University Hospitals of Morecambe Bay NHS Foundation Trust

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Development of methods for the detection Staphylococcal Protein A in ICU patient urine

Submitted for completion of Intercalated Masters of Science degree at Lancaster University

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8/19/2016

Declaration

I declare that this thesis is my own original work, and has not been submitted for a higher degree course at any other institution.

Acknowledgments

Firstly, I would like to thank the patients who kindly provided consent to have urine collected for this research. Their samples were essential for this work.

I'd like to thank my supervisor Dr Robert Lauder for his excellent guidance throughout this research, and also Dr Mark Wilkinson for his support.

Dr Michael Urbaniak provided invaluable information on Mass Spectrometry techniques and theory, without which no Mass Spectrometry work could have been undertaken for this project.

Finally, I would like to thank Dr Lisa Bishop, whose close tuition and wealth of experience made this project possible.

Abstract

Hospital acquired *Staphylococcus aureus* bacteraemia presents a serious health risk to inpatients, due to the high risk of transmission and high mortality rates, which is in part is due to the emergence of multiple drug resistant strains of *S. aureus*. Therefore, the prompt diagnosis and treatment of *S. aureus* bacteraemia is of paramount importance in the Intensive Care department.

Staphylococcal Protein A (SpA) is an immunoglobulin-binding surface protein, expressed by more than 98.9% of *S. aureus* strains, with a primary function of aiding *S. aureus* in the colonisation of the host. While many Staphylococcal toxins are excreted by the kidneys, the method of SpA processing and removal by the body has not been confirmed. If SpA is present in urine, it could provide a key marker of *S. aureus* infection.

The aim of this research was to develop techniques for the detection of SpA in samples, and evaluate the surface protein as a target for *S. aureus* urinary antigen testing. This was to be assessed in a sample population of 45 Intensive Care patients, each providing up to 8 samples over a 48 hour inpatient stay.

An effective and specific Western Blot was developed for the detection of commercial SpA in both buffer and control urine; however, this method failed to detect any SpA bands in patient urine samples. Subsequently, an optimised ELISA displayed increased sensitivity, being able to detect lower levels of SpA down to 0.78ng/ml. Using this technique, we detected an increased absorbance in 25% of patient samples tested, implying the presence of SpA. However, these samples did not display the same characteristics as commercial SpA, lacking the heat-resistance of the purified protein when ELISA samples were subject to boiling. Additionally, the application of mass spectrometry to analysing the SpA ELISA positive samples did not identify SpA. Furthermore, positive ELISA results were significantly associated with renal failure, but not with markers of infection.

This research represents a comprehensive analysis of immunoblotting and immunoassay methods for detecting SpA in urine samples. Further work is required to fully assess the route of excretion of SpA, and the techniques developed could prove useful in the testing of other clinical samples, such as serum, for SpA.

Table of Contents

List of Figures

List of Tables

1 Introduction

1.1 *Staphylococcus aureus*

Staphylococcus aureus is a gram-positive bacterium, found commonly as a commensal organism in humans. It is capable of causing life-threatening illnesses, such as major, multi-system infection and sepsis from contamination of wounds, or from invasive medical procedures. The organism possesses an extensive armoury of toxins and surface proteins, which help it to avoid eradication by the host defences. With the discovery and widespread use of antibiotics, the physicians became able to halt the progression from local *S. aureus* infection to widespread dissemination throughout the body. However, almost as soon as treatment through antibiotics became available, *S. aureus* began to evolve mechanisms of evading eradication by antibiotics.[1] Through the evolution of these various antibiotic resistant strains, S. *aureus* remains capable of causing severe, and sometimes fatal, infections, and poses a significant challenge for medical professionals, particularly in the intensive care setting.[2]

1.2 *Staphylococcus aureus* Microbiology

S. aureus is a facultative anaerobic coccal bacterium, capable of both aerobic and anaerobic respiration.[3] Most strains of *S. aureus* are positive for catalase, a digestive enzyme which characterises this particularly virulent species of *Staphylococcus*, though catalase-negative strains can also cause human disease.[4] Observed through the microscope, *S. aureus* are small, round bacteria, which resembles a 'bunch of grapes' due to their lack of separation following asexual reproduction.[5] Macroscopically, *S. aureus* forms large, circular golden-coloured colonies; hence the alternative name for the bacterium, 'Golden Staph'.[4]

Many genetic variants (or strains) of *S. aureus* exist, of which 13 have been fully gene sequenced.[6] While the majority of the genome consists of a highly conserved core-region of genes, up to 20% of genetic elements display high variability, capable of expressing a wide variety of differing virulent protein products.[7, 8] This confers on *S. aureus* a significant adaptability to develop antibiotic resistance and aid pathogenesis.

1.3 Staphylococcal Cell Wall Morphology

All Gram-positive bacteria, including Staphylococci, are encased in a thick, homogenous, cell wall formed primarily from cross-linked peptidoglycan molecules.[9] In gram-positive bacteria such as *S. aureus*, these cross-links are glycosidic covalent bonds connecting glycan strands of the peptidoglycan molecules (glycine interbridges).[5] A strongly cross-linked cell wall serves two important purposes for *S. aureus*; it provides a rigid casing to prevent osmotic lysis of the cell,[10] and it provides anchorage for surface proteins.[11] Peptidoglycan makes up approximately 50% of the cell wall mass, with the other largest component, teichoic acid, making up around 40%.[12] Teichoic acids, including cell membrane associated lipoteichoic acid, extend from the peptidoglycan layer and confer a negative charge on the *S. aureus* cell wall (Figure 1).[12, 13]

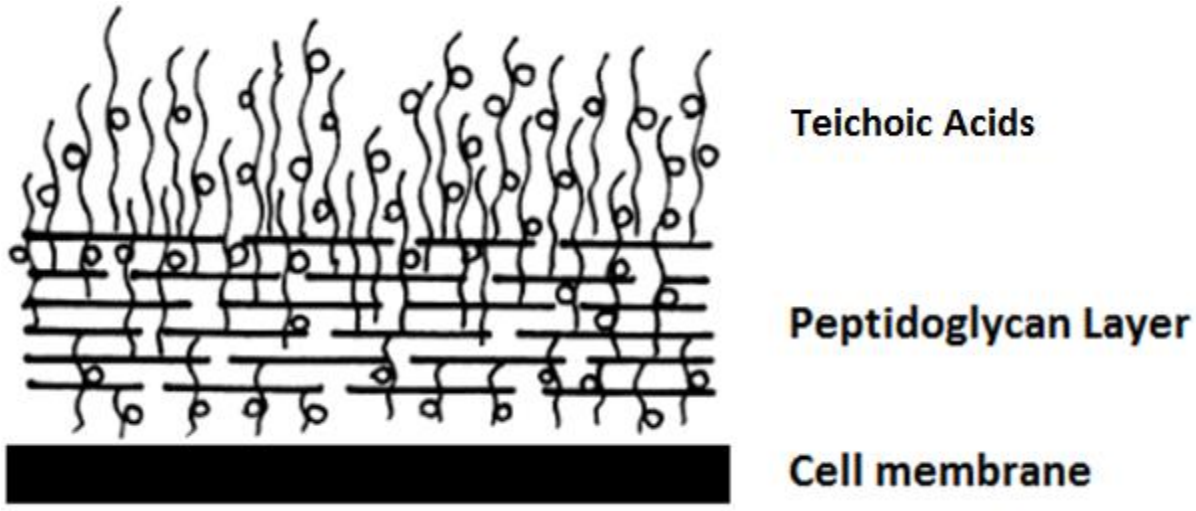


Figure 1- Schematic representation of the *S. aureus* cell wall, with the solid horizontal lines representing cross-linked peptidoglycan, and the wavy vertical lines representing teichoic acids projecting through the cell wall. Image adapted from Umeda et al.[13] Permission for use allowed by American Society of Microbiology.

The remainder of the cell wall components include surface proteins and receptors. As well as a rigid cell wall, over 90% of *S. aureus* strains posses an external polysaccharide capsule,[14] which has been shown to reduce opsonisation and phagocytosis by shielding antibody binding sites (such as immunogenic teichoic acids), from the immune system.[15]

1.4 *Staphylococcus aureus* Infection

1.5 *Staphylococcus aureus* Pathogenesis

S. aureus is human pathogen responsible for infection at a wide-range of sites, in particularly infections of the skin and soft tissues, bones and the bloodstream.[16] It is the presence of *S. aureus* in the bloodstream (bacteraemia) which disseminates the organism to sites of infection, and also leads to the most serious and life-threatening sequelae of *S. aureus* infection: endocarditis and sepsis.[17]

S. aureus bacteraemia results from invasion from the external environment, either due to breaks in the skin or from the presence of foreign bodies.[3] Evidence shows that the risk of *S. aureus* infection increases in the presence of foreign material, which in the community takes the form of contaminated dirt or clothing, and in the healthcare setting can result from sutures, intravascular devices or urinary catheters.[18, 19] Whether this initial colonisation of *S. aureus* progresses from local to metastatic infection is dependent on interactions between the host endothelial cells and the bacterium.[17] In a review of *S. aureus* infection pathogenesis by Archer et al, the progression of *S. aureus* infection is summarised in 5 stages: colonisation, local infection, systemic dissemination/sepsis, metastatic infection, and finally, toxinosis, the system-wide spread of toxins.[20]

1.6 Intracellular *S. aureus* infection

S. aureus was traditionally described as a wholly extra-cellular organism, causing local and systemic infection through adherence to endothelial cells and the release of exotoxin.[4, 21] A study investigating *S. aureus* in aortic endothelial cells showed that *S. aureus* has the ability to propagate and prolong infection through the intercellular route.[22] These findings led to the description of the primary mechanism of *S. aureus* cellular invasion, which involves the adhesion of *S. aureus* to endothelial cells through the activity of fibronectin-binding surface proteins FnbpA and FnbpB.[23]

As well as active invasion of endothelial cells, *S. aureus* has also been shown to survive in specialised immune phagocytes, such as monocytes.[24] The mechanism by which the bacterium survives phagocytosis once inside the cell is not fully understood, but is understood to involve both shielding by the polysaccharide capsule (if present), and the

release of virulence factors which can break down endosomes.[16] The clinical implications of *S. aureus* internalisation have yet to be clearly defined, with some studies suggesting that the virulence of a strain is increased by its ability to internalise, and ultimately kill, host cells,[25] while others suggest a link between recurrent *S. aureus* infection and intracellular survival.[26]

1.6.1 Staphylococcal Nasal Carriage

The most common site of colonisation of *S. aureus, a* natural commensal organism of humans, is the nose (nasal carriage).[27] The epidemiology of *S. aureus* nasal carriers has been categorised into permanent carriers (10-35%), who exhibit constant nasal carriage, transient carriers (20-75%), who only exhibit temporary carriage, and finally, permanent non-carriers (5-50%).[28] This means that at any one time, in a population of healthy individuals, around 35% of the population will harbour *S. aureus* in the nasal passage, either permanently or temporarily.[29] The nasal carriage of *S. aureus* is clinically important due to its effects on propagating and initiating *S. aureus* infection; it has been demonstrated to significantly increase hand carriage of the organism, and the risk of subsequent *S. aureus* infection following surgery is increased in patients known to be nasal carriers.[29] Persistent nasal carriers and the general population tend to demonstrate differing *S. aureus* colonisation sites (Figure 2).[30]

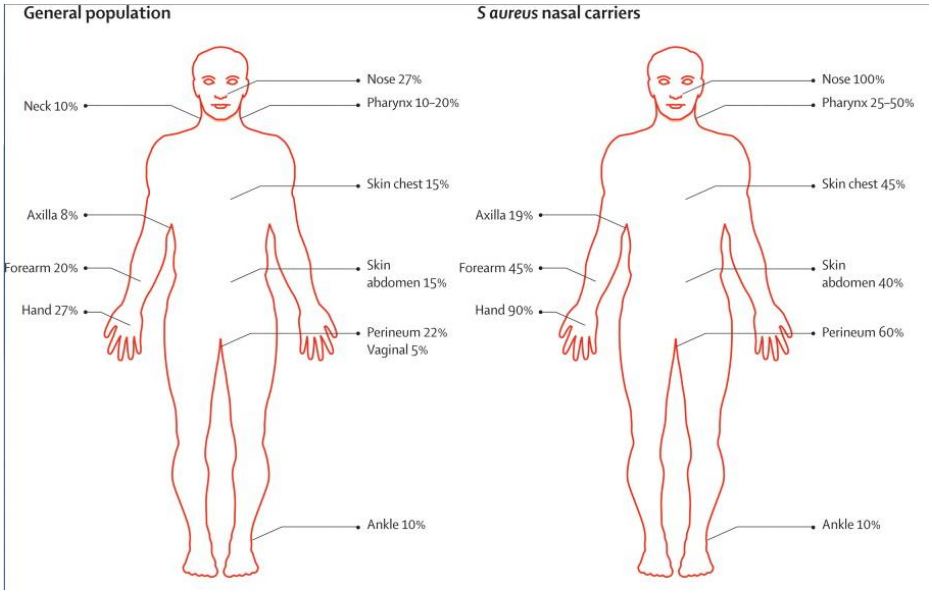


Figure 2- Sites and frequency of *S. aureus* colonisation of the general population and persistent nasal carriers. Image from Dancer at al.[30] Permission for use in MSc thesis granted by Elsevier (License number 3956002030720)

Due to the increased risk of exposure, antibodies to staphylococcal toxins have been found in significantly higher levels in permanent carriers of *S. aureus*,[31] and it has been suggested that this leads to the, paradoxically, improved infection prognosis in this group.[32]

1.6.2 *S. aureus* Bacteraemia

S. aureus is a common blood-stream pathogen, and bacteraemia is one of the most well recognised sequelae of *S. aureus* infection.[33] The annual reported rate of *S. aureus* bacteraemia in the UK was 9,533 in 2013, [34] and despite increased awareness of antibiotic resistant *S. aureus*, and widespread application of transmission prevention schemes, the mortality rate for this condition remains high. In a large US study of 6,697 patients with blood-stream infection, *S. aureus* accounted for the highest crude mortality rate (22.5%) of all bacterial causes of septicaemia.[35] In the UK, mortality amongst patients with diagnosed *S. aureus* bacteraemia is equally high (29% 30 day mortality), and higher still in those with identified MRSA infection (34% 30 day mortality).[36]

The most considerable risk to ICU patients is from *S. aureus* sepsis, the stage of infection beyond bacteraemia, where the disseminated organism provokes a damaging immune response, and, in the case of severe sepsis, causes end-organ damage through ischemia.[37]

Effective recognition, diagnosis and treatment of *S. aureus* bacteraemia are essential for reducing the burden of this disease.

1.6.3 Risk factors for *S. aureus* Bacteraemia

Traditionally, *S. aureus* bacteraemia has been an inpatient-based (particularly intensive care) complication. patients are at high risk of bacteraemia due to a combination of existing susceptibility (i.e. immunosuppression) and high risk of exposure from other patients.[38] Additionally, invasive procedures, all of which are capable of introducing organisms into the bloodstream, are commonly performed in ICU wards. The endogenous route of infection, i.e. from the patient's own flora, is thought to be responsible, (at least in part) for 80% of cases of Staphylococcal bacteraemia.[39] A large study of non-surgical patients found the relative risk of developing Staphylococcal bacteraemia was 3.0 in persistent nasal carriers compared to non-carriers.[40]

Recently, it has been identified that community antibiotic-resistance is on the rise, which is a worrying finding as it presents a reservoir for antibiotic-resistant organisms.[41] There is potential for the transmission of such organisms from the community to the hospital setting.

The primary infection which most commonly lead to *S. aureus* bacteraemia include pneumonia, osteoarticular infection, skin and soft tissue infection, infective endocarditis and intravenous catheter infection.[33, 42] Therefore, patients at risk of *S. aureus* bacteraemia include those with compromised immunity, such as HIV positive patients,[43] and those undergoing recurrent IV access, such as IV drug users or haemodialysis patients.[44 45] Additionally, these patients are among those particularly susceptible to community-acquired bacteraemia.

Considering Urinary Tract Infection (UTI) as a source of bacteraemia, *S. aureus* is rarely a direct pathogen of the urinary tract; in a study of urine samples from patients with symptomatic UTI, *S. aureus* only accounted for 0.5% of isolates.[46] However, multiple studies have demonstrated that the asymptomatic presence of *S. aureus* in the urinary tract (bacteriuria) can lead to the development of *S. aureus* bacteraemia.[47 48]

The reverse case, where an *S. aureus* bacteraemia causes a subsequent bacteriuria, does appear to occur in cases where the initial site of infection is not the urinary tract.[49] Studies demonstrate that concomitant *S. aureus* bacteriuria in patients with bacteraemia is associated with higher incidence of early complications from bacteraemia,[50] and a higher incidence of admission to ICU.[51]

Complicating the assessment of *S. aureus* bacteriuria on ICU wards is the fact that *S. aureus* urinary tract colonisation can be viewed as a common consequence of urinary catheterisation, especially from long-term indwelling catheters.[33] In one study of 127 confirmed *S. aureus* bacteriuria cases, 73% were associated with catheterisation or internal urinary tract procedures.[52] Therefore, it would be difficult to determine whether the bacterinuria is a direct result of presence of *S. aureus* in the bloodstream, or simply due to contamination from urinary tract intervention.

1.6.4 *S. aureus* antibiotic resistance

Antibiotic-resistant strains of *S. aureus* identified in ICU departments between 1996 and 2009 suggest that a rate of 12.7 new cases of MRSA bacteraemia arise every 1000 days spent on ICU.[53] In recent years, antibiotic resistance has become the single most important issue affecting *S. aureus* infection in ICU departments.

Antibiotics, in the form of penicillin, dramatically decreased mortality rates from Staphylococcal bacteraemia when they were first introduced in the early 1940s.[54] However, within 2 years, penicillin-resistant strains of *S. aureus* were discovered.[1] These were found to be caused by a Staphylococcal enzyme (β-lactamase) capable of breaking open the β-lactam ring of the penicillin molecule.[55] Widespread use of penicillin has eventually led to a resistance rate of over 90% in human isolates, effectively rendering the drug ineffective in treating *S. aureus* infection. The β-lactamase-resistant antibiotic methicillin was introduced in 1959, and the first reports of Methicillin-Resistant *Staphylococcus aureus* (MRSA) were published 2 years later.[56] The mechanism of methicillin-resistance in MRSA occurs through the action of an independent penicillin-binding protein (PBP),[57] which provides MRSA with resistance to all β-lactamase antibiotics, including cephalosporins and carbapenems. There is longitudinal and meta-analysis evidence that increased use of these antibiotics is associated with increased incidence of MRSA in the hospital setting.[58, 59] In the UK at the

turn of the 21st century, almost half of *S. aureus* strains causing bacteraemia were β-lactam antibiotic resistant.[60]

The clinical impact of this broad antibiotic resistance is of particular importance in hospital-acquired *S. aureus* infection; invasive medical devices are the most common source of MRSA infection in UK tertiary centres,[42] and infection with a resistant form of *S. aureus* increases length of stay in hospital and mortality rates.[61] In UK ICU units, MRSA accounts for 58-60% of all *S. aureus* strains isolated.[62] MRSA currently remains susceptible to glycoprotein antibiotics, such as vancomycin, and the use of vancomycin rapidly increased from 1980 onwards in order to deal with such resistant infections.[63] However, such selective pressure has led to the emergence of further resistant forms of *S. aureus*, including Vancomycin-resistant *Staphylococcus aureus* (VRSA),[64] indicating increasing difficulty in the pharmacological management of nocosomial *S. aureus* infection.

1.6.5 Treatment of *S. aureus* bacteraemia

Protocols for the initial management of *S. aureus* bacteraemia on ICU wards call for adequate and thorough clinical examination, followed by demonstrating direct evidence of bacteraemia with positive blood cultures.[65] Alongside blood cultures, transoesophagael ultrasound is recommended in order to assess heart valves for vegetations, or for other primary sites of endocarditis.[65]

Optimum treatment combines both medical management, in the form of antibiotics, and surgical removal of infectious loci, if identified.[66] Although 10-40% of patients do not have an identifiable loci of infection, such as prosthetic heart valves or joints,[67] early surgical intervention is proven to improve outcomes in appropriate patients.[66] This further emphasises the need for quick diagnosis of infection, in order to assess if surgical intervention is required. In terms of antibiotic treatment, current UK recommendations in the UK suggest a combination antibiotic therapy, using a glycoprotein, and either linezolid or daptomycin, intravenously, for at least 2 weeks.[68] However, best outcomes, with fewer recurrences of infection, are achieved with 4 or more weeks of antibiotic therapy, especially if there is concurrent endocarditis.[66]

1.6.6 Diagnosis of *S. aureus* infection

The gold standard of *S. aureus* bacteraemia diagnosis is blood cultures, of at least two samples taken from two different sites of venous access. With 2 samples, this technique has 80% sensitivity for detecting pathogenic organisms; to achieve >99% sensitivity, 4 or more samples should be taken, over a 24 hour period.[69]

As well as confirming *S. aureus* infection, blood cultures also provide useful prognostic information. Positive blood cultures for *S. aureus*, taken more than 3 days after starting optimal antibiotic therapy, is the strongest indicator of complicated bacteraemia, even more so than the antibiotic resistance status of the organism.[70]

1.7 Urinary Antigen testing

The clinical practice of testing for urinary antigens in order to diagnose infective disease is well established. Current examples include the immunochromatographic membrane test (ICT) for *Streptococcus pneumoniae* cell wall glycoprotein antigen,[71] and the indirect immunofluoresence test for *Legionella pneumophila* serogroup 1 antigen.[72] With *S. pneumoniae* antigen testing, both the sensitivity and specificity is high of antigen detection, at 90% and 94% respectively.[73] The alternative, sputum cultures, like blood cultures, can take more than 24 hours to provide diagnostic information, and organisms may be undetected in cultures following antibiotic use.[74] An additional benefit is that urine testing is non-invasive, which is important in avoiding the risk of nocosomial bloodstream infection. However, in these conditions, it has been suggested that urinary antigen tests should be used in conjunction with blood or sputum cultures, which can provide antibiotic sensitivity information and can exclude other infectious agents.[73]

Antigen testing for *S. aureus* infection has not currently been developed as a clinical test, despite the array of potential excreted and surface antigens that have been identified. In the literature, attempts to identify *S. aureus* virulence factors have been focused on detecting antigens in serum samples.[75, 76] Using ELISA techniques, Azuma et al found staphylococcal superantigens in the serum of 42% of patients with confirmed sepsis on an ICU ward (n=78),[76] providing evidence for the concurrent presence of *S. aureus* virulence factors and bloodstream infection. However, these have not progressed to clinical trials to put these tests into practice.

Similar techniques have been used to sensitively detect exogenously added Staphylococcal Enterotoxins in buffer and control urine, proving ELISA techniques capable of detecting small amounts of Staphylococcal toxin in urine.[77, 78] Testing patient urine for endogenous Staphylococcal toxins has been achieved by Harrison et al, who identified the presence of superantigens Toxin Shock Syndrome Toxin 1 (TSST-1), Staphylococcal Enterotoxin B (SEB), Staphylococcal Enterotoxin C (SEC) and Alpha-haemolysin (AH) in 9/101 infant urine samples, again using an ELISA technique.[79]

Based on the work by Harrison et al, this laboratory developed ELISA and protein immunoblotting (Western Blot) methods for detecting Staphylococcal toxins in ICU patient urine. Frances Price (MSc 2013) recruited urine samples from 45 inpatients in an ICU department and tested for four Staphylococcal toxins: TSST-1, SEB, SEC and AH.[80] The results of Western Blots found high proportions of patient samples contained AH (72%), with lower levels of other toxins SEB (21%), SEC (7.5%) and TSST (5.9%). These positive samples correlated with clinical indicators of infection; patients positive for AH had higher levels of serum white blood cells, increased temperature and a higher chance of having an infective diagnosis. These data provide evidence of *S. aureus* toxin presence in urine, as well as a foundation for the testing of ICU patient urine for other potential antigens.

1.8 *Staphylococcus aureus*- Virulence Factors

Aiding *S. aureus* in its survival and propagation in the human host, both extra- and intracellularly, are a suite of pathogenic molecules, known as virulence factors.[81] These aid *S. aureus* in killing or invading cells, anchoring to connective tissue or endothelial cells, and evading the complex human immune system, all of which are vital functions if any microorganism is to thrive within host tissues. Virulence factors are secreted from the cell, in the form of enzymes or exotoxins, or exist as surface proteins which allow *S. aureus* to directly interact with the host via the cell wall of the bacterium.

1.8.1 Enzymes

The most basic, though directly damaging, virulence factors utilized by *S. aureus* are digestive enzymes, of which this particular pathogen has a large variety, including catalase, proteases, nucleases, lipases, hyaluronate lyase and staphylokinase.[20] These are used to directly invade host tissue, or predispose conditions for bacterial growth; e.g. provide

nutrients or trigger clotting of blood around invading organisms, in order to shield them from the immune system.[4] These basic functions are vital for microorganism survival, and are also secreted by a variety of other pathogenic bacteria. However, the production of catalase is useful for distinguishing between Staphylococci and Streptococci, which is catalase-negative, and another commonly isolated Gram-positive bacteria.[82]

1.8.2 Exotoxins

Exotoxins are a family of extracellular proteins produced by *S. aureus,* as well as many other Gram-positive bacteria, which are known to have both deleterious effects on the host immune system and biological effects promoting the propagation of the bacterium.[83] As opposed to endotoxin (lipopolysaccharides), which are primarily found within Gram-negative bacteria and are released on cell lysis, exotoxin in actively secreted by bacteria.[84] There are 2 major functional groups of exotoxin, cytotoxic molecules and toxic superantigens:

1.8.2.1 Cytotoxic molecules

This functional group includes molecules capable of killing host cells; the haemolysins (alpha, beta, gamma and delta) and Panton-Valentine Leukocidin (PVL). The previously mentioned Alpha-haemolysin (AH), also known as alpha-toxin, was the first exotoxin with cell membrane pore-forming qualities discovered.[85] It binds to the membranes of a variety of host cells, including erythrocytes, monocytes and endothelial cells, and creates a 1-2nm wide hole in the membrane through the formation of a heptameric pore.[86] Pore-formation is directly damaging to the cell membrane, and disrupts the osmotic balance of the cell. In sufficient quantities (>1µg/ml), AH-pore formation can cause cell death very rapidly, especially in blood cells such as macrophages and erythrocytes.[85]

PVL is an *S. aureus* exotoxin responsible for leukocyte destruction and tissue necrosis.[87] Although the gene for PVL was found to be expressed in <5% of *S. aureus* strains, [88] it was found in 93% of strains isolated from primary necrotizing infections of the skin (such as furuncles).[87] This demonstrates a particularly specialised role for this virulence factor in skin and soft-tissue infection; the gene encoding PVL is rarely, if at all, located in *S. aureus* strains causing deep-seated infections such as pneumonia or sepsis.[89] This clearly demonstrates the

ability of *S. aureus* to vary its genetic 'loadout' in order to suit the challenges of surviving in different body tissues.

1.8.2.2 Toxic Superantigens

Toxic Superantigens (TSAgs) are a group of Staphylococcal exotoxins, which share a common set of biological functions, although separately they cause distinct clinical syndromes, without the need for overt *S. aureus* infection. Included in this group is the protein responsible for Toxic Shock Syndrome, TSST-1, and the Staphylococcal Enterotoxins (SE), which cause emesis and gastroenteritis.[90] There are up to 19 different variants of SE, though commonly studied examples include SEA, SEB, SEC, SEE, SEH.[91]

Despite their varied clinical effects, TSAgs are named after the superantigen effect which is common to this group; this is defined as the ability to bind to T-cell receptors regardless of antigen specificity. [92] The result of this superantigenic binding is to activate large numbers of T-cells in the host, and promote clonal proliferation of those cells.[93] In certain cases, TSAg stimulation of T cells causes a 'cytokine storm', defined as sustained over-production of interleukin-1, interleukin-2 and tissue necrosis factor.[94] This is the pathological basis for toxic shock syndrome, a potentially fatal complication arising from the presence of *S. aureus* growth in areas such as the genitourinary tract, or surgical sites.

The exact reason for deliberately activating adaptive immune system cells is so far unknown; subsequent T-cell anergy following clonal proliferation was long proposed as a potential pathogenic effect, following the observation of T-cell unresponsiveness *in-vitro* when subjected to SEB. [95] However, in a recent study of T-cells in active toxic shock syndrome, patient T-cells displayed as much activation in response to further exposure to TSST-1 as disease-free controls.[96] This suggests that host T-cells do not become fully anergic during active superantigen toxicity, as they are able to respond to further challenge by superantigens.

1.8.3 Surface proteins

Surface proteins, covalently bonded to the peptidoglycan of the cell wall of *S. aureus*, play a significant role in the pathogenesis of bacteraemia and sepsis, in particular by evading the immune system in the bloodstream.[97] All *S. aureus* cell-wall anchored (CWA) proteins share similar basic features allowing their incorporation into the cell wall; at the amino terminal

are secretary signal peptides which bind to the cell membrane secretory apparatus, and are subsequently removed during secretion.[98] At the carboxyl terminal, each CWA protein has a sorting signal which facilitates the adherence of the molecule to cell wall peptidoglycan.[99] The predominant family of CWA proteins is the microbial surface components recognizing adhesive matrix molecules (MSCRAMM), which contains virulence factors Clumping factor A and B (ClfA, ClfB), Fibronectin-Binding Proteins A and B (FnBPA, FnBPB) and collagen adhesin (Cna) (Table 1). [98, 100 101] These proteins, as the group name suggests, have primary roles in binding to host extra-cellular matrix (ECM) molecules, an important step in the pathogenesis of *S. aureus* soft tissue and blood stream infection.[100] Other groups include Near Iron-transporter (NEAT) motif proteins, which are proteins involved in the capture of iron from haemoglobin.[102] The sole three-helical bundle CWA protein, Protein A, which is also not an MSCRAMM, is described further in chapters below.

CWA Protein	CWA Group	Ligand	Function
Clumping factor A (ClfA)	MSCRAMM	Fibrinogen- γ-chain carboxyl terminus Complement factor I	Adhesion to immobilized fibrinogen; immune evasion by binding soluble fibrinogen
Clumping factor B (ClfB)	MSCRAMM	Fibrinogen- α-chain	Adhesion to desquamated epithelial cells; nasal colonization
Serine–aspartate repeat proteins (Sdr)	MSCRAMM	Desquamated epithelial cells. Complement Factor H	Nasal colonisation and immune evasion
Bone sialoprotein-binding proteins	MSCRAMM	Fibrinogen- α-chain	Adhesion to ECM
Fibronectin-binding proteins A (FnBPA) and B (FnBPB)	MSCRAMM	Fibrinogen- γ-chain carboxyl terminus	Adhesion to ECM
Collagen adhesin (Cna)	MSCRAMM	Collagen triple helix	Adhesion to ECM
Iron-regulated surface proteins (Isd)	NEAT protein	Haem , fibrinogen , fibronectin, cytokeratin 10, loricrin	Haem uptake and iron acquisition. Adhesion to desquamated epithelial cells
Serine-rich adhesin for platelets (SraP)	NEAT protein	Salivary agglutinin gp340, unidentified ligand on platelets	Pathogenesis of endocarditis

Table 1- Summary of *S. aureus* Cell-Wall Associated (CWA) proteins. Adapted from Foster et al.[100, 102]

1.9 Protein A

Staphylococcal Protein A (SpA) is a CWA protein found in 98.9% of coagulase-positive *S. aureus* strains,[103] and makes up 1.6% of the mass (6.7% cell wall mass) of cultured *S. aureus* colonies.[104] It is a unique product of *S. aureus*; it is not produced by other Staphylococcal species such as *S. epidermis*.[105] It is found primarily on the bacterial cell wall, and also in the culture supernatant as extracellular SpA.[106] The most distinctive feature of SpA is a strong IgG-binding capacity, which facilitated its discovery. It was initially described in 1940 by Verwey et al,[107] as a protein fraction obtained from Staphylococcal extracts, capable of precipitating antibodies from rabbit sera. Designated 'Antigen A', SpA was later confirmed to be a bacterial cell wall protein,[108] the first such discovered, and the antigen fraction as described by Verwey was given the name 'Protein A' in 1964.[109] From further antibody precipitation tests on SpA, it was found that SpA strongly binds the Fc portion of IgG antibodies, in reverse of the usual antigen-antibody binding pattern.[110] IgG is capable of penetrating the capsule of *S. aureus* to reach the cell wall, and study comparing encapsulated and non-encapsulated *S. aureus* variants showed an equal binding capacity between SpA and free IgG.[111] Later studies also confirmed an ability of SpA to also bind to human IgM, specifically the Fab VH3 subclass.[112] A description of the structure of the SpA molecule will aid in the understanding of this antibody-binding capacity.

1.9.1 Structure of Protein A

Cell-wall bound SpA, as isolated from the Cowan I strain, was found to be a 42kDa sized surface protein.[113] However, variations of SpA have been discovered, which encompass a range of molecular weights.[114] In a study by Cheung et al, cell wall extracts from 12 different strains (not including Cowen I) were purified and tested by Western Blot, which identified 7 distinct SpA variants, ranging from 45-57kDa in size.[114] This was theorized to be due to variations in the protein structure of SpA. There is also a difference in MW between cell-wall bound SpA and extracellular. The most commonly studied SpA-producing strains, Cowen I and 8325, produce cell-wall bound SpA at sizes 52kDa and 53kDa respectively, while extracellular SpA is smaller, secreted by strain A676, measured at 41kDa by equilibrium sedimentation.[100, 106, 115 116]

This main functional section of SpA is comprised of 4 or 5 IgG binding domains (IgBDs), 4 of which are highly homologous.[116] The IgBDs, designated EDABC, are proceeded by an N-

terminal signal region, and followed by an X-region, formed by 1) a variable repeat region (X_R) and 2) an end C-terminal sorting signal region (X_C).[117] As SpA is a CWA protein, the C terminal region contains the characteristic cell wall binding section. The SpA molecule is synthesised as a precursor molecule with all these regions intact (Figure 3).

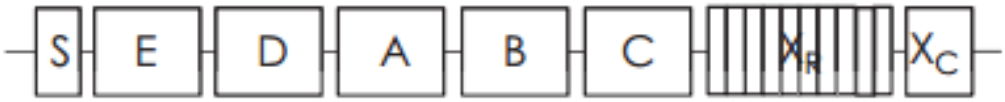


Figure 3- Structure of SpA- molecular structure consisting of an N-terminal signal peptide (S), 5 IgG binding amino-acid sequences (EDACB), and an X region, consisting of a variable region (X_R) and a C-terminal (X_C). Figure adapted from Sorum et al.[117] Permission for use in MSc thesis granted by Oxford University Press (License number 3953851085813)

1.9.1.1 N-Terminal Signal Region

The peptide found at the N-terminal is responsible for SpA joining the extracellular exportation pathway within the cell, and subsequently being incorporated into the cell-wall.[118] The particular route of secretion for SpA is known to be the general secretory (Sec) pathway, and the N-terminal signal peptide is cleaved during Sec translocation through the cell membrane.[119] This method of protein sorting to the membrane is common to many surface and excreted bacterial proteins, and a large proportion bacterial precursor proteins contain a similar sized N-terminal signal sequence for this purpose.[120]

1.9.1.2 IgG Binding regions

The Immunoglobulin binding domains (IgBD) of SpA are 58 amino-acids long, and each comprises a triple α-helix structure, with the complete tertiary structure consisting of each triple helix folding together, as opposed to a straight string of regions (Figure 4).

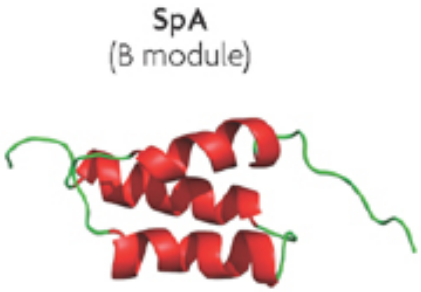


Figure 4- SpA IgBD B, ribbon diagram demonstrating triple-helical structure, image from Lambris et al.[121] Permission for use granted by Nature Publishing Group (License number 3956030424775)

The ability to bind to the Fc portion of antibodies occurs through contact on helices I and II.[121] Total binding capacity of SpA allows two IgG molecules to bind to each SpA molecule (molar ratio 2.1:1).[122] The crystal structure of this antibody-domain complex has been fully described between domain B and Fc, which has demonstrated two points of contact, one hydrophobic and one polar, between the CH2 and CH3 regions of Fc fragments and domain B.[123] All individual domains can bind to the Fc portion of IgG, as well as the Fab region.[124 125] However, not all regions bind equally; regions A, B, C and D are all highly homologous and demonstrate strong binding to IgG, while region E has a weak affinity for IgG, and was only discovered via genetic sequencing of strain 8325. This confirmed the complete Ig-binding regions of SpA to be pentameric.[116] Artificial SpA IgBDs exist, such as domain Z, engineered from naturally occurring domain B by replacing a glycine residue with an alanine residue in helix I.[126] This molecule retains the IgG-binding affinity, and is able to be polymerized to form longer chains of IgG binding regions.

Unlike the wide variety of human and animal IgG molecules which can bind SpA via the Fc region,[127] the alternative Fab binding is specific to the VH3 (variable heavy chain) region within the Fab region of IgM.[128] Binding to the Fab region occurs through IgBD helices II and III, binding to region VH3 of IgM antibodies (Figure 5).[129]

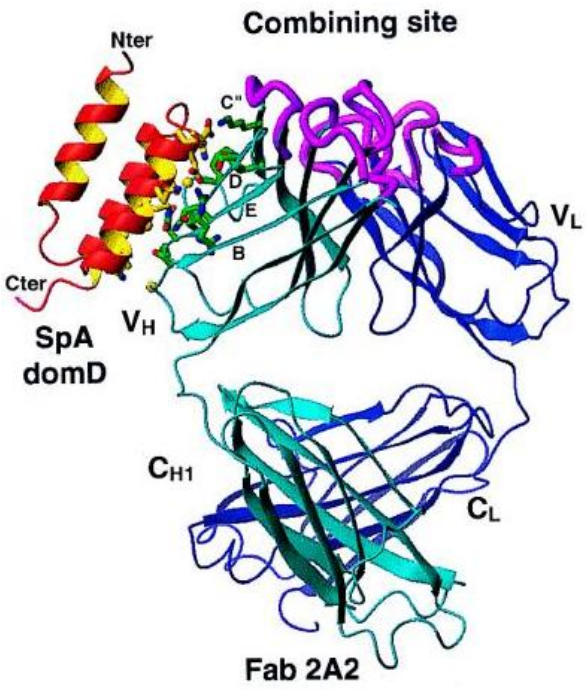


Figure 5- SpA domain D binding to the VH region of Fab 2A2. Binding sites between helices II and III, and VH are labelled D, E and B. Image from Graille et al.[129] Permission for use allowed in unpublished MSc by Proceedings of the National Academy of Sciences

Different strains of *S. aureus* can express variants of SpA molecules with different combinations of IgBDs; these can include molecules containing only 4 IgBDs, missing region A or C.[117] Referring to the SpA-producing *S. aureus* strain, Cowen I, it has been found to project 5 IgBDs from the cell surface, with the E domain closest to the N-terminal.[116]

1.9.1.3 X-region- variable repeat

The polymorphic X_R region is located immediately before the C-terminal cell-wall binding region, and consists of a variable number of 24 base-pair repeats.[130] The biological function of this region is not known, though it has been suggested that these repeats aid in extending the SpA molecule beyond the cell wall.[131] The X_R region varies between strains of *S. aureus*, and affects the molecular weight of the SpA molecule (Figure 6).[117]

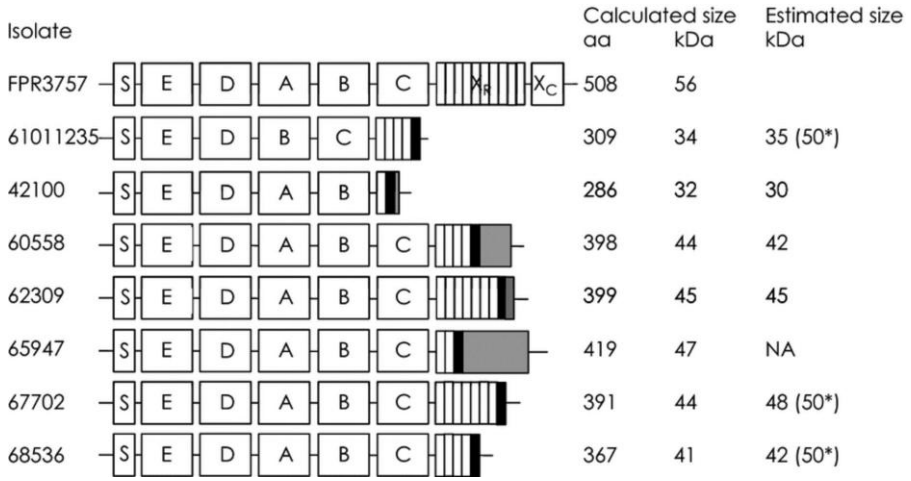


Figure 6- *S. aureus* isolates demonstrating variants of SpA, with variable X_R regions, amino acid (aa) length and estimated molecular weight. These variants of SpA genes for identified from MRSA isolates in hospital patients. Diagram from Sorum et al.[117] Permission for use in MSc thesis granted by Oxford University Press (License number 3953851085813)

This region does serve a biomedical purpose however; the short sequence repeat (SSR) region of the SpA gene (*spa*), which encodes the SpA X_R region present in most strains of *S. aureus*, can be sequenced in order to type *S. aureus*.[132] This is the most common *S. aureus* typing method, due to its speed and ease of interpretation. One disadvantage of this however, is any variability in the 5 IgBDs can lead to a 'non-typable' result, which is known to occur due in 1-2% of strains due to natural mutation.[133]

1.9.1.4 X-region- C-terminal sorting signal

The C-terminal end of the SpA molecule is responsible for attachment to the *S. aureus* cell wall, allowing the SpA to extend from the bacterium. The section directly responsible for this incorporation to the cell wall comprises a 35-residue sorting signal, in the form of the LPXTG motif.[11] This is then followed by a tail of positively charged residues. Once the molecule containing the LPXTG motif is incorporated into the cell wall, a sortase enzyme cleaves between the threonine and glycine amino acid residues, allowing the free carboxyl group of threonine to bind to the peptidoglycan cross-bridges.[99] Once this occurs, the SpA molecule is securely attached to the cell wall by the C-terminal, with the N-terminal region, including IgBDs, projected in an extended profile outside of the cell.

1.9.2 Extracellular Protein A

Most strains of *S. aureus* produce SpA as both cell wall bound and extracellular protein; approximately 2-6 times more cell wall bound SpA is produced than extracellular,[103] though some strains (particularly antibiotic-resistant strains) produce solely extracellular SpA.[134, 135] As described, known fractions of secreted extracellular SpA exist at a smaller MW than cell-wall associated.[100]

Extracellular SpA is produced and secreted during the exponential phase, as a fraction of 5% of total SpA, by Cowen I *S. aureus*.[106] However, a large proportion of SpA in culture medium is the result of release from the cell wall during cell lysis.[106] Despite this mixture of secreted and cell-wall lysed SpA, there exists evidence that the secretion of SpA is a controlled and deliberate act by *S. aureus*, induced by the external environment. One method by which SpA release is induced is through interaction with host chemokines, which has been demonstrated in MRSA skin infection in mice.[136]

The method of SpA release is through a separate biochemical process through that which incorporates SpA in to the cell wall. One such process involves the action of the LytM enzyme, a secreted autolytic enzyme which degrades the cell wall of *S. aureus* by breaking cross-bridges of peptidoglycan.[137] LytM is understood to be the final step in a process which releases SpA from the cell wall; however, LytM mutants continued to produce small amounts of secreted SpA, indicating that other enzymes facilitate the cell wall release of SpA.[138] Another, independent route of release was confirmed recently by O'Halloran et al, as released SpA was detected with an unprocessed sorting signal, indicating no prior incorporation into the cell wall.[139] This evidence shows that the secretion of free SpA is the result of a combination of enzyme-induced release from the cell wall and a distinct secretion pathway, indicating that extracellular SpA plays a useful role for *S. aureus*. Given the potent IgG binding capability of SpA , extracellular SpA may aid the cell-wall bound protein in the primary function of immune evasion.

1.9.3 Functions of Protein A

SpA is a multi-faceted virulence factor for *S. aureus*; its ability to bind host antibodies is SpA's most understood mechanism of virulence, in recent years it has been demonstrated to

provide a variety advantageous effects which enhance the propagation of *S. aureus*. These can be grouped into:

1. Preventing opsonophagocytic killing, by binding to Fc portion of host IgG
2. Evasion of host adaptive immunity, through superantigenic activation of B-cells
3. Adherence of *S. aureus* to sites of thrombosis and endothelial injury, though platelet binding
4. Promotion of *S. aureus* aggregation and biofilm formation

1.9.3.1 SpA binding to IgG Fc- prevention of opsonophagocytosis and complement activation

Opsonisation describes the process in which host antibodies (with additional aid from the complement system) bind to an invading antigen, in order to clump together pathogens and provide increased recognition by the innate and adaptive immune system. The advantage to the host is that opsonisation aids phagocytosis of invading organisms by macrophages, a process with the combined name of opsonophagocytosis.[140] The ability of SpA to bind to the Fc portion of IgG confers *S. aureus* the means to avoid opsonisation, by presenting the opposite end of the IgG molecule to the region which immune cells traditionally bind, i.e. it 'hides' the usual antigen recognised by phagocytes (Figure 7).[141]

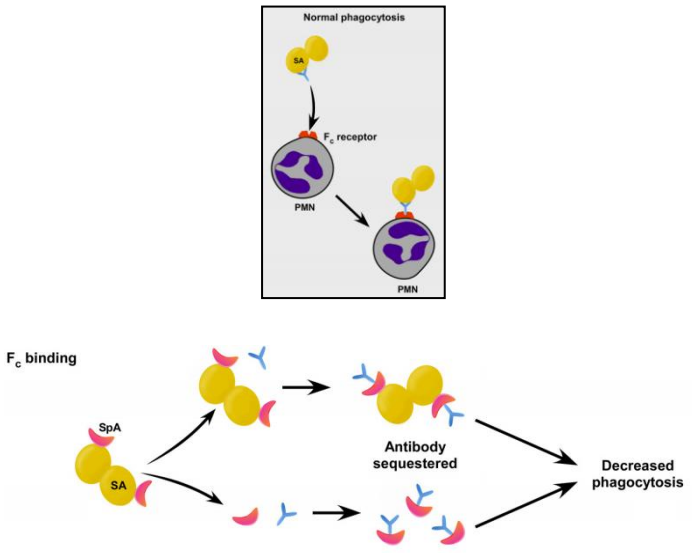


Figure 7- Comparison of normal antibody-mediated phagocytosis and SpA evasion of phagocytosis from polymorphic neutrophils (PMN). Images adapted from Kobayashi et al.[142] Permission for use allowed by American Society of Microbiology.

Experimental evidence demonstrates the effectiveness of SpA in preventing phagocytosis. Peterson et al demonstrated that *S. aureus* strains which expressed higher amounts of SpA were phagocytosed by neutrophils at a slower rate, when placed in an IgG rich environment.[143] Setting this experiment in an IgG saturated medium ensures that SpA-IgG surface binding sites are saturated, proving that the reduction in phagocytosis is as a result of IgG binding. Furthermore, *S. aureus* strains displaying a variant of SpA, specially engineered to lack specific Fc binding capacity, exhibit reduced survival capacity in mice.[141]

This technique of incapacitating the humoural immune system by reversing antibody binding patterns isn't exclusive to *S. aureus*. A study involving another Gram-positive pathogen, *Streptococcus pyogenes*, showed that the orientation of antibody binding on the surface of a bacterium does influence pathogen survival in the host; wild-type *S. pyogenes*, expressing a Streptococcal IgG Fc binding protein (protein M1), survive longer and avoid host immune response longer than mutants lacking an IgG Fc binding capacity.[144] This is additional evidence for IgG Fc binding acting as a viable immune evasion tactic for *S. aureus*, and the authors also suggest that IgG Fc binding plays a large role in the survival of pathogenic organisms as commensals in the healthy population.[144]

Another potential virulent effect of this interaction with IgG Fc is the depletion of complement factor; the complex formed between SpA and Fc fragments has been shown to reduce complement factors in rabbit sera,[145] but conversely, inhibit complement system activation.[146] This subsequent inhibition of the classical complement pathway occurs through an unknown mechanism.

1.9.3.2 SpA superantigen activity

The ability of SpA to bind to the Fab region of IgM confers another route for *S. aureus* to disrupt the host immune response; it is the method by which SpA acts a superantigen.[147] The primary target of Fab binding, the VH3 class of IgM, is specifically expressed on the cell membranes of plasma B-cells.[148] Following binding, SpA activates the B-cell and promotes clonal expansion and the release of IgG antibodies.[149, 150] This mechanism of lymphocyte interaction is similar to the *S. aureus* toxic superantigens discussed above; however, these bind to and activate T-cells only, implying a unique role for SpA in humoural immune activation. A recent study Goodyear et al, following the fate of peripheral B-cells activated

by SpA, discovered that, following a limited amount of clonal proliferation, these cells undergo programmed cell death.[151] As well as disrupting antibody responses, memory B-cells have also been proposed as a target for SpA, and murine studies has recently proven that SpA-expressing *S. aureus* ameliorates host adaptive immunity, reducing protection against *S. aureus* re-infection.[141] As up to half of mature human B cells are capable of expressing the VH3-SpA binding site,[151] this could present a significant virulence effect for *S. aureus*.

Another proposed function of these superantigen effects is the ability to trigger an exaggerated immune response towards a specific antigen; this phenomenon is termed immunodominance, and describes the predilection of targeted immune responses towards one antigen (e.g. SpA) over other potential targets.[152] SpA derived B-cell clonal expansion produces B-cell types with a bias towards the VH3 class of antibody receptor, which reduce the immune system's ability to recognise the wider range of *S. aureus* virulence factors, and therefore leads to less protection from future infection.[152]

Recently, it has been demonstrated that this B-cell superantigen effect subsequently activates CD4+ T-cells in lymphatic tissue, providing additional evidence of host immune manipulation by *S. aureus*.[153] Whether this T-cell involvement helps or hinders *S. aureus* infection is yet to be investigated.

1.9.3.3 SpA Platelet Binding

A study of Staphylococcal platelet binding by Nguyen et al, demonstrated that platelets expressing the gC1qR platelet protein show preferential binding to *S. aureus* strains expressing SpA (e.g. Cowen I), and that beads of purified SpA can precipitate gC1qR from solution.[154] This is an example of a novel method of platelet binding in *S. aureus,* an ability which aids the organism in localising to areas of endothelial damage or thrombosis.

1.9.3.4 SpA Biofilm Formation

Along with interactions with the host, SpA plays a role in multicellular behaviour in *S. aureus*, primarily the formation of biofilms. *S. aureus* mutants with the gene responsible for virulence factor expression in *S. aureus*, *agr*, deleted has been shown to form more stable and strong biofilms.[155] The expression of this gene is inversely related to production of cell surface proteins, including SpA. Recently, Merino et al demonstrated that over-expression

of SpA specifically, both attached to the cell surface and secreted into medium, contributed to the initial formation of biofilms in-vitro and in murine infection models.[156] These findings suggest a role for SpA in primary infections particularly important to ICU wards, those of intravascular and urinary catheters.

1.9.4 Evidence for SpA as a virulence factor

Several studies have confirmed the role of SpA as an important virulence factor in *S. aureus* infection. In experimental mouse models, SpA-negative mutant strains of *S. aureus* were demonstrated to produce smaller skin lesions and less severe intraperitoneal infection, than their SpA-positive counterparts.[157] A greater degree of arthritis and bone destruction is seen in wild-type *S. aureus* expressing SpA as opposed to mutants lacking SpA, when injected into the synovial cavity.[158] Further models of murine septic arthritis, demonstrate clearly that SpA expressing *S. aureus* strains give rise to more severe infection and higher mortality rates, confirming SpA as a virulence factor in osteoarticular infection.[158]

Along with direct evidence of the increased pathogenicity of SpA expressing strains, several studies have proved a correlation between SpA-depleted *S. aureus* mutants and reduced virulence.[157, 159, 160]

The pathogenic effects of SpA have also been confirmed in other manifestations of *S. aureus*, beside direct infection. High levels of SpA have been indentified in *S. aureus* isolates, collected from patients with active Kawasaki disease, an autoimmune disease of childhood with suspected *S. aureus* sensitisation playing a role in pathogenesis.[161] Similar immune disruption is seen in patients with Kawasaki Disease, as that caused by toxic superantigens (such as TSST-1).[162]

SpA has a clear, demonstrable role in the pathogenesis of *S. aureus* infection, along with a wide range of proven virulent effects which primarily act on prolonging *S. aureus* survival in the host, avoiding and disrupting the immune system and directly damaging host tissue.

1.9.5 Genetics of SpA

The structural gene for SpA, *spa,* was first cloned into *E. coli* strains by Löfdahl et al.[163] DNA sequence assays revealed 5 homologous regions corresponding to 5 IgBDs, which, when expressed, retained the ability to bind IgG. The gene was completely sequenced one year

later, using *S. aureus* strain 8325, which gave an estimated M_r for SpA of 58, 703.[164] Depending on the strain of *S. aureus*, *spa* can be found primarily on plasmids, or on bacterial chromosomes. It should be noted that coagulase-negative strains of *S. aureus*, with plasmid-encoded *spa* have been found to produce only 12-30% of the SpA expressed by chromosomal spa strains, such as Cowen I.[165] SpA is maximally produced during the exponential phase of growth, like other surface proteins; production is then down-regulated in the post-exponential phase.[166]

1.9.5.1 Control of SpA expression

S. aureus virulence factor expression, including exotoxins and surface proteins, is controlled by global regulatory systems, which are activated or deactivated at certain times of the *S. aureus* life cycle.[167] These regulate the temporal expression of virulence factors by controlling the expression of multiple genes at once, and are in turn influenced by the quorum sensing system. On such system *agr* (accessory gene regulator), works at the transcriptional level through the action of a regulatory RNA molecule (RNA III).[168] This global regulator gene was initially described as the genetic loci activated during the exponential phase of *S. aureus* growth; *agr*- knockout mutants display decreased secretion of the exotoxins AH and TSST-1, confirming its role in up-regulating virulence factor expression.[169]

The genetic control of *spa* expression involves several transcription factors, which are also involved in the expression of other virulence factors. This system involves inhibitory factors, such as SarA (staphylococcal accessory regulator), and promoting factors, such as Rot (repressor of toxins), both of which are involved in cell signalling pathways which alter rate of *spa* gene transcription.[170] Importantly, *spa* gene transcription is inversely related to the activity of quorum-sensing transcriptional factors, such as *agr*.[171] In the opposite relationship to secreted exotoxins, studies show a 25-fold increase in Protein A production displayed in mutated *agr*-knockout cells.[172] Furthermore, when RNA III expression is maximal, during the postexponential phase of bacterial growth, SpA production is inhibited (Figure 8).[168]

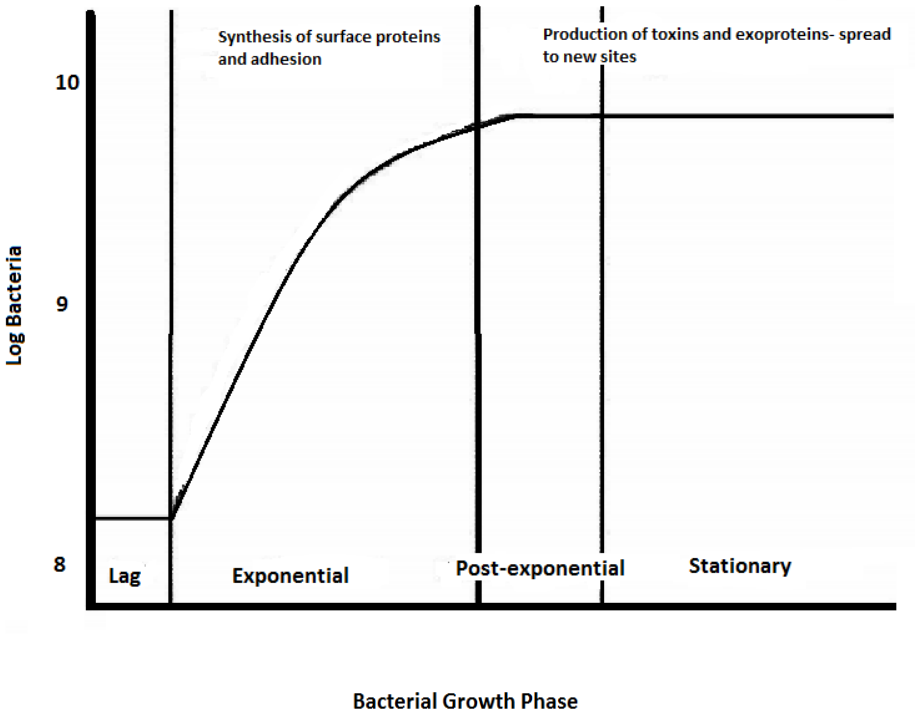


Figure 8- Diagram describing the growth phases (x axis) of *S. aureus* to bacteria number (y axis), and type of virulence factor production. Adapted from Harris et al.[12]

The control of virulence factor expression is related to the stages of *S. aureus* infection. In acute infection, with high bacterial cell density and greater nutritional need, *agr* promotes the release of toxins and digestive enzymes.[171] In initial colonisation, or chronic, low-lying infection, the *agr* system is suppressed, which in turn promotes the expression of SpA and other surface proteins.[173] This is evidence of a further adaptive response by *S. aureus* to its environment, in order to promote ongoing infection, as surface proteins are primarily involved in adherence to host tissues and biofilm formation. In the case of SpA, increased production during times of low bacterial load may correlate to its primary role in immune evasion, allowing *S. aureus* to survive as a commensal organism.[144]

1.10 Processing and excretion of Protein A

The excretion of SpA from the body is known to involve the formation and processing of SpA-IgG complexes; a study conducted using radio-labelled SpA-IgG complexes injected into rabbits showed maximal uptake of complexes by the liver and spleen.[174] This indicates that SpA is first sequestered and metabolised by the body before excretion from the urine.

Studies into the processing and excretion of naturally occurring SpA, released into the body by infection with *S. aureus*, are rare in the literature, but the potential use of SpA as a therapeutic agent has provided animal and human pharmacokinetic studies. In a study of IV SpA administration in Cynomolgus monkeys showed that a weekly dosage of 100µg/kg SpA is non-toxic and well tolerated, and clearance of purified SpA from plasma is rapid following administration.[175] Of particular note, the presence of anti-SpA antibodies was associated with quicker excretion, even after first dose, implying that there exists a pre-formed immune response in certain animals.

Tests of SpA pharmacokinetics in human subjects are rare, however, following successful animal trials, the potential for immune modulation of SpA in autoimmune disease has prompted clinical trials. A Phase I human trial was undertaken in 2013, involving 20 healthy subjects given single doses of SpA, at either 0.3µg/kg and 0.45µg/kg.[176] Generally, the doses were well tolerated by subjects, and complete clearance of SpA had occurred by 96 hours.[176] Immunological studies showed an anti-SpA antibody response in 63% of all subjects tested, and that antibody-positive patients showed increased frequency of SpA clearance. This provides further evidence that formation of anti-SpA antibodies is an important step in the excretion of SpA from the body, but not essential. This rapid clearance does not require sequestration and breakdown in the liver or spleen, as other Staphylococcal bacterial toxins found bound to antibodies in human serum, such as SEB, are cleared within 24 hours via the kidneys.[177] Other animal studies have located an accumulation of Staphylococcal exotoxins TSST-1 and SEB in rat renal tissue, from inoculated burn sites, proving that the processing and excretion of toxins from primary infection sites involve the kidneys.[178] Studies have yet to investigate whether SpA is found in the kidneys or urine during excretion, providing an area of opportunity for research.

1.10.1 Detection of naturally occurring Protein A in human samples

SpA has been used for the precipitation and purification of antibodies from serum, culture supernatants and other solutions, since its discovery, and this is usually achieved through the use of SpA-affinity columns.[179, 180] Detection of SpA by immunoassay was first achieved in order to detect SpA contaminants from commercially sourced antibody products.[181] Optimisation of ELISA methods for detection of SpA in commercial antibody products has produced techniques capable of detecting 0.2ng/ml SpA,[182] though despite this, attempts to

apply these tests to the detection of SpA in clinical samples are rare in the published literature.

In 1991, Nilsson et al described an ELISA method for the detection of commercial SpA in human serum samples, which had so far not been accomplished.[183] The main complication in detecting SpA in serum samples was the presence of IgG in serum; this was theorised to interfere with the immunoassay by competitively binding to SpA, and previous studies have demonstrated this effect by adding IgG to purified SpA samples, and producing a quenching effect on ELISA.[184] Only after boiling SpA-spiked serum samples was detection possible, implying that IgG-SpA complexes in serum could be broken by boiling. Using this preparation method, detection was possible down to a level of 5ng/ml, compared to 0.5ng/ml in buffer, implying that sensitivity of SpA detection is still reduced in serum even after boiling samples.

Endogenous SpA, produced during the course of natural *S. aureus* infection, has been detecting patient serum samples by ELISA. This was done by Steindl et al, using the method of boiling patient samples as outlined by Nilsson et al; boiling (and dilution) was again demonstrated to be the optimal method of preparing samples and dissociating SpA-antibody complexes.[185] Patients positive for SpA in this study were known to be infected with *S. aureus*, though the amount of SpA detected was small and varied between patients, from 0.25ng/ml to 7.9ng/ml.[185]

A different method of SpA testing, the detection of host antibodies raised against SpA in serum, has also been achieved through ELISA. This was developed by Greenberg et al, using SpA as capture antigen and saturating Fc binding sites with human IgG Fc fragments, prior to incubation in human serum samples and final detection using an anti-IgG (Fab-region specific) antibody.[186] Samples tested were from patients with confirmed *S. aureus* endocarditis, and results showed that the ELISA was able to distinguish between endocarditis patients and controls, but the test displayed a low sensitivity (52%) and specificity (48%) for distinguishing between the two groups. Further work into immunoassays for detecting antibody responses to SpA in human samples are currently lacking.

1.11 Summary

Staphylococcus aureus is a Gram-positive bacterium capable of causing life-threatening infection in humans, and is aided by a wide range of virulence factors which directly damage the host, and evade host defences. Peptidoglycan-anchored surface proteins allow *S. aureus* to project virulence factors from the cell wall; the first discovered surface protein, SpA, can also be found free in culture medium. SpA has a number of functions, the most important of which is the ability to bind host antibodies by both the Fc and Fab regions, providing *S. aureus* with innate and adaptive immune system evasion.

The detection of endogenous SpA in *S. aureus* infection has not been achieved in patient urine samples, though the detection of antibodies against SpA in human serum has been demonstrated. As such, the presence of SpA in human urine samples has not been assessed as a potential antigen test for *S. aureus* infection.

The emergence, and continual adaptation of, antibiotic-resistant strains of *S. aureus* continues to make *S. aureus* infection a serious consequence of ICU admission. Correct and timely treatment with antibiotics is essential for a good outcome from *S. aureus* bloodstream infection, and blood-culture diagnosis of *S. aureus* infection can be a slow process. Rapid detection of bacterial infection through antigen testing has been previously achieved for other pathogens, and has allowed rapid detection of infection through non-invasive means such as urine testing. Development of a sensitive and specific urinary antigen test for *S. aureus*, with SpA as the antigen, would allow optimal antibiotic treatment to commence sooner, improving the management of *S. aureus* infection.

1.12 Aims

This study aims to optimise and develop the work of Yue Han (MSc 2014),[187] in detecting SpA in ICU patient urine samples by Western Blot methods. Other aims include the development of an enzyme-linked immunosorbent assay (ELISA), based on the methods established by Frances Price (MSc 2013),[80] for the detection of commercial SpA and SpA in ICU patient urine. Mass Spectrometry will be utilized to detect SpA in any appropriately sized bands seen on SDS-PAGE gels, using a unique method for detecting *S. aureus* toxins by MS. The overall aim of this work is to assess the suitability of SpA as a urinary biomarker for *S. aureus* infection in ICU patients.

2 Materials and Methods

2.1 Materials:

2.1.1 Western Blot materials:

2.1.1.1 Material for SDS-PAGE gels:

- Mini-PROTEAN Tetra Cell Gel kits (BioRad- 165-8000)
- Mini-PROTEAN Glass Plates (BioRad- 1653311)
- Mini-PROTEAN Short Plates (BioRad- 1653308)
- Criterion Blotter Transfer Packs (BioRad- 170-4070)
- Acrylamide 30% Ultra-Pure Protogel (National Diagnostics- EC-890)
- N'N'N'N' Tetramethylethylenediamine (TEMED)- (Sigma- T9281)
- Ammonium Persulphate (APS) powder (Sigma- A3678)
- Sodium Dodecyl Sulphate (SDS) powder (Fisher Scientific- S/5200/53)
- TRIS-Glycine-SDS PAGE buffer (Geneflow, Staffordshire, UK- B9-0034)

2.1.1.2 Antigens

- Protein A, purified from *S. aureus* cell culture (Sigma- P6031)
- Alpha-haemolysin from *S. aureus* (Sigma- H9395)
- Staphylococcal Enterotoxin B (SEB) from *S. aureus* (Toxin Technology- BT202red)
- Toxic Shock Syndrome Toxin 1 (TSST-1) from *S. aureus* (Toxin Technology- TT606)
- Immunoglobulin G from human serum (Sigma 56834)

2.1.1.3 Antibodies:

- Anti-Protein A antibody- polyclonal chicken IgY HRP-conjugated (Abcam- ab18596)
- Anti-human IgG antibody- polyclonal rabbit IgG HRP-conjugated (Biotin- ab6758)

2.1.1.4 Protein Standards for Western Blot

- Protein Mixture ladder (GE Healthcare- 17044601)
- Precision Plus Protein Dual Colour Standards (BioRad- 161-0374)
- Kaleidoscope Prestained Standards (BioRad- 161-0324)

2.1.1.5 Washing and blocking materials:

- Phosphate-Buffered Saline Tablets (Sigma- P4417- 100 tablets)
- TWEEN® 20 (Sigma-Aldrich- P1379 500ml)
- Milk- dried milk powder (Marvel original)
- Bovine Serum Albumin, lyophilized powder (Sigma- A7906)

2.1.1.6 ECL Substrate:

- P-Coumaric Acid powder (Sigma- C9008).
- Luminol powder (Sigma- A8511).

2.1.1.7 Membranes:

- Immobilin-P PVDF Membranes (Merck- PHV00010)

2.1.2 Mass Spectrometry Materials:

2.1.2.1 Sample preparation:

- Acetonitrile (Sigma- 494445)
- Dithiothreitol (DTT)- (Melford- MB1015)
- Trypsin Singles Proteonomics Grade (Sigma- T7200)
- Trypsin Reaction Buffer (Sigma- R3527)
- Trypsin Solubilising Agent (SigmaT2073)
- Coomassie Brilliant Blue R (powder)- (Thermo-Fischer Scientific- 20278)

2.1.2.2 ELISA Materials:

- TMB Microwell Peroxidase Substrate- SureBlue™ Reserve (KPL- 53-00-00)
- Human serum from male AB plasma (Sigma- H4522- 20ml)
- Gelatine, from cold water fish (Sigma- G7765- 250ml)
- Carbonate-Bicarbonate capsules (Sigma- C3041- 100 capsules)

2.1.2.3 Bradford Assay Materials:

- Brilliant Blue Protein Reagent (Sigma- B5809- 100ml)

2.2 Methods:

2.2.1 Western Blot for Protein A samples in control and sample urine

2.2.1.1 Making polyacrylamide gels for SDS-PAGE electrophoresis

Initially, front and backing gel plates were fixed with plastic gel holders, with 1mm space between. Acrylamide gels were prepared separately as stacking and resolving gels, with varying amounts of acrylamide. A solution of 12% acrylamide resolving gel (6.3ml dH_20, 5ml 1.5M Tris HCl pH8.8, 8.3ml 30% (w/v) acrylamide, 200µl 10% (w/v) APS, 200µl 10% (w/v) SDS and 20µl TEMED- sufficient for 4 gels) was prepared in a test tube and pipetted into the space between the Mini-PROTEAN glass and short gel plates, leaving a 2cm space at the top. A small amount of isopropanol was pipetted onto the top of the resolving gel to remove bubbles. After allowing 30 minutes for the gel to set, the isopropanol was washed off with dH_20. Stacking gel (7.66ml dH_20, 1.26ml 1M Tris HCl pH 6.8, 1ml 30% (w/v) acrylamide, 0.5ml 10% (w/v) APS, 100µl 10% (w/v) SDS and 10µl TEMED- sufficient for 4 gels) was then pipetted in the remaining space to the top of the front gel plate. A 10-well comb was then immediately placed into the gel, and the combined gels were allowed to set for 30 minutes.

2.2.1.2 Preparing and loading samples

Included in Western Blots was a protein mixture used as a molecular weight protein ladder (see materials). SpA samples were prepared (1mg/ml) in either boiled control urine or Phosphate Buffered Saline (PBS), and dilutions for analysis prepared from that stock. Patient urine samples were defrosted, and then vortexed and warmed at 36^0Cto reconstitute any material in the sample which had fallen out of solution. In general, all samples and controls were mixed with loading buffer (1.15M TRIS, 10% (w/v) SDS, 0.08M DTT, 20% (v/v) glycerol, 2.5% (w/v) bromophenol blue in 25ml total volume of dH2O) at a ratio of 2/3 sample and 1/3 loading buffer. In the case of a sample being only used to load a single well, this used 8µl of sample and 4µl of loading buffer. Samples mixed with LB were vortexed and heated at 98^0C for 3 minutes. Following this, they were centrifuged for 1 minute in a bench-top centrifuge at 10,000g, to collect any sample which may have been present on the sides and lid of the microfuge tubes.

To load the gels, firstly the 10-well comb was removed from the stacking gel, and the combined plates were fitted into the Mini-PROTEAN Tetra cell system and then placed in the running tank. Tris-Glycine-SDS PAGE buffer was poured into the central reservoir and allowed to fill the wells. An aliquot 10µl of each sample was pipetted into each well.

Once loaded, the surrounding tank was filled with Tris-Glycine-SDS PAGE buffer, and electrophoresis performed at 140v for 1.5 hours. Following this, the gels were removed from the glass plates ready for transfer.

2.2.1.3 Transferring to Polyvinylidene fluoride (PVDF) membrane

The gels were transferred using the 'wet' transfer method, using membranes and filter papers soaked in transfer buffer (20mM Tris, 150mM Glycine and 20% methanol). First, PVDF membranes (Immobilion P, Millipore, UK) were cut to a size of 6cm x 9cm, then equalised by placing in pure methanol for 10 seconds, dH_20 for 2 minutes and finally transfer buffer for at least 20 minutes. The Criterion transfer pack was formed as a 'sandwich' made by stacking the following layers from bottom to top; 4 pieces of filter paper, gel, PVDF membrane and finally 4 more pieces of filter paper. The transfer packs were closed and put into a tank of transfer buffer, with an ice pack and magnetic stirrer, in order to keep the gels cool during the transfer period. An electric current (115v/700mA) was then passed from the back to the front of the tank, for 1.5 hours.

2.2.1.4 Washing and blocking

Following transfer, the membranes were removed from the transfer pack and placed in PBS-0.1% (v/v) Tween 20 (PBST), and washed on the rotary agitator at RT for 1 minute. Following this, the PBST was removed and 20ml of blocking buffer was applied; blocks used were 5% (w/v) skimmed milk, 2% (w/v) BSA or 5% (w/v) BSA. Membranes were incubated in the selected blocking solution on a rotary agitator overnight in a cold room, or for 1 hour at RT.

2.2.1.5 Incubation in antibody

The blocking solution was removed and the membranes were washed with PBS- 0.1% (w/v) Tween 20 for 1 minute, then twice more, each for a further 15 minutes. A solution of primary antibody was diluted in either 20ml 5% (w/v) milk or 2% (w/v) BSA, and applied to the membranes. Antibody concentrations used ranged from 1:500 to 1:10000. The membranes were incubated in primary antibody for 1.5 hours on a rotary agitator at RT.

2.2.1.6 Washing and Western Blot image development

Following incubation in primary antibody, the antibody solution was poured off and the membranes were washed in PBST, 3 times, for 15 minutes each. This allows final removal of non-specific or HRP-conjugated antibody displaying on development of the image. Electro-chemiluminescence substrate (ECL), comprising 1M Tris HCl pH 8.0 solution, Luminol and p-Coumaric acid (see materials), was prepared immediately prior to use, and 2-3ml was applied to each membrane. Following incubation for 1 minute, they were placed on a piece of transparent film and images were saved on a Chemidoc image reader (BioRad), using the software Image Lab (BioRad).

After viewing, membranes were transferred to a separate plate for staining with amido black (125ml dH_20, 100ml methanol, 25ml glacial acetic acid and 0.1% (w/v) Amido Black)solution, which was applied for 3 minutes at RT. They were then washed in de-stain solution (50% (v/v) dH_20, 40% (v/v)methanol and 10% (v/v) glacial acetic acid) 3 times, for 15-20 minutes, to allow for optimal viewing of bands. They were then photographed by the Chemidoc, using Brightfield settings within the Image Lab software.

2.2.2 Mass Spectrometry

2.2.2.1 SDS-PAGE gel electrophoresis and gel staining

Samples to be examined were subject to polyacrylamide gel electrophoresis, prepared as described above. Samples were prepared in the same 1/3rd ratio of loading buffer to sample, as described above for WB sample preparation, and 10µl of each were loaded in a 10-well gel and electrophoresis was performed for 1.5 hours at 140v. Following this, the gels were placed in 10ml of Brilliant Blue^{TM} Coomassie Stain (50% dH_20, 40% methanol, 10% glacial acetic acid and 0.25% (w/v) Coomassie Brilliant Blue^{TM}), for 1 hour or overnight, on an orbital mixer.

Following staining, the gels were washed for 10 minutes in destain solution (described above), and fresh destain was added every hour for 3-4 hours. The gels were rehydrated in 20ml of dH_20 overnight.

2.2.2.2 Sample Preparation

The gels were transferred to transparent PVC, where the regions to be prepared for analysis by Mass Spectrometry were identified. Prior to sample preparation, the gels were viewed and digital images captured saved using a Chemidoc, on the Coomassie Blue setting within the Image Lab software.

The chosen regions were cut out of the gel using a clean scalpel, and then divided into 1mm by 1mm squares. These were placed into clean Eppendorf microfuge tubes and centrifuged for 10 seconds to ensure their placement at the bottom of the microfuge tube. Then 150µl of Acetonitrile (ACN) was added to each sample, and they were incubated at RT for 30 minutes, with occasional vortexing. Once the gel pieces had shrunk, the ACN was removed, and 50µl of DTT solution (1M DTT in 990µl of 100Mm Ammonium Bicarbonate (ABC) pH 8.5 solution) was added to the gel pieces. The samples were incubated in DTT at 56^0C for 30 minutes. Following this, the DTT solution was removed, 150µl of ACN was reapplied to the gel pieces and they were incubated at RT for 30 minutes.

The ACN was then removed and 50µl of Iodoacetamide (10mg of Iodoacetamide in 100µl of ABC) was applied to each sample and the gel pieces kept at RT and in a dark environment for 20 minutes. The Iodoacetamide was removed and a further 150µl of ACN reapplied for 30 minutes at RT.

2.2.2.3 Trypsin Digestion

Trypsin was prepared by adding 45ul of trypsin reaction buffer and 5ul of trypsin solubilising agent to each trypsin vial. Following removal of the ACN from each sample, 25µl of this trypsin mixture was added, briefly mixed and kept at 4^0C for 30 minutes. Following this, a further 30µl of trypsin reaction buffer was added to each sample, briefly mixed and then incubated overnight (minimum 18 hours) in a 37^0C incubator.

Following overnight trypsin digestion, the trypsin was removed by pipette, and the peptides were extracted from the gel pieces with 110µl of extraction buffer (1:2 mixture of 5% Formic Acid and ACN). These were incubated at 37^0C for 20 minutes, before the extract was transferred to fresh microfuge tubes, with 4 holes cut into the lid. These were placed into a freeze-dryer, and set at a cycle of 1 hour freezing, 16 hours of drying and 1 hour final drying. The resulting samples were stored at -20^0C until use.

2.2.2.4 LC-MS Sample Processing

Freeze-dried samples were reconstituted with 20µl of 0.1% trifluoroacetic acid (TFA), before being placed in an ultra-sonicating bath for 5 minutes and centrifuged for 1 minute. Then, 20µl of reconstituted sample was transferred to micro-vials and placed within the Ultimate 3000 (LC Packings™ a Dionex™ Company) autosampler trays. Prior to sample acquisition, the computer software Esquire Control™ and Chromeleon Express™ were used to calibrate liquid chromatography settings, to a loading flow of 0.25µl/s and a voltage of ~150v. Sample was subject to ionising spray, followed by mass spectrometry analysis, for 110 minutes.

Following acquisition of data, these data were analysed using the programmes Bruker Daltronics™ and Biotools™ software, followed by hit searches using the online Mascot™ tool. Online search parameters were used to restrict results to Eurkaryotic bacteria in certain searches.

2.2.3 Enzyme-linked immunosorbent assay (ELISA) for the detection of Protein A in urine samples

An ELISA method was initially developed by Harrison et al for the detection of Staphylococcal exotoxin in infant urine samples,[79] and further optimised by Frances Price (MSc 2013),[80] for the detection of exotoxin in the same urine samples which are the subject of investigation in this study. Initial development of a sandwich ELISA for the detection of SpA was based on these methods, using IgG as a capture antibody.

Microwell plates (Nunc-Immuno F96 Maxisorp, Thermo Scientific, Hemel Hempstead, UK) were coated with 0.5µg/ml IgG in coating buffer (0.1M carbonate-bicarbonate, pH 9.6, Sigma-Aldrich) , at a volume of 100µl per well. Plates were incubated in IgG overnight, at 4^0C.

The following morning, plates were emptied and washed four times with 200µl/well PBS-0.05% (v/v) Tween 20, using a multi-channel pipette. Following each wash the wells were emptied by inverting the plates directly and tapping downward on blue roll. Following this, 200µl/well 5% (w/v) milk or 2.5% (w/v) fish gelatine was added to the plates and incubated for 1 hour at 37^0C.

Following the blocking stage, plates were washed, as described above. SpA standards, in either PBS or control urine, and patient samples were then added to the plate, 100µl/well, and plates were incubated for 1 hour at 37^0C.

Following another wash phase, 100µl of polyclonal chicken IgY anti-SpA antibody, 1:4000 or 1:8000 in blocking buffer, were added to each well and the plates incubated for 1 hour at 37^0C, then washed as previously described.

To develop the plates, 100µl of substrate (TMB Microwell Peroxidase, KPL, Gaithersburg, MD) was added to each well, and the reaction allowed to proceed for 10 minutes, before being stopped by the addition of 100µl/well of stopping solution (0.3M H_2SO_4).

The absorbance of each well was measured at 450nm, using a Wallace Victor Plate Reader (Perkin Elmer, Buckinghamshire, UK).

2.2.4 Protein Assay for total protein estimation in patient urine samples

2.2.4.1 Preparing protein standards

Total protein was measured in patient samples using an assay measuring the absorbance of samples following application of a Coomassie Brilliant Blue assay solution, as compared to a blank standard. A blank standard was produced by adding 50µl of NaCl solution to 2.5ml of Protein Assay Standard (PAS). The blank was measured for absorbance by placing 1ml of the standard in a cuvette and measured at 595nm using an Eppendorf™ BioPhotometer. This produced a calibrated absorbance of 0.000. A standard solution of 300mg/ml BSA was prepared in dH_20. A Protein Assay Solution (PAS) was made by combining Coomassie Brilliant Blue R with dH_20 in a mixture of 1 to 4. A volume of 50µl of 300mg/ml BSA solution was added to 2.5ml PAS, to give a final concentration of 6mg/ml, and left to react for 2 minutes. A volume of 1.25ml of this solution was added to 1.25ml of fresh PAS, in order to divide the protein concentration by half. This solution was again left for 2 minutes. This process was repeated 12 times in order to generate a protein curve down to a concentration of 0.073mg/ml. These standards where then measured in the same way as the blank, in order to produce a curve.

2.2.4.2 Testing patient samples for total protein

Once the protein curve was established, patient samples were created by adding 50µl of undiluted patient sample to 2.5ml of PAS. This was left to stand for 2 minutes and measured in the same way as the standard protein samples.

2.3 Patient sample collection and characteristics

Patient samples from 47 patients were collected from the Lancaster Royal Infirmary ICU ward, by a previous MSc student,[80] in 2012-2013, over an inpatient period of at least 48 hours. Aims of collection were to acquire 8 samples per patient, at intervals of 4 hours, giving a full inpatient sample range. The average sample number per patient was 7, with some patients providing less than the required number. This was due to a failure to collect urine, lack of urine production by the patient, or death of the patient during admission. Samples were collected from the patient catheter port, and placed immediately into sterile containers at 4^0C. They were then stored at -80^0C, until use.

With each patient sample range, demographic, clinical and biochemical data were collected into a database. Patient age and diagnosis at admission and prognosis (death during admission) are summarised in the appendix.

2.3.1 Statistical Analysis

Statistical analysis, based upon the findings of this primary research and the pre-existing clinical database, was performed with the statistical program Stata (Edition 12- StataCorp). The statistical tests performed on these data comprised of:

- Student's T-test- for continuous data
- Logistic regression- for categorical data

2.4 Ethical Approval

This study received a favourable opinion from Greater Manchester South Ethics Committee, with reference: 12/NW/0783.

An amendment to the above approval was required to allow research to be carried out by successive students on patient urine samples; amendment reference number 12/NW/0136, amendment number 2.

3 Results

3.1 Method Development- Western Blot for commercial Protein A

Initial method development focused on the detection of commercial SpA by Western Blot (WB), using primary HRP-conjugated anti-SpA antibody. The basis for the WB technique was that developed for the detection of *S. aureus* exotoxin by Frances et al, which was to use SDS-PAGE electrophoresis to separate the proteins in patient urine samples and probe with toxin specific antibody.[80] To ensure the positive identification of SpA, the WB method would first have to be capable of detecting small amounts of the target protein in standard samples. The detection of commercially purified SpA by WB had previously been performed by Yue Han (MSc 2014),[187] however, that work achieved this by using high amounts of antibody which proved to be highly sensitive for SpA, but displayed high background. The initial aim of our WB method development was to ensure that the test was both sensitive and specific for biologically relevant levels of SpA, to measure and confirm the standard MW of commercial SpA, and to determine the ideal antibody concentration and blocking solutions for testing patient samples for endogenous SpA.

3.1.1 Western Blot to detect commercial SpA in PBS and control urine

The detection of commercial SpA by WB was confirmed in control urine, at concentrations starting at 10µg/ml, and decreasing by 10 times dilution to 0.001µg/ml (Figure 10--13). Primary antibody was used at high concentrations (1:500 and 1:1000), and suspended in both 5% (w/v) milk and 2% (w/v) BSA. These concentrations of antibody were those used by Yue Han (MSc 2014) in the screening of patient samples for SpA, so were seen as an appropriate baseline for WB detection (Figure 9-12).[187]

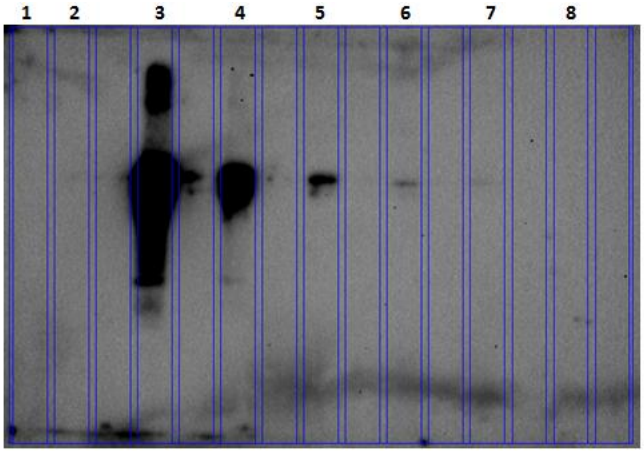


Figure 9- Western Blot of SpA controls of varying concentrations constituted in control urine, using a 1:500 concentration anti-SpA antibody constituted in 5% (w/v) milk. Standards of SpA are electrophoresed on SDS-PAGE gel, before being transferred to membrane as per protocol. Membrane blocked in 5% (w/v) milk, and incubated in 1:500 concentration of anti-SpA antibody, constituted in 5% (w/v) milk. Ladder proteins are not visible.

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Sample	Ladder	Control Urine	10µg/ml SpA	1µg/ml SpA	0.1µg/ml SpA	0.01µg/ml SpA	0.001µg/ml SpA	0.0001µg/ml SpA

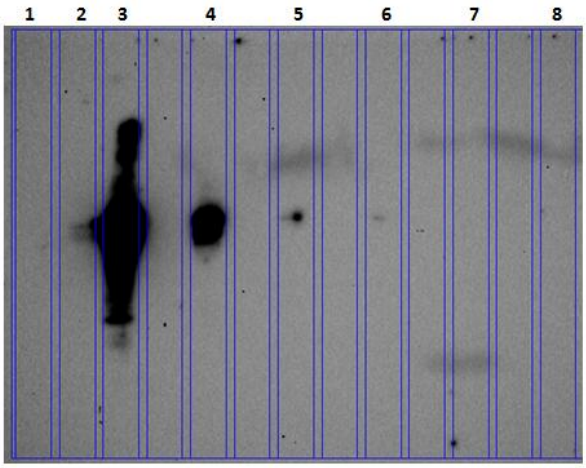


Figure 10- Western Blot of SpA controls of varying concentrations constituted in control urine, using a 1:1000 concentration anti-SpA antibody constituted in 5% (w/v) milk. Standards of SpA are electrophoresed on SDS-PAGE gel, before being transferred to membrane as per protocol. Membrane blocked in 5% (w/v) milk, and incubated in 1:1000 concentration of anti-SpA antibody, constituted in 5% (w/v) milk. Ladder proteins are not visible.

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Sample	Ladder	Control Urine	10µg/ml SpA	1µg/ml SpA	0.1µg/ml SpA	0.01µg/ml SpA	0.001µg/ml SpA	0.0001µg/ml SpA

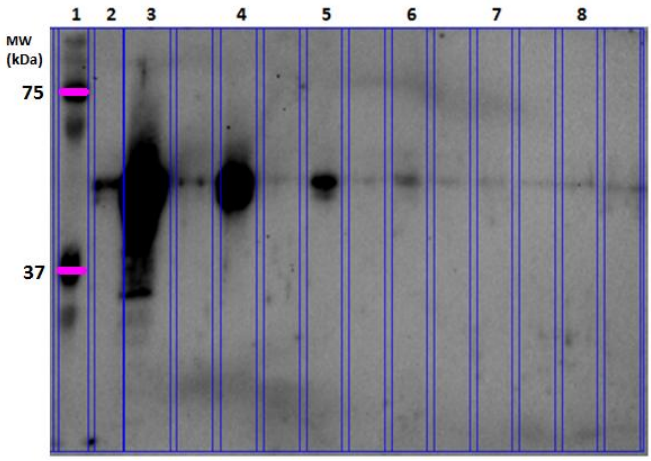


Figure 11- Western Blot of SpA controls of varying concentrations constituted in control urine, using a 1:500 concentration anti-SpA antibody constituted in 2% (w/v) BSA. Standards of SpA are electrophoresed on SDS-PAGE gel, before being transferred to membrane as per protocol. Membrane blocked in 2% (w/v) BSA, and incubated in 1:500 concentration of anti-SpA antibody, constituted in 2% (w/v) BSA.

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Sample	Ladder	Control Urine	10µg/ml SpA	1µg/ml SpA	0.1µg/ml SpA	0.01µg/ml SpA	0.001µg/ml SpA	0.0001µg/ml SpA

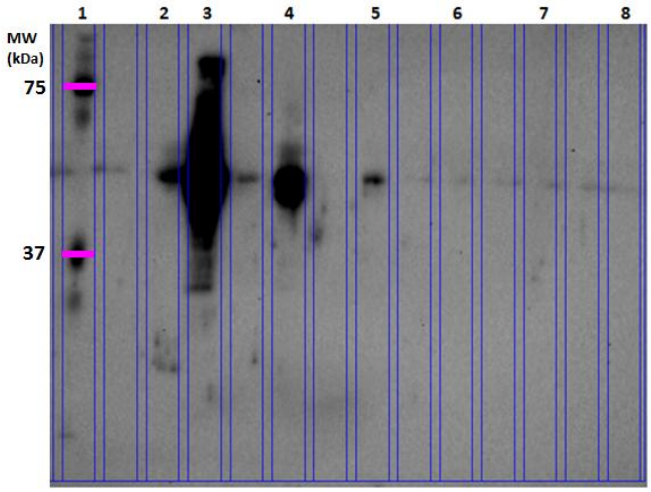


Figure 12- Western Blot of SpA controls of varying concentrations constituted in control urine, using a 1:1000 concentration anti-SpA antibody constituted in 2% (w/v) BSA. Standards of SpA are electrophoresed on SDS-PAGE gel, before being transferred to membrane as per protocol. Membrane blocked in 2% (w/v) BSA, and incubated in 1:1000 concentration of anti-SpA antibody, constituted in 2% (w/v) BSA.

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Sample	Ladder	Control Urine	10µg/ml SpA	1µg/ml SpA	0.1µg/ml SpA	0.01µg/ml SpA	0.001µg/ml SpA	0.0001µg/ml SpA

These initial WBs resulted in a clear and strongly positive detection of SpA at 10µg/ml, 1µg/ml and 0.1µg/ml, with faint and variable detection of a band in the 0.01µg/ml lane. All of these bands were produced from SpA diluted in control urine, confirming the viability of SpA detection in the matrix under investigation in this research.

However, as each of these samples are further diluted by preparation of a 1/3 loading buffer sample at 10µl per well, these initial concentrations are equivalent to approximately 70ng, 7ng and 0.7µg total volume of SpA in each lane. The definitive detection limit was therefore the lowest possible amount detectable by this method under optimal conditions, which was 0.7ng. However, it was recognised that the signal was weak for this amount of SpA, especially at lower antibody concentration, and that the likely condition of patient urine samples did not represent ideal testing conditions for WB. As a 0.01µg/ml SpA solution could not be relied upon as a reliable standard during sample testing, the decision was made to utilise commercial SpA in standards at a concentration of 0.1µg/ml (70ng total SpA).

Healthy control urine, without added SpA, was found to have no positive bands detected by the antibody, proving its suitability as a control lane substance in WB experiments.

At this initial phase, unwanted background and non-specific signal was found to be more effectively diminished by blocking, and incubating antibody, in 2% (w/v) BSA.

Following this, a series of experiments were carried out, which tested the detection limit of the primary anti-SpA antibody, by reducing the concentration for primary antibody to 1:8000, and incubating a serial dilution of SpA in this concentration of antibody (Figure 13). Also included with each WB was a mixture of protein standards (ladder) to provide the MW measurement of SpA.

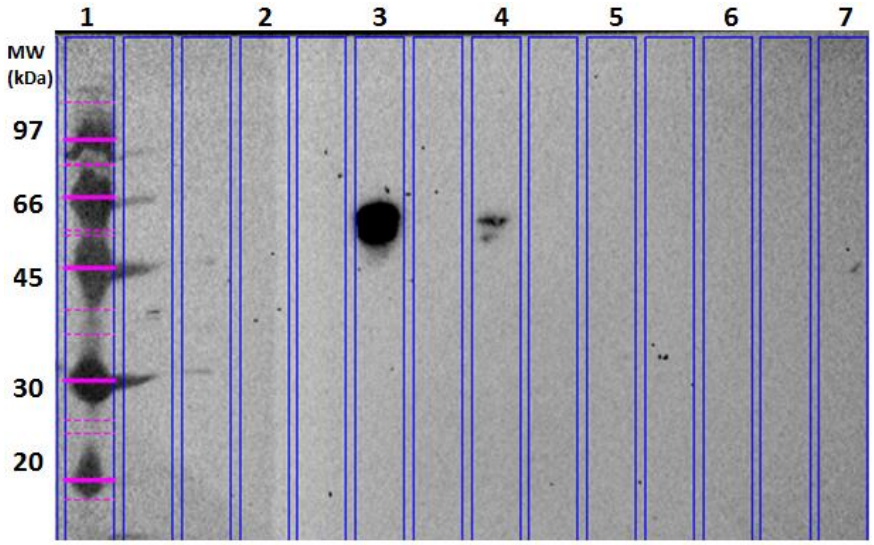


Figure 13- Western Blot of SpA standards constituted in control urine, detected by 1:8000 anti-SpA antibody concentration. Standards were constituted at varied concentrations in control urine, before being loaded into SDS-PAGE gels and being subject to electrophoresis and transferred to membrane as per protocol. Membranes were blocked in 2% (w/v) BSA and then incubated in 1:8000 anti-SpA antibody in 2% (w/v) BSA.

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Sample	Ladder	Control Urine	1µg/ml SpA	0.1µg/ml SpA	0.01µg/ml SpA	0.001µg/ml SpA	0.001µg/ml SpA

Detection of SpA was found to be possible using lower antibody concentrations, down to 1:8000, to reveal the SpA from a 0.1µg/ml solution. This level of detection was achieved in control urine, showing this method was appropriate for the medium in which we were aiming to detect endogenous SpA. Detection of SpA concentrations of 0.01µg/ml or below was only seen when using 1:500 antibody concentration, amounts which caused considerable background and bleaching of patient sample lanes when used by Yue Han (MSc 2014) on patient samples.[187] It was therefore decided that the reliable detection of SpA in 0.1µg/ml solutions, or total SpA of 7ng per sample well, with the lowest concentration of anti-SpA antibody, would be the main outcome of optimising the WB method.

Additional information provided by these blots were the MW of commercial SpA and the appearance of SpA when electrophoresed on SDS-PAGE gels. The MW of SpA was estimated using the BioTools Image Lab software, using protein standards of known weight in order to calculate the estimated MW of SpA bands detected. The MW of SpA was consistent between blots, and was found to run at 50-55 kDa, as opposed to its documented MW of 42kDa, a figure which was provided from the product information . However, this MW is

measured from the Cowen I strain of *S. aureus*, and SpA from different strains display heterogeneity.[114] Our findings are consistent with findings on Western Blots by other studies using SpA from *S. aureus* strain 8325, the strain cultured to obtain the purified SpA used in this study.[114, 117] This MW is evident in the following Western Blot (Figure 14):

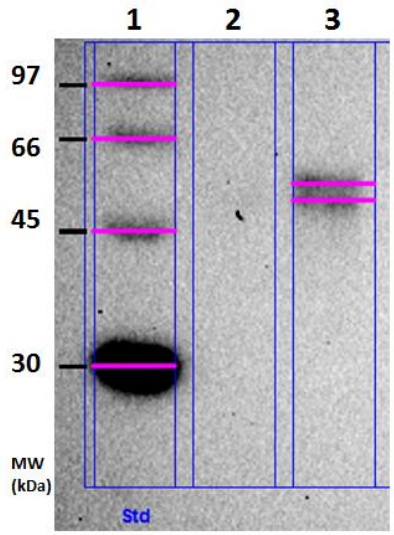


Figure 14- Western Blot- Protein Standards ladder, tested with 0.1µg/ml (7ng total volume) SpA standards. The prepared samples were loaded into SDS-PAGE gel, and electrophoresed and transferred to membrane as per protocol. Membranes were blocked in 2% (w/v) BSA, and incubated in 1:2000 anti-SpA antibody in 2% (w/v) BSA. 1= protein standards ladder, 3= 0.1µg/ml SpA.

Band	**Molecular Weight**
Band 1	54.8
Band 2	51.1

On the WB, SpA consistently displayed the appearance of 2 close bands at the position of ~50-55 kDa. These bands are of similar MW, the higher band appearing to run to ~55kDa and the lower band to 50kDa, and show similar signal strength (a slightly stronger signal on the heavier band was noted in certain experiments). This indicates that both molecules which produce the bands are of similar size and relatively at the same concentration in the commercial SpA standards. At concentrations of 1µg/ml and above, these SpA double bands merge into a single large band. Work by Yue Han (MSc 2014), which used mass spectrometry (MS) to examine samples from the 2 gel bands stained with Coomassie Blue,

found that both these bands match the molecular structure of SpA.[187] It is very likely therefore, that these bands both represent variants of SpA at different MWs, though the reason for the resolution of two differently sized SpA molecules within a commercially produced sample was unknown.

Based on these initial experiments on SpA in control urine, it was revealed that the chosen lowest reliable concentration of SpA, 0.1µg/ml was visualised well at antibody concentrations 1:1000 and 1:2000, and provides a clear view of the SpA double bands without bleaching adjacent lanes (see Figure 15).

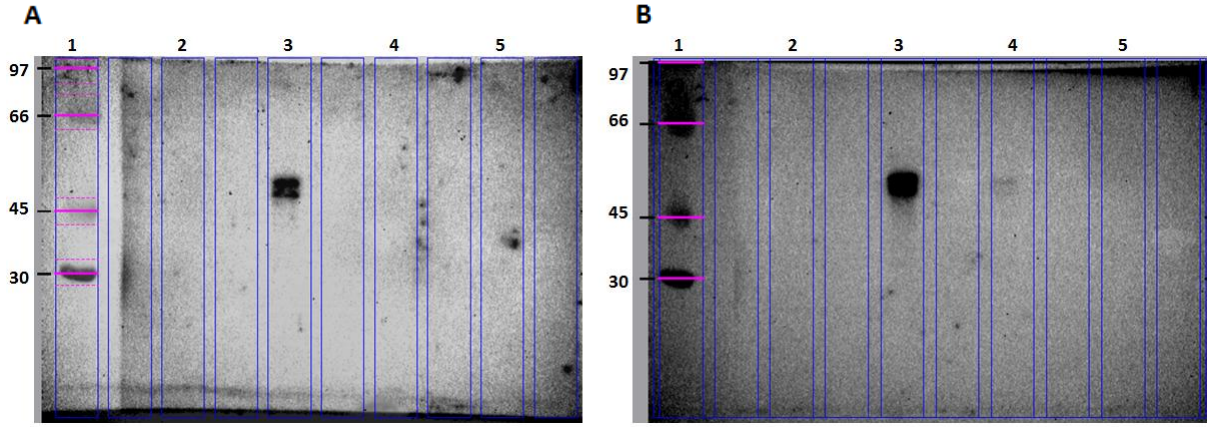


Figure 15- Western Blot of SpA standards, constituted in control urine, and incubated in different antibody concentrations. SpA standards were constituted at a variety of concentrations. The prepared samples were loaded into SDS-PAGE gel, and electrophoresed and transferred to membrane as per protocol. Membranes were blocked in 2% (w/v) BSA, and incubated in 1:1000 concentration anti-SpA antibody (A), and 1:2000 concentration anti-SpA antibody (B). Both antibody concentrations were constituted in 2% (w/v) BSA.

Lane	**1**	**2**	**3**	**4**	**5**
Sample	Ladder	Control Urine	0.1µg/ml SpA	0.01µg/ml SpA	0.001µg/ml SpA

The reliable detection of SpA in control urine samples, when spiked with commercial SpA to a concentration of 0.1µg/ml, was demonstrated. An additional series of blots were done to demonstrate this in both healthy control urine and a sample of ICU patient urine, to a level of 0.1µg/ml and 0.01µg/ml SpA (Figure 16 and 17):

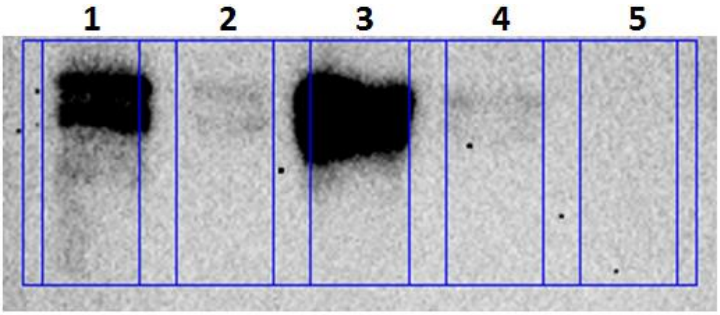


Figure 16- Western Blot of SpA standards (0.1µg/ml - 0.01µg/ml concentration, total volume 7ng – 0.7ng) in control urine and patient urine (Patient 18, sample number 5). Samples were loaded into SDS-PAGE gel, and electrophoresed and transferred to membrane as per protocol. Membranes were blocked in 5% (w/v) milk, and incubated in 1:4000 anti-SpA antibody in 5% (w/v) milk.

Lane	1	2	3	4	5
Sample	0.1µg/ml SpA (control urine)	0.01µg/ml SpA (control urine)	0.1µg/ml SpA (Patient 18 urine)	0.01µg/ml SpA (patient 18 urine)	Patient 18 urine (no SpA)

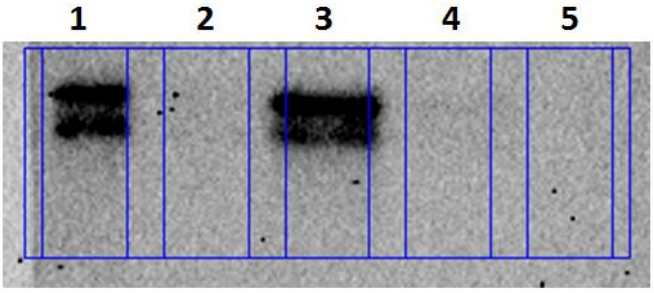


Figure 17- Western Blot of SpA standards (0.1µg/ml - 0.01µg/ml concentration, total volume 7ng – 0.7ng) in control urine and patient urine (Patient 18, sample number 5). Samples were loaded into SDS-PAGE gel, and electrophoresed and transferred to membrane as per protocol. Membranes were blocked in 5% (w/v) milk, and incubated in 1:8000 anti-SpA antibody in 5% (w/v) milk.

Lane	1	2	3	4	5
Sample	0.1µg/ml SpA (control urine)	0.01µg/ml SpA (control urine)	0.1µg/ml SpA (Patient 18 urine)	0.01µg/ml SpA (patient 18 urine)	Patient 18 urine (no SpA)

Following these experiments to assess the limits of sensitivity for the anti-SpA antibody, a WB experiment was performed to investigate the specificity of the anti-SpA antibody in relation to previously tested *S. aureus* toxins by this laboratory.[80] This experiment tested for SpA, SEB, TSST and AH to a standard sample concentration of 1µg/ml, incubated in 1:1000 and 1:2000 anti-SpA antibody. These standards concentrations of Staphylococcal toxins

were based on standard solutions used by Frances Price (MSc 2013),[80] and are significantly higher than the concentrations of toxin found in patient urine in the same research. The antigens were also made in 2 different solutions, PBS and control urine, to find out if the solutions exhibited any differences in SpA signal strength. The subsequent WB shows no cross-reactivity with SEB, TSST and AH at the same concentrations of SpA. SpA at 1µg/ml also shows evidence of attenuated signal when diluted in control urine (Figure 18).

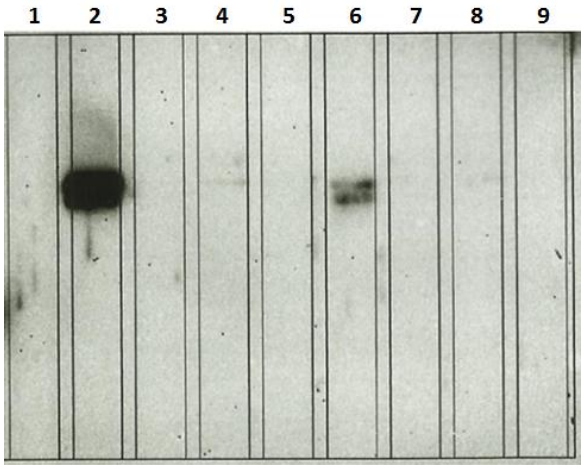


Figure 18- Western Blot of PBS and control urine standards containing SpA, SEB, TSST and AH. Each toxin was prepared in solution to a concentration of 1µg/ml (total volume 70ng). Each prepared sample was loaded into SDS-PAGE gel, and electrophoresed and transferred to membrane as per protocol. Membranes were blocked in 2% (w/v) BSA, and incubated in 1:2000 anti-SpA antibody in 2% (w/v) BSA.

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Load	Control Urine	SpA 1µg/ml	SEB 1µg/ml	TSST 1µg/ml	AH 1µg/ml	SpA 1µg/ml	SEB 1µg/ml	TSST 1µg/ml	AH 1µg/ml

The additional finding of this experiment, that the 1µg/ml concentration SpA shows evidence of signal attenuation when detected in control urine and not PBS, prompted further experiments to investigate this finding. This was repeated when 0.1µg/ml SpA was made with PBS and a variety of healthy control urines, and re-examined together on the same gel. WB techniques also display an increased sensitivity for SPA when it is diluted in PBS, as is shown by the detection of 0.01µg/ml SpA in PBS, but not control urine. These

effects can be seen on the following WB, which utilizes 3 different healthy control urine samples in comparison to PBS samples (Figure 19):

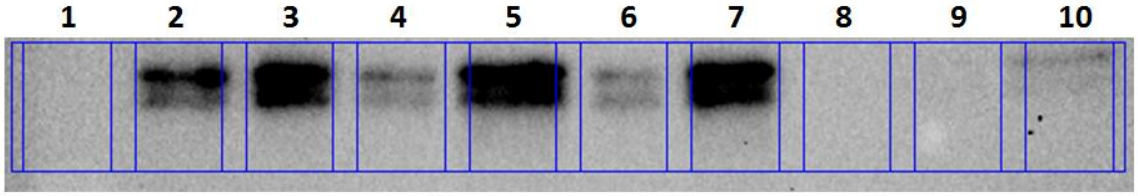


Figure 19- Western Blot of SpA standards prepared with PBS or healthy control urine, loaded intermittently. Each prepared sample was loaded into SDS-PAGE gel, and electrophoresed and transferred to membrane as per protocol. Membranes were blocked in 5% (w/v) milk, and incubated in 1:2000 anti-SpA antibody in 5% (w/v) milk.

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample	Control Urine	0.1µg/ml SpA (CU 1)	0.1µg/ml SpA (PBS)	0.1µg/ml SpA (CU 2)	0.1µg/ml SpA (PBS)	0.1µg/ml SpA (CU 3)	0.1µg/ml SpA (PBS)	0.01µg/ml SpA (CU 1)	0.01µg/ml SpA (CU 2)	0.01µg/ml SpA (PBS)

This blot also demonstrates an increased sensitivity by WB for SpA when made in PBS solution; the final three wells contain 0.01µg/ml SpA, wells 8 and 9 contained this amount in control urine and the final well, which contains a faint double-band, the SpA standard was constituted PBS solution. It was decided to investigate this effect further prior to completing WB method optimisation, in order to determine if the same effect would occur in patient urine samples.

The attenuation of commercial SpA detection appeared to be specific to control urine. IgG is one component known to be present in urine, including healthy samples. Therefore, its strong binding to SpA was hypothesised to be disrupting either the sample preparation, or the binding of anti-SpA in the antibody incubation phase of WB. To test the IgG hypothesis in disrupting commercial SpA detection via WB, an experiment was set up which used only PBS as sample solution, with samples containing SpA alone, and SpA with added human IgG (Figure 20).

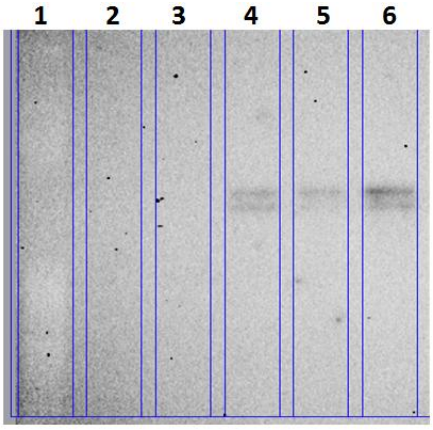


Figure 20- Western Blot of SpA standards in PBS solution, spiked intermittently with Human IgG 10µg/ml. Each prepared sample was loaded into SDS-PAGE gel, and electrophoresed and transferred to membrane as per protocol. Membranes were blocked in 5% (w/v) milk, and incubated in 1:2000 anti-SpA antibody in 5% (w/v) milk. Ladder proteins are not visible.

Lane	**1**	**2**	**3**	**4**	**5**	**6**
Samples	Ladder	10µg/ml IgG	1µg/ml IgG	0.1µg/ ml SpA	0.1µg/ml SpA (+IgG 10µg/ml)	0.1µg/ml SpA

This experiment demonstrates a lack of binding between IgG and anti-SpA antibody, as there are no positive bands seen on WB in the 10µg/ml IgG concentration lane. It also demonstrates a lack of association between signal strength of SpA and IgG contained within the sample solution. To test this theory further, it was decided that prior to WB experiments in patient urine, the control urine and patient urine should be tested directly for human IgG, using WB and incubating in anti-human IgG antibody. The resulting experiment confirmed the fact that both control and patient urine contains IgG, present on WB as heavy and light chains (see text below for approximate MW) (Figure 21).

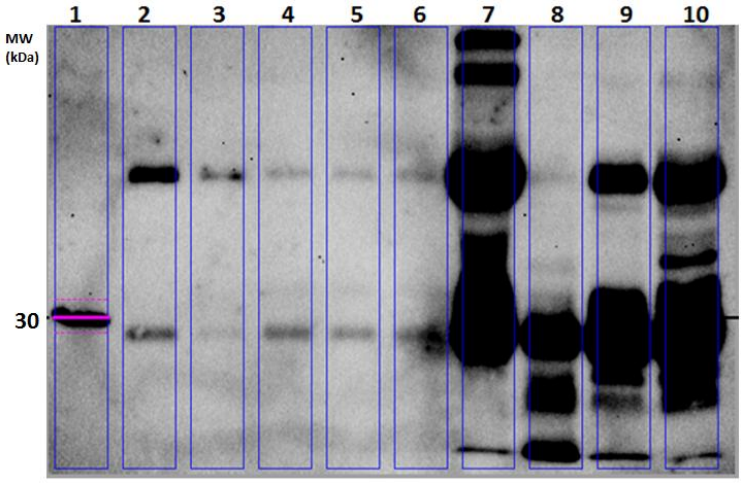


Figure 21- Western Blot of control urine and patient urine samples, identifying IgG with anti-human IgG antibody. IgG spiked standards, control and patient urine samples were prepared and loaded into SDS-PAGE gel, and electrophoresed and transferred to membrane as per protocol. Membranes were blocked in 2% (w/v) BSA, and incubated in 1:4000 anti-human IgG antibody in 2% (w/v) BSA.

Lane	1	2	3	4	5	6	7	8	9	10
Sample	Ladder	10µg/ml IgG (PBS)	1µg/ml IgG (PBS)	Control Urine	Control Urine (with 1µg/ml SpA)	Control Urine (with 0.1µg/ml SpA)	Patient 4 urine	Patient 20 urine	Patient 33 urine	Patient 40 urine

This result is a clear indicator to the large amounts of IgG in patient urine samples, and even small amounts in healthy urine. These correlate to the correct MW as described for human IgG; IgG light chains (F) are ~25kDa, heavy chains (H) are ~50kDa, making a full molecule weight of ~100kDa.[188] As was previously demonstrated, SpA binding in WB is not impaired in patient samples, including the samples used in Figure 14. This is further evidence that the presence of IgG in patient samples does not attenuate the signal of SpA when it is added to those samples and detected by WB. As attenuation did not appear to occur in the testing matrix under investigation (only control urine) this effect was not investigated further, allowing application of the method to patient samples.

3.1.2 Optimising Western Blotting for detection of SpA in patient samples

Following this period of optimising for the detection of commercial SpA by WB, it was decided to further optimise the method, based on the presence of multiple, strong bands seen in patient samples when tested previously by Yue Han (MSc 2014). While we had

determined the optimal antibody concentration to be 1:2000 for detecting commercial SpA, an initial experiment was performed to demonstrate the effect of differences in the primary antibody concentration on patient samples. Of the 45 patients who provided urine samples, we tested the first 5 patients, selecting a sample from the middle of their inpatient period, using primary antibody at a range of concentrations, from 1:1000 to 1:8000, diluted in 2% (w/v) BSA.

A single sample from the middle of each patient series was tested due to previous data by Frances Price (MSc 2013) demonstrating that the middle sample of a series contained higher levels of Staphylococcal toxins. Included in sample testing were SpA standards diluted in both PBS and control urine, due to the potential, recognised, difference in signal strength which may occur in WB. The following series of blots demonstrates the detection of both SpA standards and multiple bands of higher and lower molecular weight and intensity in patient urine, when using anti-SpA in 2% (w/v) BSA (Figure 22- 25).

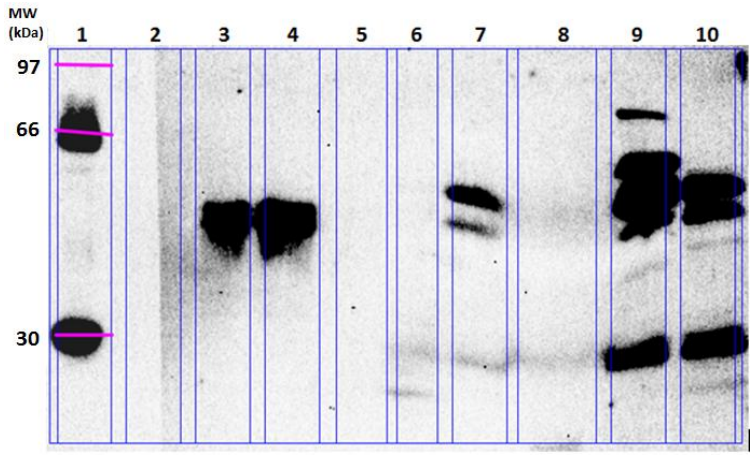


Figure 22- Western Blot of patient 1 – 5 urine samples, undiluted, with 0.1µg/ml SpA (total volume 7ng) standards, tested by 1:1000 concentration of antibody. Included in Western Blot was a protein mixture as MW ladder. Patient samples and standards were electrophoresed in an SDS-PAGE gel and transferred to membrane as per protocol. Membrane was blocked in 2% (w/v) BSA and incubated in 1:1000 concentration anti-SpA antibody, also constituted in 2% (w/v) BSA.

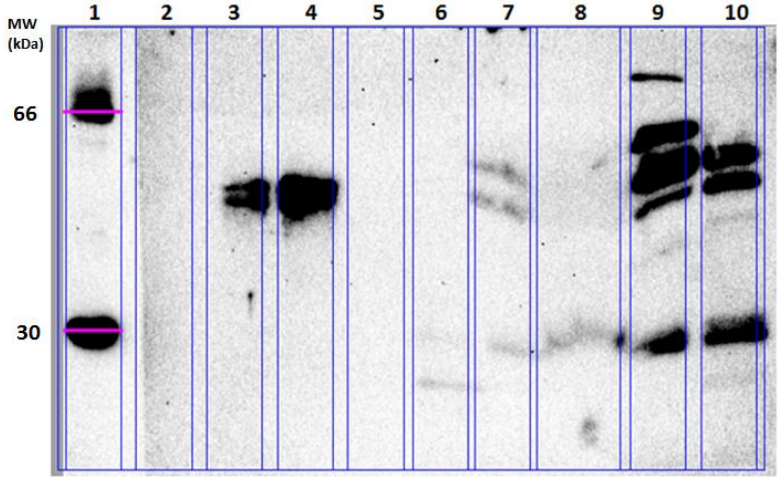


Figure 23- Western Blot of patient 1 – 5 urine samples, undiluted, with 0.1µg/ml SpA (total volume 7ng) standards, tested by 1:2000 concentration of antibody. Included in Western Blot was a protein mixture as MW ladder. Patient samples and standards were electrophoresed in an SDS-PAGE gel and transferred to membrane as per protocol. Membrane was blocked in 2% (w/v) BSA and incubated in 1:2000 concentration anti-SpA antibody, also constituted in 2% (w/v) BSA.

Lane	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample	Ladder	0.1µg/ml SpA (CU)	0.1µg/ml SpA (PBS)	Control Urine	Patient 1 sample 5	Patient 2 sample 6	Patient 3 sample 1	Patient 4 sample 5	Patient 5 sample 5

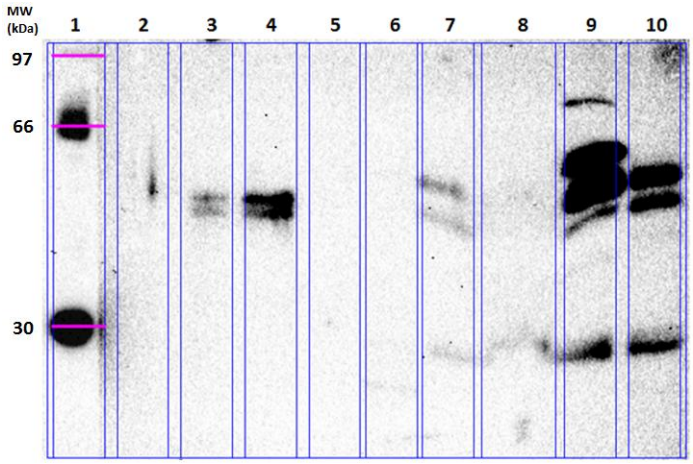


Figure 24- Western Blot of patient 1 – 5 urine samples, undiluted, with 0.1µg/ml SpA (total volume 7ng) standards, tested by 1:4000 concentration of antibody. Included in Western Blot was a protein mixture as MW ladder. Patient samples and standards were electrophoresed in an SDS-PAGE gel and transferred to membrane as per protocol. Membrane was blocked in 2% (w/v) BSA and incubated in 1:4000 concentration anti-SpA antibody, also constituted in 2% (w/v) BSA.

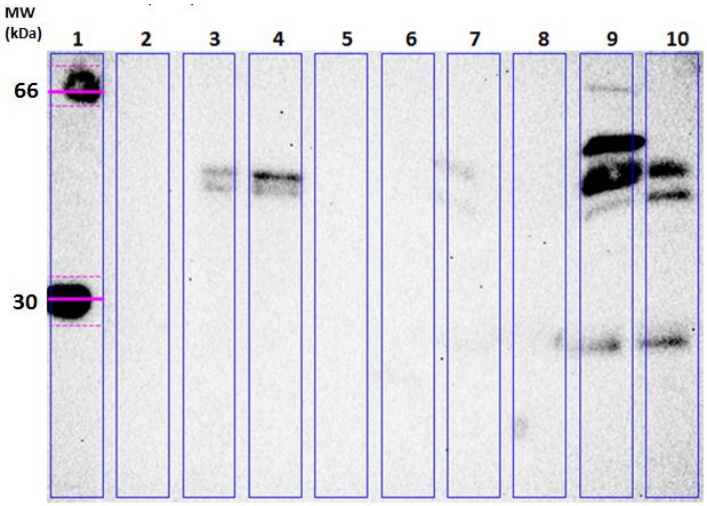


Figure 25- Western Blot of patient 1 – 5 urine samples, undiluted, with 0.1µg/ml SpA (total volume 7ng) standards, tested by 1:8000 concentration of antibody. Included in Western Blot was a protein mixture as MW ladder. Patient samples and standards were electrophoresed in an SDS-PAGE gel and transferred to membrane as per protocol. Membrane was blocked in 2% (w/v) BSA and incubated in 1:8000 concentration anti-SpA antibody, also constituted in 2% (w/v) BSA.

Lane	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample	Ladder	0.1µg/ml SpA (CU)	0.1µg/ml SpA (PBS)	Control Urine	Patient 1 sample 5	Patient 2 sample 6	Patient 3 sample 1	Patient 4 sample 5	Patient 5 sample 5

This series of WB results show that the patient urine samples display a variety of bands, of different molecular weights, and of varying intensity. This can be clearly seen in patient 4, sample 5, with particularly strong bands seen in patient urine at ~60kDa and ~30kDa. Initially, it was thought that these bands may either entirely be due to non-specific binding, i.e. urinary proteins such as albumin exhibiting cross-reactivity with anti-SpA antibodies, or they are variants of SpA at different MWs. A mixture of SpA and non-specific binding was thought to be responsible for the large number of bands seen, so further optimisation work was carried out in order to reduce any non-specific binding on urinary proteins.

The fainter of these additional patient bands are seen to disappear at lower antibody concentrations, as well as lower band intensity for SpA standards, which is to be expected. Following this series of blots, 1:2000 was decided as the most appropriate concentration of antibody for reducing the amount of non-specific binding, while also retaining a strong signal for SpA standards in PBS and control urine.

Sample testing optimisation was continued by diluting patient samples; specifically, to examine whether the dilution of patient urine would reduce the number of additional bands seen on WB, while retaining binding to any endogenous SpA, and so allowing detection, in those samples. Patient samples were loaded undiluted and also diluted with PBS 2, 10, 20 and 100 times. Patients 4, 30 and 33 samples were chosen as the sample volumes associated with these patients were amongst the largest in the series, allowing large amounts of urine to be used during a dilution. Also, patient 4 had previously demonstrated many strong additional bands in WB experiments and so would provide insight into the impact of this approach on the most challenging of patient samples. The subsequent WB results demonstrated significant attenuation in patient band signal when diluted 10 times (Figure 26 - 28).

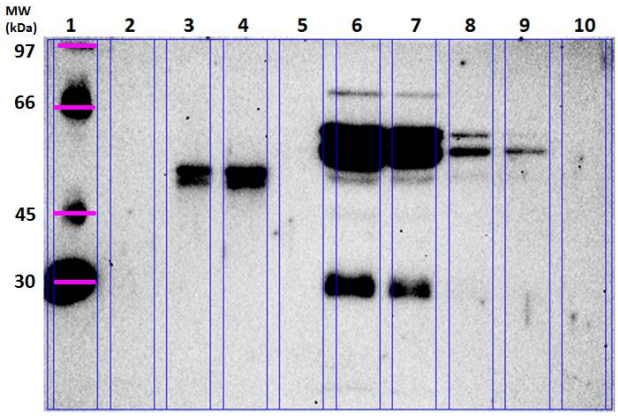


Figure 26- Western Blot of Patient 4, urine sample 5, undiluted and also diluted in PBS; 2, 10, 20 and 100 times. Each prepared sample, and SpA standards (0.1µg/ml concentration, total volume 7ng), was loaded and electrophoresed in SDS-PAGE gel and transferred as per protocol. Membranes were blocked in 2% (w/v) BSA and incubated in 1:2000 anti-SpA antibody (constituted in 2% (w/v) BSA).

Lane	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample	Ladder	0.1µg/ml SpA (CU)	0.1µg/ml SpA (PBS)	Control Urine	Patient 4 sample 5 undiluted	Patient 4 sample 5 (2x dilution)	Patient 4 sample 5 (10x dilution)	Patient 4 sample 5 (20x dilution)	Patient 4 sample 5 (100x dilution)

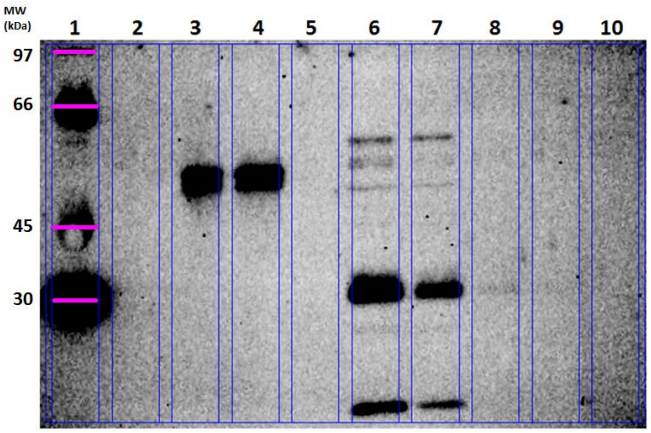


Figure 27- Western Blot of Patient 30, urine sample 5, undiluted and diluted in PBS; 2, 10, 20 and 100 times. Each prepared sample, and SpA standards (0.1µg/ml concentration, total volume 7ng), was loaded and electrophoresed in SDS-PAGE gel and transferred as per protocol. Membranes were blocked in 2% (w/v) BSA and incubated in 1:2000 anti-SpA antibody (constituted in 2% (w/v) BSA).

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample	Ladder	Control Urine	0.1µg/ml SpA (CU)	0.1µg/ml SpA (PBS)	Control Urine	Patient 30 sample 5 undiluted	Patient 30 sample 5 (2x dilution)	Patient 30 sample 5 (10x dilution)	Patient 30 sample 5 (20x dilution)	Patient 30 sample 5 (100x dilution)

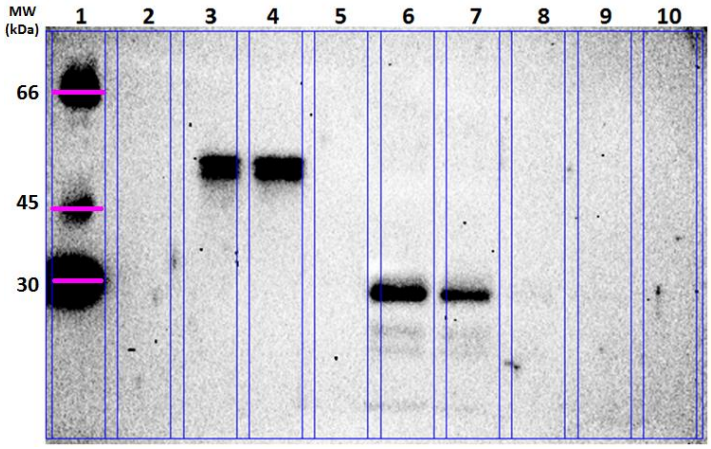


Figure 28- Western Blot of Patient 33, urine sample 5, undiluted and diluted in PBS 2, 10, 20 and 100 times. Each prepared sample, and SpA standards (0.1µg/ml concentration, total volume 7ng), was loaded and electrophoresed in SDS-PAGE gel and transferred as per protocol. Membranes were blocked in 2% (w/v) BSA and incubated in 1:2000 anti-SpA antibody (constituted in 2% (w/v) BSA).

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample	Ladder	Control Urine	0.1µg/ml SpA (CU)	0.1µg/ml SpA (PBS)	Control Urine	Patient 33 sample 5 undiluted	Patient 33 sample 5 (2x dilution)	Patient 33 sample 5 (10x dilution)	Patient 33 sample 5 (20x dilution)	Patient 33 sample 5 (100x dilution)

The results demonstrate the losses of patient band detection as the samples are diluted, losing the majority of band detection at 10 times dilution. More importantly to the detection, no bands of equal size to the SpA controls remain and/or appear once samples are diluted, meaning that these additional bands are also not diminishing the effect of SpA bands in the undiluted samples, at least in patients 4, 30 and 33. As there is no increasing signal seen for any particular band following dilution, it was decided to continue using patient samples undiluted when testing for SpA.

Further challenges exist in the analysis of patient samples, as many contained insoluble material, which may be sequestering proteins in patient sample WBs. Some of this solid matter was in fine grain form, while others were found to exist in the urine as large particles larger than 1mm in size. Before continuing to screen patient samples, we performed an experiment to finalise the optimal method of preparing samples; either mixing and

therefore reconstituting the solid material into the sample, or avoiding it when possible for creating a sample for WB. Patients 30 and 33 had a significant amount of solid material in some samples, in particular samples 3 (patient 30) and sample 5 (patient 33). These samples were each separated into two; one sample contained the pure liquid, which was retrieved by spinning down the solid matter into the bottom of the microfuge tubes in a bench-top centrifuge. The remaining solid parts of the samples were subject to heating and reconstitution, by vortexing, in 100 µl of sterile PBS. Both liquid and solid samples were diluted 2, 10, 20 and 100 times with PBS. These samples were both subject to WB on two separate membranes, incubating in 1:2000 anti-SpA antibody, in 2% (w/v) BSA (Figure 29 and 30):

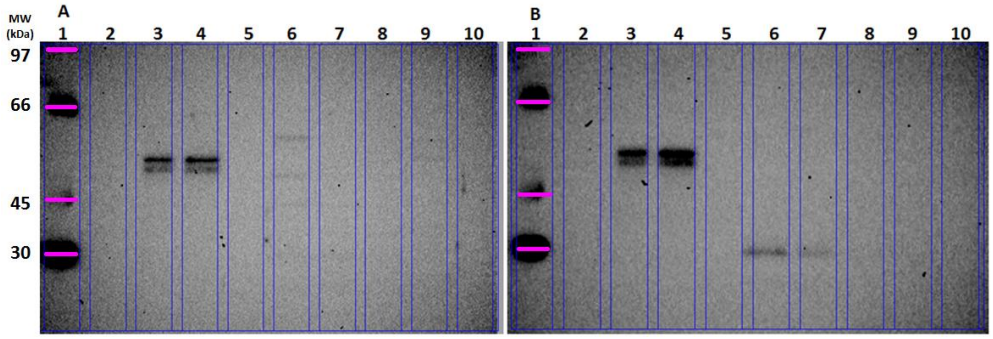


Figure 29- Western Blot of Patients 30 (A) and 33 (B) liquid portion of urine reconstituted in PBS. Each patient urine sample was diluted 2, 10, 20 and 100 times in PBS. Each prepared sample, and SpA standards (0.1µg/ml concentration, total volume 7ng), was loaded and electrophoresed in SDS-PAGE gel and transferred as per protocol. Membranes were blocked in 2% (w/v) BSA and incubated in 1:2000 anti-SpA antibody (constituted in 2% (w/v) BSA).

Lane	**1**	**3**	**4**	**6**	**7**	**8**	**9**	**10**
Sample	Ladder	0.1µg/ml SpA (CU)	0.1µg/ml SpA (PBS)	Patient sample undiluted	Patient sample (2x dilution)	Patient sample (10x dilution)	Patient sample (20x dilution)	Patient sample (100x dilution)

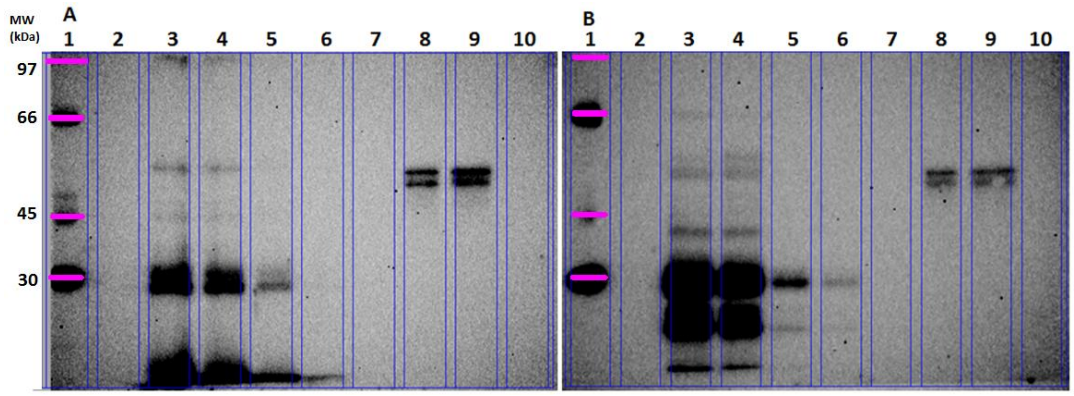


Figure 30- Western Blot of Patients 30 (A) and 33 (B) solid portion of urine reconstituted in PBS, by heating and vortexing. Each patient urine sample was diluted 2, 10, 20 and 100 times in PBS. Each prepared sample, and SpA standards (0.1µg/ml concentration, total volume 7ng), was loaded and electrophoresed in SDS-PAGE gel and transferred as per protocol. Membranes were blocked in 2% (w/v) BSA and incubated in 1:2000 anti-SpA antibody (constituted in 2% (w/v) BSA).

Lane	**1**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Sample	Ladder	Patient sample- undiluted	Patient sample (2x dilution)	Patient sample (10x dilution)	Patient sample (20x dilution)	Patient sample (100x dilution)	0.1µg/ml SpA (CU)	0.1µg/ml SpA (PBS)

In these sample blots, it is evident that the separated liquid produced far fewer bands than the reconstituted solid, and no bands at the level concurrent with commercial SpA. Due to this paucity of bands seen in the liquid, without prior mixing of solid material, further screening of samples would involve vortexing of patient urine in order to retain as much solid material as possible. This was seen as giving the highest chance of detecting SpA in patient samples, as it may be present as a precipitate or bound to other proteins in the solid material.

Initial observations in the WB method development stage showed 2% (w/v) BSA was a superior agent for use in blocking and as antibody solution, as it was more effective in removing background signal from purified SpA blots (see Figure 10 and 12). However, these experiments only evaluated the WB detection of commercial SpA at different concentrations. Given the large number of strong bands of different molecular weight being detected in patient urine samples, attempts were made to further define the best agent for blocking, when testing patient samples in particular. Retaining the WB sample preparation as described, two WB membranes containing SpA controls (PBS and control urine) and a

selection of patient urine samples, were both blocked with 5% (w/v) milk. Following this, one membrane was incubated in 1:2000 antibody in 2% (w/v) BSA, and the other with 1:2000 antibody 5% (w/v) milk. The differences in the resulting blots were therefore as a result of the antibody binding solution only (Figure 31 and 32).

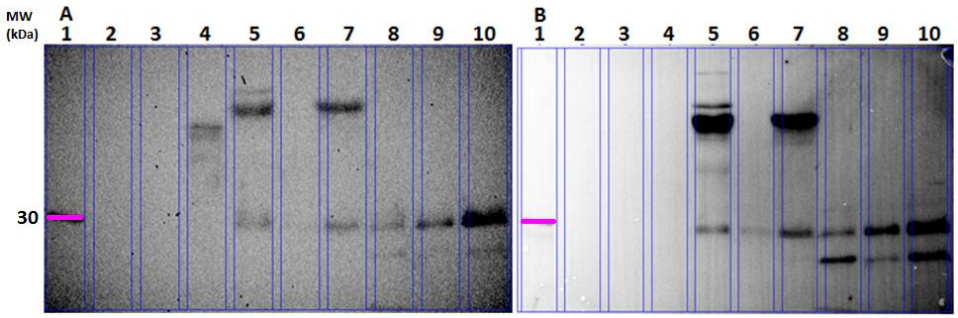


Figure 31- Western Blot of SpA controls and select patient urine samples, incubated antibody in BSA, with associated Amido Black stain. SpA standards (concentration 0.1µg/ml, total volume 7ng), and patient urines were electrophoresed in SDS-PAGE gels and transferred to membrane as per protocol. Membranes were blocked in 5% (w/v) milk and incubated in 1:2000 anti-SpA antibody, except in 2% (w/v) BSA. This figure includes developed Western Blot (A), and Amido black staining of the membrane (B).

Lane	**1**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample	Ladder	0.1µg/ml SpA (CU)	Urine Sample Patient 4	Urine Sample Patient 7	Urine Sample Patient 15	Urine Sample Patient 30	Urine Sample Patient 33	Urine Sample Patient 44

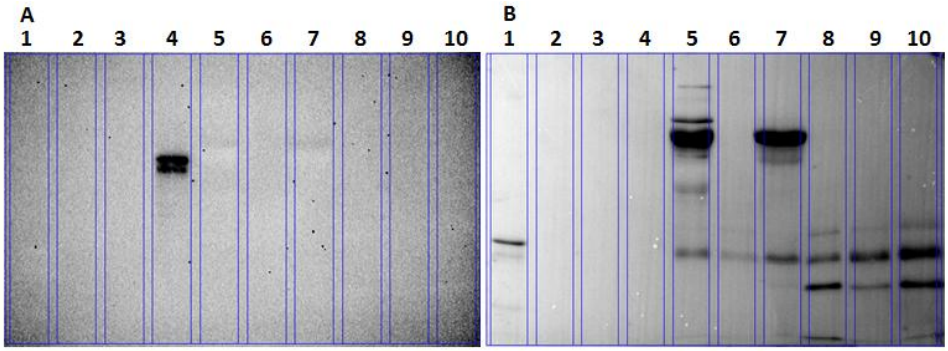


Figure 32- Western Blot of SpA controls and select patient urine samples, incubated antibody in milk, with associated Amido Black stain. SpA standards (concentration 0.1µg/ml, total volume 7ng), and patient urines were electrophoresed in SDS-PAGE gels and transferred to membrane as per protocol. Membranes were blocked in 5% (w/v) milk and incubated in 1:2000 anti-SpA antibody, in 5% (w/v) milk. This figure includes developed Western Blot (A), and Amido black staining of the membrane (B).

Lane	**1**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample	Ladder	0.1µg/ml SpA (PBS)	Urine Sample Patient 4	Urine Sample Patient 7	Urine Sample Patient 15	Urine Sample Patient 30	Urine Sample Patient 33	Urine Sample Patient 44

These WB results clearly demonstrate a significantly increased blocking effect when anti-SpA antibody was incubated in milk as opposed to BSA. This effect is seen entirely in the multiple patient bands that had been previously detected using BSA as antibody solution. Not only does diluting antibody in milk prevent the detection of all protein bands in patient samples, the ladder bands (which are a mixture of proteins), which were previously detected are absent. The 0.1µg/ml SpA standards also appeared to display increased signal. These findings together provide strong evidence that the bands previously seen on patient sample WB are in fact non-specific urinary proteins, displaying similar binding with anti-SpA antibody as the ladder proteins. These non-specific bands appear to correspond to the protein bands seen when the membrane is stained with Amido Black. Given the ability of the WB method for reliably detecting 0.1µg/ml SpA, and the retention of these SpA standard bands when using 5% (w/v) milk as antibody solution, it is reasonable to suggest that there is no SpA at, or above, this concentration in the patient samples tested.

This method of using 5% (w/v) milk as both blocking agent and antibody solution, was adopted as the optimised method for the detection of SpA. This retained the sensitivity of detecting 0.1µg/ml (or 7ng total volume), SpA, with the beneficial removal of the additional bands seen in original patient sample blots. A further experiment was devised in order to test the limit of this enhanced blocking effect of milk antibody solution. This was achieved by testing SpA standards and patient samples by WB, and increasing antibody concentration in 5% (w/v) milk, from 1:1000 to 1:500. This would prove whether the non-specific bands in patient urine would once again become visible at higher antibody concentrations, indicating that the blocking effect is dose-dependent (Figure 33 and 34).

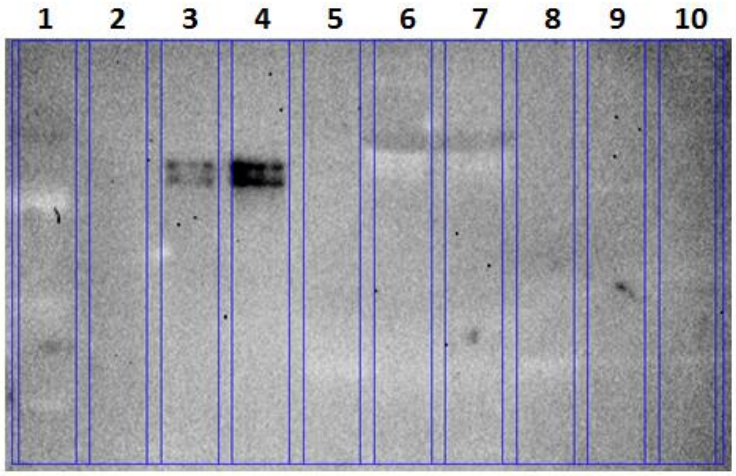


Figure 33- Western Blot of SpA standards and patient urine samples, incubated in 1:1000 concentration anti-SpA antibody. SpA standards (concentration 0.1µg/ml, total volume 7ng), and patient urines were electrophoresed in SDS-PAGE gels and transferred to membrane as per protocol. Membranes were blocked in 5% (w/v) milk and incubated in 1:500 anti-SpA antibody (also in 5% (w/v) milk). Ladder proteins not stained.

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample	Ladder	Control Urine	0.1µg/ml SpA (CU)	0.1µg/ml SpA (PBS)	Urine Sample Patient 1	Urine Sample Patient 4	Urine Sample Patient 15	Urine Sample Patient 30	Urine Sample Patient 33	Urine Sample Patient 44

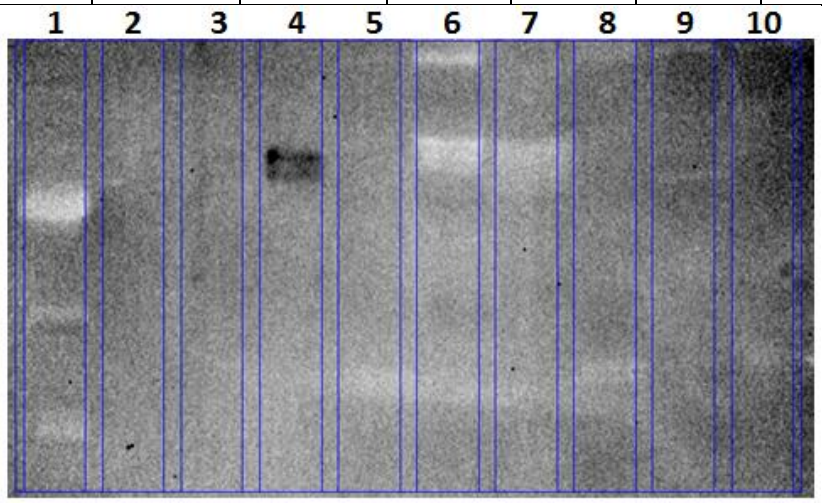


Figure 34- Western Blot of SpA standards and patient urine samples, incubated in 1:500 concentration anti-SpA antibody. SpA standards (concentration 0.1µg/ml, total volume 7ng), and patient urines were electrophoresed in SDS-PAGE gels and transferred to membrane as per protocol. Membranes were blocked in 5% (w/v) milk and incubated in 1:500 anti-SpA antibody (also in 5% (w/v) milk). Ladder proteins not stained.

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample	Ladder	Control Urine	0.1µg/ml SpA (CU)	0.1µg/ml SpA (PBS)	Urine Sample Patient 1	Urine Sample Patient 4	Urine Sample Patient 15	Urine Sample Patient 30	Urine Sample Patient 33	Urine Sample Patient 44

These blots demonstrate that the blocking of additional bands is not negated by increasing the concentration of antibody incubated in 5% (w/v) milk. However, at higher antibody concentrations, white bands, or 'bleached' bands appear at positions analogous with the additional bands seen when the antibody is in a BSA solution. Because of this bleaching effect, further testing of patient samples would utilize anti-SpA at a 1:2000 concentration.

3.1.3 Patient sample testing by Western Blot

A full sample screening of single patient samples was undertaken, using the optimised WB technique and 1:2000 antibody in 5% (w/v) milk. As in previous optimisation experiments, samples from the middle of the range were used per patient, with a total of 8 patient samples per gel. Similarly to previous blots using milk as antibody solution, these patient sample WBs did not produce any positive results for SpA, nor were any additional bands present as seen in previous blots of patient samples. These were confirmed as viable experiments due to the continued detection of 0.1µg/ml SpA standards, in both control urine and PBS (Figure 35).

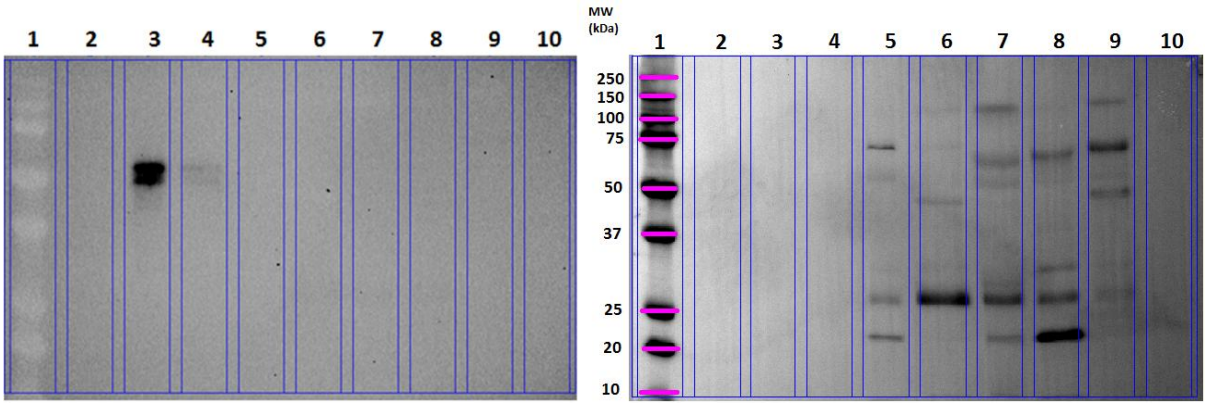


Figure 35- WB- Western Blot of SpA standards and patient number 37 – 41 urine samples, with associated Amido Black stain. SpA standards (concentration 0.1µg/ml, total volume 7ng), and patient urines were electrophoresed in SDS-PAGE gels and transferred to membrane as per protocol. Membranes were blocked in 5% (w/v) milk and incubated in 1:2000 anti-SpA antibody (also in 5% (w/v) milk). This figure includes developed Western Blot (A), and Amido black staining of the membrane (B).

Lane	1	2	3	4	5	6	7	8	9
Sample	Ladder	Control Urine	0.1µg/ml SpA (PBS)	0.1µg/ml SpA (CU)	Urine Sample Patient 37	Urine Sample Patient 38	Urine Sample Patient 39	Urine Sample Patient 40	Urine Sample Patient 41

In order to fully assess the validity of these negative findings, it was important to rule out variability of sample composition within the same patient range; i.e. the chances that individual samples in a patient sample range were positive, while others were negative. TO achieve this, it was decided to test the full sample ranges of particular patients which would provide the highest chance of detecting SpA. Patients 4 and 12 were found to contain many additional protein bands on Amido Black staining, so were candidates for full sample testing. Urine samples from patients 18, 19 and 24 were also fully tested by WB, due to a high probability of exposure to *S. aureus* (Table 2).

Patient	Rationale for full sample range testing
18	Gram positive bacteria in blood culture
19	Necrotising fasciitis
24	Necrotising fasciitis, Gram positive bacterial growth from wound swab

Table 2- Patient number and diagnosis indicating likely presence of *S. aureus*.

These full sample range blots, tested using the same method as the previous screening experiments, also did not yield any positive results, again appearing blank apart from the SpA standards. These negative WB are contrasted with the Amido Black stain displaying multiple protein bands (Figure 36).

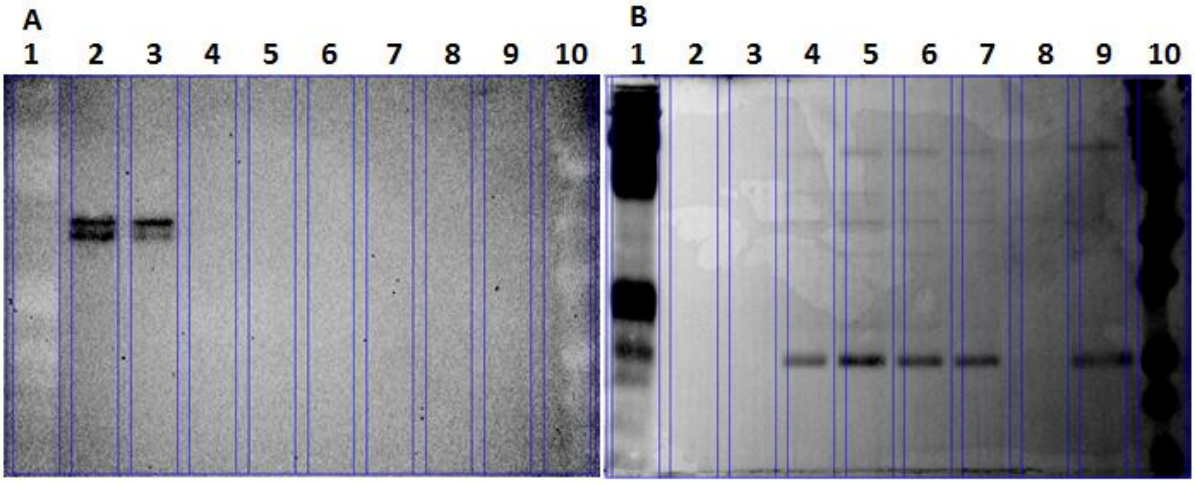


Figure 36- Western Blot of SpA standards and patient 18 full sample range, with associated Amido Black stain. SpA standards (concentration 0.1µg/ml, total volume 7ng), and patient 18 urine sample range were electrophoresed in SDS-PAGE gels and transferred to membrane as per protocol. Membranes were blocked in 5% (w/v) milk and incubated in 1:2000 anti-SpA antibody (also in 5% (w/v) milk). This figure includes developed Western Blot (A), and Amido black staining of the membrane (B. Ladder proteins are non-distinct on the stain.

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample	Ladder	0.1µg/ml SpA (PBS)	0.1µg/ml SpA (CU)	Patient 18 Sample 1	Patient 18 Sample 2	Patient 18 Sample 3	Patient 18 Sample 4	Patient 18 Sample 5	Patient 18 Sample 6	Ladder

Due to these negative WB results, the decision was made to not continue screening patient samples by WB. Despite optimising the method and removing almost all non-specific binding, the detection limit of our SpA WB, in patient sample testing conditions (7ng SpA) was considered insufficient to entirely rule out the presence of SpA in patient samples. This prompted the development of a sensitive ELISA technique, in order to continue our search for endogenous SpA.

3.1.4 Western Blot Results Summary

This work represents the development of a highly specific WB method for the detection of commercial, cell-culture purified SpA in sterile PBS and control urine solutions. However, application of this technique to testing patient urine has found it to be incapable of detecting any endogenous SpA above a solution concentration of 0.1-0.01µg/ml. Optimisation of this technique has revealed that multiple strong bands, initially detected in patient samples at a variety of MWs using WB, are likely to correspond to non-specific

urinary proteins. This is due to their elimination from WB following the switch from BSA to milk as a blocking agent and antibody solution, a method which continued to detect SpA standards.

3.2 ELISA Results

3.2.1 ELISA development for detection of SpA in patient urine

The development of an ELISA technique for the detection of SpA was based on the method developed by Harrison et al and Frances Price (MSc 2013), using an indirect 'sandwich' ELISA technique. These ELISA techniques have shown high sensitivity for Staphylococcal toxins, as previous work by this laboratory had achieved detection of TSST, SEB, SEC and AH, to a concentration of 1-3ng/ml.[80]

The method chosen for the detection of SpA was an indirect ELISA, using a capture antibody to bind to SpA, before using an HRP-conjugated anti-SpA antibody to bind to the IgG-bound SpA. Human IgG was used as capture antibody, due to its previously discussed affinity for SpA. Several parameters were to be assessed in order to identify the optimal method:

1. Capture antibody concentration- each microwell was coated in 100µl of 0.5µg/ml or 1µg/ml commercial human IgG
2. Blocking solution- following capture antibody, blocking buffer, similar to those used in WB, are placed in wells to reduce the non-specific binding ability of the capture antibody. Blocking buffers compared in our ELISA were 5% (w/v) milk, and 2.5% (v/v) cold water fish gelatine.
3. Anti-SpA antibody concentration- lower amounts of antibody are usually required for ELISAs rather than WB; initial tests compared 1:4000 and 1:8000 anti-SpA concentration

Initial ELISA method development focused on first; establishing the efficacy of the method as described for the detection of SpA, and second; to determine the sensitivity limits of the methods for SpA detection in PBS solution and urine. To determine if the technique was viable for the detection of small amounts of SpA , known concentrations of SpA from 100ng/ml to 25ng/ml were added to both PBS and control urine, and tested by ELISA using a variety of capture IgG concentrations and blocking buffers (Figure 37-40).

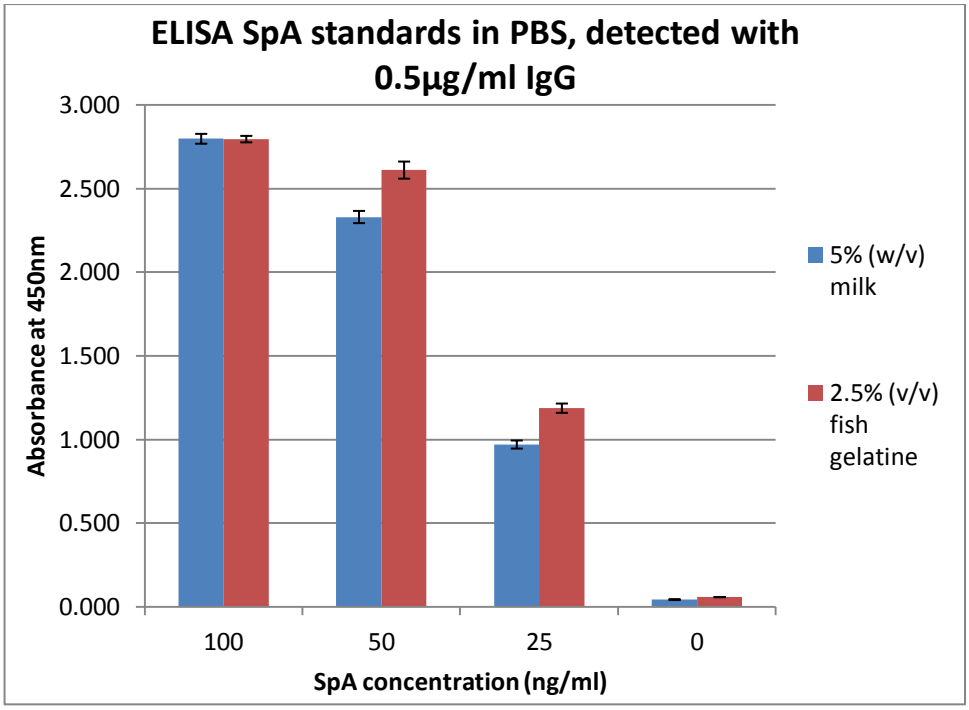


Figure 37- SpA standards in PBS, tested by SpA ELISA, with varying blocking materials. Microwell plates were coated with 0.5µg/ml IgG, and blocked with either 5% (w/v) milk or 2.5% (v/v) fish gelatine. SpA standards were then added, and finally incubated with 1:4000 anti-SpA antibody. Error bars are standard deviation.

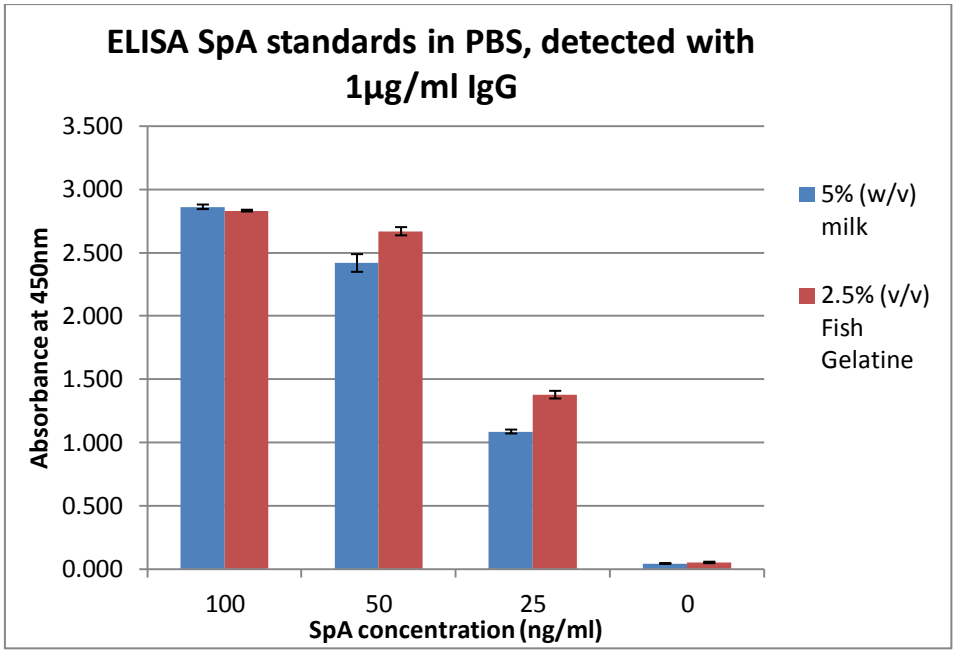


Figure 38- SpA standards in PBS, tested by SpA ELISA, with varying blocking materials. Microwell plates were coated with 1µg/ml IgG, and blocked with either 5% (w/v) milk or 2.5% (v/v) fish gelatine. SpA standards were then added, and finally incubated with 1:4000 anti-SpA antibody. Error bars are standard deviation.

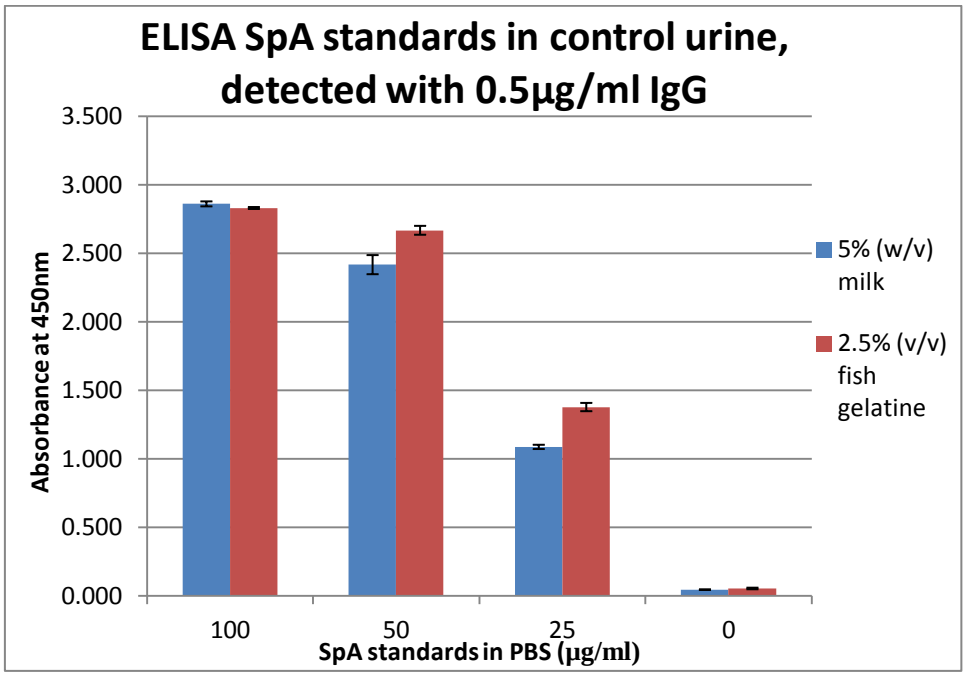


Figure 39- SpA standards in control urine, tested by SpA ELISA, with varying blocking materials. Microwell plates were coated with 0.5µg/ml IgG, and blocked with either 5% (w/v) milk or 2.5% (v/v) fish gelatine. SpA standards were then added, and finally incubated with 1:4000 anti-SpA antibody. Error bars are standard deviation.

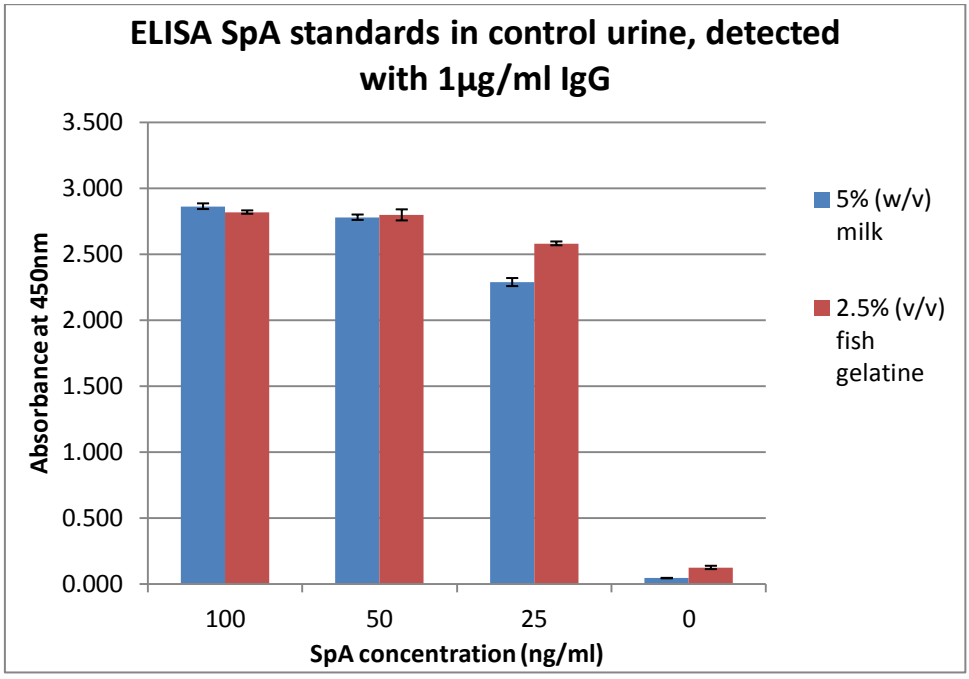


Figure 40- SpA standards in control urine, tested by SpA ELISA, with varying blocking materials. Microwell plates were coated with 1µg/ml IgG, and blocked with either 5% (w/v) milk or 2.5% (v/v) fish gelatine. SpA standards were then added, and finally incubated with 1:4000 anti-SpA antibody. Error bars are standard deviation.

The ability to detect SpA on ELISA was confirmed in both PBS and urine, and background was low across the plate; average PBS absorbance was 0.052 (SD 0.005).

In terms of SpA sample absorbance, there was little appreciable difference between blocking in 5% (w/v) milk and 2.5% (v/v) fish gelatine; however, control urine background was raised when blocked with fish gelatine (0.124) when compared with milk (0.043). This corroborates with the superior blocking effect of milk seen in WB, when incubating with polyclonal anti-SpA antibody. It was decided to use milk as a blocking agent for ELISA, due to a lower background and to maintain consistency with WB method.

The concentration of primary antibody was investigated by comparing 1:4000 and 1:8000 solutions, based on concentrations used in ELISA techniques for the detection of other Staphylococcal toxins by Frances Price (MSc 2013).[80] The 1:4000 antibody solution provided higher absorbance range when testing SpA standards in control urine, as well as displaying lower standard deviation error bars at the higher-middle range of SpA concentration (Figure 41).

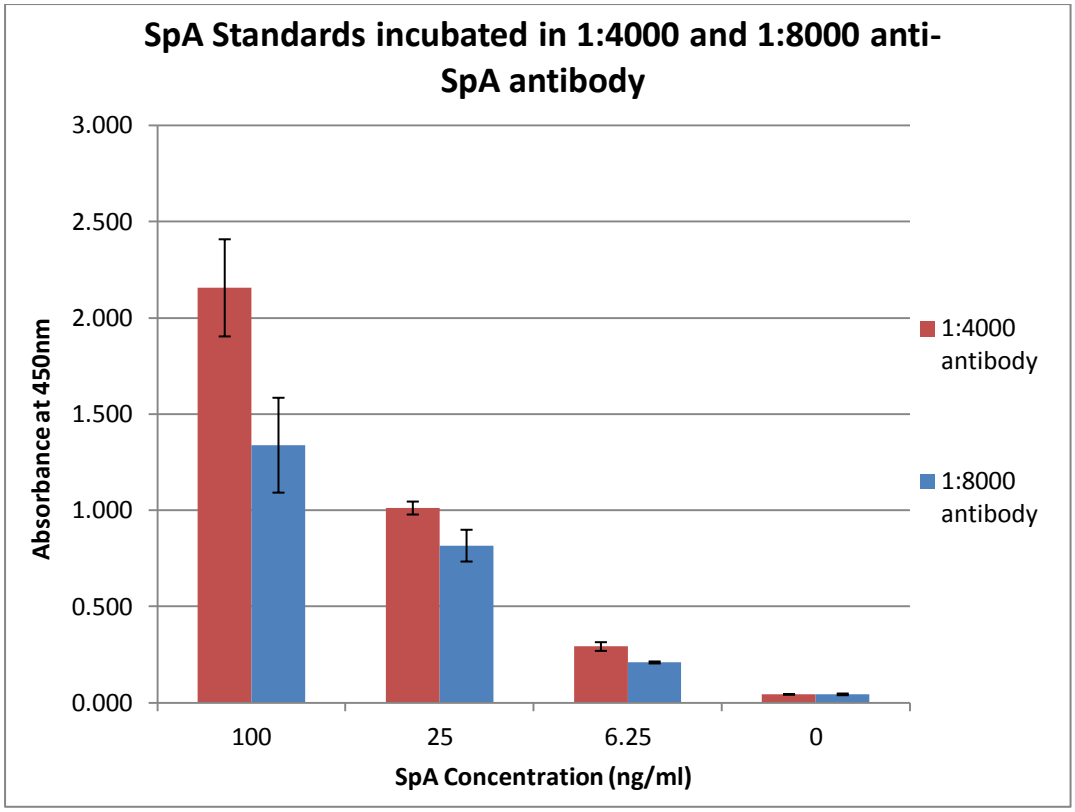


Figure 41- SpA standards (in control urine), tested by SpA ELISA, with varying primary antibody concentration. Microwell plates were coated in 0.5µg/ml IgG, then blocked in 5% (w/v) milk. SpA standards were added and incubated in either 1:4000 or 1:8000 anti-SpA antibody. Error bars are standard deviation.

As expected, the absorbance of SpA standards on ELISA was higher when using an primary antibody at the higher concentration. This was particularly evident at higher standard concentrations (100ng/ml SpA) which produced an absorbance of 2.156 with 1:4000 antibody concentration, compared to 1.338 with 1:8000 antibody concentration. However, SpA negative standards produced the same average absorbance (0.043), meaning that 1:4000 antibody concentration displayed a greater useful range of SpA detection.

Based on these optimisation experiments, an SpA concentration curve was created by SpA ELISA, utilising 0.5µg/ml IgG as capture antibody, blocked in 5% (w/v) milk and detected with 1:4000 anti-SpA antibody. The sensitivity of commercial SpA detection in PBS and urine can be demonstrated by a concentration curve of SpA samples, starting at 400ng/ml to 0.35ng/ml, in a series of 2x dilutions (see Figure 42-43).

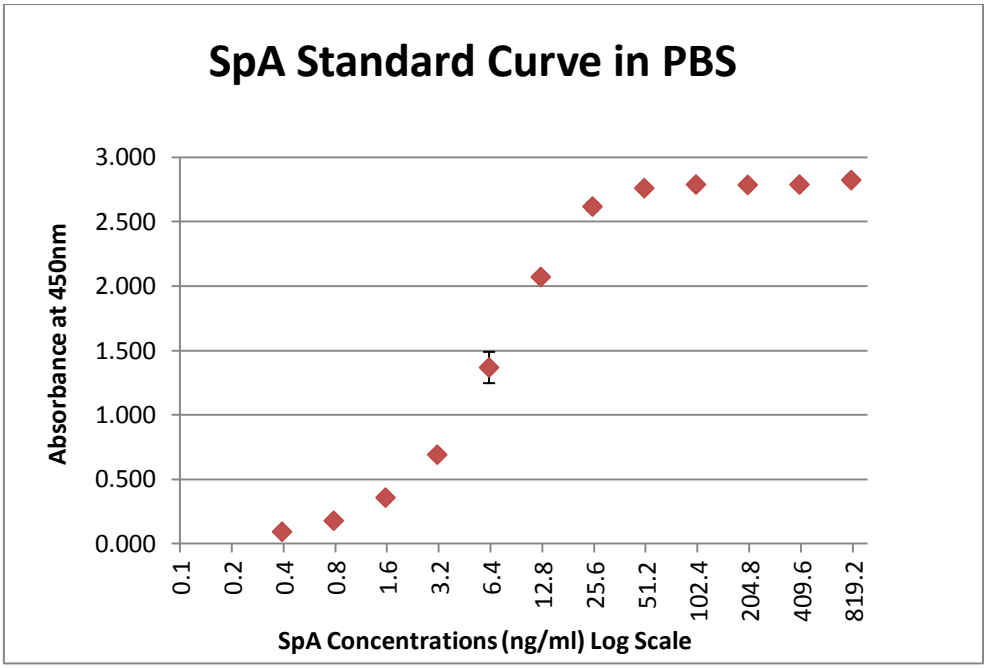


Figure 42- SpA concentration curve, with SpA standards constituted in PBS. Commercial SpA standards were tested by ELISA using 0.5µg/ml IgG as capture antibody, then blocked in 5% (w/v) milk and incubated in 1:4000 anti-SpA antibody. Error bars are standard deviation. SpA concentrations were created in a 2x dilution series.

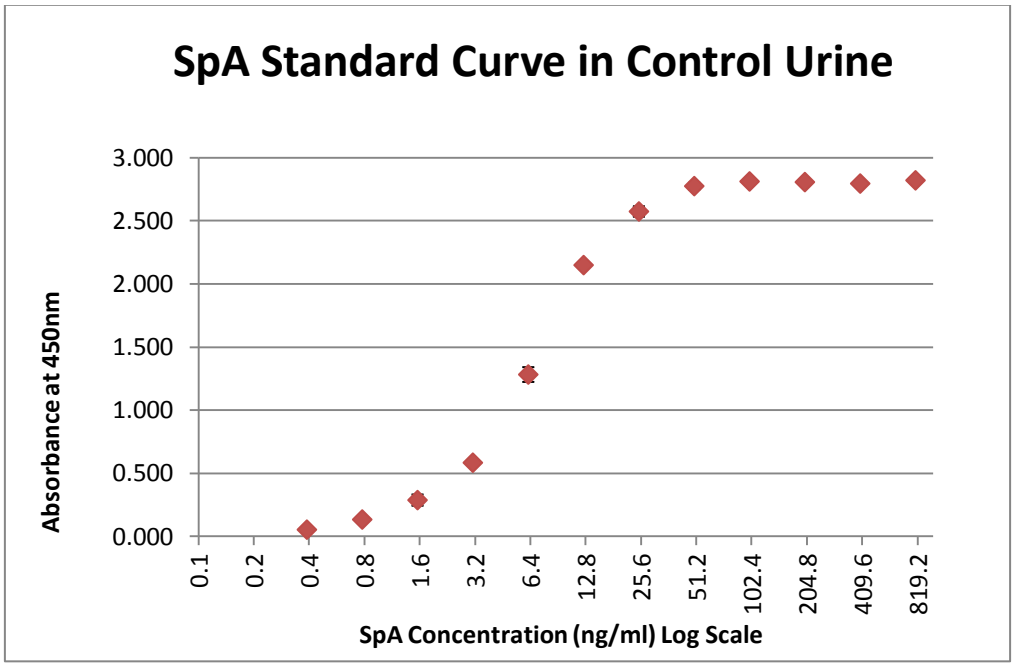


Figure 43- SpA concentration curve, with SpA standards constituted in control urine. Commercial SpA standards were tested by ELISA using 0.5µg/ml IgG as capture antibody, then blocked in 5% (w/v) milk and incubated in 1:4000 anti-SpA antibody. Error bars are standard deviation. SpA concentrations were created in a 2x dilution series.

These results demonstrate the ELISA technique to be effective and sensitive for the detection of SpA, in both PBS and control urine. Standard deviation was very low throughout this curve, confirming the ELISA method optimised to be reliable and sensitive for detecting nanogram amounts of SpA. Both PBS and control urine SpA concentration curves roughly equate to each other; upper asymptotes are reached between 25-50ng/ml, and lower asymptotes are reached at approximately 0.78ng/ml, creating a linear region from 25-0.78ng/ml (EC50= 9.37ng/ml). This produced the following linear standard SpA curve in control urine, which would allow quantification of SpA in patient samples (Figure 44).

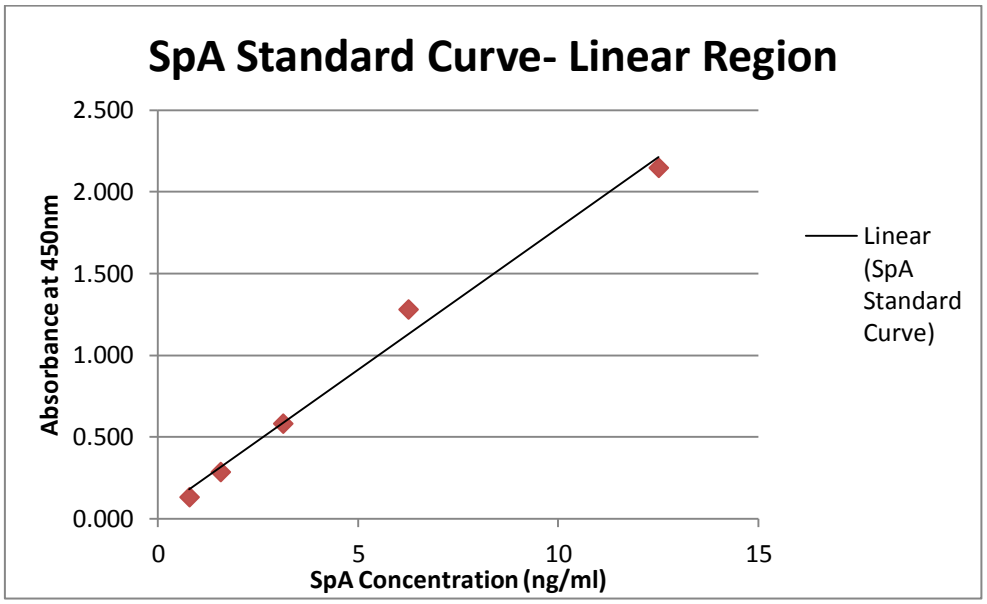


Figure 44- SpA ELISA of SpA standards in PBS, creating a standard curve with trend line through linear region. SpA standards were tested by ELISA using 0.5µg/ml IgG as capture antibody, then blocked in 5% (w/v) milk and incubated in 1:4000 anti-SpA antibody.

3.2.2 Screening Patient Urine Samples for SpA

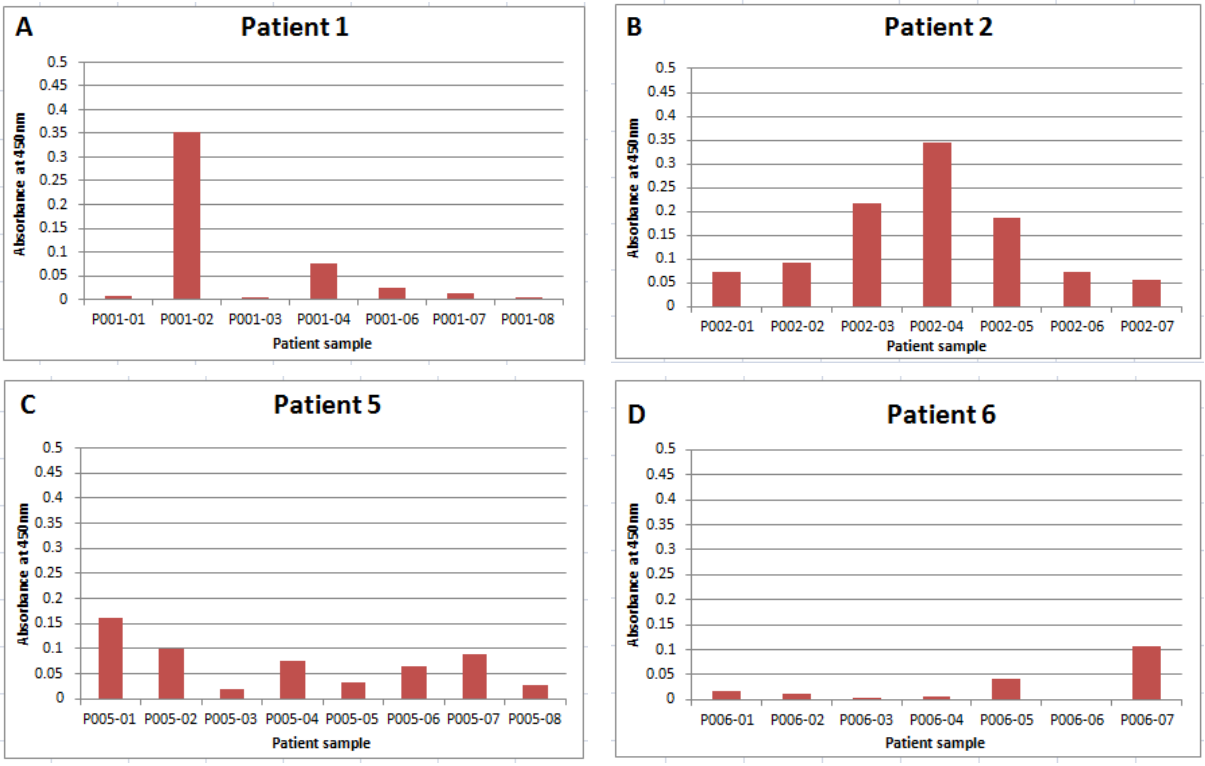
Following the development of a method for the detection of 1ng/ml of SpA in control urine, we performed a screening experiment by testing a single sample from each available patient range (patient n=45). This was done as an initial pilot experiment due to the potential for interaction between the large amounts of protein in patient urine, and the anti-SpA antibody. An initial ELISA would be useful to rule out this result, and in order to confirm this technique as sensitive and specific.

Plates were prepared as per the optimised protocol, incubated first in 0.5µg/ml human IgG,

blocked in 5% (w/v) milk, and incubated in 1:4000 anti-SpA antibody. Only 1 well was used per patient sample, at 100µl/well, in an attempt to limit overuse of patient samples.

Results of the initial experiment were promising, with an average background of 0.053. Several samples displayed a raised absorbance corresponding to low levels of SpA (1.562 - 3.125ng/ml), while others showed absorbance no greater than background (PBS) level. Given that only 1 well was used per patient sample, the experiment was repeated with 2 wells per patient sample, using the same patient samples as previously. This experiment concurred with the increased absorbance seen in certain patient samples, compared to control urine and PBS background, leading to the decision to test more patient samples.

Full sample-range testing was conducted for 241 (79%) of patient urine samples. Patient samples were not tested if there was insufficient volume of sample, or there were unavailable samples in that range. The figures below display the absorbance on ELISA for the patient ranges containing 1 or more samples positive for SpA. Patients with entirely negative sample ranges are not displayed (Figure 45A – 45L, 46A - 46D and 47).



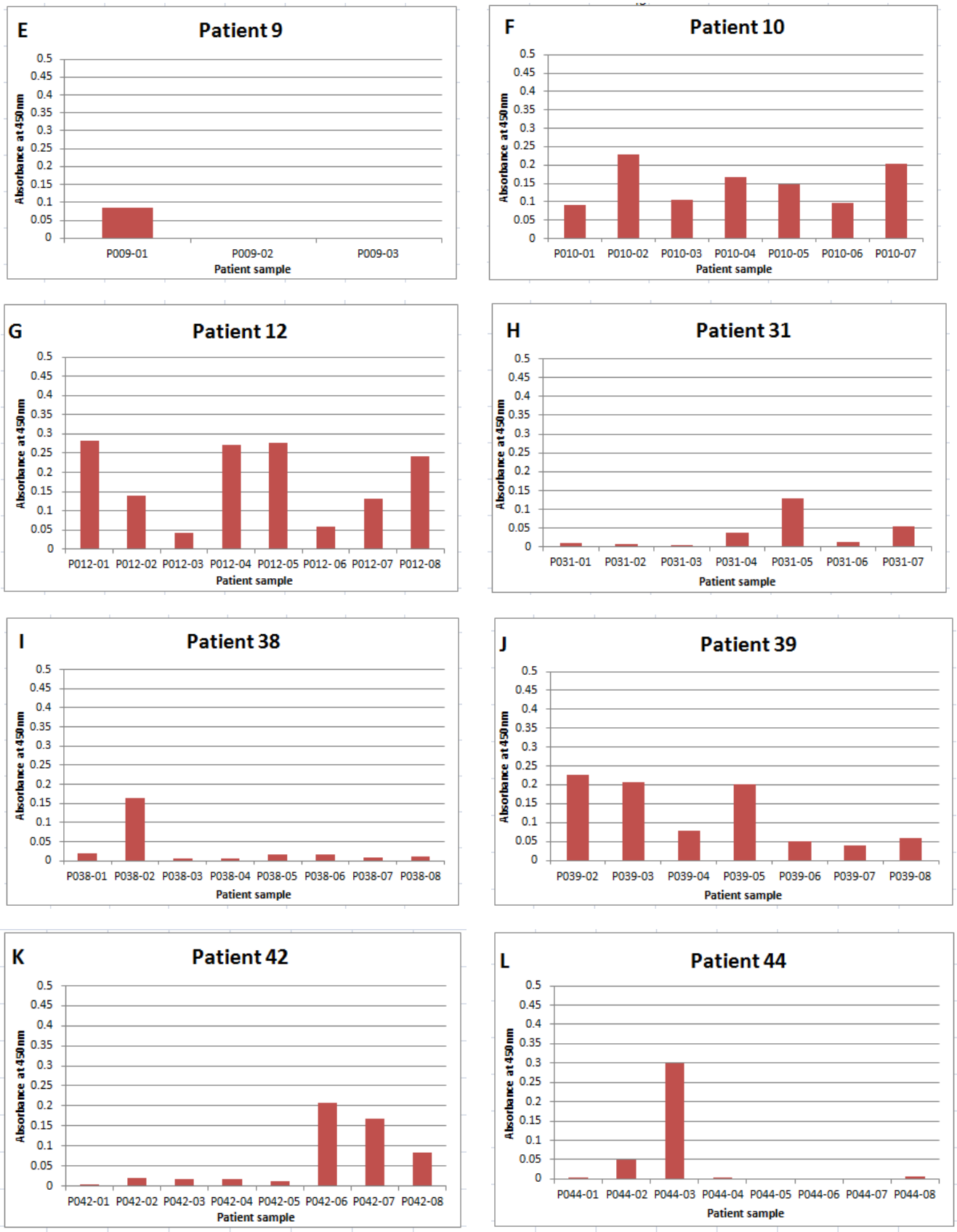


Figure 45A- 45L - Patient urine sample ranges, tested by ELISA, with mildly raised absorbance (0.0- 0.5 at 450nm). SpA ELISA was performed with 0.5µg/ml IgG as capture antibody, blocked in 5% (w/v) milk and incubated in 1:4000 anti-SpA antibody. Patient samples were tested at a volume of 100µl, undiluted.

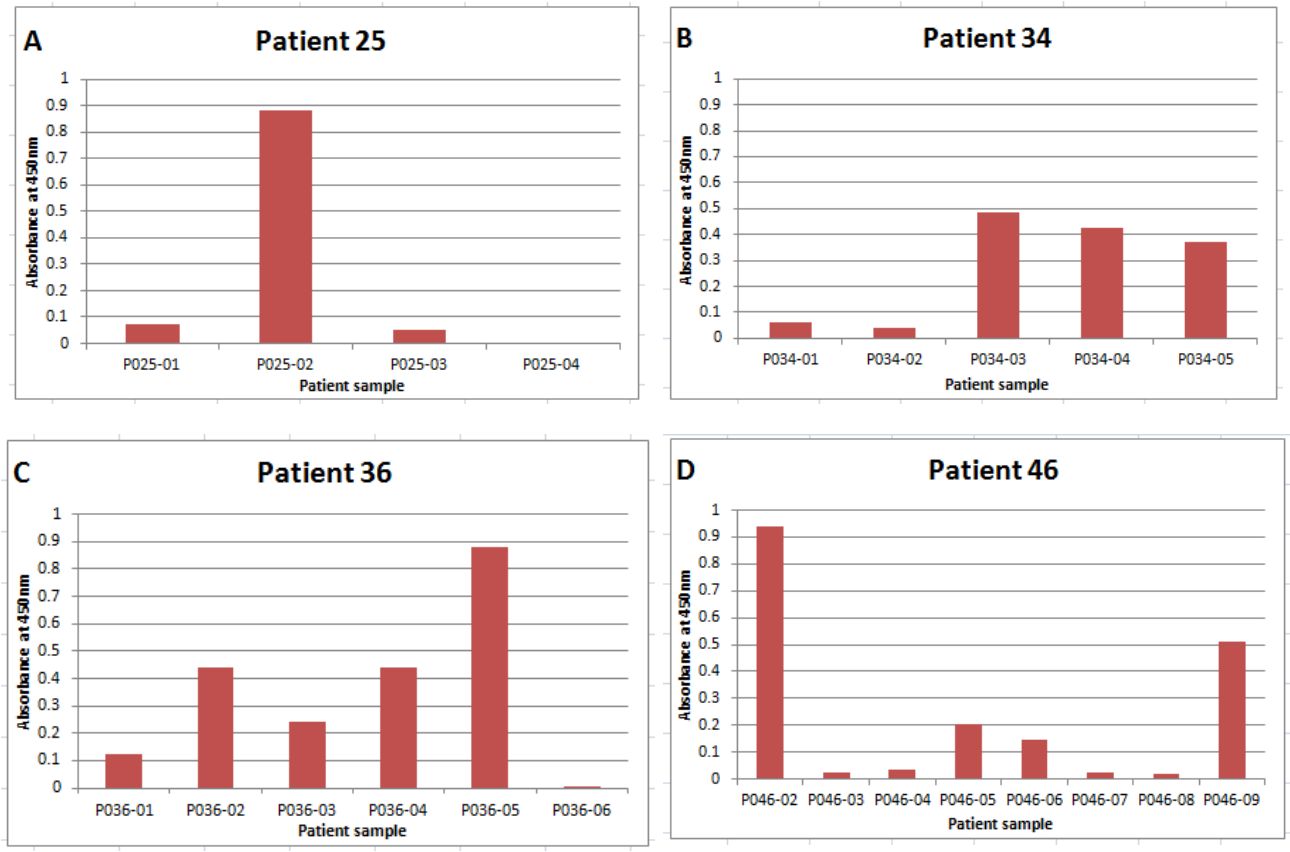


Figure 46A- 46D- Patient urine sample ranges, tested by SpA ELISA, with moderate raised absorbance (0.0- 1.0 at 450nm). SpA ELISA was performed with 0.5µg/ml IgG as capture antibody, blocked in 5% (w/v) milk and incubated in 1:4000 anti-SpA antibody. Patient samples were tested at a volume of 100µl, undiluted.

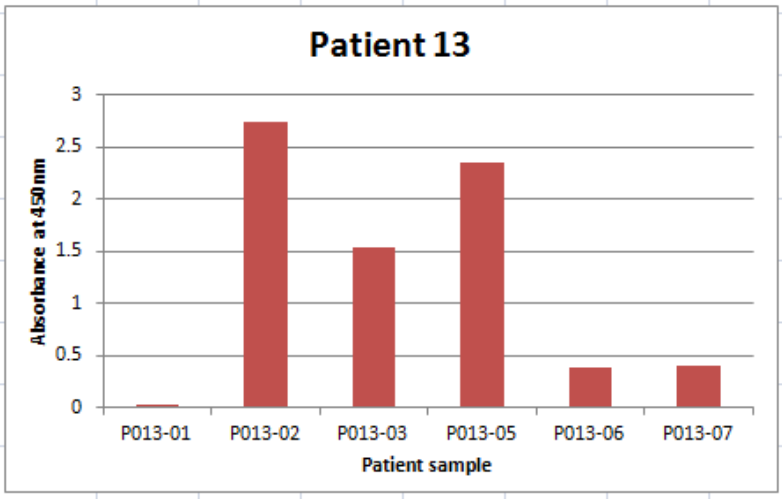


Figure 47- Patient 13 sample range absorbances tested by SpA ELISA. Patient 13 is the only sample with absorbance between 0.0 - 3.0 at 450nm. SpA ELISA was performed with 0.5µg/ml IgG as capture antibody, blocked in 5% (w/v) milk and incubated in 1:4000 anti-SpA antibody. Patient samples were tested at a volume of 100µl, undiluted.

Of the 241 patient samples tested for SpA, 55 samples (22.81% of those tested), derived from a total of 16 patients, showed a significantly raised absorbance, indicating the presence of SpA. The majority of these patients (e.g. Figure 48A- 48L) displayed an absorbance corresponding to very low concentrations of SpA (<1ng/ml). Only 5 patient ranges contained samples which displayed an absorbance above this level (1-5ng/ml), while one patient (patient 13) displayed an absorbance significantly above this level (5-15ng/ml). Of the patients with full sample ranges tested for SpA by ELISA, 16 patients did not contain a single sample with a significantly raised absorbance.

These patient sample range graphs also provide temporal information about the variation of SpA ELISA result over the course of the 48 hour ICU admission. In this patient group, there did not appear to be any relationship over time, with adjacent urine samples (taken 6 hours apart) displaying high variability.

3.2.3 Comparison of ELISA with Western Blot results

Following full sample range ELISAs, the patient samples with highest SpA ELISA absorbance were selected for re-testing by WB, in order to discover if bands of SpA could be visualised. Patients tested by both ELISA and WB were 13 and 36, as these were found to contain the highest potential concentration of SpA. Each patient sample range was treated as per WB methods described above, and incubated in 1:2000 anti-SpA antibody (Table 3-4, Figure 48-49).

Patient and sample number	Absorbance on SpA ELISA	Estimated concentration of SpA (ng/ml)
P013-01	0.055	0.11
P013-02	2.802	15.765
P013-03	1.584	8.826
P013-05	2.409	13.526
P013-06	0.429	2.239
P013-07	0.453	2.377

Table 3- Patient 13 full sample range absorbance on SpA ELISA, and estimated concentration of SpA based on SpA standard concentration.

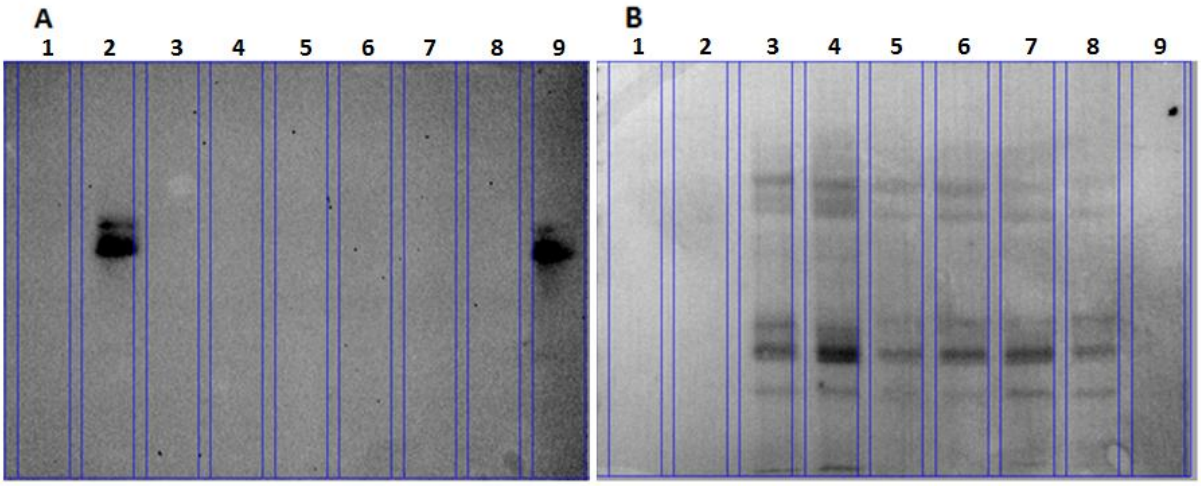


Figure 48- Western Blot of Patient 13 samples 1-7, with SpA standards and Precision Plus protein standards (ladder). Patient urine samples and standards were electrophoresed and transferred to membrane as per Western Blot protocol, blocked in 5% (w/v) milk and incubated with 1:2000 anti-SpA antibody. This figure displays the developed blot (A) and Amido Black stain of the same membrane (B).

Lane	1	2	3	4	5	6	7	8	9
Sample	Control urine	0.1µg/ml SpA (PBS)	Pt 13 (1)	Pt 13 (2)	Pt 13 (3)	Pt 13 (5)	Pt 13 (6)	Pt 13 (7)	0.1µg/ml SpA (PBS)

Patient and Sample Number	Absorbance on SpA ELISA	Estimated concentration of SpA (ng/ml)
P036-01	0.156	0.685
P036-02	0.475	2.502
P036-03	0.276	1.369
P036-04	0.475	2.503
P036-05	0.916	5.018
P036-06	0.042	0.035

Table 4- Patient 36 full sample range absorbance on SpA ELISA, and estimated concentration of SpA based on SpA standard concentration.

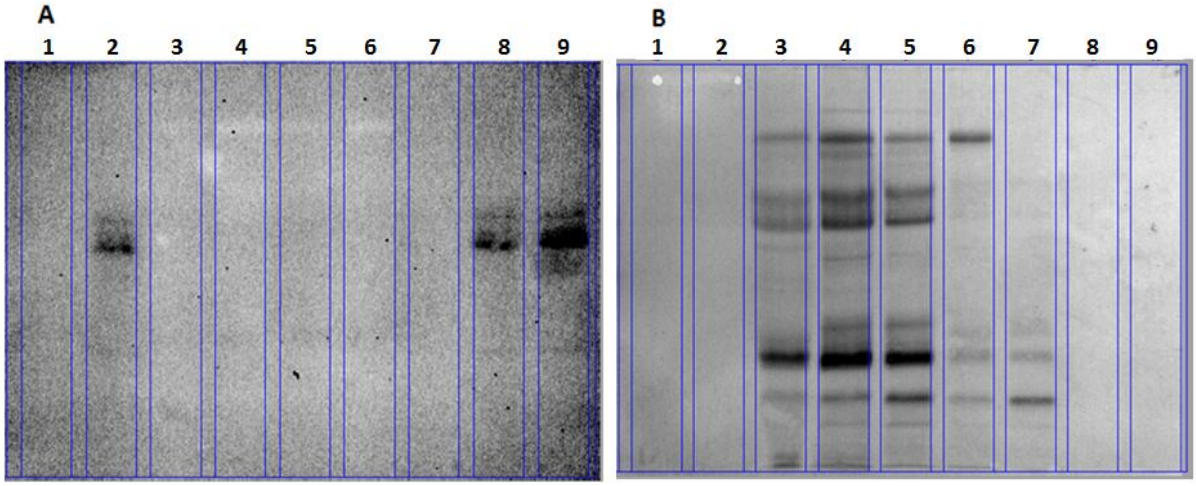


Figure 49- Western Blot of Patient 36 samples 1-6, with SpA standards and Precision Plus protein standards (ladder). Patient urine samples and standards were electrophoresed and transferred to membrane as per Western Blot protocol, blocked in 5% (w/v) milk and incubated with 1:2000 anti-SpA antibody. This figure displays the developed blot (A) and Amido Black stain of the same membrane (B).

Lane	1	2	3	4	5	6	7	8	9
Sample	Control urine	0.1µg/ml SpA (PBS)	Pt 36 (1)	Pt 36 (2)	Pt 36 (3)	Pt 36 (4)	Pt 36 (5)	0.1µg/ml SpA (PBS)	0.1µg/ml SpA (PBS)

These results demonstrate definitively that the high absorbance results seen when testing patient urine by ELISAs, a raised absorbance does not translate to positive findings on WB using the same antibody. Amido Black stains of the membranes following WB display the presence of protein bands, and the identification of 0.1µg/ml SpA standards show that any SpA at that concentration should have been identified. However, in terms of total SpA identified, WB can detect ~7ng, while ELISA is more sensitive, capable of detecting SpA standards of <1ng/ml. Only patient 13 contains samples with ELISA results which may indicate levels of SpA that may cross the WB detection limit (~15ng/ml), however, these samples do not display bands on WB.

These results suggests that either our WB developed for SpA does not have the sensitivity to detect biologically relevant levels of SpA, or SpA is not present in the urine samples, and the raised absorbance seen on ELISA is the result of non-specific binding with urinary proteins. Further experiments which could provide evidence of non-specific binding were undertaken before the results of our ELISA could be definitively stated as endogenous SpA.

3.2.4 ELISA for detection of total IgG in patient urine

We were aware of the potential for the cross-reaction of the antibody with urinary proteins and IgG, as seen on initial patient sample WBs. Therefore, we decided to quantify total IgG and protein in a certain ELISA positive and negative patient urine samples, to statistically determine if these contributed to the raised absorbance seen in positive SpA ELISA results.

3.2.4.1 ELISA technique development for detection of total IgG

An IgG ELISA was theorised to be achievable through two methods; direct adsorbance of IgG to the microwell plates, followed by incubation in anti-human IgG antibody (HRP-conjugated), or initial adsorbance of SpA to the microwell plates for use as a capture antigen, followed by incubation in sample, and finally incubation in anti-Human IgG antibody. IgG curves were created from an initial concentration of 500µg/ml, with the concentration reduced by half in 18 steps in order to reach the lowest concentration of 3.8ng/ml.

For the experiments investigating SpA as capture antigen, either 1µg/ml or 0.5µg/ml SpA was adsorbed onto microwell plates, overnight at 4^0C. Following the formation of IgG curve samples in carbonate-bicarbonate buffer, and incubation in anti-Human IgG antibody, an

IgG curves was produced for each adsorption technique. All IgG concentrations had background absorbance subtracted before inclusion into the curve (Figure 50).

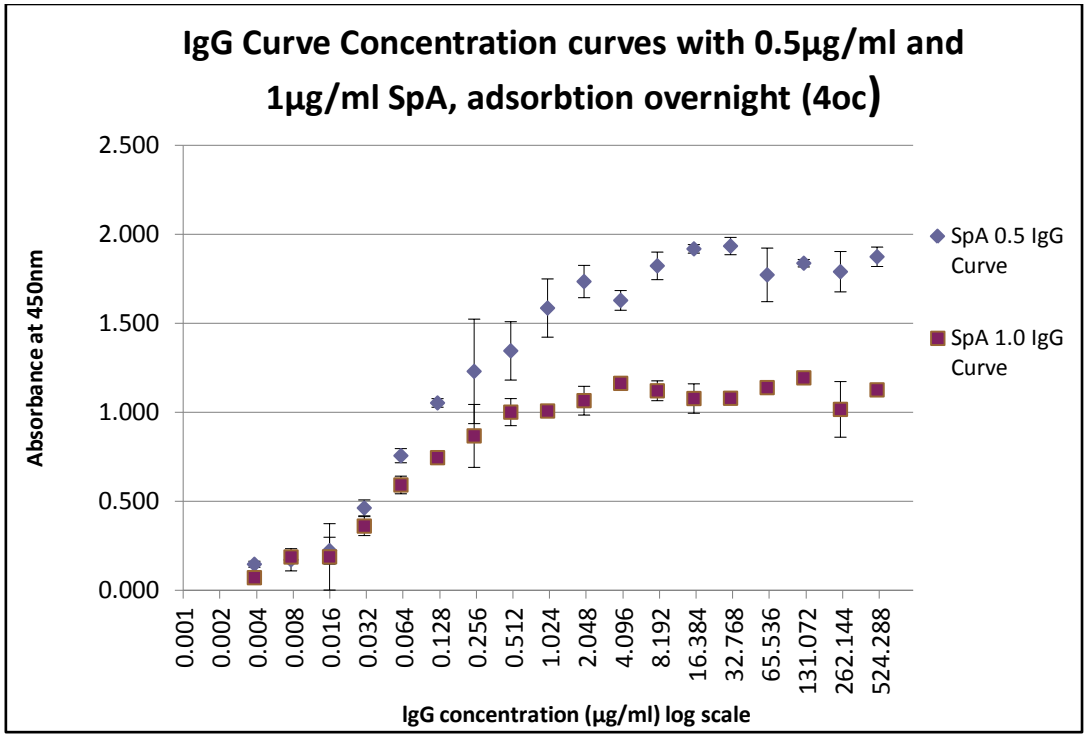


Figure 50- IgG ELISA concentration curves, tested on a microwell plate coated with either 0.5µg/ml or 1µg/ml SpA. Standards were incubated on microwell plates overnight at 4^0C. Following incubation, plates with blocked in 5% (w/v) milk and incubated in 1:4000 concentration anti-human IgG antibody. Error bars are standard deviation. IgG standards were created from a 500µg/ml solution in a 2x dilution series, and are

PBS background absorbance was significantly raised on the plate containing 1µg/ml, hence the lower absorbance of the curve after subtraction of the background. For the IgG curve created with 0.5µg/ml SpA as capture antigen, background was 0.712, and for 1µg/ml SpA background was 1.417.Low ELISA absorbances seen in these curves were the result of subtracting these high background from standard absorption. As well as this high background, standard deviation error bars through the linear region were large. Both of these limiting factors meant that the method of using SpA as capture antigen was deemed unacceptable for detection of endogenous IgG in patient urine.

An alternative method of IgG detection, would be to IgG adherence to microwell plates without capture antibody, developing a direct ELISA. To achieve this, IgG concentration curve samples were incubated overnight at 4^0C in order to give highest chance of IgG

adherence to microwell plates. Following adherence, 5% (w/v) milk was used as blocking agent, and anti-IgG antibody was then applied (Figure 51).

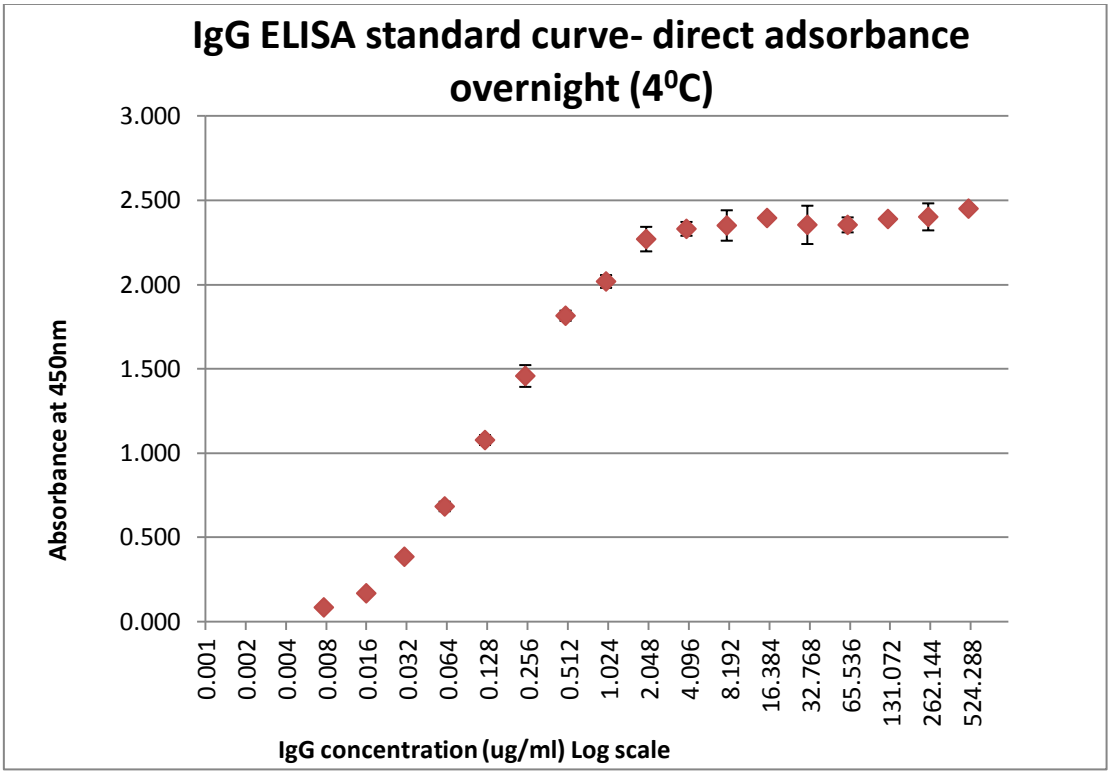


Figure 51- IgG ELISA concentration curve, tested by IgG ELISA, on a microwell plate coated directly with sample overnight at 4^0C. Following standards adsorption to plates, they were blocked with 5% (w/v) milk and incubated in 1:4000 anti-human IgG antibody. Error bars are standard deviation.

Background was low, with an average of 0.047 for PBS, similar to levels seen in ELISA experiments testing for SpA using IgG as capture antibody, while remaining sensitive for IgG down to 7ng/ml. It was decided to use the overnight direct adsorbance method for detecting IgG in patient samples. However, the standard curve would ideally be in the same solution as the samples being tested, which would be urine.

If patient samples would to be constituted in carbonate-bicarbonate buffer, as in the curve, a 1:1 solution with 2 times carbonate-bicarbonate buffer would be used. This would mean that the true concentration of IgG in patient samples would be double that measured by ELISA. This finalised IgG ELISA technique produced the following IgG standard curve (Figure 52).

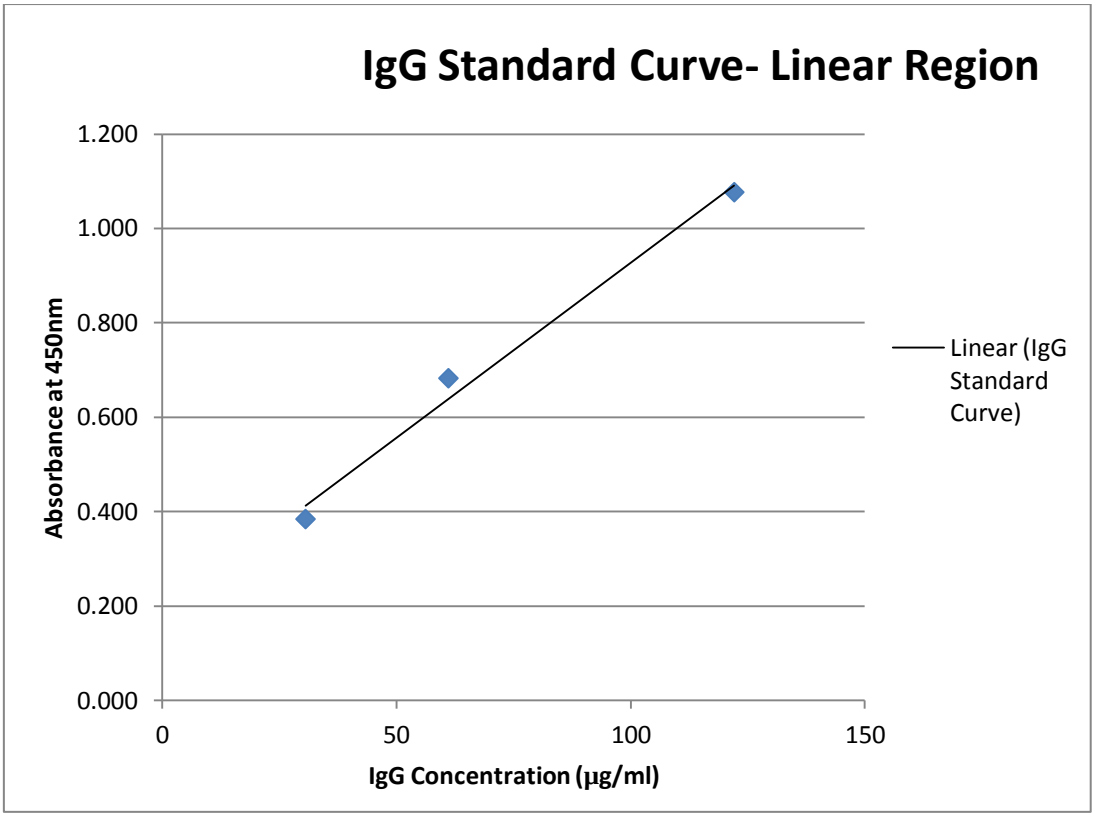


Figure 52- IgG Standard Curve, of human IgG standards constituted, in carbonate-bicarbonate buffer. Linear region of curve is displayed. IgG standards were tested by direct IgG ELISA, blocked with 5% (w/v) milk and incubated with 1:4000 anti-human IgG antibody.

3.2.5 ELISA for total IgG in patient urine

Using the method developed for detecting total IgG on ELISA, samples which were positive on SpA ELISA were retested for IgG, alongside a control group of negative SpA ELISA samples. This could be a possible cause of false-positive results in SpA ELISA; if large numbers of urinary IgG molecules remain bound to capture IgG, it could non-specifically bind to anti-SpA antibody. Total number of patient samples tested for IgG was 33, 15 SpA positive samples, and 18 SpA negative samples (Table 5 and 6).

Positive Patient (Sample Number) N=15	SpA ELISA Absorbance	Total IgG Absorbance	Estimated concentration of IgG (ng/ml)
1(2)	0.063	1.787	428.4
2(5)	0.091	1.687	401.8
5(6)	0.122	1.515	239
9(1)	0.700	0.363	48.6
10(2)	0.063	1.893	456.6
12(5)	0.117	1.987	481.8
13(2)	1.992	0.337	41.6
25(1)	0.109	1.465	342.6
31(5)	0.076	2.581	640.2
34(3)	0.229	0.528	92.6
36(5)	1.044	0.445	70.4
38(2)	0.059	1.047	231.2
39(2)	0.150	1.995	483.8
42(6)	0.102	1.568	239
44(3)	0.060	1.846	444.2

Table 5- Patient samples displaying highest absorbance for SpA, retested by SpA and IgG ELISA. Estimated concentration of IgG in patient urine samples is calculated by IgG ELISA.

Negative Patient (Sample Number) N=18	SpA ELISA Absorbance-retest	Total IgG Absorbance	Estimated concentration of IgG (ng/ml)
4(3)	0.053	2.106	513.5
6(5)	0.056	1.804	433.1
11(5)	0.051	0.464	75.6
18(5)	0.047	2.136	521.6
19(3)	0.049	1.761	421.6
21(1)	0.091	0.525	91.8
22(1)	0.061	2.380	586.7
24(3)	0.066	1.847	444.6
28(6)	0.056	1.109	247.7
29(3)	0.072	1.719	410.3
30(3)	0.046	1.042	229.9
32(1)	0.047	1.704	406.3
33(2)	0.059	1.462	341.7
35(1)	0.053	1.490	349.2
37(1)	0.054	1.508	353.9
40(4)	0.053	1.496	350.9
43(7)	0.050	0.563	101.9
47(3)	0.051	1.359	314.4

Table 6- Patient samples displaying lowest absorbance for SpA, retested by SpA and IgG ELISA. Estimated concentration of IgG in patient urine samples is calculated by IgG ELISA.

Statistical analysis on the levels of IgG absorbance revealed no difference between these 2 groups. Mean absorbance for IgG in positive samples was 1.471 (95% CI 1.196-1.746), and for negative samples was 1.403 (95% CI 1.017-1.789), giving a non-significant p-value of 0.7570. Given the significant difference between the two groups for absorbance when tested for SpA, this was seen as evidence that the presence or absence of IgG in the patient samples did not affect the absorbances seen when testing for SpA by ELISA.

These IgG ELISAs proved that IgG is present in variable amounts in patient urine samples, and the presence of IgG was not significantly raised in the patient samples with highest SpA ELISA absorbance.

3.3 Bradford Assay of Patient Samples

Following indication that the amount of total IgG is not producing false positive absorbances in patient samples, the same sample range was tested for total protein content. This was to rule out non-specific interaction with urinary proteins (such as albumin) as the sole cause of positive SpA ELISA results. It was previously shown that the polyclonal anti-SpA antibody used in WB had the potential for non-specific binding, especially when in BSA solution. However, whether this phenomenon also occurred with non-denatured samples, tested by ELISA, was unknown.

To estimate total protein in patient samples, a standard protein curve was made, as detailed in the methods. Following measurement of absorbance, a standard protein curve was created (Figure 53).

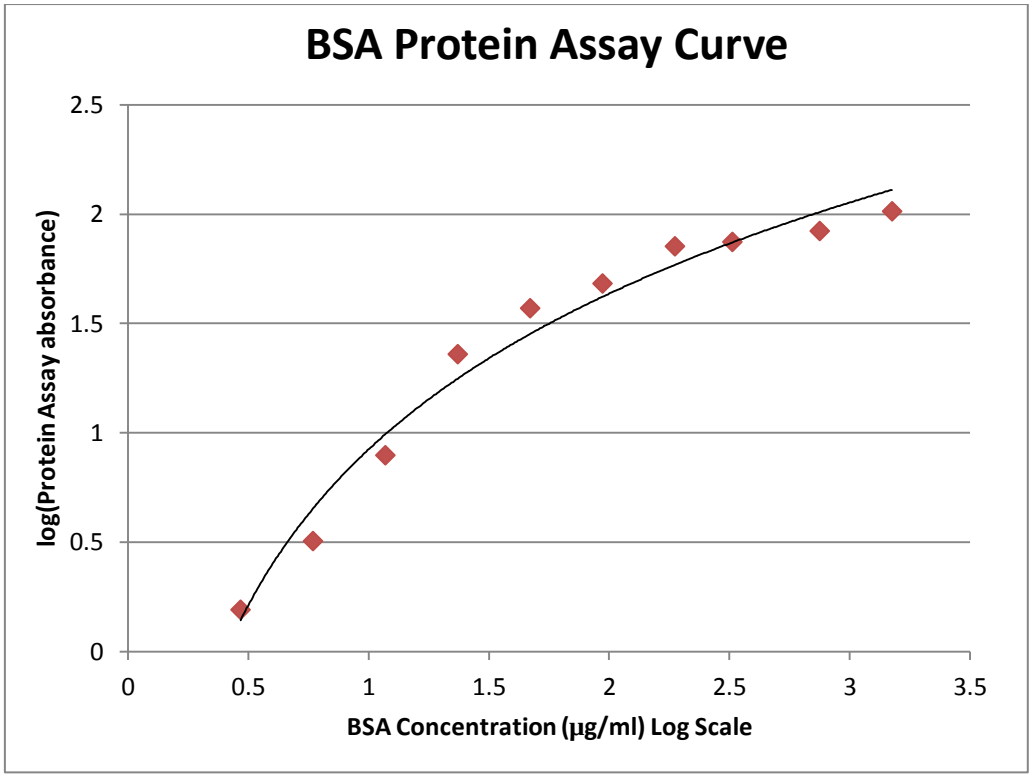


Figure 53- BSA standard curve, equalised against a blank of NaCl. BSA standards were tested by Bradford assay as per protocol. BSA standards were created from a 1.5mg/ml BSA solution, in a 2x dilution series.

Each patient sample, previously tested for SpA and IgG by ELISA, was retested for total protein, using the method as described for creating protein standards. The results are displayed as absorbance and concentration of protein (Table 7 and 8).

Positive Patient (Sample Number) N=15	Bradford Assay Absorbance	Estimated concentration of protein (mg/ml)
1(2)	0.112	0.292
2(5)	0.68	1.774
5(6)	0.91	2.375
9(1)	1.35	3.523
10(2)	0.722	1.884
12(5)	0.284	0.741
13(2)	0.703	1.834
25(1)	0.549	1.432
31(5)	0.254	0.662
34(3)	0.045	0.116
36(5)	0.543	1.417
38(2)	0.192	0.501
39(2)	0.803	2.095
42(6)	0.81	2.114
44(3)	0.802	2.093

Table 7- Patient samples most positive for SpA by ELISA, tested for total protein content by Bradford Assay absorbance, with estimated protein concentration.

Negative Patient (Sample Number) N=17	Bradford Assay Absorbance	Estimated concentration of protein (mg/ml)
4(3)	1.66	4.333
6(5)	0.354	0.924
11(5)	0.311	0.812
18(5)	0.266	0.694
19(3)	0.513	1.339
21(1)	0.253	0.660
22(1)	0.245	0.639
24(3)	0.494	1.289
28(6)	0.422	1.101
30(3)	0.28	0.730
32(1)	0.572	1.492
33(2)	0.507	1.323
35(1)	0.794	2.072
37(1)	0.299	0.781
40(4)	0.933	2.435
43(7)	0.76	1.983
47(3)	0.376	0.981

Table 8- Patient samples negative for SpA by ELISA, tested for total protein content by Bradford Assay absorbance, with estimated protein concentration

Statistical analysis performed on the Bradford Assay absorbances showed no significant difference between positive and negative patient groups. Mean protein assay absorbance for the positive group was 0.583 (95% CI 0.388-0.779), and for the negative group was 0.532 (95% CI 0.348-0.715), giving a non-significant p-value of 0.6808 for the difference in the means, as tested by Student's T-Test. This provided evidence that the total amount of protein in patient samples was not the sole contributing factor to a raised absorbance seen on SpA ELISAs. However, given the small sample population tested for both total IgG and protein, only limited conclusions can be drawn from this information.

3.4 Boiling patient urine and serum samples

As the patient samples and control urine are known to contain IgG, it was hypothesised that this may be found bound to endogenous SpA, reducing its ability to bind to the capture IgG during the initial phase of an SpA ELISA. This sample attenuation by IgG would also apply to SpA concentration curves, as they have been constituted in control urine in the course of this research. The ELISA method for SpA detection in human serum, developed by Nilsson et al, showed clear evidence of SpA-IgG binding in human serum, which could be broken by boiling the serum for 10 minutes.[183] In this study, it was found that without boiling, the absorbance of SpA standards constituted in human serum was not raised above background level, while following boiling, the SpA concentration curve in serum equated to levels seen in PBS solution.[183]

Using this information, we repeated this experiment by creating an SpA concentration curve in control urine, and we subjected one curve's samples to heating at 98^0C for 10 minutes. Both sets of SpA standards in control urine were treated as per SpA ELISA method, with the only independent variable being the heating of samples (Figure 54).

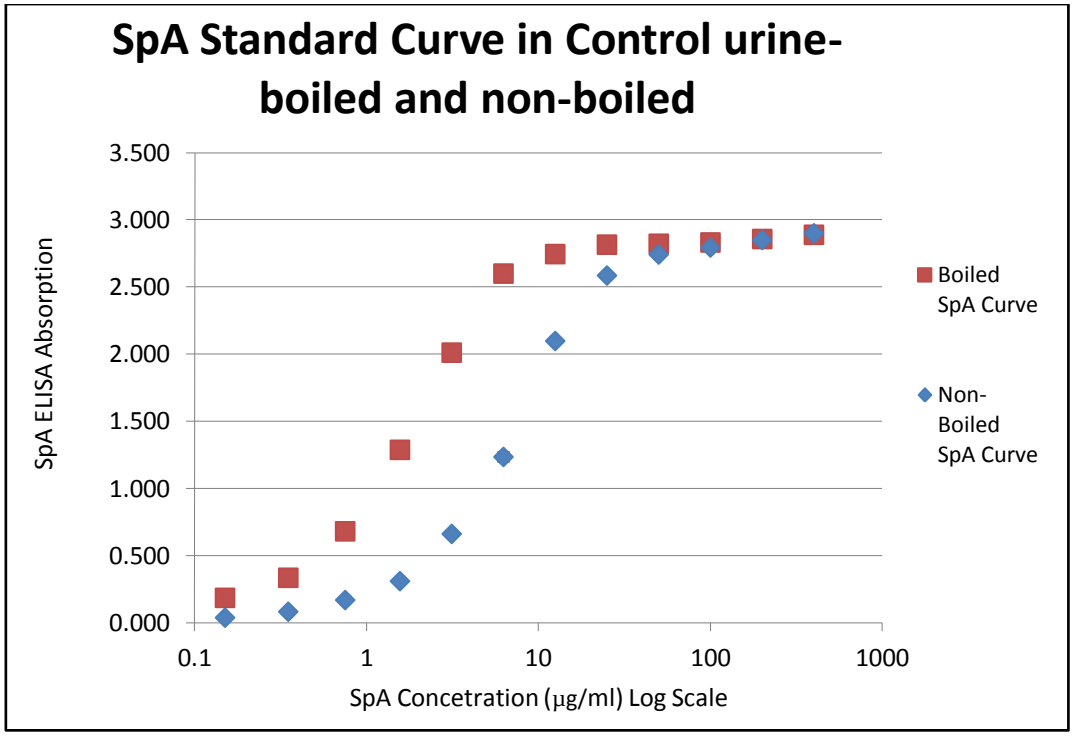


Figure 54- SpA concentration curves, constituted in control urine, tested non-boiled and following boiling for 10 minutes. Commercial SpA was added to undiluted control urine to create a concentration curve. SpA ELISA was performed with 0.5µg/ml IgG as capture antibody, blocked in 5% (w/v) milk and incubated in 1:4000 anti-SpA antibody. SpA standards were constituted in a 2x dilution series, in control urine.

These curves show that the sensitivity for SpA in ELISA is increased when control urine standards are boiled prior to testing, with the concentration curve displaying a shift to the right. This correlates with the findings of Nilsson et al, and demonstrates that a certain amount of SpA in patient urine samples may be quenched by the binding action of human IgG.[183] Furthermore, it shows that the SpA molecule is capable of tolerating boiling for 10 minutes, with anti-SpA binding retained.

3.4.1 Protein A Standards in serum

In order to confirm the potential quenching effect of IgG in commercially sourced healthy human serum on detecting SpA by ELISA, a series of SpA standards was made up in a sample of commercial human serum. These standards were prepared at 400ng/ml, 40ng/ml and 4ng/ml concentration, and subsequently tested by SpA ELISA.

3 samples were tested non-boiled, and the same samples were also tested after 10 minutes of heating at 98^0C. Each serum sample was diluted 1:5 with dH_20, in order to avoid solidification of the serum sample on heating (Figure 55).

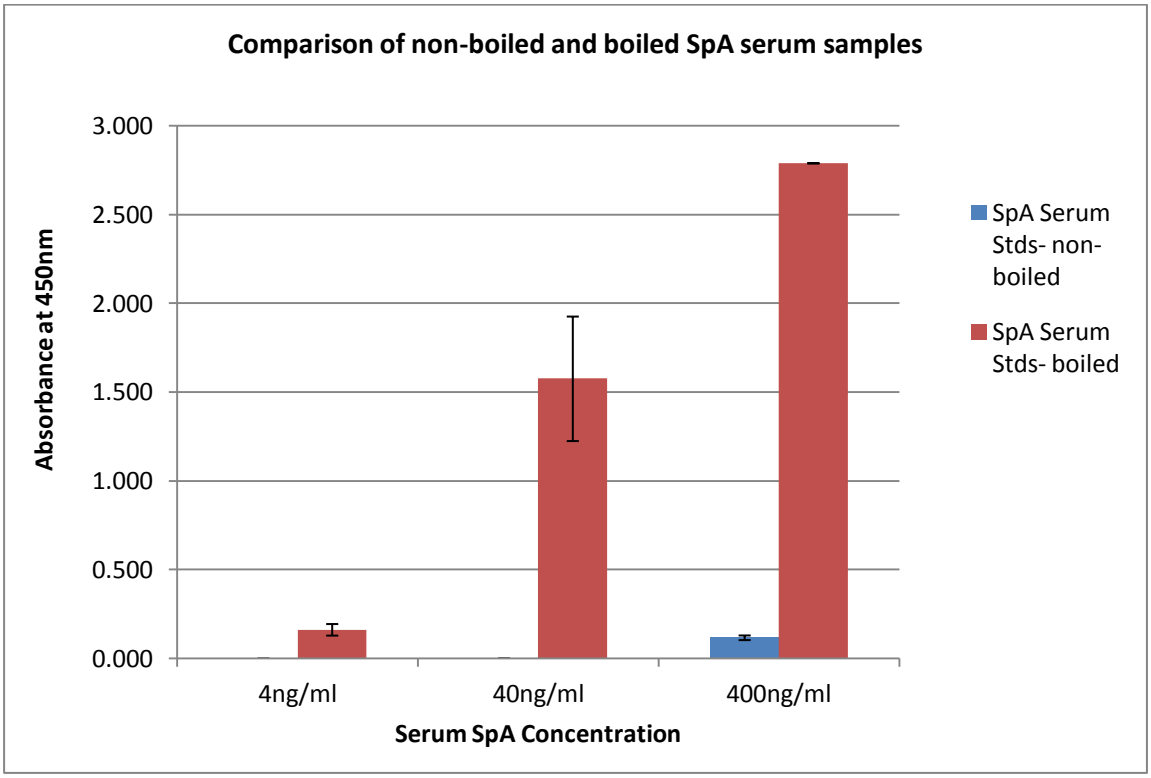


Figure 55- Comparison of commercial SpA detected by SpA ELISA, when added to human control serum. Standards were tested non-boiled and following boiling for 10 minutes. SpA standards were constituted in human control serum, and diluted 1:5. SpA ELISA was performed with 0.5µg/ml IgG as capture antibody, blocked in 5% (w/v) milk and incubated in 1:4000 anti-SpA antibody.

This result clearly demonstrates the quenching effect seen in serum, most likely due to the effect of IgG binding to SpA and blocking binding to the capture antibody. On boiling, these existing bonds are likely to be broken, freeing up SpA binding sites. To confirm the presence of IgG in the commercial serum, a separate ELISA dilution test was performed, which was positive; the healthy serum was calculated to contain 9.85mg/ml total IgG. Again, this also demonstrates the viability of commercial SpA detection by ELISA after boiling for 10 minutes.

3.4.2 Testing patient samples after boiling

The logical next step in investigating this effect was an ELISA retest of SpA positive and negative patient urine samples, following boiling. If endogenous IgG is capable of quenching

SpA ELISA signal, then patient urine samples which were initially negative may become positive, while positive ELISA samples may display increased signal. This experiment was conducted with two groups of urine samples, three SpA ELISA positive samples and 3 SpA ELISA negative samples. These were tested both untreated, and after heat treatment for 10 minutes, meaning each urine sample was tested twice (Table 9 and 10).

Positive Samples	Non-boiled Absorbance	Boiled absorbance
9	0.068	0.046
9	0.102	0.051
34	0.571	0.042
34	0.777	0.041
36	2.763	0.041
36	2.769	0.067

Table 9- Positive urine samples tested by SpA ELISA, with samples tested non-boiled and following boiling.

Negative Samples	Non-boiled Absorbance	Boiled absorbance
4	0.047	0.041
4	0.041	0.040
18	0.046	0.042
18	0.051	0.044
24	0.071	0.043
24	0.064	0.039

Table 10- Negative urine samples tested by SpA ELISA, with samples tested non-boiled and following boiling.

These samples demonstrate that after 10 minutes of boiling, these patient samples display no higher absorbance than background (PBS background 0.047- SD 0.011). This is the opposite to what was expected from this experiment, if we are detecting SpA, as it is heat tolerant and should have raised signal following dissociation from IgG.

Not only is heating capable of eliminating positive ELISA results, but it was found to be capable of this effect after only a short amount of heating time. Within only 3 minutes of boiling, 8 patient urine samples, which initially showed a range of absorbance on SpA ELISA, became uniformly negative, which indicated that the non-specific binding, or protein, is denatured quickly (Figure 56).

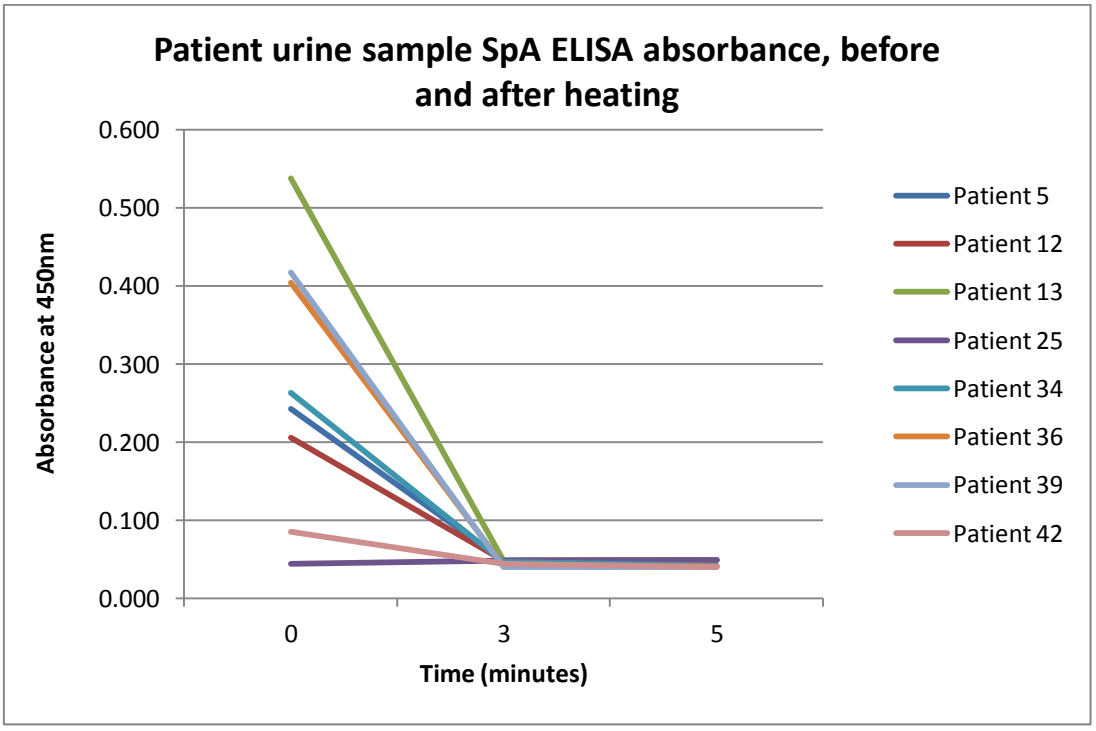


Figure 56- Patient urine samples tested by SpA ELISA, before heating, after 3 minutes heating and 5 minutes heating (including background). Each patient urine sample was tested once at each temperature. SpA ELISA was performed with 0.5µg/ml IgG as capture antibody, blocked in 5% (w/v) milk and incubated in 1:4000 anti-SpA antibody.

This figure shows the complete reduction in absorbance following a short amount of heating at 98^0C.

Concurrent with the collection of urine samples, a single sample of serum was collected at the start of each patient ICU admission. This preparation of SpA ELISA samples by boiling was tested in these patient serum samples, to identify if signal increases in certain samples after boiling. A collection of 40 patient serum samples (1 serum sample per patient) was independently tested for SpA by ELISA, and the same samples were tested for SpA following 10 minutes of boiling. Like the control serum used to constitute SpA standards (Figure 58), each patient sample was diluted 1:5 with dH_20. Following SpA ELISA, prior to boiling, none

of the patient samples displayed an absorbance above that of background (PBS background 0.064- SD 0.005). These patient samples displayed no increase in absorbance following boiling; no samples displayed a significant increase in absorbance from background.

3.4.3 ELISA Conclusions

We have developed a sensitive ELISA for SpA, using IgG as capture antibody, which was capable of detecting commercial SpA down to physiological levels in PBS and control urine. It is also able to be detected in serum by the same method, but only after serum samples are boiled. IgG is suspected to cause a quenching effect on commercial SpA detection in both urine and serum, an effect which is reversed fully by heating.

Initial positive ELISA results in patient urine samples, thought to be due to low levels of endogenous SpA, become negative on heating, indicating that the protein or molecule which caused a raised absorbance in these samples was not heat-resistant. From this, we can conclude that the substance responsible for a raised absorbance in these samples is not the same molecule as the commercially purified SpA.

3.5 Mass Spectrometry Results

In order to identity the substance causing a raised ELISA absorbance in patient urine, an LC-MS method using samples created by in-gel digestion was devised. The aim was to identify the presence of SpA in positive ELISA samples, and to achieve this, 14 patient samples were prepared by on two SDS-PAGE gels. Following staining with Coomassie Blue, a band, selected on the gel at the same MW as commercial SpA, was excised from each patient sample well, and subject to in-gel digestion. Excised bands were sufficiently sized in order to encompass a range of MWs from ~47-53kDa (Figure 57).

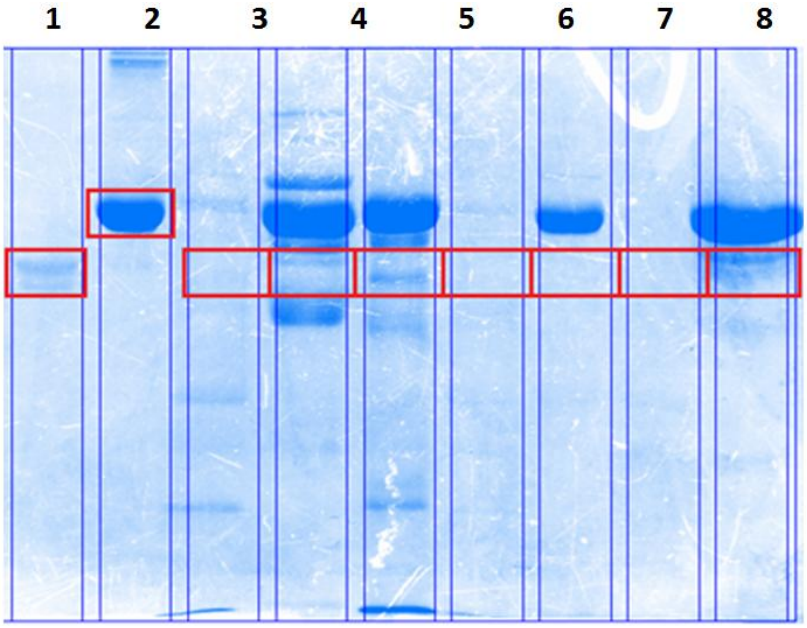


Figure 57- SDS-PAGE gel of SpA and BSA standards, and 6 patient samples, electrophoresed for 1.5 hours. Gels were then stained with Coomassie Brilliant Blue. Red boxes indicate bands excised to form MS samples.

Lane	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Sample	SpA 100µg/ml	BSA 1mg/ml	Patient 1	Patient 4	Patient 6	Patient 9	Patient 10	Patient 12	Patient 15

For Mascot search results, each search result is given a score, and the significance level is a cut-off score above which results have a >5% chance of not being a random event. In this chapter, the score will only be presented if it is significantly higher than this level.

In order to confirm LC-MS as capable of detecting SpA, and what form it is able to detect it, a sample of commercial SpA at concentration 500µg/ml was tested, following SDS-PAGE electrophoresis and in-gel digestion. Also in order to confirm the efficacy of the machine, BSA standards at 1mg/ml were processed and tested with each sample run (Table 11).

Standard Sample	Top Hits	Score	Molecular Weight (kDa)
SpA 100µg/ml	IMMUNGLOBULIN G BINDING PROTEIN A PRECURSOR (*Staphylococcus aureus*)	1153.34	53.7
	Structural Protein A (*Staphylococcus aureus*)	1065	53.2
	Protein A (*Staphylococcus aureus*)	1018	35.1
	Protein A (*Staphylococcus aureus*)	795	38.02
BSA 1mg/ml	Albumin (*Bos taurus*)	2107.87	71.2

Table 11- SpA and BSA standards, from SDS-PAGE and in-gel digestion, with LC-MS protein identification, score and estimated mass of identified proteins.

The results of the SpA standard experiments confirmed the efficacy of the method of in-gel digestion used for processing patient samples, and the ability for the BioTools program to confirm the presence of SpA. Interestingly, the commercial SpA from *S. aureus* strain 8325 was shown to be constituted of different forms of SpA, of different molecular weights. Mainly these constituted SpA at 53kDa and another protein, identified at SpA, at 35kDa and 38kDa.

The top hits found by MS for each patient individual patient sample, tested from the middle of the sample range (14 samples- see Figure 60 and 61), were found to contain no SpA in any form recognised by the BioTools software. The most common detectable protein product, with no search filters applied, was alpha-1 glycoprotein and albumin, two proteins commonly found in urine. Furthermore, only these proteins were above the significance levels for these searches (chance of random detection >5%). When search filters were applied to detect only bacterial protein products, a wide variety of different species molecules were detectable, but with no hits at scores higher than the significance level. This indicated a need for further, more encompassing testing of samples.

The decision was made to test certain patients sample ranges, based on the patients which displayed positive SpA ELISA results, and diagnostic information. These patients were:

1. Patients 13 and 36- chosen as they displayed the highest absorbance on SpA ELISA
2. Patient 18 and 24- both independently positive for Gram-positive bacterial infection, from blood culture and wound swab respectively.

These patients full sample range were run on a single SDS-PAGE gel, and excised as one large band, at MW ~47-54kDa. The top results for these samples are displayed below, with only the significant hits displayed (Table 12).

Patient sample	Top Hits- all results	Score	Top hits- bacterial proteins	Score	SpA Identified?
13	Alpha-1 acid glycoprotein	242.9	Collagen-like protein 3 (*Streptococcus equi*)	57	No
18	Albumin (*Bos taurus*)	468.9	No significant hits	N/A	No
24	Unnamed product protein (*Homo sapiens*)	162.3	Fusion Protein: Heavy Chain (*E. coli*)	75	No
36	Alpha-1 Antitrypsin (*Homo sapiens*)	475.2	Fusion Protein: Heavy Chain (*E. coli*)	148	No
	Alpha-1 Acid Glycoprotein precursor	258.2			
	Immunoglobulin Gamma: heavy Chain (*Homo sapiens*)	126.9			

Table 12- Full sample ranges of patient tested, and band excised at level of commercial SpA. Top hits with scores are displayed.

As stated, these patient samples demonstrated clear detection of expected urinary proteins (such as alpha-1 acid glycoprotein), but no significant detection of SpA. As these samples represented the best chance of detection, based on previous research, this suggests the absence of SpA in these patients at the MW of commercial SpA.

Due to the chance of SpA being present in urine samples at different MW, or small products of SpA breakdown being present lower in the gel, it was decided to create samples for LC-MS which would contain all the products of a urine sample. This was achieved by placing patient samples into SDS-PAGE gels, and applying electrophoresis for 10 minutes only, thereby eliminating small charged molecules and leaving all large molecules and proteins in a single compressed region. This region was excised and halved to create two samples per patient range, which would contain all proteins present in the sample (Figure 58-59).

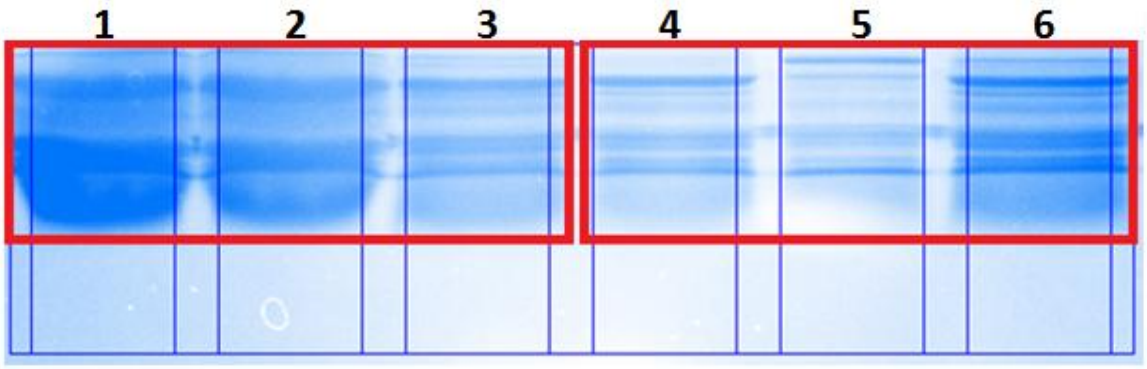


Figure 58- Patient 36 samples on SDS-PAGE to create MS samples for complete urine samples. Patient urine samples were electrophoresed for 10 minutes, before staining with Coomassie Brilliant Blue. Red boxes indicate excision boundaries, the subsequent gel pieces formed became MS samples 36-1 and 36-2.

Lane	**1**	**2**	**3**	**4**	**5**	**6**
Sample	Patient 36, Sample 2	Patient 36, Sample 3	Patient 36, Sample 4	Patient 36, Sample 5	Patient 36, Sample 6	Patient 36, Sample 7

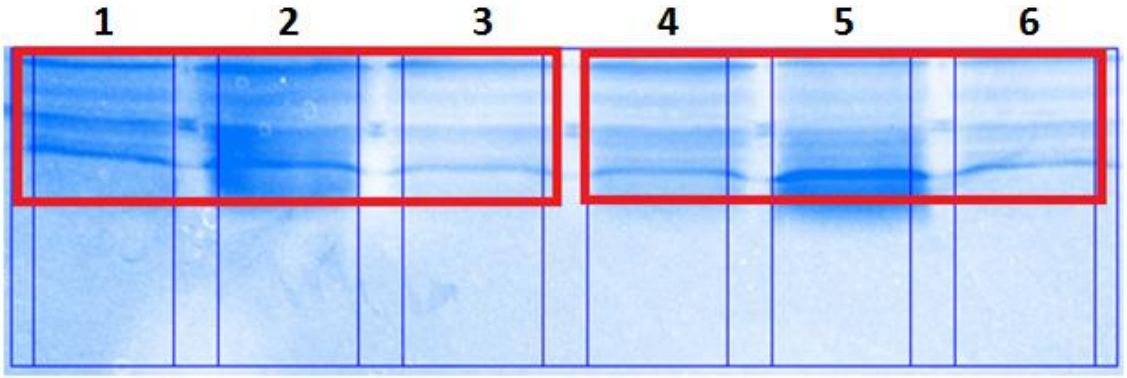


Figure 59- Patient 13 samples on SDS-PAGE to create MS samples for complete urine samples. Patient urine samples were electrophoresed for 10 minutes, before staining with Coomassie Brilliant Blue. Red boxes indicate excision boundaries, the subsequent gel pieces formed became MS samples 13-1 and 13-2.

Lane	**1**	**2**	**3**	**4**	**5**	**6**
Sample	13 (1)	13 (2)	13 (3)	13 (5)	13 (6)	13 (7)

Once these samples were analysed, as expected, many significant hits for urinary protein were found, though again, none of these included SpA. This occured even when anlysis was restricted to bacterial proteins (Table 13).

Patient sample	Top Hits- all results	Score	Top hits- bacterial proteins	Score	SpA Identified?
13-1	Human Albumin	566	Unnamed Protein Product (E. coli)	151.36	No
	Human Serum Albumin	532			
13-2	Serum albumin precursor (Homo sapiens)	1145.7	Unnamed Protein Product (E. coli)	120	No
	Alpha-1 acid glycoprotein precursor	225.2			
36-1	Sequence 92 from Patient US 6663485	627.6	Unnamed Protein Product (E. coli)	91	No
	Human Serum Albumin	534.3			
36-2	Serum Albumin precursor	892	Unnamed Protein Product (E. coli)	76	No
	Unnamed protein product (Homo Sapiens)	837			

Table 13- Full range of samples with all bands excised, allowing the complete sample to be tested. Only top hits and scores displayed.

In summary, these LC-MS sample analyses have shown no evidence of the presence of SpA, in a small sample of patient urine samples. However, as these samples have been chosen specifically for their relation to *S. aureus* infection, and their positive ELISA results, we have given ourselves the highest chance of detecting the molecule. The lack of positive SpA identification may be due to number of factors, which are discussed in the limitations of this study.

3.6 Statistical results and patient characteristics

In order to obtain further information which might help determine the identity of the non-specific binding molecules found in ELISA testing, statistical analysis was performed, looking at clinical and biochemical variables obtained when urine samples were collected. Basic statistics of the 47 patients, such as mortality rates, diagnosis and treatment, were performed by Frances et al.[80]

Statistical analysis was performed using the Stata (Edition 12) program, with inputted data from ELISA experiments and collected clinical data. The aim of this was to reveal which characteristics, clinical or biochemical, were significantly associated with a raised absorbance seen on SpA ELISA, and whether these results indicated a link to infection.

In order to perform statistical analysis, ELISA results were dichotomised as 'positive' (absorbance >0.100) and 'negative' (absorbance <0.100). Absorbance was set at the high level of 0.100, compared to an average background absorbance of 0.038-0.055, in order to ensure all true positives are contained within the positive group. Based on these groupings, basic statistics on SpA ELISA results were collected (Table 14).

Category	Total number of patient samples	Total number of samples tested for SpA by ELISA	Samples with raised absorbance (>0.100)	Samples without raised absorbance (<0.100)
Number (percentage)	**305**	**241 (79.02%)**	**60 (24.9%)**	**181 (75.1%)**

Table 14- proportion of positive and negative patient urine samples on SpA ELISA, based on a cut-off value of 0.100 at 450nm.

Statistical analysis was performed comparing these two groups by continuous variables, related to clinical status. Based on 6 clinical findings, heart rate, respiratory rate, temperature, pH, white cell count and CRP, there was no statistical significant differences in these variables between 'positive' and 'negative' patient samples (Table 15).

Variable	Mean-positive group	Mean-negative group	Mean Difference between groups	Standard Error of the Mean	P Value
Heart Rate	92.268	93.517	1.251	2.546	0.624
Respiratory Rate	19.811	21.624	1.812	1.813	0.128
Temperature	37.436	37.461	0.025	0.11	0.82
pH	7.382	7.393	0.011	0.015	0.454
White Cell Count	13.412	12.936	0.477	0.924	0.606
CRP	211.518	229.86	18.342	20.702	0.377

Table 15- statistical analysis of clinical variables between positive and negative groups on SpA ELISA.

This indicates that the substance which is causing a raised absorbance in SpA ELISA is not related to traditional markers of infection. When patient diagnosis was categorised as infectious or non-infectious, comparison between ELISA result groups showed that there were more non-infective diagnoses within the 'positive' group. This is the opposite result to what could reasonably be assumed, if SpA was the cause of the raised ELISA absorbance, providing further evidence that the results are due to non-specific binding.

Further statistical analysis revealed a strong, significant, association between a raised SpA ELISA result, and evidence for renal failure. These variables were serum urea and creatinine (Table 16).

Variable	Mean-positive group	Mean-negative group	Mean Difference between groups	Standard Error of the Mean	P Value (significant?)
Serum Urea	13.665	9.098	4.566	0.969	0.001 (yes)
Serum Creatinine	169.55	86.209	83.34	12.906	0.001 (yes)

Table 16- variables associated with renal failure, compared between SpA ELISA positive and negative samples, tested by Student's T-test.

Patients with eGFR > 60 (normal renal function), and <60 (renal failure) were split into two groups, and the groups were subject to logistic regression to predict the odds of renal failure being present in samples with a positive SpA ELISA result. Incidence of renal failure was found to be significantly increased in SpA ELISA positive patients (odds ratio: 4.28, p-value 0.001).

In summary, patients with evidence of renal failure were likely to produce urine which caused a raised absorbance on SpA ELISA. However, whether those patients exhibited kidney damage (and therefore glomerular leakage), which would allow large molecules into the urine, is not known. It is also a possibility, given the association with a reduced eGFR, that these patients produced more concentrated urine, implying an increased concentration of protein or other macromolecules which could exhibit non-specific binding with anti-SpA antibody.

4 Discussion

The primary aim of this research was to develop methods to detect commercially sourced SpA via WB and ELISA, and apply those techniques to the detection of endogenous SpA in ICU patient urine samples. Mass spectrometry methods were developed to provide an additional insight into patient samples, in order to confirm the results of WB and ELISA. This information would then be used to assess the viability of SpA as a biomarker for *S. aureus* infection or bacteraemia in the ICU setting. The research to be conducted would involve three main stages:

1. The development of a sensitive WB technique for detection of commercial SpA, and the application of the technique to testing ICU patient samples.
2. The development of a sensitive ELISA technique for detection of commercial SpA, and the screening of patient samples by ELISA for endogenous SpA.
3. Preparation and testing of patient samples by LC-MS.

4.1 Detection of Protein A by Western Blot

Previous work conducted by Yue Han proved that detection of commercial SpA by a WB method is possible,[187] and many other studies investigating the structure of SpA have also utilised this approach to confirm the presence of SpA, for example in cell culture.[114, 117] The work reported here has confirmed this technique as efficacious and specific for commercial SpA detection, using a primary (HRP conjugated) polyclonal anti-SpA antibody. The detection of SpA was possible down to the nanogram range, shown to be physiological relevant, based levels of other Staphylococcal urinary toxins (TSST-1, SEB) quantified by Harrison et al.[79] Solutions containing 0.1µg/ml SpA produce a reliable and strong signal on WB, while there is variable and weak detection of solutions of 0.01µg/ml SpA. These concentrations represent a total of 7ng – 0.7ng of SpA per sample. The higher of these two figures was set as the lower limit of detection when applying the method to patient samples, as it could be certain that 0.1µg/ml SpA could be detected even in less than optimal sample conditions.

During the course of method development, we observed a number of issues which required resolving prior to using the method to test patient samples; these included variations in

commercial SpA band size seen on WB, differences in signal strength of SpA when diluted in buffer or urine, and the effect of antibody incubation in milk or BSA.

4.1.1 Differences in Protein A size and bands

The commercially sourced SpA used in WB standards was purified from cell culture of SpA-producing *S. aureus* strain 8325, using an IgG-affinity column. This was documented in the product information as MW 42kDa; however, as seen in our SpA WB (e.g. Figures 16 and 17), the product was detected as two distinct bands at the larger size of ~50-55kDa. We initially thought this could be due to phenomenon known as 'gel shifting', a phenomenon primarily seen when cell membrane proteins are subject to electrophoresis in SDS-PAGE gels, and can result in bands at MWs higher than expected.[189] However, other reports, in the literature, including genetic studies on SpA secretion by strain 8325, established the MW of SpA as ~58kDa.[115] This was confirmation that our WB results were displaying the correct MW for the particular *S. aureus* strain, though 'gel-shifting' may explain slight variation in MW seen between blots.

Commercially sourced SpA was consistently identified as a double band upon examination by WB, with the higher MW band displaying marginally increased signal, implying a higher concentration (e.g. Figure 20). These multiple bands were initially believed to be contamination from a separate protein; IgG was a likely candidate, as this was used to purify the SpA from culture, and the heavy chain of IgG is of approximate MW of 50kDa.[188] However, LC-MS investigation of multiple SpA bands, separated by electrophoresis, showed that each band contains SpA, but of different MWs (38-55kDa). This experiment also excluded the contamination of IgG (or other proteins) in the commercially sourced SpA.

One explanation for multiple bands of the same protein is protease degradation during storage of SpA, which can cause of proteins to run at a different MW or appear as a double band.[190] However, another possibility, backed by evidence in the literature, is that the commercial SpA culture medium contains different variants of SpA.. One study identified an enzyme (LytM) which actively cleaves SpA from the cell wall, producing a secreted form of SpA which included peptidoglycan amino acid residues; these residues contribute approximately 3-5kDa to the whole SpA molecule.[137] Another study by O'Halloran et al, into the structure of secreted forms of SpA, discovered that the protein can be found secreted

without evidence of prior incorporation into the cell wall, with precursor molecule regions (e.g. C-terminal sorting signal) unprocessed.[139] That study found that this purely secretory form of SpA measured at 51.9kDa, while variants of SpA, displaying prior inclusion to the cell wall, measured between 52kDa and 54kDa. This is evidence that SpA can be found in a number of forms in culture, at different MWs. The commercial purified SpA used in our WB experiments is from a single culture source, we suggest that we are seeing a mixture of cell-wall associated SpA and a secreted variant, also explaining the identification of both bands as SpA by LC-MS.

4.1.2 Effect of sample solution on detection of Protein A

In the WB method optimisation stage, signal attenuation of commercial SpA was noted when SpA was examined in a control urine solution, as opposed to PBS (e.g. Figure 22). As no studies have been identified that have attempted to detect SpA in urine by WB, this is a novel observation. Previous work on WB development for the detect Staphylococcal toxins by Frances Price (MSc 2013), showed no attenuation in the signal of purified toxin in control urine compared to PBS.[80]

We theorised that a component of control urine may be contributing to the reduced signal, as both urine and PBS SpA samples were prepared identically and to the same concentration. A molecule which is known to bind to SpA and is present in healthy urine is IgG, which is also found along with small amounts of IgA and IgD.[191] SpA ELISA results show that there is approximately 0.4-0.5µg/ml IgG in the control urine used for creating WB samples. The interaction between SpA and IgG may affect the ability for anti-SpA antibodies to bind to the protein once transferred to membrane, potentially reducing signal on WB. However, the mechanism by which this effect occurs following heating and coating of samples in SDS, which should eliminate SpA-IgG bonds, is not known. It was decided to attempt to replicate the potential quenching effect of IgG on SpA, in PBS.

Despite the repeated observation of a quenching effect in control urine, this was not reproducible by adding 1µg/ml commercially sourced human IgG to sterile PBS samples containing SpA. This result confirms that a collection of human IgG, at higher concentrations found in urine, is unable to reproduce this effect. Further experiments demonstrated that patient urine, which can contain up to 10 times the amount of IgG than healthy urine (as

demonstrated by WB and ELISA), showed no quenching effect on samples when spiked with SpA (Figure 19- 20). Ultimately, this attenuation in SpA signal was not reliably reproduced by WB of patient samples, and IgG levels did not appear to influence the effect. It was decided that we could not conclude the reason for this difference in signal between different standard solutions.

4.1.3 Optimal blocking and antibody solution for Protein A detection

In WB method development, important factors to consider are the blocking buffer, and the subsequent concentration of antibody solution, which is constituted in the same blocking solution. These factors proved to greatly influence the sensitivity and specificity of the WB technique developed to detect SpA.

Firstly, blocking solution was compared in WBs of commercial SpA, between BSA (2% (w/v) or 5% (w/v)) and 5% (w/v) milk. BSA was initially chosen as the blocking and antibody solution of choice, due to its purity; it is made up of a single serum protein as opposed to milk, which is a mixture of proteins and other macromolecules. Literature evidence also shows that milk, due to the presence of casein, could interfere with the detection of antibodies by ECL.[190] During initial WB experiments detecting purely SpA standards, BSA-blocked membranes demonstrated lower background signal. However, when adapting the method to testing patient samples, it was revealed that blocking and incubating in 5% (w/v) milk greatly reduced the additional bands seen in patient samples. This was restricted to ladder proteins and patient urinary proteins, with 0.1µg/ml SpA standards still displaying strong signal (e.g. Figure 38). By exchanging milk for BSA in the antibody incubation stage of WB, we developed a specific test for SpA.

Literature describing this increased blocking potential of milk is scanty, with most sources describing equal efficacy for both milk and BSA as blocking buffers,[190] or reduced background and increased sensitivity when using BSA.[192] However, we found that the elimination of non-specific binding was only seen when milk was used as antibody solution (not blocking specifically). Milk may be responsible for this effect in a number of ways:

1. Increased variety of proteins in milk- as stated, milk contains a mixture of proteins, including casein and lactoglobulin, which allows a greater variety of blocking sites. Given the wide variety of potential proteins in patient urine, including plasma

proteins, immunoglobulins and enzymes, this may make milk a very effective blocking substance for WB of this matrix.

2. Competitive binding with anti-SpA antibody- as we have demonstrated, the reduction in non-specific binding does not occur during the blocking stage, but is specific to the antibody incubation stage. This implies a degree of interaction with anti-SpA for the blocking effect to be achieved, which could be of a competitive nature. Anti-SpA may be directly inhibited from non-specific binding sites by the presence of milk proteins, or, it may be that anti-SpA antibodies bind to a milk protein preferably to the non-specific urinary proteins. Given that that the sensitivity of the WB for SpA standards remained unchanged by incubating antibody in milk, the level of interaction between milk protein and antibody would have to not interfere with commercial SpA binding.

4.2 Detection of Protein A by Western Blot- sample experiments

When applying the optimised SpA WB method to testing patient samples, it was decided to perform screening experiments, using a sample from each patient, followed by in-depth experiments on certain patients most likely to produce positive results. Following this series of WB tests, no positive detection of SpA was achieved, meaning no bands of proteins were detected in patient samples, at the correct MW for commercial SpA, or at any other MW.

During initial screening, using BSA as blocking and incubating solution, multiple bands of different MW were detected in patient samples (e.g. Figure 25- 26), which showed promise that SpA might be present in some form in patient urine. However, for these bands to be SpA, it would have to be at a variety of MWs not documented in the literature. The highest estimate for *S. aureus* toxin rates in the same patient samples was 72% (specifically for Staphylococcal AH),[80] and as almost every patient sample displayed a band using the non-optimised WB method, these additional bands were unlikely to all represent a form of SpA. This was confirmed during optimised testing (using milk), as none of these additional bands were detected, in any patient sample.

The lack of detected SpA, even those later found to be positive on SpA ELISA, can have arisen for a number of reasons. Firstly, there may be no SpA present in these samples. This is a likely eventuality, as corroborating evidence (e.g. LC-MS) also concur that no SpA is

present in patient samples. However, given a reliable detection limit of 0.1µg/ml (~7ng) SpA, it may be that SpA is present, but at limits below that which is detectable by WB. Physiological levels of regular urinary proteins (e.g. albumin) is around <300µg/ml,[193] but bacterial toxins and other excreted products can be significantly lower; Harrison et al estimated levels of Staphylococcal toxins in the range of nanograms to picograms (e.g. 1-0.1ng).[79] If SpA is present in samples at the picogram level, then our WB method would not have sufficient sensitivity to detect it.

Some patient samples were later estimated to contain levels of SpA which approach the detection level of WB, such as patient 13, indicating that SpA bands could be detected by WB. This patient's sample range demonstrated an ELISA result corresponding to a level of approximately 15ng/ml (1.5ng total), but a repeat WB of this patient samples did not yield any positive bands.

Finally, it may be the case that SpA is present in patient urine at appropriate levels for WB detection, but in an altered form or variant which is unable to bind to the primary antibody used. Given the previously discussed range of SpA variants, depending on strain and protein secretion method, it may be that SpA is present in urine in one such form. Using a primary antibody known to bind to a different epitope of SpA may be helpful in ruling out this eventuality.

4.2.1 Optimal sample preparation and method

A series of experiments were carried out in order to optimise the preparation of patient samples for WB testing, to allow an effective screening process for SpA. This process elucidated important information about the patient samples which were carried into the screening and testing.

Many patient samples carried a substantial amount of solid material suspended in the urine. This is seen amongst urine collected from patients on ICU wards, due to sloughed material from the urinary tract, as well as contaminant material from urinary catheters. However, it was not clear as to how to prepare samples containing substantial material; either the solid was to be avoided, to reduce non-specific protein contamination of WB, or it should be mixed in with the liquid urine, to obtain greatest chance of detecting SpA. We carried out an experiment testing these two methods of preparation found that the vast majority of

material detectable by SpA WB, which was later thought to be mostly non-specific proteins, is contained within the solid particulate matter. It was decided that in order to give the best chance of finding SpA in patient urine, this proteinacious material should be incorporated in WB samples, by vortexing. SpA, if present in patient urine, may have been bound to a number of antibodies, which may have experienced aggregation with other proteins in the samples. This method of sample preparation also extended to SpA ELISA sample application, and LC-MS sample analysis.

4.3 Conclusions from Western Blots

An effective and specific WB method, developed to detect commercial SpA, was unable to detect any positive results in a wide range of patient samples which had previously shown to be positive for AH, SEB, SEC and TSST-1 by WB methods. This does not rule out the presence of SpA in these samples, or in its presence in urine in a form undetectable by WB. The development of an ELISA with higher sensitivity for SpA would aid in interpreting the negative WBs.

4.4 Detection of Protein A by ELISA

An ELISA method was developed for the detection of SpA, utilizing the strong SpA-IgG binding affinity, by using IgG as capture antibody, followed by detection of IgG bound SpA by use of anti-SpA antibody. During optimisation, it was found that primary antibody at 1:4000 concentration, and using milk as a blocking agent and antibody solution, lead to the greatest useful range of SpA detection. Using this method, we were able to detect commercial SpA diluted in both PBS and control urine; this was a sensitive and specific test for SpA, with a linear detection range of 0.78-25ng/ml SpA. Background, in both PBS and control urine, was consistently low.

Screening of patient samples by this method was promising, with 25% of patient samples displaying an increased absorbance, the majority containing potentially low levels of SpA, e.g. 1-5ng/ml. In each ELISA microwell, 100µl of sample is added for testing, meaning that the total amount of SpA potentially being detected in sample is one tenth of this concentration, equalling 0.1-0.5ng. These protein levels are in keeping with physiological levels of Staphylococcal toxin found in patient urine by work carried out by Harrison et al,[79] and Frances Price (MSc 2013).[80] Not only did concentrations of antigen correspond to

previous studies, but the percentage of positive samples fell within the range of samples estimated to be positive for other Staphylococcal toxins (5.9-72%).[80] If these results were proven to be specific for low levels of SpA, this represents a sensitive technique for detecting a Staphylococcal surface protein in a novel matrix.

However, we were aware of a number of methodological shortcomings which may call into question the specificity of this ELISA. These include the use of a mixture of human IgG as capture antibody, and detecting SpA with a primary anti-SpA antibody known to be capable of non-specific interaction with urinary proteins (shown on WB). This raises the suggestion that the positive results we see on SpA ELISA may be due to non-specific interaction, which prompted a series of experiments to confirm the specificity of this SpA ELISA method.

4.4.1 Tests of protein and IgG content

Unlike using an antigen specific antibody, using IgG as capture antibody means that a number of different proteins capable of binding to IgG, could be present in the microwell plates when primary antibody is added. This presents the chance of non-specific binding, as human IgG is capable of binding to a host of antigens and proteins. In order to find out if the presence of additional proteins attached to IgG is the cause of the increased absorbance on SpA ELISA, an experiment was carried out to estimate the levels of the two most likely candidates causing cross-reactivity; IgG and total protein.

Following a further stage of method development, an IgG ELISA was underdone which measured directly adsorbed IgG (direct ELISA). Interestingly, the initial section of our method development discovered that using SpA as a capture antigen for IgG, which has been suggested as efficacious by other studies,[194] proved to be non-specific and unreliable for commercial IgG estimation.

A select number of patient samples were collated, based on previous ELISA results, in order to test a cohort of the most positive patient urine samples on a single microwell plate, as well as negative patient controls. These same patient samples were retested for SpA, along with a fresh test for IgG and total protein content. We identified no statistical difference in IgG and total protein absorbance between positive and negative patient SpA groups. The sample size for each group was small (n=15 positive group, n=18 negative group), lending these tests limited statistical power. Despite this, it was found that certain samples with

large amounts of IgG and protein displayed very little absorbance on SpA ELISA, confirming that the positive results seen on this ELISA were not solely influenced by these factors. We propose this as evidence of a high specificity of the SpA ELISA method, and if the positive SpA ELISA results are in fact the result of antibody cross-reaction, this is likely due to a specific urinary protein, not level of total protein or IgG. Further work needs to be done in order to confirm the specificity of the anti-SpA antibody, and therefore confirm the SpA ELISA as a viable test.

4.4.2 ELISA of boiled samples

As discovered from previous WB and ELISA, as well as the literature, IgG is present in both healthy and patient urine. Previous studies have theorised that the presence of IgG in human samples, such as serum, interferes with immunoassays for SpA by binding to SpA and blocking binding sites.[183, 184] We were able to corroborate these findings by attempting to detect SpA in healthy human serum, which showed a large quenching effect, large enough to almost completely mask the SpA in the serum (Figure 63). The absorbance of SpA standards became co-incident with PBS standards following boiling of the serum SpA samples, which would provide the energy to break apart SpA-IgG bonds. This quenching effect was seen at a greatly reduced level in control urine, but an increase in sensitivity was still observed following boiling of urine (Figure 62).

So far, this information was consistent with the nature of SpA-IgG binding, though this proves that saturation of SpA binding sites can occur in IgG solutions, and this effect is dose dependent. Also, importantly, these boiling experiments proved that the commercially sourced SpA used to create the standard ELISA curve is heat resistant. When ELISA-positive patient samples were boiled, we expected to see either an increase, or no change, to absorbance of patient samples. As our ELISAs show, this did not occur, and all patient samples became uniformly negative following boiling. This means that the molecule, or protein, which was causing the raised absorbance, was denatured fully after boiling after 3 minutes.

We propose that any SpA produced during the course of infection should remain heat resistant following excretion into urine; therefore, the positive ELISA results are most likely not due to the presence of SpA.

This leads to the primary question arising from our ELISA results; what have we detected by this technique, if not purely SpA? The most likely answer is a different protein which is denatured by boiling, but other options include particular antibody complexes or whole cells, or cell fragments. If these are present in the urine, unaltered, they can be immobilised by IgG in the first step of the ELISA, and subsequently picked up non-specifically by anti-SpA. The substance which is binding to both the IgG and anti-SpA in the ELISA, is unlikely to be a pure form of SpA released by *S. aureus* infection, a conjecture which is backed up by the results of both LC-MS of positive samples, and also statistical analysis displaying a lack of association with infective status.

4.5 Protein A detection by LC-MS

By using LC-MS techniques, our aim was to rule out, or confirm, the presence of SpA in patient urine samples, by using targeted in-gel digestion sample preparation. By excising and digesting visible bands of commercial SpA on an SDS-PAGE gel, we were able to confidently identify SpA. These data confirmed the viability of an in-gel digestion method for detecting Staphylococcal toxins, and also identified the presence of SpA variants, of differing MW, contained within the commercial SpA product. This confirmed previous speculation on the cause of the SpA double-band consistently seen on WB.

Following this positive identification of the target protein, we tested a range of patient samples by excising bands at the approximate MW of commercial SpA. This targeted approach aimed to avoid the detection of more abundant urinary proteins, present at different MWs. However, none of the sample analyses by LC-MS provided identification for SpA, including testing whole samples which were positive by ELISA. The lack of detection could not be attributed to preparation or processing errors, due to the identification of commercial SpA and BSA, analysed concurrently with samples, as well as significant detection of known urinary proteins such as albumin and alpha 1 acid glycoprotein (orosomucoid) in the patient samples themselves.

The method devised to test full samples, using a limited amount of electrophoresis to create a single large band of all proteins, allowed the identification of most abundant proteins in a sample. This did not detect SpA, only finding *E. coli* proteins at significant levels. These proteins may be from the urinary tract, urinary catheter or catheter bag, or from

contamination while handling samples. Waiting time between sample preparation and processing by MS was minimal (<1 day), so it is unlikely to be from Gram-negative growth from storage. This full sample testing method provides a promising route of investigation for urinary Staphylococcal antigens. However, to take full advantage of this method, a machine capable of detecting proteins at very small concentrations would have to be used.

The use of LC-MS in indentifying Staphylococcal toxins is a technique used extensively in the investigation of the *S. aureus* proteome, using either in-gel digestion techniques, or whole cell culture sample digestion, to catalogue the proteins found in bacterial culture.[195] The use of LC-MS to detect antigens in human samples has not been studied extensively, so could represent a potentially fruitful area of research. However, the presence of other bacterial proteins from the urinary tract or, or contamination from handling of samples, as well as the large amount of other urinary proteins, are issues which complicate the detection of scarce bacterial toxins.

4.6 Statistical Analysis

A basis for statistical analysis on the patient cohort was formed by characterising ELISA results as positive (1) or negative (0), based on a cut-off absorbance of 0.100, which would correspond to a hypothetical concentration of 0.1ng/ml SpA. Using this cut-off absorbance was deliberately high (>10 SD away from background absorbance), due to the likely chance of non-specific antibody interaction marginally raising absorption. Choosing a high cut-off was a strategy to avoid potential false positive results being analysed within the SpA positive group. This created a positive group of patient samples, which consisted of 25% of samples tested. Statistical analysis, performed by dichotomisation of samples into positive and negative and testing mean difference, would help to highlight any factors which may be contributing to a raised absorbance in these samples. Identifying a relationship between SpA ELISA result and infective status of patient was also an important aim for this research.

To achieve this, markers of infection (HR, RR, BP, temperature, pH, WCC and CRP) were compared between the two groups, then analysed by Student's T-test and logistic regression. Results showed no significant differences between the 25% of patients with positive ELISA and the rest of the sample population. As the raised absorbances seen on SpA ELISAs are strongly suspected to be of a non-specific nature, these findings are further

indication of these results being false positive. Previous work on this patient cohort, by Frances Price (MSc 2013) had revealed a weak association between markers of infection and presence of Staphylococcal toxins (particularly AH). This is in keeping with previous work on ICU patient status and urinary Staphylococcal toxins, by Azuma et al, which found a greatly increased incidence of toxins in patients with confirmed sepsis (42%), compared to other diagnosis (6%).[76] Based on this, it should be expected that a positive SpA ELISA, detecting endogenous SpA, would relate to increased markers of infection. However, this particular patient group was collected from a general ICU population, without prerequisites for sepsis or infection, making the relationship of the ELISA results to infective status difficult.

However, despite small patient numbers with an infectious diagnosis (n=22, 49% of total samples), and a smaller number with confirmed sepsis (n=6, 13% of total samples), limited conclusions can be drawn based on this diagnostic information. These mostly concur with the absence of SpA in patient urine. Of the 6 patients with confirmed sepsis, 3 produced samples with a significantly raised ELISA result. Two patients at highest risk of *S. aureus* infection, 18 (Gram-positive blood culture) and 24 (Gram-positive wound swab), were negative for SpA in all modes of testing; WB, ELISA and LC-MS. The patients with the most positive ELISA results, 13 and 36, were admitted with non-infectious diagnosis, diabetic ketoacidosis and bowel perforation respectively. Admittedly, these are single case examples and a number of factors could have influenced this pattern of ELISA results (e.g. urinary contamination, infection during ICU admission), but does provide some evidence against the SpA ELISA for predicting *S. aureus* infection in the ICU setting.

Statistical analysis did reveal a significant association between the positive ELISA result group, and evidence of renal failure, including raised serum urea, creatinine and a reduced eGFR. This could be a result of antigen deposition in the kidneys, an event which is known to occur with Staphylococcal toxins,[196] TSST-1,[197] and also Streptococcal toxins.[198] This leads on to the prospect that the anti-SpA antibody used in ELISA is detecting SpA in a form of SpA-IgG complexes, which are known to form in response to SpA in the bloodstream.[175, 176] These would be large molecules, which would only be able to be excreted in the event of glomerular basement membrane leakage. However, for this to explain the our positive SpA ELISA results, it would depend on the SpA-IgG complexes being detected by an anti-SpA antibody, but not the unbound SpA molecule which would be released by boiling. We

believe this is unlikely, as it would involve the alteration of the SpA molecule by interaction with IgG, which is not known to occur.

It has been shown that proteinuria, caused by damage to the glomerular basement membrane, increases over the course of chronic renal failure,[199] as well as being a direct result of kidney damage in diseases such as acute tubular necrosis.[200] As we have clear evidence of the lack of SpA in patient urine from WB, and to a more limited extent, LC-MS results, the presence of proteins causing non-specific binding is the more likely explanation for the link between raised absorbance in SpA ELISA and markers of renal failure.

4.7 Limitations of this study

Given the negative outcome of this study in terms of identifying a novel antigen target for *S. aureus* infection status, it is important to highlight areas in which research required improvement, before ruling out SpA as potential testing candidate. While we have developed a sensitive and specific ELISA, as well as a specific (but less sensitive) WB, certain aspects could be improved to optimise these methods further.

4.7.1 Antibody specificity

As demonstrated during the WB method development, and during preliminary work by this laboratory, polyclonal HRP-conjugated anti-SpA antibodies have the potential to exhibit extensive non-specific binding to various proteins which may be found in patient urine. While through the process of this research we were able to remove much of this effect in WB, through the use of lower amount of antibody diluted in 5% (w/v) milk, there remained the possibility that non-specific binding could have been reducing the sensitivity of the WB technique. The use of an HRP-conjugated primary antibody ('direct detection' WB) could also be associated with reduced sensitivity as signal amplification is less pronounced without the use of a primary-specific secondary antibody.[201] However, using a secondary antibody (such as HRP-conjugated anti-rabbit IgG), has been associated with an increased background and increased likelihood of non-specific binding.[202] It was decided that the detection of SpA on WB to a concentration of 0.1µg/ml (7ng per sample), was a sensitive enough detection level to rule out the presence of SpA, however, we were unable to rule out whether an indirect WB method would provide a greater level of sensitivity.

In the ELISA experiments, antibody specificity is likely to have contributed to the raised absorbance results seen in patient samples. Due to the lack of protein separation by SDS-PAGE electrophoresis, a positive result seen in ELISA may be due to high-protein levels in the samples, especially in ICU patient urine. Because of this lack of protein differentiation, it has been recommended that ELISA is best utilised when using an antibody with high specificity to the target antigen.[203] As the antibody used in these experiments has shown to be capable of exhibiting non-specific binding, it would be difficult to rule cross-reaction as a complicating factor in interpreting results. Further experiments to demonstrate the specificity of a variety of anti-SpA antibodies, monoclonal and polyclonal, would be required in order to improve the development of an effective SpA ELISA method.

The direct comparison of antibody binding in WB and ELISA is difficult due to the differences in sample preparation between the methods. Antibody-binding in ELISA samples occurs in more natural conditions than in WB; this is due to the SDS-coating process which is integral to the separation of proteins on SDS-PAGE. During WB sample preparation, SDS binds to hydrophobic regions on proteins and induces 'reconstructive denaturation', forming proteins into alpha-helices.[189] While it may be that the anti-SpA would have had more available sites of binding in WB samples due to this denaturation process, unaltered urine may have contained more large contaminants, such as complexes, biofilms or whole cells, which may have facilitated antibody binding, or produced false positive ELISA results.

Regardless of sample preparation, the fact remains that the primary antibody used was a polyclonal IgY, meaning the potential exists for a monoclonal anti-SpA antibody to be more specific, if not necessarily more sensitive.

4.7.2 Patient sample group

For this research, as well as the earlier work by Frances Price (MSc 2013), 305 urine samples, from 46 patients, were collected for this research from the local ICU ward at Royal Lancaster Infirmary. In order to test potential candidate molecules for *S. aureus* antigen testing, a wide selection of patients, with varying diagnoses, was included in the sample series. However, despite providing the opportunity to test antigen levels in a realistic hospital population, it may be the case that the amount of samples available to test did not provide a higher enough incidence of *S. aureus* bacteraemia or transient colonisation, in order to detect SpA

in sufficient quantities. Previous published work on detecting an antibody response to SpA in serum has been limited to patients with confirmed *S. aureus* endocarditis,[186] which would guarantee the highest chances of detecting Staphylococcal toxins.

To fully assess the data found by this research, the small sample size must be taken into account. A relatively small number of patients, and specifically from the ICU department, may limit generalisation to the rest of the hospital, or general, population.

Roughly 80% (241) of the available samples were tested by ELISA, and substantially less by LC-MS, which was primarily undertaken in order to confirm the constituents the urine samples which were positive on ELISA. While we can be reasonably confident that our ELISA results are influenced by non-specific antibody binding, in order to confirm the absence of SpA in the ICU patient samples, a more complete screening of samples, using the LC-MS method capable of detecting proteins of low concentration, would need to undertaken.

4.7.3 Mass spectrometry sensitivity

Interpreting LC-MS data, specifically the ability to rule out the presence of SpA in patient urine samples, must take into account the sensitivity and resonance of the MS machine. The sensitivity of the instrument used in this research was capable of generating an identification score of >1000 for BSA, when a sample of 1.5pmol of BSA was analysed, indicating a very high sensitivity. Positive identification (e.g. scores of ~100-1000) of very small amounts of a target molecule could be 'masked' by the abundance of other proteins within the samples. This ability to discern wide ranging concentrations of proteins in a sample is known as the dynamic range. Unfortunately, this figure is low in our MS instrument, measuring approximately 10^2 to 10^3, meaning any molecule with concentration less than 1-0.1% of a sample will not be (significantly) detected. This represents a significant problem in interpreting MS data of full patient samples, given the amount of total proteins present in some samples, hence the requirement to run samples on an SDS-PAGE gel and excise the band of desired MW range. While this may reduce the amount of additional protein in a sample, the problem of SpA variants of different MW going undetected remains an issue.

Also of concern is the MS resonance, which is the degree in which peaks of similar weight can be distinguished as separate proteins. As commonly identified urinary proteins include

albumin (MW 66kDa) and orosomucoids (MW 22-25kDa), there is reduced chance of interference with the entire SpA protein (MW 50-55kDa). However, if SpA is present as a smaller breakdown product, or in a variant at different MW, the MS resonance may be playing a role in the negative SpA identification seen.

As such, our findings from LC-MS data should be analysed with the detection limits of the machine taken into account, when confirming, or ruling out, the presence of a protein. This is particularly the case in test solutions containing a high number of additional proteins, and if the target protein or toxin is believed to be present at very low concentrations (e.g. nanograms).

4.8 Wider Implications

In an attempt to locate SpA in ICU patient urine by a variety of commonly used biomedical techniques, we have developed a sensitive and specific WB for SpA, a sensitive ELISA for SpA, and a method for screening patient urine samples for bacterial proteins through in-gel digestion and LC-MS. Despite this, we have been unable to detect any SpA by WB and LC-MS, or confidently rule out the chance of false positive ELISA results.

Previous work, by other groups and this laboratory, has revealed the presence of Staphylococcal toxins in the general and hospital population. Healthy infant urine displayed a pyogenic toxin rate of ~10%,[79] indicating early exposure to *S. aureus*, while Frances Price (MSc 2013) found a high proportion of exotoxins in the same cohort of ICU patients, especially AH (72%).[80] If these results are indicative of *S. aureus* colonisation or infection, then SpA should be present in these same patients. Aside from methodological factors, such as non-specific binding, the fact that these results do not correlate with previous findings may be due to:

- Lack of urinary excreted SpA- SpA is shown to induce IgG antibody responses in the host, which increase rate of SpA elimination from the bloodstream,[175] and is known to involve primarily the liver and spleen.[174] These are common sites of phagocytosis, which serves the function of removing unwanted serum components, the breakdown products of which are able to be excreted in the urine. However, it may be the case that SpA is entirely digested in the liver and spleen, and the eventual excreted product bears no resemblance to the original SpA molecule. SpA-IgG complexes are too large (~200kDA) to pass through the

intact glomerular basement membrane, so it is reasonable to suggest that most SpA goes through a stage of processing before eventual secretion.

- Low production of SpA by *S. aureus*- studies attempting to quantify the amount of SpA produced by *S. aureus* strains are limited, however, an early study produced a figure of 1.6% of cell culture mass of *S. aureus*, which gives an indication that production can be significant.[104] However, different *S. aureus* strains produce variable amounts of SpA,[135] making the possibility that even if infection was present, sufficient quantities of SpA might not be released in order to be detectable. Genetic control of SpA production indicates that there is up-regulation of SpA during times of low bacterial load,[204] which implies that SpA may be found in higher proportions during early infection or healthy colonisation. However, levels may also rise during times of Staphylococcal cell lysis (releasing cell wall SpA), and secretion of SpA also differs between strains.[106] Therefore is it difficult to accurately suggest when during the course of *S. aureus* infection SpA production is maximal.

4.9 Future work

Previous discussion has illuminated areas of this research which could be continued, along with new areas of research which would provide more information on the presence of SPA in the human body. For example, the SpA WB developed by this research could be extended to testing urine from different patient populations, where the concentrations of SpA may be significantly higher, such as *S. aureus* soft tissue infection or endocarditis patients. The concurrent collection of serum and urine samples from such patients would allow the testing for SpA in both, in order to learn more about SpA excretion from the body.

4.9.1 Further patient sample testing

While the search for SpA in ICU patient urine may have proved ineffective by WB, future work could focus on ELISA of other patient samples, given the increased sensitivity of this method. Evidence in the literature proves that antibody responses to SpA can be elucidated in the bloodstream of bacterial endocarditis patients, using a sensitive and specific ELISA technique for anti-SpA.[186] This demonstrates that SpA, either extracellular or cell-wall bound, is located in the bloodstream during active infection. Furthermore, the ability for *S. aureus*, particularly antibiotic resistant strains, to secrete SpA,[117] provides a basis for more widespread serum testing .The ability to detect an antibody response, or to directly detect

SpA, in the serum of ICU patients could provide a viable biomarker for the presence of *S. aureus* in the bloodstream.

As our patient population contains no confirmed *S. aureus* bacteraemia or sepsis cases, this research would involve the collection of serum samples from patients with these conditions. Development of WB and ELISA tests optimised for anti-SpA in serum could also be developed, though this matrix may prove more difficult than urine due to high protein content of serum samples.

However, the potential benefits of a serum SpA test are great; the identification of SpA, or response to SpA, in the bloodstream could provide rapid information on the presence of *S. aureus* in the actual location of importance in sepsis, bypassing the need to take renal excretion into account. Aside from diagnostic information, the frequent use of invasive interventions on ICU wards exposes patients to the risk of bloodstream infection, and a rapid serum test for SpA could prove valuable in determining if *S. aureus* exposure has occurred. However, the disadvantage of a serum test is the invasive route required for sampling, over the ease and non-invasive nature of urine testing.

Another potential candidate for future sample testing would be nasal isolates taken from ICU patients, followed by culture and WB testing for SpA. As ~30% of the population is colonised by *S. aureus* at any one time,[205] this could provide useful quantification information into SpA production in patient ICU population, compared to the general population.

4.9.2 Continued mass spectrometry of patient samples

The LC-MS method developed to test entire patient samples (e.g. Figure 63- 64) carries potential for cataloguing the constituents of the unique subset of ICU patients. The samples tested in this research revealed a number of human proteins traditionally found in urine, as well as some significant evidence of *E. coli* proteins. As discussed, these may be due to contamination, either from sample preparation or from the storage and collection of urine from the catheter. However, these preliminary findings prove the potential effectiveness of this LC-MS technique in screening this sample population for bacterial toxins, if features such as dynamic range and resonance are improved.

Future work could aim to establish the presence of other infectious biomarkers by MS, and determine whether these relate to, or predict, infection status. Ideally, these samples should be analysed with an LC-MS machine with greater ability to detect proteins of lower concentration. Follow up experiments could attempt to confirm these markers using the biochemical tests used in this research; WB and ELISA.

5 Conclusion

Staphylococcus aureus is a common cause of serious infection on Intensive Care wards, and ensuring prompt treatment of bloodstream infection is important in avoiding potentially fatal outcomes. Techniques involving urinary testing by ELISA have allowed swift diagnostic tools for a variety of infections, such as pneumococcal and *Leigionella* pneumonia.[73] Recent research by this laboratory has revealed the presence of Staphylococcal toxins and superantigens in ICU patient urine using similar techniques. We decided that it would be beneficial to test these samples for the presence of additional Staphylococcal antigens, with the aim of ascertaining whether a suitable diagnostic test for *S. aureus* infection could be developed in the future.

Staphylococcal Protein A is an abundant surface protein found in almost all *S. aureus* strains. It has been shown to play a role in overt *S. aureus* infection and also promoting healthy carriage of the organism,[205] and it is conceivable that its detection in urine could provide a marker for these conditions.

Our aim was to develop methods for detecting SpA by Western Blot and ELISA. These laboratory techniques were optimised for the detection of commercial SpA spiked in buffer and urine, which included work to reduce the cross-reactivity of anti-SpA antibody. However, when our optimised Western Blot was performed on patient urine sample ranges, we could not detect bands at a MW which could correspond to SpA.

Subsequently, an effective ELISA using IgG as capture antibody was developed, which proved more sensitive than Western Blot, allowing detection of SpA levels as low as 0.78ng/ml (0.07ng total SpA). Once applied to patient sample screening, this technique identified a raised absorbance level in approximately 25% of the ICU urine samples, suggesting the presence of SpA. However, follow-up experiments indicated that non-specific

binding of SpA antibody may have contributed to the increased absorbance levels seen in these samples. For example, whilst commercial SpA spiked samples retained their levels of SpA following heat treatment, in the positive patient samples, SpA levels appeared to entirely disappear on heat treatment. Additionally, LC-MS analysis of the same patient urine samples could not identify SpA. It could be the case that our ELISA technique has detected a heat sensitive SpA variant in the positive samples, or, more likely these results are due to non-specific binding to abundant urinary proteins.

A potential reason for our inability to detect SpA in the urine could be that SpA binds to plasma IgG, generating SpA-IgG complexes, which have an approximate MW of 200kDa. Interestingly, statistical analysis revealed that while a positive SpA ELISA result did not significantly correlate with patient markers of infection, there was a correlation between SpA detection and evidence of renal failure, specifically raised serum creatinine and urea, and lowered eGFR. It appears that kidney dysfunction has led to positive ELISA results in these patients, conceivably because of increased passage of SpA-IgG complexes, or simply total protein, through damaged glomerular membranes.

Limitations of this study include the small sample size (<241 samples). While we can infer that our positive ELISA samples may be the result of non-specific interaction of the SpA antibody, investigation of a wider range of patient samples would allow us to add weight to this suggestion. We could also employ different anti-SpA antibodies in Western blotting and ELISA to see if these allow a more sensitive detection of SpA in our positive samples, or whether they confirm absence of SpA in these samples.

Using optimised immunoblotting and immunoassay techniques, the Staphylococcal surface protein SpA has been determined to currently be an elusive candidate for these methods of testing in a general ICU patient cohort. This research presents opportunity for future work to investigate the primary route of excretion of SpA, with *S. aureus* diagnosis through SpA testing a viable opportunity for future research. The focused collection of additional patient serum and urine samples with known *S. aureus* bacteraemia would provide useful confirmation of these methods. Attempts to identify potential markers of Staphylococcal infection are rare in the literature, despite the great need for improving our knowledge of, and management of, this disease.

References

1. Rammelkamp CH, Maxon T. Resistance of Staphylococcus aureus to the Action of Penicillin. Experimental Biology and Medicine. 1942;51(3):386-9.
2. Xia J, Gao J, Kokudo N, Hasegawa K, Tang W. Methicillin-resistant Staphylococcus aureus antibiotic resistance and virulence. Bioscience Trends. 2013;7(3):113-21.
3. Goering RV. Mims' medical microbiology 2013. Available from: https://www-dawsonera-com.ezproxy.lancs.ac.uk/readonline/9780702050299/startPage/16.
4. Ryan K.J. Ray GC. Sherris Medical Microbiology: 4th Edition. McGraw-Hill; 2003.
5. Madigan TM, Stahl DA. Martinko JM. Bender KS. Buckley DH. Brock T. Brock Biology of Microorganisms. 12th Edition. Pearson International; 2011.
6. Rolain J-M, François P, Hernandez D, Bittar F, Richet H, Fournous G, et al. Genomic analysis of an emerging multiresistant Staphylococcus aureus strain rapidly spreading in cystic fibrosis patients revealed the presence of an antibiotic inducible bacteriophage. Biology Direct. 2009;4:1-.
7. Holden MTG, Feil EJ, Lindsay JA, Peacock SJ, Day NPJ, Enright MC, et al. Complete genomes of two clinical Staphylococcus aureus strains: Evidence for the rapid evolution of virulence and drug resistance. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(26):9786-91.
8. Baba T, Bae T, Schneewind O, Takeuchi F, Hiramatsu K. Genome Sequence of Staphylococcus aureus Strain Newman and Comparative Analysis of Staphylococcal Genomes: Polymorphism and Evolution of Two Major Pathogenicity Islands. Journal of Bacteriology. 2008;190(1):300-10.
9. Giesbrecht P, Kersten T, Maidhof H, Wecke J. Staphylococcal Cell Wall: Morphogenesis and Fatal Variations in the Presence of Penicillin. Microbiology and Molecular Biology Reviews. 1998;62(4):1371-414.
10. Sidow T, Johannsen L, Labischinski H. Penicillin-induced changes in the cell wall composition of Staphylococcus aureus before the onset of bacteriolysis. Archives of Microbiology. 1990;154(1):73-81.
11. Schneewind O, Fowler A, Faull K. Structure of the cell wall anchor of surface proteins in Staphylococcus aureus. Science. 1995;268(5207):103-6.
12. Harris LG, Foster SJ, Richards RG. An introduction to Staphylococcus aureus, and techniques for identifying and quantifying S. aureus adhesins in relation to adhesion to biomaterials: review. European Cells and Materials. 2002;4:39-60.
13. Umeda A, Ueki Y, Amako K. Structure of the Staphylococcus aureus cell wall determined by the freeze-substitution method. Journal of Bacteriology. 1987;169(6):2482-7.
14. Thakker M, Park J-S, Carey V, Lee JC. Staphylococcus aureus Serotype 5 Capsular Polysaccharide Is Antiphagocytic and Enhances Bacterial Virulence in a Murine Bacteremia Model. Infection and Immunity. 1998;66(11):5183-9.
15. Wilkinson BJ, Holmes KM. Staphylococcus aureus cell surface: capsule as a barrier to bacteriophage adsorption. Infection and Immunity. 1979;23(2):549-52.
16. Sinha B, Fraunholz M. Staphylococcus aureus host cell invasion and post-invasion events. International Journal of Medical Microbiology. 2010;300(2–3):170-5.
17. Lowy FD. Staphylococcus aureus Infections. New England Journal of Medicine. 1998;339(8):520-32.
18. Zimmerli W, Lew PD, Waldvogel FA. Pathogenesis of foreign body infection. Evidence for a local granulocyte defect. Journal of Clinical Investigation. 1984;73(4):1191-200.
19. Kornbau C, Lee KC, Hughes GD, Firstenberg MS. Central line complications. International Journal of Critical Illness and Injury Science. 2015;5(3):170-8.
20. Archer GL. Staphylococcus aureus: A Well-Armed Pathogen. Clinical Infectious Diseases. 1998;26(5):1179-81.
21. Ogawa SK, Yurberg ER, Hatcher VB, Levitt MA, Lowy FD. Bacterial adherence to human endothelial cells in vitro. Infection and Immunity. 1985;50(1):218-24.

22. Hamill RJ, Vann JM, Proctor RA. Phagocytosis of Staphylococcus aureus by cultured bovine aortic endothelial cells: model for postadherence events in endovascular infections. Infection and Immunity. 1986;54(3):833-6.
23. Fowler T, Wann ER, Joh D, Johansson S, Foster TJ, Höök M. Cellular invasion by Staphylococcus aureus involves a fibronectin bridge between the bacterial fibronectin-binding MSCRAMMs and host cell β1 integrins. European Journal of Cell Biology. 2000;79(10):672-9.
24. Fraunholz M, Sinha B. Intracellular staphylococcus aureus: Live-in and let die. Frontiers in Cellular and Infection Microbiology. 2012;2:43.
25. Krut O, Utermöhlen O, Schlossherr X, Krönke M. Strain-Specific Association of Cytotoxic Activity and Virulence of Clinical Staphylococcus aureus Isolates. Infection and Immunity. 2003;71(5):2716-23.
26. Jarry TM, Cheung AL. Staphylococcus aureus Escapes More Efficiently from the Phagosome of a Cystic Fibrosis Bronchial Epithelial Cell Line than from Its Normal Counterpart. Infection and Immunity. 2006;74(5):2568-77.
27. Kluytmans JAJW, Wertheim HFL. Nasal Carriage of Staphylococcus aureus and Prevention of Nosocomial Infections. Infection. 2005;33(1):3-8.
28. Williams REO. HEALTHY CARRIAGE OF STAPHYLOCOCCUS AUREUS: ITS PREVALENCE AND IMPORTANCE. Bacteriological Reviews. 1963;27(1):56-71.
29. Kluytmans J, van Belkum A, Verbrugh H. Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks. Clinical Microbiology Reviews. 1997;10(3):505-20.
30. Dancer SJ. Importance of the environment in meticillin-resistant Staphylococcus aureus acquisition: the case for hospital cleaning. The Lancet Infectious Diseases. 2008;8(2):101-13.
31. Holtfreter S, Roschack K, Eichler P, Eske K, Holtfreter B, Kohler C, et al. Staphylococcus aureus Carriers Neutralize Superantigens by Antibodies Specific for Their Colonizing Strain: A Potential Explanation for Their Improved Prognosis in Severe Sepsis. Journal of Infectious Diseases. 2006;193(9):1275-8.
32. Wertheim HF, Vos MC, Ott A, van Belkum A, Voss A, Kluytmans JA, et al. Risk and outcome of nosocomial Staphylococcus aureus bacteraemia in nasal carriers versus non-carriers. The Lancet. 2004;364(9435):703-5.
33. Tong SYC, Davis JS, Eichenberger E, Holland TL, Fowler VG. Staphylococcus aureus Infections: Epidemiology, Pathophysiology, Clinical Manifestations, and Management. Clinical Microbiology Reviews. 2015;28(3):603-61.
34. England PH. Voluntary reporting of Staphylococcus aureus bacteraemia in England, Wales and Northern Ireland, 2013. In: England PH, editor. 2014.
35. Shorr AF, Tabak YP, Killian AD, Gupta V, Liu LZ, Kollef MH. Healthcare-associated bloodstream infection: A distinct entity? Insights from a large U.S. database*. Critical Care Medicine. 2006;34(10):2588-95.
36. Wyllie DH, Crook DW, Peto TEA. Mortality after Staphylococcus aureus bacteraemia in two hospitals in Oxfordshire, 1997-2003: cohort study. British Medical Journal. 2006;333(7562):281-.
37. Wheeler AP, Bernard GR. Treating Patients with Severe Sepsis. New England Journal of Medicine. 1999;340(3):207-14.
38. Thompson RL, Cabezudo I, Wenzel RP. Epidemiology of Nosocomial Infections Caused by Methicillin-Resistant Staphylococcus aureus. Annals of Internal Medicine. 1982;97(3):309-17.
39. von Eiff C, Becker K, Machka K, Stammer H, Peters G. Nasal Carriage as a Source of Staphylococcus aureus Bacteremia. New England Journal of Medicine. 2001;344(1):11-6.
40. Wertheim HFL, Vos MC, Ott A, van Belkum A, Voss A, Kluytmans JAJW, et al. Risk and outcome of nosocomial Staphylococcus aureus bacteraemia in nasal carriers versus non-carriers. The Lancet.364(9435):703-5.
41. Rasmussen RV, Fowler VG, Skov R, Bruun NE. Future challenges and treatment of Staphylococcus aureus bacteremia with emphasis on MRSA. Future microbiology. 2011;6(1):43-56.

42. Lewis T, Chaudhry R, Nightingale P, Lambert P, Das I. Methicillin-resistant Staphylococcus aureus bacteremia: epidemiology, outcome, and laboratory characteristics in a tertiary referral center in the UK. International Journal of Infectious Diseases. 2011;15(2):e131-e5.
43. Larsen MV, Harboe ZB, Ladelund S, Skov R, Gerstoft J, Pedersen C, et al. Major but differential decline in the incidence of Staphylococcus aureus bacteraemia in HIV-infected individuals from 1995 to 2007: a nationwide cohort study*. HIV Medicine. 2012;13(1):45-53.
44. Craven DE, Rixinger AI, Goularte TA, McCabe WR. Methicillin-resistant staphylococcus aureus bacteremia linked to intravenous drug abusers using a “shooting gallery&#x201d. The American Journal of Medicine.80(5):770-6.
45. FitzGerald SF, O'Gorman J, Morris-Downes MM, Crowley RK, Donlon S, Bajwa R, et al. A 12-year review of Staphylococcus aureus bloodstream infections in haemodialysis patients: more work to be done. Journal of Hospital Infection.79(3):218-21.
46. Barrett SP, Savage MA, Rebec MP, Guyot A, Andrews N, Shrimpton SB. Antibiotic sensitivity of bacteria associated with community-acquired urinary tract infection in Britain. Journal of Antimicrobial Chemotherapy. 1999;44(3):359-65.
47. Asgeirsson H, Kristjansson M, Kristinsson KG, Gudlaugsson O. Clinical significance of Staphylococcus aureus bacteriuria in a nationwide study of adults with S. aureus bacteraemia. Journal of Infection.64(1):41-6.
48. Muder RR, Brennen C, Rihs JD, Wagener MM, Obman A, Stout JE, et al. Isolation of Staphylococcus aureus from the Urinary Tract: Association of Isolation with Symptomatic Urinary Tract Infection and Subsequent Staphylococcal Bacteremia. Clinical Infectious Diseases. 2006;42(1):46-50.
49. Lee BK, Crossley K, Gerding DN. The association between staphylococcus aureus bacteremia and bacteriuria. The American Journal of Medicine.65(2):303-6.
50. Perez-Jorge EV, Burdette SD, Markert RJ, Beam WB. Staphylococcus aureus bacteremia (SAB) with associated S. aureus bacteriuria (SABU) as a predictor of complications and mortality. Journal of Hospital Medicine. 2010;5(4):208-11.
51. Huggan PJ, Murdoch DR, Gallagher K, Chambers ST. Concomitant Staphylococcus aureus bacteriuria is associated with poor clinical outcome in adults with S. aureus bacteraemia. Journal of Hospital Infection. 2008;69(4):345-9.
52. Demuth PJ, Gerding DN, Crossley K. STaphylococcus aureus bacteriuria. Archives of Internal Medicine. 1979;139(1):78-80.
53. Thompson D. Hospital infection control and the reduction in intensive care unit-acquired MRSA between 1996 and 2009. Journal of Hospital Infection. 2010;76(3):271-2.
54. Peacock SJ, Paterson GK. Mechanisms of Methicillin Resistance in Staphylococcus aureus. Annual Review of Biochemistry. 2015;84(1):577-601.
55. Barber M, Rozwadowska-Dowzenko M. INFECTION BY PENICILLIN-RESISTANT STAPHYLOCOCCI. The Lancet.252(6530):641-4.
56. Jevons MP. "Celbenin" - resistant Staphylococci. British Medical Journal. 1961;1(5219):124-5.
57. Pinho MG, de Lencastre H, Tomasz A. An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(19):10886-91.
58. Nakamura A, Miyake K, Misawa S, Kuno Y, Horii T, Hori S, et al. Association between antimicrobial consumption and clinical isolates of methicillin-resistant Staphylococcus aureus: a 14-year study. J Infect Chemother. 2012;18(1):90-5.
59. Tacconelli E, De Angelis G, Cataldo MA, Pozzi E, Cauda R. Does antibiotic exposure increase the risk of methicillin-resistant Staphylococcus aureus (MRSA) isolation? A systematic review and meta-analysis. Journal of Antimicrobial Chemotherapy. 2008;61(1):26-38.
60. Kluytmans J, Struelens M. Meticillin resistant Staphylococcus aureus in the hospital. British Medical Journal. 2009;338.

61. Neidell MJ, Cohen B, Furuya Y, Hill J, Jeon CY, Glied S, et al. Costs of Healthcare- and Community-Associated Infections With Antimicrobial-Resistant Versus Antimicrobial-Susceptible Organisms. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2012;55(6):807-15.
62. Johnson AP, Pearson A, Duckworth G. Surveillance and epidemiology of MRSA bacteraemia in the UK. Journal of Antimicrobial Chemotherapy. 2005;56(3):455-62.
63. Levine DP. Vancomycin: A History. Clinical Infectious Diseases. 2006;42(Supplement 1):S5-S12.
64. Gardete S, Tomasz A. Mechanisms of vancomycin resistance in Staphylococcus aureus. The Journal of Clinical Investigation. 2014;124(7):2836-40.
65. Holland TL, Arnold C, Fowler VG. Clinical Management of Staphylococcus aureus Bacteremia. Journal of the American Medical Associaton. 2014;312(13):1330-41.
66. Thwaites GE, Edgeworth JD, Gkrania-Klotsas E, Kirby A, Tilley R, Török ME, et al. Clinical management of Staphylococcus aureus bacteraemia. The Lancet Infectious Diseases. 2011;11(3):208-22.
67. Fowler VG, Jr., Olsen MK, Corey GR, Woods CW, Cabell CH, Reller LB, et al. Clinical identifiers of complicated Staphylococcus aureus bacteremia. Archives of Internal Medicine. 2003;163(17):2066-72.
68. Gould FK, Brindle R, Chadwick PR, Fraise AP, Hill S, Nathwani D, et al. Guidelines (2008) for the prophylaxis and treatment of methicillin-resistant Staphylococcus aureus (MRSA) infections in the United Kingdom. Journal of Antimicrobial Chemotherapy. 2009;63(5):849-61.
69. Lee A, Mirrett S, Reller LB, Weinstein MP. Detection of Bloodstream Infections in Adults: How Many Blood Cultures Are Needed? Journal of Clinical Microbiology. 2007;45(11):3546-8.
70. Khatib R, Johnson LB, Fakih MG, Riederer K, Khosrovaneh A, Shamse Tabriz M, et al. Persistence in Staphylococcus aureus bacteremia: incidence, characteristics of patients and outcome. Scandinavian Journal of Infectious Diseases. 2006;38(1):7-14.
71. Dominguez J, Gali N, Blanco S, Pedroso P, Prat C, Matas L, et al. Detection of Streptococcus pneumoniae antigen by a rapid immunochromatographic assay in urine samples. Chest. 2001;119(1):243-9.
72. Domínguez JA, Manterola JM, Blavia R, Sopena N, Belda FJ, Padilla E, et al. Detection of Legionella pneumophila serogroup 1 antigen in nonconcentrated urine and urine concentrated by selective ultrafiltration. Journal of Clinical Microbiology. 1996;34(9):2334-6.
73. Couturier MR, Graf EH, Griffin AT. Urine Antigen Tests for the Diagnosis of Respiratory Infections: Legionellosis, Histoplasmosis, Pneumococcal Pneumonia. Clinics in Laboratory Medicine. 2014;34(2):219-36.
74. Musher DM, Montoya R, Wanahita A. Diagnostic Value of Microscopic Examination of Gram-Stained Sputum and Sputum Cultures in Patients with Bacteremic Pneumococcal Pneumonia. Clinical Infectious Diseases. 2004;39(2):165-9.
75. Miwa K, Fukuyama M, Kunitomo T, Igarashi H. Rapid assay for detection of toxic shock syndrome toxin 1 from human sera. Journal of Clinical Microbiology. 1994;32(2):539-42.
76. Azuma K, Koike K, Kobayashi T, Mochizuki T, Mashiko K, Yamamoto Y. Detection of circulating superantigens in an intensive care unit population. International Journal of Infectious Diseases. 2004;8(5):292-8.
77. Poli MA, Rivera VR, Neal D. Sensitive and specific colorimetric ELISAs for Staphylococcus aureus enterotoxins A and B in urine and buffer. Toxicon. 2002;40(12):1723-6.
78. Kijek TM, Rossi CA, Moss D, Parker RW, Henchal EA. Rapid and sensitive immunomagnetic-electrochemiluminescent detection of staphyloccocal enterotoxin B. Journal of Immunological Methods. 2000;236(1-2):9-17.
79. Harrison LM, Morris JA, Lauder RM, Telford DR. Staphylococcal pyrogenic toxins in infant urine samples: a possible marker of transient bacteraemia. Journal of Clinical Pathology. 2009;62(8):735-8.

80. Price F. Analysis of urine as a marker of Staphylococcus aureus bacteraemia during intensive care treatment 2013. Masters Thesis, Lancaster Univeristy
81. Powers ME, Wardenburg JB. Igniting the Fire: Staphylococcus aureus Virulence Factors in the Pathogenesis of Sepsis. PLoS Pathogens. 2014;10(2):1-4.
82. Foster T. Staphylococcus. Medical Microbiology. 4th edition ed. Brown S, editor. University of Texas Medical Branch at Galveston 1996.
83. Dinges MM, Orwin PM, Schlievert PM. Exotoxins of Staphylococcus aureus. Clinical Microbiology Reviews. 2000;13(1):16-34.
84. Ramachandran G. Gram-positive and gram-negative bacterial toxins in sepsis: A brief review. Virulence. 2014;5(1):213-8.
85. Bhakdi S, Tranum-Jensen J. Alpha-toxin of Staphylococcus aureus. Microbiological Reviews. 1991;55(4):733-51.
86. Valeva A, Palmer M, Bhakdi S. Staphylococcal α-Toxin: Formation of the Heptameric Pore Is Partially Cooperative and Proceeds through Multiple Intermediate Stages. Biochemistry. 1997;36(43):13298-304.
87. Lina G, Piémont Y, Godail-Gamot F, Bes M, Peter M-O, Gauduchon V, et al. Involvement of Panton-Valentine Leukocidin—Producing Staphylococcus aureus in Primary Skin Infections and Pneumonia. Clinical Infectious Diseases. 1999;29(5):1128-32.
88. Prevost G, Couppie P, Prevost P, Gayet S, Petiau P, Cribier B, et al. Epidemiological data on Staphylococcus aureus strains producing synergohymenotropic toxins. Journal of Medical Microbiology. 1995;42(4):237-45.
89. Shallcross LJ, Fragaszy E, Johnson AM, Hayward AC. The role of the Panton-Valentine leucocidin toxin in staphylococcal disease: a systematic review and meta-analysis. The Lancet Infectious Diseases. 2013;13(1):43-54.
90. Bohach GA, Fast DJ, Nelson RD, Schlievert PM. Staphylococcal and Streptococcal Pyrogenic Toxins Involved in Toxic Shock Syndrome and Related Illnesses. Critical Reviews in Microbiology. 1990;17(4):251-72.
91. Thomas D, Chou S, Dauwalder O, Lina G. Diversity in Staphylococcus aureus enterotoxins. Chemical Immunology and Allergy. 2007;93:24-41.
92. Fleischer B. Schrezenmeier H. T cell stimulation by staphylococcal enterotoxins. Clonally variable response and requirement for major histocompatibility complex class II molecules on accessory or target cells. The Journal of Experimental Medicine. 1988;167(5):1697-707.
93. Xu SX, McCormick JK. Staphylococcal superantigens in colonization and disease. Frontiers in Cellular and Infection Microbiology. 2012;2:52.
94. Ikejima T, Okusawa S, van der Meer JW, Dinarello CA. Induction by toxic-shock-syndrome toxin-1 of a circulating tumor necrosis factor-like substance in rabbits and of immunoreactive tumor necrosis factor and interleukin-1 from human mononuclear cells. Journal of Infectious Diseases. 1988;158(5):1017-25.
95. Miller C, Ragheb JA, Schwartz RH. Anergy and Cytokine-Mediated Suppression as Distinct Superantigen-Induced Tolerance Mechanisms in Vivo. The Journal of Experimental Medicine. 1999;190(1):53-64.
96. Rasigade J-P, Thomas D, Perpoint T, Peyramond D, Chidiac C, Etienne J, et al. T-cell response to superantigen restimulation during menstrual toxic shock syndrome. FEMS Immunology & Medical Microbiology. 2011;62(3):368-71.
97. Foster TJ. Immune evasion by staphylococci. Nature Reviews Microbiology. 2005;3(12):948-58.
98. Foster TJ, Geoghegan JA, Ganesh VK, Höök M. Adhesion, invasion and evasion: the many functions of the surface proteins of Staphylococcus aureus. Nature Reviews Microbiology. 2014;12(1):49-62.
99. Navarre WW, Schneewind O. Proteolytic cleavage and cell wall anchoring at the LPXTG motif of surface proteins in gram-positive bacteria. Mol Microbiol. 1994;14(1):115-21.

100. Foster TJ, Höök M. Surface protein adhesins of Staphylococcus aureus. Trends in Microbiology. 1998;6(12):484-8.
101. J M Patti, B L Allen, M J McGavin a, Hook M. MSCRAMM-Mediated Adherence of Microorganisms to Host Tissues. Annual Review of Microbiology. 1994;48(1):585-617.
102. Hammer ND, Skaar EP. Molecular mechanisms of Staphylococcus aureus iron acquisition. Annual Review of Microbiology. 2011;65:10.1146/annurev-micro-090110-102851.
103. Forsgren A. Significance of Protein A Production by Staphylococci. Infection and Immunity. 1970;2(5):672-3.
104. Sjöquist J, Movitz J, Johansson I-B, Hjelm H. Localization of Protein A in the Bacteria. European Journal of Biochemistry. 1972;30(1):190-4.
105. Kronvall G, Dossett JH, Quie PG, Williams RC. Occurrence of Protein A in Staphylococcal Strains: Quantitative Aspects and Correlation to Antigenic and Bacteriophage Types. Infection and Immunity. 1971;3(1):10-5.
106. Movitz J. Formation of extracellular protein A by Staphylococcus aureus. European Journal of Biochemistry. 1976;68(1):291-9.
107. Verwey WF. A TYPE-SPECIFIC ANTIGENIC PROTEIN DERIVED FROM THE STAPHYLOCOCCUS. The Journal of Experimental Medicine. 1940;71(5):635-44.
108. Löfkvist T, Sjöquist J. CHEMICAL AND SEROLOGICAL ANALYSIS OF ANTIGEN PREPARATIONS FROM STAPHYLOCOCCUS AUREUS. Acta Pathologica Microbiologica Scandinavica. 1962;56(3):295-304.
109. Grov A, Myklestad B, Oeding P. Immunochemical Studies on Antigen Preparations from Staphylococcus Aureus. 1. Isolation and Chemical Characterization of Antigen A. Acta Pathol Microbiologica Scandinavica. 1964;61:588-96.
110. Forsgren A, Sjöquist J. “Protein A” from S. Aureus: I. Pseudo-Immune Reaction with Human γ-Globulin. The Journal of Immunology. 1966;97(6):822-7.
111. King BF, Wilkinson BJ. Binding of human immunoglobulin G to protein A in encapsulated Staphylococcus aureus. Infection and Immunity. 1981;33(3):666-72.
112. InganÄS M. Comparison of Mechanisms of Interaction between Protein A from Staphylococcus aureus and Human Monoclonal IgG, IgA and IgM in Relation to the Classical Fcγ and the Alternative F(ab')2γ Protein A Interactions. Scandinavian Journal of Immunology. 1981;13(4):343-52.
113. Björk I, Petersson BÅ, Sjöquist J. Some Physicochemical Properties of Protein A from Staphylococcus aureus. European Journal of Biochemistry. 1972;29(3):579-84.
114. Cheung AL, Bayer AS, Peters J, Ward JI. Analysis by gel electrophoresis, Western blot, and peptide mapping of protein A heterogeneity in Staphylococcus aureus strains. Infection and Immunity. 1987;55(4):843-7.
115. SjÖDahl J. Repetitive Sequences in Protein A from Staphylococcus aureus. European Journal of Biochemistry. 1977;73(2):343-51.
116. Moks T, Abrahmsen L, Nilsson B, Hellman U, Sjoquist J, Uhlen M. Staphylococcal protein A consists of five IgG-binding domains. European Journal of Biochemistry. 1986;156(3):637-43.
117. Sorum M, Sangvik M, Stegger M, Olsen RS, Johannessen M, Skov R, et al. Staphylococcus aureus mutants lacking cell wall-bound protein A found in isolates from bacteraemia, MRSA infection and a healthy nasal carrier. Pathology and Disease. 2013;67(1):19-24.
118. Blobel G. Intracellular protein topogenesis. Proceedings of the National Academy of Sciences of the United States of America. 1980;77(3):1496-500.
119. Sibbald MJ, Ziebandt AK, Engelmann S, Hecker M, de Jong A, Harmsen HJ, et al. Mapping the pathways to staphylococcal pathogenesis by comparative secretomics. Microbiology and Molecular Biology Review. 2006;70(3):755-88.
120. Rapoport TA. Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes. Nature. 2007;450(7170):663-9.

121. Deisenhofer J. Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from Staphylococcus aureus at 2.9- and 2.8-A resolution. Biochemistry. 1981;20(9):2361-70.
122. Sjöquist J, Meloun B, Hjelm H. Protein A Isolated from Staphylococcus aureus after Digestion with Lysostaphin. European Journal of Biochemistry. 1972;29(3):572-8.
123. Deisenhofer J, Jones TA, Huber R, Sjodahl J, Sjoquist J. Crystallization, crystal structure analysis and atomic model of the complex formed by a human Fc fragment and fragment B of protein A from Staphylococcus aureus. Hoppe Seylers Z Physiological Chemistry. 1978;359(8):975-85.
124. Jansson B, Uhlén M, Nygren P-Å. All individual domains of staphylococcal protein A show Fab binding. FEMS Immunology & Medical Microbiology. 1998;20(1):69-78.
125. Lambris JD, Ricklin D, Geisbrecht BV. Complement evasion by human pathogens. Nature Review Microbiology. 2008;6(2):132-42.
126. Nilsson B, Moks T, Jansson B, Abrahmsén L, Elmblad A, Holmgren E, et al. A synthetic IgG-binding domain based on staphylococcal protein A. Protein Engineering. 1987;1(2):107-13.
127. Lindmark R, Thoren-Tolling K, Sjoquist J. Binding of immunoglobulins to protein A and immunoglobulin levels in mammalian sera. Journal of Immunological Methods. 1983;62(1):1-13.
128. Sasso EH, Silverman GJ, Mannik M. Human IgM molecules that bind staphylococcal protein A contain VHIII H chains. Journal of Immunology. 1989;142(8):2778-83.
129. Graille M, Stura EA, Corper AL, Sutton BJ, Taussig MJ, Charbonnier J-B, et al. Crystal structure of a Staphylococcus aureus protein A domain complexed with the Fab fragment of a human IgM antibody: Structural basis for recognition of B-cell receptors and superantigen activity. Proceedings of the National Academy of Sciences of the United States of America. 2000;97(10):5399-404.
130. Shopsin B, Gomez M, Montgomery SO, Smith DH, Waddington M, Dodge DE, et al. Evaluation of Protein A Gene Polymorphic Region DNA Sequencing for Typing of Staphylococcus aureus Strains. Journal of Clinical Microbiology. 1999;37(11):3556-63.
131. von Heijne G, Uhlen M. Homology to region X from staphylococcal protein A is not unique to cell surface proteins. Journal of Theoretical Biology. 1987;127(3):373-6.
132. Hallin M, Friedrich AW, Struelens MJ. spa typing for epidemiological surveillance of Staphylococcus aureus. Methods in Molecular Biology. 2009;551:189-202.
133. Baum C, Haslinger-Löffler B, Westh H, Boye K, Peters G, Neumann C, et al. Non-spa-Typeable Clinical Staphylococcus aureus Strains Are Naturally Occurring Protein A Mutants. Journal of Clinical Microbiology. 2009;47(11):3624-9.
134. Winblad S, Ericson C. SENSITIZED SHEEP RED CELLS AS A REACTANT FOR STAPHYLOCOCCUS AUREUS PROTEIN A. Acta Pathologica Microbiologica Scandinavica Section B Microbiology and Immunology. 1973;81B(1):150-6.
135. Cohen S, Sweeney HM. Modulation of Protein A Formation in Staphylococcus aureus by Genetic Determinants for Methicillin Resistance. Journal of Bacteriology. 1979;140(3):1028-35.
136. Yung SC, Parenti D, Murphy PM. Host Chemokines Bind to Staphylococcus aureus and Stimulate Protein A Release. The Journal of Biological Chemistry. 2011;286(7):5069-77.
137. Ramadurai L, Jayaswal RK. Molecular cloning, sequencing, and expression of lytM, a unique autolytic gene of Staphylococcus aureus. Journal of Bacteriology. 1997;179(11):3625-31.
138. Becker S, Frankel MB, Schneewind O, Missiakas D. Release of protein A from the cell wall of Staphylococcus aureus. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(4):1574-9.
139. O'Halloran DP, Wynne K, Geoghegan JA. Protein A Is Released into the Staphylococcus aureus Culture Supernatant with an Unprocessed Sorting Signal. Infection and Immunity. 2015;83(4):1598-609.
140. Coleman DL. Regulation of macrophage phagocytosis. European Journal of Clinical Microbiology. 1986;5(1):1-5.
141. Falugi F, Kim HK, Missiakas DM, Schneewind O. Role of Protein A in the Evasion of Host Adaptive Immune Responses by Staphylococcus aureus. mBio. 2013;4(5):e00575-13.

142. Kobayashi SD, DeLeo FR. Staphylococcus aureus Protein A Promotes Immune Suppression. mBio. 2013;4(5).
143. Peterson PK, Verhoef J, Sabath LD, Quie PG. Effect of protein A on staphylococcal opsonization. Infection and Immunity. 1977;15(3):760-4.
144. Nordenfelt P, Waldemarson S, Linder A, Mörgelin M, Karlsson C, Malmström J, et al. Antibody orientation at bacterial surfaces is related to invasive infection. The Journal of Experimental Medicine. 2012;209(13):2367-81.
145. Wright C, Willan KJ, Sjödahl J, Burton DR, Dwek RA. The interaction of protein A and Fc fragment of rabbit immunoglobulin G as probed by complement-fixation and nuclear-magnetic-resonance studies. Biochemical Journal. 1977;167(3):661-8.
146. Kronvall G, Gewurz H. Activation and inhibition of IgG mediated complement fixation by staphylococcal protein A. Clinical and Experimental Immunology. 1970;7(2):211-20.
147. Sasso EH, Silverman GJ, Mannik M. Human IgA and IgG F(ab')2 that bind to staphylococcal protein A belong to the VHIII subgroup. The Journal of Immunology. 1991;147(6):1877-83.
148. Hakoda M, Hayashimoto S, Yamanaka H, Terai C, Kamatani N, Kashiwazaki S. Molecular basis for the interaction between human IgM and staphylococcal protein A. Clinical Immunology and Immunopathology. 1994;72(3):394-401.
149. Romagnani S, Giudizi MG, del Prete G, Maggi E, Biagiotti R, Almerigogna F, et al. Demonstration on protein A of two distinct immunoglobulin-binding sites and their role in the mitogenic activity of Staphylococcus aureus Cowan I on human B cells. Journal of Immunology. 1982;129(2):596-602.
150. Kristiansen SV, Pascual V, Lipsky PE. Staphylococcal protein A induces biased production of Ig by VH3-expressing B lymphocytes. Journal of Immunology. 1994;153(7):2974-82.
151. Goodyear CS, Silverman GJ. Death by a B Cell Superantigen: In Vivo V(H)-targeted Apoptotic Supraclonal B Cell Deletion by a Staphylococcal Toxin. The Journal of Experimental Medicine. 2003;197(9):1125-39.
152. Pauli NT, Kim HK, Falugi F, Huang M, Dulac J, Henry Dunand C, et al. Staphylococcus aureus infection induces protein A–mediated immune evasion in humans. The Journal of Experimental Medicine. 2014;211(12):2331-9.
153. Kim HK, Falugi F, Missiakas DM, Schneewind O. Peptidoglycan-linked protein A promotes T cell-dependent antibody expansion during Staphylococcus aureus infection. Proceedings of the National Academy of Sciences of the United States of America. 2016;113(20):5718-23.
154. Nguyen T, Ghebrehiwet B, Peerschke EIB. Staphylococcus aureus Protein A Recognizes Platelet gC1qR/p33: a Novel Mechanism for Staphylococcal Interactions with Platelets. Infection and Immunity. 2000;68(4):2061-8.
155. Boles BR, Horswill AR. agr-Mediated Dispersal of Staphylococcus aureus Biofilms. PLoS Pathogens. 2008;4(4):e1000052.
156. Merino N, Toledo-Arana A, Vergara-Irigaray M, Valle J, Solano C, Calvo E, et al. Protein A-Mediated Multicellular Behavior in Staphylococcus aureus. Journal of Bacteriology. 2009;191(3):832-43.
157. Patel AH, Nowlan P, Weavers ED, Foster T. Virulence of protein A-deficient and alpha-toxin-deficient mutants of Staphylococcus aureus isolated by allele replacement. Infection and Immunity. 1987;55(12):3103-10.
158. Gemmell CG, Goutcher SC, Reid R, Sturrock RD. Role of certain virulence factors in a murine model of Staphylococcus aureus arthritis. Journal of Medical Microbiology. 1997;46(3):208-13.
159. Jonsson P, Lindberg M, Haraldsson I, Wadström T. Virulence of Staphylococcus aureus in a mouse mastitis model: studies of alpha hemolysin, coagulase, and protein A as possible virulence determinants with protoplast fusion and gene cloning. Infection and Immunity. 1985;49(3):765-9.
160. Cheng AG, Kim HK, Burts ML, Krausz T, Schneewind O, Missiakas DM. Genetic requirements for Staphylococcus aureus abscess formation and persistence in host tissues. The FASEB Journal. 2009;23(10):3393-404.

161. Wann ER, Fehringer AP, Ezepchuk YV, Schlievert PM, Bina P, Reiser RF, et al. Staphylococcus aureus Isolates from Patients with Kawasaki Disease Express High Levels of Protein A. Infection and Immunity. 1999;67(9):4737-43.
162. Matsubara K, Fukaya T. The role of superantigens of group A Streptococcus and Staphylococcus aureus in Kawasaki disease. Current Opinions in Infectious Diseases. 2007;20(3):298-303.
163. Löfdahl S, Guss B, Uhlén M, Philipson L, Lindberg M. Gene for staphylococcal protein A. Proceedings of the National Academy of Sciences of the United States of America. 1983;80(3):697-701.
164. Uhlén M, Guss B, Nilsson B, Gatenbeck S, Philipson L, Lindberg M. Complete sequence of the staphylococcal gene encoding protein A. A gene evolved through multiple duplications. Journal of Biological Chemistry. 1984;259(3):1695-702.
165. Uhlén M, Guss B, Nilsson B, Götz F, Lindberg M. Expression of the gene encoding protein A in Staphylococcus aureus and coagulase-negative staphylococci. Journal of Bacteriology. 1984;159(2):713-9.
166. Cheung AL, Bayer AS, Zhang G, Gresham H, Xiong Y-Q. Regulation of virulence determinants in vitro and in vivo in Staphylococcus aureus. FEMS Immunology & Medical Microbiology. 2004;40(1):1-9.
167. Heinrichs JH, Bayer MG, Cheung AL. Characterization of the sar locus and its interaction with agr in Staphylococcus aureus. Journal of Bacteriology. 1996;178(2):418-23.
168. Novick RP, Ross HF, Projan SJ, Kornblum J, Kreiswirth B, Moghazeh S. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. The EMBO Journal. 1993;12(10):3967-75.
169. Peng HL, Novick RP, Kreiswirth B, Kornblum J, Schlievert P. Cloning, characterization, and sequencing of an accessory gene regulator (agr) in Staphylococcus aureus. Journal of Bacteriology. 1988;170(9):4365-72.
170. Gao J, Stewart GC. Regulatory Elements of the Staphylococcus aureus Protein A (Spa) Promoter. Journal of Bacteriology. 2004;186(12):3738-48.
171. Le KY, Otto M. Quorum-sensing regulation in staphylococci—an overview. Frontiers in Microbiology. 2015;6:1174.
172. Recsei P, Kreiswirth B, O'Reilly M, Schlievert P, Gruss A, Novick RP. Regulation of exoprotein gene expression in Staphylococcus aureus by agr. Molecular and General Genetics MGG. 1986;202(1):58-61.
173. Cheung GY, Wang R, Khan BA, Sturdevant DE, Otto M. Role of the accessory gene regulator agr in community-associated methicillin-resistant Staphylococcus aureus pathogenesis. Infection and Immunity. 2011;79(5):1927-35.
174. HÄllgren R, StÅlenheim G, Bill A. ELIMINATION OF PROTEIN A-IgG COMPLEXES FROM THE BLOOD CIRCULATION IN RABBITS: ROLE OF SPLEEN AND LIVER. Acta Pathologica Microbiologica Scandinavica Section C Immunology. 1977;85C(6):435-40.
175. Bernton E, Haughey D. Studies of the Safety, Pharmacokinetics and Immunogenicity of Repeated Doses of Intravenous Staphylococcal Protein A in Cynomolgus Monkeys. Basic & Clinical Pharmacology & Toxicology. 2014;115(5):448-55.
176. Ballow C, Leh A, Slentz-Kesler K, Yan J, Haughey D, Bernton E. Safety, Pharmacokinetic, Immunogenicity, and Pharmacodynamic Responses in Healthy Volunteers Following a Single Intravenous Injection of Purified Staphylococcal Protein A. The Journal of Clinical Pharmacology. 2013;53(9):909-18.
177. Vabulas R, Bittlingmaier R, Heeg K, Wagner H, Miethke T. Rapid clearance of the bacterial superantigen staphylococcal enterotoxin B in vivo. Infection and Immunity. 1996;64(11):4567-73.
178. Mino MJ, Ortiz RT, Randad P, Moffatt LT, Jordan MH, Shupp JW. Localization of superantigen virulence factors in kidney tissue of animals with Staphylococcus aureus-infected burn wounds. Journal od Burn Care Research. 2013;34(1):142-50.

179. Hnasko RM, McGarvey JA. Affinity Purification of Antibodies. In: Hnasko R, editor. ELISA: Methods and Protocols. New York, NY: Springer New York; 2015. p. 29-41.
180. Langone JJ. Applications of immobilized protein A in immunochemical techniques. Journal of Immunological Methods. 1982;55(3):277-96.
181. Dertzbaugh MT, Flickinger MC, Lebherz WB. An enzyme immunoassay for the detection of staphylococcal protein A in affinity-purified products. Journal of Immunological Methods. 1985;83(1):169-77.
182. Godfrey MA, Kwasowski P, Clift R, Marks V. A sensitive enzyme-linked immunosorbent assay (ELISA) for the detection of staphylococcal protein A (SpA) present as a trace contaminant of murine immunoglobulins purified on immobilized protein A. Journal of Immunological Methods. 1992;149(1):21-7.
183. Nilsson R, Davidsson B. Quantitation of protein A in human plasma is possible after heat inactivation of the samples. Journal of Immunological Methods. 1990;135(1-2):77-80.
184. Knicker SM, Profy AT. Immunoassay to measure staphylococcal protein A in the presence of murine immunoglobulins. Journal of Immunological Methods. 1991;142(1):53-9.
185. Steindl F, Armbruster C, Hahn R, Armbruster C, Katinger HWD. A simple method to quantify staphylococcal protein A in the presence of human or animal IgG in various samples. Journal of Immunological Methods. 2000;235(1–2):61-9.
186. Greenberg DP, Bayer AS, Turner D, Ward JI. Antibody responses to protein A in patients with Staphylococcus aureus bacteremia and endocarditis. Journal of Clinical Microbiology. 1990;28(3):458-62.
187. Han Y. Analysis of protein A and Bacterial toxins in patients in ICU 2014. Masters thesis Lancaster University
188. Yang L, Biswas ME, Chen P. Study of Binding between Protein A and Immunoglobulin G Using a Surface Tension Probe. Biophysical Journal. 2003;84(1):509-22.
189. Rath A, Glibowicka M, Nadeau VG, Chen G, Deber CM. Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. Proceedings of the National Academy of Sciences. 2009;106(6):1760-5.
190. Mahmood T, Yang P-C. Western Blot: Technique, Theory, and Trouble Shooting. North American Journal of Medical Sciences. 2012;4(9):429-34.
191. Burdon DW. Immunoglobulins of normal human urine and urethral secretions. Immunology. 1971;21(2):363-8.
192. Kothari V, Mathews ST. Detection of Blotted Proteins: Not All Blockers Are Created Equal. In: Kurien TB, Scofield HR, editors. Detection of Blotted Proteins: Methods and Protocols. New York, NY: Springer New York; 2015. p. 27-32.
193. Simerville JA, Maxted WC, Pahira JJ. Urinalysis: a comprehensive review. American Family Physician. 2005;71(6):1153-62.
194. O'Keefe E, Vennett V. Use of immunoglobulin-loaded protein A-bearing staphylococci as a primary solid phase immunoadsorbent in radioimmunoassay. Journal of Biological Chemistry. 1980;255(2):561-8.
195. Francois P, Scherl A, Hochstrasser D, Schrenzel J. Proteomic approaches to study Staphylococcus aureus pathogenesis. Journal of Proteomics. 2010;73(4):701-8.
196. Pertschuk LP, Woda BA, Vuletin JC, Brigati DJ, Soriano CB, Nicastri AD. Glomerulonephritis Due to Staphylococcus aureusAntigen. American Journal of Clinical Pathology. 1976;65(3):301-7.
197. Newbould MJ, Malam J, McIllmurray JM, Morris JA, Telford DR, Barson AJ. Immunohistological localisation of staphylococcal toxic shock syndrome toxin (TSST-1) antigen in sudden infant death syndrome. Journal of Clinical Pathology. 1989;42(9):935-9.
198. Batsford SR, Mezzano S, Mihatsch M, Schiltz E, Rodriguez-Iturbe B. Is the nephritogenic antigen in post-streptococcal glomerulonephritis pyrogenic exotoxin B (SPE B) or GAPDH? Kidney International. 2005;68(3):1120-9.

199. Abbate M, Zoja C, Remuzzi G. How Does Proteinuria Cause Progressive Renal Damage? Journal of the American Society of Nephrology. 2006;17(11):2974-84.
200. West BL, Picken MM, Leehey DJ. Albuminuria in acute tubular necrosis. Nephrology Dialysis Transplantation. 2006;21(10):2953-6.
201. Alegria-Schaffer A, Lodge A, Vattem K. Chapter 33 Performing and Optimizing Western Blots with an Emphasis on Chemiluminescent Detection. In: Richard RB, Murray PD, editors. Methods in Enzymology: Academic Press; 2009. p. 573-99.
202. Bergendahl V, Glaser BT, Burgess RR. A fast Western blot procedure improved for quantitative analysis by direct fluorescence labeling of primary antibodies. Journal of Immunological Methods. 2003;277(1–2):117-25.
203. Shields JG, Turner MW. The importance of antibody quality in sandwich ELISA systems. Journal of Immunological Methods. 1986;87(1):29-33.
204. Cheung AL, Eberhardt K, Heinrichs JH. Regulation of protein A synthesis by the sar and agr loci of Staphylococcus aureus. Infection and Immunity. 1997;65(6):2243-9.
205. Hong X, Qin J, Li T, Dai Y, Wang Y, Liu Q, et al. Staphylococcal Protein A Promotes Colonization and Immune Evasion of the Epidemic Healthcare-Associated MRSA ST239. Frontiers in Microbiology. 2016;7:951.

Appendix

Patient diagnosis and characteristics.

Patient number- 45

Patient number	Diagnosis on admission	Age	Death during admission?
1	Addisonian Crisis	77	N
2	Ruptured Aortic Aneurysm	83	N
3	Pneumococcal Pneumonia	66	Y
4	Hodgkin's Lymphoma	19	N
5	Ruptured Aortic Aneurysm	73	N
6	Viral Encephalitis	67	N
7	Trauma	40	N
8	Spinal Cord Ischemia	54	N
9	Urinary Sepsis	67	N
10	Acute Kidney Injury	61	N
11	Pneumonia	64	N
12	Bowel Perforation	80	N
13	Diabetic Ketoacidosis	37	N
14	Ventilation Acquired Pneumonia	30	N
15	Gram-negative sepsis	71	N
16	Pneumonia	82	N
17	Ischaemic Bowel	86	N
18	Not Known	54	N
19	Necrotising Fascitis	48	N
21	Perforated Stomach	18	N
22	Pneumonia	77	N
23	Deep Vein Thrombosis	25	N
24	Necrotising Fascitis	72	N
25	Ischaemic Bowel	67	N
26	Sepsis	63	Y
27	Not Known	38	N
28	Caecal Volvulus	46	N
29	Upper GI haemorrhage	80	Y
30	Pneumococcal Pneumonia	61	N
31	Pneumonia	74	N
32	Multiple Organ Failure	37	Y
33	Pneumonitis	77	Y
34	Sepsis	Not collected	Y
35	Pneumonia	35	N
36	Bowel Perforation	Not collected	N
37	Diabetic Ketoacidosis	69	N

38	Intra-abdominal haemorrhage	61	Y
39	Pneumonia	65	Y
40	Meningitis	56	N
41	Pneumonia	83	N
42	Sepsis	70	N
43	Necrotising Fascitis	33	N
44	Pancreatitis	46	Y
46	Sepsis	34	N
47	Gram-negative Sepsis	76	N

For further patient details, the following information is available in Frances Price (Msc 2013):[80]

- Vital Signs:
    - Heart rate
    - Blood pressure
    - Temperature
    - Respiratory Rate
- Ventilation
    - Invasive/ Non Invasive
- Invasive lines and catheters:
    - Central Venous Catheter
    - Arterial Catheter
    - Urinary Catheter
    - Abdominal Drain
    - Chest Drain
    - Other lines
- Gender