 2013).

High levels of antimicrobial resistance were found in species identified on the hospital ward and also on patients, with many isolates being MDR. Although, none of these were found to cause SSIs following HPB surgery, potentially they could cause other nosocomial infections. A way to prevent this has been suggested to isolate infected patients in side rooms; this is already done for those colonised by MRSA but this measure could also be taken when patients develop SSIs, particularly those with MDR or

XDR infections.

This research did influence policy change within the hospital that this study took place. Due to the nurses' computer keyboard being contaminated with many bacteria, including a MDR *Pseudomonas stutzeri*, cleaning protocol in the hospital was changed and computer keyboards were cleaned more frequently.

It is clear that more needs to be done to prevent SSIs following HPB surgery. One preventative method could be the introduction of patient predicative care plans in patients who have risk factors for SSIs, although most of the risk factors identified were intraoperative and postoperative factors. Extra measures could be taken in those patients who are having non-laparoscopic pancreatic procedures, especially those which also involve the stomach. These extra-measures could include the use of antimicrobial wound dressings, antimicrobial sutures or a different antimicrobial prophylaxis regime. Seeing as the SSIs were caused by endogenous gut bacteria, in high-risk patients, stool cultures and antibiotic resistance profiles of the cultured bacteria could be taken before surgery. Prophylaxis could then be personally tailored to each patient by identifying potential pathogens found in their stool. This would not only help patient outcomes but also might prevent unnecessary antibiotic use and thus help prevent the development of antimicrobial resistance.

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In progress:

- Bacterial colonization of a hepatopancreatobiliary hospital ward.
- Case studies of six patients who developed surgical site infections following hepatopancreatobiliary surgery.

AIMS *Bioengineering*

Review

A systematic review on the incidence and risk factors of surgical site infections following hepatopancreatobiliary (HPB) surgery

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Abstract: Background: Surgical site infections (SSI) are one of the most common hospital acquired infections and result in increased morbidity, mortality and financial burden on health services. The incidence of SSIs are not clearly defined and infection rates as varied as 20%–40% have been reported. The aim of this study was to systematically review the incidence and risk factors of SSI following HPB surgery. Methods: The database of Medline (via PubMed) was systematically searched from 2013–2022. Articles were screened using the PRISMA statement and those that met the inclusion criteria were included in the study. Results: Sixteen studies were eligible for inclusion in this systematic review. The average incidence of SSI was 29.8%. Key risk factors identified included male gender, open surgery, preoperative biliary stenting and obesity. Conclusions: The incidence of SSI following HPB surgery varied, but it is generally high. A variety of pre-disposing patient factors can affect infection rates following HPB surgery. The results from this study suggest that perhaps laparoscopic surgery should be used where possible, and that there should be an awareness that gender, obesity and the use of stents may increase the incidence of SSIs following these operations.

1. Introduction

Surgical site infections (SSIs) are the most common type of hospital acquired infections (HAI) [1]. The centre for disease control and prevention (CDC) defines a surgical site

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infection (SSI) as an infection that occurs after surgery in the part of the body where the surgery took place [2]. SSIs are divided into three categories: 1) superficial incisional SSIs that infect the skin and subcutaneous tissue; 2) deep incisional SSIs that affect the deep soft tissue; and 3) organ/space SSIs where the infection involves any other part of the anatomy including organs and excluding the incision [3]. It has been suggested up to 60% of SSIs are preventable, [4] yet incidences of SSIs can be as high as 20%–40%, depending on the procedure and methods of data collection [5]. SSIs also increase mortality rates, and it has been suggested that patients with an SSI are 2–11 times more at risk of death compared to patients without a SSI [6]. Approximately 16% of patients that receive HPB surgery are thought to be re-admitted [7] with pancreaticoduodenectomy having the highest re-admission rates of all surgery (15%–20%) [8]. Furthermore, the incidence of SSIs after hepatectomy has been reported to be as high as 20%–40% [5]. Alongside the deleterious physiological and psychological issues of patient infection, SSIs increase the length of hospital stay, [9] which results in an increased financial burden, when considering the extended costs of bed stay (length of stay, LOS), treatment, nursing care and further diagnostics that are required [10]. In addition, when antimicrobial resistant organisms cause SSIs, this can result in a higher financial burden and prolonged hospital stay since they are more difficult to treat [9]. The aim of this work was to determine if there was an association between incidence and risk factors of SSI following HPB surgery.

2. Materials and methods

Medline (via PubMed) was searched using the term "(HPB) OR (pancreatic surgery) OR (liver resection) OR (pancreaticoduodenectomy) OR (pancreatectomy) OR (cholecystectomy) AND (surgical site infection) AND (incidence)". Only studies between 2013–2022 were included and only observational studies including adults. Transplant studies were also excluded. The search was conducted within the Preferred Reporting Items for Meta-Analyses (PRISMA) guidelines [11].

Data on methods, country, surgery type, samples size, total SSI incidence, laparoscopic surgery, SSI definition, type of SSI (superficial, deep, organ and space), the three most frequent bacteria causing SSIs and significant reported risk factors were recorded.

Where enough data was available to determine risk factors of SSIs, odds ratios (95% CI) were calculated, and forest plots were made. These factors were gender, age, weight, open surgery, smoking status, diabetes and use of preoperative biliary drains.

3. Results

The initial search resulted in 25 research papers [12–36]. After screening the titles and abstract three articles were excluded (Figure 1) [15,19,29]. These papers were excluded because one looked at the incidence of hernias following HPB surgery and not wound infection, one only focused on pancreatic transplant surgery and one because all of the participants were children. The full texts were then analysed, and six further research papers were excluded [16,17,22,32,35,36]. Two included other surgeries and incidence data on SSI incidence after HPB surgery could not be extracted. Three articles were excluded as they did not specify the type of infection, so SSI incidence could not be distinguished from other postoperative infections. One research paper could not accessed and thus was not included. A total of 16 papers were then eligible for use in this systematic review.
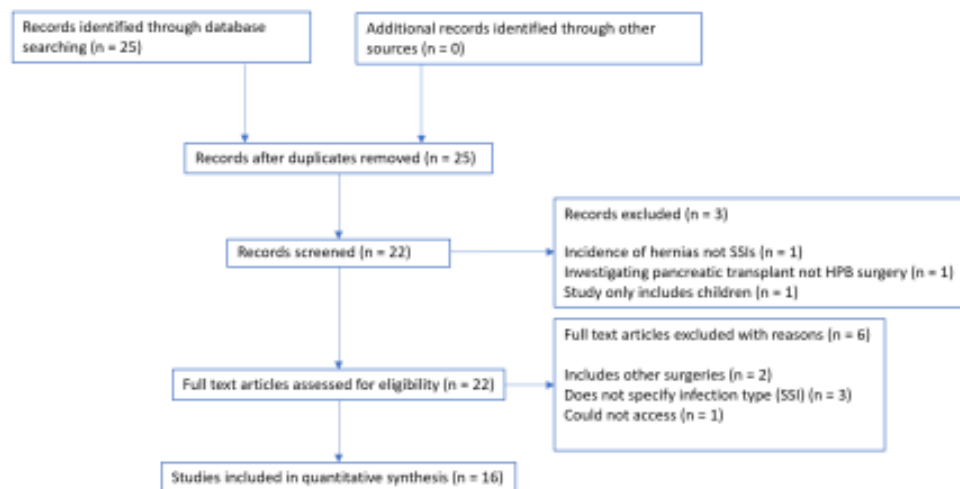
Figure 1. PRISMA eligibility flowchart.

3.1. Incidence

The incidence of SSI in the 16 studies varied from 2.0%–54.7% (Table 1). The average incidence of SSI was 29.8%.

3.2. Odds ratio for risk factors of SSIs following HPB surgery

3.2.1. Gender

Odds ratio of male gender as a risk factor for SSI was available in 8 studies. Three research papers found male gender to be a significant risk factor of SSI following HPB surgery. Liu et al., 2019 [18] OR 1.17 (95% CI: 1.03, 1.33), Laviano et al., 2020 [12] OR 2.21 (95% CI: 1.25, 2.6) and Algado-Sellés et al., 2022 [14] OR 1.54 (95% CI: 1.05, 2.26) (Figure 2).

Table 1. Methods, country, sample size, SSI incidence and surgical factors of the 16 studies. NR = Not recorded.

First author, year	Methods	Country	Surgery type(s)	Sample size	Total SSI	Laparoscopic	SSI definition	Superficial	Deep SSI	Superficial and organ space	Organ space SSI
Laviano et al., 2020 [12]	Observational, prospective	Spain	Cholecystectomy, pancreaticoduodenectomy, total pancreatectomy, segmentectomy, hepatectomy, hepaticojejunostomy and exploratory laparotomy	321	25.80%	35%	NR	4%	4%		92%
Joliat et al., 2018 [13]	Observational, retrospective	Switzerland	Pancreatic	529	26%	NR	CDC	48.60%	NR	34.70%	16.70%
Algado-Sellés et al., 2022 [14]	Observational, prospective, cohort	Spain	Cholecystectomy	2,200	5%	88.70%	CDC	NR	NR	NR	NR
Bortolotti et al., 2021 [17]	Observational, retrospective, monocentric	France	Pancreaticoduodenectomy	129	14.80%	0%	CDC				100%

First author, year	Methods	Country	Surgery type(s)	Sample size	Total SSI	Laparoscopic	SSI definition	Superficial	Deep SSI	Superficial and organ space	Organ space SSI
Liu et al., 2019 [18]	Observational, cohort	USA	Pancreatoduodenectomy	5969	20.30%	0%	CDC	7.20%			14.10%
Sert et al., 2022 [20]	Observational	Turkey	Pancreaticoduodenectomy	45	40%	0%	NR	NR	NR	NR	NR
Gyoten et al., 2021 [21]	Observational, prospective	Japan	Pancreaticoduodenectomy, distal pancreatectomy for pancreatic ductal adenocarcinoma (PDAC), total pancreatectomy, major hepatectomy of three segments or more, anatomical sectionectomy and subsectionectomy, common bile duct resection for congenital biliary disease, and liver transplantation	66	30.30%	0%	CDC	NR	NR	NR	NR
Bednarsch et al., 2021 [23]	Observational, cohort	Germany	Liver resection with mandatory portal vein reconstruction (and hepatoduodenopancreatectomy on demand)	95	54.70%	0%	Postoperative abdominal infection	NR	NR	NR	NR

First author, year	Methods	Country	Surgery type(s)	Sample size	Total SSI	Laparoscopic	SSI definition	Superficial	Deep SSI	Superficial and organ space	Organ space SSI
Wagle et al., 2020 [24]	Retrospective, observational	India	Hepatectomy	19	36.80%	0%	CDC	NR	NR	NR	NR
Herzog et al., 2015 [25]	Retrospective, observational, cohort	Germany	Pancreatic head resection or palliative bypass procedures	887	10%	NR	CDC	NR	NR	NR	NR
Li et al., 2017 [26]	Observational, retrospective	China	Hepatectomy combined with hepaticojejunostomy	335	10.15%	0%	CDC	0	0	0	100%
Bhayani et al., 2014 [27]	Observational, retrospective	USA	Pancreaticoduodenectomy, total pancreatectomy	6512	19.30%	0%	NR	NR	NR	NR	NR
Gavazzi et al., 2016 [28]	Observational, retrospective	Italy	Pancreaticoduodenectomy	178	20.80%	NR	CDC	11.80%	9%		26.80%
Rodriguez-Caravaca et al., 2016 [30]	Observational, retrospective	Spain	Cholecystectomy	766	1.96%	77%	NR	0.91%	0.52%		0.52%

First author, year	Methods	Country	Surgery type(s)	Sample size	Total SSI	Laparoscopic	SSI definition	Superficial	Deep SSI	Superficial and organ space	Organ space SSI
Rodríguez-Sanjuán et al., 2013 [31]	Observational, prospective	Spain	Cholecystectomy	287	8.40%	73.90%	CDC	5.20%			3.10%
Comajuncosas et al., 2014 [33]	Observational, prospective	Spain	Cholecystectomy	220	17.70%	100%	CDC	NR	NR	NR	NR
De Pastena et al., 2018 [34]	Observational, retrospective	Italy	Pancreaticoduodenectomy	387	18%	NR	Clavien–Dindo classification	NR	NR	NR	NR
Huang et al., 2015 [36]	Observational, retrospective	China	Pancreaticoduodenectomy	270	35.60%	NR	Clavien–Dindo classification	16.60%	NR	NR	18.90%
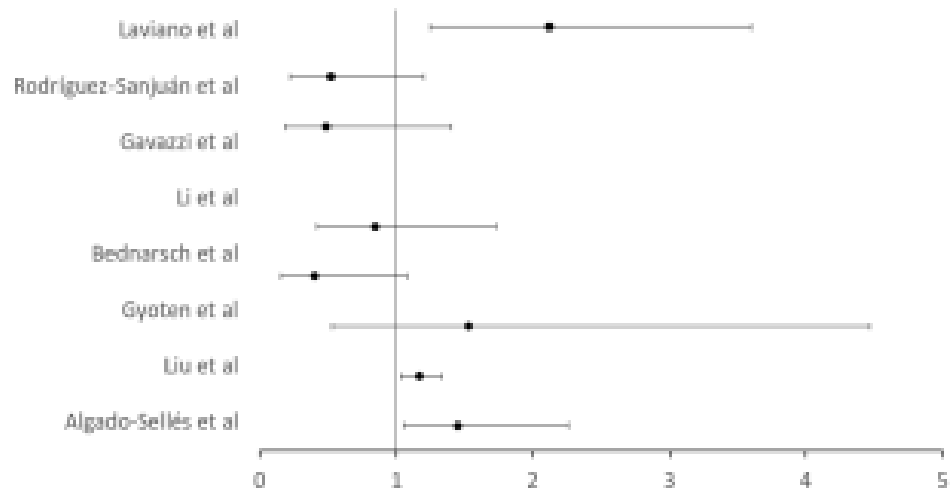
Figure 2. Forest plot of odds ratio of male gender and SSI (95% CI).

3.2.2. Age

Three studies were suitable for the determination of odds ratio of an age of >65. Of these, only Algado-Sellés et al., 2022 [14] found older age to be a significant risk factor of SSIs (OR 2.5 95% CI: 1.7, 3.7) (Figure 3).
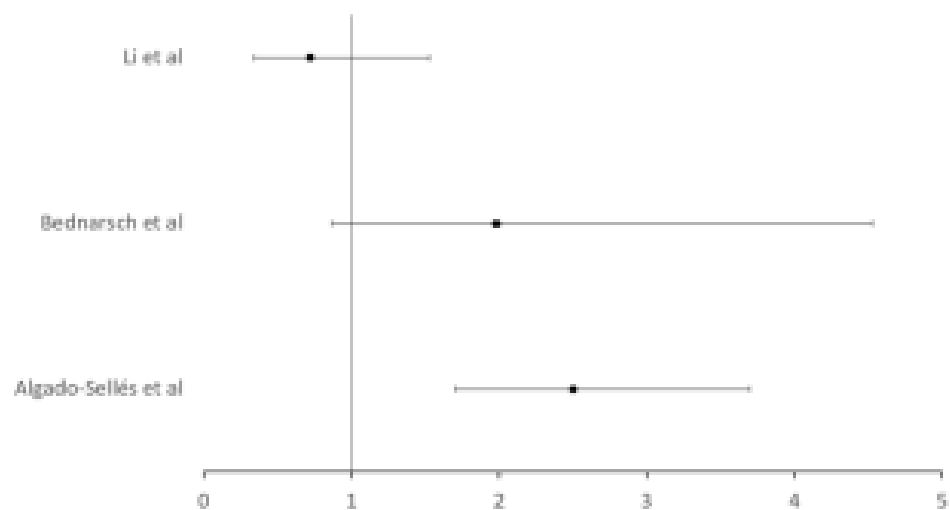
Figure 3. Forest plot of odds ratio of age >65 and SSI (95% CI).

3.2.3. Obesity

Although other articles than the four used, contained details of weight, a BMI >25 was the only measurement of weight used that was identical in multiple studies. A BMI of over 25 includes obese and overweight patients. Two of the four studies analysed found BMI >25 to be a significant risk factor (Figure 4). Gavazzi et al., 2016 [28] OR 2.99 (95% CI: 1.03, 8.64) and Liu et al., 2019 [18] OR 1.75 (95% CI: 1.52, 2.0). Rodríguez-Caravaca et al., 2016 [30] also found obesity to be a risk factor.
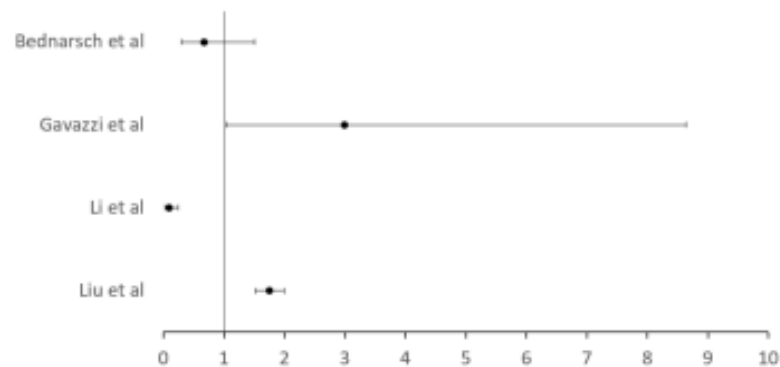
Figure 4. Forest plot of odds ratio of BMI >25 and SSI (95% CI).

3.2.4. Open surgery

Eight of the 16 studies did not include any laparoscopic procedures. Four research articles had the required data to do an odds ratio analysis. Of these, Algado-Sellés et al., 2022 [14] (OR 7.01 (95% CI: 4.68, 10.51)) and Laviano et al., 2020 [12] (OR 4.36 (95% CI: 2.25, 8,47)) found open surgery to be a significant risk factor of SSIs following HPB surgery (Figure 5).
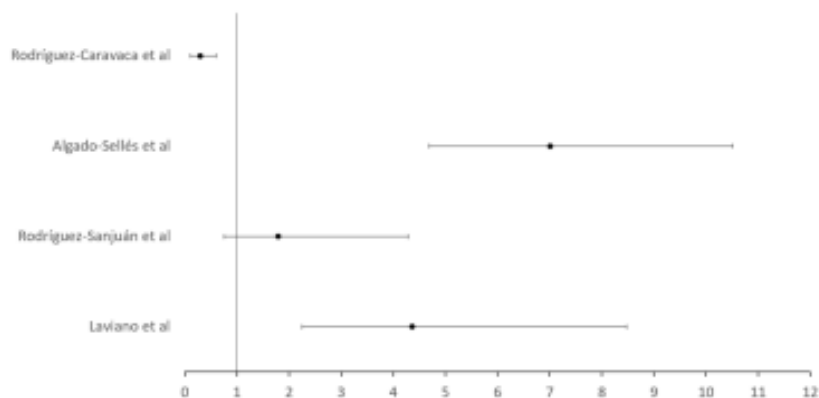
Figure 5. Forest plot of odds ratio of open surgery and SSI (95% CI).

3.2.5. Smoking status

Of the three eligible studies for calculating odds ratio of smoking as a risk factor for SSI, none showed this as a significant risk factor (Figure 6). Furthermore, none of the 18 articles in this review reported smoking as significant risk factor for SSI following HPB surgery.
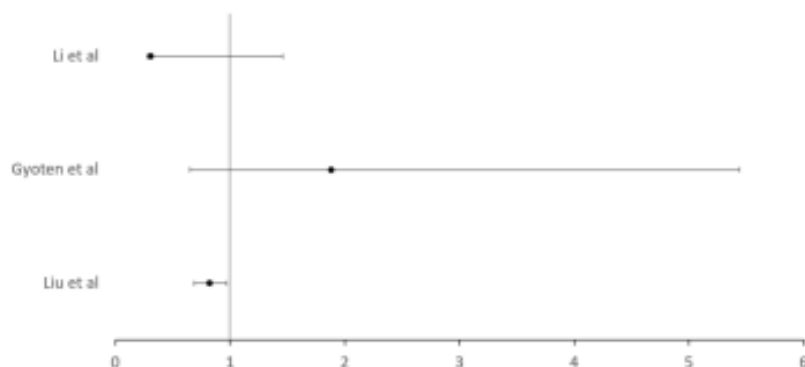
Figure 6. Forest plot of odds ratio of smoking status and SSI (95% CI).

3.2.6. Diabetes

Six research articles were eligible to be included in the odds ratio calculation for diabetes as a risk factor of SSI. Diabetes type 1 and type 2 were differentiated in the studies used. None of these four studies found diabetes to be a significant risk factor of SSIs following HPB surgery (Figure 7). However, Rodríguez-Caravaca et al., 2016 [30] identified diabetes mellitus as an intrinsic risk factor (14.8%).
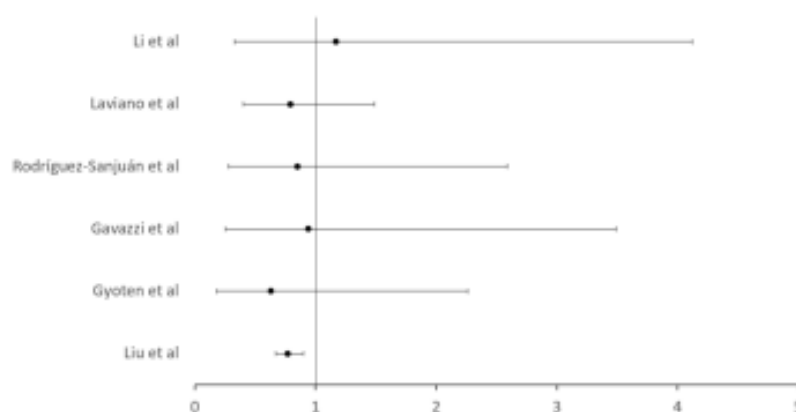
Figure 7. Forest plot of odds ratio of diabetes and SSI (95% CI).

3.2.7. Preoperative biliary stent

Four studies were eligible to perform odds ratio analysis on preoperative biliary stenting as a risk factor of SSIs. Of these, only Laviano et al., 2020 [12] found preoperative biliary stenting to be a significant risk factor of SSI (OR 9.01, 95% CI: 4.04, 21.8) (Figure 8).
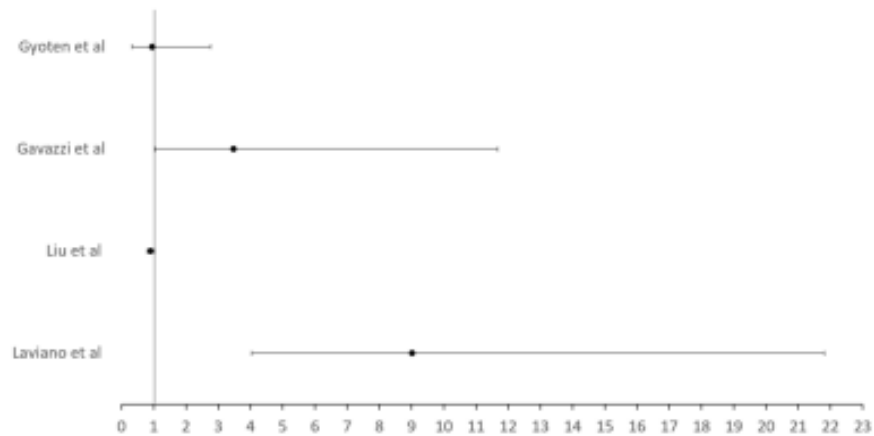
Figure 8. Forest plot of odds ratio of preoperative biliary stent and SSI (95% CI).

3.2.8. Microorganisms

E. coli and *Enterococcus* spp. were the most frequently identified causes of SSIs (Table 2). One study found that following HPB surgery (hepatectomy with and without biliary tract resection, pancreatectomy [pancreaticoduodenectomy (PD), others], and open cholecystectomy) *Enterococcus* spp. (36%) were the leading cause of SSIs followed by *S. aureus* (14%) (methicillin resistant *Staphylococcus aureus* (MRSA) 8.6%), *Klebsiella* spp. (11%), *Pseudomonas aeruginosa* (8%) and *Enterobacter* spp. (6%) [37]. Both *E. coli* and *Enterococcus* spp. are part of the normal gastrointestinal flora. The fact that these species along with other Enterobacteriaceae species were identified as the cause of SSI might imply that the infection occurred due to contamination from the patient's own gastrointestinal flora.

Table 2. The most commonly reported causative organisms of SSIs and the reported risk factors for SSIs in the 16 research papers.

First Author, year	Three most commonly reported organisms of SSIs	Key risk factors of SSIs
Laviano et al., 2020 [12]	Other GN (22.1%), *E. coli* (18.3%), Other GPs (16.3%)	Male, protein malnutrition, neoplasms, hospitalization in last 18 months, open surgery, transfusions, vasopressors, elective, pancreaticoduodenectomy
Joliat et al., 2018 [13]	NR	Male, biliary stenting, anastomosis
Algado-Sellés et al., 2022 [14]	*E. coli* (35%), *E. faecalis* (13.3%), *E. faecium* (8.3%)	Age, pre-surgical glycemia, laparoscopic technique, time of the intervention, type of surgery and NNISS index.
Bortolotti et al., 2021 [17]	(Bile cultures) *E. coli* (19%), *Klebsiella* spp. (14%), *Enterococcus* spp. (12.47%)	NR
Liu et al., 2019 [18]	NR	Male, non-White, hispanic, obese, small pancreatic duct, longer operation.
Sert et al., 2022 [20]	NR	NR
Gyoten et al., 2021 [21]	*E. faecalis*, Coagulase negative *Staphylococci*, *Enterobacter* spp.	Gastric Candida colonization
Bednarsch et al., 2021 [23]	*Enterococcus faecium* 71.2%), *Enterococcus faecalis* (30.8%), *Enterobacter cloacae* (25%)	Reduced susceptibility to perioperative antibiotic prophylaxis, Portal vein embolization, Other postoperative infections, increased hospital and ICU stay
Wagle et al., 2020 [24]	NR	NR

First Author, year	Three most commonly reported organisms of SSIs	Key risk factors of SSIs
Herzog et al., 2015 [25]	*Enterococcus* spp. (41%) *E. coli* (17%) MRSA (12%)	Positive bile duct cultures
Li et al., 2017 [26]	*E. coli* (25%), *S. epidermidis* (12.5%), *Pseudomonas* spp./*Streptococcus* spp./MRSA (8.3%)	Coexisting cholangiolithiasis, blood loss >1500mL, previous abdominal surgical history, bile leak
Bhayani et al., 2014 [27]	NR	NR
Gavazzi et al., 2016 [28]	(Drain fluid) *Enterococcus* spp. (69.1%), *E. coli* (26.8%), *Staphylococcus* spp. (26.8%)	BMI ≥25 kg/m2, biliary stenting, cardiac disease
Rodríguez-Caravaca et al., 2016 [30]	*E. coli* (47.8%), *Klebsiella pneumoniae* (13.1%). *E. faecium* (13.1%)	Open surgery, renal failure, diabetes mellitus, malignancy, chronic obstructive pulmonary disease, liver cirrhosis, obesity, neutropenia, neoplasia
Rodríguez-Sanjuán et al., 2013 [31]	*E. coli* (26.5%), *Streptococcus* spp. (19.4%), *Enterococcus* spp. (17.3%)	Open surgery, conversion to open surgery
Comajuncosas et al., 2014 [33]	NR	NR
De Pastena et al., 2018 [34]	(Bile cultures) *E. coli* (19.9%), *E. faecalis* (18.8%), *Klebsiella* spp. (17.7%)	Positive rectal swab, preoperative biliary drain
Huang et al., 2015 [36]	NR	Endoscopic retrograde biliary stent

*Note: NR = Not recorded; GP = Gram positive; GN = Gram negati

4. Discussion

4.1. Risk factors

4.1.1. Gender

Male sex was found to be a risk factor of SSI following HPB surgery in three of the eight studies analysed. Indeed, it has been it had been previously demonstrated that men were generally at a higher risk of SSI following various surgeries [38]. A surveillance study in Germany found that SSI rates were significantly higher for male patients who had abdominal surgeries, including cholecystectomies [39], and a prevalence study investigating predictors of colonization with *Staphylococcus* spp. in patients undergoing cardiac and orthopaedic surgery found significantly higher colonization rates in men [40]. However, Enterobacteriaceae are the predominant bacteria that cause SSIs following HPB surgery and hence this area requires further investigation.

4.1.2. Age

One of the three studies included in the analysis found that people of an older age (>65) were more likely to be at a risk factor of SSIs [41,42]. For example, Ansari et al., 2019 found that SSIs were more common in older participants (11.4% vs. 6.4%; p = 0.009) [43]. Conversely, others have found that the risk of SSI decreases as age increases, although these studies have small sample sizes [44]. It is difficult to determine if older age results in comorbidities which may be risk factors of SSIs or immunologic senescence as patients age is a risk factor of SSI [45]. Older patients are more likely to have surgery and the population is progressively aging, therefore surgeries and surgical site infection incidence in older patients is likely to increase [46].

4.1.3. Obesity

Three studies found obesity to be a risk factor of SSIs. Due to unhealthy lifestyle habits obesity is becoming more prevalent, particularly in western countries. Obesity is a known risk factor for many types of SSI [5], although many obese patients may also have other comorbidities such as type II diabetes (T2DM), coronary heart disease and osteoarthritis; this makes it difficult to determine if obesity is a single causative risk factor of SSIs. Another factor to consider is that operating times in obese patients are often longer and this is an independent risk factor in the development SSIs. Thelwall et al., 2015 found that in patients undergoing abdominal hysterectomy, knee replacement and large bowel surgery, the risk of SSI increased approximately linearly with increasing BMI [47]. However, HPB surgery was not specifically included in this research and laparoscopic procedures were not included in the cohort due to a recognised lower risk of infection [47].

A study in Shanghai (China) between 2010 and 2011, aimed to identify the risk factors for SSIs following hepatic resection in 7,388 patients and of these participants, 27.3% were obese, and hence the results showed that obesity significantly predicted incisional SSI but no other forms of SSIs [48]. It is thought that high infection rates in obese patients occurs due to tissue oxygen pressure and Kabon et al., (2004) concluded that wound and tissue hypoxia commonly occurred in obese patients perioperatively [49]. SSIs may also occur in obese patients due to reduced blood circulation in the fat tissues which results in a reduced circulation of the immune cells and hence a reduced propensity of the body to eradicate bacteria [50].

4.1.4. Type of surgery

The steady transition from open to minimally invasive surgery (laparoscopic) or keyhole surgery is becoming apparent with more operations now undertaken via laparoscopic techniques. There is evidence to suggest that infections rates are lower in patients following laparoscopic procedures rather than open surgery. Indeed, this metanalysis found two of the four studies analysed showed open surgery to be risk factors of SSIs. Another meta-analysis found that when laparoscopic abdominal surgery was compared to open surgery, the incidence of SSIs was reduced by 70%–80% following laparoscopic surgery in obese patients [51]. In a cased-matched control study of 50 patients, López-Ben et al. (2014) found that the rates of SSIs in laparoscopic surgery patients was 2%, whilst 18% of open surgery patients developed a SSI [52]. However, this study also found that the mean operating time for laparoscopic surgery was 95 minutes longer than for open surgery. Thus, the relationship between the

incidence of an SSI, length of operation time and type of operation carried out needs further investigation, but it appears that a larger wound may have an effect on increasing wound infection rates when considered along with the length of the operation.

Although less frequent SSIs can still occur following laparoscopic surgery these are referred to as port site infections (PSI). Similar species cause SSIs and PSIs although Mir et al., found that *Pseudomonas* spp. (42.2%) was the common offending organism in PSIs following laparoscopic cholecystectomy [53]. The source of these infections was found to be the water used to wash surgical instruments.

4.1.5. Smoking

None of the 16 studies highlighted smoking a risk factor for SSI and hence the three studies included in statistical analysis did not identify smoking as a significant risk factor. In contrast, a number of studies have found that smoking increases the risk of SSIs. A meta-analysis identified a range of cohort studies and randomized controlled trials that found a higher incidence of SSIs in smokers [54]. Nicotine use is known to delay primary wound healing [55] and thus the longer a wound takes to heal, the greater the propensity for it to become infected. Nicotine can cause vasoconstriction resulting in reduces cutaneous blood flow; stimulate the release of proteases that accelerate tissue destruction and supress the immune response, increasing the risk of bacterial infection [56]. However, another factor to take into account is that smoking is known to cause respiratory and cardiovascular disease and thus it might be these clinical manifestations that increase the risk of developing a SSI and not primarily smoking alone [57]. Furthermore, other factors such as how long an individual has smoked and the amount of cigarettes smoked may influence SSI occurrence.

4.1.6. Diabetes

Diabetes is considered a risk factor for many infectious diseases and infections. Diabetes is becoming more prevalent with the number of people with diabetes more than doubling in the last 20 years in the UK [58]. In the studies included in this review only Rodríguez-Caravaca et al., 2016 [30] found diabetes to be an intrinsic risk factor of SSI following HPB surgery.

There is a large body of evidence suggesting diabetes is a risk factor. Barreto et al., (2015) found that when patients with T2DM underwent surgery, they were at a greater risk of developing a SSI [59]. Indeed, a meta-analysis of 14 studies found that patients with diabetes were almost twice as likely to develop a SSI when compared to non-diabetic patients [60]. A number of reasons can explain the higher rates of SSIs in diabetic patients; firstly, diabetic patients often suffer from small vessel disease where there is a decrease in nutrients and oxygen flow to peripheral tissues and thus reduced systemic ability to fight infections [61]. Secondly, high blood glucose levels impair the function of monocytes and leukocytes, resulting in decreased phagocytosis of bacterial cells [62]. Finally, diabetic patients often experience peripheral neuropathy, and this decreases the release of neuropeptides, disrupting the healing response [63]. Furthermore, T2DM has been found to reduce bacterial diversity of the skin microbiome [64]. The skin microbiome protects against infection due to competitive exclusion and direct inhibition.

4.1.7. Preoperative biliary drains

Preoperative biliary stenting was significantly associated with SSI in two of the four research articles included in this analysis. De Pastena et al., 2018 [34] and Joliat et al., 2018 [13] also recorded preoperative biliary stenting as a risk factor of SSIs. In HPB surgery, drains may also be used to remove bile and pancreatic juice, as these are toxic to surrounding tissues. Results from randomised control trials have shown that in hepatic surgery, the use of drains may increase the risk of infections in some patient undergoing a hepatectomy [65]. A meta-analysis found that prophylactic drains did not reduce the occurrence of bile collection which is interesting since this contradicts the objective of this technique [56]. Furthermore, drains may act as a channel for bacteria to spread to the wound, thus increasing the risk of SSIs [66]. Late removal of surgical drains has also been suggested to increase the risk of infections including wound infections, since it has been demonstrated that retrograde drain infections increased when drain placement was prolonged for more than 4 days postoperatively [67]. An explanation for this is that if a drain left in place for more than 4 days bacteria are able to form a biofilm on the foreign object.

4.1.8. Microorganisms

In the 16 studies included, the frequency of the different bacteria found to be causing SSIs varied although there were similarities in the three most commonly found species causing SSIs. In no particular order, the most commonly identified causative species were *E. coli*, *E. faecalis*, *E. faecium*, CoNS, *Klebsiella* spp., *Enterobacter* spp., MRSA, *Pseudomonas* spp. and *Streptococcus* spp. Other researchers have shown similar findings, for example, Shirata et al., 2017 found that incisional SSIs were caused by MRSA (29%), CoNS (21%), *Enterobacter clocae* (12.5%), methicillin susceptible *Staphylococcus aureus* (MSSA) (8%), *Klebsiella* spp. (4%) and *Enterococcus faecalis* (4%) [68]. Shirata et al., 2017 also found that organ and space SSIs were caused by CoNS (33%), *Enterococcus faecalis* (14%), MRSA (12%), *Enterococcus faecium* (10%), MSSA (8%), *Enterobacter clocae* (5%), *Streptococcus* spp., *Bacteroides* spp., *Escherichia coli*, *Klebsiella* spp., *Candida* spp. (3%), *Serratia* spp., *Pseudomonas* spp. and other *Enterococcus* spp. (1%) [68]. Another study found that *Enterococcus* spp. (n = 59) were the leading cause of SSIs following HPB surgery followed by *S. aureus* (n = 23 MSSA, n = 14 MRSA), *Klebsiella* spp. (n = 18), *Pseudomonas aeruginosa* (n = 13) and *Enterobacter* spp. (n = 10) [37]. The prevalence of different bacteria on patients and on hospital wards may vary between geographical locations, although gastrointestinal and skin commensals may be the most likely cause of SSIs following HPB surgery.

4.2. Limitations and future work

Eleven of the 16 studies presentenced in this review were conducted in Europe with five of these being Spain. This could mean that the results are not representative of the SSI incidence in the world due to a location bias. As the studies included were initially screened for incidence results and not risk factors a further metanalysis searching for each separate risk factor would be useful in adding to this body of research.

5. Conclusions

A variety of pre-disposing patient factors can affect infection rates following HPB surgery. Pre, intra and post-surgical factors also influence the occurrence of a SSI following HPB surgery. The results from this study suggest that perhaps there is an association between the use of laparoscopic surgery and infection, and that there should be an awareness that gender, obesity and the use of stents may increase the incidence of SSIs following these surgeries. Further, confounding factors could be responsible for the development of an SSI. This complicated relationship between surgical interventions and SSIs merits further investigation and understanding if the incidences of such infections are to be reduced.

Author contributions

Lucy E. Chambers was responsible for the acquisition, analysis and interpretation of data for the work. Aali J. Sheen and Kathryn A. Whitehead were responsible for the conception and design of the work. All authors were responsible for the drafting and revision of the data and final approval of the version to be published.

Conflict of interest

The author declares no conflicts of interest in this paper.

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Photodynamic antimicrobial chemotherapy coupled with the use of the photosensitizers methylene blue and temoporfin as a potential novel treatment for *Staphylococcus aureus* in burn infections

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Abstract

Photodynamic antimicrobial chemotherapy (PACT) is a novel alternative antimicrobial therapy that elicits a broad mechanism of action and therefore has a low probability of generating resistance. Such properties make PACT ideally suited for utilization in localized applications such as burn wounds. The aim of this study was to determine the antimicrobial activity of MB and temoporfin against both a *S. aureus* isolate and a *P. aeruginosa* isolate in light (640 nm) and dark conditions at a range of time points (0–20 min). A *Staphylococcus aureus* isolate and a *Pseudomonas aeruginosa* isolate were treated *in vitro* with methylene blue (MB) and temoporfin under different conditions following exposure to light at 640 nm and in no-light (dark) conditions. Bacterial cell viability [colony-forming units (c.f.u.) ml^{-1}] was then calculated. Against *P. aeruginosa*, when MB was used as the photosensitizer, no phototoxic effect was observed in either light or dark conditions. After treatment with temoporfin, a reduction of less than one log (7.00×10^7 c.f.u. ml^{-1}) was observed in the light after 20 min of exposure. However, temoporfin completely eradicated *S. aureus* in both light and dark conditions after 1 min (where a seven log reduction in c.f.u. ml^{-1} was observed). Methylene blue resulted in a loss of *S. aureus* viability, with a two log reduction in bacterial viability (c.f.u. ml^{-1}) reported in both light and dark conditions after 20 min exposure time. Temoporfin demonstrated greater antimicrobial efficacy than MB against both the *S. aureus* and *P. aeruginosa* isolates tested. At 12.5 µM temoporfin resulted in complete eradication of *S. aureus*. In light of this study, further research into the validity of PACT, coupled with the photosensitizers (such as temoporfin), should be conducted in order to potentially develop alternative antimicrobial treatment regimes for burn wounds.

INTRODUCTION

Widespread antibiotic misuse, coupled with an increasingly mobile global population, has facilitated an alarming increase in the rates of emerging antimicrobial-resistant (AMR) bacteria. The treatment of AMR bacteria results in both a decline in the physiological and psychological well-being of patients (including morbidity and mortality) and serious financial burdens to healthcare providers and their respective countries worldwide [1]. In Europe alone, multidrug-resistant

(MDR) bacteria are estimated to be responsible for ~25000 deaths per year [2]. Furthermore, it is estimated that by 2050 mortality rates attributed to AMR bacterial infections will surpass 10 million people per annum, superseding cancer as the leading cause of global mortality [3, 4]. Commonly isolated AMR bacteria from patients include methicillin-resistant *Staphylococcus aureus* (MRSA) [5], vancomycin-resistant *Enterococcus* spp. (VRE) [6], carbapenem-resistant *Enterobacteriacea*e spp. [7] and MDR *Pseudomonas* spp. [8].

The main therapeutic strategies that are currently used to control AMR include antimicrobial stewardship, improved infection control and the development of new antimicrobials (including novel antibiotics) [9]. However, since the 'golden era' of antibiotic discovery (~1950–1970) [10], the development and approval of novel antibiotic classes has decreased significantly. This is mainly due to the high cost (>USD $1 billion for new molecular entities) involved in antibiotic development, the low success rate and a lengthy process time (10–15 years) [11, 12]. In addition, the limited mechanism of action of most antibiotics has indicated that resistance is likely to develop and therefore novel antibiotics potentially have a limited shelf life [9].

Burns patients are at high risk of nosocomial infection due to compromised innate host defences (in this instance damage to the epidermidis) [13]. Bacterial colonization of burns can result in invasive infection, septicaemia, multi-organ failure and ultimately death [14]. *Pseudomonas aeruginosa* is the most commonly isolated bacteria from burn wounds, followed by *S. aureus* [15].

The antimicrobial effect of photodynamic antimicrobial chemotherapy (PACT) relies on three components: the presence of oxygen (O_2), a photosensitizer and a wavelength of light that coincides with the peak absorption of the photosensitizers [16]. Methylene blue (MB) is a well-established photosensitizer that has been extensively documented throughout the past decade [17, 18]. Due to the antimicrobial efficacy of MB against a broad range of micro-organisms it is often utilized as a potent photodynamic therapy (PDT) drug for the local treatment of periodontal diseases [19, 20]. The efficiency of MB-mediated PACT has also been confirmed on antibiotic-resistant polymicrobial biofilms of *P. aeruginosa* and MRSA in a maxillary sinus model [21]. In addition, several *in vitro* studies have assessed its antimicrobial efficacy against a range of bacteria commonly isolated from burn infections [22, 23].

Temoporfin is a second-generation photosensitizer that has been utilized successfully in PDT to treat squamous cell carcinoma of the head and neck and has been investigated for use as a treatment for other cancers, such as biliary tract carcinomas [24, 25]. Temoporfin has been shown to achieve the same PDT response at lower concentrations and with lower light doses than its first-generation predecessors [26, 27]. In addition, temoporfin has a better safety profile than other photosensitizers, as it does not cause damage to underlying anatomical structures [26, 27]. Therefore, temoporfin has potential as a promising photosensitizer, although its antibacterial efficacy has not yet been thoroughly characterized in the context of burn infections.

Novel therapies to treat burn infections are urgently needed; particularly therapies that will not facilitate the development of antimicrobial resistance. One potential avenue to be explored is PACT. The current study aimed to assess the antimicrobial efficacy of methylene blue- and temoporfin-mediated PACT against both Gram-positive and Gram-negative bacterial species (namely *S. aureus* and *P. aeruginosa*) that are commonly isolated from burn infections

METHODS

Bacterial cultures

S. aureus (NCTC 6571) and *P. aeruginosa* (B9T2436) were utilized throughout this study. Both species of bacteria were cultured aerobically in Luria–Bertani broth (LB) (Fisher Scientific, USA) in a shaking incubator at 180 r.p.m. for 24 h at 37 °C. Following incubation, the bacterial cultures were normalized in LB broth to achieve an optical density ($OD_{600\,nm}$) of 0.05 (±0.01), equating to approximately 1.0×10^6 colony-forming units (c.f.u.) ml^{-1}.

Photosensitizers and light source

Methylene blue (Sigma Aldrich, UK) was dissolved in sterile water to produce a 1% stock solution (w/v) (10 mg ml^{-1}). Temoporfin (Sigma Aldrich, UK) was dissolved in absolute ethanol (≥99.8%; Sigma Aldrich, UK) at a concentration of 1 mM and stored at −20 °C prior to use. Both photosensitizers were stored in a dark environment to minimize light exposure prior to experimentation. For the MB PACT experiments, the concentration of MB used was 1 mg ml^{-1} (3.13 mM) and the concentration of temoporfin was 50 µM for *P. aeruginosa* and 12.5 µM for *S. aureus*. A portable light-emitting diode (LED) PDT light source that had a red wavelength (λ) (640 nm) was utilized throughout this study. Previous studies have determined that the maximum absorption for methylene blue and temoporfin is 668 and 650 nm, respectively [27, 28].

PACT assays

Photodynamic antimicrobial chemotherapy experiments were conducted in clear, flat-bottom, 96-well microtitration plates (Fisher Scientific, UK). *S. aureus* and *P. aeruginosa* were exposed to four different parameters in the presence of both MB and temoporfin, and red light. A maximal light exposure time of 20 min was used, due to the assumption that patients would tolerate longer treatment times poorly. All PACT experiments were conducted in triplicate alongside a LB broth (negative control) (*n*=3). The bacteria were tested in the presence of the light and a photosensitizer (L+P+) – methylene blue (1 mg ml^{-1}) or temoporfin (50 µM used for *P. aeruginosa* and 12.5 µM for *S. aureus*). The bacterial suspensions (~1.0×10^6 c.f.u. ml^{-1}) were incubated in the dark for 20 min by covering the sterile microtitre plates with aluminium foil. Samples were illuminated using red light (λ=640 nm) for up to 20 min. Serial dilutions were performed at intervals of 1, 10 and 20 min of light exposure and plated onto LB agar plates (Fisher Scientific, USA). The inoculated agar was incubated overnight at 37 °C in the dark. After incubation, the bacterial colonies were enumerated and the c.f.u. ml^{-1} determined. The antimicrobial efficacy testing for the light and the photosensitizer was also carried out without the light and a photosensitizer (L−P−) as a negative control, with no light but with a photosensitizer (L−P+) or with light but with no photosensitizer (L+P−).
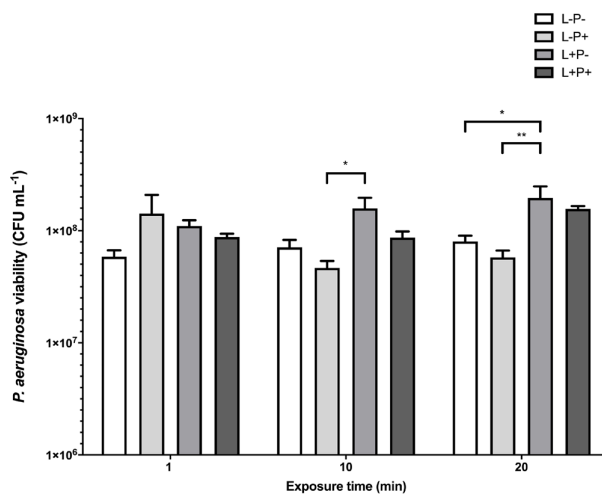
Fig. 1. Effect of MB (1 mg ml⁻¹) on *P. aeruginosa* (B9T2436) after 1, 10 and 20 min of red light exposure (λ=640 nm; *n*=3). Group L+P+, incubated with MB for 20 min, and then irradiated with red light. Group L+P−, no incubation with MB but exposed to red light. Group L−P−, no incubation with MB or exposure to red light. Group L−P+, incubated with MB, but not exposed to red light. Bars represent median value +range of three biological replicates. Two-way ANOVA tests were performed between experimental groups at different time points. Asterisks denote significance (*$P \leq 0.05$, **$P \leq 0.01$).

Statistical analysis

Statistical analysis was conducted by performing two-way analysis of variance (ANOVA) coupled with Tukey's multiple comparison tests for post hoc analysis using GraphPad Prism (version 8.4.2; GraphPad Software, USA) to determine significant differences at a confidence level of 95% ($P<0.05$). Error bars represent the standard error of the mean. Asterisks denote significance, *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.0001$.

RESULTS

The effect of MB- and temoporfin-mediated PDT on *P. aeruginosa*

Initially, the effect of PACT using MB on *P. aeruginosa* was determined. It was demonstrated that the number of viable cells increased with increased light exposure in the untreated experimental group (L−P−) with a mean of 5.44×10^7 c.f.u. ml⁻¹ at 1 min and 8.00×10^7 c.f.u. ml⁻¹ by 20 min (Fig. 1). There was also a similar pattern observed with the L+P+ and L+P− groups. The L−P + group, representing the dark control and hence the antimicrobial activity of MB alone, was the only group to show a decrease in the number of viable cells with increasing time. However, no statistical difference was found between the negative control (L−P−) and (L−P+) at 20 min ($P=0.9434$) (Fig. 1).

The effect of temoporfin-mediated PACT on *P. aeruginosa* was determined. In contrast to the MB-mediated PACT experiment with *P. aeruginosa*, the L+P+ group demonstrated a

decrease in cell viability from 1.49×10^8 c.f.u. ml⁻¹ at 1 min to 7.00×10^7 c.f.u. ml⁻¹ by 20 min. The number of bacterial colonies present at 20 min was significantly lower than for all other experimental groups. The bacterial viability (c.f.u. ml⁻¹) in the L−P− group was 2.89×10^8 c.f.u. ml⁻¹ at 20 min, and the antimicrobial effect of temoporfin with 20 min of red light exposure resulted in 7.00×10^7 c.f.u. ml⁻¹ (Fig. 2).

The effect of MB- and temoporfin-mediated PDT on *S. aureus*

The MB-mediated PACT experiments demonstrated that the Gram-positive bacterium, *S. aureus*, was more susceptible to MB than the Gram-negative bacterium, *P. aeruginosa*. Cell viability was determined at 2.83×10^7 c.f.u. ml⁻¹ and 2.05×10^6 c.f.u. ml⁻¹ between 1 and 20 min in the L−P + and L+P+ groups, respectively (Fig. 3). The viable bacterial counts were higher (with statistical significance) in the experimental controls compared to the L+P+ and L−P + groups at 1, 10 and 20 min, indicating that MB demonstrated antimicrobial efficacy under both light and dark conditions against *S. aureus*. The toxicity of MB alone when no light was applied had a greater effect on *S. aureus* than when illuminated, with the c.f.u. ml⁻¹ being consistently lower at 1, 10 and 20 min in the L−P + group when compared to the L+P+ group. Relative to the control (L−P−) at 20 min (1.79×10^7 c.f.u. ml⁻¹), when MB was used without exposure to light (L−P+), a reduction in viable *S. aureus* (1.50×10^5 c.f.u. ml⁻¹) was achieved, whilst

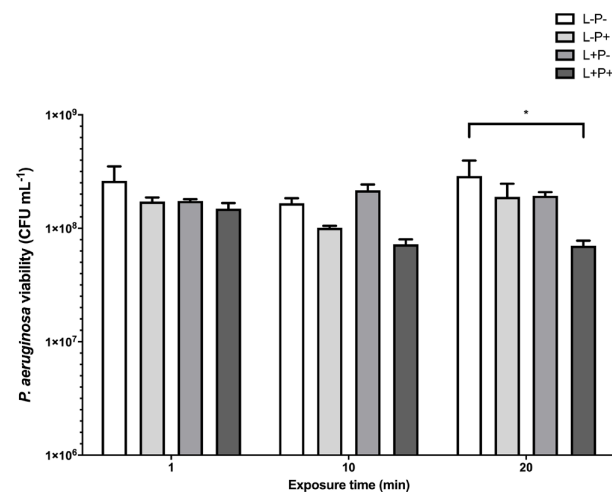**Fig. 2.** Effect of temoporfin (50 µM) on *P. aeruginosa* (B9T2436) after 1, 10 and 20 min of red light exposure (λ=640 nm; *n*=3). Group L+P+, incubated with temoporfin for 20 min, and then exposed to red light. Group L+P−, not incubated with temoporfin but exposed to red light. Group L−P−, not incubated with temoporfin or exposed to red light. Group L−P+), incubated with temoporfin but not exposed to red light. Bars represent the mean of three biological replicates whilst error bars denote standard error of mean (sem). Two-way ANOVA tests were performed between experimental groups at different time points. Asterisks denote significance (*$P \leq 0.05$).
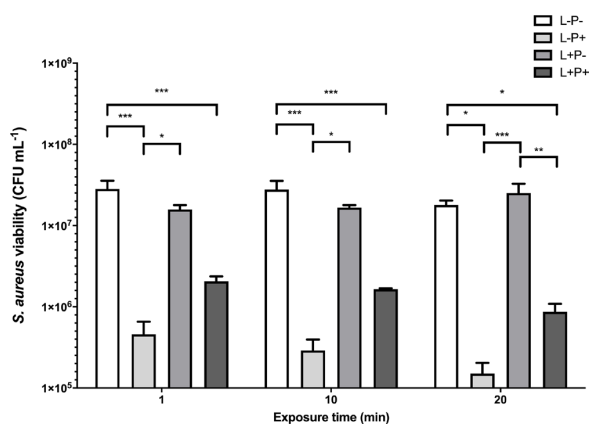
Fig. 3. Effect of MB (1 mg ml^{-1}) on *S. aureus* c.f.u. ml^{-1} after 1, 10 and 20 min of red light exposure (λ=640 nm; *n*=3). Group L+P+, incubated with MB for 20 min, and then exposed to red light. Group L+P−, no incubation with MB but exposed to red light. Group L−P−, no exposure to MB and no exposure to red light. Group L−P+, incubation with MB but no exposure to red light. Bars represent the mean of three biological replicates whilst error bars denote standard error of mean (sem). Two-way ANOVA tests were performed between experimental groups at different time points. Asterisks denote significance (*P≤0.05, **P≤0.01 and ***P≤0.001).

the phototoxicity group, L+P+, reported 8.67×10^5 c.f.u. ml^{-1} of viable *S. aureus* (Fig. 3).

Temoporfin also demonstrated greater antimicrobial efficacy against the Gram-positive bacterium, *S. aureus* (Fig. 4). The killing effect of temoporfin at 12.5 μM was substantially greater than that of MB (which was tested at a higher concentration of 3.13 mM), with a complete eradication of *S. aureus* observed in both the L−P + and L+P+ groups after 1 min (Fig. 4). The L−P + and L+P+ groups both showed statistically significant differences from the L−P− and L+P− groups at 1, 10 and 20 min. This indicated that temoporfin had an antimicrobial effect against *S. aureus* in the dark at 12.5 μM, with complete eradication observed after 1 min of incubation (Fig. 4).

DISCUSSION

This study aimed to determine the efficacy of light-activated photosensitizers against bacterial species commonly found in burn wound infections. The results from this *in vitro* study demonstrated that *S. aureus* (a Gram-positive bacterium) was more susceptible to killing by the photosensitizers in the absence of light than *P. aeruginosa* (a Gram-negative bacterium). Temoporfin demonstrated a photodynamic effect against *P. aeruginosa* and did not demonstrate an antimicrobial effect in the absence of light against *P. aeruginosa*. Incubation of *S. aureus* with temoporfin at 12.5 μM (but no light exposure) demonstrated antimicrobial activity, with complete bacterial eradication after 1 min. Temoporfin at 12.5 μM combined with red light exposure

also resulted in the complete loss of *S. aureus* viability after

1 min, and therefore exclusive phototoxicity activity could not conclusively be determined. The toxicity of MB when tested against *S. aureus* in the dark was greater than its antimicrobial activity following exposure to light. MB did not demonstrate an antimicrobial effect in the absence of light against *P. aeruginosa*

The greater sensitivity of Gram-positive bacteria to photo-sensitizers has been reported by other *in vitro* studies. In 2001, Usacheva *et al.* detailed the photobactericidal effi- cacy of the photosensitizers, MB and toluidine blue (TB), which was assessed against a range of Gram-positive and Gram-negative bacteria [20]. It was reported that the concentrations of both temoporfin and MB required to achieve complete eradication of Gram-negative bacteria with light were in general 3- to 30-fold higher than those required to kill the Gram-positive bacteria tested. Another *in vitro* study conducted by Yang *et al.* (2012) reported complete eradication of MRSA with temoporfin after a 90 min incubation period followed by continuous expo- sure to 100 J cm^{-2} of light (λ=652 nm) [29]. The discrep- ancy in sensitivity is believed to be due to differences in cell wall structure, with Gram-negative bacteria having an additional negatively charged outer membrane that impedes the diffusion of non-cationic photosensitizers [30]. However, this does not fully explain the decreased efficacy of MB, as it is a positively charged photosensi- tizer. An alternative explanation was provided in a study
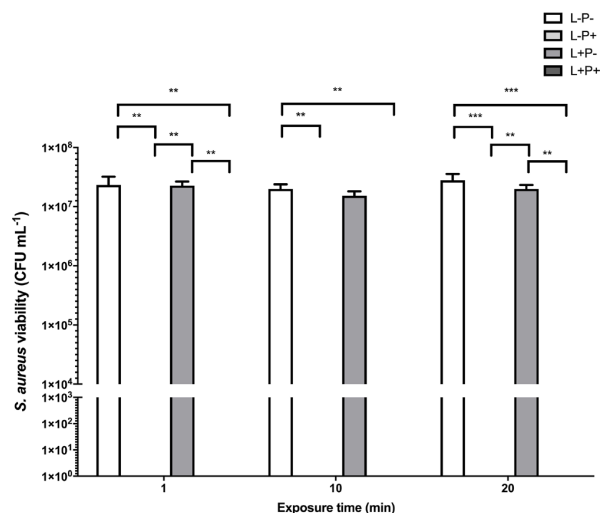

Fig. 4. Effect of temoporfin (12.5 μM) on *S. aureus* c.f.u. ml^{-1} after 1, 10 and 20 min of red light exposure (λ=640 nm; *n*=3). Group L+P+, incubated with temoporfin for 20 min, and then exposed to red light. Group L+P−, no incubation with temoporfin but exposed to red light. Group L−P−, no incubation with temoporfin or exposure to red light. Group L−P+, incubated with temoporfin but not exposed to red light. Bars represent the mean of three biological replicates whilst error bars denote standard error of mean (sem). Two-way ANOVA tests were performed between experimental groups at different time points. Asterisks denote significance (*P≤0.05, **P≤0.01 and ***P≤0.001).

by Rineh *et al.* (2018), which reported a potential efflux mechanism against MB [31]. In this study it was shown that a NorA efflux pump inhibitor–methylene blue (EPI– MB) hybrid compound displayed a greater PDT against the Gram-negative bacteria *Escherichia coli* and *Acinetobacter baumannii* than MB alone. The antimicrobial activity against Gram-negative bacteria may therefore be enhanced by mitigating the effect of efflux pumps, through the use of shorter incubation times with photosensitizers, or by repeated doses of photosensitizers.

Another potential explanation for the poor photodynamic efficacy of MB against *P. aeruginosa* is a phenomenon called the self-shielding effect [30]. This arises when high concentrations of the photosensitizer are present in solution and absorb a significant proportion of the light, thereby reducing the light exposure to photosensitizer-loaded cells [30]. For many photosensitizers this self-shielding effect is observed when the concentration reaches $\geq 300\ \mu M$ [30]. The concentration of MB used throughout this study was 3.13 mM and was greater than that of the temoporfin, and was selected since studies in this area use a range of MB concentrations from $\leq 25\ \mu g\ ml^{-1}$ to 10 mg ml^{-1} and hence the MB concentration selected for use in this study was taken for use at a conservative mid-range [30, 32–34]. The use of this higher concentration may explain the potential shielding effect demonstrated.

The current study demonstrated that a temoporfin concentration of 50 μM enabled a photodynamic effect to be observed against *P. aeruginosa*. *P. aeruginosa* cell viability at this concentration reduced from 2.89×10^8 to 7.00×10^7 c.f.u. ml^{-1}. In a previous study by Yang *et al.* (2012), a similar phenomenon was observed; no overall significant reduction in *P. aeruginosa* viability was observed when temoporfin was utilized at 12.5 μM, and the authors stated that this was likely due to the neutral charge of temoporfin, which meant that penetration of the outer membrane was less probable [29]. The threshold required by the American Society of Microbiology for a treatment to be termed antimicrobial is when it can achieve at least a three log reduction in c.f.u. ml^{-1} (killing efficiency of 99.9%) [35]. It would therefore appear to be an ineffective antimicrobial treatment against antibiotic-resistant Gram-negative bacteria when used at this concentration.

Future research may involve the use of temoporfin as a photosensitizer against resistant strains of bacteria, in particular MRSA, as this species commonly colonizes burn wounds. This research has shown that temoporfin is effective in the eradication of a Gram-positive *S. aureus* species, meaning that it may result in the killing of other Gram-positive species causing burn infections, such as *Enterococcus* spp. This has been shown by Kranz *et al.* (2011), who described a six log reduction in *Enterococcus faecalis* c.f.u. ml^{-1} after treatment with 30 μM of a liposomal formulation of temoporfin, subjected to a light dose of 100 J cm^{-2}, at a wavelength of 652 nm [36].

CONCLUSIONS

Temoporfin demonstrated greater antimicrobial efficacy than MB against a *S. aureus* isolate and a *P. aeruginosa* isolate tested *in vitro*. At 12.5 μM, temoporfin resulted in complete eradication of *S. aureus*. Although the use of light and temoporfin decreased the numbers of *P. aeruginosa*, viable cells were still present following treatment. The results of this study demonstrate that the antimicrobial activity of temoporfin as a photosensitizer could be more suited to Gram-positive bacterial infections. In light of this study, further research is warranted for the development of an alternative treatment option for burn wound infections.

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