Detection of Staphylococcal Enterotoxins in Patients with Rheumatoid Arthritis or Closed Fractures

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Abstract

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Aim: To develop protocols to digest staphylococcal enterotoxins B and C (SEB/SEC), toxic shock syndrome toxin 1 (TSST-1) and alpha haemolysin (AH), members of the pyrogenic toxin superantigen family (PTSAg). To develop a novel mass spectrometry method to analyse and compare urine samples from patients with rheumatoid arthritis (RA) and orthopaedic fracture patients for the presence of *Staphylococcus aureus*.

Background: RA is a disease of unknown etiology; with a pathogenesis that is due to a mixture of genetic, immunological and environmental factors. A T-cell immune response to the presence of PTSAgs in the joints of RA patients has previously been described.

A link has been proposed between pathogenic micro-organisms and the development of chronic, autoimmune conditions. Potential pathogenic mechanisms include the hygiene hypothesis and molecular mimicry. Due to the widespread prevalence of RA, it has been hypothesised that the pathogenesis could involve a common bacterium. In RA, one potential bacterial candidate that has been suggested is *S.aureus.*

Current published data averages the presence of *S.aureus* in the general population at 30% (nasal/nasopharyngeal swabs). However, our unpublished data suggests immune complexes containing *S.aureus* antigens are detectable in urine.

Methods: Mid-stream urine samples were collected from the rheumatology and orthopaedic departments of the Royal Lancaster Infirmary (RLI), UK. Urine samples were analysed by western blot and mass spectrometry.

Results: 56.4% of RA patients showed the presence of at least one staphylococcal toxin in their urine compared with 27.1% of fracture patients.

Conclusion: Our work demonstrates an increased presence of bacterial toxins in urine from RA patients, compared to the fracture controls and the current literature. This study is the first to demonstrate the presence of common staphylococcal enterotoxins in RA patient urine, raising the question of what role they may have in the disease pathogenesis, given that these patients have no active infections.

This raises questions of whether the bacteria and their toxins are involved in an individual's likelihood of getting RA; are those people with RA more likely to have *S.aureus* infections due to their immunological state? The presence of *S.aureus* in RA patient tissues warrants further investigation to determine if it is causative of, or a result of RA diagnosis.

Acknowledgements

This thesis is dedicated to the memory of my father, Colin William Grace, whose final request to me was that I completed my PhD on time. He supported me wholeheartedly, right from the beginning but was unfortunately unable to see the finished thesis. He is missed everyday and I am sure he would be proud of my accomplishments.

To my parents, Kathryn and Colin, for their love and support, for making the right choices for me and for making me believe that nothing is out of my reach. To my sister, Hannah, for always challenging me. To Dean, for his unwavering love, encouragement, patience and understanding.

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Declaration

I, Laura Grace, declare that this thesis was composed by me and that, except where explicitly stated otherwise in the text, the work contained therein is my own and has not been submitted substantially in the same form for the award of a higher degree elsewhere.

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αAH = Anti Alpha Haemolysin Primary Antibody

αSEB = Anti Staphylococcal Enterotoxin B Primary Antibody

αSEC = Anti Staphylococcal Enterotoxin B Primary Antibody

αTSST-1 = Anti Toxic Shock Syndrome Toxin 1 Primary Antibody

ACR = American College of Rheumatology

AH = Alpha Haemolysin

AN = Anorexia Nervosa

Anti – CCP = Anti-Cyclic Citrullinated Peptide

APC = Antibody Presenting Cell

BLAST = Basic Local Alignment Search Tool

BR = Bronchiectasis

cALL = Childhood Acute Lymphoblastic Leukaemia

CFA = Complete Freunds Adjuvant

CFS = Chronic Fatigue Syndrome

CI = Confidence Interval

CIA = Collagen Induced Arthritis

CRP = C Reactive Protein

CVD = Cardiovascular Disease

CXCL1 = Chemokine (C-X-C motif) Ligand 1

DAS = Disease Activity Score

DHEAS = Dehydroepiandrosterone

DMARDs = Disease-Modifying Anti-Rheumatic Drugs

EBV = Epstein Barr Virus

ESI-MS = Electrospray Ionisation Mass Spectrometry

ESR = Erythrocyte Sedimentation Rate

EULAR = European League Against Rheumatism

GCF = Ginigval Crevicular Fluid

GWAS = Genome Wide Association Studies

HAQ = Health Assessment Questionnaire

HBVP = Hepatitis B virus polymerase

HCS = Hertfordshire Cohort Study

HIV = Human Immunodeficiency Virus

HLA = Human Leukocyte Antigen

HSV = Herpes Simplex Virus

HZV = Herpes Zoster Virus

HuAChR = Human Acetylcholine Receptor

IBS = Irritable Bowel Syndrome

ILs = Interleukins

ILD = Interstitial Lung Disease

IFN-γ = Interferon Gamma

LC-MS = Liquid Chromatography Mass Spectrometry

LCMS-IT-TOF = Liquid Chromatography Mass Spectrometry Ion-Trap Time-Of-Flight

LLOD = Lower Limit of Detection

LPS = Lipopolysaccharide

LT = Lymphotoxin

M/Z = Mass to Charge Ratio

MBP = Myelin Basic Protein

MHC = Major Histocompatibility Complex

MRM = Multiple Reaction Monitoring

mRNA = messenger RNA

MS = Multiple Sclerosis

NCBI = National Center for Biotechnology Information

NO = Nitric Oxide

NOAR = Norfolk Arthritis Register

NSAID = Non-Steroidal Anti-Inflammatory Drug

OA = Osteoarthritis

OR = Odds ratio

PAD = Peptidylarginine Deaminase

PAGE = Polyacrylamide Gel Electrophoresis

PMN = Polymorphonuclear Neutrophils

PPAD = *P.gingivalis* Peptidylarginine Deaminase

Ppm = parts per million

PTSAg = Pyrogenic Toxin Superantigen

RαS = Rabbit Anti-Sheep HRP Conjugate Secondary Antibody

RA = Rheumatoid Arthritis

RBC = Red Blood Cell

RF = Rheumatoid Factor

SDS = Sodium Dodecyl Sulphate

SEB = Staphylococcal Enterotoxin B

SEC = Staphylococcal Enterotoxin C

SIDS = Sudden Infant Death Syndrome

SIM = Selected Ion Monitoring

SIR = Standard Incidence Ratio

SLE = Systemic Lupus Erythromatosus

SMR= Standard Mortality Ratios

SSSS = Staphylococcal Scalded Skin Syndrome

T1D = Type 1 Diabetes

TEMED = Tetramethylethylenediamine

TIC = Total Ion Current

TFA = Trifluoroacetic Acid

TCR = T Cell Receptor

TNF = Tumour Necrosis Factor

TSS = Toxic Shock Syndrome

TSST-1 = Toxin Shock Syndrome Toxin 1

TQ = Triple Quadrupole

UTI = Urinary Tract Infection

VZV = Varicella Zoster Virus

WBC = White Blood Cell

XIC = Extracted Ion Chromatogram

Chapter 1 INTRODUCTION

1.1 Motivation

Rheumatoid arthritis (RA) is a chronic and disabling multifactorial autoimmune disease of unknown aetiology, affecting approximately 0.5-1% of the world's population, and 690,000 people in the UK alone (Arthritis Research UK, 2012). An effective cure has not been developed, primarily because a definite cause of the condition is yet to be identified. It has been hypothesised that an environmental factor such as pathogenic bacteria could be a trigger for autoimmune disease.

Numerous pathogens have been researched as a possible cause of RA; however, *Staphylococcus Aureus*, a very common bacterium, seems considerably understudied. Furthermore, there is an evident gap in the literature in relation to the complete digestion of staphylococcal enterotoxins and the ability to detect very small amounts of such toxins in bodily fluids such as urine.

1.2 Aims & Hypothesis

- Hypothesis: patients with RA are more likely to have microbial toxins in their urine.
    - Aim 1: To develop a protocol to digest staphylococcal toxins.
    - Aim 2: develop a protocol using mass spectrometry to detect digested staphylococcal enterotoxin fragments/peptides at low levels – in urine.

- Aim 3: To perform a case control study, analysing urine from patients with RA and control patients, for the presence of staphylococcal enterotoxins, using the methodologies developed (as stated above).

1.3 The Immune System

Innate immunity is the body's initial defence against pathogenic microorganisms, such as bacteria and viruses. The innate immune system is generally non-specific and includes physical barriers, such as skin, chemicals in the blood, such as cytokines and chemokines, and immune cells, primarily neutrophils and macrophages (Flagarone, 2005). The cells of the innate immune system are activated within hours of the initial infection. Neutrophils and macrophages release chemokines that attract more immune cells to the site of infection and cytokines that initiate inflammatory processes. Cytokines such as tumour necrosis factor alpha (TNFα) and interleukin 1 (IL-1), isolated from synovial joints of RA patients, are known to play roles in the inflammatory and destructive nature of the disease (Murphy, 2008; Lubberts, 2000; Flagarone, 2005). Macrophages are able to phagocytose pathogens, digesting and disposing of the proteins. Furthermore, plasma proteins are able to lyse microorganisms by way of the complement cascade. Cytokines produced in the cascade further contribute to the presence of inflammation. For example, macrophage migration inhibitory factor (MIF) is a cytokine that plays a role in fundamental events in both innate and adaptive immunity. There is evidence that MIF could play a role in RA, amongst other inflammatory diseases; therefore its antagonism may be a potential therapeutic option. MIF is abundant in the serum and synovium of patients with RA

and is known to induce the expression of pro-inflammatory genes and to be implicated in the proliferation and apoptosis of synoviocytes via p53 (Morand, 2005).

As well as bridging the time gap, the innate immune system contributes to the development of adaptive immune responses by increasing the flow of lymph containing antigen to the lymphoid tissues. Here, specialised dendritic cells become activated antigen presenting cells (APCs) that present the particular antigen to T lymphocytes. T cells are able to proliferate and generate highly specific clones that detect only the pathogen that induced them. Adaptive immunity is specific and long lasting. There are two types of response, antibody response mediated by B lymphocytes and cell-mediated responses, governed by T lymphocytes. B cells produce antibodies/immunoglobulins that act by binding to antigens, inhibiting their binding with host cells. T cells on the other hand can act to the antigen directly by killing the host cell whose surface they are presented on or by signalling and activating macrophages to destroy the microorganisms that they have phagocytosed (Alberts, 2002).

Strong evidence indicates that autoantigen recognition by specific T cells is crucial to the pathophysiology of synovitis in RA. T cells were found to participate in a complex network of cell- and mediator-driven events leading to joint destruction. Such T cells may be stimulated by an autoantigen - a highly conserved foreign protein cross-reacting with its human homolog, or a neo-antigen expressed as a result of posttranslational events. As well as the direct effects of autoimmunity by effector T cells, RA could result from defective homeostatic control of immunity by regulatory T cells (Fournier, 2005).

1.4 Rheumatoid Arthritis

RA is a chronic, progressive and disabling multifactorial autoimmune disease of unknown aetiology. The disease causes chronic systemic inflammation, particularly of the joints, and is thought to be sustained by environmental and genetic factors (Bond, 1996). RA affects approximately 690,000 people in the UK, making it the 2nd most common form of arthritis after osteoarthritis (OA), which affects 8.5 million Britons (Arthritis Research UK, 2012). The reported financial cost of RA to the UK was estimated to be £3.8-4.75 billion per year in 2010; this includes indirect costs and work-related disability benefit (NRAS, 2010).

Common symptoms of RA include: stiffness (particularly morning stiffness), fatigue, depression, irritability, anaemia and flu-like symptoms. Less common effects include: weight loss, uveitis, rheumatoid nodules and systemic inflammation (Arthritis Research UK, 2012). RA does not affect fibrous and cartilaginous joints, only synovial joints; most commonly, joints of the hands and feet, knee, elbow, shoulder and pelvis are affected (Lubberts, 2000). It is often noted in the diagnosis of RA that symmetrical joints are often affected at the same time (NICE, 2009). As RA is a systemic inflammatory disease, uncontrolled disease can lead to an increased risk of heart attacks and strokes in some patients (NRAS, 2010).

RA is classed as an autoimmune disease due to the presence of auto-antibodies that cause and sustain the chronic inflammation (Shipley, 2009; Firestein, 2003). The 2010 ACR/EULAR Rheumatoid Arthritis Classification Criteria is the most up to date RA criteria, overruling the criteria of 1987 (Arnett, 1988; Aletaha, 2010). To classify a patient as having or not having RA the following must be obtained:, a history of

symptom duration, a thorough joint evaluation, and at least one serologic test (rheumatoid factor [RF] or anti-citrullinated peptide antibodies [ACPA]) and one acute-phase response measure (erythrocyte sedimentation rate [ESR] or C-reactive protein [CRP]) (Aletaha, 2010).

The presence and detection of autoantibodies, in particular, RF and ACPA, allows for diagnosis up to six years before onset of RA symptoms (Aletaha, 2010; Rantapaa-Dahlqvist, 2003). ACPA is tested for as anti-cyclic citrullinated peptide (anti-CCP) antibodies. Citrullination is a type of post-translational modification that changes the amino acid arginine in a protein, for the amino acid citrulline. Enzymes called peptidylarginine deaminases (PADs) replace the primary ketamine group (=NH) by a ketone group (=O) (Basu, 2011). RF is an auto-antibody directed against the Fc fragment of immunoglobulin (Ig). These autoantibodies are thought to arise due to a mistake in the response to micro-organisms, switching between Th1 (pro-inflammatory) and Th2 (anti-inflammatory) responses (Shipley, 2009).

The disease can progress rapidly after the initial onset of symptoms, causing painful swelling of synovial joints due to an excess of synovial fluid produced by inflamed synovium. The hyperplastic synovium migrates from the joint margins to the cartilage, blocking the normal route between chondrocytes and their nutrition, causing irreversible damage to the synovial joint. Direct effects are also exerted by the cytokines, such as IL-1 and TNFα, released from the inflammatory cells (Murphy, 2008; Lubberts, 2000). The normal lining of a synovial joint is very thin and has very few blood vessels and no white blood cells. However, a rheumatoid joint lining becomes increasingly thick and dense with numerous new blood vessels and an infiltration of white blood cells. The inflamed synovial cells excrete chemicals, including enzymes, such as collagenase and stromelysin, which reduces the integrity

and resilience of the joint cartilage (Lubberts, 2000). Auto reactive T cells are thought to play a large role in the immunology of RA by reacting and binding to antigens in the joint synovium. This leads to prolonged inflammation, tissue damage and subsequent destruction of tissue (Murphy, 2008).

RA has proved to have been a difficult disease to establish in the past as the 1987 ACR criteria for diagnosis was not deemed sensitive enough for diagnosis of RA in its early disease stages (Aletaha, 2010). Moreover, a number of patients may also have been in remission at the time of a prevalence study and will therefore have been missed out in the data set. Sometimes, individuals may have been given a differential diagnosis like idiopathic RA or polyarthritis, particularly in the very early stages when it can be less clear that they are definitely suffering from RA. Currently, the underlying aetiology and detailed pathogenesis of RA is unknown.

1.5 Epidemiology

1.5.1 Prevalence

Worldwide, RA affects 0.5-1% of the population, with an incidence of around 20–50 cases per 100,000 annually. The majority of these are women; however the incidence of RA seems to be declining overall (Carmona, 2010). Prevalence varies between populations and is, for example, high in the Pima Indian population of North America and Alaskan Indians, where it can be as high as 7.1% of the whole population (Power, 1999; Acres, 2012; Bond, 1996) and much lower in black Africans and Chinese populations (Shipley, 2009; Gottlieb, 1974). The epidemiological studies conducted in Europe have produced data to give intermediate prevalence rates (Aho, 1986; Power, 1999). The incidence of RA in developing countries is lower than in developed countries. For example, it has been reported that China, Indonesia and South Africa all show a prevalence of RA lower than the aforementioned 0.5%; whereas developed countries have been described as having a prevalence of around 1% (Darmawan, 1993; Lau, 1993; Silman 1993b, Solomon, 1975). However, the incidence of RA in developed countries does appear to be decreasing (Doran[b], 2002; Kaipiainen-Seppanen, 1996; Hochberg, 1990; Symmons, 2002). Doran *et al.* conducted a population cohort study over four decades in Rochester, Minnesota, USA. The incidence of RA decreased throughout the study, almost halving over forty years from 62.1/100,000 in the first decade (1955-1964) to 32.7/100,000 in the last (1985-1994) (Doran[b], 2002). Furthermore, in Finland it was reported that there was a reduction of

approximately 40% (p=0.008) in the number of RF negative RA cases over a single decade (1980-1990) (Kaipiainen-Seppanen, 1996).

1.5.2 Morbidity

Extra-articular features of RA include rheumatoid nodules and interstitial lung disease, which are seen to occur at a much higher frequency in patients with RA than in controls; a clear correlation is shown to the activity and severity of the disease (Massardo, 1995; Sany, 2004). Cardiovascular disease (CVD) is considered as major co-morbidity, likely due to the systemic inflammatory nature of RA. Other co-morbidities, known to be significantly raised in patients with RA include congestive heart failure, chronic pulmonary disease, dementia, and peptic ulcer disease (Gabriel, 1999). Adverse effects due to treatment have more recently been considered as a type of comorbidity in RA (Michaud, 2007).

1.5.3 Current Treatment

The first line treatments for early stage and mild RA are analgesic and anti-pyretic agents such as low dose corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs), for instance: paracetamol, ibuprofen, aspirin, naproxen and diclofenac (Ruderman, 2015). As the disease becomes severe, anti-inflammatory therapy is combined with a high dose corticosteroid plus immunosuppressive and cytotoxic drugs such as azathioprine, cyclophosphamide and mycophenolate (Murphy, 2008).

Long acting corticosteroid injections such as methylprednisolone are usually administered into large joints, which are defined as shoulders, elbows, hips, knees and ankles (Aletaha, 2010); whereas the use of hydrocortisone injections is the preferred option when treating superficial joints and flexor tendon sheaths. Hydrocortisone is a weaker preparation and reduces the chance of side effects such as subcutaneous inflammation and skin atrophy (Moots, 2004).

Aside from treating the pain, doctors prescribe disease-modifying anti-rheumatic drugs (DMARDs); usually methotrexate is given first. However if methotrexate has little or no effect, sulfasalazine (and more recently so, leflunomide) can be given (Murphy, 2008). As with the treatment of other complex diseases, such as cancer; it is considered that RA is best targeted by combination therapy rather than with a single drug (Dale, 2007). 'Triple therapy' constitutes the addition of hydroxychloroquine to DMARDs methotrexate and sulfasalazine and has been shown to treat severe RA better than a single agent alone (Dougados, 2002). The vast majority of current RA treatments treat the symptoms of disease rather than the disease itself. The rise of DMARDs and new biologics has been paving the way towards more tailored treatment. DMARDs have been able to slow disease progression and new biologics such as tocilizumab, an anti-IL6 receptor monoclonal antibody, have shown to greatly benefit patients and could be a potentially superior treatment option (Choy, 2011). However, side effects include a reduction in circulating neutrophils, increased incidence of infections and increases in lipid and liver transaminases (Choy, 2011).

1.5.4 Cancer

There has been no overall association found between RA and cancer. A patient with RA seems to have the same risk of developing cancer, as the general population; however there appears to be an increased incidence of haematopoietic (leukaemia and non-Hodgkin's lymphoma) and lung cancers within the RA population. Predictive factors for an increased risk of cancer as a co-morbidity of RA is old age, a male sex, chronic disease duration and the use of cytotoxic drugs other than methotrexate (Abasolo, 2008; Khurana, 2008). The risk of malignant lymphoma is significantly increased in RA (standard incidence ratio (SIR) =2.0) (Askling, 2005) and relates to the degree of inflammation (Carmona, 2010; Ekstrom, 2003; Franklin, 2006; Zintzaras, 2005). Furthermore, there is also some evidence to suggest that delayed exposure to viruses in early life can lead to an increased chance of developing childhood acute lymphoblastic leukaemia (cALL) (Brown, 1961). One possible mechanism may be due to the fact that lymphocytes are stem cells that can acquire somatic mutations as a result of viraemia (Morris, 2012). This links us back to the possibility of RA developing as a result of pathogenic invasion. If stem cells can mutate as a result of viraemia to cause leukaemia, the defective T cell response characteristic of RA may also develop from alterations caused by bacterial or viral infection in early life.

1.5.5 Mental Health

Many studies have reported an increase of depression and anxiety disorders in patients with RA (Covic, 2009; Lok, 2010). Patients who have little social support and who rely on social welfare assistance are more prone to developing a psychiatric disorder. Conversely, the incidence of schizophrenia is reduced in RA and there is sufficient evidence to confirm the negative relationship between both diseases (Gorwood, 2004).

1.5.6 Infection as a Result of RA

The rate of infection, specifically of opportunistic infections, is increased in RA compared to that of the general population. For example, tuberculosis (TB) infection is increased fourfold (Carmona, 2003) and herpes zoster twofold (Wolfe, 2006). More recently it has been documented that the incidence of *Pneumocystis jirovecii* (formerly *Pneumocystis carinii*) infections in RA patients are on the rise, especially since the use of new biologics and they also show a mortality rate higher than that of human immunodeficiency virus (HIV)-positive individuals with this fungal pneumonia-causing infection (Mori, 2012). These increased rates are evidently attributed to the amount of systemic inflammation, the immunomodulatory effect of RA and the immunosuppressive drugs used in its treatment; e.g. corticosteroids, methotrexate, infliximab. The risk of death from infection is ten times higher in individuals with RA (Meune, 2009; Naz, 2007).

1.5.7 Mortality

Studies often report an increased mortality rate in RA patients compared to the general population. Standard Mortality Ratios (SMRs) can differ dramatically from 0.8 to 3.0; however, depending on the exclusion criteria, RA associated SMRs fall between the range of 1.5-1.8 (Meune, 2009). In a recent Dutch study, van Nies *et al.* compared the mortality rates of three RA cohorts with that of the general population of the Netherlands. The cohorts represented three different time periods: 1993-1995 (mainly NSAID use), 1996-1998 (mainly hydroxychloroquine and salazopyrin use) and 1996-2006 (methotrexate and new biologics). The SMR for the most recent period, when treatment was most aggressive, was 0.49. This was significantly reduced from the two previous periods which were 1.35 and 1.23 respectively (van Nies, 2010; Carmona, 2010). Life expectancy has been calculated to be reduced by 8 years in men and 9 years in women (Lassere, 2012).

The risk of CVD is 60% higher in individuals with RA and is the major killer amongst the RA population, as it is in the general population, and accounts for 40% of deaths in RA patients (Meune, 2009). Other RA-associated causes of death include: cancer (17%), infection (14%), musculoskeletal disease (9%), respiratory disease (9%) and renal disease (6%) (Meune, 2009). Significant predictors of mortality are older age, male sex, a comorbidity and functional status (measured by the Health Assessment Questionnaire (HAQ)) (Meune, 2009). Disease evaluation may extend to the assessment of socio-economic factors, extra-articular disease and measurements of disease severity, including joint count, RF positivity and ESR (Naz, 2007; Sokka, 2008).

1.6 Hygiene Hypothesis

1.6.1 Background

Over the twentieth century, a number of factors have led to a reduction in the amount of cross-infection in young families; such as a lower birth rate, a smaller family size, higher standard of personal cleanliness and improvement to household sanitation and amenities. The idea of a hygiene hypothesis was initially generated by David Strachan, who observed an inverse relationship between individuals that suffered from hay fever and the number of older siblings that they had. This study involved 17,414 British children born in the same week in March 1958 (Strachan, 1989).

The hygiene hypothesis was developed when a decrease in infectious burden in urbanised and industrialised countries was associated with an increase in autoimmunity and allergic diseases, such as RA, type 1 diabetes (T1D) and multiple sclerosis (MS). Criteria for a possible autoimmune disease are: an incidence and/or prevalence that is rising in developed countries; a disease more common in females than males; a disease more common in higher socio-economic groups and seasonal variation (Acres, 2012). According to the hygiene hypothesis, delayed exposure to certain pathogens means that the immune system is not able to mature properly; which can lead to an inappropriate immune response later in life.

Infections, bacterial and viral, appear to produce the most severe symptoms, on first exposure. Following this exposure, the initial innate immune reaction converts to a more specific, acquired immunity. Therefore, subsequent exposure to the same virus or bacterium produces only a mild or asymptomatic condition (Morris, 2012). It is

thought that delayed exposure to a pathogen often leads to a more severe infection. The more severe infection can be the result of two dynamic processes: a decreasing exponential curve of the incidence of the primary exposure to a common bacterium and increasing deterioration of immune functions in an ageing human body (Morris, 1987). Morris describes autoimmunity: a decision theory model based on concepts from statistical decision theory. The probability of the first exposure to a common organism decreases exponentially as we age. For example, 50% of the population are initially exposed to the organism in the first year of life, 25% are primarily exposed in the second year, 12.5% in the third, 6.25% in the fourth and so on. The resulting curve peaks early for more common organisms and later for rarer organisms (Morris, 1987; Morris, 2012). Not only is the timing of infection important, but also the dose and route of primary infection. It is thought that the preferred exposure, in order to reduce the chance of severe disease, is a low dose of the organism via the mucosal route, at an early age (Morris, 2012).

An example of a disease being more severe after delayed exposure is that of paralytic poliomyelitis, seen in epidemics of the 19th century. Typically, children would suffer a mild infection with little or no paralysis. Yet, as social standards improved and technology progressed at the turn of the twentieth century, children began to encounter the virus for the first time after infancy. This lead to an increase in the incidence of paralysis related to this infection (Nathanson, 1979). Furthermore, the incidence of glandular fever is a relevant example, caused by Epstein Barr Virus (EBV). This viral infection is very common in Africa and as children contract it at an early age, infection is so mild that it is rarely diagnosed as glandular fever. In developed countries, lower socio-economic groups are, like the Africans, often exposed early; however for individuals in higher socio-economic groups, the initial

encounter is in the teenage years (Heath, 1972). An infection of EBV at this age usually results in severe disease, often lasting months and causing severe inflammation of a number of organs such as the liver and spleen.

1.6.2 Molecular Mimicry

There are approximately 25,000 human protein-coding genes, resulting in the coding of more than 250,000 proteins. The huge amount of proteins, coded for by 10-fold fewer genes, is possible due to alternative splicing of messenger RNA (mRNA) and post-translational modifications such as phosphorylation, acetylation and citrullination (Morris, 2012). Bacteria only have between 3000 and 5000 genes coding for a similar number of proteins; viral genomes are considerably smaller still. However, due to the huge number of bacterial and viral species, as well as gene conservation throughout evolution, there are many genes that share very similar sequences across species (Tlaskalova-Hogenova, 2011). This makes it easier for an immature immune system to make a mistake and recognise a foreign peptide as self. If a peptide in unable to appropriately fit into the groove of the major histocompatibility complex (MHC), recognition can be compromised due to impaired molecular presentation (Morris, 2012).

It is possible that similar sequences between foreign and self-peptides can be sufficient enough to result in the cross-activation of auto-reactive T or B cells (Getts, 2010). For example, the QKRAA amino acid motif found in human leukocyte antigen subunit DRB1*0401 (HLA-DRB1*0401) occurs 37 times in the UniProt database, a comprehensive database of protein sequences and functional information. As well as

occurring naturally in the human body, the motif has also been found to occur in glycoprotein 110 of EBV and in *E.Coli* (Balandraud, 2004), meaning that a response to the QKRAA motif of either of these pathogens could cause the immune system to mistake HLA-DRV1*0401 for an invader, initiating autoimmunity.

The concept of molecular mimicry was initially suggested by Fujinami and Oldstone in 1985, when describing 6 consecutive amino acids shared by encephalitogenic site of rabbit myelin basic protein (MBP) and Hepatitis B virus polymerase (HBVP) (Fujinami, 1985). T cell receptors recognise antigens, both self and foreign, and are understood to be degenerate. This allows T cell activation by a number of peptides bound to one or numerous MHC molecules. The flexibility of the T cell receptor's (TCR) recognition is thought to be a key characteristic that allows the immune system to detect, recognize and react to the vast majority of pathogen-derived peptides. However, this apparent degeneracy leaves the mechanism vulnerable to the induction of autoimmunity driven by microbial antigens. It has been shown that viral/bacterial peptides with a certain degree of homology with self-antigens are able to stimulate auto-reactive T cells, *in vitro* (Wucherpfennig, 1995). Cross-reactive self-antigens are known to activate complement pathways that cause tissue damage due to cytotoxic products released during the process of inflammation (Wucherpfennig, 1995).

For example, infection of a group A beta-haemolytic streptococci such as *streptococcus pyogenes* can induce rheumatic fever, a persistent autoimmunity following infection (Murphy, 2008). This is thought to occur via the mechanism of molecular mimicry, where infected individuals produce autoantibodies to the heart, brain, skin and joints (Fujinami, 2006). Monoclonal antibodies from these patients cross react with antigens of the bacteria such as the group A carbohydrate antigen and the group M protein, as well as, myosin, a superfamily of human motor proteins. It has

been shown in murine models of rheumatic heart disease, that cross-reactivity between M protein and cardiac myosin can result in autoimmune disease (Fujinami, 2006). It is thought that for autoimmunity to occur, the virus must cross-react with a 'disease-related' epitope. This epitope can be presented by MHC class II on APCs such as macrophages, dendritic cells and B cells to auto-reactive $CD4^+$ T cells. Induction of epitope-sufficient activation of APCs appears to be necessary, as autoimmunity only occurs *in vivo* with the addition of complete Freund's adjuvant (CFA) or a dose of the infection itself (Fujinami, 2006).

Other examples of instances of molecular mimicry include EBV as a cause for Guillain – Barré syndrome (Grose, 1972); and Hepatitis C virus and Herpes Simplex Virus (HSV) have been linked to the development of Myasthenia Gravis (Eddy, 1999; Schwimmbeck, 1989). Myasthenia Gravis sufferers present with characteristic antibodies to the human acetylcholine receptor (HuAChR). The HuAChR α-subunit residues 160-167 show 'specific immunological cross-reactivity' with a homologous domain present as part of HSV glycoprotein D, residues 286-293. It was concluded that the cross-reactivity of the 'self-epitope' with HSV could suggest a viral role in the pathogenesis of Myasthenia Gravis (Schwimmbeck, 1989).

Mechanisms similar to, but aside from molecular mimicry, include bystander activation and persistent viral infection with or without epitope spreading. Such mechanisms are not mutually exclusive and are dynamic. It has been difficult to establish a causal relationship between RA and a pathogen-derived trigger of human autoimmunity. This is due to the problem that symptomatic onset of RA usually occurs after a considerable period of subclinical immune responses. After such a long time, the initial pathogen is likely to have been cleared by the immune system or immune responses will have subsided. This has also been deemed the 'hit-and-run'

hypothesis (Getts, 2010). Repeated and chronic viral infection can cause damage mediated by the immune system. After recovery from acute disease, the infection can persist. The virus, its proteins and genome, can be detected throughout life. T cell responses promote inflammation. A key example of this is persistent CNS infection of Theiler's murine encephalomyelitis virus in susceptible mice (Fujinami, 2006).

1.6.3 Bystander activation

It has been shown in established murine models, for T1D and MS, that active APCs have the ability to activate auto-reactive T cells that are already present in the body. Furthermore virus-specific T cells can be activated, which are able to migrate to an organ containing infected cells where APCs present viral peptides on MHC class I which are recognised by CD8+ T cells. Following this, the CD8+ cytotoxic T cells, APCs and already dying infected cells release a deadly cocktail of cytokines such as tumour necrosis factor alpha and beta (TNF α and β), nitric oxide (NO), lymphotoxin (LT), interferon-gamma (IFN-γ) and interleukins (IL); which consequently kill neighbouring, non-infected cells. This results in further inflammation in the infected area (Fujinami, 2006). CD4+ T cells recognise peptides presented by MHC class II and are able to directly kill uninfected cells, as can macrophages (Yasukawa, 1993).

1.6.4 Infection as a cause of RA

There is a good possibility of an infectious cause of RA; therefore its epidemiology is being studied alongside the hygiene hypothesis in order to establish if there is any association. The surge in incidence of autoimmune diseases, such as asthma, MS and Crohn's disease in developed countries has been linked with the hygiene hypothesis (Okada, 2010). Currently, a viral or micro-organism infection is the strongest candidate for an environmental RA trigger. Both RF and ACPA have been found in individuals who do not suffer from RA after a viral, bacterial or parasitic infection (Rashid, 2012). This shows that when these antibodies are found in RA sufferers, they could perhaps have originated from an earlier infection which has left their products behind, allowing them to play a role in the pathogenesis of RA. Due to the worldwide distribution of RA, it has been suggested that it may not be just a single agent that is responsible (Silman, 1997). Also, due to the spatial and non -temporal clustering nature of the incidence of RA, there is an argument against an acute epidemic infection and more towards an endemic agent (Silman, 1997).

EBV has been suspected as of one the causal agents of RA for the last 35 years, with good evidence. EBV-specific suppressor T cell function is known to be defective in RA sufferers and patients are shown to have higher serum levels of anti-EBV antibodies. The EBV load in peripheral blood lymphocytes of RA patients (median 8.84 copies/50ng DNA) was found to be considerably increased from that of healthy individuals (median 0.6 copies/50ng DNA) (Balandraud, 2004). The virus is highly recognised by the immune system; however it is never eliminated and can remain latent for long periods of time. EBV has also been associated with the initiation and

development of MS and Systemic Lupus Erythromatosus (SLE) via the proposed mechanism of molecular mimicry (Getts, 2010).

A suggested candidate bacterium is *Porphyromonas gingivalis*, a periodontal pathogen that when tested along with numerous other oral bacteria, showed a unique ability to citrullinate proteins. Results of a study by Wegner *et al.* suggest that the citrullination mediated by *P.gingivalis* occurs in both the bacterial proteins and host proteins, thus providing a potential molecular mechanism to generate epitopes to which the body has no immunologic tolerance (Wegner, 2010).

Laugisch *et al.* investigated peptidylarginine deiminase (PAD) and *P.gingivalis*-derived enzyme (PPAD) activity in the gingival crevicular fluid (GCF); looking at serum levels of antibodies against citrullinated epitopes in RA and periodontitis. Citrullination, PAD and PPAD activities were present in similar amounts within the RA and non-RA populations. The enzyme activities, derived from both human and bacteria, were raised in patients with periodontitis regardless of RA disease (Laugisch, 2016). PPAD secreted by *P. gingivalis* residing in epithelial cells may be able to citrullinate peptides in distant regions of the periodontium or other distant tissues, generating ACPAs after breaching immunotolerance in susceptible individuals.

Both the presence of foreign (bacterium) citrullinated proteins and the foreign mode of proteolytic processing and post-translational modification of host protein, give the potential as a causative mechanism from *P.gingivalis*.

Another candidate bacterium is *Proteus mirabilis*, a regular member of commensal gut flora that can become pathogenic when given the opportunity to infect a wound or the urinary tract. Urinary tract infections (UTI) appear to be much more common in patients with RA (Tishler, 1992). Studies have shown that isolation of *P.mirabilis* in

the urine of RA patients can be two-fold higher than controls. Levels of *P.mirabilis* are particularly increased in females (63%) with RA compared with males (50%); and even more so than in healthy female (32%) and male (11%) controls (Ebringer, 1996). In relation to a genetic link to RA, a study using rabbits injected with HLA-DR4 positive lymphocytes showed that they produced antibodies to *P.mirabilis* and none of the further eighteen microbes tested for (Ebringer, 1985). Furthermore, such antibodies were found to be in higher titres in the sera and urine of RA patients compared to controls (Rashid, 2007; Ebringer, 2010). Although the evidence regarding *P.mirabilis* is convincing, it is important to note that the majority of papers published are by the same group.

1.7 Staphylococcus Aureus

1.7.1 Staphylococcus Aureus

The polio and EBV examples previously described (Chapter 1.5.1) give reason to contemplate *S.aureus* infection as a possible cause or contributor to the pathogenesis of RA in relation to the hygiene hypothesis. As mentioned earlier, *S.aureus* is a common pathogen, often initially encountered early in life. However, delayed exposure may lead to an inappropriate immune response to the pathogenic peptides. For example, the human T cells may mistake *S.aureus* peptides for self; as part of the phenomenon of molecular mimicry.

S.aureus is a very common bacterium, which has evolved to become both a component of the commensal flora and a major cause of invasive infection, like *P.mirabilis* (Chapter 1.6.5). *S.aureus* is not always pathogenic but is known to be a common cause of respiratory infections (sinusitis), skin infections (boils, atopic dermatitis and staphylococcal scalded skin syndrome (SSSS)) (Curran, 1980) and food poisoning. The bacterium can also play a role in or cause life threatening diseases such as endocarditis, toxic shock syndrome (TSS) and sepsis. It has been estimated that 25-30% of healthy, asymptomatic humans are permanent nasal carriers, with a further 30% carrying the bacterium intermittently (Vandenbergh, 1999). More than 50% of *S.aureus* can produce one or more of the SAgs (Kluytmans, 1997). SAgs are superior to regular antigens as they do not need to endure processing by APCs and are also able to independently induce polyclonal T cell expansion of T cells that contain specific Vβ chains in their TCR (Kageyama, 2001; Choi, 1990).

Staphylococci were first described by Alexander Ogston in 1880, identified in pus from a knee joint. The term *S.aureus* was then coined by Friedrich Julius Rosenbach in 1884 (etymologia: staphylococcus, 2013). It took another 50 years however for the prevalence of *S.aureus* to be noted for the first time (Hallman, 1937; Hart, 1937). This delay was not due to a lack of recognition of healthy carriers but because the numerous staphylococci and micrococci could not be differentiated until the development of the coagulase test in 1934 (Chapman, 1934; Cruickshank, 1937).

Severe respiratory infection due to staphylococci is on the increase. The ability of *S. aureus* to adapt to the environment of the respiratory tract has enabled its persistence in the airways, via adaptations such as the expression of surface adhesins (Parker, 2012). Its metabolic versatility, the ability to scavenge iron, coordinate gene expression, and the horizontal acquisition of useful genetic elements have all

contributed to its success as a component of the respiratory flora, in both ill and healthy hosts (Parker, 2012).

There are numerous strains of *S. aureus*, and dependent on type, they are capable of secreting a number of enzymes, haemolysins (alpha, beta, delta and gamma) and exoproteins. The exoproteins include: leucocidin, exfoliative toxins (EFA and EFB), staphylococcal enterotoxins (SEA-E, G-I) and TSST-1. The SEs (A-E, H), TSST-1 and streptococcal pyrogenic exotoxins (A-C, F-H, J and streptococcal superantigen) are also part of a family called the pyrogenic toxin superantigens (PTSAgs) which share a number of functional characteristics, as well as, genetic and biochemical characteristics. To be included in the group, each of the toxins needs to show a number of biological properties, including: superantigenicity, pyrogenicity and the capacity to enhance the lethality of endotoxin in rabbits up to 100,000-fold (Bohach, 1990; Bohach, 1994; Marrack, 1990).

Mature PTSAgs, cleaved of their precursor proteins, are small, non-glycosylated polypeptide molecules with molecular weights between 20 – 30 kDa. They are moderately stable and are relatively resistant to denaturation by boiling or enzymatic lysis (Dinges, 2000). An abundance of the TCR β-chain variable regions (Vβ14+) have been isolated from T cell populations in synovial fluid taken from the joints of patients with RA compared to the peripheral blood, where they are almost absent. Results from Paliard *et al*. suggest that the aetiology of RA may involve the initial activation of Vβ 14+ T cells by a Vβ 14-specific superantigen. This could indicate the detrimental presence of PTSAgs (Paliard, 1991). PTSAgs have the ability to activate autoreactive T cell clones, meaning that they could induce autoimmune disease in humans. However, a definite mechanistic link between autoimmunity and the presence

of PTSAgs has not been confirmed. The aforementioned Vβ skewing of T cells is not typical in normal infection.

1.7.2 *S.aureus* toxins

1.7.2.1 Alpha Haemolysin (AH)

AH is the dominant cytotoxic agent secreted by *S.aureus* bacteria (Bhakdi, 1991). The toxin is a water-soluble, monomeric protein and has a pore-forming beta-barrel structure; which binds to specific, but unidentified cell surface receptors to create heptameric pores that lead to DNA fragmentation and subsequent apoptosis (Tanaka, 2011). This is due to the rapid loss of vital molecules, such as adenosine triphosphate (ATP), dissipation of the membrane potential and ionic gradients and osmosis-induced cell wall rupture (Tweten, 1983).

At low concentrations, AH induces pro-inflammatory mediators, promoting a breach of the epithelial barrier (Frank, 2012). At these low concentrations, AH binds to an as yet unidentified receptors that have been reported on the outer cell membranes of a number of cells, including human monocytes, platelets and endothelial cells, as well as, rabbit erythrocytes, which have been found to be particularly susceptible. Low concentrations of 1µg/ml (less than 100nM) can be lethal in this instance (Bhakdi, 1991). The rabbit erythrocytes are much more susceptible to lysis by AH than other cells; at least 100 times more than other mammals and 1000 times more than human erythrocytes (Bernheimer, 1963; Bhakdi, 1984). However, at higher concentrations

(>1μM), AH binds non-specifically by adhering to the cell membrane and utilises its pore-forming ability to disrupt the ion gradients and affect the membrane integrity, subsequently leading to direct lysis (Foletti, 2013).

Although pore formation and cellular lysis appear to be the main consequential characteristics of AH secretion, a number of studies have suggested that sub-lytic concentrations produce unfavourable cellular responses, particularly by altering the cell signalling pathways governing cell proliferation, cytokine secretions and inflammatory responses (Bhakdi, 1991). Furthermore, there is evidence that indicates that AH induces the rapid processing and secretion of interleukin1β (IL-1β) if there is an accumulation of the IL precursor intracellularly. Increased production of IL-1β has been linked to the development of a number of autoinflammatory syndromes (Masters, 2009).

1.7.2.2 Staphylococcal Enterotoxin B (SEB)

SEB is an exotoxin superantigen that is able to regulate the activity of immunomodulatory and pro-inflammatory cell types (Li, 2015). SEB is a relatively stable toxin that can be resistant to proteolytic enzymes; remaining active after its journey through the digestive tract and even once the staphylococcal infection itself has been eradicated (Nema, 2007). A study by Argudín *et al.* indicates that SEB can penetrate the intestinal wall, inhibit intestinal absorption of water and electrolytes, and trigger a local or systemic immune response (Argudin, 2010).

SEB has a strong immuno-regulatory function and plays a vital role in the pathogenesis of autoimmune disease either by initiating the autoimmune process or by inducing relapse in a person in clinical remission from an autoimmune disease. SEB, itself, can directly activate a large number of T lymphocytes without the traditional antigen presenting mechanism. Compared with normal antigen-induced immune response in which 0.001-0.0001% of the body's T cells are activated, SEB is capable of activating up to 20% of T cells, because it has a unique ability as an SAg to bridge the MHC class II on the APCs and the TCRs in both CD4 and CD8 T cells; forming a tri-molecular complex (Papageorgiou, 2000; Li, 1998). This bridging effect causes the release of massive amounts of cytokines and chemokines, specifically interleukin 2 (IL-2), TNFα, interferon-γ (IFN-γ) and chemokine (C-X-C motif) ligand 1 (CXCL1) (Fujiki, 1999; Ito, 2000; Liu, 2004, Marrack, 1990). The mechanisms described are shown in Figure 1.1.

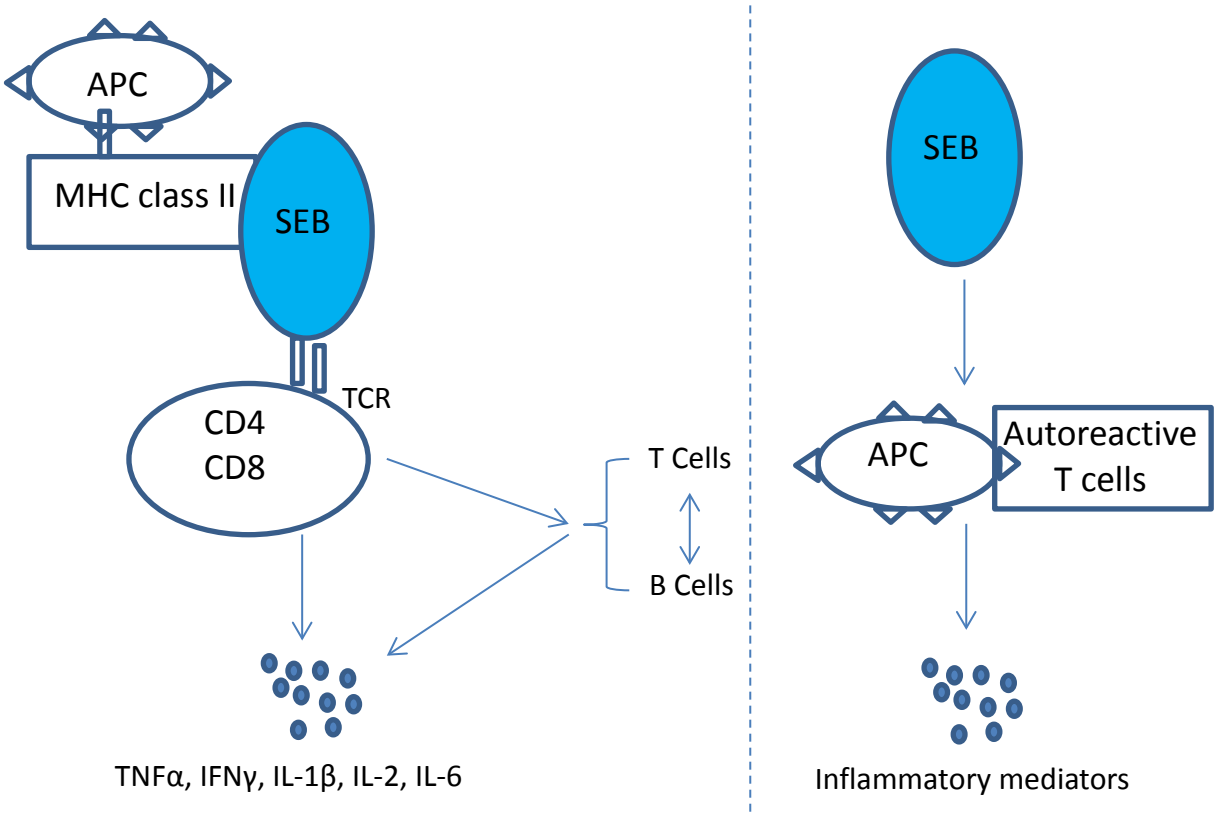


Figure 1.1 **Diagram showing two ways in which SEB may be involved in the pathogenesis of RA.**

SEB has been implicated in the pathogenesis of RA by activating T and B cells in combination with Vβ-TCR and activating polyclonal B cells as a bridge between T and B cells at the same time. Histological studies of synovium have shown that RA patients had increased levels of serum IgM SEB antibody compared with normal subjects, and synovial cells could also serve as APCs for SEB and induce further T cell activation (Sybre, 2012; Origuchi, 1995).

Omata *et al.* studied collagen-induced arthritis (CIA), the experimental disease model of human RA. Their results confirmed that SEB in implicated in activating specific autoreactive Vβ8+ T cell clones, producing Th1 cytokines, IL-2 and IFN-γ, which are all important inflammatory mediators implicated in the joint destruction typical in RA.

1.7.2.3 Staphylococcal Enterotoxin C (SEC)

The SEC SAgs are a group of highly conserved proteins with significant immunological cross-reactivity. There are three antigenically distinct SEC subtypes - SEC1, SEC2, and SEC3 - distinguishable by their isoelectric point (pI) values: approximately 8.6, 7.8, and 8.2, respectively (Reiser, 1984). Amongst the SAgs, SEC and SEB are the most homologous and share antibody binding epitopes (Schmidt, 1983; Spero, 1978), which lead to antibody cross-reactivity.

1.7.2.4 Toxic Shock Syndrome Toxin 1 (TSST-1)

TSST-1 is a SAg known to be produced by 5-25% of *S.aureus* bacteria (Kageyama, 2001; Choi, 1990).

As well as making APCs redundant, SAgs may have other distinctive qualities (Kageyama, 2001; Choi, 1990). For example, TSST-1 is unique, in that it has the ability to cross mucosal surfaces (Hamad, 1997). This is of particular interest as *S.aureus* often occupies mucosal areas, such as the nasal cavity and this ability means that TSST-1 does not need a break in the skin in order to affect the host. Furthermore, TSST-1 is the only PTSAg known to reactivate bacterial cell wall-induced arthritis (Schwab, 1993) and has been implicated in the pathogenesis of a number of human diseases, both acute and chronic (Kotzin, 1993). Examples include TSST-1 being found or isolated in 60% of cases with Kawasaki syndrome (Leung, 1993) and in the kidneys of 18% of cases of sudden infant death syndrome (SIDS) (Newbould, 1989).

Higher serum levels of TSST-1 have been reported in RA patients (Tabarya, 1996), which was further confounded by an experiment showing an increase in incidence and severity of arthritis in mice, compared with control mice lacking TSST-1 (Kageyama, 2001). However, TSST-1 is not thought to be a causal agent of RA when acting alone, it is thought to play a more adjuvant role. For example, bacterial cell walls, exotoxins, endotoxins or DNA may heighten the current status of the disease. Experiments conducted by Kageyama *et al.* suggest that products from bacteria could potentially have a strong association with the induction and maintenance of autoimmune disease (Kageyama, 2001).

1.8 Aetiology

As mentioned earlier, RA is of unknown aetiology, however, a huge number of factors are being investigated such as: genetics, socio-economic status (SES), geographical location, urbanization, seasonal variation and infectious exposures during childhood. The latter of these gives rise to the consideration of the hygiene hypothesis (Section 1.5). A number of autoimmune diseases have already been linked with the hygiene hypothesis such as asthma, T1D, MS and Crohn's disease (Okada, 2010). It is hypothesised that delayed exposure to particular pathogens could increase the risk of developing an incorrect immune response in later life. Therefore, this review of current literature looks to explore what is already known about the epidemiology of RA, with a specific focus on the relevance to the hygiene hypothesis.

1.8.1 Age

People of any age can develop RA, however, epidemiological studies in the UK, USA and Norway have described the average age of onset as around 55-64 years in women and 65-75 years in men. Over the last half decade the general age of onset has increased, while the overall incidence and prevalence of RA has decreased (Symmons, 2002). It is also important to note, that there are approximately an extra 12,000 individuals in the UK under the age of 16, suffering from juvenile RA (Arthritis Research UK, 2012).

1.8.2 Gender

Numerous studies have consistently reported that RA affects many more women, specifically premenopausal women, than men (Doran[a], 2002; Kaipiainen-Seppanen, 1996; Symmons, 2002; Bond, 1996; Iikuni, 2007). A study conducted by Doran *et al.* found that the incidence of RA peaked at an earlier age in women than men and even claimed that women were three times more likely than men to have RA (73.1% female to 26.9% male) (Doran[a], 2002; Shipley, 2009). Although incidence and prevalence of RA is higher in women than men, it has been shown that RA-related mortality is significantly higher in men than women ($p<0.001$) (Kuo, 2012). It is also important to note here that of the general population, men have a 50% greater risk of dying of natural causes, at any age. This has been partly linked to the fact that men have at least 1000 less genes than women due to only having one X chromosome (Morris, 2009). Furthermore, the lack of the heterozygous advantage of X leaves them more susceptible to, otherwise recessive, genetic disorders. It appears that men are less susceptible to disease overall, however women are able to live with disease and disability for longer.

The gender difference has led questions as to whether hormones may play a role in the development of RA. A small group of seropositive (IgM-RF positive) women with RA were assessed for the concentration of a number of hormones. Normal hormone levels were observed in the premenopausal women, however the postmenopausal women displayed significantly increased levels of testosterone ($p<0.05$), androstenedione ($p<0.05$) and dehydroepiandrosterone (DHEAS) ($p<0.01$) (Cutolo, 1986). Still, this observation of hypergonadism relative to osteoarthritis (OA) controls is not fully explained. Nevertheless, there have also been reports of a reduction in

plasma DHEAS in female RA patients (Spector, 1988). Similarly, serum levels of free testosterone and plasma androgens were found to be decreased in male RA patients compared to OA age matched controls.

Alternatively, it has been suggested that the difference in incidence between the sexes is not down to the direct effects of the sex hormones themselves, but rather it may be due to the behavioural and associated differences in exposure to environmental pathogens (Bond, 1996).

1.8.3 Birth Order

Believed to be the first study into the association of birth order with the incidence of RA; Sayeeduddin *et al.* studied 115 patients attending a rheumatology clinic in Punjagutta, Hyderabad, India. It was reported that being the 1^{st}-3^{rd} child born into a family meant that there was an increased likelihood that the individual would develop RA. Of the 115 patients studied, 65-70% were the 1^{st}-3^{rd} born and 32.2% of the patients with diagnosed RA were the first born child (Sayeeduddin, 1994).

The Hertfordshire Cohort Study (HCS), assessed each of the 1397 participants involved (born between 1930 and 1939) for markers of infectious exposure during their childhood, compared with each individual's RF measurement. They found a trend towards a lower birth order (2^{nd}-5^{th}+) as being associated with a lower chance of becoming RF positive, in women only (Edwards, 2006).

1.8.4 Sharing a bedroom

As part of the HCS, it was also investigated as to whether sharing a bedroom had any significant impact on RF positivity in both men and women. They documented that sharing a bedroom during early childhood significantly reduced the likelihood of being RF positive later in life (Odds Ratio (OR) 0.48, 95% Confidence Interval (CI) 0.30 to 0.78, p=0.003). This was only the case in women, there was no significance amongst the male population studied (Edwards, 2006).

1.8.5 Exposure to animals

Bond *et al.* carried out a case-control, retrospective study in Adelaide, South Australia in order to establish if there was an association between RA and exposure to animals in three distinct periods of an individual's lifetime: the first 5 years from birth, the 5 year period before puberty and the 5 year period before RA disease onset. Firstly, using questionnaires they looked into the history of each patient from birth to onset of rheumatic disease (n=122 RA and 114 controls). It was found that prior exposure to cats (OR 3.04 95% CI 1.69-5.50) and budgerigars (OR 1.74 95% CI 0.83-3.66) was associated with the occurrence of RA (Bond, 1996). This study follows up from an earlier study by Gottlieb *et al.* that stated that patients with RA appeared to have had greater exposure to dogs, cats or birds (combined), dogs alone and sick animals in the 5-year period before disease onset compared to controls (Gottlieb, 1974).

Bond *et al.* also describe a dose-response effect of a longer extent of prior exposure leading to a greater risk of developing RA (OR 3.60 95% CI 2.01-6.47 p=0.0001). Furthermore, they investigated the three periods mentioned earlier. There was a statistically significant number of RA patients who had been intimately exposed to cats during the 5 year period before puberty (OR 3.10 95% CI 1.74-5.55). These data may suggest that pets can act as reservoirs for infection that could, after a latency period, trigger RA (Bond, 1996). Furthermore, nasal carriage of *S.aureus* is common in dogs and guinea pigs, giving support to the hygiene hypothesis (Section 1.2) (Morrison, 1961; Freeman, 1956).

1.8.6 Socioeconomic status (SES)

Numerous studies have described a higher prevalence of RA in more affluent and highly educated areas compared to poorer and lower social classes. A trend was found associating a higher social class with an increased chance of testing RF positive, in women only in Hertfordshire, UK (n=1343) (Edwards, 2006). Social class was determined however, by the father's occupation at the time of the child's birth and during early childhood, which may not properly determine the social class of the family or the lifestyle that they led. These results were not statistically significant (OR 0.8 95% CI 0.4401.48). In Karachi, Pakistan a study was undertaken of 4232 adults, evenly distributed between a squatter settlement, characterised by poverty and poor sanitation; and an affluent district consisting of a large professional population (Hameed, 1995). Therefore, it was assumed that the affluent population had a better standard of living, better sanitation, a certain level of education and better personal

hygiene. The prevalence of RA was determined to be higher in the affluent area (1.98/1000) compared to the poorer settlement (0.9/1000). Access to healthcare amenities may affect these data as it is assumed that wealthier communities would be more willing and have easier access to a doctor in order to express any health complaints.

Education is an established marker of SES (Reckner Olsson, 2001; Pedersen, 2006; Bengtsson, 2005; Bergstrom, 2011). Both Olsson *et al.* (Reckner Olsson, 2001) and Pedersen *et al.* (Pedersen, 2006) carried out studies in Denmark that described an association of RA, particularly RF-positive RA, to the level of education. Pederson even went on to report a 2-fold lower risk of developing RA in the lowest bracket of education compared to those in formal education for the longest duration. It is, however, self-criticised by both studies that a higher educational level may affect lifestyle and patterns of healthcare use. A Swedish case-control study of 930 cases (1126 controls) between 1996 and 2001 found that those with a university degree had an increased risk of developing RA compared to those with no degree (Bengtsson, 2005). Furthermore, it was reported that there was a 20% increased risk of RA in manual and intermediate manual workers compared to non-manual (white collar) workers. Once again, results were more pronounced for seropositive RA and an increased risk linked to women. A much more recent study of 290 cases of RA identified in Sweden between 1974 and 1994 further supports the inverse association described. Bergström *et al.* reported that blue collar workers (manual, skilled and unskilled) had a significantly greater risk of developing RA compared to individuals classed as white collar workers (non-manual workers and professionals) (OR 1.54 95% CI 1.112-2.10) (Bergstrom, 2011).

1.8.7 Smoking

Cigarette smoking has been shown to be a major environmental risk factor for RA in numerous studies and is often associated with the lower socio-economic classes (Sugiyama, 2010). Smoking has been shown to have a causal link to rheumatoid vasculitis and extra-articular disease, particularly rheumatoid nodules and pulmonary disease (Wolfe, 2000; Albano, 2001). Both past and current smokers are at an increased risk of RA; seen in both sexes, particularly men, for whom there is a significant linear dose-response relationship with the number of tobacco pack years ($p < 0.005$) (Reckner Olsson, 2001; Wolfe, 2000). Here, it is also important to note that as the worldwide use of tobacco has reduced, so have RA manifestations (Albano, 2001). Quantitative relationships have been established between the extent to which one smokes and RF positivity, RF concentration, radiographic progression, nodule formation and pulmonary disease; the latter three of which are independent to RF positivity and RF concentration (Wolfe, 2000). The increased RF measurement may be due to the effect of RF production being able to mediate some of the detrimental effects of smoking (Naz, 2007). Furthermore, Hensvold *et al.* studied genetic and environmental factors in the development of ACPAs and ACPA positive RA in twins. It was concluded that variability in the ACPA status between twins was mostly accounted for by non-shared environmental or stochastic factors rather than shared environmental and genetic factors. However, an association was specified between smoking, the shared epitope (HLA-DRB1) and the presence of ACPAs. Furthermore, twins with ACPA-positive RA were shown to have the shared epitope more frequently than twins with ACPAs but without RA (Hensvold, 2015).

1.8.8 Urbanisation

In the Java, Indonesia study the prevalence of RA was found to significantly differ between rural and urban populations (0.2% in rural areas, 0.3% in urbanized areas) .It was also found that there was a low prevalence of RA compared to that found in developed countries. This could be explained in part by the differing age structures of each population and the lower life expectancy of the population in Indonesia as a whole (Darmawan, 1993). Further studies conducted in both developed and developing countries have reported a higher prevalence of RA in urbanised areas (Solomon, 1975; Chou, 1994; Carmona, 2002). In the study of the Norfolk Arthritis Register (NOAR), in Norwich, UK between 1990 and 1994 Silman *et al.* described 'clustering in space'. In the northwest area of the region studied the prevalence of RA was higher in a market town compared to a local rural village. Migration in this area was deemed to be low however there were concerns over the variation of GP compliance (Silman, 1997).

Solomon *et al.* reported that the prevalence of RA in a black South African community in Orlando, a large and long established township in the metropolitan area of Johannesburg, was significantly higher than a similar population in rural South Africa ($p<0.01$) (Solomon, 1975). A more detailed study conducted in Taiwan, carried out a 2-staged population survey of 8998 people living in rural, suburban, and urban areas of the country. The results showed that the prevalence of RA was 0.26 (rural), 0.78 (suburban) and 0.93 (urban). The prevalence of RA in the rural area was shown to be significant compared to that of the suburban and urban areas ($p<0.05$) (Chous, 1994). A study of an adult population of Spain reported an increased prevalence of RA in cities compared to rural villages. Although not statistically significant,

prevalence was shown to be 0.6% in urban areas and 0.2% in rural areas, with a case ratio of urban to rural as 4:1. It is considered that many cases of RA go undiagnosed; however, the results seen in the Spanish population studied is comparable to other Mediterranean countries (Carmona, 2002).

1.8.9 Race and Culture

RA, although a worldwide endemic autoimmune disease, has an uneven distribution of prevalence. A hut-to-hut survey was carried out in Venda, a rural community in South Africa that still maintains a traditional way of living. The study received a response from 97% of the village population (n=543) and revealed no cases of RA. The study also revealed that together, the three major local hospitals had only encountered fourteen cases of RA in the population of 520,000 people that they serve. This lead to a reported prevalence of just 0.0026% (Brighton, 1988), however, this figure needs to be taken with caution as access to healthcare may be limited, allowing cases in certain groups to slip up the radar. An earlier study also found that RA was rarer in black South Africans compared to their Caucasian counterparts and that the overall prevalence of RA in South Africa was lower than that in Europe and North America (Solomon, 1975). Consistent with these data, is a more recent study comparing RA in Caucasians and people of black Caribbean descent in inner city Manchester, UK. The age and sex adjusted results for 1046 black Caribbean's and 997 Caucasians showed the prevalence of RA to be 2.9/1000 and 8/1000 respectively (MacGregor, 1994).

1.8.10 Seasonal Variation

It is often reported by RA patients that their joints are stiffer and more painful in the winter. This is not thought to be related to cold or damp but to a fall in barometric pressure (associated with an increase in bad weather) detected by barometric nerves in the body that respond to pressure change. This pattern has also been found to occur in warmer climates when there is a decrease in barometric pressure (Moots, 2004). However, there is conflicting evidence as some studies have observed no association between barometric pressure change and RA disease activity (Iikuni, 2007). Similarly, no seasonal variation was described in a study by Silman *et al.* who looked at patients who presented as 'new cases' on the NOAR between 1990 and 1994. Over the three year period studied, no trend was found to associate RA with seasonal variation or time within this patient cohort (n=687) (Silman, 1997).

On the contrary, a study conducted by Doran *et al.* reported a cyclical pattern in annual incidence of RA. Their research is based upon a population-based cohort study of the residents of Rochester, Minnesota over a 40 year period (January 1955 to December 1994)(n=609) (Doran[b], 2002). Additionally, a large observational cohort study in Tokyo, Japan, assessed 1665 RA patients and asked them to fill in questionnaires bi-annually (Iikuni, 2007). The April/May assessment represented spring and the October/November assessment, autumn. Ten criteria were set, collected and analysed, for example: CRP, ESR, RF, the physician's assessment of disease and the patient's assessment of pain. It was reported that there was a statistically significant seasonal variation in disease activity ($p<0.05$ for 9/10 criteria). Disease activity was found to decrease in autumn and increase again in spring.

1.8.11 Genetic Influence

Familial genes have been implicated in the increased risk of developing of RA, however they are not considered to be causal. Even if an individual with an identical twin has RA, their sibling only has a 1 in 5 chance of also developing the disease. Disease severity can also vary between identical twins (Arthritis Research UK, 2012).

Important genetic influences on RA disease susceptibility have been suggested by a four times greater monozygotic than dizygotic twin concordance rate (Silman, 1993a). A Finnish study conducted of more than 15,000 twins born before 1958, found that the age and sex adjusted ratio of observed per expected number of concordant pairs (relative risk) was 8.6 for monozygotic twins and 3.4 for dizygotic twins (Aho, 1986). Furthermore, inheritability of RA has been estimated at about 60%, based on current published studies (MacGregor, 2000). Genome Wide Association Studies (GWAS) have identified a number genes contributing to a genetic predisposition to RA (Perricone et al., 2011), where the strongest association was found with a gene polymorphism in the MHC region of the genome (Spector, 1988). Within this region, the strongest association for RA susceptibility is with the alleles of the HLA-DRB1 locus, with the QK/RRAA or RRRAA motifs in the third hyper variable region (HVR3) (Balandraud, 2004). HLAs of the DR subtype encode the beta chain of MHC class II. These alleles have been found to share a common sequence of amino acids, encoding HLA-DR4, also known as, ‘the shared epitope’, where the polymorphism codes for a specific part of the peptide binding groove. Individuals presenting with the shared epitope have a higher risk of developing RA (Cutolo, 1986).

A study to determine the specificity of the HLA-DR4 shared epitope for antibodies to citrullinated proteins was conducted in a Dutch population cohort containing 408 RA patients. A statistically significant association was reported, between possessing the shared epitope and the risk of developing RA (OR 4.37, $p<0.0001$). Furthermore, a dose effect was described, where the possession of more than one shared epitope allele led to an even further increased risk of RA (OR (2 copies) 11.79, $p<0.0001$). There is evidence that anti-CCP antibodies primarily mediate association of the shared epitope with joint damage and disease persistence (Cutolo, 1986). Both RF and ACPA display a positive correlation to the increase in virus/bacteria specific antibodies and the HLA-DR4 shared epitope (Rashid, 2012).

The association of RA with HLA-DR1, -DR4 and -DR10 alleles in different populations has further encouraged the shared epitope hypothesis (Hameed, 1995; Fujinami, 1985). In northern European populations, HLA-DR4 subtypes, DW14 and DW4, show a very strong association to RA and these alleles, along with double alleles of the shared epitope are shown to be good severity markers for clinical RA (Massardo, 1995). Stastry *et al.* were able to show an increase in the presence of HLA- D subtype DW4 in RA patients (70%) compared to healthy controls (28%) (Spector et al., 1988). It has been shown that HLA-DRB1 alleles can be segregated into three groups: Susceptible – e.g. DR1 and DR4 (except DRB*0402 and *0403); Neutral (positive charge in P4 pocket) – DR3, DR15; Protective (negative/neutral charge) – D7, D8 and DRB1*0403 (Fujinami, 2006).

In the past, autoimmune diseases linked to the hygiene hypothesis have often been shown to be associated with MHC polymorphisms (Wucherpfennig, 1995). This highlights the importance of MHC molecules when trying to establish a suitable response from the immune system.

Even though the shared epitope it seen to be increased in 90% of patients with RA, such a high association with genetic haplotypes cannot explain the comparatively low incidence of the disease. Genes appear to only play a limited part in disease development, exhibited by the 15% concordance rate between monozygotic twins. This gives an indication that an exogenous entity is playing a vital causal role that is likely sometimes linked to a genetic predisposition (Rashid, 2012).

1.9 Conclusion

The possible link between the hygiene hypothesis, late microbial /bacterial /viral infection in childhood and the onset of autoimmune disease in later life has been well documented; and over the last half a century, the theory has become widely accepted. A huge number of papers and studies back EBV as a good candidate for the initiation of autoimmune disease, and many others show substantial links with bacterial infections of *Porphyromonas gingivalis, Proteus mirabilis* and *Staphylococcus aureus.*

Currently, there is little published literature discussing any possible link between *S.aureus* toxins and RA. Due to this apparent gap in the literature we will analyse patient samples of urine in order to establish if SEB, SEC, AH and TSST-1, are present. TSST-1, SEB and SEC are the most abundant enterotoxins (respectively) secreted by the bacterium and AH is produced by all *S.aureus* strains (Kohler, 2012).

Additionally, it would be interesting to create a patient questionnaire that includes questions linking to the epidemiological areas discussed in this chapter. The data will

be used to identify an association between lifestyle, the hygiene hypothesis and RA. Although this is not part of the initial project, it would be a wasted opportunity to have access to a large number of patients and to not gain some extra information to describe the cohort.

Chapter 2 MATERIALS

2.1 Chemicals & Reagents

Antibodies bought from Abcam

αSEB (ab15919), αSEC (ab), αTSST-1(SLT1101), αAH (ab50536) (ab15948), goat anti-rabbit HRP conjugate, rabbit anti-sheep HRP conjugate.

Chemicals bought from Fisher Scientific

Pierce ECL western blotting substrate (10005943), Pierce ECL Plus western blotting substrate (11557910), Protogel acrylamide 30% (12381469), Film CL-Xposure 180mmx240mm (10465145), Sodium dodecyl sulphate (SDS) (10090490).

Chemicals bought from GE Healthcare

Calibration kit low molecular weight (SDS) for electrophoresis (10606515).

Reagents bought from Geneflow

SDS PAGE Tris-Glycine 10x tank buffer (B9-0032).

Chemicals bought from Insight Biotechnology

SureBlue.

Chemicals bought from Lyreco

Lyreco A4 plain write-on transparency film.

Chemicals bought from Merck Millipore

Immobilon PVDF 0.45UM 26.5 X 3.75M roll (P2938).

Chemicals and reagents bought from Scientific Lab Supplies

Tween 20 (CHE3852), whatman 3mm chromatography paper 580 x 680 nm (CHR1134), glacial acetic acid (CHE1012), coomassie brilliant blue (27815-100g-F), ammonium bicarbonate (09830), Eppendorf Protein LoBind tubes 1.5ml PCR clean (100pk) (E0030108116), Acetonitrile for HPLC gradient grade (00683-2), Water lc-ms chromasolv (39253-1L-R).

Chemicals and enzymes bought from Sigma Aldrich (Dorset, UK)

Bovine Serum Albumin (A7906), N,N,N,N, - Tetramethylethylenediamine (T9281), GBX developer/replenisher (P7042), GBX fixer/replenisher (P7167), Ammonium persulfate (A3678), 1-butanol (B7906), Extravidin, peroxidase conjugate (E2886), Fish gelatin (G7765), Carbonate buffer (C3041), Trypsin singles kit (T7575), trypsin from bovine pancreas (T8658), Pepsin from porcine gastric mucosa (P7000), Papain from papaya latex (P3125), α-chymotrypsin from bovine pancreas (P6423), trifluoroacetic acid (TFA) (T6508), endoproteinase lysine c from lysobacter enzymogens (P3428).

Reagents bought from Toxin Technology (Florida, USA)

Alpha haemolysin (AH), staphylococcal enterotoxin B (SEB), staphylococcal enterotoxin C1 (SEC1), toxic shock syndrome toxin 1 (TSST-1).

Chemicals bought from Total Lab Systems

Glycine (G0709), Tris (B2005).

Equipment bought from Web Scientific

1.5mm gel spacers (SHS08-150), Alumina Plates 8.3cmx10.2cm (10 plates) (AHS-0810-10).

2.1.1 Toxins

All toxins were reconstituted to 1mg/ml in distilled water and stored as 100µl aliquots at -20^{o}C until use, as described in the manufacturer's instructions.

- Staphylococcal enterotoxin B (SEB)
- Staphylococcal enterotoxin C (SEC)
- Toxic Shock Syndrome Toxin-1 (TSST-1)
- Staphylococcal alpha haemolysin (AH)

2.1.2 Antibodies

Antibodies were divided into 50µl aliquots to reduce freeze-thaw cycles.

	Antibody	Conc	Specificity	Animal	Dilution
Primary	Anti-AH IgG antibody	1mg/ml	Polyclonal	Rabbit	1:1000
	Anti-AH IgG antibody	1mg/ml	Polyclonal	Sheep	1:1000
	Anti-SEB IgG antibody	1mg/ml	Monoclonal	Rabbit	1:2000
	Anti-SEB IgG antibody	1mg/ml	Polyclonal	Sheep	1:2000
	Anti-SEC IgG antibody	1mg/ml	Monoclonal	Rabbit	1:2000
	Anti-SEC IgG antibody	1mg/ml	Monoclonal	Sheep	1:2000
	Anti-TSST-1 IgG antibody	1mg/ml	Monoclonal	Rabbit	1:2000
Secondary	Anti-rabbit HRP conjugate	1mg/ml	Polyclonal	Goat	1:4000
	Anti-sheep HRP conjugate	2mg/ml	Polyclonal	Rabbit	1:8000

2.1.3 Enzymes

All enzymes were purchased from Sigma-Aldrich and prepared and stored as instructed by the manufacturers (Section 2.3).

- Trypsin Profile IGD (T7575-1kt) kit and trypsin from bovine pancreas (T8658).
- Pepsin from porcine gastric mucosa (P7000).
- Papain from papaya latex (P3125).
- α-chymotrypsin from bovine pancreas (P6423).

2.1.4 Buffers

Phosphate buffered saline (PBS)

PBS was made up as a 10x solution in the laboratory and stored at room temperature (8g sodium chloride, 0.2g potassium chloride, 1.44g sodium phosphate dibasic, 0.24g potassium phosphate monobasic, 1L distilled water, pH adjusted to 7.4 using 1M hydrochloric acid.). A working solution of 1x PBS was made by making a 1 in 10 dilution with distilled water.

PBS-Tween20 (0.1%) (PBST)

PBST was made with 1ml Tween (Sigma Aldrich CHE3852) in 1L of 1x PBS.

SDS-PAGE buffers

Resolving buffer

To make the resolving gel: 1.5M tris HCl pH8.8 (72.6g tris, 400ml dH_2O).

Stacking buffer

To make the stacking gel: 1M tris HCl pH6.8 (24g tris, 400ml dH_2O).

Running buffer (for gel electrophoresis)

100ml tris glycine buffer (Sigma Aldrich 93015-10L-F) and 900ml dH_2O.

Transfer buffer

9.7g Tris, 45.04g glycine and 800ml methanol – made up to 4L with dH_2O.

ELISA buffers

PBS-Tween (0.05%)

500µl Tween in 1L 1x PBS.

Carbonate buffer

Empty 1 carbonate capsule (Sigma Aldrich C304)1 into 100ml dH_2O.

Blocking buffer

Dissolve 2.5ml fish gelatin (Sigma Aldrich G7765) in 100ml dH_2O.

Stop solution

40ml 10% H_2SO_4 and 200ml dH_2O.

2.1.5 Gels

All recipes for both resolving and stacking gels are enough to make two 1.5mm thick gels.

Stacking gel

7.66ml dH_2O, 1.26ml stacking buffer, 1ml 30% acrylamide (Thermo Scientific, Hemel Hempstead, UK), 100µl 10% SDS (w/v), 0.5ml 0.15% ammonium persulfate (Sigma Aldrich A3678) (w/v), and 10µl TEMED (Sigma Aldrich T9281).

12.5% acrylamide resolving gel

6.3 ml dH_2O, 5ml resolving buffer, 8.3ml 30% acrylamide, 200µl 10% (w/v) SDS, 200µl 0.15%(w/v) ammonium persulfate and 20µl TEMED.

17% acrylamide resolving gel

1.48g sucrose, 5.56ml resolving buffer, 8.4ml 30% acrylamide, 148µl 10% (w/v) SDS, 880µl 0.15%(w/v) ammonium persulfate and 12µl TEMED.

20% acrylamide resolving gel

1.48g sucrose, 10ml 30% acrylamide, 148µl 10% (w/v) SDS, 880µl 0.15%(w/v) ammonium persulfate and 12µl TEMED.

2.1.6 Reduction & Alkylation

Dithiothreitol (DTT) was made up:

- In-gel: 10mM DTT (from a 1M stock) in 100mM ammonium bicarbonate.
- In-solution: 15mM DTT in dH_2O used 1:3 to result in a 5mM DTT solution.

Iodoacetamide (IAA) was made up (fresh each time):

- In-gel: 55mM iodoacetamide in 100mM ammonium bicarbonate.
- In-solution: 60mM iodoacetamide in dH_2O used 1:4 to result in a 15mM iodoacetamide solution.

2.1.7 Mass Spectrometry Sample Preparation

Bovine serum albumin (BSA) bought as lyophilised powder (Sigma Aldrich) was made up at 2mg/ml in dH_2O and stored in the freezer (-20^oC).

Ammonium bicarbonate (ABC) was made up as 100mM solution in dH_2O. This was always made on the day of use and discarded.

Dithiothreitol (DTT) was made up at 10mM DTT (from a 1M stock, dH_2O) in 100mM ammonium bicarbonate immediately prior to use.

Iodoacetamide (IAA) was made up as 55mM Iodoacetamide in 100mM ammonium bicarbonate immediately prior to use.

2.1.8 Mass Spectrometry

Lyophilised samples for mass spectrometry analysis were re-suspended in 0.1% TFA (Sigma Aldrich).

HPLC-MS materials

Liquid chromatography (LC) was carried out using a Dionex Ultimate 3000. Subsequent electrospray ionisation mass spectrometry (ESI-MS) used a Bruker HCT ultra mass spectrometer.

LC Buffers: 5% MeCN, 95% MeCN , 5% Formic acid

Software to control the LC-MS includes Hystar, esquireControl, Bruker daltonics and Chromeleon Xpress. Subsequent MS data analysis was carried out using Biotools and MASCOT.

LCMS-IT-TOF materials

MS analysis was performed using an LCMS-IT-TOF mass spectrometer (Shimadzu, Kyoto) coupled to a NexeraX2 HPLC system (Shimadzu, Kyoto Japan).

Chromatographic separation was performed using a Shim Pack XR-ODS 2.2 μm (3.0 x 50 mm) analytical column with mobile phase conditions of solution A; ultrapure water + 0.1% formic acid (v/v), and solution B; acetonitrile + 0.1% formic acid (v/v). A total flow rate of 0.5 mL/min was used during the analytical segment of the instrument method, with gradient LC conditions, (see Table 7.6).

HPLC gradient grade acetonitrile (Fisher), ultrapure water (Milli-Q purification) and LCMS grade formic acid (Sigma Aldrich) were used as received.

METHODOLOGY

2.2 Sample Population

The RA sample population was derived from rheumatology clinics at Medical Unit 2, Royal Lancaster Infirmary, under the supervision of consultant, Dr Marwan Bukhari.

When choosing a control population for the case/control comparison, we needed a cohort of individuals without a diagnosis of RA. Patients presenting with closed fractures attending the fracture clinic in the orthopaedic department of the same hospital were chosen as good candidates. Clinical supervision was provided by Mr Paul Marshall and Mr Shyam Kumar.

As both the RA and fracture patients were attending the RLI as outpatients, it could be assumed that their exposure to hospital pathogens was similar. Furthermore, both populations were in a clinical setting and the collection of urine and personal data could be carried out under the supervision of the appropriate consultant(s).

2.2.1 Sample Size Calculation

The sample size issue for unmatched case control studies having dichotomous exposures, dichotomous disease status and no stratification, is as follows: you need to determine the number of cases and controls necessary to have 100 x (1-β)% confidence of identifying a Relative Risk (exposure odds ratio) of R or larger at the

100x(1-α)% level of statistical significance, if the rate of exposure in the controls is equal to p_0.

It can be shown that the exposure rate, p_1, among many cases would then be equal to:

$$p_1 = \frac{p_0 R}{\left[1 + p_0 (R-1)\right]}$$

Therefore, the following equation is necessary for the cases and controls (corresponds to PEPI results without continuity correction factor):

$$n = \frac{\left(z_\alpha \sqrt{2\bar{p}\bar{q}} + z_\beta \sqrt{p_1 q_1 + p_0 q_0}\right)^2}{(p_1 - p_o)^2}$$

For 95% significance, two tailed, $Z_\alpha = 1.96$

For 80% power, $Z_\beta = 0.84$

There is no published data that reports the prevalence of *S.aureus* in urine, to determine the exposure to the general population. The current literature was searched and an average prevalence of 30% determined for individuals testing positive for *S.aureus* by nasal or nasopharyngeal swab:

$P_0 = 0.3$

R = OR = 2

The calculation resulted in a number of ~142 subjects per population for statistical significance (calculated manually and by computer).

2.2.2 Inclusion/Exclusion Criteria

Table 2.1 Inclusion criteria

RA	Fracture
Rheumatology clinic, Medical Unit 2, RLI	Fracture clinic, Centenary Building, RLI
RA diagnosis	No RA diagnosis
>30 years old	
No infection	
-	Closed fracture
Able to read and understand the patient information	
Able to give consent	
Able to give a urine sample	

2.2.3 Risks/Benefits

Taking part in the study should not involve any risk to the health of the participants. There were no further risks other than those already associated with the patients' scheduled appointment and subsequent assessments and/or procedures.

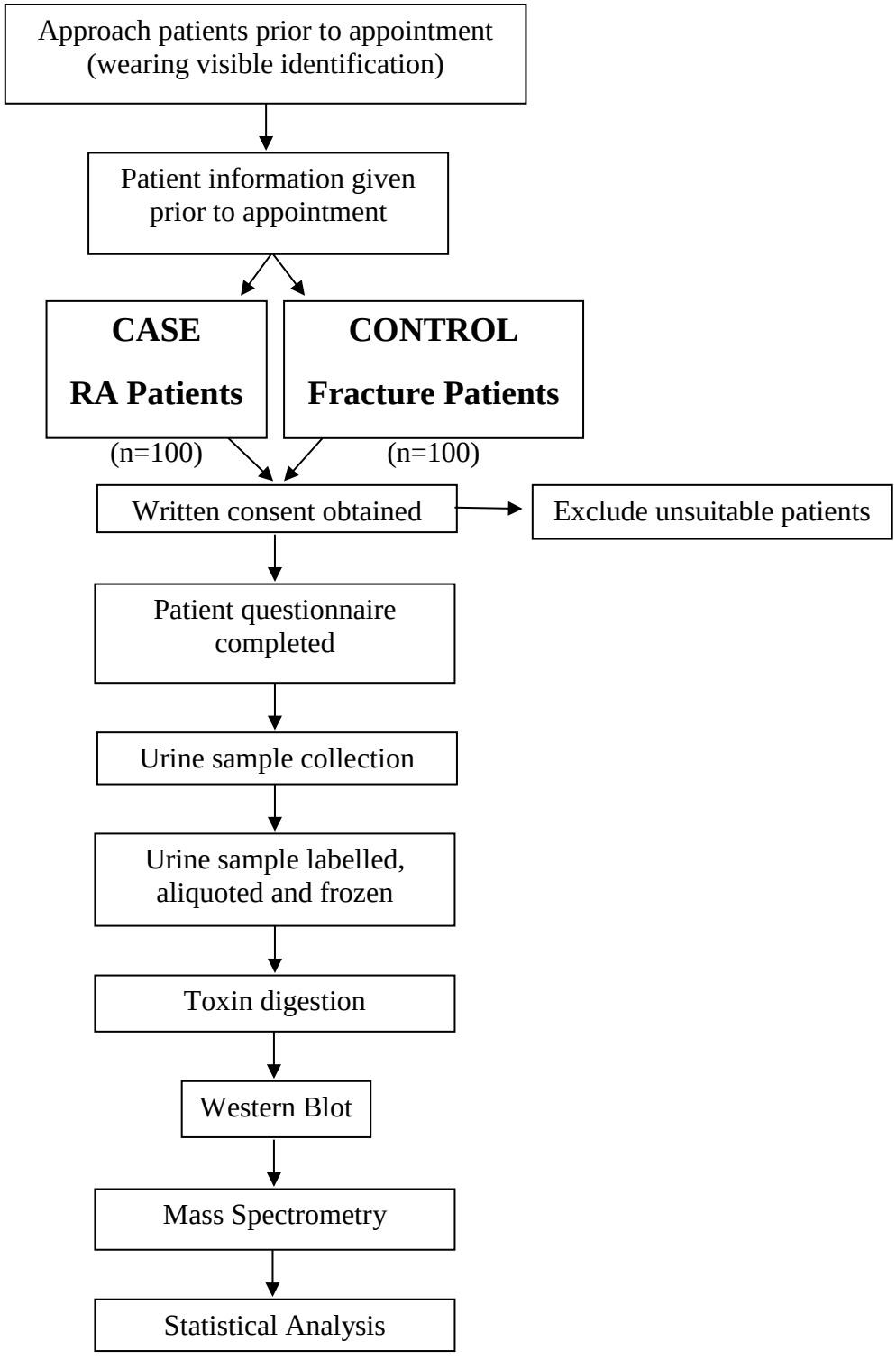
There was no intended immediate clinical benefit to the participants. By taking part in the study individuals were helping us to gather valuable information that will allow us to gain a better understanding and more insight into the development of RA.

2.2.4 Questionnaire Design

Based on the epidemiological studies discussed in Chapter 1, a patient questionnaire was designed to generate data about our study populations. These results can be extensively compared to each patient's positive or negative result for *S.aureus* presence. An example of each questionnaire (RA and Fracture) can be seen in appendix D.

2.2.5 Study Design

This project will be a case-control study, looking at the case population of RA patients and the control population of fracture patients.



2.3 Anonymisation

Study participants were assigned a unique study number – e.g. LR001 or LF001 - which was used to label the urine sample given, and to identify the subsequent data.

2.4 Sample Collection and Storage

Mid-stream urine samples were obtained and immediately stored in a refrigerated environment. Samples were consequently transported on ice to the university laboratories. There, they were aliquoted into 1.5ml eppendorfs and stored at -20^{o}C in a locked freezer.

2.5 Enzyme Digestion

All lyophilized enzymes were reconstituted in a fume cupboard, in line with health and safety guidelines.

2.5.1 Trypsin

2.5.1.1 Trypsin Singles, proteomics grade

'Trypsin singles'- pre-prepared trypsin kit, stored at 4^{o}C, contains the required reagents and instructions.

In-solution digestion

To each 1µg vial, 1µl of Trypsin Solubilisation Reagent was added. Next, 50µl of protein sample was added and the vial was vortexed to mix. 49µl of prepared Trypsin Reaction Buffer was then added to each vial. The final NH_4HCO_3 buffer concentration was ~20mM. The sample can be incubated at 37^{o}C for 2-18 hours to digest the target protein(s).

In-gel digestion

To each 1µg vial, 5µl of Trypsin Solubilisation Reagent was added and vortexed to ensure all of the trypsin had been dissolved. Next, 45µl of the Trypsin Reaction Buffer was added and vortexed; the final trypsin concentration was 20µg/ml. 25µl (0.4µg of trypsin) of the trypsin single is added to each Protein LoBind Eppendorf containing the dried gel sample. Following the rehydration of the gel sample, a further 30µl of the prepared Trypsin Reaction Buffer was added to each vial. The sample was incubated at 37^{o}C for 18 hours/overnight to digest the target protein(s).

2.5.1.2 Trypsin

The lyophilised trypsin (T8658) was soluble in 1 mM HCl (1 mg/ml) (pH3), yielding a clear solution. Before and after reconstitution in HCl, the trypsin was stored at -20^{o}C. For trypsin digestion of peptides, use of a ratio (w/w) of 1:100 to 1:20 for trypsin: peptide is desired.

Solutions in 1 mM HCl (pH 3) are stable for approximately 1 year when aliquoted and stored at -20°C. The presence of Ca^{2+} (20 mM) reduced trypsin's ability to autolyse and therefore maintained the stability of the trypsin in solution (Sipos, 1970; Walsh, 1970).

2.5.2 Pepsin

Pepsin was made up at a concentration of 1%. Ratio of substrate: pepsin was 1:100.

Pepsin (1%) dissolved easily in 3M guanidine hydrochloride (GuHCl) (Sigma Aldrich G3272). 3M GuHCl was mixed at 30^{o}C for 30 minutes. Pepsin can also be dissolved in distilled water, as it is soluble in water at 1% (10mg/ml).

Pepsin, in its powdered form, is stable at room temperature; however, pepsin made up in solution is best stored at -20^{o}C.

The enzyme solution used was kept between pH 2.0-3.0, as literature has described this pH range as the optimum for experimental peptide digestion (Bohak, 1969).

Pepsin solutions are stable at pH 6-7, however, if the pH is increased to between 8 and 11 at room temperature, the pepsin will be irreversibly inactivated (Ryle, 1970).

2.5.3 Papain

The papain was supplied as a buffered aqueous suspension of 2x crystallized papain purified from papaya latex in 0.05M sodium acetate, pH 4.5, containing 0.01% thymol. It was stored at 2-8^{o}C. Immediately prior to use, the papain was diluted in buffer containing ~5mM L-cysteine (Sigma Aldrich W326305). A stock solution of 10mM L-cysteine was made and used 1:1 with the papain (21mg/ml). Control toxin (1mg/ml) was added to the enzyme solution at a ratio of 1:100 and incubated at 25^{o}C for 1-18 hours.

2.5.4 Chymotrypsin

Chymotrypsin is supplied as a lyophilised powder that is reconstituted in 1ml of distilled water and stored at 2-8^{o}C until needed. Chymotrypsin was made up to a working solution immediately prior to use: 10µl stock chymotrypsin, 10µl 1mg/ml substrate protein, 40µl Tris HCl (0.2M) and 40µl calcium chloride (20mM). The chymotrypsin was used at pH7.8 and incubations occurred at 30^{o}C.

2.5.5 Lysine C & Trypsin

Lysine C was supplied as a lyophilised powder and stored in the fridge. When needed, the powder was reconstituted in 100µl dH_2O and stored at -20^{o}C. Lysine C was used at approximately 1:100 with the target peptide and incubated at 37^{o}C. Following this, the sample(s) was further incubated with trypsin, as described in 2.5.1.1.

2.6 Western Blot for Detection of Staphylococcal Toxins

12.5% acrylamide resolving gels (1.5mm) were poured and allowed to set for thirty minutes at room temperature with a layer of isobutanol-saturated water on top to prevent air bubbles. Once set, a stacking gel and comb were added and allowed to set at room temperature for a further thirty minutes.

Samples were prepared by dilution with dissociation buffer (3.5g 1M Tris pH 6.8, 2.5g SDS, 0.3085g DTT, 5ml glycerol, 0.05g bromophenol blue in 25ml total volume with dH_2O) to achieve a solution containing two thirds sample, one third dissociation buffer. These samples were heated at 98^{O} C using a dry heat block for three minutes. A low molecular weight standard (GE Healthcare, Buckinghamshire, UK) was diluted 1/4 in dissociation buffer.

20µl of the low molecular weight ladder mixture was added to the first lane then 28µl of each sample to the remaining lanes. The gels were electrophoresed using running

buffer (Tris-Glycine- SDS PAGE buffer, Geneflow, Staffordshire, UK) at 60mA/300V for approximately one hour.

After electrophoresis, the proteins were transferred to a PVDF membrane (Immobilion P, Millipore, UK) using a transfer tank containing Towbin Buffer (20mM Tris, 150mM Glycine and 20% methanol) for one hour at 700mA/115V. After transfer, the membrane was washed with PBS for 5 minutes then blocked in 20 ml milk or BSA (5% (w/v) skimmed milk powder or BSA in PBST (0.1%)) for one hour at room temperature on a rotary agitator. After blocking, the membrane was again washed with PBS before incubation overnight at $4^{O}C$ on a rotary agitator, in the appropriate antibody diluted in 2% (w/v) BSA (Sigma-Aldrich, Dorset, UK) in PBST (0.1%). Anti-SEB and anti-SEC primary antibodies were used at 1:2000; anti-TSST-1 and anti-AH primary antibodies were used at 1:1000.

The following morning, each membrane was washed in PBST (0.1%) once for 1 minute and twice for 15 minutes. The secondary antibody was diluted at 1:4000 (GαR) or 1:8000 (RαS) in 2% (w/v) BSA in PBST (0.1%). The membrane was incubated in 20ml of antibody at room temperature for 1h. Finally, each membrane was washed with PBS, once for 1 minute and twice for 15 minutes.

Imaging the membrane

To enable visualisation of the protein(s), 2ml electrochemiluminescence (ECL) substrate (Thermo Scientific, Hemel Hempstead, UK) was pipetted over each membrane, at room temperature, for 2 minutes. The membranes were sandwiched between two sheets of acetate and any bubbles squeezed out. Two different imaging methods were used: X-ray film (Thermo Scientific, Hemel Hempstead, UK) and a BioRad Chemidoc.

Using the X-ray film: In a dark room, X-ray film was placed over the membrane for sufficient time to gain exposure (2 minutes for SEB and SEC, 4-5 minutes for TSST-1). The X-ray film was developed manually using developer and fixer.

Using the Chemidoc: membranes were exposed for up to 3 minutes in the Chemidoc. Protein bands were usually visible within seconds.

Staining the membrane

Each membrane was stained with amido black stain (250ml H_2O, 200ml methanol, 50ml acetic acid and 0.1% (w/v) amido black) for five minutes and then rinsed with distilled water.

2.7 ELISA for Detection of Staphylococcal Toxins

Blocking buffer: (2.5% (v/v) fish gelatin in phosphate buffered saline-0.05% (v/v) Tween 20.

Microwell plates (Nunc-Immuno F96 Maxisorp, Thermo Scientific, Hemel Hempstead, UK) were coated using 100µl/well of solution containing 0.5µg/ml of capture antibody in coating buffer (0.1 M Na_2CO_3, pH 9.6). Plates were incubated overnight at 4^oC.

The following morning plates were washed four times using a multi-channel pipette with 200µl of wash buffer (Phosphate buffered saline (PBS) 0.05% (v/v) Tween20). Once all wells were filled, the plates were inverted sharply over a sink and tapped on

paper to ensure all remaining wash buffer was removed. After the washing, 200µl of blocking buffer (PBS-0.05% (v/v) Tween20 with 2.5% (v/v) cold water fish gelatin) was added and plates incubated at 37^{O}C for one hour. Next, TSST-1 samples digested by pepsin were added at 100µl/ well. Standards containing known concentrations of the toxin to be detected were treated in the same way. Any blank wells were filled with blocking buffer. After a 45 minute incubation at 37^{O}C the wash step as described previously was repeated.

100µl of biotinylated anti-toxin antibody in blocking buffer was added to each well. For TSST, the biotinylated antibody was diluted to 0.5µg/ml. After a further 45 minute incubation a third wash step was performed. ExtrAvidin®−Peroxidase (Sigma-Aldrich) diluted 1:2000 in blocking buffer was added at 100µl/well and incubated at 37^{O}C for 30 minutes.

After a final wash stage, 100µl/well of substrate (TMB Microwell Peroxidase, (KPL, Gaithersburg, MD) was added and colour allowed to develop for 10-15 minutes. The reaction was stopped with 100µl/well stop solution (0.3M H_2SO_4).

Absorbance was read at 450nm for one second using a Wallac Victor^2 Plate Reader (Perkin Elmer, Buckinghamshire, UK).

2.8 Mass Spectrometry Sample Preparation

Gels were made, as the western blot method describes: 1.5mm12.5% resolving gels were poured and allowed to set for thirty minutes at room temperature with a layer of

isobutanol-saturated water on top to prevent air bubbles. Once set, the stacking gel and comb were added and allowed to set at room temperature for thirty minutes.

Samples were prepared by dilution with dissociation buffer (3.5g 1M Tris pH 6.8, 2.5g SDS, 0.3085g DTT, 5ml glycerol, 0.05g bromophenol blue in 25ml total volume with dH_2O) to achieve a solution containing two thirds sample. These samples were heated at 98^{O} C using a dry heat block for three minutes. A low molecular weight standard (GE Healthcare, Buckinghamshire, UK) was diluted 1/4 in dissociation buffer.

30µl of low molecular weight ladder was added to the first lane then 30µl of sample to remaining lanes. The gels were electrophoresed using running buffer (Tris-Glycine-SDS PAGE buffer, Geneflow, Staffordshire, UK) at 60mA/300V for approximately 70 minutes.

The gels were transferred to a petri dish and incubated for 1 hour at room temperature with Coomassie brilliant blue (2g coomassie brilliant blue in 500ml - 50% methanol, 40% dH_2O, 10% acetic acid). Following this, they were incubated with destain (50% dH_2O, 40% methanol, 10% glacial acetic acid) until the background of the gels became clear. They were left overnight in dH_2O to rehydrate.

The bands of interest were excised using a sterile scalpel. Each band was diced into 1mm cubes. The gel pieces were transferred into Protein LoBind microcentrifuge Eppendorfs and spun down in a bench-top microcentrifuge. 100mM ammonium bicarbonate (ABC) was made up immediately prior to use. The gel pieces were destained using 100µl of destain solution, comprising 100mM ABC/acetonitrile (ACN) (1:1, v/v) and incubate with occasional vortexing for 60 minutes. This can take longer, depending on staining intensity.

500µl ACN was added and the samples were incubated at room temperature with occasional vortexing, until gel pieces become white and shrink. The ACN was removed. The dithiothreitol (DTT) solution (1:100 in 100mM ammonium bicarbonate) was made up immediately prior to use. 50µl of the DTT solution was added to each of the dehydrated gel pieces (to completely cover) and incubated for 30 minutes at 56^{o}C in an air thermostat.

Samples were left to cool down to room temperature, all liquid was removed and 500µl ACN was added. They were incubated at room temperature for 10 minutes, until dehydrated. The 55mM iodoacetamide solution was made up immediately prior to use. All liquid was removed and 50µl of the iodoacetamide solution was added and the samples were incubated for 20 minutes at room temperature, in the dark. Again, all liquid was removed and 500µl ACN was added to the gel pieces until white and shrunken.

Trypsin digestion, using trypsin singles kit (Sigma), was carried out according to the manufacturer's instructions. 5µl of trypsin solubilisation reagent (1mM) was added to each vial and vortexed. 45µl of trypsin reaction buffer was added and mixed (final trypsin concentration of 20µg/ml). 25µl (0.5µg trypsin) of the prepared mixture was added to each sample. All samples were incubated at 4^{o}C for 30 minutes. Following hydration of the gel pieces, a further 30µl trypsin reaction buffer was added to completely cover the gel pieces. Samples were incubated at 37^{o}C overnight in an air thermostat.

All of the remaining liquid was removed and 110µl of extraction buffer (1:2, (v/v) 5% formic acid/acetonitrile) was added to each eppendorf and incubated for 15 minutes at 37^{o}C in a shaker. For samples with much larger (or smaller) volume of gel matrix, the

extraction buffer should be added such that the approximate ratio 1:2 between volumes of the digest and extraction is achieved. The supernatants were transferred into fresh Protein LoBind eppendorfs and the lid pierced 3-4 times. The samples were frozen in a freeze-dryer and left to dry overnight.

Each sample was re-suspended in 20µl 0.1% trifluoroacetic acid (TFA), ensuring that the dry matter was completely re-suspended and mixed. The samples were incubated in an ultra-sonic water bath for 5 minutes. The samples were spun down in a table-top microcentrifuge to ensure the entire sample was at the bottom. The 20µl of each sample was transferred into sterilised micro-volume sample vials.

Each sample was inserted into the automated high performance liquid chromatography (HPLC) machine and in the position defined in esquireControl, the system used to control the HPLC/MS.

2.9 mMass

The accession number for each toxin needs was found using the European Bioinformatics Institute (EBI), part of the European Molecular Biology Laboratory (EMBL) (http://www.ebi.ac.uk/). The accession number was input into a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the UniProt database chosen. The BLAST search came with an abundance of results and the relevant toxin was chosen. The amino acid code of the toxin was then copied into mMass – an open source mass spectrometry tool (http://www.mmass.org/). From here, the digest button was clicked and the relevant enzyme chosen. mMass then gave an output of expected peptides;

their amino acid code, length and mass:charge ratio (m/z). When clicking on an individual peptide, a separate window opened to show what the expected peaks would actually look like in the MS.

2.10 Mass Spectrometry

2.10.1 HPLC-ESI-MS

In the initial experiments, MS sample solutions were made up of 50µl acetonitrile, 45µl 0.1% formic acid and 5µl of the digested protein sample. The sample was then taken up by a syringe and fitted into an automatic injector that fed the sample into the MS at a constant rate.

Biotools was used to process the raw data from the MS by finding the compounds (AutoMS(n)), de-convoluting the subsequent mass spectra and exporting as an .mgf file. The .mgf produced was run through the MASCOT database to identify the proteomic composition of the sample.

The defined method used as involved the following settings:

Scan mode: standard enhanced; divert valve: to source; range: 5-3000m/z; speed: 8100m/z/sec; polarity: positive; Trap: ICC (smart target = 200000; max accumulation time = 200.00ms; scan: 50-2000 m/z).

2.10.2 LCMS-IT-TOF

Following the protocol for mass spectrometry preparation (2.1.7), the freeze-dried samples were diluted with 20µl of mobile phase A (ultrapure water + 0.1% formic acid) to provide sufficient sample for accurate collection from the autosampler. An estimated concentration of 50ng/ml was hypothesised prior to analysis.

MS^n analysis was performed using electrospray ionisation (ESI) using an LCMS-IT-TOF mass spectrometer (Shimadzu, Kyoto) coupled to a NexeraX2 HPLC system (Shimadzu, Kyoto Japan). Mobile phase consisted of solution A; ultrapure water + 0.1% formic acid (v/v), and solution B; acetonitrile + 0.1% formic acid (v/v). LC conditions are described in Table 7.6.

Electrospray ionisation was employed with probe voltages of 4.5 kV, curve desolvation line temperature 200°C, heater block temperature 200°C, nebuliser gas flow 1.5 L/min. Collision energies were not optimised for each transition or fragmentation experiment; therefore, fragmentation efficiencies were a product of the instrument default settings; collision energy set at 50%, collision gas set at 50%, q value 45.0 kHz. Ion accumulation times were consistent in all MS1 and MS2 scans set at 10 milliseconds and 200 milliseconds respectively. A full description of the instrument method parameters is reported in Table 7.6.

UniProt Knowledgebase (UniProtKB, Magrane and the UniProt consortium) was searched for SEB, SEC1, TSST1, AH and BSA amino acid sequences. Protein sequences were digested *in silico* using PeptideMass (ExPASy) with trypsin digestion enzyme, cysteines treated with iodoacetamide and up to 2 missed cleavages allowed. Monoisotopic peptide masses were reported, and data searched for the masses of the

resultant peptide sequences. Peptides observed in analysis of standard compounds were checked for uniqueness using NCBI protein BLAST online tool to check if targets are proteotypic, peptides that are unique to the target protein (Kuster 2001). An online tool (FragmentIonCalculator) was used to generate fragment ions of the targeted peptides, generating monoisotopic masses of y/b fragment ions in the 1+, 2+ and 3+ charged states.

The standard compounds of SEB, SEC1 and TSST-1 were combined in the same standard sample having undergone the sample preparation procedure proposed for the patient samples, reported in section 2.1.7. Each toxin was added to the sample preparation procedure as 20 μL of 10 μg/mL; the reclaimed sample band from the SDS-Page gel was assumed as 100% recovery as tryptic peptide material. Stock solutions containing all three toxins were diluted with mobile phase A (ultrapure water + 0.1% formic acid) to achieve an external serial dilution standards used to generate the calibration curve. All solutions were frozen when not in use and once defrosted kept at 4°C using the chilled sample compartment of the instrument autosampler until injection.

Injection volume of 10μl was used throughout. Periodic blank and standard analytical runs were performed every 5 analytical injections confirming no effects from carryover or loss of instrument sensitivity. However, anecdotal evidence of sample degradation was observed from analyses performed over the entire course of method development; the impact of which was not investigated, but may have had an effect on the quantitation values or toxin detection in cases where concentration levels were border line detectable.

2.11 Statistical Analysis

Initially patients with RA will be compared to fracture controls using the students T test for continuous variables and chi squared test for categorical variables.

A logistic model will be fitted to determine the odds of positivity in the RA group.

In the RA group, we will investigate whether patients with higher disease activity (using either DAS >5.2 or DAS >2.6) had a higher prevalence of positivity.

2.12 Ethical Approval

This study was given a favourable opinion on 17th February 2015 by Northwest Research Ethics Committee – Lancaster [15/NW/0038].

Chapter 3 TOXIN DIGESTION

3.1 Staphylococcal Toxins

Staphylococcal enterotoxins SEB, SEC, TSST-1 and AH needed to be digested by enzymes in order to be analysed by mass spectrometry. Western blot and ELISA were used to ascertain whether digestion had occurred.

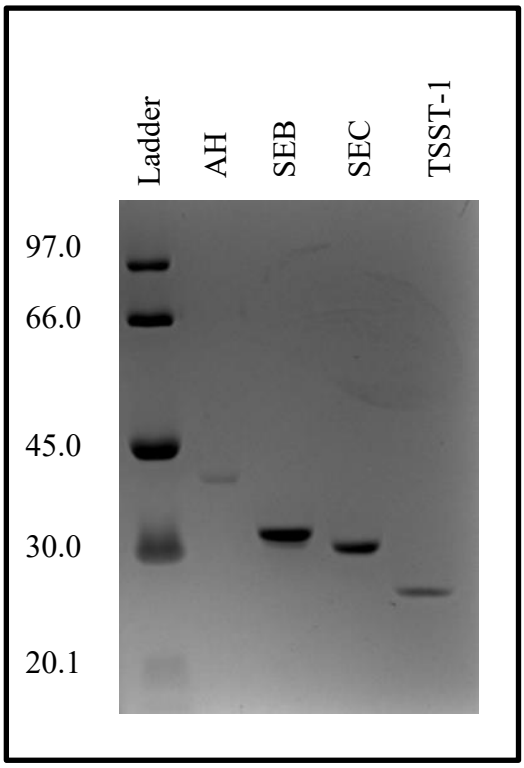


Figure 3.1 Coomassie stain of ladder (kDa), AH, SEB, SEC and TSST-1 (125ug/ml). 1.5mm 12.5% acrylamide gel.

Figure 3.1 shows each of the four staphylococcal toxins analysed and their molecular weights relative to a low molecular weight ladder. The molecular weights of the toxins (according to the manufacturers) are:

- AH = 33kDa
- SEB = 28kDa
- SEC = 30kDa
- TSST-1= 24kDa

3.2 Sensitivity of Western Blotting

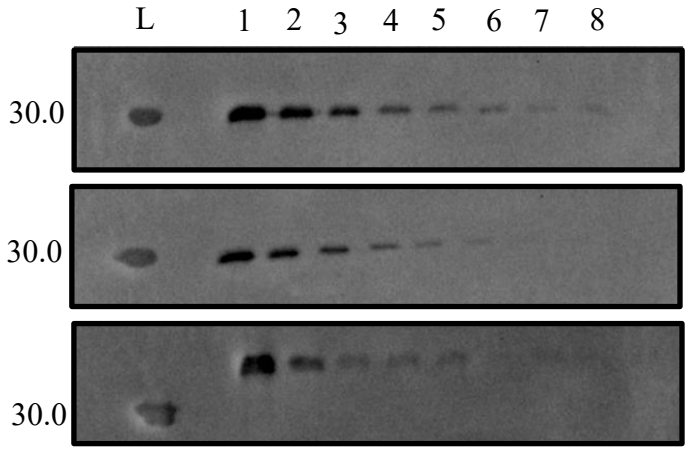


Figure 3.2 Western blot of SEB. Ladder (L) (30kDa) and two-fold serial dilution from 5µg/ml - 39ng/ml.

Figure 3.3 Western blot of SEC. Ladder (L) (30kDa) and two-fold serial dilution from 5µg/ml - 39ng/ml.

Figure 3.4 Western blot of AH. Ladder (L) (30kDa) and two-fold serial dilution from 5µg/ml - 39ng/ml.

As part of the aim of the project was to develop a mass spectrometry technique of superior sensitivity to current detection methods, it was important to show the sensitivity of the current method. The western blot membranes above (Figure 3.2-3.4) show two-fold serial dilutions of SEB, SEC and AH. The first band in each blot is carbonic anhydrase, the 30kDa band of the low molecular weight ladder used. The concentration of each toxin band (shown in corresponding lane) is as follows: 5µg/ml (1), 2.5µg/ml (2), 1.25µg/ml (3), 625ng/ml (4), 312.5ng/ml (5), 156.25ng/ml (6), 78.1ng/ml (7), 39ng/ml (8). Although faint, all bands from 5µg/ml to 39ng/ml (100ng-78fg in 20µl load) can be seen for each of the 3 toxins.

3.3 Sensitivity of Electrochemiluminescence (ECL) Substrate

From the beginning of the project, the standard ECL from Fisher Scientific (Pierce ECL western blotting substrate 10005943) was used in order to develop the membranes. The sensitivity of this product has proved suitable for SEB, SEC and AH toxins, however, TSST-1 proved difficult to develop, even if it was left to expose for 15 minutes or more. This was a problem when developing on film and when using the BioRad ChemiDoc. Therefore, a more sensitive ECL substrate. ECL 2 (Pierce ECL Plus western blotting substrate 11557910) from Fisher Scientific was purchased.

For ECL1: The ratio of substrate 1 (stable peroxidase solution): substrate 2 (luminal solution) is recommended at 1:1, therefore, 1ml of each solution was combined to develop the membrane. When using film, exposures upwards of 15 minutes were needed to visualise TSST-1. A long exposure time and a long duration in the developer risks making the film dark, thus making bands harder to see. The Chemidoc was used to overcome the need to develop film, however a similar exposure time was needed and any bands were still faint.

For ECL2: The ratio of substrate 1 (stable peroxidase solution): substrate 2 (luminal solution) is recommended at 40:1, therefore, 2ml of solution 1 and 50µl of solution 2 was combined to develop the membrane.

In order to see the bands in Figure 3.5, an exposure of over 10 minutes is needed. However, this considerably over-exposes the ladder (highlighted in red), even following dilution.

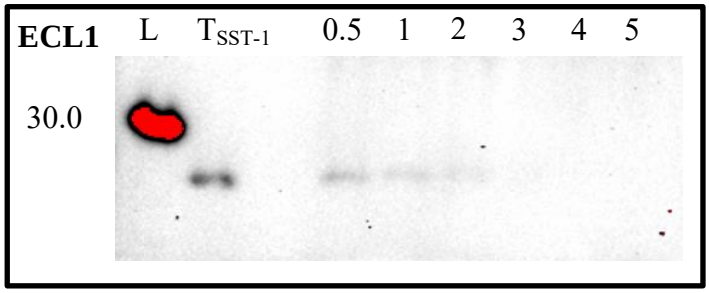


Figure 3.5 Western blot of TSST-1 (250ng/ml), 600 second exposure. Lanes contain ladder (L), undigested TSST-1 and samples incubated with pepsin for 0.5-5 hours. ECL1.

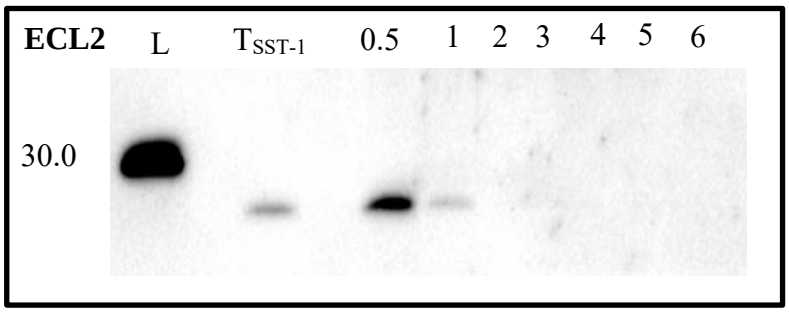


Figure 3.6 Western blot of TSST-1 (250ng/ml), 60 second exposure. Lanes contain ladder (L), undigested TSST-1 and samples incubated with pepsin for 0.5-6 hours. ECL2.

Figure 3.6, shows a repeat of the membrane in Figure 3.5, however, on this occasion ECL 2 has been used to develop the membrane, with just a 1 minute exposure.

The image is much clearer and the bands are easier to see, even with the exposure duration being 10 times less than in Figure 3.5.

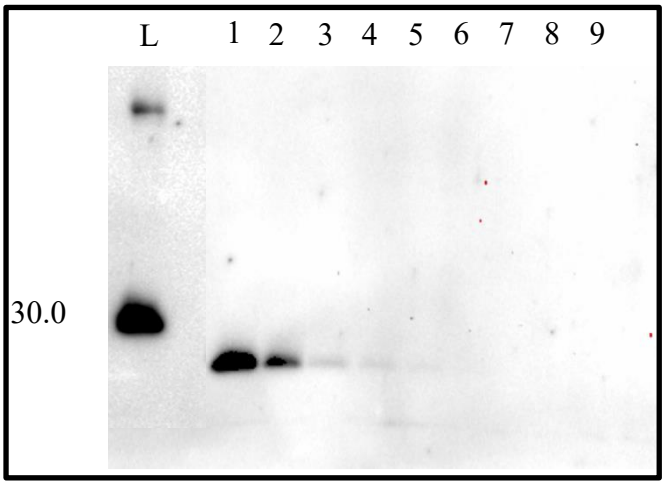


Figure 3.7 Serial (1:1) dilution of TSST-1. (1) 500, (2) 250, (3) 125, (4) 62.5, (5) 31.25, (6) 15.6, (7) 7.8, (8) 3.9, (9) 1.9ng/ml. Exposed for 240 seconds. ECL2.

Figure 3.7 shows the sensitivity capabilities of ECL 2 when developing blots of TSST-1. Concentrations of 500-125ng/ml [1-3] can be seen easily, with the possibility

of detecting concentrations as low as 31.25ng/ml [5]. To achieve this, a longer exposure time would be necessary (i.e. longer than 4 minutes).

3.4 Digestion of SEB

3.4.1 Trypsin Digestion of SEB

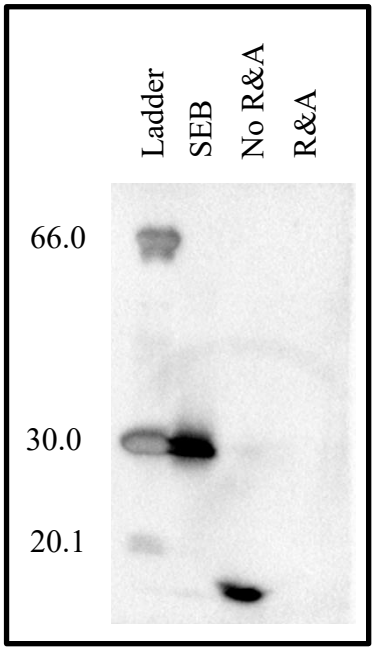


Figure 3.8 Western blot of SEB (1µg/ml). Exposure = 5 seconds. Lanes contain low molecular weight ladder, undigested SEB and two samples of SEB incubated with trypsin 18 hours at 37^{o}C; the second of which has been reduced and alkylated (R&A).

The product information sheet recommended an incubation of between 2-18 hours. Therefore, the initial digestion experiments using trypsin included an incubation time of 18 hours, to maximise the chance of complete digestion.

The membrane shows a 30kDa band in the ladder and a 28kDa band in the lane containing whole SEB. There is no band at 28kDa in the lanes containing SEB incubated with trypsin. However, there is a band at around 22kDa, in the 'No R&A' (no reduction and alkylation) lane, meaning there is still a rather large peptide fragment present. Following reduction, alkylation and 18h incubation with trypsin (37^{o}C), SEB has been completely digested.

3.5 Digestion of SEC

3.5.1 Trypsin Digestion of SEC

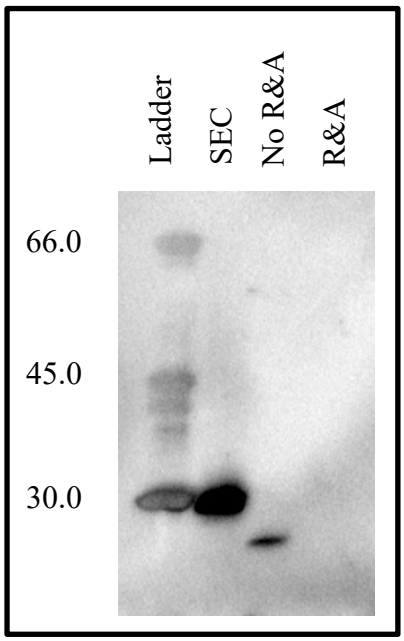


Figure 3.9 Western blot of SEC (1µg/ml). Exposure = 5 seconds. Lanes contain low molecular weight ladder, undigested SEC and two samples of SEC incubated with trypsin 18 hours at 37^{o}C; the second of which has been reduced and alkylated (R&A).

The membrane shows a 30kDa band in the ladder and a 30kDa band in the lane containing whole SEC. The whole SEC is clearly seen, to the right of the ladder, at the

level of 30kDa. The membrane shows no 30kDa band in the lanes containing SEC incubated with trypsin. However, as with SEB, there is still a large molecular weight protein in the 'No R&A' lane, where the SEC has not been reduced or alkylated prior to enzymatic digestion. Such a band is not present in the 'R&A' lane, showing that SEC is fully digested following reduction, alkylation and 18h incubation with trypsin (37^{o}C).

3.6 Digestion of TSST-1

3.6.1 Trypsin Digestion of TSST-1

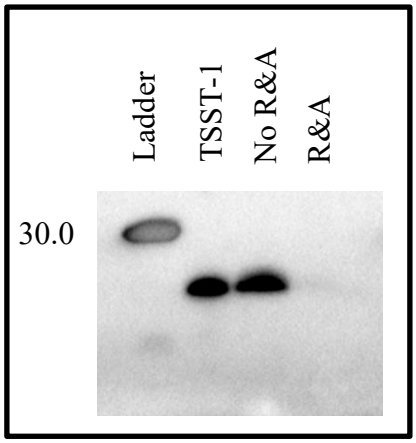


Figure 3.10 Western blot of TSST (1µg/ml). Exposure = 10 seconds. Lanes contain low molecular weight ladder, undigested TSST-1 and two samples of TSST-1 incubated with trypsin 18 hours at 37^{o}C; the second of which has been reduced and alkylated (R&A).

As shown in Figure 3.10, there is still a strong band present at 24kDa in the 'No R&A' lane containing TSST-1 incubated with trypsin. However, following reduction, alkylation and 18h incubation (37^{o}C), TSST-1 is sufficiently digested. Complete digestion is not stated as there does appear to be a very faint band at 24kDa in the

'R&A' lane. Although not completely digested, sufficient unique peptide fragments have been generated for MS analysis.

3.6.2 Pepsin Digestion of TSST-1

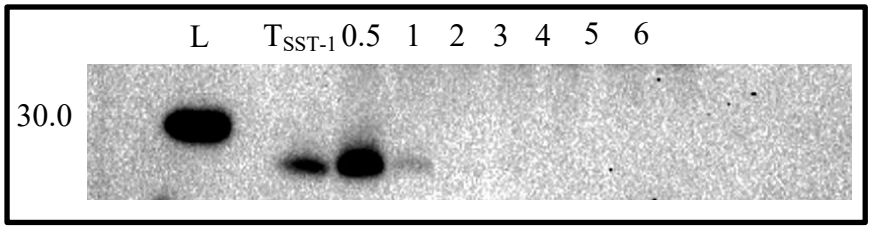


Figure 3.11 Western blot of TSST-1 (250ng/ml) incubated with pepsin for 0.5-6 hours at 37°C. Exposure = 180 seconds. Lanes contain ladder (L), whole TSST-1 and samples incubated with pepsin for 'n' hours.

ɔlot membrane of TSST-1 incubated with pepsin. Bands of TSST-1 are seen at 24kDa after 0.5 and 1 hour of incubation. Bands are no longer visible after 2 hours of incubation with pepsin. From this, it can be concluded that TSST-1 is completely digested after 2 hours of incubation. In order to confirm this result, an ELISA was carried out.

[a]

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.5h	1h	4h	8h	12h	B	B	B	100ng/ml	10ng/ml	1ng/ml	0.1ng/ml
B	0.5h	1h	4h	8h	12h	B	B	B	100ng/ml	10ng/ml	1ng/ml	0.1ng/ml
C	0.5h	1h	4h	8h	12h	B	B	B	100ng/ml	10ng/ml	1ng/ml	0.1ng/ml
D	B	B	B	B	B	B	B	B	B	B	B	B

[b]

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.103	0.107	0.099	0.106	0.101	0.097	0.091	0.099	1.164	0.269	0.160	0.150
B	0.102	0.093	0.086	0.092	0.094	0.078	0.095	0.089	1.144	0.270	0.149	0.146
C	0.096	0.100	0.096	0.092	0.096	0.087	0.088	0.090	1.139	0.254	0.160	0.162
D	0.311	0.209	0.079	0.084	0.083	0.370	0.085	0.093	0.089	0.091	0.126	0.101

Figure 3.12 ELISA of TSST-1 incubated with pepsin. Wells contain blanks (B), incubation intervals (columns 1-5) and repeats (A-C), and undigested TSST-1 of concentrations 100, 10, 1, 0.1ng/ml (columns 9-12 respectively) and repeats (A-C). [a] the sample is in each well. [b] the absorbance results of each sample (A^{450nm}).

The ELISA (Figure 3.12) has a sensitivity of 0.2 A^{450nm}, therefore it is assumed that anything below that threshold of detection is negative. The ELISA confirms the results of the western blot – TSST-1 has been digested by pepsin.

3.7 Digestion of AH

3.7.1 Trypsin Digestion of AH

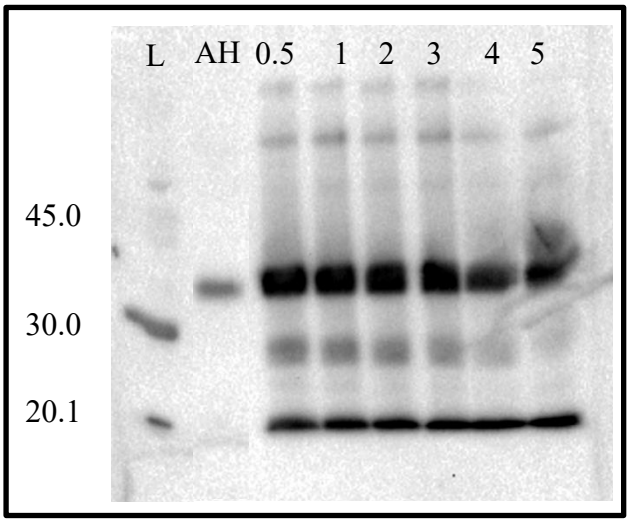


Figure 3.13 Western blot of AH (5µg/ml) incubated with trypsin for 0.5-5 hours at 37°C. Exposure = 20 seconds. Lanes contain ladder (L), undigested AH and AH incubated with trypsin (0.5-5 hours).

In Figure 3.13, bands of AH are consistently present at ~36kDa, even after 5 hours of incubation with trypsin. The enzyme has had no effect on AH and does not digest the toxin.

3.7.2 Pepsin Digestion of AH

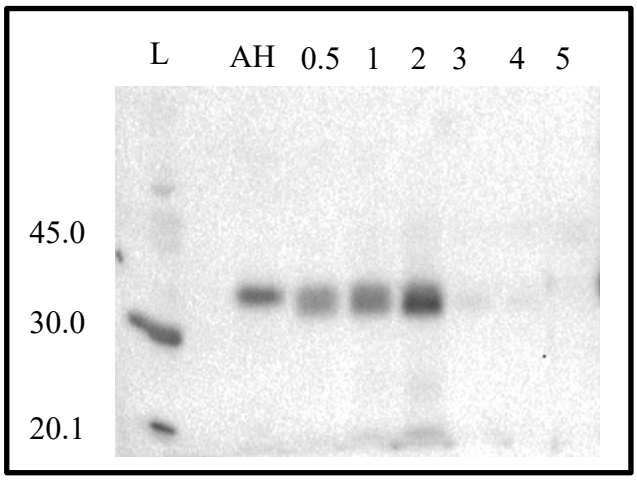


Figure 3.14 Western blot of AH (250ng/ml) incubated with pepsin for 0.5-5 hours at 37°C. Exposure = 20 seconds. Lanes contain ladder (L), undigested AH (A_w) and samples incubated with pepsin for 0.5-5 hours.

AH was incubated with pepsin. Bands of AH are present throughout at ~36kDa, after incubation with pepsin. Although the bands in lanes containing samples incubated for 0.5-2 hours are considerably stronger, there are also faint bands in the lanes of the 3-5 hour samples. Pepsin does not appear to completely digest AH.

Therefore, the search for an enzyme to digest AH continues. The enzymes chymotrypsin and papain were chosen to be tested next as they are both enzymes found naturally in the human body.

3.7.3 Chymotrypsin Digestion of AH

AH was incubated with chymotrypsin for up to 24 hours (Figure 3.15). Bands of AH are present consistently at 36kDa, even after 24 hours of incubation with chymotrypsin. The bands are faint in this instance but it is clear to see that the AH toxin has not been digested. It can be concluded that chymotrypsin does not digest AH either.

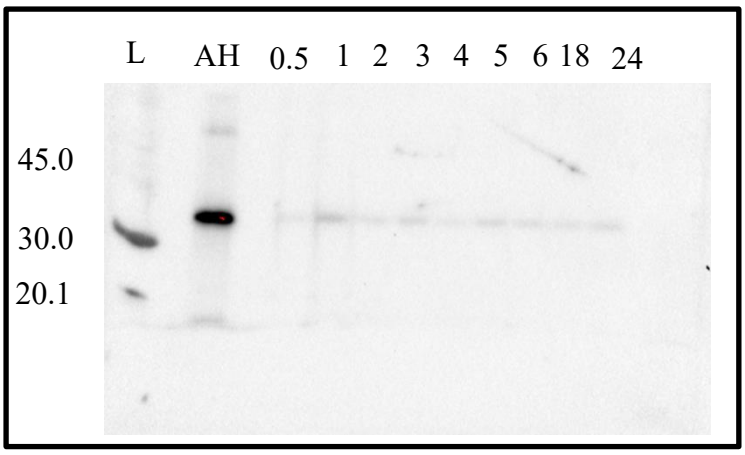


Figure 3.15 Western blot of AH (250ng/ml) incubated with chymotrypsin for 0.5-24 hours at 25°C. Exposure = 30 seconds. Lanes contain ladder (L), undigested AH and samples incubated with chymotrypsin (hours).

3.7.4 Papain Digestion of AH

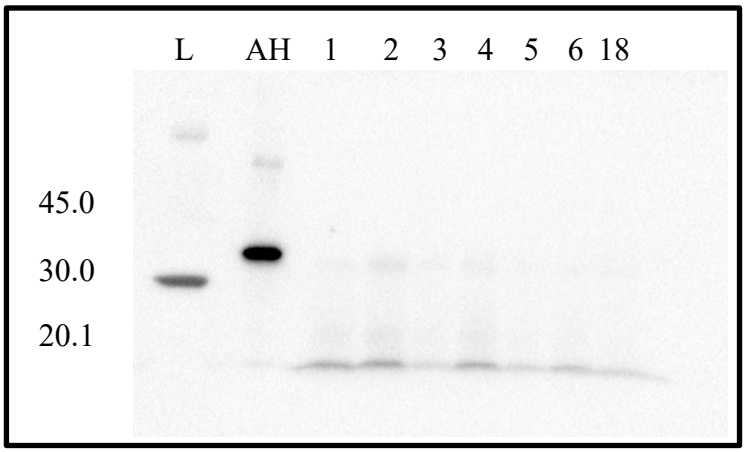


Figure 3.16 Western blot of AH (250ng/ml) incubated with papain for 1-18 hours at 30°C. Exposure = 30 seconds. Lanes contain ladder (L), undigested AH and samples incubated with papain (hours).

After discovering that trypsin, pepsin and chymotrypsin do not digest AH, papain was the next proteolytic enzyme chosen. As shown in Figure 3.16, bands of AH are present at ~36kDa, up until ~5 hours of incubation with papain. The bands are very faint on this membrane but it can be seen that the AH toxin has not been digested in lanes containing AH incubated for 1-4 hours. It can be concluded that papain does digest completely digest AH after 6 hours.

3.7.5 Lysine-C and Trypsin Digestion of AH

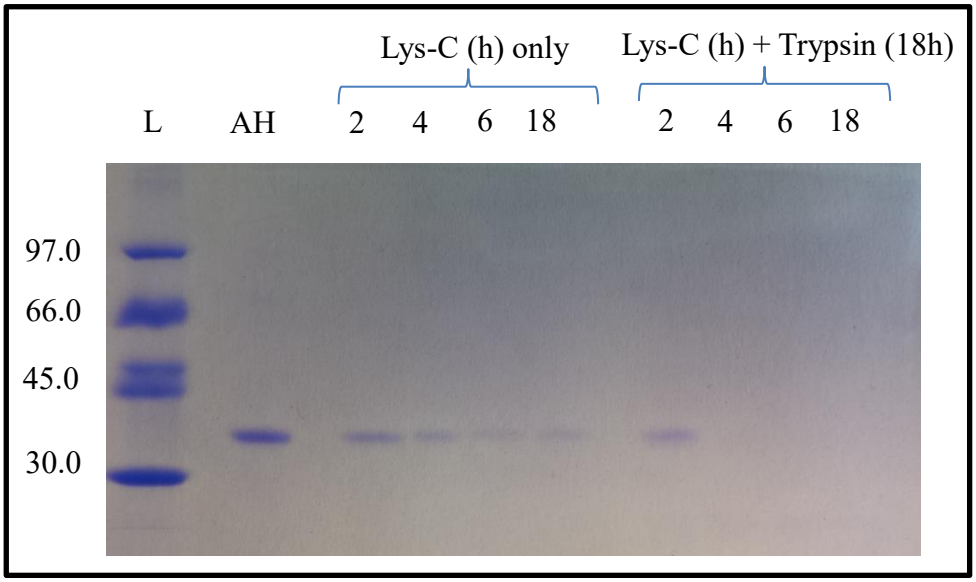


Figure 3.17 Coomassie stain of AH (62.5ug/ml), initially incubated with Lysine C only for 2-18 hours (37°C); followed by incubation with trypsin for18 hours (37°C). Lanes contain ladder (L), undigested AH (AH) and AH incubated with lysine C only followed by the same samples incubated with trypsin.

In Figure 3.17, AH was firstly incubated for 2, 4, 6 and 18 hours with lysine c only. These samples are shown under the 'Lys-C (h) only' bracket. These samples were then all incubated with trypsin for 18h following their incubation with lysine c for 2, 4, 6 or 18 hours. These samples are shown under the 'Lys-C (h) + Trypsin (18h)' bracket.

Following staining with coomassie brilliant blue, bands of AH can be seen in all lanes after incubation with lysine c only; although the bands appear fainter over time. After the subsequent trypsin incubation, there is still a band present in the AH sample that had a 2 hour incubation and 18 hour trypsin incubation. However, there are no bands

of AH present in the lanes of 4, 6 and 18 hours of lysine c incubation and 18 hours of trypsin incubation.

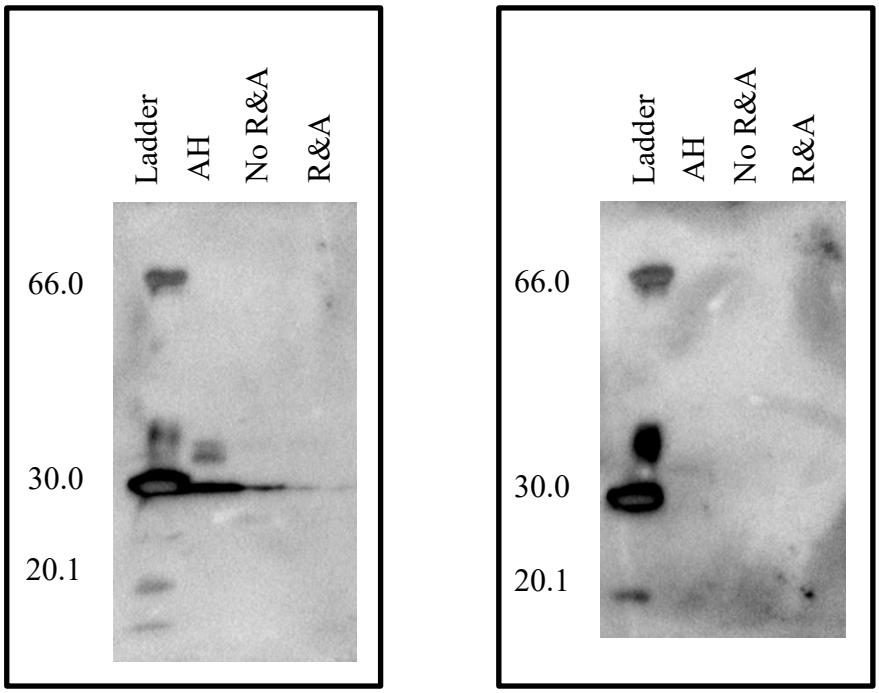


Figure 3.18 Western blot (x2) of AH (1µg/ml). Exposure = 10 seconds. Lanes contain low molecular weight ladder, undigested AH (AH) and two samples of AH incubated with lysine c for 4 hours and trypsin for a further 18 hours at 37°C; the second sample of which has been reduced and alkylated.

Figure 3.18 shows a comparison of AH digested with and without R&A prior to incubation with lysine c and trypsin. Both of the images are of the same samples. There is no band at 36kDa in both the 'No R&A' and 'R&A' lanes.

3.8 Urine Spiked with Toxin

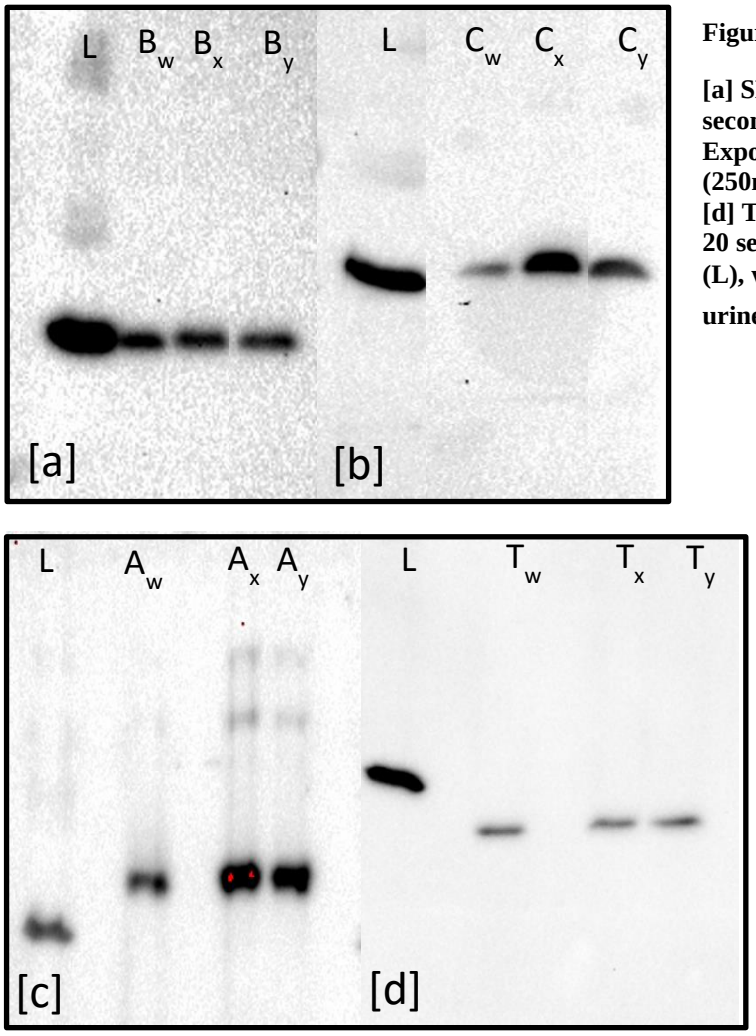


Figure 3.19 Western blots of:

[a] SEB (100ng/ml). Exposure = 60 seconds. [b] SEC (100ng/ml). Exposure = 80 seconds. [c] AH (250ng/ml). Exposure = 30 seconds. [d] TSST-1 (250ng/ml). Exposure = 20 seconds. Lanes contain ladder (L), whole toxin (?$_w$), un-centrifuged urine (?$_x$) and centrifuged urine (?$_y$).

So far, all toxin samples have been in buffer, which would not have any adverse effects on the western blotting process. As the patient samples will be bodily fluids, samples in a solution more complex than buffer needed to be tested. Therefore, urine of a healthy male was collected and spiked with the toxins (SEB, SEC, TSST-1 and AH), in order to establish the effects of urine on the detection process. As Figure 3.20 shows, the whole toxins are clearly visible in all of the lanes. The uncentrifuged urine (x) and centrifuged urine (y) do not appear to show any differences.

3.9 Discussion

3.9.1 The Toxins

Each of the toxins – AH, SEB, SEC, TSST-1 – are said to be of a particular molecular weight, according to each datasheet. As shown in Figure 3.1, the proteins do not always appear to be the exact weight described. This discrepancy can be caused by the type of gel used and the parameters used in the running of it. When comparing each toxin to the low molecular weight ladder SEB, SEC and TSST-1 appear to be approximately the same weight as described on the data sheets; 28kDa, 30kDa and 24kDa, respectively. However, AH appears at approximately 40kDa, rather than 33kDa. AH has also been described as 36kDa (Parimon et al., 2013). This may be due to a precursor or additional protein binding with the main protein, making it heavier and appear as a band higher up the 12.5% acrylamide gel.

3.9.2 WB sensitivity

Following the serial dilution of each toxin, bands were seen in lanes containing 39ng/ml toxin. The sensitivity of this western blot protocol is good, showing albeit faint bands of a total 780pg protein load. Bands of SEB and SEC appeared stronger than the bands of AH throughout, this may be due to differences in the binding affinity of the antibodies used for each. For TSST-1 however, the technique was not so sensitive, as shown in Figure 3.7. A more sensitive ECL (ECL2) was used and strong

bands were seen to 250ng/ml (5ng protein load) and very faint bands down to 31.25ng/ml sample (625pg protein load).

3.9.3 Toxin Digestion

A number of proteolytic enzymes were trialled in the digestion of the four staphylococcal toxins, including trypsin, pepsin, chymotrypsin, papain and a lysine c - trypsin combination. Trypsin is currently the most commonly used enzyme in proteomics and provides a reliable method for effective and accurate protein digestion. For this reason, trypsin was used first. Initially unaltered (not reduced or alkylated) toxin samples of SEB and SEC were sufficiently digested and AH and TSST-1 were not. Subsequently, pepsin was used to digest AH and TSST-1. Pepsin dissolves well in an acid, such a GuHCl, but only at very low concentrations in water. It was found that the samples dissolved in acid did not stay in the wells during the western blot. Therefore, after running the gel, there were no bands in any of the samples containing GuHCl. Gels were run using the 3M pepsin that we had tried to dissolve in water (heating, vortexing) and they did give some results; however it was impossible to know what the actual concentration of pepsin in the sample was. It was therefore concluded that pepsin was not the best enzyme to be used. Chymotrypsin and papain were each incubated with AH and gave similar results. Both partially digested AH however, a faint band remained in most of the lanes at the MW of AH. With AH proving difficult to successfully digest, a combination of lysine c and trypsin was eventually used. This dual enzyme incubation proved to be very successful as AH was completely digested. Lysine c appears to open up the tertiary protein structure of AH,

allowing the trypsin more access to completely digest the toxin. The 293 amino acid long mature protein of AH is preceded by a 26 residue signal sequence that is cleaved during secretion (Gray, 1984). The signal peptide contains 2 amino-terminal charged residues, followed by 16 neutral, hydrophobic amino acids, a helix-bending proline and a further four neutral residues (Gray, 1984).

For trypsin to be most effective, the protein needs to be completely denatured and contain a sufficient number of arginine and lysine residues (Shah, 2010). Once this was realised, all of the staphylococcal toxins were reduced and alkylated prior to incubation with trypsin (prior to lysine c for AH) and compared to toxin incubated with trypsin only. The results proved how vital reduction and alkylation was the digestion of these toxins using trypsin. Although SEB and SEC had no band at 28kDa and 30kDa, respectively, there was a strong band in each of a lower molecular weight. TSST-1 remained seemingly untouched by trypsin. The AH results were inconclusive due to very faint bands, even for the whole control toxin. Following reduction and alkylation, SEB and SEC showed no bands remaining and TSST-1 only showed a very faint band at 24kDa and none elsewhere, allowing us to conclude that all three toxins were completely or sufficiently digested, suitable for mass spectrometry.

Reduction and alkylation in more detail:

DTT ($C_4H_{10}O_2S_2$) is a small molecule, strong reducing agent. Once oxidised, a stable 6-membered ring forms, with an internal disulphide bond. The reduction of a typical disulphide bond proceeds be two sequential thiol-disulfide exchange reactions, resulting in oxidised DTT and a reduced disulphide bond(s).

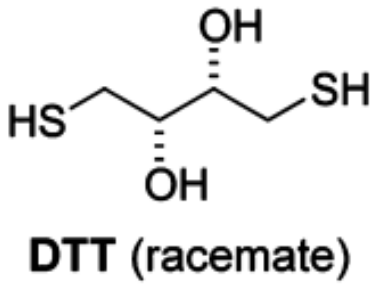


Figure 3.20 Chemical structure of dithiothreitol (DTT) (Lukesh, 2012).

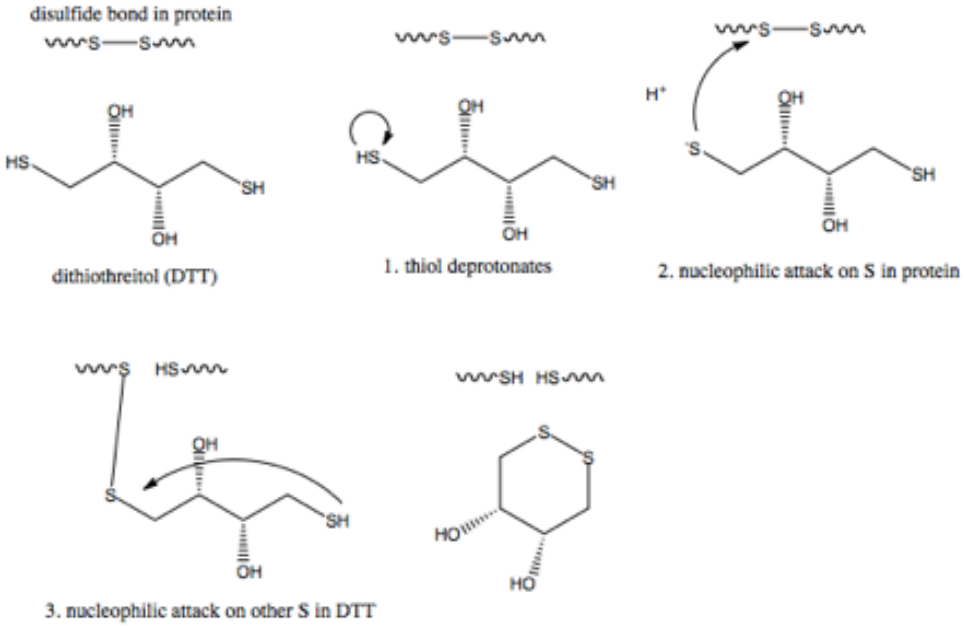


Figure 3.21 Mechanism of action of DTT (Minikel, 2015).

Iodoacetamide (C_2H_4INO) binds covalently with the thiol group of cysteine so that the amino acid cannot form disulphide bonds.

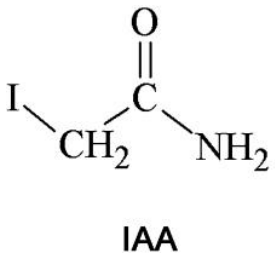


Figure 3.22 Mechanism of action of iodoacetamide (Schmidt, 2009).

The thiol group is the functional unit of cysteine, which plays a role in protein folding. Oxidation generates a cysteine unit containing a disulfide bond. When the cysteines are within the same peptide, the tertiary structure is affected; when cysteines are in different peptides, quaternary structure is affected (strong covalent bonds). Iodoacetamide binds to the thiol group, inhibiting such bonds, preventing reformation following reduction (Boja, 2001).

Chapter 4 MASS SPECTROMETRY: TOXIN DIGESTION

4.1 Introduction

The work in this chapter was carried out using the HPLC-ESI-MS method. The result(s) of mass spectrometry (MS) were determined using the MASCOT server. The raw data generated by the LC-MS is opened into ‘Esquire’. There, the compounds are found using ‘Find – Compounds AutoMSn’. The resultant mass spectrum is subsequently de-convoluted and the compounds are exported and saved as an ‘.mgf’ file. Such ‘.mgf’ files can be opened in ‘Biotools’ – the software used to run the data through the MASCOT database.

The MASCOT database can be discriminatory and we chose to only search for staphylococcal peptides, so as not to detect proteins that could have implications for the participants taking part in the study.

MASCOT scores are generated for each peptide detected. A threshold of 50 has been set and any score over this value is considered a correct and confident identification. The higher the score, the more confident the identification.

The one letter amino acid abbreviations used are explained in appendix A.

The amino acid sequences for each toxin are available in appendix B (1-4).

4.2 Toxin Alignments

Using EMBOSS Pairwise Alignment software, each toxin was run against one another to determine the similarity of their amino acid sequences. Similarity within their amino acid sequences could result in similar tertiary protein structures which may lead to cross-reactivity.

4.2.1 Comparison of SEB and SEC1

```
SEB    1   MYKRLFISHVILIFALILVISTPNVLAESQPDPKPDELHKSSKFTGLMEN   50
           |.|..|||.||||||||||:.|||||||||||.||||||:|||||||||
SEC1   1   MNKSRFISCVILIFALILVLFTPNVLAESQPDPTPDELHKASKFTGLMEN   50

SEB    51  MKVLYDDNHVSAINVKSIDQFLYFDLIYSIKDTKLGNYDNVRVEFKNKDL  100
           |||||||::|||..|||:|:||..||||:|.|.||.|||.|:.|..|:.|
SEC1   51  MKVLYDDHYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEGL  100

SEB    101 ADKYKDKYVDVFGANYYYQCYFSKKTNDINSHQTDKRKTCMYGGVTEHNG  150
           |.||||:.|||:|:|||..||||.|.|   ..:....|||||||:|:|.|
SEC1   101 AKKYKDEVVDVYGSNYYVNCYFSSKDN---VGKVTGGKTCMYGGITKHEG  147

SEB    151 NQLDK--YRSITVRVFEDGKNLLSFDVQTNKKKVTAQELDYLTRHYLVKN  198
           |..|.  .:::.:||:|:.:|.:|||:|||:||.|||||||...|::|:..
SEC1   148 NHFDNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELDIKARNFLINK  197

SEB    199 KKLYEFNNSPYETGYIKFIENE-NSFWYDMMPAPGDKFDQSKYLMMYNDN  247
           |.|||||:|||||||||||||. |:|||||||||||||||||||||||||
SEC1   198 KNLYEFNSSPYETGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDN  247

SEB    248 KMVDSKDVKIEVYLTTKKK  266
           |.||||.|||||:||||..
SEC1   248 KTVDSKSVKIEVHLTTKNG  266
```

Identity: 182/269 (67.7%)

Similarity: 213/269 (79.2%)

Gaps: 6/269 (2.2%)

4.2.2 Comparison of SEB and TSST-1

```
SEB    1   MYKRLFISHVILIFALILVISTPNVLAESQPDPKPDELHKSSKFTGLMEN   50
           |.|:|.::..|:         :|.:||.:..|..|..|..:.    :::.
TSST-1 1   MNKKLLMNFFIV---------SPLLLATTATDFTPVPLSSNQ----IIKT   37

SEB    51  MKVLYDDNHVSAINVKSIDQFLYFDLIYSIKDTKLGNYDNVRVEFKNKDL  100
           .|...:||....::..|.....:.:.  .:.|..||:     :..||.|.
TSST-1 38  AKASTNDNIKDLLDWYSSGSDTFTNS--EVLDNSLGS-----MRIKNTDG   80

SEB    101 ADKYKDKYVDVFGANYYYQCYFSKKTNDINSHQTDKRKTCMYG-------  143
           :..     :.:|.:.||...:..:..|:|:.:|.|.:....|
TSST-1 81  SIS-----LIIFPSPYYSPAFTKGEKVDLNTKRTKKSQHTSEGTYIHFQI  125

SEB    144 -GVTEHNGNQLDKYRSITVRVFEDGKNL-LSFDVQTNKKKVTAQELDYLT  191
            |||  |..:|.....:.::|...||:. |.:..:.:||::....||:..
TSST-1 126 SGVT--NTEKLPTPIELPLKVKVHGKDSPLKYGPKFDKKQLAISTLDFEI  173

SEB    192 RHYLVKNKKLYEFNNSPYETGYIKFIENENSFWYDMMPAPGDKFDQSKYL  241
           ||.|.:...||.  :|....||.|...|:.|.:.         |.||..
TSST-1 174 RHQLTQIHGLYR--SSDKTGGYWKITMNDGSTYQS---------DLSKKF  212

SEB    242 MMYNDNKMVDSKDVKIEVYLTTKKK  266
           ....:...::..::|.   :..:..
TSST-1 213 EYNTEKPPINIDEIKT---IEAEIN  234
```

Identity: 56/275 (20.4%)

Similarity: 107/275 (38.9%)

Gaps: 50/275 (18.2%)

4.2.3 Comparison of SEB and AH

```
SEB    1   MYKRLFISHVILIFALILVISTPNV-LAESQPDPKPDELHKSSKFTGLME   49
           |..|: :|.|.....|..::..|.. .|:|..:.|.......|..|....
AH     1   MKTRI-VSSVTTTLLLGSILMNPVAGAADSDINIKTGTTDIGSNTTVKTG   49

SEB    50  NMKVLYDDNH-------VSAINVKSIDQFLYFDLIYSIKDTKLGNYDNVR   92
           :: |.||..:       .|.|:.|:.::.|   |:...|.|..|.|....
AH     50  DL-VTYDKENGMHKKVFYSFIDDKNHNKKL---LVIRTKGTIAGQYRVYS   95

SEB    93  VEFKNKD------------LADKYKDKYVDVFGANYY-YQCYFSKKTND  128
           .|..||.             |.|....:..|.:..|.. .:.|.|..|..
AH     96  EEGANKSGLAWPSAFKVQLQLPDNEVAQISDYYPRNSIDTKEYMSTLTYG  145
```

```
SEB    129 INSHQTDKRKTCMYGGVTEHN---GNQLDKYRSITVRVFEDGKNL-----  170
           .|.:.|.. .|...||:...|   |:.|...:.....:.|...:.
AH     146 FNGNVTGD-DTGKIGGLIGANVSIGHTLKYVQPDFKTILESPTDKKVGWK  194

SEB    171 LSFDVQTNKK----------KVTAQELDYLTRHYLVKN-------KKLYE  203
           :.|:...|:.          .|...:|...||:..:|.       .|...
AH     195 VIFNNMVNQNWGPYDRDSWNPVYGNQLFMKTRNGSMKAADNFLDPNKASS  244

SEB    204 FNNSPYETGYIKFI--ENENSFWYDMMPAPGDKFDQSKYLMMYNDN-KMV  250
           ..:|.:...:...|  :.:.|.....:...::.....|...:.| |..
AH     245 LLSSGFSPDFATVITMDRKASKQQTNIDVIYERVRDDYQLHWTSTNWKGT  294

SEB    251 DSKD---------VKIEVYLTTKKK  266
           ::||         .||:........
AH     295 NTKDKWTDRSSERYKIDWEKEEMTN  319
```

Identity: 60/325 (18.5%)

Similarity: 101/325 (31.1%)

Gaps: 65/325 (20.0%)

4.2.4 Comparison of SEC1 and TSST-1

```
SEC1   1 MNKSRFISCVILIFALILVLFTPNVLAESQPDP-TPDELHKASKFTGLME     49
           |||.     :::.|.::..|.......:..|.| :.:::.|.:| ....:
TSST-1 1 MNKK-----LLMNFFIVSPLLLATTATDFTPVPLSSNQIIKTAK-ASTND     44

SEC1   50 NMKVLYDDHYVSATKVKSVDKFLAHDLIYN-ISDKKLKNYDKVKTELLNE    98
          |:|.|.| .|.|.:     |.|...:::.| :...::||.|...:.::
TSST-1 45 NIKDLLD-WYSSGS-----DTFTNSEVLDNSLGSMRIKNTDGSISLII--    86

SEC1   99 GLAKKYKDEVVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGN   148
           ....|..... ..|....:|...:.|..............|:|..|
TSST-1 87 -FPSPYYSPAF-TKGEKVDLNTKRTKKSQHTSEGTYIHFQISGVTNTE--   132

SEC1   149 HFDNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELDIKARNFLINKK  198
           .........:.::|: .|.:.:.:..:.|||.:....||.:.|:.|....
TSST-1 133 KLPTPIELPLKVKVH-GKDSPLKYGPKFDKKQLAISTLDFEIRHQLTQIH  181

SEC1   199 NLYEFNSSPYETGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNK  248
           .||  .||....||.|...|:|:|:..|:    ..||:.:......|.::
TSST-1 182 GLY--RSSDKTGGYWKITMNDGSTYQSDL----SKKFEYNTEKPPINIDE  225

SEC1   249 TVDSKSVKIEVHLTTKNG  266
           .   |::::.|::
TSST-1 226 I---KTIEAEIN------  234
```

Identity: 53/268 (19.8%)

Similarity: 103/268 (38.4%)

Gaps: 36/268 (13.4%)

4.2.5 Comparison of SEC1 and AH

```
SEC1   1   MNKSRFISCVILIFALILVLFTPNVLAESQPD-----PTPDELHKASKFT   45
           | |:|.:|.|.....|..:|..| |...:..|     .|.|.....:..|
AH     1   M-KTRIVSSVTTTLLLGSILMNP-VAGAADSDINIKTGTTDIGSNTTVKT   48

SEC1   46  GLM-----EN---MKVLY---DD--HYVSATKVKSVDKFLAHDLIY----   78
           |.:     ||   .||.|   ||  |......::.........:|
AH     49  GDLVTYDKENGMHKKVFYSFIDDKNHNKKLLVIRTKGTIAGQYRVYSEEG   98

SEC1   79  -NISDKKLKNYDKVKTELLNEGLAK---KYKDEVVDVYGSNYYVNCYFSS  124
            |.|.....:..||:.:|.:..:|:   .|....:|.......:...|:.
AH     99  ANKSGLAWPSAFKVQLQLPDNEVAQISDYYPRNSIDTKEYMSTLTYGFNG  148

SEC1   125 K---DNVGKVTG--GKTCMYGGITKHEGNHF----DNGNLQNVLIRV-YE  164
           .   |:.||:.|  |.....|...|:....|    ::...:.|..:| :.
AH     149 NVTGDDTGKIGGLIGANVSIGHTLKYVQPDFKTILESPTDKKVGWKVIFN  198

SEC1   165 NKRNTISFEVQTDK-KSVTAQELDIKARNFLINKK-NLYEFN------SS  206
           |..|........|. ..|...:|.:|.||..:... |..:.|      ||
AH     199 NMVNQNWGPYDRDSWNPVYGNQLFMKTRNGSMKAADNFLDPNKASSLLSS  248

SEC1   207 PYETGYIKFI---------ENNGNTFWYDMMPAPGDKFDQSKYLMMYNDN  247
           .:...:...|         :.|.:..:..:.......:..:.:......:
AH     249 GFSPDFATVITMDRKASKQQTNIDVIYERVRDDYQLHWTSTNWKGTNTKD  298

SEC1   248 KTVD--SKSVKIEVHLTTKNG  266
           |..|  |:..||:........
AH     299 KWTDRSSERYKIDWEKEEMTN  319
```

Identity: 61/321 (19.0%)

Similarity: 104/321 (32.4%)

Gaps: 57/321 (17.8%)

4.2.6 Comparison of TSST-1 and AH

```
TSST-1   1 MNKK--------LLMNFFIVSPLLLATTAT---DFTPVPLSSNQIIKTAK  39
             |..:        ||:...:::|:..|..:.   ......:.||..:||..
AH       1 MKTRIVSSVTTTLLLGSILMNPVAGAADSDINIKTGTTDIGSNTTVKTGD  50

TSST-1  40 ASTNDNIKDLLD--WYSSGSDTFTNSEVL-----DNSLGSMRIKNTDGSI  82
           ..|.|....:..  :||...|...|.::|     ....|..|:.:.:|:.
AH      51 LVTYDKENGMHKKVFYSFIDDKNHNKKLLVIRTKGTIAGQYRVYSEEGAN  100

TSST-1  83 -SLIIFPSPY-YSPAFTKGEKVDLNTKRTKKSQHTSEG----TY-IHFQI  125
            |.:.:||.: ........|...::...:.|..|.|.    || .:..:
AH     101 KSGLAWPSAFKVQLQLPDNEVAQISDYYPRNSIDTKEYMSTLTYGFNGNV  150

TSST-1 126 SG---------------VTNTEKLPTP-----IELPLKVKV---------  146
           :|               :.:|.|...|     :|.|...||
AH     151 TGDDTGKIGGLIGANVSIGHTLKYVQPDFKTILESPTDKKVGWKVIFNNM  200

TSST-1 147 -------HGKDS--PLKYGPKFDKKQ-----------------------  163
                  :.:||  |:.....|.|.:
AH     201 VNQNWGPYDRDSWNPVYGNQLFMKTRNGSMKAADNFLDPNKASSLLSSGF  250

TSST-1 164 ----LAISTLDFEIRHQLTQIHGLY-RSSDKTGGYWKITMNDGSTYQSDL  208
               ..:.|:|.:...|.|.|..:| |..|....:|..|...|:..:.
AH     251 SPDFATVITMDRKASKQQTNIDVIYERVRDDYQLHWTSTNWKGTNTKD--  298


TSST-1 209 SKKFEYNTEKPPINIDEIKTIEAEIN  234
            |..:.::|:..|:.::    |...|
AH     299 -KWTDRSSERYKIDWEK----EEMTN  319
```

Identity: 57/326 (17.5%)

Similarity: 104/326 (31.9%)

Gaps: 99/326 (30.4%)

Table 4.1 The similarity of each toxin - AH, SEB, SEC1, TSST - as determined by EMBOSS pairwise alignment.

	SEB	SEC1	TSST-1	AH
SEB		79.2 %	38.9%	31.1%
SEC1			38.4%	32.4%
TSST-1				31.9%
AH				

4.3 ExPASy: Expected Peptide Fragments

Using the gi number of each toxin, a theoretical enzymatic digestion was carried out using ExPASy enzyme cutter.

The amino acids highlighted in blue (Section 4.4.1 – 4.4.4) are amino acids that have been identified successfully by the MS. The detection may have been of the exact peptide shown or a combination of 2 or more.

Table 4.3 shows the gi number of each toxin and the enzyme(s) used to digest each one. The enzyme choice for the digestion of each toxin was explained previously in Chapter 3. Amino acids that were identified by the MS and matched to a peptide in the 'expected' peptides list generated by ExPASy were counted and totalled up. The % of amino acids of identified for each toxin are shown.

Table 4.2 The toxins used (gi number) and the enzyme(s) used to digest each one. Total number of amino acids in each toxin and the number of amino acids detected by the MS.

Toxin	gi number	Enzyme	Total amino acids (n)	Matched amino acids (n)	Matched amino acids (%)
AH	gi|46765	Lys-C/Trypsin	319	132	41.38
SEB	gi|153000	Trypsin	266	137	51.50
SEC	gi|46567	Trypsin	266	144	54.14
TSST-1	gi|153123	Trypsin	234	108	46.15

4.3.1 AH & Lysine C/Trypsin

Position	Peptide Cleavage
137-157	EYMSTLTYGFNGNVTGDDTGK
112-130	VQLQLPDNEVAQISDYYPR
242-262	ASSLLSSGFSPDFATVITMDR
195-210	VIFNNMVNQNWGPYDR
211-224	DSWNPVYGNQLFMK
280-292	DDYQLHWTSTNWK
158-173	IGGLIGANVSIGHTLK
267-277	QQTNIDVIYER
35-47	TGTTDIGSNTTVK
64-72	VFYSFIDDK
232-241	AADNFLDPNK
102-111	SGLAWPSAFK
48-56	TGDLVTYDK
181-189	TILESPTDK
93-101	VYSEEGANK
174-180	YVQPDFK
27-34	ADSDINIK
85-92	GTIAGQYR
57-62	ENGMHK
310-314	IDWEK
131-136	NSIDTK
315-319	EEMTN
78-82	LLVIR
300-303	WTDR
227-231	NGSMK
293-297	GTNTK
73-76	NHNK
191-194	VGWK
304-307	SSER
308-309	YK
264-266	ASK
225-226	TR
278-279	VR
298-299	DK
83-84	TK
63-63	K
77-77	K
190-190	K
263-263	K

4.3.2 SEB & Trypsin

Position	Peptide Cleavage
216-234	FIENENSFWYDMMPAPGDK
108-124	YVDVFGANYYYQCYFSK
139-155	TCMYGGVTEHNGNQLDK
67-81	SIDQFLYFDLIYSIK
201-215	LYEFNNSPYETGYIK
53-66	VLYDDNHVSAINVK
28-40	ESQPDPKPDELHK
182-192	VTAQELDYLTR
169-179	NLLSFDVQTNK
126-136	TNDINSHQTDK
240-248	YLMMYNDNK
44-52	FTGLMENMK
257-264	IEVYLTTK
85-92	LGNYDNVR
163-168	VFEDGK
193-197	HYLVK
235-239	FDQSK
249-253	MVDSK
158-162	SITVR
99-103	DLADK
93-96	VEFK
82-84	DTK
254-256	DVK
156-157	YR
41-43	SSK
104-105	YK
106-107	DK
97-98	NK
198-199	NK
137-137	R
125-125	K
138-138	K
180-180	K
181-181	K
200-200	K
265-265	K
266-266	K

4.3.3 SEC1 & Trypsin

Position	Peptide Cleavage
106-125	DEVVDVYGSNYYVNCYFSSK
215-234	FIENNGNTFWYDMMPAPGDK
145-161	HEGNHFDNGNLQNVLIR
199-214	NLYEFNSSPYETGYIK
71-83	FLAHDLIYNISDK
28-40	ESQPDPTPDELHK
53-64	VLYDDHYVSATK
168-178	NTISFEVQTDK
240-248	YLMMYNDNK
180-189	SVTAQELDIK
93-102	TELLNEGLAK
44-52	FTGLMENMK
136-144	TCMYGGITK
257-264	IEVHLTTK
192-197	NFLINK
162-166	VYENK
235-239	FDQSK
249-253	TVDSK
87-90	NYDK
126-130	DNVGK
131-135	VTGGK
67-70	SVDK
254-256	SVK
104-105	YK
41-43	ASK
85-86	LK
65-66	VK
91-92	VK
190-191	AR
265-266	NG
167-167	R
84-84	K
103-103	K
179-179	K
198-198	K

4.3.4 TSST-1 & Trypsin

Position	Peptide Cleavage
5-36	LLMNFFIVSPLLLATTATDF TPVPLSSNQIIK
48-74	DLLDWYSSGSDTFTNSEVLD NSLGSMR
112-133	SQHTSEGTYIHFQISGVTNT EK
77-98	NTDGSISLIIFPSPYYSPAF TK
212-227	FEYNTEKPPINIDEIK
196-210	ITMNDGSTYQSDLSK
163-174	QLAISTLDFEIR
175-185	HQLTQIHGLYR
134-143	LPTPIELPLK
40-47	ASTNDNIK
228-234	TIEAEIN
190-195	TGGYWK
102-107	VDLNTK
150-154	DSPLK
155-158	YGPK
146-149	VHGK
186-189	SSDK
159-161	FDK
1-3	MNK
99-101	GEK
37-39	TAK
75-76	IK
109-110	TK
144-145	VK
108-108	R
4-4	K
111-111	K
162-162	K
211-211	K

4.4 Initial Mass Spectrometry

Mass spectrometry has never been used to detect these staphylococcal enterotoxins in urine before, but it has been used to analyse urine and toxins separately (Callahan, 2006; Andjelkovic, 2016; Storer, 2011).

4.4.1 MS of Bovine Serum Albumin

In order to analyse patient samples for the presence of staphylococcal enterotoxins, a highly sensitive and robust method needs to be developed. Currently, ELISA and western blot methods are able to detect the toxins at ng/ml levels. Mass spectrometry has the potential to detect proteins of picomolar concentration.

Firstly, a protocol needed to be established to run the samples through the MS and to be able to identify the peaks that could be peptide fragments of a staphylococcal enterotoxin. To do this, bovine serum albumin (BSA) was used as a standard; as it is an inexpensive protein that has been extensively used in MS, meaning that well-established spectra were already available, Figure 4.1.

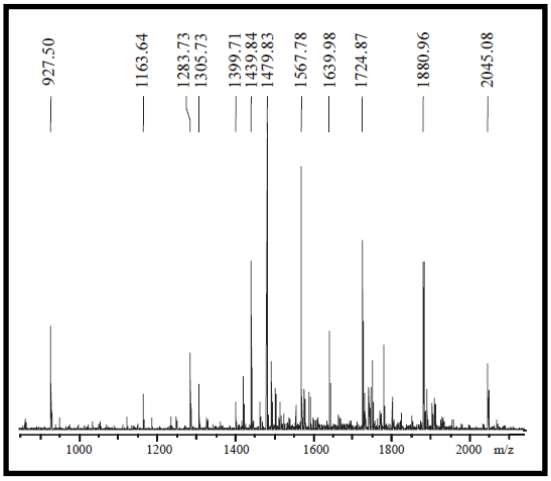


Figure 4.1 Resolved spectra of BSA. (Bruker Daltonics).)

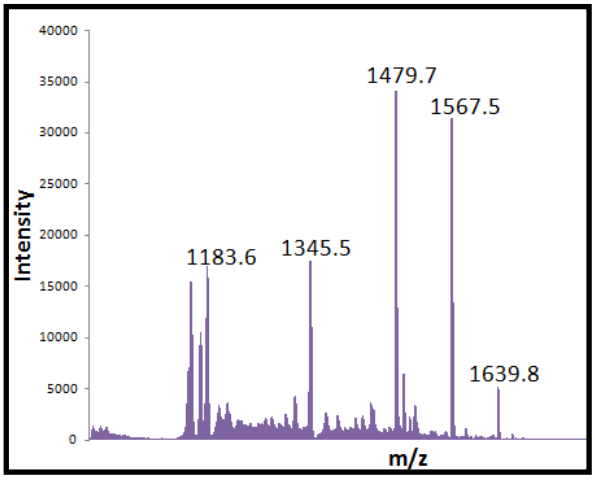
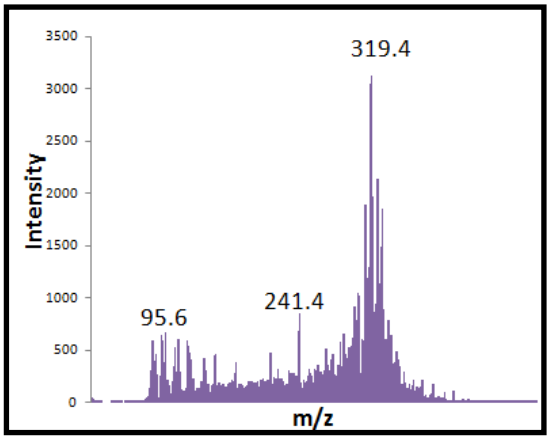


Figure 4.2 Resolved spectra of BSA (1mg/ml).

Results of this showed that milli-, nano- and picomolar concentrations can be observed. Although the spectra are not the same, some of the expected peaks have been seen in the spectra of BSA (Figure 4.2).

4.4.2 Initial MS of Staphylococcal Toxins

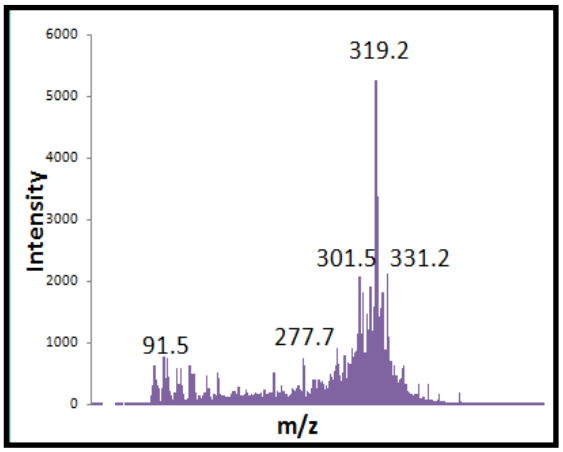
Figures 4.3-4.5 show the spectra of SEB (1ng/ml), SEC1 (1pg/ml) and TSST-1 (1pg/ml), digested with trypsin for 18 hours at 37^{o}C. The list of numbers to the right of each figure, are the expected m/z (mass to charge) values of the resultant peptides, according to mMass. The numbers coloured purple are where the expected peak has been matched with a peak in the experimental spectra.



Expected m/z

2290.978	1191.517	522.2922
2129.931	1070.501	363.1874
1866.811	966.5506	361.2081
1864.973	950.469	338.1823
1837.864	694.3406	321.1768
1586.817	659.3875	310.1761
1519.739	624.2987	262.1397
1308.679	579.2807	261.1557
1278.669	575.3511	175.1189
1272.582	561.2878	147.1128

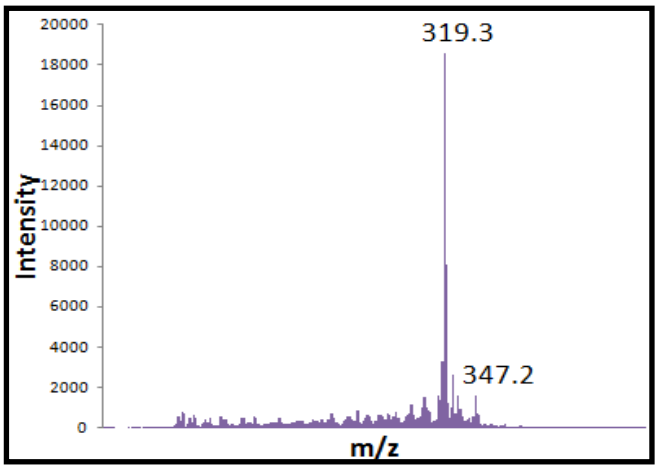
Figure 4.3 MS spectra of SEB (1µg/ml) and expected m/z peaks according to mMass. Samples were digested with trypsin at 37°C for 18h then diluted to 1ng/ml for analysis.



Expected m/z

2351.017	1087.599	448.2402
2347.016	1070.501	333.2132
1976.969	973.4481	310.1761
1924.896	940.5462	305.1819
1548.806	748.4352	260.1968
1492.691	652.33	246.1812
1410.69	624.2987	246.156
1281.632	549.2878	190.0822
1191.517	539.246	175.1189
1103.594	532.2725	147.1128
	461.2718	

Figure 4.4 MS spectra of SEC (1µg/ml) and expected m/z peaks according to mMass. Samples were digested with trypsin at 37°C for 18h then diluted to 1pg/ml for analysis.



Expected m/z

3009.342	559.3086
2464.174	464.2503
2418.223	440.2616
1949.985	436.2038
1659.753	409.2081
1405.769	333.1768
1365.739	260.1968
1120.698	248.1605
791.3894	246.1812
789.3988	175.1189
711.346	147.1128
689.3828	

Figure 4.5 MS spectra of TSST-1 (1µg/ml) and expected m/z peaks according to mMass. Samples were digested with trypsin at 37°C for 18h then diluted to 1pg/ml for analysis.

The MS spectra of the trypsinised toxins did not prove as successful as expected. These samples were not reduced and alkylated and these data were generated before the importance of reduction and alkylation in these tryptic digests was realised. As shown in Chapter 3, western blots compared the trypsinised toxins with and without prior reduction and alkylation.

4.5 MS of BSA and Staphylococcal Toxins in Water

BSA is used as a standard in the protocol for enzymatic digestion using trypsin and the subsequent LC-MS spectra are well established and defined. The table below summarises the identifications of each protein and the highest score produced. A score of <50 means that the MASCOT database is quite certain of the identification; the higher the score, the higher the certainty.

Table 4.3 Each sample, concentration, proteolytic enzyme(s) used and MASCOT identification based on MS raw data.

Sample	Concentration	Enzyme(s)	MASCOT Identification	gi number	Score
BSA	200ng	Trypsin	Albumin [Bos taurus]	gi|162648	2323
SEB	20µg	Trypsin	Enterotoxin B	gi|153000	1507
SEC	20µg	Trypsin	Unnamed protein product [Staphylococcus aureus]	gi|46567	1122
TSST-1	20µg	Trypsin	Toxic shock syndrome toxin precursor	gi|153123	615
AH	20µg	Lysine C & Trypsin	Unnamed protein product [Staphylococcus aureus]	gi|46765	585

The mascot identifications stated in the table above are the gi numbers that have the highest score, most often. However, sometimes, slightly different gi numbers are given for a similar peptide; i.e. a precursor or smaller derivative.

Other gi numbers included:

- BSA (ALB protein [Bos Taurus] gi|74267962, Albumin gi|164318)
- SEC (Enterotoxin C3 precursor gi| 153004)

4.6 MS of Urine

Samples of urine – from a healthy individual - were analysed by mass spectrometry in order to understand what proteins exist and can be detected in 'normal' urine. The urine was centrifuged for 10 minutes at 5000 x g; no other 'clean-up' steps were taken. The samples were incubated with trypsin for 18 hours.

Table 4.4 The proteins detected in the urine samples and the MASCOT scores obtained.

MASCOT Identification	Score
PK-120 precursor [Homo Sapiens] gi|1402590	167
unnamed protein product [Homo sapiens] gi|35148	135
prostaglandin D2 synthase [Homo Sapiens] gi|189772	114
Sequence 40 from patent US 6680209 gi|42686962	79
immunoglobulin heavy chain, constant region [Homo sapiens] gi|2414494	76
10B5 scFv [synthetic construct] gi|7230486	75
Sequence 62 from patent US 6576896 gi|33738626	69
trypsinogen 7 [Mus musculus] gi|2358072	69
Bovine CD44 [Bos taurus] gi|187	63
lambda-chain precursor (AA -20 to 215) [Homo sapiens] gi|33395	60
Sequence 17 from patent US 6165745 gi|14107669	57

4.7 MS of Spiked Urine

Following the results of the MS of urine and of each protein in dH_2O, urine was spiked with individual staphylococcal toxins.

Table 4.5 MS results of urine spiked with BSA or toxin, including the identification (gi number) and MASCOT score.

Sample	Concentration	Enzyme(s)	MASCOT Identification	gi number	Score
BSA	200ng	Trypsin	Albumin [Bos taurus]	gi|162648	958
SEB	10µg	Trypsin	Enterotoxin B	gi|153000	918
SEC	10µg	Trypsin	Unnamed protein product [Staphylococcus aureus]	gi|46567	667
TSST-1	10µg	Trypsin	Toxic shock syndrome toxin precursor	gi|153123	369
AH	10µg	Lysine C & Trypsin	Unnamed protein product [Staphylococcus aureus]	gi|46765	633

4.8 MS Repeatability

To analyse the repeatability of the HPLC-MS method, BSA was diluted to 200ng/ml in dH_2O. Three identical vials were made up, treated identically and run consecutively in the LC-MS. As shown in Table 4.7, the BSA samples were constistantly identified as BSA, with a very high MASCOT score.

Table 4.6 MS repeatability of BSA (200ng), first analysis cycle. MASCOT identification and score >50.

Sample	Concentration	Enzyme(s)	MASCOT Identification	gi number	Score
BSA	200ng	Trypsin	Albumin [Bos taurus]	gi|162648	1696
BSA	200ng	Trypsin	Albumin [Bos taurus]	gi|162648	1286
BSA	200ng	Trypsin	Albumin [Bos taurus]	gi|162648	1256

The exact same three BSA samples shown in Table 4.7 were run through the LC-MS again, once the first cycle had finished. The results are shown in Table 4.8. Again, BSA was correctly and strongly identified by the MASCOT database.

Table 4.7 MS repeatability of BSA (200ng), second analysis cycle. MASCOT identification and score >50.

Sample	Concentration	Enzyme(s)	MASCOT Identification	gi number	Score
BSA	200ng	Trypsin	Albumin [Bos taurus]	gi|162648	1487
BSA	200ng	Trypsin	Albumin [Bos taurus]	gi|162648	1191
BSA	200ng	Trypsin	Albumin [Bos taurus]	gi|162648	1256

Further to the repeatability results of BSA, repeatability was further tested using one of the toxins. SEC1 was diluted to 10µg/ml with dH_2O. Three samples were made, treated identically and run consecutively in the LC-MS.

Table 4.8 MS repeatability of SEC1 (10µg). MASCOT identification and score >50.

Sample	Concentration	Enzyme(s)	MASCOT Identification	gi number	Score
SEC	10µg	Trypsin	Enterotoxin C3 precursor	gi|153004	363
SEC	10µg	Trypsin	Unnamed protein product [Staphylococcus aureus]	gi|46567	412
SEC	10µg	Trypsin	Enterotoxin C3 precursor	gi|153004	386

Two of the three samples have been identified as SEC3 precursor. This is most likely due to the homology within the SEC family. The scores recorded are similar to one another and considerably more than the >50 threshold. It has been concluded that the LC-MS method shows good repeatability.

4.9 MS Sensitivity

To test the sensitivity of the HPLC-MS method, SEC1 was diluted from 1mg/ml in dH_2O, reduced and alkylated then trypsinised (37^{o}C, 18 hours).

Table 4.9 MS sensitivity of SEC1, at various concentrations. MASCOT identification and score >50.

Sample	Concentration	Enzyme(s)	MASCOT Identification	gi number	Score
SEC	5µg/ml	Trypsin	Unnamed protein product [Staphylococcus aureus]	gi|162648	312
SEC	1µg/ml	Trypsin	Unnamed protein product [Staphylococcus aureus]	gi|162648	327
SEC	500ng/ml	Trypsin	Unnamed protein product [Staphylococcus aureus]	gi|162648	235
SEC	100ng/ml	Trypsin	\	\	\
SEC	10ng/ml	Trypsin	\	\	\
SEC	1ng/ml	Trypsin	\	\	\

SEC (gi|162648) was correctly identified at concentrations of 5µg/ml to 500ng/ml. There was no detection of SEC in the 100ng/ml and less samples.

4.10 SEC1-3

There is strong sequence homology within the SEC family (Reiser, 1984). The toxin used was SEC1, however it is important to include information about SEC2 and SEC3 due to their similarity. The amino acid sequences of SEC2 and SEC are available in Appendix B (5-6). SEC1-3 were compared for similarity by alignment and the results are shown below:

4.10.1 Comparison of SEC1 and SEC2

```
SEC1     1 MNKSRFISCVILIFALILVLFTPNVLAESQPDPTPDELHKASKFTGLMEN  50
           |||||||||||||||||||||||||||||||||||||||:|:||||.|.|
SEC2     1 MNKSRFISCVILIFALILVLFTPNVLAESQPDPTPDELHKSSEFTGTMGN  50

SEC1    51 MKVLYDDHYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEGL 100
           ||.|||||||||||.|||||||||||||||||||||||||||||||||.|
SEC2    51 MKYLYDDHYVSATKVMSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDL 100
```

```
SEC1   101 AKKYKDEVVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHF 150
           |||||||||||||||||||||||||||||||||||||||||||||||||||
SEC2   101 AKKYKDEVVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHF 150

SEC1   151 DNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELDIKARNFLINKKNL 200
           |||||||||||||||||||||||||||||||||||||||||||||||||||
SEC2   151 DNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELDIKARNFLINKKNL 200

SEC1   201 YEFNSSPYETGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTV 250
           |||||||||||||||||||||||||||||||||||||||||||||||||||
SEC2   201 YEFNSSPYETGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTV 250

SEC1   251 DSKSVKIEVHLTTKNG 266
           ||||||||||||||||
SEC2   251 DSKSVKIEVHLTTKNG 266
```

Identity: 259/266 (97.4%)

Similarity: 261/266 (98.1%)

Gaps: 0/266 (0.0%)

4.10.2 Comparison of SEC1 and SEC3

```
SEC1     1 MNKSRFISCVILIFALILVLFTPNVLAESQPDPTPDELHKASKFTGLMEN  50
           |.|..|||.||||||||||:.||||||||||||.||:||||:|:|||.|.|
SEC3     1 MYKRLFISRVILIFALILVISTPNVLAESQPDPMPDDLHKSSEFTGTMGN  50

SEC1    51 MKVLYDDHYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEGL 100
           ||.|||||||||||||||||||||||||||||||||||||||||||||.|
SEC3    51 MKYLYDDHYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDL 100

SEC1   101 AKKYKDEVVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHF 150
           |||||||||||||||||||||||||||||||||||||||||||||||||||
SEC3   101 AKKYKDEVVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHF 150

SEC1   151 DNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELDIKARNFLINKKNL 200
           |||||||||:||||||||||||||||||||||||||||||||||||||||||
SEC3   151 DNGNLQNVLVRVYENKRNTISFEVQTDKKSVTAQELDIKARNFLINKKNL 200

SEC1   201 YEFNSSPYETGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTV 250
           |||||||||||||||||||||||||||||||||||||||||||||||||||
SEC3   201 YEFNSSPYETGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTV 250

SEC1   251 DSKSVKIEVHLTTKNG 266
           ||||||||||||||||
SEC3   251 DSKSVKIEVHLTTKNG 266
```

Identity: 251/266 (94.4%)

Similarity: 256/266 (96.2%)

Gaps: 0/266 (0.0%)

4.10.3 Comparison of SEC2 and SEC3

```
SEC2     1 MNKSRFISCVILIFALILVLFTPNVLAESQPDPTPDELHKSSEFTGTMGN  50
           |.|..|||.|||||||||:.|||||||||||.||:|||||||||||||
SEC3     1 MYKRLFISRVILIFALILVISTPNVLAESQPDPMPDDLHKSSEFTGTMGN  50

SEC2    51 MKYLYDDHYVSATKVMSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDL 100
           |||||||||||||||.||||||||||||||||||||||||||||||||||
SEC3    51 MKYLYDDHYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDL 100

SEC2   101 AKKYKDEVVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHF 150
           ||||||||||||||||||||||||||||||||||||||||||||||||||
SEC3   101 AKKYKDEVVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHF 150

SEC2   151 DNGNLQNVLIRVYENKRNTISFEVQTDKKSVTAQELDIKARNFLINKKNL 200
           |||||||||:||||||||||||||||||||||||||||||||||||||||
SEC3   151 DNGNLQNVLVRVYENKRNTISFEVQTDKKSVTAQELDIKARNFLINKKNL 200

SEC2   201 YEFNSSPYETGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTV 250
           ||||||||||||||||||||||||||||||||||||||||||||||||||
SEC3   201 YEFNSSPYETGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTV 250

SEC2   251 DSKSVKIEVHLTTKNG 266
           ||||||||||||||||
SEC3   251 DSKSVKIEVHLTTKNG 266
```

Identity: 256/266 (96.2%)

Similarity: 259/266 (97.4%)

Gaps: 0/266 (0.0%)

Table 4.10 Comparison of the similarities of the amino acid structures of SEC1, SEC2 and SEC3.

	SEC1	SEC2	SEC3
SEC1		98.1%	96.2%
SEC2			97.4%
SEC3			

4.11 Comparison of Bovine Serum Albumin and Human Albumin

Finally, the amino acid make-up of BSA and human albumin (HA) were compared to see if they held much similarity to one another. BSA has been used as a standard protein and it is possible to see HA in human urine. The amino acid sequences for BSA and HA are available in Appendix B (7-8). The theoretical tryptic digest peptides are included in Appendix C (1-2).

4.11.1 Comparison of BSA and HA

```
BSA   1  MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHFKGLVLIA  50
         ||||||||||.|||||||||||||||.||||:||||||||||:||.|||||
HA    1  MKWVTFISLLFLFSSAYSRGVFRRDAHKSEVAHRFKDLGEENFKALVLIA  50

BSA  51  FSQYLQQCPFDEHVKLVNELTEFAKTCVADESHAGCEKSLHTLFGDELCK 100
         |:|||||||||::|||||||:|||||||||||||...|:||||||||:||.
HA   51  FAQYLQQCPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCT 100

BSA 101 VASLRETYGDMADCCEKQEPERNECFLSHKDDSPDLPKL-KPDPNTLCDE  149
         ||:||||||:|||||.||||||||||.||||:|:||:| :|:.:.:|..
HA  101 VATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTA  150

BSA 150 FKADEKKFWGKYLYEIARRHPYFYAPELLYYANKYNGVFQECCQAEDKGA  199
         |..:|:.|..||||||||||||||::|.:|...|.|||||.||.|
HA  151 FHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAA  200

BSA 200 CLLPKIETMREKVLASSARQRLRCASIQKFGERALKAWSVARLSQKFPKA  249
         |||||::.:|::..||||:|||:|||:||||||.|||:|||||:||||
HA  201 CLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKA  250

BSA 250 EFVEVTKLVTDLTKVHKECCHGDLLECADDRADLAKYICDNQDTISSKLK  299
         ||.||:||||||||||.||||||||||||||||||||:|||:||||||
HA  251 EFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLK  300

BSA 300 ECCDKPLLEKSHCIAEVEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAF  349
         |||:|||||||||||||.|.:|.:||.|.|||.|.|||||||.||||.|
HA  301 ECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVF  350

BSA 350 LGSFLYEYSRRHPEYAVSVLLRLAKEYEATLEECCAKDDPHACYSTVFDK  399
         ||.|||||:||||:|:|.:|||||||.||.|||:|||..|||.||:.|||:
HA  351 LGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDE  400

BSA 400 LKHLVDEPQNLIKQNCDQFEKLGEYGFQNALIVRYTRKVPQVSTPTLVEV  449
         .|.||:||||||||||:.|||:|||||.||||||:|||||||||||||||
H   401 FKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEV  450

BSA 450 SRSLGKVGTRCCTKPESERMPCTEDYLSLILNRLCVLHEKTPVSEKVTKC  499
         ||:||||||::||..||::|||||.||||||:||:||||||||||::||||
HA  451 SRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKC  500

BSA 500 CTESLVNRRPCFSALTPDETYVPKAFDEKLFTFHADICTLPDTEKQIKKQ  549
         |||||||||||||||..|||||||.|:.:.||||||||||.:.|:|||||
HA  501 CTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQ  550

BSA 550 TALVELLKHKPKATEEQLKTVMENFVAFVDKCCAADDKEACFAVEGPKLV  599
         ||||||:|||||||:||||.||::|.|||:|||.|||||.|||.||.|||
HA  551 TALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLV  600

BSA 600 VSTQTALA-  607
         .::|.||.
HA  601 AASQAALGL  609
```

Identity: 465/609 (76.4%)

Similarity: 536/609 (88.0%)

Gaps: 2/609 (0.3%)

4.12 Summary

4.12.1 Amino Acid Sequences

All of the toxins share some degree of similarity (31.1-38.9%); however the similarity between SEB and SEC1 is clearly very high (79.2%). Amongst the SAgs, SEC and SEB are the most homologous and share antibody binding epitopes (Schmidt, 1983; Spero et al., 1978), which lead to antibody cross-reactivity. The SEC1 family showed similarity of 96.2-98.1%, often leaving them indistinguishable. BSA and HA were also found to be very similar (88%).

4.12.2 MS Detection

MS analysis of each toxin detected 40-55% of the amino acids within the derived peptides. Each protein was successfully identified, in both LCMS grade water and healthy urine, with scores well over the MASCOT threshold of 50. The scores for the toxins in spiked urine were considerably lower than those in water but more than 50.

This may have been due to the toxins in urine being half as concentrated; although this should not really affect the score too much as MS is a sensitive method that is detecting the presence of specific peptides. As shown in Section 4.7, there is a number of other protein components in urine and these may hinder the detection of the specific peptides that we are looking for. Overall, the number of resultant m/z peaks identified was disappointing and is likely due to the fact that these samples were not reduced and alkylated. The importance of this step was eventually realised (Chapter 3.9.3). For trypsin work most effectively, the protein needs to be completely denatured (Shah, 2010).

4.12.3 MS Repeatability & Sensitivity

As shown in Table 4.7 the LC-MS method showed good repeatability. The results shown in Table 4.7 are re-runs of Table 4.8. Two of the BSA scores are slightly lower than in the first round of running cycles. This may be due to the fact that they have been in the HPLC autosampler (10^{o}C) for up to 6 hours. This may allow the sample time to degrade or evaporate as the first round of analysis will have punctured a hole in the vial lid.

Similarly, SEC1 repeatability results shown in Table 4.9 agree with the repeatability results for BSA. SEC1 was also used to test the sensitivity of the method. The toxin was only detected down to 500ng/ml, whereas the MS is capable of detecting much lower concentrations of peptides.

CHAPTER 5 SUBJECT POPULATION

5.1 Introduction

Following the initial research carried out in the literature review (Chapter 1), a questionnaire was designed to be completed by each study participant. The information collected would also describe each of the populations that we have sampled. Additionally, some of these data could be compared to current literature that has explored the pathogenesis of RA and factors that could contribute to its development.

The risk of developing RA is increased by an average age (of onset) of 55-64 (Chapter 1.8.1), being female (Chapter 1.8.2) and of Caucasian ethnicity (Chapter 1.8.9). A higher birth order (1^{st} – 3^{rd}) (Chapter 1.8.3), not sharing a bedroom (Chapter 1.8.4) and exposure to animals (Chapter 1.8.5.), particularly birds, cats and sick animals, are also linked with an increased chance of RA. Socioeconomic status (SES) (Chapter 1.8.6), smoking (Chapter 1.8.7) and urbanisation (Chapter 1.8.8) also seem to influence the chance of developing RA. Genetics appear to predispose individuals to RA (Chapter 1.8.11) and seasonal variation of disease severity has also been discussed (Chapter 1.8.10).

A 3 page questionnaire was completed by each participant. The study populations are described in the results below.

5.2 Age & Gender

Table 5.1 Table to show the age and gender of participants. Age was recorded in age brackets of 10 years.

	RA (n=149)	**Fracture** (n=70)
Female (%)	110 (73.8)	52 (74.3)
Age 25-34 years (%)	4 (2.7)	10 (14.3)
Age 35-44 years (%)	5 (3.4)	3 (4.3)
Age 45-54 years (%)	21 (14.1)	20 (28.6)
Age 55-64 years (%)	42 (28.2)	7 (10.0)
Age 65-74 years (%)	49 (32.9)	14 (20.0)
Age 75-84 years (%)	24 (16.1)	12 (17.1)
Age 85-94 years (%)	4 (2.7)	2 (2.9)

The average age of the RA patients fell within the 65-74 years bracket. The fracture population were slightly younger, between 55-64 years old.

5.3 Ethnicity

Of the total RA population sampled (n=149), 146 (98%) described themselves as white British, 1(0.7%) as white Irish, 1(0.7%) as black Caribbean and 1(0.7 %) as Indo-Caribbean.

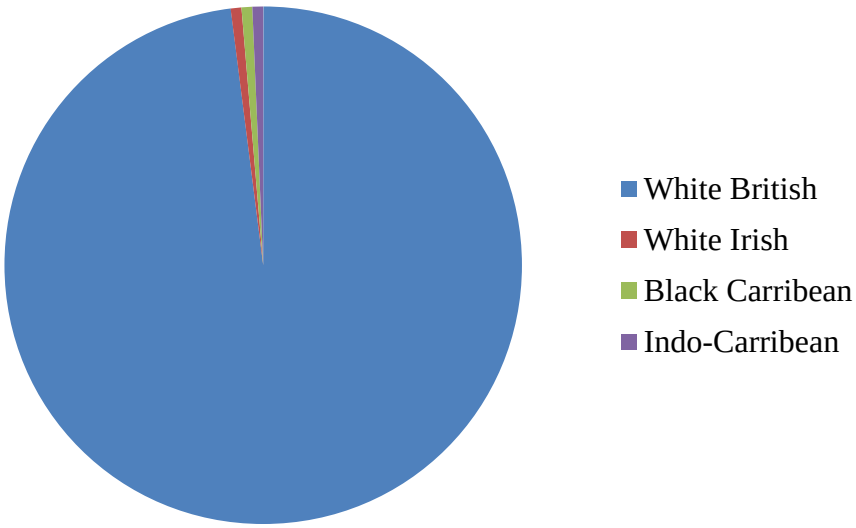


Figure 5.1 Pie chart to show the ethnicity of the RA patient cohort.

The fracture patient cohort (n=70) all identified themselves as white; 68 (97.1%) as white British, 1 (1.4%) as white Irish and 1(1.4%) as white Other.

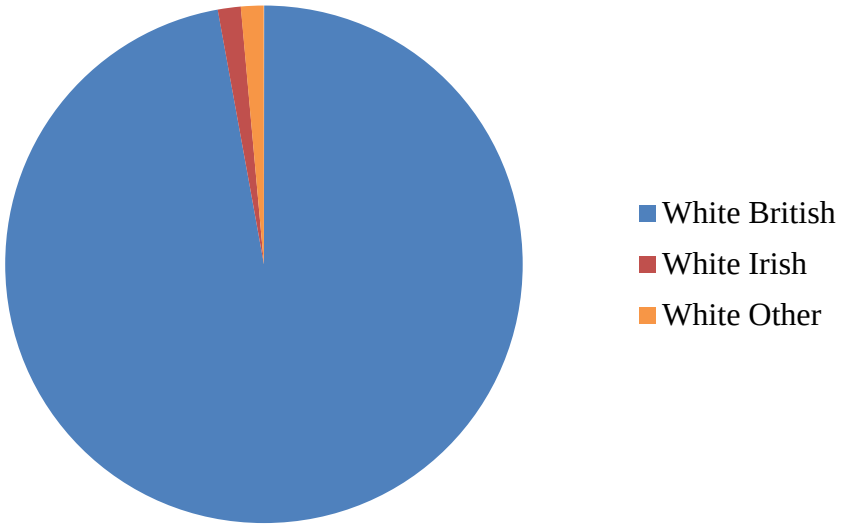


Figure 5.2 **Pie chart to show the ethnicity of the fracture patient cohort.**

5.4 Smoking Status

Of the 149 participants 64 (43.0%) had never smoked and 58 (39.0%) had smoked in the past. Of the participants that still smoke (n=27(18.1%)), 14 (9.4%) smoked less than 10 cigarettes per day, 10 (6.7%) smoked 10-20 per day, 2 (1.3%) smoke 20-30 per day and 1 (0.7%) smoke more than 30 per day.

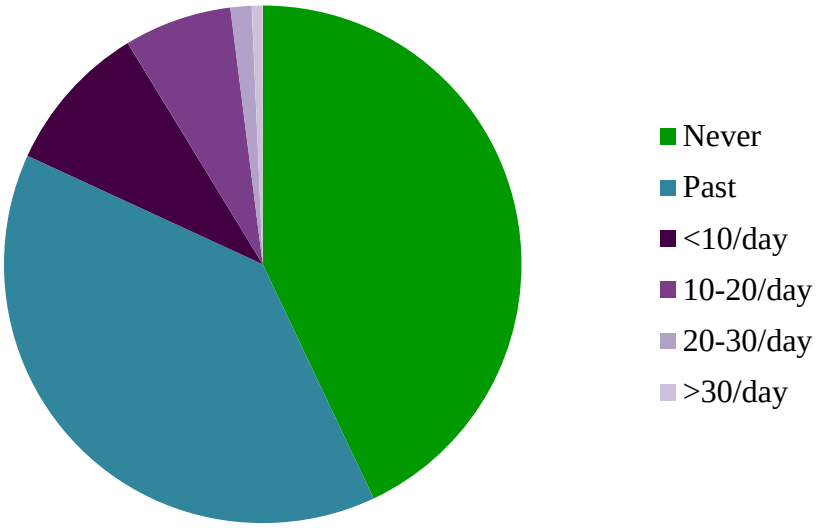


Figure 5.3 Pie chart to show the proportion of the rheumatology cohort who have never smoked, have smoked in the past or currently smoke (number of cigarettes/day).

Of the 70 participants from the fracture cohort, 41 (58.6%) had never smoked and 22 (31.4%) had smoked in the past. Of the participants that still smoke (n=7(10.0%)), 4 (5.7%o of total) smoked less than 10 cigarettes per day and 3 (4.3%) smoked 10-20 per day. No patients said that they currently smoked more than 20 cigarettes per day.

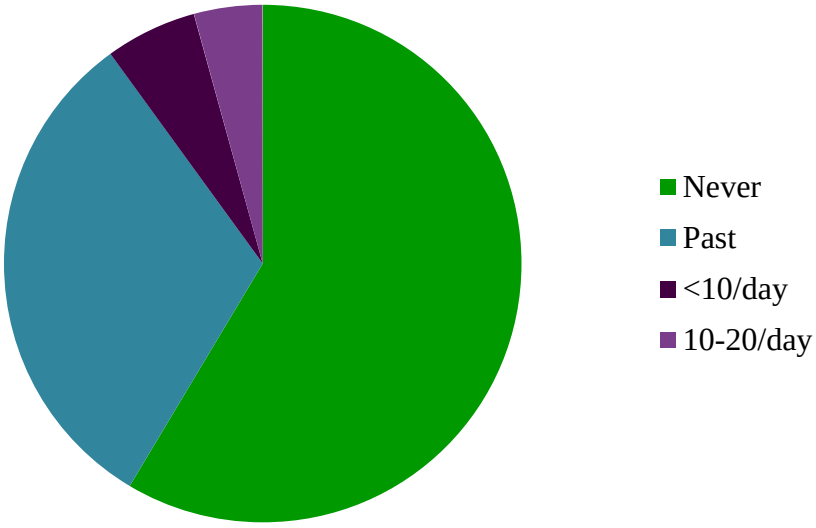


Figure 5.4 Pie chart to show the proportion of the fracture patient cohort who have never smoked, have smoked in the past or currently smoke (number of cigarettes/day).

5.5 Genetics

Of the 149 participants surveyed, 72 (48.3%) people said that there was or had been a case of RA in their blood-related family. 74 (49.7%) people said that there were no cases in their family or, at least, any that they knew of. The remaining 3 (2%) people were adopted, fostered or did not answer the question. Participants were able to identify as many relations as they wanted to/could. The relatives identified are shown below:

Table 5.2 Family members identified as having RA; number, percentage of the total number of people saying 'Yes' to a familial link and the percentage of the total RA cohort.

Family Member	Number	% of 'Yes' (n=72)	% of Total (n=149)
Great Grandmother	1	1.4%	0.7%
Great Aunt	3	4.2%	2.0%
Grandmother	18	25.0%	12.1%
Grandfather	5	6.9%	3.4%
Mother	25	34.7%	16.8%
Father	16	22.2%	10.7%
Aunt	9	12.5%	6.0%
Uncle	1	1.4%	0.7%
Sister	8	11.1%	5.4%
Half Sister	1	1.4%	0.7%
Brother	7	9.7%	4.7%
Cousin	3	4.2%	2.0%
Daughter	4	5.6%	2.7%
Son	1	1.4%	0.7%

Of the participants that answered 'Yes' (n=72), 102 family members were indicated to have/have had RA. 70.6% of those indicated were female and 29.4% male, much like the gender ratio of the sample cohort. 'Mother' made up a third of the answers and 'Grandmother', a quarter. This was followed closely by 'Father'.

Of the participants that answered 'Yes' (n=12) in the fracture patient cohort, 13 family members were indicated to have/have had RA. 92.3% of those indicated were female and 7.7% male. As with the rheumatology cohort, 'Mother' and 'Grandmother' made up the majority of the answers.

Table 5.3 Family members identified as having RA; number, percentage of the total number of people saying 'Yes' to a familial link and the percentage of the total fracture cohort.

Family Member	**Number**	**% of 'Yes'** (n=12)	**% of Total** (n=70)
Grandmother	5	41.7%	7.1%
Grandfather	1	8.3%	1.4%
Mother	5	41.7%	7.1%
Sister	1	8.3%	1.4%
Daughter	1	8.3%	1.4%

5.6 Birthplace

Participants were asked in what region of the UK they were born, the results from the RA patients (Table 4.4) and fracture patients (Table 4.5) are shown below.

Table 5.4 Birthplace (by region) of each RA patient; number and percentage of the total RA population.

Birthplace (UK Region)	**Number** (n=149)	**% of Total** (n=149)
Scotland	7	4.7%
North West	102	68.5%
North East	4	2.7%
Yorkshire	14	9.4%
West Midlands	6	4.0%
East Midlands	2	1.3%
South West	3	2.0%
South East	7	4.7%
Norther Ireland	2	1.3%
Outside of the UK	2	1.3%

Table 5.5 Birthplace (by region) of each fracture patient; number and percentage of the total fracture population.

Birthplace (UK Region)	**Number** (n=70)	**% of Total** (n=70)
Scotland	1	1.4%
North West	49	70.0%
North East	3	4.3%
Yorkshire	6	8.6%
West Midlands	2	2.9%
East Midlands	1	1.4%
South West	1	1.4%
South East	4	5.7%
Northern Ireland	1	1.4%
Outside of the UK	2	2.9%

5.7 Birth Order

Participants were asked, amongst their siblings, where in the birth order were they born. Only children were considered as 1st born. Participants who had had a sibling before them in the birth order, who had died at birth were not to include them in this instance. The results are as follows:

Table 5.6 The birth order of each RA and fracture patient.

Birth Order	Total RA (n=149)	Total RA (%)	Total Fracture (n=70)	Total Fracture (%)
1st Born	63	41.6%	28	40.0%
2nd Born	33	22.1%	22	31.4%
3rd Born	31	20.8%	10	14.3%
4th Born	13	9.4%	5	7.1%
5th Born	8	5.4%	5	7.1%
Twin	1	0.7%	0	0.0%

5.8 Sharing a bedroom

RA	N	Minimum	Maximum	Mean	Std. Deviation
Bedroom Age	83	0	10	2.54	3.005
Bedroom Years	83	3	23	10.89	5.097
Bedroom Total People	83	2	10	2.51	1.162

Table 5.7 The age at which the RA patient started sharing a bedroom, for how many years and how many people shared the room.

Fracture	N	Minimum	Maximum	Mean	Std. Deviation
Bedroom Age	40	0	12	2.03	2.759
Bedroom Years	40	2	18	9.85	4.860
Bedroom Total People	40	1	4	2.20	.516

Table 5.8 The mean age at which fracture patients started to share a bedroom, the mean duration and mean number of total people in the bedroom.

In the RA population 83 of 149 (55.7%) participants shared a bedroom, compared to 40 of 70 (57.1%) of the fracture patient cohort.

5.9 Parent's Occupation during childhood

Participants were asked to state the occupations of their mother and father during their early childhood. Occupations were divided into three divisions: white, blue and pink collar. A white collar worker is "a person engaged in non-manual work, esp. in office work of an administrative, managerial, or clerical nature; an office worker" (Oed.com, 2016). Blue collar workers are defines as "A manual worker, *esp.* one employed in industry" (Oed.com, 1968). Pink collar work is defined as "relating to employment traditionally associated with women (as nursing, hairdressing, secretarial work, etc.), or workers engaged in such employment" (Dictionary.com, 2016). This could also be extended to when a person's labour is related to customer interaction, entertainment, sales or other service orientated work.

Table 5.9 The occupation type of each patient's father; divided into white collar, pink collar or blue collar or absent.

	White Collar	Pink Collar	Blue Collar	Absent
RA	19.5%	3.4%	71.8%	5.4%
Fracture	24.3%	4.3%	64.3%	7.1%

5.10 Area/Farm

Participants were asked to state the type of area they lived during their early childhood and the type of area that they currently live in.

Table 5.10 Respondents' 'Yes' or 'No' answers to the type of area they lived in during their childhood and the type of area in which they currently live; number and percentage of RA and fracture patients.

CHILDHOOD	RA	Fracture	CURRENT	RA	Fracture
Urban	36.9%	25.7%	**Urban**	22.8%	32.9%
Suburban	27.5%	18.6%	**Suburban**	38.2%	22.9%
Rural	35.6%	55.7%	**Rural**	37.5%	44.3%

Table 5.11 Respondents' 'Yes' or 'No' answers to spending time on a farm during their childhood; number and percentage of RA and fracture patients.

FARM	Total RA (n=149)	Total RA (%)	Total Fracture (n=70)	Total Fracture (%)
'Yes'	34	22.8%	24	34.3%
'No'	115	77.2%	46	65.7%

5.11 Pets

The majority of RA participants had pets during their early childhood (n=123; 82.6%). Similarly, the majority of fracture participants had pets during their early childhood (n=60; 85.7%). Many participants lived with a combination of pets.

Table 5.12 Pet ownership during childhood.

	RA Total (n=149)	**RA Total (%)**	**Fracture Total (n=70)**	**Fracture Total (%)**
Dog(s)	86	69.9%	45	64.2%
Cat(s)	65	52.8%	28	40.0%
Rabbit(s)	33	26.8%	15	21.4%
Guinea pig(s)	6	4.9%	6	8.6%
Bird(s)	28	22.8%	15	21.4%
Total	123	82.6%	60	85.7%

5.12 DAS28

The disease activity score (DAS) for each RA patient was recorded.

Table 5.13 The mean and range of the DAS28 scores for the RA patients.

	N	Minimum	Maximum	Mean	Std. Deviation
DAS28	149	1.11	7.73	3.6134	1.32178

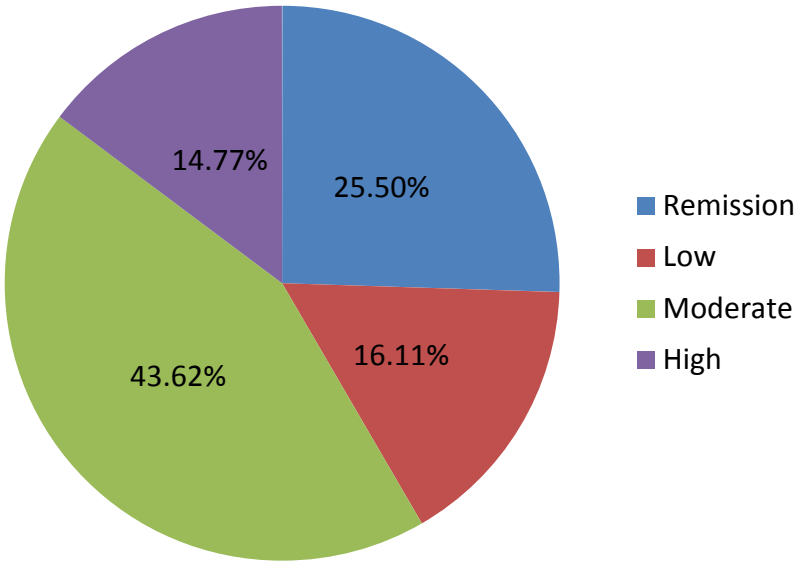


Figure 5.5 Pie chart to show the proportion of RA patients in each disease category based on DAS28 score. Remission (<2.6); Low (2.6-3.2); Moderate (3.2-5.1); High (>5.1).

5.13 Duration of RA

RA patients were asked at what age their symptoms began, at what age they were diagnosed with RA and how many years it had been since diagnosis.

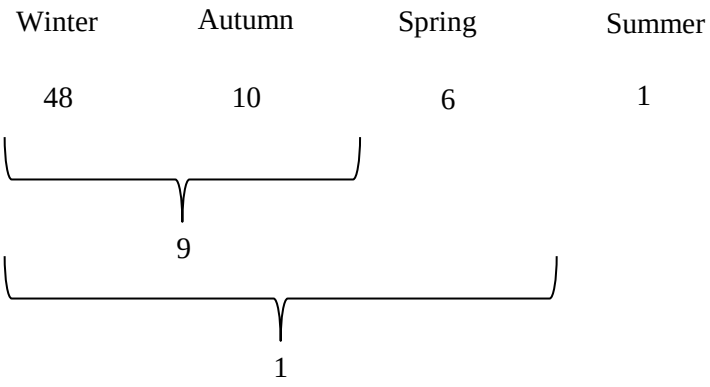
	N	Minimum	Maximum	Mean	Std. Deviation
Symptom Age	149	15	85	49.46	15.032
Diagnosis Age	149	16	85	51.12	14.526
RA Duration (years)	149	0	52	13.09	11.598

Table 5.14 The age at which RA symptoms started, the age of RA diagnosis and the number of years since diagnosis.

5.14 Season

52 (34.9%) of RA participants said that they felt that their RA symptoms were more severe in one or more seasons.

Figure 5.6 The number of RA patients that said that their symptoms appeared to be worse in each season or seasons.



CHAPTER 6 RESULTS: PATIENT SAMPLES

6.1 Western Blot of RA Patient Samples

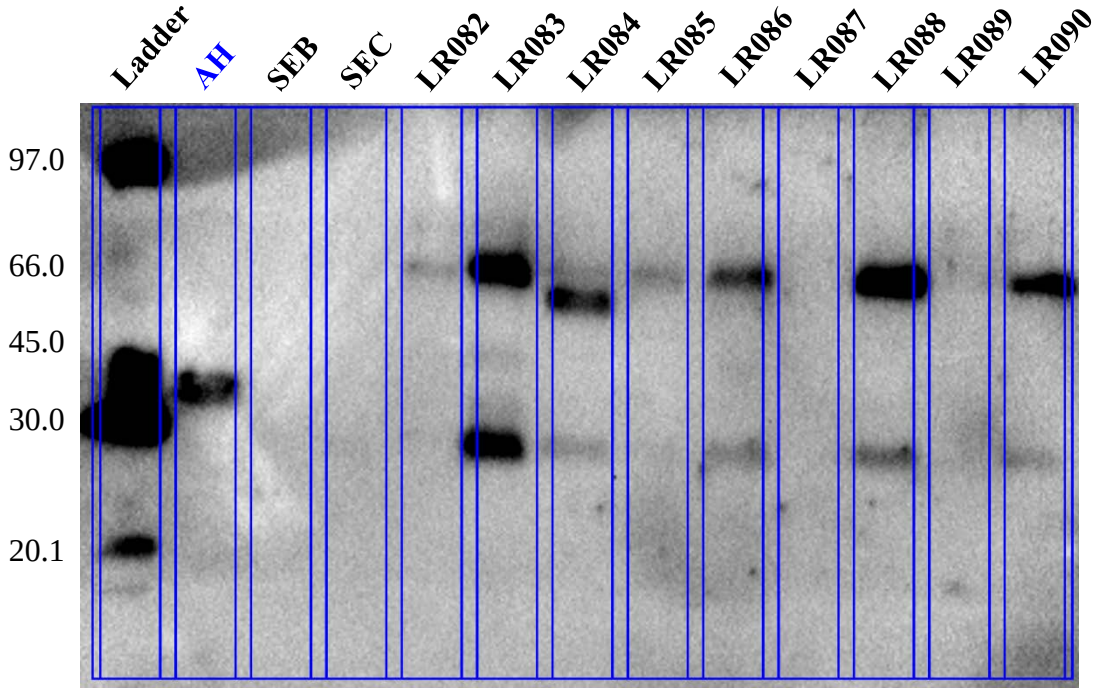


Figure 6.1 Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR082-090 (28µl) (Lane 5-13).

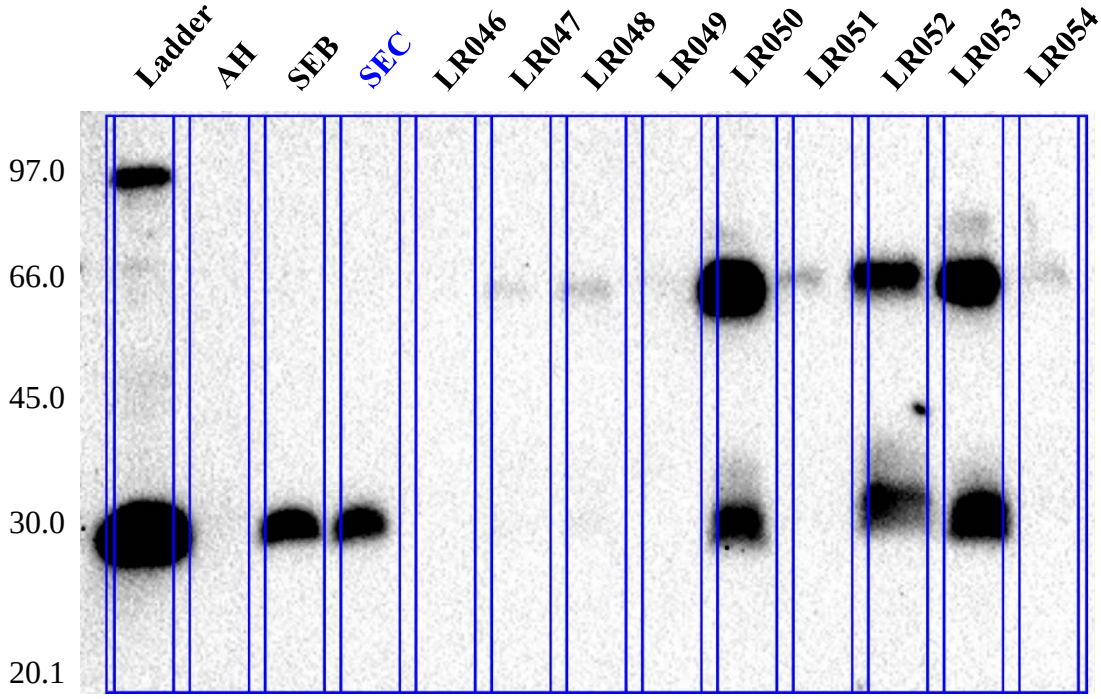


Figure 6.2 Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR0046-054 (28µl) (Lane 5-13).

Figure 6.1 is an example of a western blot membrane of urine from RA patients, analysed using a sheep αAH primary antibody and a rabbit anti-sheep HRP-conjugated secondary antibody. The low molecular weight protein ladder shows bands at 20.1, 30.0, 45.0, 66.0 and 97.0 kDa. There is a band visible in the AH lane, between the 30.0 and 45.0 kDa bands in the ladder. AH is approximately 33 kDa, however the band of control AH was often seen higher (heavier) than this in our blots. There are no bands seen in the lanes of control SEB and SEC as the αAH has little specificity to these toxins. The blot in Figure 6.1 does not seem to have run perfectly even and runs down to the right. There are bands at approximately 30 kDa in lanes LR082, LR083, LR086, LR088 and LR090. Heavier proteins are detected in the lanes containing urine, except LR087 and LR089.

Figure 6.2 is a western blot membrane of urine from RA patients, analysed using a sheep αSEC primary antibody and a rabbit anti-sheep HRP-conjugated secondary antibody. There is a band visible in the both control lanes of SEB and SEC; the toxins are approximately 30 and 28 kDa, respectively. It is no surprise that both toxins were detected by αSEC, as there is cross-reactivity between the toxins and corresponding antibodies due to the structural similarities of SEB and SEC (see Table 4.1). There is no band seen in the lane of control AH as the αSEC has no specificity to AH. There are bands at approximately 30 kDa in lanes LR050, LR052 and LR053. As seen in Figure 6.1, Figure 6.2 shows heavier proteins that have been detected in all of the lanes containing urine.

6.2 Western Blot of Fracture Patient Samples

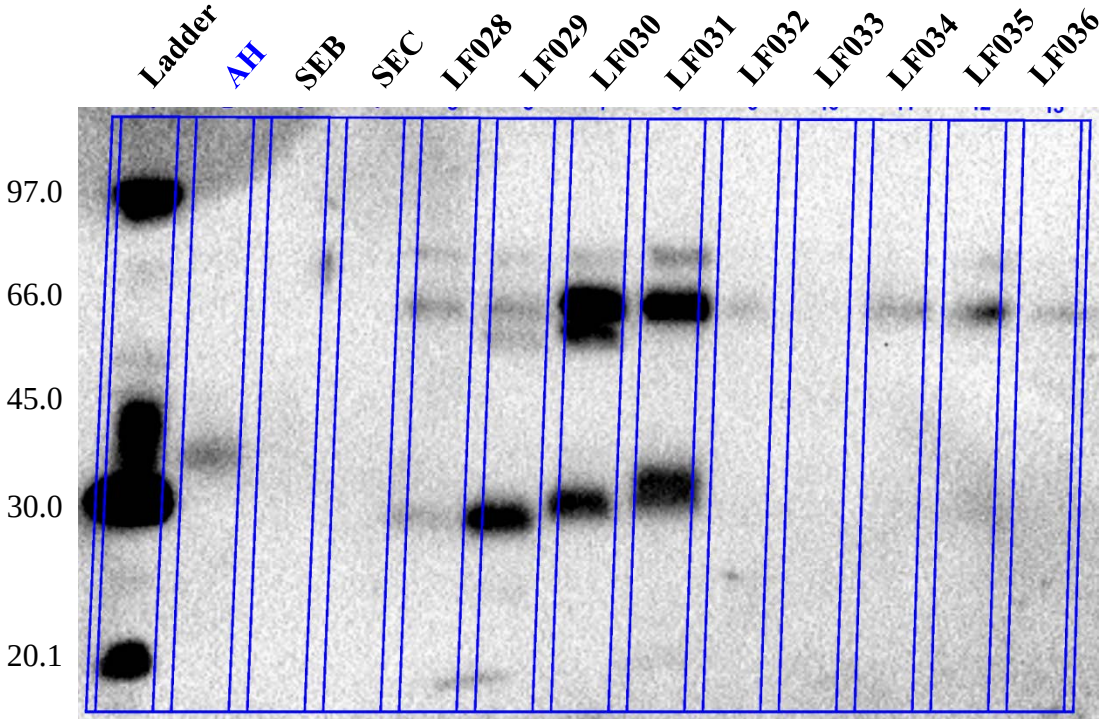


Figure 6.3 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF028-036 (28µl) (Lane 5-13).**

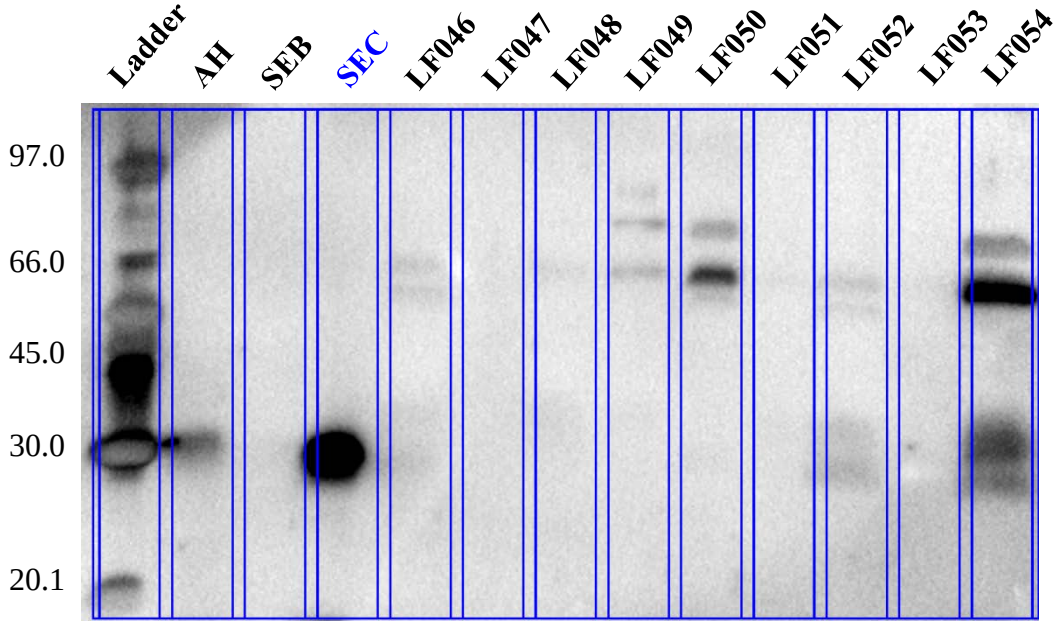


Figure 6.4 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF046-054 (28µl) (Lane 5-13).**

Figure 6.3 is an example of a western blot membrane of urine from fracture patients, analysed using a sheep αAH primary antibody and a rabbit anti-sheep HRP-conjugated secondary antibody. As for the RA urine western blots, the low molecular weight protein ladder shows bands at 20.1, 30.0, 45.0, 66.0 and 97.0 kDa. Again, there is a band visible in the AH lane, between the 30.0 and 45.0 kDa bands in the ladder. AH is approximately 33kDa, however the band of control AH was often seen higher (heavier) than this in our blots. There are no bands seen in the lanes of control SEB and SEC. As in Figure 6.1, the blot in Figure 6.3 runs down to the right. There are bands at approximately 30 kDa in lanes LF028, LF029, LF030 and LF031. Each of the bands does appear to be at a different level but all are within the range of the molecular weight of AH. As in the RA blots, heavier proteins are detected in the lanes containing urine, except in LF033.

Figure 6.4 is a western blot membrane of urine from fracture patients analysed using a sheep αSEC primary antibody and a rabbit anti-sheep HRP-conjugated secondary antibody. There is a strong band visible in the control lane SEC. There are bands at approximately 30 kDa in lanes LF052 and LF054. As in all of the blots shown, heavier proteins have been detected in most of the lanes containing urine.

6.3 Western Blot Summary

6.3.1 RA Patient Samples

Table 6.1 RA patient results showing whether samples LR001-152 were positive or negative for each toxin: AH, SEB, SEC and TSST-1.

Patient Sample	AH	SEB	SEC	TSST-1
LR001	-	-	-	-
LR002	-	-	-	-
LR003	+	-	+	-
LR004	+	+	+	-
LR005	-	-	-	-
LR006	-	-	-	-
LR007	+	-	+	-
LR008	+	+	+	-
LR009	-	-	-	-
LR010	-	-	-	-
LR011	+	-	+	-
LR012	-	-	-	-
LR013	-	+	+	-
LR014	+	-	+	-
LR015	+	+	+	-
LR016	+	+	+	-
LR017	-	-	-	-
LR018	-	-	-	-
LR019	-	-	-	-
LR020	-	-	+	-
LR021	+	-	+	-
LR022	+	-	+	-
LR023	-	-	-	-

LR024	-	-	-	-
LR025	+	-	-	-
LR026	+	-	-	-
LR027	+	-	+	-
LR028	+	-	+	-
LR029	-	-	+	-
LR030	+	+	+	-
LR031	-	-	-	-
LR032	-	-	-	-
LR033	-	-	-	-
LR034	+	-	-	-
LR035	+	-	-	-
LR036	-	-	-	-
LR037	-	-	-	-
LR038	+	+	+	-
LR039	+	-	-	-
LR040	+	+	+	-
LR041	-	-	-	-
LR042	+	+	+	-
LR044	-	-	-	-
LR045	+	-	-	-
LR046	-	-	-	-
LR047	+	-	-	-
LR048	+	-	-	-
LR049	-	-	-	-
LR050	+	+	+	-
LR051	+	-	-	-
LR052	+	+	+	-
LR053	+	+	+	-
LR054	+	-	-	-
LR055	+	-	+	-
LR056	-	-	-	-
LR057	+	-	+	-

LR058	+	-	+	-
LR059	-	-	-	-
LR060	-	-	-	-
LR061	+	+	+	-
LR062	-	+	-	-
LR063	-	-	-	-
LR064	-	-	-	-
LR065	-	-	-	-
LR066	-	-	-	-
LR067	+	+	+	-
LR068	-	-	-	-
LR069	-	-	-	-
LR070	+	+	+	-
LR071	+	-	-	-
LR072	+	-	-	-
LR073	+	+	+	-
LR074	-	-	-	-
LR075	-	-	-	-
LR076	-	-	-	-
LR077	-	-	-	-
LR078	+	-	+	-
LR079	+	+	+	-
LR080	+	+	+	-
LR081	+	+	-	-
LR082	-	-	-	-
LR083	+	-	+	-
LR084	+	-	-	-
LR085	-	-	-	-
LR086	+	+	+	-
LR087	-	-	-	-
LR088	+	-	-	-
LR089	-	-	-	-
LR090	+	-	-	-

LR091	-	+	-	-
LR092	-	-	-	-
LR094	-	-	+	-
LR095	+	+	+	-
LR096	+	-	-	-
LR097	+	+	-	-
LR098	-	-	-	-
LR099	-	-	-	-
LR100	-	-	+	-
LR101	-	-	-	-
LR102	-	-	-	-
LR103	-	-	-	-
LR104	-	-	-	-
LR105	-	-	-	-
LR106	-	-	-	-
LR107	+	+	-	-
LR108	-	-	-	-
LR109	+	-	+	-
LR110	+	-	+	-
LR111	+	-	+	-
LR112	-	-	+	-
LR113	+	-	+	-
LR114	-	-	-	-
LR115	-	+	+	-
LR116	-	+	+	-
LR118	-	-	-	-
LR119	-	-	-	-
LR120	+	+	+	-
LR121	-	-	-	-
LR122	-	-	-	-
LR123	-	-	-	-
LR124	+	+	-	-
LR125	-	-	-	-

LR126	-	-	-	-
LR127	+	+	+	-
LR128	+	-	+	-
LR129	-	+	+	-
LR130	+	-	+	-
LR131	-	-	+	-
LR132	+	-	+	-
LR133	-	-	+	-
LR134	-	+	+	-
LR135	-	-	+	-
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LR137	+	+	+	-
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LR139	-	-	-	-
LR140	-	-	-	-
LR141	+	+	-	-
LR142	+	+	+	-
LR143	+	+	-	-
LR144	-	-	-	-
LR145	+	+	+	-
LR146	-	-	-	-
LR147	+	+	-	-
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LR151	+	+	-	-
LR152	-	-	-	-

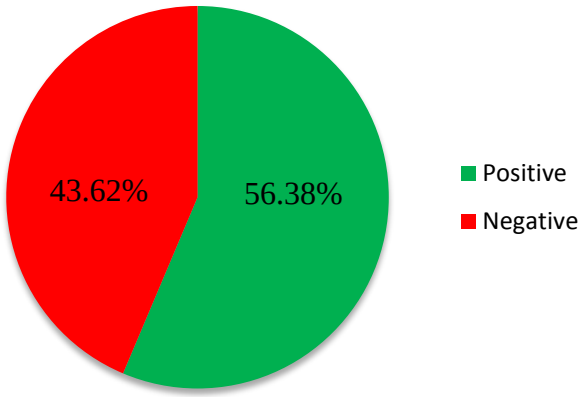


Figure 6.5 Pie chart to show the percentage of RA patients testing positive and negative for one or more of the staphylococcal toxins.

Overall, the presence of at least one staphylococcal toxin was found in 84 of the 149 samples (56.4%).

69 urine samples obtained from patients with RA were positive for AH. This accounts for 82.1% of the total positive samples (n= 84) and 46.3% of the whole patient cohort (n=149).

40 urine samples obtained from patients with RA were positive for SEB. This accounts for 47.6% of the total positive samples (n= 84) and 26.8% of the whole patient cohort (n=149).

57 urine samples obtained from patients with RA were positive for SEC. This accounts for 67.9% of the total positive samples (n= 84) and 38.3% of the whole patient cohort (n=149).

0 of 149 urine samples obtained from patients with RA were positive for TSST-1.

In total, 26 urine samples tested positive for 1 toxin only [AH] [SEB] [SEC]; 31% of the total positive samples (n=84) and 17.4% of the whole patient cohort (n=149).

34 urine samples tested positive for 2 toxins in the following combinations: 9 [AH, SEB], 20 [AH, SEC], 5 [SEB, SEC]; 40.5% of the total positive samples (n=84) and 22.8% of the whole patient cohort (n=149).

24 urine samples tested positive for 3 toxins [AH, SEB, SEC]; 28.6% of the total positive samples (n=84) and 16.1% of the whole patient cohort (n=149).

6.3.2 Fracture Patient Samples

Table 6.2 Fracture patient results showing whether samples LF001-070 were positive or negative for each toxin: AH, SEB, SEC and TSST-1.

Patient Sample	AH	SEB	SEC	TSST
LF001	-	-	-	-
LF002	-	-	-	-
LF003	-	-	-	-
LF004	-	-	-	-
LF005	-	-	-	-
LF006	-	-	-	-
LF007	-	-	-	-
LF008	-	+	+	-
LF009	-	+	-	-
LF010	-	-	+	-
LF011	-	-	+	-
LF012	-	-	-	-
LF013	-	-	-	-
LF014	-	-	-	-
LF015	-	-	-	-
LF016	-	-	-	-
LF017	-	-	-	-

LF018	-	-	-	-
LF019	-	-	-	-
LF020	-	+	+	-
LF021	-	-	-	-
LF022	-	+	+	-
LF023	-	+	+	-
LF024	-	-	-	-
LF025	-	-	-	-
LF026	-	-	-	-
LF027	-	-	-	-
LF028	-	-	-	-
LF029	-	+	+	-
LF030	-	+	+	-
LF031	+	+	+	-
LF032	-	-	-	-
LF033	-	-	+	-
LF034	-	-	-	-
LF035	-	-	+	-
LF036	-	-	-	-
LF037	-	-	-	-
LF038	+	+	+	-
LF039	-	-	-	-
LF040	-	-	-	-
LF041	-	-	-	-
LF042	-	-	-	-
LF043	-	-	-	-
LF044	-	-	-	-
LF045	-	-	-	-
LF046	-	-	-	-
LF047	-	-	-	-
LF048	-	-	-	-
LF049	-	-	-	-
LF050	-	-	-	-

LF051	-	-	-	-
LF052	-	+	+	-
LF053	-	-	-	-
LF054	+	+	+	-
LF055	-	+	-	-
LF056	-	+	-	-
LF057	-	-	-	-
LF058	-	-	-	-
LF059	-	-	-	-
LF060	-	-	-	-
LF061	-	-	-	-
LF062	-	-	-	-
LF063	-	-	-	-
LF064	-	+	-	-
LF065	-	-	-	-
LF066	+	-	-	-
LF067	-	-	-	-
LF068	-	-	-	-
LF069	-	-	-	-
LF070	-	-	-	-

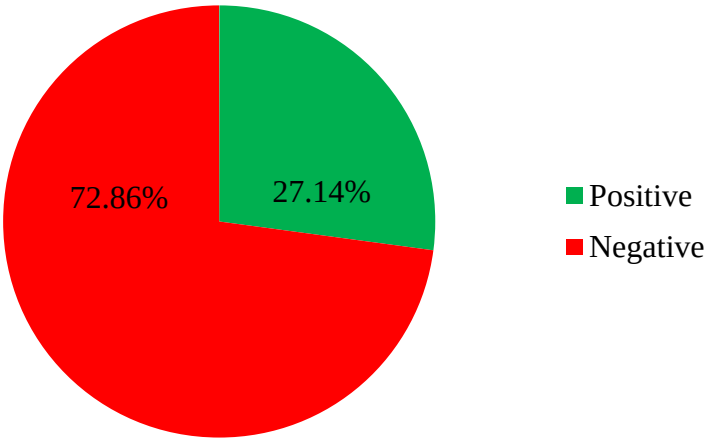


Figure 6.6 Pie chart to show the percentage of fracture patients testing positive and negative for one or more of the staphylococcal toxins.

Overall, the presence of at least one staphylococcal toxin was found in 19 of the 70 samples (27.1%).

4 urine samples obtained from fracture patients were positive for AH. This accounts for 21.1% of the total positive samples (n= 19) and 5.7% of the whole patient cohort (n=70).

14 urine samples obtained from fracture patients were positive for SEB. This accounts for 73.7% of the total positive samples (n= 19) and 20.0% of the whole patient cohort (n=70).

14 urine samples obtained from fracture patients were positive for SEC. This accounts for 73.7% of the total positive samples (n= 19) and 20.0% of the whole patient cohort (n=70).

0 of 70 urine samples obtained from fracture patients were positive for TSST-1.

In total, 9 urine samples tested positive for 1 toxin only [AH] [SEB] [SEC]; 42.9% of the total positive samples (n=19) and 12.9% of the whole patient cohort (n=70).

7 urine samples tested positive for 2 toxins appearing in the following combination: [SEB, SEC]; 36.8% of the total positive samples (n=19) and 10% of the whole patient cohort (n=70).

3 urine samples tested positive for 3 toxins [AH, SEB, SEC]; 15.8% of the total positive samples (n=19) and 4.3% of the whole patient cohort (n=70).

6.4 Questionnaire Data

6.4.1 Age

Table 6.3 The number of RA patients in each age category; of the positive population and total population.

RA Age	**Number Positive** (n=84)	**Total Number** (n=149)	**% of Positive** (n=84)	**% of Total** (n=149)
25-34	4	4	4.8%	2.7%
35-44	2	5	2.4%	3.4%
45-54	8	21	9.5%	14.1%
55-64	21	42	25.0%	28.2%
65-74	34	49	40.5%	32.9%
75-84	14	24	16.7%	16.1%
85-94	1	4	1.2%	2.7%

Table 6.4 The number of fracture patients in each age category; of the positive population and total population.

Fracture Age (years)	**Number Positive** (n=19)	**Total Number** (n=70)	**% of Positive** (n=19)	**% of Total** (n=70)
25-34	3	10	14.3%	14.3%
35-44	0	3	0.0%	4.3%
45-54	4	20	19.1%	28.6%
55-64	2	7	9.5%	10.0%
65-74	2	14	9.5%	20.0%
75-84	7	12	33.3%	17.1%
85-94	1	2	4.8%	2.9%

6.4.2 Birth Order

Participants were asked, amongst their siblings, where in the birth order did they come. Only children were considered as 1st born. Participants who had had a sibling before them in the birth order, who had died at birth were not to include them in this instance. The results are as follows:

Table 6.5 Where each RA patient appeared in the birth order amongst their siblings; number and percentage of positive RA samples and number and percentage of the total RA population.

Birth Order	Number Positive (n=84)	Total Number (n=149)	% of Positive (n=84)	% of Total (n=149)
1st Born	36	63	42.9%	41.6%
2nd Born	21	33	25.0%	22.1%
3rd Born	15	31	17.9%	20.8%
4th Born	7	13	8.3%	9.4%
5th + Born	4	8	4.8%	5.4%
Twin	1	1	1.2%	0.7%

Table 6.6 Where each fracture patient appeared in the birth order amongst their siblings; number and percentage of positive fracture samples and number and percentage of the total fracture population.

Birth Order	Number Positive (n=19)	Total Number (n=70)	% of Positive (n=19)	% of Total (n=70)
1st Born	9	28	42.9%	40.0%
2nd Born	9	22	38.1%	31.4%
3rd Born	1	10	0.0%	14.3%
4th Born	1	5	5.8%	7.1%
5th Born	1	5	5.8%	7.1%

6.4.3 Sharing a bedroom

The comparison of 'Yes' and 'No' answers versus positivity/negativity for *S.aureus* are shown below:

Table 6.7 RA patients that responded 'Yes' or 'No' to sharing a bedroom as a child compared with their *S.aureus* positivity/negativity.

RA	Positive	Negative
'Yes'	29.5%	26.2%
'No'	26.8%	17.4%

Table 6.8 Fracture patients that responded 'Yes' or 'No' to sharing a bedroom as a child compared with their *S.aureus* positivity/negativity.

FRACTURE	Positive	Negative
'Yes'	14.3%	14.3%
'No'	45.7%	27.7%

In the RA population 83 of 149 (55.7%) participants shared a bedroom, compared to 41 of 70 (58.6%) of the fracture patient cohort. After looking in more detail, the percentage of RA patients positive for *S.aureus* is similar if they shared a bedroom or not. However there is a notable difference in the fracture patient cohort where 45.7% of the respondents had said that they did not share a bedroom but were positive for *S.aureus*. This is compared to just 15.7% of whom were positive but did share a bedroom during their childhood.

6.4.4 Parent's Occupation During Childhood

Table 6.9 The occupation type of each RA patient's father; divided into white collar, pink collar or blue collar or absent and compared to *S.aureus* positivity.

RA	White Collar	Pink Collar	Blue Collar	Absent
Positive	8.1%	0.7%	43.6%	4.0%
Negative	11.4%	2.7%	28.2%	1.3%
Total	19.5%	3.4%	71.8%	5.4%

Table 6.10 The occupation type of each fracture patient's father; divided into white collar, pink collar or blue collar or absent and compared to *S.aureus* positivity.

Fracture	White Collar	Pink Collar	Blue Collar	Absent
Positive	5.1%	0.0%	18.6%	2.9%
Negative	18.6%	4.3%	45.7%	4.3%
Total	24.3%	4.3%	64.3%	7.1%

6.4.5 Area/Farm

Participants were asked to state the type of area they lived during their early childhood and the type of area that they currently live in.

Table 6.11 The area of residence during childhood and currently, compared to S.aureus positivity/negativity – of the RA cohort.

CHILDHOOD	Positive	Negative	CURRENT	Positive	Negative
Urban	8.6%	17.1%	**Urban**	10.0%	22.9%
Suburban	4.3%	14.3%	**Suburban**	4.3%	18.6%
Rural	14.3%	41.4%	**Rural**	12.9%	31.4%

Of the rheumatology patient cohort, 34 of the 149 participants lived on or spent a lot of time on a farm during their early childhood. Of these, 61.8% were positive for *S.aureus*. Of the patients that responded 'No', 54.8% tested positive.

Table 6.12 RA respondents' 'Yes' or 'No' answers to spending time on a farm during their childhood, compared to *S.aureus* positivity; number and percentage of total positive samples and total RA population.

FARM	Positive (n=84)	Total number (n=149)	% of positive (n=84)	% of total (n=149)
'Yes'	21	34	25%	22.8%
'No'	63	115	75%	77.2%

Table 6.13 The area of residence during childhood and currently, compared to S.aureus positivity/negativity – of the fracture cohort.

CHILDHOOD	Positive	Negative	CURRENT	Positive	Negative
Urban	20.1%	16.8%	**Urban**	16.1%	6.7%
Suburban	16.8%	10.7%	**Suburban**	22.8%	15.4%
Rural	19.5%	16.1%	**Rural**	17.4%	20.1%

One third (34.4%) of the fracture patients said that they had lived on or spent a lot of time on a farm during their early childhood. Of the 24 patients that answered 'Yes', 29.2% were positive for *S.aureus*. Of the patients that responded 'No', 30.4% tested positive.

Table 6.14 Fracture respondents' 'Yes' or 'No' answers to spending time on a farm during their childhood, compared to *S.aureus* positivity; number and percentage of total positive samples and total fracture population.

FARM	Positive (n=19)	Total number (n=70)	% of positive (n=19)	% of total (n=70)
'Yes'	6	24	28.6%	34.3%
'No'	13	46	61.9%	65.7%

Table 6.15 Summary of RA and fracture patients, positive for *S.aureus*, compared with time spent on a farm during childhood.

POSITIVE	Rheumatoid Arthritis (n=84)	Fracture (n=19)
'Yes'	25.0% (n=21)	28.6% (n=6)
'No'	75.0% (n=63)	61.9% (n=13)

6.4.6 Pets

Table 6.16 The number and percentage of positive RA samples divided by pet ownership during childhood.

RA	**N positive of pet total**	**% positive of pet total**	**% of positive total** (n=84)
Dog(s) (n=87)	52	59.8%	61.9%
Cat(s) (n=65)	34	52.3%	40.5%
Rabbit(s) (n=33)	16	48.5%	19.0%
Guinea pig(s) (n=6)	3	50.0%	3.6%
Bird(s) (n=28)	18	64.3%	21.4%

Table 6.17 The number and percentage of positive fracture samples divided by pet ownership during childhood.

FRACTURE	**N positive of each pet total**	**% positive of each pet total**	**% of positive total** (n=19)
Dog(s) (n=45)	14	31.1%	73.7%
Cat(s) (n=28)	9	32.1%	47.4%
Rabbit(s) (n=15)	4	26.7%	21.1%
Guinea pig(s) (n=6)	1	16.7%	5.3%
Bird(s) (n=15)	4	26.7%	21.1%

6.4.7 DAS28

Table 6.18 The proportion of RA patients testing positive or negative for *S.aureus*, divided by disease category. Remission (<2.6); Low (2.6-3.2); Moderate (3.2-5.1); High (>5.1). OR Table 6.18 The proportion of RA patients testing positive or negative for *S.aureus*, divided by disease category. Remission (<2.6); Low (2.6-3.2); Moderate (3.2-5.1); High (>5.1).

	Positive	**Negative**
Remission (DAS<2.6)	14.1%	11.4%
Low (DAS 2.6-3.2)	10.7%	5.4%
Moderate (DAS 3.2-5.1)	23.5%	20.1%
High (DAS>5.1)	8.1%	6.7%

As shown in Figure 6.18, the distribution of DAS28 category, within the positive and negative results are very similar. DAS28 does not seem to correlate with any increased likelihood of testing positive for *S.aureus*.

6.5 Statistical Analysis

Table 6.19 Descriptive characteristics of the population(s).

All characteristics	**All (n=219)**	**RA (n=149)**	**Fracture (n=70)**	**Difference P value***
Female (%)	162 (74.0)	110 (73.8)	52 (74.3)	-
Mean Age (SD)	62.1 (14.3)	63.9 (12.4)	58.1 (17.1)	<0.0045
Positive AH (%)	73 (33.3%)	69 (46.3%)	4 (5.7%)	**<0.001**
Positive SEB (%)	54 (24.7%)	40 (26.9%)	14 (20%)	0.27
Positive SEC (%)	71 (32.4%)	57 (38.23%)	14 (20%)	**0.007**
Positive TSST (%)	0 (0)	0 (0)	0 (0)	-
Any Toxin (%)	103 (47.0%)	84 (56.4%)	19 (27.1%)	-
Mean DAS (SD)	-	3.6 (1.3)	-	-

*p value obtained by chi squared test.

No date of birth was obtained from the participants. Instead, participants indicated their age based on 10 year age brackets. It was assumed that there was an even spread of age within each bracket. The ages halfway through each age bracket were used in a t test to calculate the mean age.

Table 6.20 Odds ratio of *S.aureus* positivity in RA.

Odds of positivity	OR	95% CI		P value*
Unadjusted	3.47	1.87	6.44	**0.000**
Adjusted for age	3.25	1.74	6.09	**0.000**
Adjusted for age and gender	3.38	1.78	6.43	**0.000**
Adjusted for age, gender and smoking	3.56	1.87	6.76	**0.000**

*p value obtained by chi squared test.

In patients with RA, is disease activity associated with toxin positivity? Initially a t-test was carried out to calculate the mean DAS score of the positive and negative RA samples:

Mean DAS (positive) = 3.6 (1.36)

Mean DAS (negative) = 3.61 (1.30)

Furthermore, a logistic model was used to see if the DAS score can explain positivity. This result was not significant (OR 1.00; CI 0.78, 1.28). We can conclude that DAS has no bearing on the likelihood of a patient tasting positive for a staphylococcal enterotoxin.

Table 6. 21 Number of positive samples divided by disease activity status.

DAS score	Total n	Positive n (%)
<2.6	43	24 (55.8)
2.6 – 5.19	87	50 (57.5)
>5.2	19	10 (52.6)

Table 6.22 Odds ratio of *S.aureus* positivity by toxin.

Positive Toxin		OR	95% CI		P value*
AH	Unadjusted	14.23	4.93	41.05	**0.000**
	Adjusted for age	13.65	4.71	39.60	**0.000**
	Adjusted for age and gender	15.33	5.15	45.68	**0.000**
	Adjusted for age, gender and smoking	16.63	5.52	50.10	**0.000**
SEB	Unadjusted	1.47	0.74	2.92	0.275
	Adjusted for age	1.34	0.66	2.71	0.414
	Adjusted for age and gender	1.34	0.65	2.76	0.423
	Adjusted for age, gender and smoking	1.42	0.69	2.94	0.346
SEC	Unadjusted	2.48	1.27	4.85	**0.008**
	Adjusted for age	2.41	1.22	4.78	**0.012**
	Adjusted for age and gender	2.48	1.23	4.95	**0.011**
	Adjusted for age, gender and smoking	2.62	1.29	5.31	**0.008**

*p value obtained by chi squared test.

6.6 Questionnaire Results Discussion

6.6.1 Age and Gender

The mean age group of both populations was similar; the average age was 65-74 years in the RA cohort and 55-64 years in the fracture cohort.

Each patient population is made up of a similar ratio of females: males; 3:1. It has been consistently reported that women are three times more likely than men to have RA (73.1% female to 26.9% male) (Doran, 2002; Shipley, 2009). Our results, in Table 6.19, support this with the gender split being 73.8% female to 26.2% male in the RA population. However, the gender distribution is also very similar in the fracture cohort (74.3% female to 25.7%). According to the most recent census (ONS, 2011), Lancaster's population was 52% female, therefore the high number of subject females cannot simply be explained by the proportion in the general population. The gender distribution of both populations sampled may be explained in a couple of ways. There may just have been more female patients within each hospital department; this could have been at random or because women are more likely to have RA or sustain fractures. As the average age of the fracture population was 55-64 years old, it can be assumed that most of the women were post-menopausal and more prone to suffering from osteoporosis (Cawthon, 2011). Men also suffer from osteoporosis and low bone mass in old age but at a much lower rate; women are more susceptible at a rate of 3:1 (NOF, 2002).The second option is that female adherence to the study was better in each population; women may be more inclined to take part.

6.6.2 Ethnicity

According to the 2011 census, Lancaster had a population of 138,375, of which 95.6% identified as white. The high percentage of white people in both sample populations- 98.7% in RA and 100% in fracture – is similar to the demographic of the general population in Lancaster.

6.6.3 Smoking

Overall, the rheumatology cohort consisted of fewer people who had never smoked, more people who had smoked in the past, and of the individuals who still smoked, they smoked more cigarettes per day. This may agree with previous findings, that smoking plays a role in the development of RA (Sugiyama, 2010). Smoking is linked to infection, which may explain why *S.aureus* was detected in more of the patients with RA (Stampfli, 2009).

6.6.4 Genetics

GWAS have identified a number of genes that contribute to a genetic predisposition to RA and inheritability of RA is estimated at 60% (MacGregor, 2000; Perricone, 2011). Of the RA respondents, 72 (48.3%) individuals indicated that 102 of their blood-relatives have/had RA, whereas only 12 (17.1%) of the fracture patients said that 13 of their relatives have/had RA. Coupled with the literature, these data infer that genetics may play a role in the development of RA.

6.6.5 Birthplace

The information gathered on the region that each patient was born in shows us that, in both populations, people were born all over the UK, however the vast majority (68-70%) were born in, and have remained in the North West.

6.6.6 Birth order

It has been hypothesised that being 1st-3rd born in a family increases the chances of developing RA. This is based on the hygiene hypothesis and the assumption that having more older siblings means that an individual will be introduced to pathogens earlier in life (Strachan, 1989). In both populations, the majority (40-42%) of participants were 1st born, followed by 2nd born, 3rd born, 4th born and 5th+ born. Of these, similar percentages were positive for *S.aureus* within each category. Sayeeduddin *et al.* reported that of their rheumatology cohort (n=115), 65-70% were 1st-3rd born children, with 32.2% 1st born. In comparison, our rheumatology cohort is 85.2% 1st-3rd born, with 41.6% (Sayeeduddin, 1994). On the face of it, there seems to be an association with an early birth order and the chance of developing RA. However, if we also compare these data to our fracture cohort, the results are very similar, with 85.7% 1st-3rd born; 40.0% 1st born. According to these data, birth order does not seem to show any difference in the *S.aureus* positivity between the two populations.

6.6.7 Sharing a bedroom

In the literature, it has been described that women who shared a bedroom as a child, were less likely to be RF-positive (Edwards, 2006). It would be expected that those who shared a bedroom during their childhood would have more opportunity to come into contact with pathogens. In both the RA and fracture populations, if the

participants answered “Yes” to sharing a bedroom, roughly similar percentages of the total of each population were positive or negative for *S.aureus* toxins. However, of all the participants that said “No”, the vast majority, tested positive for *S.aureus*. The presence of the bacterium may be because they encountered *S.aureus* later on in life, when they weren’t able to mount the correct immune response to clear it. If they had shared a bedroom, they would have been more likely to have been ‘infected’ at a younger age (Strachan, 1989).

6.6.8 Fathers’ occupation

Fathers’ occupation has been used as a measure of SES (Edwards, 2006). It has been said that children of fathers with a white collar job, who were considered to have a higher SES, were more likely to be RF positive (in women only) (Edwards, 2006). The majority of the fathers’ occupations were classed as “blue collar”. This also reflects the average age of the sample populations, which are 55-74 collectively. These patients would have been born between the 1930s and 1960s; decades where there were a lot of manual jobs. There seems to be little difference between the RA and fracture patient populations with regards to the influence of father’s occupation on producing a urine sample positive for *S.aureus*. The use of the father’s occupation as a marker for SES is controversial. The status of a father’s job does not always determine the standard of the home environment and living standards, particularly in more modern times.

6.6.9 Area

Studies have shown that people living in urban areas are more likely to develop an RA than individuals in rural areas (Soloman, 1975; Chou, 1994; Carmona, 2002). The questionnaire data collected, seems to show that the type of area lived in as a child or currently as an adult, does not influence whether an individual will be positive for *S.aureus* or develop RA.

6.6.10 Farm

In summary, the percentage of fracture patients who answered “Yes” or “No” and who were positive for *S.aureus*, were very similar. However, the percentage of rheumatology patients testing positive for *S.aureus*, regardless of their answer, was nearly double. Also, within this cohort, patients answering “Yes” were 7% more likely to have *S.aureus* in their urine.

6.6.11 Pets

On comparison of each population, there is little difference between the proportions of the animals within each household during the participants’ early childhoods. There is no particular pet that stands out as contributing to an increased chance in *S.aureus* positivity or to the development of RA.

6.6.12 RA population

The DAS28 value did not, in this case, show any correlation to the presence of *S.aureus* in the urine of the RA patients.

On average, patients start to develop symptoms at 49 years of age, gaining a formal diagnosis of RA at the age of 51. Diagnosis within this population occurred at a slightly younger age than on average (Symmons, 2002). In the sample population, patients had had their RA diagnosis for 0-52 years, 13 years on average.

The vast majority of the RA respondents named winter as the/a season where they felt that their RA was worse. This could be due to the low temperatures endured during a UK winter, which make even a healthy individual's joint more stiff and immobile. Additionally, some patients felt that their RA symptoms were worse when there was a change in the barometric pressure.

6.7 Discussion

6.7.1 Toxins

The odds ratio for each individual toxin, unadjusted, adjusted for age, adjusted for age and gender and adjusted for age, gender and smoking status, are shown in Table 6.22. TSST-1 is not shown in the table as the western blot analysis didn't show any protein bands at the level of TSST-1 (24kDa). The odds ratio for the presence of both AH and SEC were significant (p value <0.05), and there was little difference once results were adjusted for age, gender and smoking status.

AH is a 33kDa pore forming toxin that is secreted by the majority of *S. aureus* strains and is active against a wide range of mammalian cells (Bhakdi, 1991; Gray, 1984). AH also induces the release of cytokines and chemokines such as IL-6, IL-1β, IL-1α, IL-8, TNF-α, KC and MIP-2 (Bhakdi, 1989; Dragneva, 2001; Hruz, 2009); many of which are strongly implicated in RA (Brenner, 2015). Along with pro-inflammatory cytokines such as TNF-α and IL-6, large amounts of IL-1β released from macrophages and monocytes in response to AH (in cultured cells) (Bhakdi, 1989). Such mediators are shown to cause joint damage; directly by activating osteoclasts or indirectly by stimulating synovial macrophages to produce further inflammatory cytokines. Increased production of IL-1β has even been linked to the development of a number of autoinflammatory syndromes (Masters, 2009). TNF-α and IL-1 are considered to work synergistically to induce the body into a shock-like state (Ikejima, 1988). However, human leukocytes, although originally considered to have high resistance to endotoxins, have shown to be particularly vulnerable to the effects of AH (Bhakdi,

1989). It is also understood that AH promotes the adherence of neutrophils to endothelial cells (Fast, 1988), an important step in the early inflammatory reaction.

AH is secreted as a monomeric protein and has a pore-forming beta-barrel structure; which binds to specific, but unidentified cell surface receptors to create heptameric pores that lead to DNA fragmentation and subsequent apoptosis (Tanaka, 2011). AH is particularly defined by its ability to kill healthy cells by apoptosis. Once the pore inserts itself in the membrane, rapid loss of vital molecules occurs; there is dissipation of the membrane potential and ionic gradients and osmosis-induced cell wall rupture (Tweten, 1983).

AH is encoded by the *hla* gene and is an important virulence factor for *S. aureus*, which can lead to ailments such as sepsis and septic arthritis (Nilsson, 1999). For example, Nilsson *et al.* inoculated mice with AH and describe inflammation, joint damage and weight loss as a consequence (Nilsson, 1999). Furthermore, Fast *et al.* have suggested that strains of staphylococci that produce exotoxin, such as TSST-1, are able to inhibit the migration of polymorphonuclear neutrophils (PMN) to sites of infection by the production of TNF (Fast, 1988).

AH induces colloid osmotic lysis in erythrocytes (Bhakdi, 1984) and necrosis or apoptosis in nucleated cells (Essmann, 2003; Jonas, 1994). Pore formation does not always lead to cell death and studies have shown that cells can survive and recover from a limited number of small pore formations (Thelestam, 1983; Walev, 1994; Valeva, 2000). *S.aureus* AH pores are relatively small and are calcium impermeable and can therefore not be repaired by the wounded membrane response (Walev, 1994). Conversely, streptolysin O inflicts mechanical lesions and large pores that rely on the rapid calcium-dependent replacement of ions in the attacked cell (McNeil, 2005;

Idone, 2008). The latter repair mechanism is faster than that for the AH pores and both differ at a molecular level, particularly in the role played by p38 (Husmann, 2006).

Compared to AH, there is much less in the literature about SEC and its possible link to autoimmune development. Our results have shown a significant presence of SEC in the urine of patients with RA or closed fractures; although the OR for SEC is considerably lower than AH. The pathological roles of SEC in relation to inflammation have been discussed by Kuroishi *et al.* in relation to bovine staphylococcal mastitis (Kuroishi, 2003). Strains isolated from cases of bovine staphylococcal mastitis were shown to produce SEs, the main one of which was SEC. Results concluded that SEC stimulates mononuclear cells to activate PMNs that migrate to the mammary glands, causing inflammation. Previous cases have shown staphylococci to cause subclinical and/or chronic mastitis because of persistent infection in the mammary gland (Sutra, 1994). It has been proposed that the chronic nature of bovine staphylococcal mastitis suggests that *S.aureus* or (part of) its products, in particular the SAgs, could interfere with the proper development of a protective immune system (Ferens, 1998). This appears to show that staphylococcal enterotoxins are able cause subclinical inflammation and remain in the body, exerting their effects, for a long period of time.

6.7.2 Inflammation

The literature suggests that up to 20% of the general population are permanent, asymptomatic carriers of *S.aureus*, 60% are intermittent carriers and 20% never carry *S.aureus*; based on results from nasal and nasopharyngeal swabs (Kluytmans, 1997;

Ansari, 2016). A number of studies were compared and a mean of 30% of the general population were positive for the presence of *S.aureus* (Fall, 2014; Treesirichod, 2014; Lucia Preoţescu, 2013; Kuehnert, 2006; Weidenmaier, 2012; Graham, 2006, den Heijer, 2013; Ateba Ngoa, 2012).

The presence of the bacterium in the nasal passage is not unexpected as *S.aureus* is a commensal bacterium of the normal flora found on the moist squamous epithelium of the anterior nares (Ansari, 2016; Williams, 1963). The nasal flora of the upper respiratory tract is made up of nearly 200 species of bacteria and is established within 48 hours of birth. The normal flora is influenced by an individual's age, sex, race, genetics, diet, nutrition and stress levels. Staphylococci are generally non-pathogenic and produce fatty acids to prevent the growth of fungi and yeast on the skin (Todar, 2012). The lower respiratory tract does not have commensal bacteria however, as it is virtually pathogen free. This is due to the action of the ciliated epithelium that lines the tract. Any microbes that manage to reach the lower respiratory tract are swept upwards by the action of the mucociliary blanket that lines the bronchi (Todar, 2012). Consequently, in a healthy individual, the lungs are pathogen-free. Defective host immunity and the ability of staphylococci to evade host innate immunity results in the ability of the nasal passages to harbour pathogenic *S.aureus* that may see it's opportunity to infect (Ansari, 2016; Quinn, 2007). This reservoir promotes the multiplication and spread of the bacterium.

Further to the figure of 30% positivity in the general population, unpublished data has suggested that *S.aureus* toxins can only be detected in the urine of 18% of the general population (Bull, 2014). Compared with our result of 56.4% *S.aureus* positivity in RA patients, there is a substantial difference when compared with either base figure. As the unpublished figure, of 18%, was generated when detecting the same four

staphylococcal toxins in urine, it is perhaps a better comparison. This would make the presence of *S.aureus* three times higher in the urine of the RA patients than the general population.

The urine from patients with closed fractures has demonstrated the presence of at least one staphylococcal enterotoxin in 27.1% of the samples. This is similar to the figures for the general population, according to the data associated with nasopharyngeal carriage (Fall, 2014; Treesirichod, 2014; Lucia Preoţescu, 2013; Kuehnert, 2006; Weidenmaier, 2012; Graham, 2006, den Heijer, 2013; Ateba Ngoa, 2012). However, these figures are not comparable due to the differing route and conditions that the bacterium and/or toxins have gone through. Treatment of a fracture requires frequent visits back to the hospital for outpatient appointments. It is well known that the *S.aureus* carrier rates of hospital personnel and patients can be high, approximately 50% (Millian, 1960; Kluytmans, 1997; Rongpharpi, 2013). Furthermore, in the study conducted by Rongpharpi *et al.*, the orthopaedic department had the highest *S.aureus* presence in the hospital (Rongpharpi, 2013). This added exposure could contribute to the increased prevalence of *S.aureus* toxins in the urine of patients with closed fractures.

It is not known exactly why the presence of *S.aureus* in urine is so much higher in patients with RA. Due to the fact that all of the patients recruited already had RA, we can only speculate whether the *S.aureus* presence could be a cause or an effect of the disease. It may be that individuals were infected with a strain of *S.aureus* at some point earlier in life, since which time, the bacteria have remained in the body, avoiding clearance by the immune system by hiding in immune privileged sites. The evasion of bacteria/pathogens from the immune system is not a new concept (Finlay, 2006). This phenomenon has been described in relation to viruses, bacteria and

parasites; for example, Ebola, HIV, TB and malaria (Morens, 2004; Fauci, 2005; Audet, 2015). Pathogens can evade the immune system using a number of strategies, such as antigenic hyper variability, subverting or killing immune cells/phagocytes or inhibiting complement; to name but a few (Finlay, 2006). Bacterial pathogens have also shown an ability to alter downstream inflammatory cytokines, however, the molecular mechanisms by which this is achieved is not known in most cases due to the complexity of bacteria and the diverse array of effectors and other immune modulators produced by these organisms (Tato, 2002). However, *S.aureus* is an example of a pathogen that can specifically target a cytokine pathway to enhance pathogenesis. The bacterium produces protein A which is able to bind directly to the TNFα receptor, TNFR1, on respiratory epithelium, which then potentiates a chemokine and cytokine cascade and subsequent disease (Gomez, 2004). TNFα is known to play a major role in RA pathogenesis (Brenner, 2015).

As an exact molecular cause of RA is unknown, part of the *S.aureus* bacterium or the toxins that it produces could perhaps be an antigen that stimulates the immune system into eliciting the inappropriate immune response, stimulating the production of the autoantibodies that attack the synovial joints (Cusick, 2012). Autoantibodies cause the chronic and systemic inflammation characteristic of RA. SEB in particular has been implicated in the disease pathogenesis of RA and other autoimmune diseases (Li, 2015).

Typically, small proteins (<70kDa) can be filtered through the glomeruli of the kidneys and are found in the urine of a healthy individual (Wartiovaara, 2004; Pavenstadt, 2003; Akilesh, 2008). Some of the toxins are small enough to be filtered through into the urine; however, their amino acid structure may have been altered whilst in the body and/or they may become part of a larger protein complex. As shown

in Chapter 3, the toxins are susceptible to degradation by proteolytic enzymes such as trypsin and pepsin. Both of these enzymes are naturally present in the human body as part of the gastric and pancreatic juices of the digestive system (Boundless Biology, 2016). Assuming that at least some of the bacterial toxins pass through the digestive system, it is likely that these activated enzymes could alter the amino acid makeup of the proteins. Alternatively, the kidneys can become leaky, allowing large proteins and larger protein complexes into the urine (Chamberlain, 2003). This can happen when an individual's body is under stress, such as chronic inflammation. Diabetes, SLE, RA and infection have all been described as being able to cause damage to glomeruli, resulting in nephrotic syndrome (leaky kidneys) (Hull, 2008; Kodner, 2009). It can also occur as a side-effect of medicines and treatments, as well as a consequence of various poisons or toxins. Nephrotic syndrome is characterised by proteinuria that was described by Richard Bright's series of descriptions of "albuminous urine" (Hull, 2008; Cameron, 2002). In our western blots, strong bands were seen in many of the lanes at the level of albumin, approximately 66.5kDa (Sigma Aldrich – Human Albumin). This supports a claim that many of the patients sampled were perhaps suffering from nephrotic syndrome, possibly caused by inflammation, which was allowing the filtration of staphylococcal toxin complexes through the kidney and into the urine.

Alternatively, the inability to clear *S.aureus* from individuals with RA may be due to the fact that such patients already have a lot of demand on their immune system, which is both attacking and trying to defend itself. The white blood cells (WBCs) may not be present in the quantities necessary to kill and phagocytose enough of the bacteria and toxin products. Neutrophils circulate in the blood for approximately 10 hours, during which time all of an individual's blood passes through the capillary

venules of the lungs numerous times. Here, the neutrophils move more slowly than the red blood cells (RBCs), meaning that an estimated 50% of all neutrophils are present in the lung at any one time; which is a very effective mechanism for clearing the lungs of pathogens (Morris, 2007). During inflammation or fever, neutrophils and RBCs appear to flow through the venules at the same speed, thus reducing the concentration of WBCs in the lung, leaving the lungs more vulnerable to invasion by pathogens (Morris, 2007).

Similarly, the treatment of RA often involves the prescription of a combination of drugs that suppress the immune system. Severe RA is treated with high dose corticosteroids alongside immunosuppressive and cytotoxic drugs such as azathioprine, cyclophosphamide and mycophenolate (Murphy, 2008). The drug combinations are used to suppress the immune system, reducing the symptoms rather than treating the cause. The use of glucocorticoids (GCs) as an immunosuppressant have been shown to increase the risk of infection 4-fold (in a dose dependent manner) (Listing, 2013). The use of DMARDs and biologics has led to side effects such as a reduction in circulating neutrophils and increased incidence of infection (Choy, 2011).

Since the introduction of biologic treatments 15 years ago, at least 2-fold increased rates of infection in RA patients have been reported (Strangfeld, 2009; Thomas, 2004). New biologics and the more recent biosimilars are the most advanced treatments for RA. TNF-α inhibitors are often prescribed and are used to reduce the effect of TNF-α, a major pro-inflammatory cytokine implicated in the disease pathogenesis of RA (Brenner, 2015). Infliximab and adalimumab are monoclonal anti-TNF-α antibodies and etanercept is a TNF-α fusion protein (Strangfeld, 2009). The introduction of biologics and biosimilars has proven to be a good treatment option for RA sufferers, particularly those for whom conventional DMARDs do not sufficiently

control their disease. However, these benefits have also brought with them an apparent increased risk of infection compared to patients prescribed DMARDs alone (Listing, 2005). In particular, increased risk and reactivation of HZV and TB have been documented (Keane, 2001; Mohan, 2004).

Due to the inflammatory nature of RA and the immunosuppressive treatment, infection in RA is not uncommon. However, infection is not normally attributed to *S.aureus*, except in cases with congestive heart failure and following an indwelling catheter (Sams, 2015). Fewer studies have looked at the presence of *S.aureus* in RA, with research often focussing on the presence of pathogens perceived to be more life-threatening (Sams, 2015; Carmona, 2003; Wolfe, 2006). Although *S.aureus* can be a member of the commensal flora, pathogenic strains can be opportunistic (Cohen, 2016). This can result in severe bacteraemia, particularly in the immunocompromised (Richards, 2015). Bacteraemia caused by *S.aureus* is associated with higher morbidity and mortality than bacteraemia caused by other pathogens (Naber, 2009).

Other bacteria possibly linked to RA include *P.gingivalis* and *P.mirabilis*. *P.gingivalis* is a periodontal pathogen with a unique ability to citrullinate proteins. Anti-CCP antibodies are a characteristic of RA, included in the 2010 RA classification criteria (Shipley, 2009; van Venrooij, 2011; Aletaha, 2010). However, it is possible to have anti-CCPs without having RA (Lima, 2010). *P.gingivalis* can be detected in 25% of healthy individuals but is not thought to be a normal inhabitant of a healthy periodontal dentition (Griffen, 1998). Results of a study by Wegner *et al.* suggest that the citrullination mediated by *P.gingivalis* occurs in both the bacterial proteins and host proteins, thus providing a potential molecular mechanism of generating the epitopes to which the body has no immunologic tolerance (Wegner, 2010). Anti-CCP

antibodies have been seen following various microbial infections, particularly following 37% of TB cases (Lima, 2010; Mori, 2009).

P.mirabilis is a member of the commensal gut flora but, like *S.aureus*, can become pathogenic when given the opportunity to infect a wound or the urinary tract. UTIs are much more common in patients with RA (Tishler, 1992). Studies have shown that isolation of *P.mirabilis* in the urine of RA patients can be two-fold higher than controls. Levels of *P.mirabilis* are particularly increased in females with RA (63%) compared with males with RA (50%); and even more so than in healthy female (32%) and male (11%) controls (Ebringer, 1996). Furthermore, such antibodies were found to be in higher titres in the sera and urine of RA patients compared to controls (Rashid, 2007; Ebringer, 2010). It is likely that *P.mirabilis* infection is a consequence of RA, taking advantage of the compromised immune system.

EBV has been linked to the pathogenesis of RA. Similar to *S.aureus*, EBV is abundantly present and is often first encountered early in life. In developing countries, most children have encountered the virus before the age of 5, whereas this is often delayed in developed countries (Haahr, 2004; Henle, 1967; de-The, 1975). Haahr *et al.* looked at the prevalence of EBV in an area with a high incidence of MS, an autoimmune disease whose pathogenesis shows some similarities to that of RA. Interestingly, all of the MS patients were seropositive for EBV antibodies (Haahr, 2004).

A basic overview of the immunological response to an antigen and a simplified diagram of the process of inflammation are described in Figure 6.7 and 6.8.

Figure 6.7 A basic diagram to show the process of inflammation - adapted from K.Wassung (Wassung, 2012).

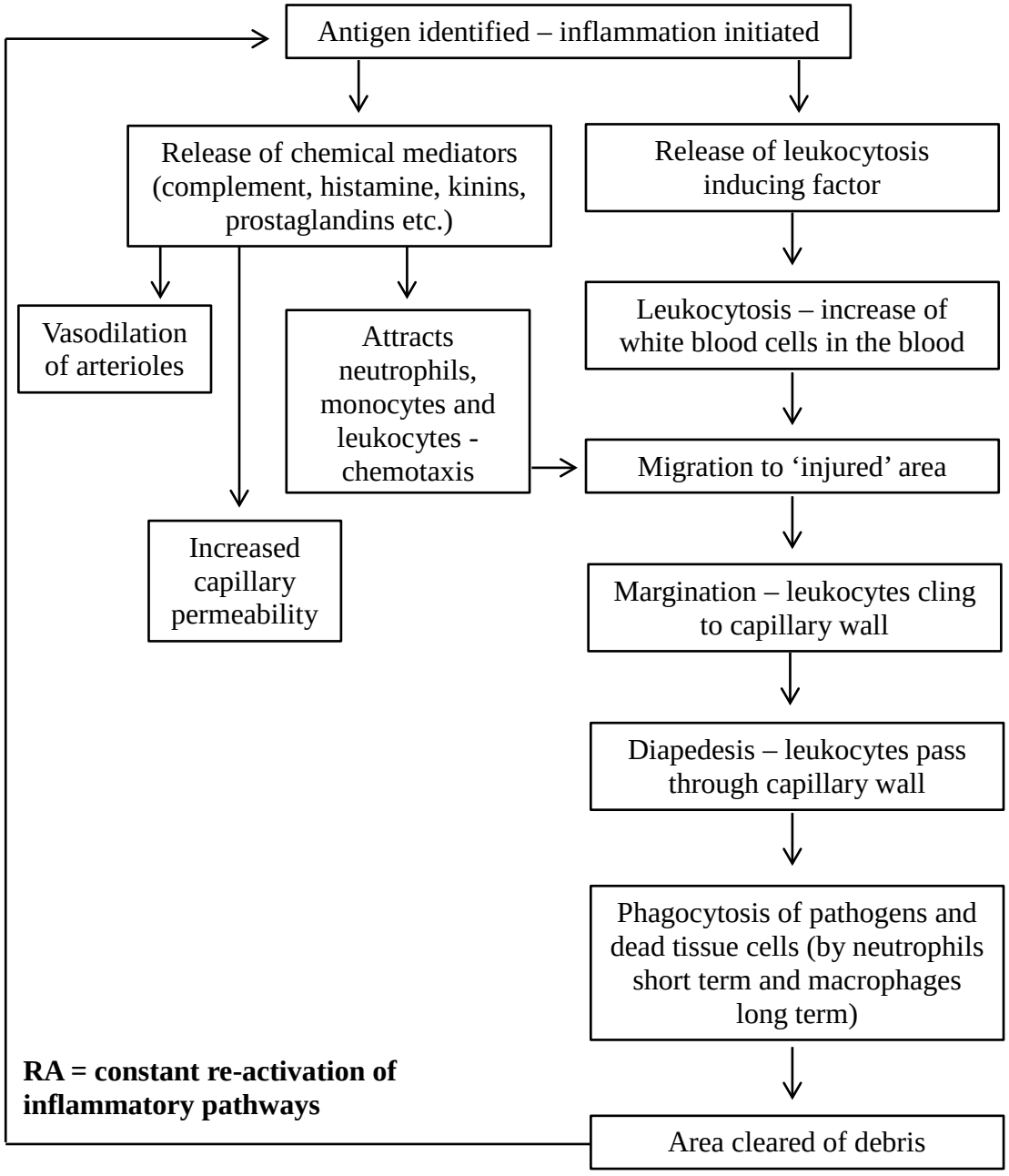
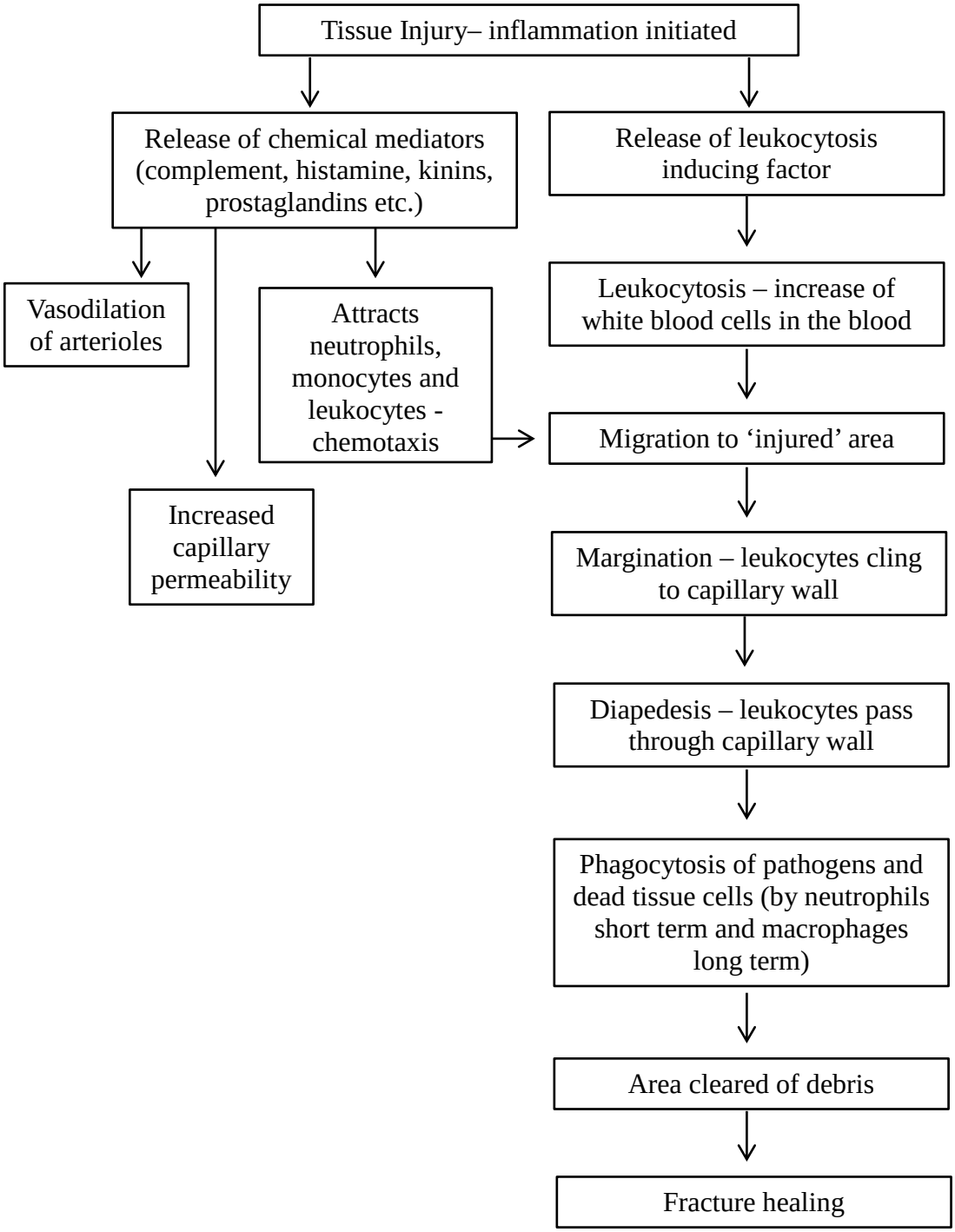


Figure 6.8 A basic diagram to show the process of inflammation following a fracture - adapted from K.Wassung (Wassung, 2012).



Macrophages are derived from monocytes following monocytopoesis. Macrophages reside in almost all of the tissues in the body, in numerous forms, scavenging for potential pathogens by amoeboid movement (Ovchinnikov, 2008). Similarly, mast cells are also found in most tissues of the body, particularly in locations that are in close contact with the external environment; such as skin, airways, and intestines. Activation of mast cells results in the release of a variety of soluble factors including TNF-α (Urb, 2012).

White blood cells such as monocytes and neutrophils reside in the blood, continuously circulating through the body. Monocytes are highly plastic and heterogeneous, and they are able to change their functional phenotype in response to an environmental stimulus; they also have the ability to differentiate into inflammatory or anti-inflammatory subsets. Upon infection, monocytes are rapidly recruited to the affected tissue, where they differentiate into macrophages or dendritic cells (Yang, 2014). Macrophages are present in almost all of the tissues in the body, scavenging for potential pathogens by amoeboid movement (Ovchinnikov, 2008). Mast cells are also found in most human tissues, particularly in locations that are in close contact with the external environment. Activation of mast cells results in the release of a variety of soluble factors including TNF-α (Urb, 2012).

Inflammation is a major and important part of the healing process. Ordinarily, aside from the pain and restricted movement caused by inflammation, the process is beneficial and repairs the damage. However, the immune system of fracture patients is under a considerable burden due to the trauma-induced inflammation. Furthermore, an individual with a fracture needs to repeatedly visit the hospital, where the chance of bacterial cross-infection is high. These two factors together go some way to explain why staphylococcal enterotoxins can be detected in the urine of patients with closed fractures. Similarly, in those individuals suffering from RA, the inflammation is triggered repeatedly, causing irreparable damage and chronic pain. The immune system is compromised, as a consequence of both the chronic inflammation and immunosuppressive drugs.

There is an apparent correlation between inflammation and a dysfunctional immune response within autoimmune disease. Vella *et al.* determined that inflammation in conjunction with the activation of other T cell stimulatory molecules can help T cell

growth and increase the number of helper T cells. In experiments, the researchers injected mice with an antigen that caused significant death of the antigen-responsive T cells; however, when inflammation was present, the death of these cells was prevented. In addition, some bacteria have a lipopolysaccharide (LPS). It appears that LPS can play a role in causing inflammation, and keeping activated T cells alive (Vella, 1998; Wassung, 2012). This mechanism could mean that T cells that have come into contact with, and mounted an immune response towards staphylococcal toxins could actually multiply and remain active; exerting their immunological effect repeatedly and long after the toxin is removed.

The healing of fractures and broken bone is a well-orchestrated sequence of biological events (Bigham-Sadegh, 2015). Inflammation occurs immediately at the site of the fracture and persists for up to 5 days. This event is important as the damaged bone tissue dies back and is removed by osteoclasts. The dead cells stimulate the release of cytokines, initiating the healing process (Bigham-Sadegh, 2015). Fibroblasts in the blood lay down granulation tissues, leading to the early formation of cartilage and fibrocartilage. As time goes on, the inflammation subsides and soft callus formation commences (4 days to 3 weeks post fracture), followed by hard callus (6-12 weeks post fracture) and eventually bone remodelling (years post fracture) (Bigham-Sadegh, 2015).

Other than external factors, there may also be internal immunological factors in the fracture patients to consider. The increased presence of staphylococcal toxins in urine when suffering from a fracture compared to the general population can only be speculated. As discussed previously, when the body is under stress the kidneys may become leaky as a way of ridding the body of surplus pathogens and toxins, relieving the immune system of some of the burden (Chamberlain, 2003). The trauma causing the fractured bone(s) will no doubt have inflamed the tissue surrounding it. This acute inflammation may, like the chronic inflammation associated with RA, have an effect

on the kidneys, allowing them to permit larger proteins through the glomeruli and into the urine. The patients included in this study had closed fractures; therefore for the chance of the *S.aureus* bacterium entering the body through a break in the skin is highly unlikely and does not explain the increased number of patients with staphylococcal toxin in their urine.

Alternatively, the inability to clear *S.aureus* in individuals with closed fractures may be due to the fact that such patients already have a demand on their immune system, due to acute inflammation. The WBCs may not be present in the quantities necessary to kill and phagocytose the toxin. As described previously, neutrophils circulate in the blood for approximately 10 hours, during which time all of on individuals blood passes through the capillary venules of the lungs numerous times. Here, the neutrophils move more slowly than the RBCs, meaning that an estimated 50% of all neutrophils are present in the lung; which is a very effective mechanism for clearing the lungs of pathogens (Morris, 2007). During inflammation or fever, neutrophils and RBCs appear to flow through the venules at the same speed, thus reducing the concentration of WBCs in the lung, leaving the lungs more vulnerable to invasion by pathogens (Morris, 2007).

Chapter 7 RESULTS: MASS SPECTROMETRY

7.1 Theoretical Target Proteins

Detection of SEB, SEC1 and TSST-1 in food and patient samples is typically performed using immunological techniques; however, commercial kits available for the detection of enterotoxins, SEA to SEE, suffer from serious limitations with respect to sensitivity, specificity and suitability for complex matrices. A number of reports have been published on the detection of staphylococcal enterotoxins in food matrices using mass spectrometry as the detection method, with the aim of selective and sensitive identification and quantitation of such toxins (Andjelkovic, 2016; Callahan, 2006; Sospedra, 2011).

Herein we report the analysis of staphylococcal enterotoxins, SEB and SEC1, along with TSST-1 in patient samples via mass spectrometric detection using a Shimadzu ion-trap time-of-flight tandem mass spectrometer (LCMS-IT-TOF), to selectively confirm presence of the toxins where immune assays tests have returned a positive result.

7.1.1 Toxin Identification

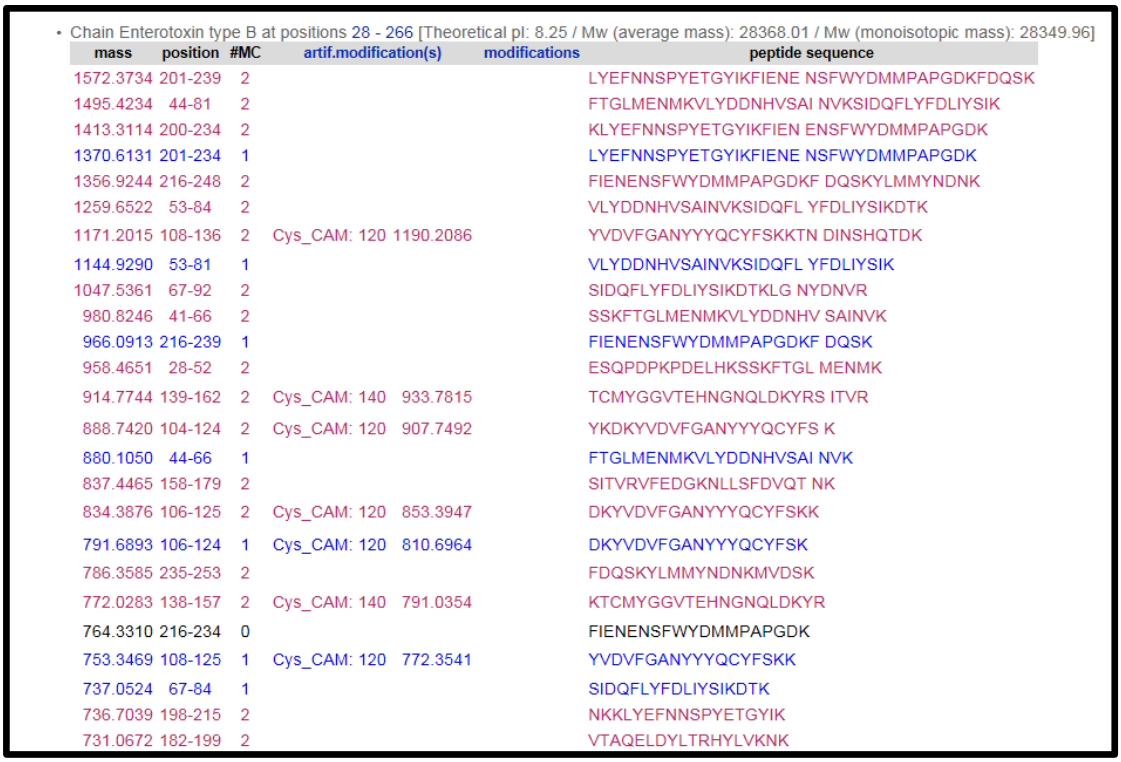
7.1.1.1 *In silico* digests

Peptide sequences for SEB, SEC1 and TSST-1 were obtained from the UniProtKB online database, reference codes and links shown in Table 7.1. Each UniProtKB sequence was used to generate peptide masses for tryptic digests *via* ExPASy peptide mass calculation tool. PeptideMass calculations were performed with iodoacetamide treatment of cysteines, digested with trypsin enzyme allowing for up to 2 missed cleavage sites. Monoisotopic mass-to-charge values were obtained for $[M+H]^{+}$, $[M+2H]^{2+}$ and $[M+3H]^{3+}$ peptides between 500 – 1,000 Da. Example data generated *via* UniProtKB and ExPASy is shown below, Figure 7.1.

Table 7.1 UniProtKB protein database identification code for target proteins AH, SEB, SEC1 and TSST-1, full amino acid sequence presented in appendix.

Toxin	**Uniprot Reference Code**	
SEB	P01552	http://www.uniprot.org/uniprot/P01552
SEC1	P01553	http://www.uniprot.org/uniprot/P01553
TSST-1	P06886	http://www.uniprot.org/uniprot/P06886
AH	P09616	http://www.uniprot.org/uniprot/P09616

Figure 7.1 ExPASy *in silico* theoretical tryptic digestion of SEB with iodoacetamide treatment and up to 2 missed cleavages. Data displays amino acid sequences, cleavage sites and monoisotopic mass from $[M + 3H]^{3+}$ peptides.



• Chain Enterotoxin type B at positions 28 - 266 [Theoretical pI: 8.25 / Mw (average mass): 28368.01 / Mw (monoisotopic mass): 28349.96]

mass	position	#MC	artif.modification(s)	modifications	peptide sequence
1572.3734	201-239	2			LYEFNNSPYETGYIKFIENE NSFWYDMMPAPGDKFDQSK
1495.4234	44-81	2			FTGLMENMKVLYDDNHVSAI NVKSIDQFLYFDLIYSIK
1413.3114	200-234	2			KLYEFNNSPYETGYIKFIEN ENSFWYDMMPAPGDK
1370.6131	201-234	1			LYEFNNSPYETGYIKFIENE NSFWYDMMPAPGDK
1356.9244	216-248	2			FIENENSFWYDMMPAPGDKF DQSKYLMMYNDNK
1259.6522	53-84	2			VLYDDNHVSAINVKSIDQFL YFDLIYSIKDTK
1171.2015	108-136	2	Cys_CAM: 120	1190.2086	YVDVFGANYYYQCYFSKKTN DINSHQTDK
1144.9290	53-81	1			VLYDDNHVSAINVKSIDQFL YFDLIYSIK
1047.5361	67-92	2			SIDQFLYFDLIYSIKDTKLG NYDNVR
980.8246	41-66	2			SSKFTGLMENMKVLYDDNHV SAINVK
966.0913	216-239	1			FIENENSFWYDMMPAPGDKF DQSK
958.4651	28-52	2			ESQPDPKPDELHKSSKFTGL MENMK
914.7744	139-162	2	Cys_CAM: 140	933.7815	TCMYGGVTEHNGNQLDKYRS ITVR
888.7420	104-124	2	Cys_CAM: 120	907.7492	YKDKYVDVFGANYYYQCYFS K
880.1050	44-66	1			FTGLMENMKVLYDDNHVSAI NVK
837.4465	158-179	2			SITVRVFEDGKNLLSFDVQT NK
834.3876	106-125	2	Cys_CAM: 120	853.3947	DKYVDVFGANYYYQCYFSKK
791.6893	106-124	1	Cys_CAM: 120	810.6964	DKYVDVFGANYYYQCYFSK
786.3585	235-253	2			FDQSKYLMMYNDNKMVDSK
772.0283	138-157	2	Cys_CAM: 140	791.0354	KTCMYGGVTEHNGNQLDKYR
764.3310	216-234	0			FIENENSFWYDMMPAPGDK
753.3469	108-125	1	Cys_CAM: 120	772.3541	YVDVFGANYYYQCYFSKK
737.0524	67-84	1			SIDQFLYFDLIYSIKDTK
736.7039	198-215	2			NKKLYEFNNSPYETGYIK
731.0672	182-199	2			VTAQELDYLTRHYLVKNK

7.1.1.2 Toxin Standards MS^1 Screening

Tryptic digests of standard compounds for SEB, SEC1 and TSST-1 were combined in a single sample and analysed *via* a generic 30 minute LCMS method using a C18 column with a water/acetonitrile gradient with 0.1% (v/v) formic acid modifier. Resultant LCMS Total Ion Current (TIC) MS^1 data was mined for extracted ion chromatograms (XIC) corresponding to the monoisotopic m/z values obtained from the *in silico* digestions, example shown in Figure 7.2. Mass spectra peaks that could be assigned within 5 ppm mass accuracy to a predicted monoisotopic peptide mass and which also displayed the correct isotopic distributions were considered for further confirmation *via* MS^2 fragmentation experiments. The monoisotopic masses observed in the MS^1 of the peptides from toxin standard scans are reported below in in Table

7.2, Table 7.3 and Table 7.4, additional tryptic peptides are expected but were not observed.

Figure 7.2 Extracted ion chromatogram of LCMS MS^1 analysis of standard sample containing SEB, SEC1 and TSST-1 toxins. Target peptides predicted, in order from top to bottom (423.25; 760.37; 564.77; 746.85; 789.40; 705.37; 552.30; 529.61; 485.25; 654.84; 774.91; 560.85; SEB, SEC1 and TSST-1 peptides respectively).

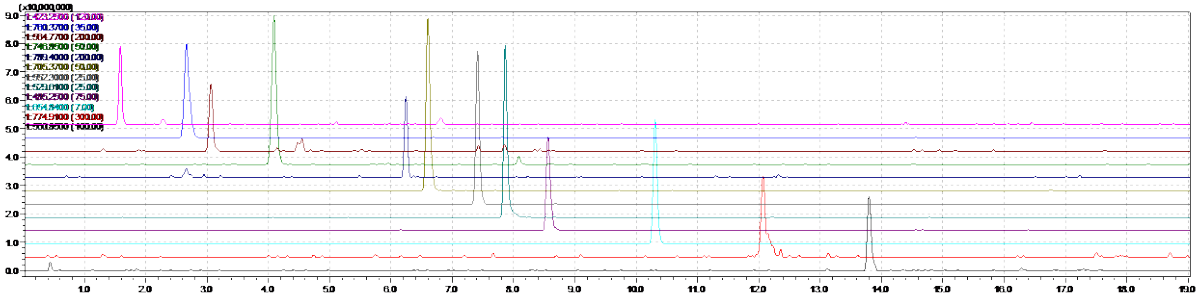


Table 7.2 SEB peptides observed from LCMS analysis of peptide standard compounds; matched against predicted peptides generated *in silico*.

SEB Tryptic Digest Peptide Sequence	Mass to Charge [m/z]	Charge [+]	Start	Finish	Missed Cleavages
VLYDDNHVSAINVK	793.9123	2	53	66	0
KTNDINSHQTDKR	778.8924	2	125	137	2
TNDINSHQTDKRK	778.8924	2	126	138	2
ESQPDPKPDELHK	760.3730	2	28	40	0
SIDQFLYFDLIYSIKDTK	737.0524	3	67	84	1
LGNYDNVRVEFK	727.3753	2	85	96	1
DVKIEVYLTTKK	718.9216	2	254	265	2
KVTAQELDYLTR	714.8449	2	181	192	1

VFEDGKNLLSFDVQTNKK	694.7004	3	163	180	2
HYLVK	659.3875	1	193	197	0
VTAQELDYLTR	654.8433	2	182	192	0
VLYDDNHVSAINVK	529.6112	3	53	66	0
LGNYDNVRVEFK	485.2532	3	85	96	1

Table 7.3 SEC1 peptides observed from LCMS analysis of peptide standard compounds; matched against predicted peptides generated *in silico*.

SEC1 Tryptic Digest Peptide Sequence	Mass to Charge [m/z]	Charge [+]	Start	Finish	Missed Cleavages
FLAHDLIYNISDK	774.9064	2	71	83	0
ESQPDPTPDELHK	746.8493	2	28	40	0
NTISFEVQTDKK	705.3672	2	168	179	1
SVTAQELDIKAR	665.8699	2	180	191	1
SVTAQELDIK	552.3008	2	180	189	0
DNVGK	532.2725	1	126	130	0
DNVGKVTGGK	487.7669	2	126	135	1
IEVHLTTK	470.7767	2	257	264	0
NTISFEVQTDKK	470.5805	3	168	179	1
SVTAQELDIKAR	444.2490	3	180	191	1
NFLINKK	438.7687	2	192	198	1
NYDKVK	383.7083	2	87	92	1
NFLINK	374.7212	2	192	197	0

Table 7.4 TSST-1 peptides observed from LCMS analysis of peptide standard compounds; matched against predicted peptides generated *in silico*.

TSST-1 Tryptic Digest Peptide Sequence	Mass to Charge [m/z]	Charge [+]	Start	Finish	Missed Cleavages
TIEAEIN	789.3988	1	228	234	0
KFEYNTEKPPINIDEIK	693.3650	3	211	227	1
HQLTQIHGLYR	683.3729	2	175	185	0
SSDKTGGYWK	564.7696	2	186	195	1
LPTPIELPLK	560.8524	2	134	143	0
VDLNTKR	423.2456	2	102	108	1

7.1.1.3 MS^2 Fragmentation Experiments

For the initial MS^1 scan the instrument method performed a single scanning event measuring m/z values between 300 – 1000 Da, Figure 7.2. Individual peaks were allocated retention time windows called "Segments", each Segment was then expanded to include an MS^2 fragmentation experiment which isolated a 3 Da wide ion packet which contained the tryptic peptide eluted in that Segment. Fragmentation experiments were conducted on this ion packet to generate an MS^2 product ion scan containing fragment ions of the isolated peptide precursor. Peptide molecules fragment predictably and MS^2 spectra were compared against a list of masses corresponding to calculated fragment ions generated *in silico*, Figure 7.3. Toxin compounds could be assigned unequivocally through matching MS^2 ions with the

predicted fragments generated from the peptide sequences described in Table 7.2, Table 7.3 and Table 7.4. Table 7.5.contains a list of peptides that were identified through the observed product ions that correspond to predicted y/b fragments. Figure 7.4 shows an example MS^1 scan with the peptide precursor selected for the MS^2 experiment highlighted. Figure 7.5 shows the resultant MS^2 spectrum obtained from the fragmentation of 552.3014 ± 3 Da, the assigned y/b fragment ions are also highlight. Peaks found during the MS^1 scan that did not generated predicted fragment product ions could not be assigned to have originated from the target toxins; it is likely that these peaks contain the same amino acid composition, but do not match the amino acid sequence of the target peptide.

Fragment Ion Calculator Results

Sequence: **LPTPIELPLK**, pI: **6.00141**

Fragment Ion Table, monoisotopic masses

Seq	#	B	Y	# (+1)
L	1	114.09139	1120.69766	10
P	2	211.14415	1007.61360	9
T	3	312.19183	910.56084	8
P	4	409.24459	809.51316	7
I	5	522.32865	712.46040	6
E	6	651.37125	599.37633	5
L	7	764.45531	470.33374	4
P	8	861.50807	357.24968	3
L	9	974.59214	260.19691	2
K	10	1102.68710	147.11285	1

Figure 7.3 Screen shot of predicted y/b fragmentation ions along the peptide chain of LPTPIELPLK from TSST-1. Only amino acids in this exact sequence will generate MS^2 spectra containing the above masses from the isolation and fragmentation of $[M+2H]^{2+}$.

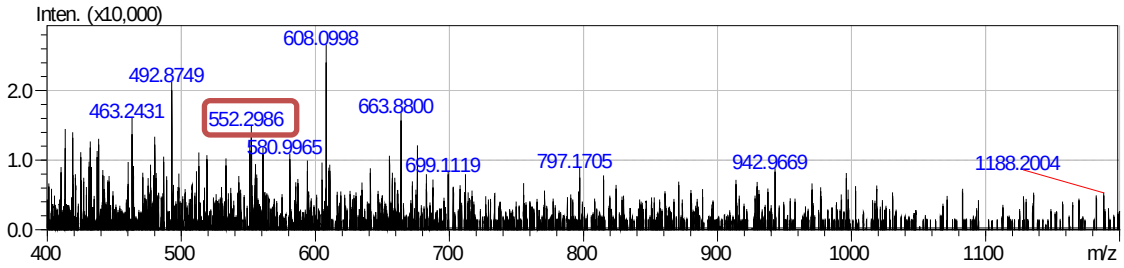


Figure 7.4 **MS[1] full scan spectrum of SEB, SEC1, TSST-1 standard peptide mixture, data averaged across a peak position located within a XIC targeted at 552.30 Da, highlighted in red.**

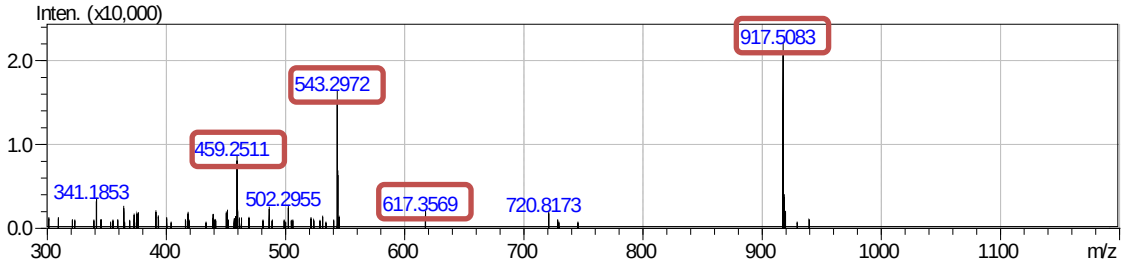


Figure 7.5 **MS[2] fragmentation spectrum of precursor 552.80 ± 3.0 Da (isotopic envelope); target fragments 917.49, 617.35, 543.30, 459.25 Da, Table 7.5.**

Table 7.5 **Tryptic peptides observed in LCMS analysis of combined SEB, SEC1 and TSST-1 standards; fragment ions generated from selected precursors, matched against fragmentation position along the peptide chain.**

Seg.	Toxin	Peptide Sequence	Mass to Charge [m/z]	Charge	Fragment Ions [m/z]	Fragment Type	Charge	Proteotypic [Sequence Match]
1	TSST-1	VDLNTKR	423.2456	2+	746.4120 631.3820 518.3030 404.2560 316.1940	Y6 Y5 Y4 Y3 Y5	1+ 1+ 1+ 1+ 2+	No [Fruit fly myosin heavy chain 95F]
2	SEB	ESQPDPKPDELHK	760.3736	2+	963.5264 751.3683 557.2208 539.3706 526.2990 482.2671	Y8 B13 B5 Y9 Y4 Y8	1+ 2+ 1+ 2+ 1+ 2+	Yes
3	TSST-1	SSDKTGGYWK	564.7702	2+	711.3466 610.2990	Y6 Y5	1+ 1+	Yes
4	SEC1	ESQPDPTPDELHK	746.8499	2+	936.4791	Y8	1+	No

					737.8447 574.7830 526.2990 468.7435	B13 Y10 Y4 Y8	2+ 2+ 1+ 2+	[SEC2; ATP protease]
5	TSST-1	TIEAEIN	789.3990	1+	657.3460 575.2677 544.2619	B6 Y5 B5	1+ 1+ 1+	Yes
6	SEC1	NTISFEVQTDKK	705.3677	2+	847.4526 718.4100 597.8225 275.2084	Y7 Y6 Y10 Y2	1+ 1+ 2+ 1+	No [SEC2; SEC3]
7	SEC1	SVTAQELDIK	552.3014	2+	917.4944 844.4053 816.4468 745.4096 617.3511 543.2961 488.3085 459.2512 375.2244 260.1975	Y8 B8 Y7 Y6 Y5 B10 Y4 Y8 Y3 Y2	1+ 1+ 1+ 1+ 1+ 2+ 1+ 2+ 1+ 1+	No [SEC2; SEC3]
8	SEB	VLYDDNHVSAINVK	529.6112	3+	687.8366 744.3787 631.3779 606.3050 548.7915 491.2780 360.2247	Y12 Y13 Y6 Y11 Y10 Y9 Y3	2+ 2+ 1+ 2+ 2+ 2+ 1+	Yes
9	SEB	LGNYDNVRVEFK	485.2532	3+	670.8339 642.3232 585.3017 446.2566 294.1818	Y11 Y10 Y9 Y7 Y2	2+ 2+ 2+ 2+ 1+	Yes
10	SEB	VTAQELDYLTR	654.8439	2+	909.4682 780.4256 667.3416 552.3146 389.2513 276.1672	Y7 Y6 Y5 Y4 Y3 Y2	1+ 1+ 1+ 1+ 1+ 1+	Yes
11	SEC1	FLAHDLIYNISDK	774.9071	2+	965.5308 852.4468 739.3627 609.3123 349.1724	Y8 Y7 Y6 Y10 Y3	1+ 1+ 1+ 2+ 1+	No [SEC2; SEC3]
12	TSST-1	LPTPIELPLK	560.8530	2+	974.5927 910.5614 809.5137 712.4609 599.3769 551.8478 470.3343 455.7846 405.2608 357.2502 312.1924	B9 Y8 Y7 Y6 Y5 B10 Y4 Y8 Y7 Y3 B3	1+ 1+ 1+ 1+ 1+ 2+ 1+ 2+ 2+ 1+ 1+	Yes

7.1.1.4 Protein BLAST Search

Once target peptides and fragment markers were identified peptides were tested for uniqueness *via* an NCBI (National Center for Biotechnology Information) protein BLAST (Basic Local Alignment Search Tool) search referenced against the UniProt/SwissProt database, the results of which are shown in Table 7.5. Peptides are checked that the amino acid composition and sequence are unique to that protein and therefore the presence in samples cannot be attributed to other compounds. Peptides which display a unique sequence are described as proteotypic; peptides that created more than one possible source, with the exception of SEC1, were excluded from the analytical method and not used as a detection marker for the presence of a given toxin. Proteotypic peptides were observed for both SEB and TSST-1, however, SEC1 peptides share a large amount of their peptide sequence with other SEC toxins and therefore proteotypic peptides could not be assigned for this target.

The use of combined tryptic peptides derived from standard protein toxins of SEB, SEC1 and TSST-1 in a combined sample generated four unique peptides with a minimum of three, and up to eleven, fragment product ions with accurate mass confirmation for the detection of the three described toxin compounds known to us as products of staphylococcus aureus. Figure 7.6 and Figure 7.7 show overlay spectra of XICs generated from both MS^1 and MS^2 scans of peptide and fragment ions that come from the same origin and were used to unequivocally confirm peptide structure and consequently the presence of a toxin.

Figure 7.6 Extracted ion chromatograms of standard compound mixture containing SEB, SEC1 and TSST-1; targeted to Segment 12, TSST-1 peptide LPTPIELPLK (m/z 560.8524) MS^1 XIC and overlapping XICs of product ions from MS^2 fragmentation experiment.

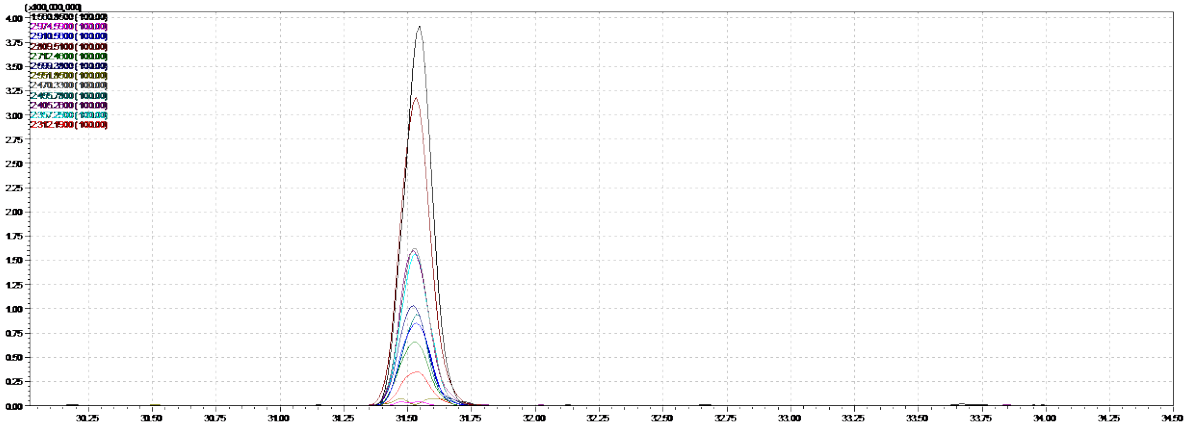


Figure 7.7 Extracted ion chromatograms (XICs) of Segment 7 of the standard compound mixture containing SEB, SEC1 and TSST-1; the displayed Segment analyses SEC1 peptide SVTAQELDIK (m/z 552.3008); MS^1 and MS^2 XIC data.

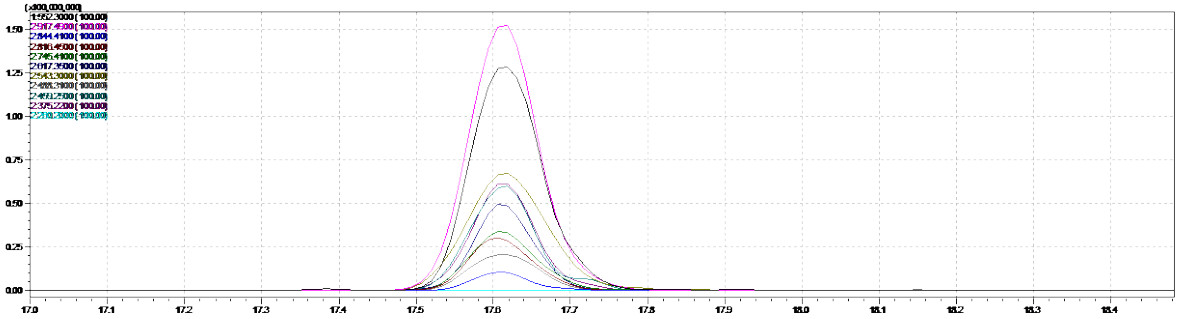


Figure 7.8 Extracted ion chromatograms (XICs) of Segment 7 of the standard compound mixture containing SEB, SEC1 and TSST-1; the displayed Segment analyses SEC1 peptide SVTAQELDIK (m/z 552.3008); MS^1 and MS^2 XIC data.

7.2 Method Development

The analytical method underwent further development to improve sensitivity and increase target peak resolution; this included increasing the length of the chromatographic run from 30 to 60 minutes total elution time. Retention times, mass

assignment and fragmentation experiments were repeated to confirm correct instrument settings for toxin assignment. The final analytical method used to analyse patient samples is fully described in Table 7.6. Instrument settings are specific to software and operating protocols of the Shimadzu LCMS-IT-TOF although a number of common analytical parameters are transferable between similar instruments.

7.3 Quantitation

The main objective of this work was to detect the presence of SEB, SEC1 and TSST-1 in patient samples both sensitively and selectively using mass spectrometry. Quantitation of the protein toxins was not a requirement; however, some quantitation was achieved *via* an external calibration curve generated from the SEB, SEC1 and TSST-1 combined external standard. It is important to stress that whilst data is presented with concentration values obtained from the intensity of the XIC MS^2 product ions spectra, Table 7.7 and Table 7.8, a number of requirements were not undertaken that would enable quantitation of patient samples to be accurately reported. Therefore, whilst concentration values are reported they are done so under the caveat that quantitation is beyond the scope of this study.

The analytical method targeted four peptides from each toxin; each peptide was then fragmented to generate MS^2 product ions that can be used to create XIC data targeting a MS/MS transition with an m/z tolerance of 0.005 Da. XIC data was then integrated and quantitated using a calibration curve for that particular MS/MS transition. The number of MS^2 fragments per peptide were limited only by the number of matching

product ions observed consistently from the standard compounds during method development. In some cases as many as 10 product ion peaks were targeted and integrated, the MS^1 peptide precursors and corresponding MS^2 product ions monitored during analysis are reported in Table 7.5. Figure 7.6 and Figure 7.7 show example spectra of multiple XICs from MS^1 and corresponding MS^2 scans. All XICs overlap exactly as the MS^2 scans originate solely from the fragmentation of the MS^1 precursor. Again, the number of MS^2 markers that confirm the presence of a specific toxin was limited only by the number of MS^2 products assigned from the peptide fragment experiment; obviously a larger number of assigned fragments creates an increase in peptide assignment.

All XICs were generated to an accuracy of 2 decimal places, data smoothing was not adjusted from the instrument default settings. The automatic peak picking and integration parameters could not be optimised to fit all of the target peptide XICs in a single analytical run due to the large differences in instrument response for each target, therefore manual peak integration of MS^2 XICs was performed in the majority of cases. Furthermore, the patient samples proved to be very weak, sometimes beyond the detection limits of the instrument, which proved too difficult for the instrument software to perform peak integration reproducibly; therefore, some automatic peak integration had to be corrected manually to obtained linear response curves with a minimum R^2 value of 0.99 or greater.

Table 7.6 LCMS conditions in the analysis of patient samples for SEB, SEC1 and TSST-1.

Column	Shimpack XR-ODS (3.0 x 50 mm, 2.2 μm, 12.5 nm)
Oven Temperature	40°C
Mobile Phase A	water + 0.1% formic acid (v/v)
Mobile Phase B	Acetonitrile + 0.1% formic acid (v/v)
Gradient Program	0.00 min 5%B; 5.00 min 5%B; 50.00 min 30%B;

		50.01 100%B; 55.00 min 100%B; 55.01 min 5%B; 60.00 5%B		
Flow Rate		0.5 mL/min		
Flow Rate Gradient Program		0.00 min 0.5 mL/min; 50.00 min 0.5 mL/min; 50.01 1.0 mL/min; 55.00 min 1.0 mL/min; 55.01 min 0.5 mL/min; 60.00 0.5 mL/min		
Injection Volume		10 µL		
Ionisation Mode		ESI(+)		
Nebulising Gas		1.5 L/min		
Drying Gas Pressure		128 bar		
Probe Voltage		+4.5 kV		
CDL Temperature		200°C		
Heat Block Temperature		200°C		
MS Acquisition Parameters				
Segment 1	Event 1	400 – 1200	10	
0.00 – 5.00 min	Event 2	300 – 1200	200	423.55
Segment 2	Event 1	400 – 1200	10	
5.00 – 7.00 min	Event 2	300 – 1200	200	760.87
Segment 3	Event 1	400 – 1200	10	
7.00 – 10.00 min	Event 2	300 – 1200	200	565.27
Segment 4	Event 1	400 – 1200	10	
10.00 – 12.00 min	Event 2	300 – 1200	200	747.35
Segment 5	Event 1	400 – 1200	10	
12.00 – 15.00 min	Event 2	300 – 1200	200	789.90
Segment 6	Event 1	400 – 1200	10	
15.00 – 17.00 min	Event 2	300 – 1200	200	705.87
Segment 7	Event 1	400 – 1200	10	
17.00 – 18.50 min	Event 2	300 – 1200	200	552.80
Segment 8	Event 1	400 – 1200	10	
18.50 – 20.00 min	Event 2	300 – 1200	200	530.11
Segment 9	Event 1	400 – 1200	10	
20.00 – 22.00 min	Event 2	300 – 1200	200	485.75
Segment 10	Event 1	400 – 1200	10	
22.00 – 26.00 min	Event 2	300 – 1200	200	655.35
Segment 11	Event 1	400 – 1200	10	
26.00 – 30.00 min	Event 2	300 – 1200	200	775.41
Segment 12	Event 1	400 – 1200	10	
30.00 – 37.00 min	Event 2	300 – 1200	200	561.35
Segment 13	Event 1	400 – 1200	0.5	
37.00 – 60.00 mins	Event 2			

Table 7.7 Patient samples analysed by proteomics based MS method generated a list of observed peptides with accompanying assignment of fragment ions. The XIC for the product ion intensity in the MS^2 scan is integrated against an external calibration standard. Data field *blank* – no observed target signal;

Sample Name	Toxin Detected	Calc. Precursor [m/z]	Calc. Product Mass [m/z]	Conc [ng/mL]	Sample Name	Toxin Detected	Calc. Precursor [m/z]	Calc. Product Mass [m/z]	Conc [ng/mL]
LR014					**LF008**				
LR028	TSST-1	423.2456	746.4120	28.9	**LF009**	TSST-1	423.2456	746.4120	16.5
			631.3820	36.6				631.3820	5.1
			518.3030	31.2				518.3030	21.2
			404.2560	32.6				404.2560	11.2
			316.1940	41.7				316.1940	25.8
	TSST-1	789.3988	657.3460	104.5		SEC1	746.8493	936.4791	36.0
			575.2677	58.0				737.8447	100.8
			544.2619	116.3				574.7830	11.3
	TSST-1	560.8524	910.5614	125.0				468.7435	36.2
			809.5137	143.0		TSST-1	789.3988		47.6
			712.4609	77.9		SEC1	705.3672	847.4526	34.6
			599.3769	111.9				718.4100	30.1
			551.8478	793.1		SEC1	552.3008	917.4944	29.1
			470.3343	111.6				816.4468	13.4
			455.7846	149.9				745.4096	11.1
			405.2608	138.9				617.3511	14.0
			357.2502	111.6				543.2961	24.4
LR038	SEC1	746.8493						459.2512	11.7
	TSST-1	560.8524	910.5614	8.5				375.2244	17.6
			809.5137	3.5		TSST-1		910.5614	23.2
LR050	TSST-1		910.5614	7.3				809.5137	44.1
			809.5137	9.7				712.4609	38.2
			712.4609	6.3				599.3769	24.1
LR052	TSST-1	560.8524	809.5137	26.9				470.3343	40.5
			599.3769	27.9				405.2608	52.5
			405.2608	40.0	**LF010**	TSST-1	423.2456	746.4120	20.6
LR053	TSST-1	560.8524	910.5614	57.2				631.3820	52.4
			809.5137	75.6				518.3030	45.9
			712.4609	29.9				404.2560	62.8
			599.3769	61.4				316.1940	61.2
			551.8478	315.7		SEC1	746.8493	936.4791	26.9
			470.3343	59.8				737.8447	100.4
			405.2608	83.7				574.7830	23.8
			357.2502	96.2				526.2990	19.2

LR061								468.7435	23.1
LR073	TSST-1	423.2456	746.4120	38.2		TSST-1	789.3988	657.3460	94.4
			631.3820	46.3				544.2619	63.6
			518.3030	51.5		SEC1	705.3672	847.4526	15.9
			404.2560	45.3				718.4100	9.8
			316.1940	64.9				597.8225	27.2
	SEC1	746.8493	936.4791	13.9		SEC1	552.3008	917.4944	11.9
			737.8447	37.5				816.4468	12.5
LR073			574.7830	13.7	**LF010**			617.3511	8.0
	SEC1	552.3008	917.4944	16.6				543.2961	15.4
			844.4053	3.7				459.2512	10.4
			816.4468	7.2				375.2244	12.1
			745.4096	12.4		SEB		744.3787	2.3
			617.3511	7.3				687.8366	16.3
			543.2961	8.4				548.7915	11.7
			459.2512	7.1		TSST-1	560.8524	910.5614	13.4
			375.2244	8.9				809.5137	58.9
	TSST-1	560.8524	910.5614	94.2				712.4609	23.0
			809.5137	118.8				599.3769	48.7
			712.4609	107.6				551.8478	199.8
			599.3769	88.0				470.3343	48.2
			551.8478	631.4				405.2608	92.5
			470.3343	112.5	**LF011**				
			455.7846	75.6	**LF022**				
			405.2608	143.2	**LF023**				
			357.2502	126.8	**LF029**				
			312.1924	119.2	**LF030**	TSST-1		631.3820	
LR079								316.1940	4.5
LR083					**LF031**				2.9
LR110					**LF054**	TSST-1		631.3820	
LR111								518.3030	8.9
LR113								316.1940	6.6
LR133					**LF056**	TSST-1		746.4120	17.9
LR142								631.3820	6.1
LR151								518.3030	12.8
								316.1940	10.7
						SEC1	746.8493	936.4791	11.3
								737.8447	14.8
								574.7830	46.4
								468.7435	21.3
						TSST-1	789.3988	657.3460	17.8
						SEC1	552.3008	917.4944	56.6
								816.4468	12.0
								745.4096	10.0
								617.3511	15.8

								543.2961	9.2
								488.3085	17.2
								459.2512	15.3
								375.2244	8.9
						SEB	654.8433	909.4682	9.2
								780.4256	3.3
								667.3416	3.7
								552.3146	3.2
								389.2513	4.4
						TSST-1	560.8524	910.5614	2.6
								809.5137	6.1
								599.3769	36.6
								405.2608	8.9
					LF064				30.5

7.4 Detection

Four patient samples, including LR003, LR004 and LR008, which all displayed a positive response for at least one of SEB, SEC1 and TSST-1 *via* western blot, were analysed using the original 30 minute LCMS method described early. Unfortunately, no signals were observed in any of the samples, neither MS^1 peptides nor the corresponding MS^2 fragment ions, Figure 7.8. It was clear from the TIC data alone that sufficient biological material was present in the patient samples and in reasonably high concentrations. However, MS^2 scans gave no assignable fragment ions, and mining data for XICs of the peptide fragments described in Table 7.2, Table 7.3 and Table 7.4 gave no indication that the target peptides were eluting at an alternative retention time. LR004 spiked with a concentration of combined standard within the known detection range of the instrument yielded peak detection as predicted from the analysis of the toxin standards, retention time matching was achieved as expected.

Assuming that the patient samples did indeed contain a reasonable quantity of the target peptide mixtures, instrument and method sensitivity was the likely candidate for lack of signal. Ion suppression from matrix components is an understood issue for proteomics work in complex matrices (Callahan, 2006). Improving separation of the sample mixture could reduce co-elution of target compounds with sample matrix components. Further development of the method resulted, as described earlier; extension of the LC gradient time from 30 to 60 minutes was hypothetically chosen to improve method sensitivity. However, whilst this hypothesis was not validated, further studies with more concentrated samples yielded useful results.

The next batch of patient samples were selected because they gave the most concentrated positive response in the western blot analysis, coupled with a possible reduction in ion suppression; these next batch of samples, Table 7.7 and Table 7.8, returned positive confirmation for the presence of toxin peptides.

TSST-1 was the most commonly observed toxin, LR028, LR038, LR050, LR052, LR053, LR073, LF009, LF010, LF054, LF056. Peptides corresponding to SEC1 were observed in three patient samples LF009, LF010, and LF056; whilst SEB toxin was observed only twice at best, LF010 and LF056. Observed peaks in MS^1 and MS^2 scans with estimated quantitation, Table 7.7, and observed peaks with mass accuracy against theoretical values, Table 7.8, are reported. However, partial reporting of data, see LF030 Table 7.7, or blank entries, see LR151, had limited either evidence of toxin peptides or no evidence respectively.

The concentration of toxin peptides within the patient samples was low in all cases, which made detection difficult, and perhaps ambiguous in a few samples, see LF022, LF023 and LF029 in Table 7.8. Sensitivity of the analysis was complicated further by

the presence of multiple co-eluting compounds from the sample matrix. Figure 7.9 and Figure 7.10 show typical TIC and XIC data obtained from some of the higher concentration batch of samples, the cluster of MS^2 peaks from the XICs of target fragment ions shows the presence of tryptic peptide material indicative of a specific toxin compound. Noise, or isobaric peaks in MS^2 XIC data is not uncommon in proteomics based analysis; Figure 7.9 contains an intense MS^1 peak in blue corresponding to a match with the measured 423.25 m/z, a targeted peptide mass. However, multiple co-eluting MS^2 peaks with a retention time consistent with a peptide from a standard compound is observed to elute later at 1.5 min, the multiple points of confirmation gave certainty to the assignment of the detection of a given toxin in a patient sample. Similar data are shown in Figure 7.10. Figure 7.11 shows the assignment of MS^2 product ions that match *in silico* fragmentation products for the proteotypic peptide of TSST-1. In this case, six points of confirmation are obtained, peak retention time matching against known standards, MS^1 mass confirmation (typically sub 10 ppm, but more discussion is required, see Table 7.8), MS^2 matching of multiple fragment ions each with accurate mass confirmation (sub-10 ppm would be ideal, see Table 7.8). The complete method of assignment is observed in Figure 7.12, Figure 7.13 and Figure 7.14.

As mentioned above, an accurate mass measurement was used to assign elemental composition consistent with the peptides derived from toxins in patient samples. It is common to apply a requirement of a sub-10 ppm measurement error to such an assignment (Webb, 2004). The data shown in Table 7.8 occasionally assigns target peaks with a measurement error as high as 25 ppm, and whilst this is not ideal, a number of factors are known to affect the accuracy of mass measurements in time-of-flight instruments. In this instance both the temperature stability of the lab (a current

known issue) and the intensity of the measured ions play an important role in observed mass accuracy. An excessively weak signal can yield poor ion statistics and can therefore be unreliable; low intensity peaks, such as those observed in some of the samples reported here, are known to suffer from poor mass accuracy (Webb, 2004).

Signal-to-noise levels of ions are not always above the 3:1 level required to define lower limit of detection, but in most cases at least three diagnostic ions with reasonable mass accuracy are observed, reported in Table 7.8.

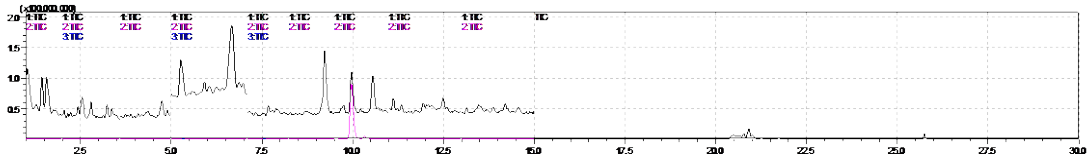


Figure 7.9 LCMS chromatogram of LR004 patient sample; the black trace represents the TIC for the MS^1 scans, whilst the pink trace represents the TIC for MS^2 scans (Event 2 of each Segment only). The sample contains a significant quantity of peptide like material, as shown in the intensity of Event 1 (MS^1) TIC. None of the detected material matched well with targeted method for detection of the toxins.

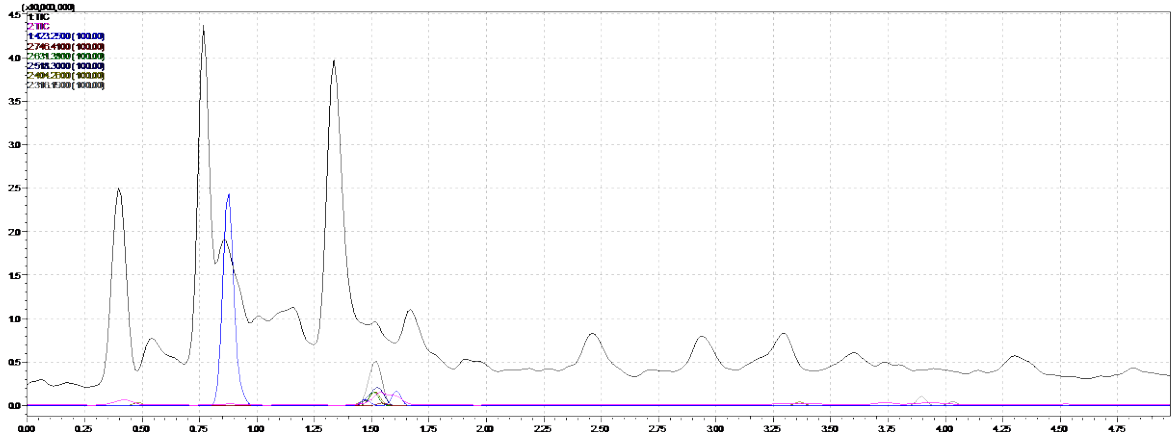


Figure 7.10 LCMS chromatogram of LR053 Segment 1 showing the total ion chromatogram (TIC) for MS^1 full scan (black), the TIC MS^2 scan from precursor 423.75 ± 3 Da (t_R = 1.56 min); all other colours represent XICs of targeted y/b fragment ions from MS^2 of precursor mass. XIC data enhanced x100.00 to improve visualisation, therefore a weak cluster of fragment peaks confirming the presence of TSST-1 peptide VDLNTKR.

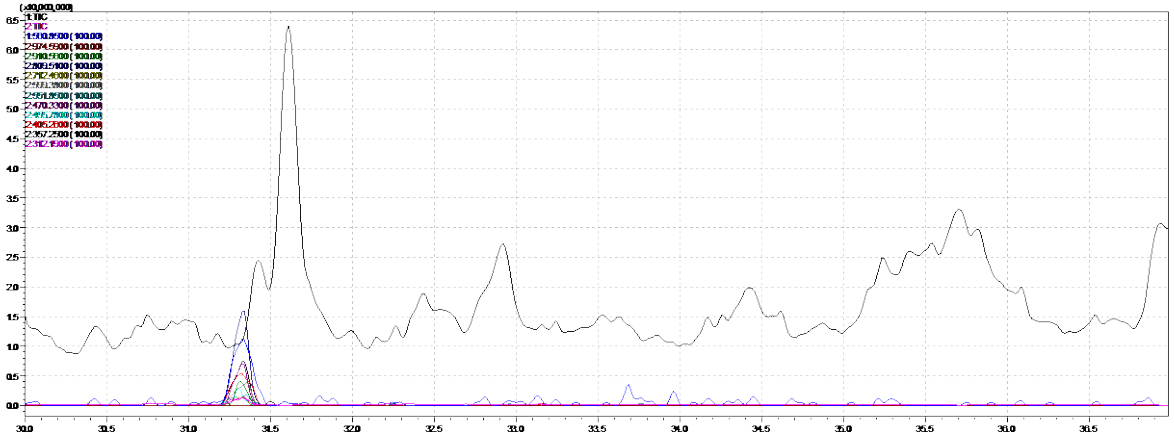


Figure 7.11 LCMS chromatogram of LR053 Segment 12 showing TIC for MS^1 full scan (black); TIC MS^2 scan for precursor mass 561.3524 ± 3 Da (t_R = 31.4 min); all other colours represent XIC of targeted y/b fragment ions from MS^2 of precursor. XIC data enhanced x100.00 to improve visualisation, therefore a weak cluster of fragment peaks confirming the presence of TSST-1 proteotypic peptide LPTPIELPLK.

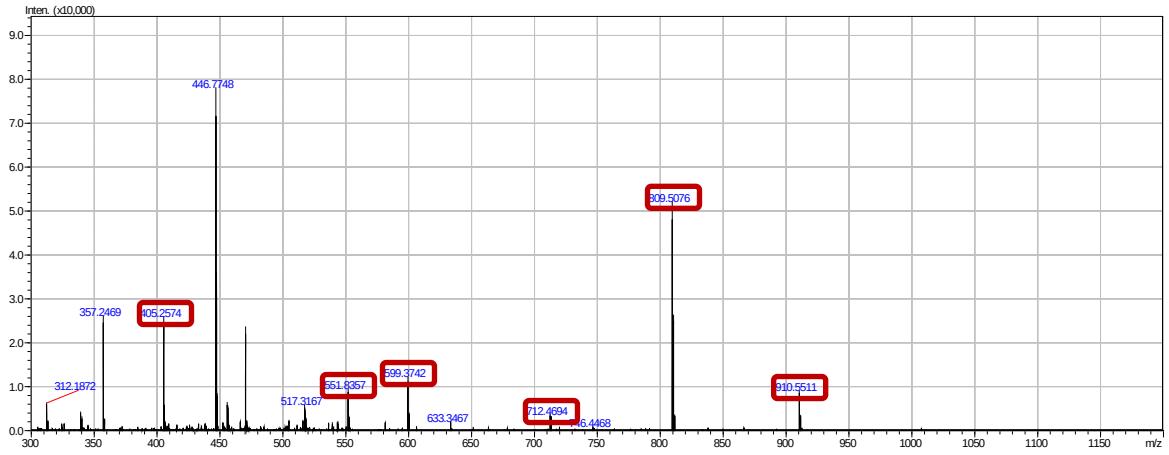


Figure 7.12 LR053 Segment 12; MS^2 full scan of product ions from fragmentation experiments of 561.35 ± 3 Da precursor. Highlighted peaks represent ions matched with theoretical y/b fragment ions for the proteotypic peptide LPTPIELPLK of TSST-1.

No attempt was made to calculate a lower limit of detection in this project. The use of external standards and the calibration curve used to generate the approximations in concentration were less than ideal. However, a lower limit of detection was applied with some basic assumptions and the understanding of likely inaccuracy. Data presented in Table 7.7 and Table 7.8 contains various comments relating to observations made about the intensity of target peaks within these sample data; this

approach formed the basis of how the lower limit of detection was estimated. For example, MS^1 and MS^2 XICs for LR050 displayed peaks capable of reasonable integration using the instrument software; however, it was not possible to locate the peak responsible in the averaged mass spectrum to assign mass accuracy. On such occasions, signals were considered too weak to positively confirm the presence of a target compound. Further complications to this approach were evident from the method of data collection used for MS^1 and MS^2 scans respectively. On occasion MS^1 scans were too weak to assign an observed mass to the target, whereas the high ion accumulation times used in the MS^2 scans, see Table 7.6, meant that ion intensity of MS^2 fragments were much higher and thus provided target confirmation. On such occasions, MS^2 fragment detection and assignment was sufficient to consider the target peak as being above the limit of detection. From this approach, the lower limit of detection (LLOD) of target peptides in the LCMS samples was 10ng/ml. Quoted values in the literature use more stringent approaches to determine the LLOD and values such as 80-100ng (Callahan, 2006) have been reported; although much depends on the particulars of the experimental procedure including type and manufacturer of the analytical instrument, the extent of sample preparation and the accompanying sample loss, to a name only a few obvious variants.

It is noteworthy, that whilst the LC gradient conditions were optimised to create maximum resolution between eluting target peptides, the method could not be further developed to remove co-elution of background matrix compounds from the patient samples prior to them being run. As a consequence it is likely that the co-elution of multiple matrix components will influence the ionisation efficiency of the target peptides and thus make any quantitative data obtained via an external calibration method uncertain.

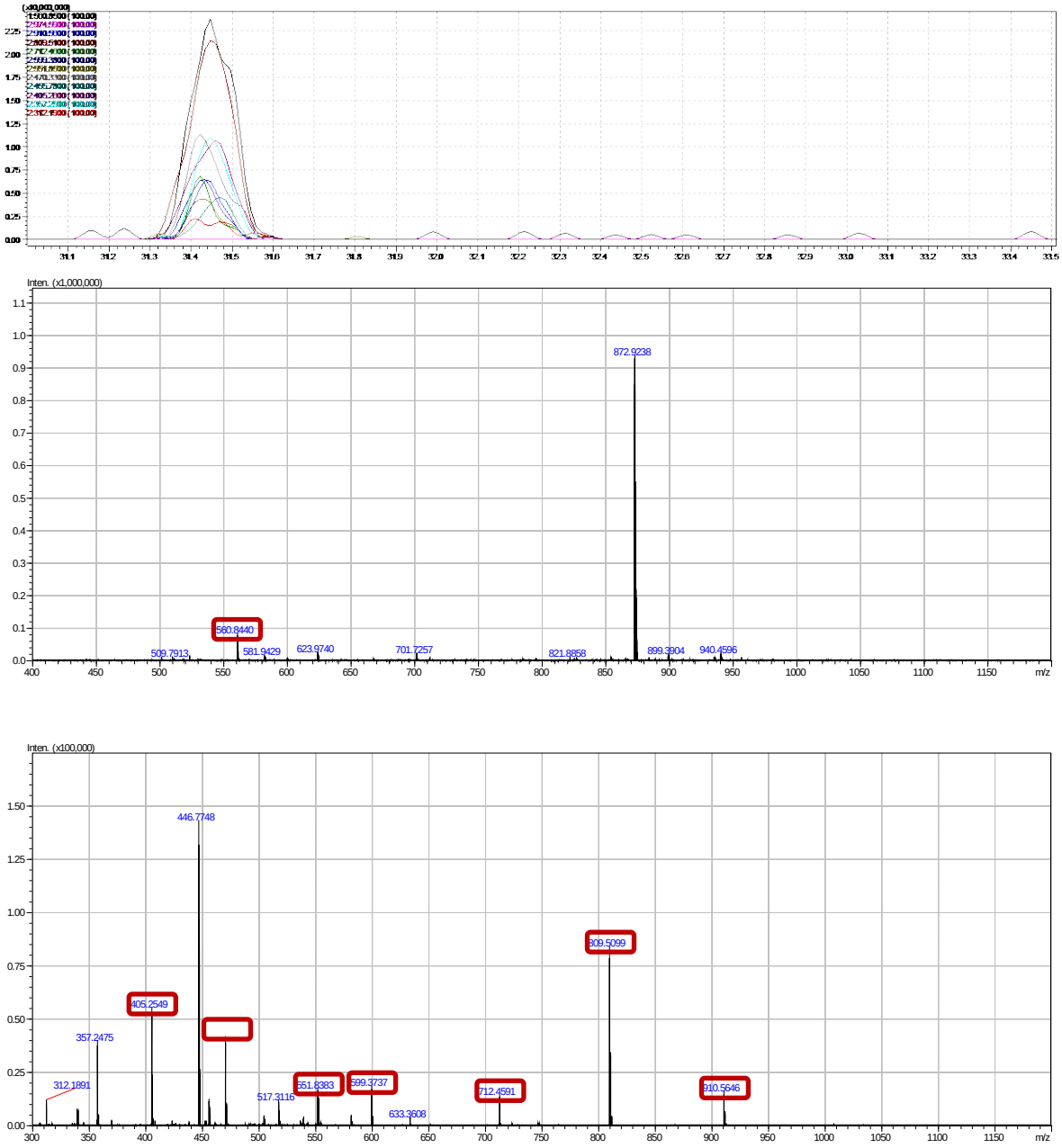


Figure 7.13 LR073 Segment 12 XIC of MS^1 and MS^2 targets (top); MS^1 scan of peptide mass 560.85 Da (middle); MS^2 precursor 561.35 + 3 Da, 10 separate product ions matched to predict y/b fragments (bottom). Three separate data sets confirming the presence of TSST-1 proteotypic peptide LPTPIELPLK.

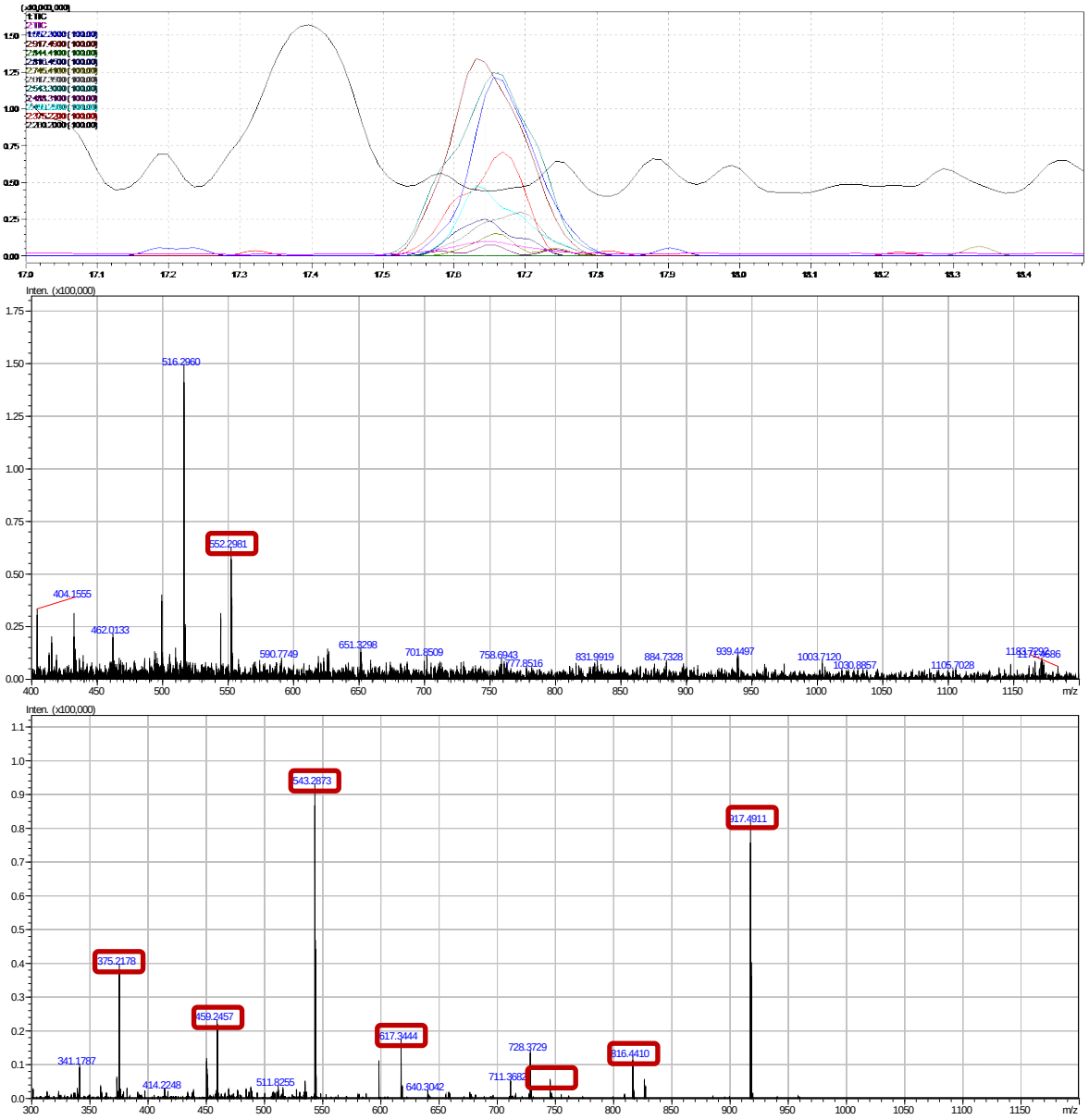


Figure 7.14 LF009 Segment 7 TIC (black line) with enhanced (x100) XIC of MS^1 and MS^2 targets (top); MS^1 scan of peptide mass 552.30 Da (middle); MS^2 precursor 552.80 + 3 Da, 7 separate product ions matched to predict y/b fragments (bottom). Three separate data sets confirming the presence of SEC1 peptide SVTAQELDIK.

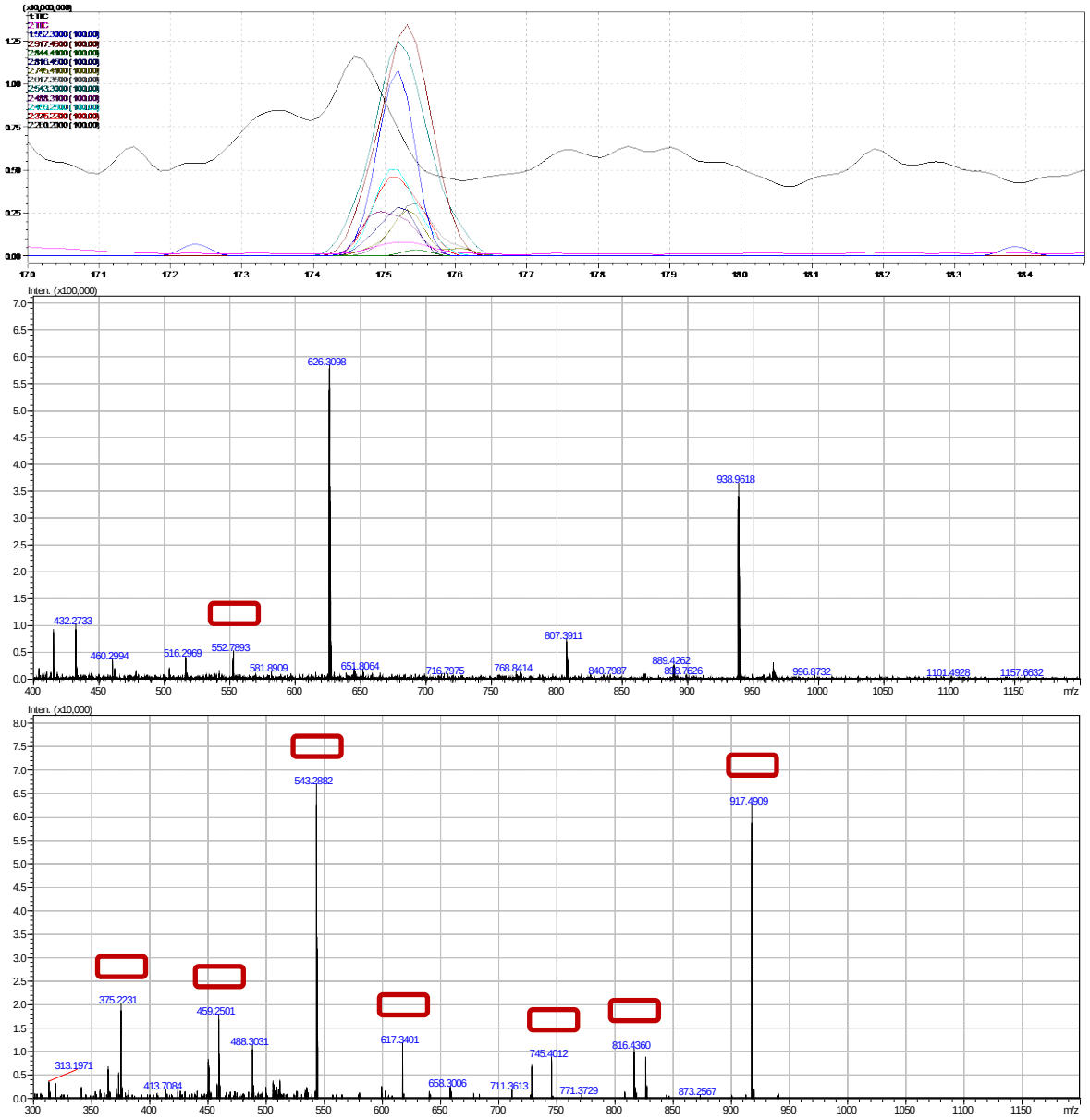


Figure 7.15 LF056 Segment 7 TIC (black line) with enhanced (x100) XIC of MS^1 and MS^2 targets (top); MS^1 scan of peptide mass 552.30 Da (middle); MS^2 precursor 552.80 + 3 Da, 7 separate product ions matched to predict y/b fragments (bottom). Three separate data sets confirming the presence of SEC1 peptide SVTAQELDIK.

7.5 Conclusion

An LCMS method for the sensitive and selective detection of toxins SEB, SEC1 and TSST-1 has been developed using an ion-trap time-of-flight mass spectrometer. The LCMS-IT-TOF allows the detection of target peptides with a high degree of certainty as a large number of fragment ions are unequivocally assigned simultaneously due to the time-of-flight detection. This approach using similar instruments in often referred to as parallel reaction monitoring.

The results directly confirm molecular identity of toxin peptides in samples where immune assay tests have already returned a positive result. However, the detection of toxin peptides was not confirmed in all samples analysed creating some doubt as to whether the current analytical method is of sufficient sensitivity to detect the relatively lower concentrations of toxin peptides found in patient samples. Whilst the limits of detection for the target peptides have not been calculated, standard compounds demonstrated a detection limit of approximately 5ng/ml in LCMS samples. This appears to be consistent with MS examples in the literature where selected ion monitoring (SIM) and multiple reaction monitoring (MRM) has been used with triple quadrupole mass detection to achieve LLOD values of 5 – 10 ng/ml (Callahan, 2006), but are higher than detection limits already observed in biological based assays.

Toxin standards used to investigate the instrument's response were prepared from purified compounds and therefore not matrix matched with the patient samples which are the focus of this study. The complex sample matrices observed in the patient samples was likely to have had a significant influence on the ionisation efficiency of the target peptide as they are eluted from the LC column, quantitative data derived

from this external calibration method will be inaccurate as a result. Alternative approaches utilising internal standards have been reported (Adrait, 2012; Dupuis, 2008; Dupre, 2015) that overcome this issue, but such an approach was beyond the capabilities of this project. However, using isotopically labelled synthetic internal standards, literature examples have reported LLOD for toxin peptides ~0.4ng/ml (Adrait, 2012).

Further improvements to the sensitivity of the analytical method can be made through judicial adjustments made to sample preparation and some of the LCMS parameters. The principle limitation of the current technique is likely to be the ion suppression effects induced by the high levels of background signals from the complicated matrix. Efficient sample preparation prior to mass spectrometric analysis is essential to counteract ion suppression/matrix effects observed for complex biological samples. Therefore, decomplexification of the patient samples coupled with improvements in LC methods, such as nanoLCMS (Callahan, 2016), could yield improved LLODs.

Chapter 8 DISCUSSION & CONCLUSION

8.1 Staphylococcal Toxins in Urine

Chronic autoimmune diseases are the consequence of the immune system recognising self-antigens as foreign, leading to inflammation and destruction of specific tissues and organs (Abou-Raya, 2006; Cusick, 2012).

Table 8.1 Summary of *S.aureus* presence in human samples. Comparison of general population, medical students, acute ischemic heart disease (AIHD) (on admission & 6 week follow-up), RA and closed fractures.

Source	Sample Type	Positive for *S.aureus*
General population (Published literature)	Nasal/nasopharyngeal swabs	30.0%
Medics (Bull,2014)	Urine	18.0%
AIHD –admission (Bull,2014)	Urine	29.0%
AIHD – 6 weeks (Bull, 2014)	Urine	19.0%
RA patients	Urine	56.4%
Fracture patients	Urine	27.1%

In the patient cohorts that we analysed, *S.aureus* was found to be present in 56.4% of RA patients and 27.1% of fracture patients. Similar work by Bull *et al*. looked for the presence of *S.aureus* toxin in medical students and patients suffering an episode of acute ischaemic heart disease (AIHD) (Bull, 2014). A urine sample was taken from the AIHD patients, in the same way as for the RA/fracture study, before any intervention in A+E (AIHD UA samples). A further sample was taken at an outpatient clinic appointment 6 weeks later (AIHD UB sample). The AIHD UB sample would represent a sample taken under similar conditions to the RA and fracture patients as all three populations have attended scheduled appointments as outpatients. The population of medical students consisted of 50 individuals between 20-25 years old. It was assumed that by this age and following a lot of time spent in the hospital environment due to their education and training, this population would most likely have been infected or colonised by *S.aureus* at some point.

The positive results were as follows: medical students (18%); AIHD UA (29%); AIHD UB (19%) (Bull, 2014). The results for the medical students and AIHD UB samples were very similar, which is understandable as an AIHD patient 6 weeks post-episode would probably be returning back towards the health of a healthy individual, which is the group that the medical students represent. Both of these populations would have attended hospital, giving them a similar amount of exposure.

Similarly, 27.1% of fracture patients and 29% of AIHD patients (on admission) were shown to have *S.aureus* toxins in their urine. Although we do not have data for exactly how long after the fracture each and every sample was taken, most were asked to take part within a week of the initial trauma. It can be said that these two populations are also comparable as they are both at the initial contact stage with the hospital.

Although there is a substantial difference between the numbers of patients testing positive for staphylococcal enterotoxin in each of our populations (RA and fracture), the question must be asked; why does at least one quarter of the patients appear to have toxin present in their urine (RA, fracture and AIHD UA)? This observation suggests that an inflammation event – acute or chronic – increases the chance of an individual testing positive for staphylococcal enterotoxin(s) in their urine. Using current evidence, we can only speculate, however there are a couple of potentially credible explanations.

The kidney filters small molecules such as glucose, urea, ions and water into the nephrons. However, larger molecules are too big to diffuse through the capillary membrane and therefore remain in the blood. We hypothesise that: (i) the opportunistic bacteria are more of a burden when under stress and/or; (ii) the immune cells are elsewhere in the body.

Neutrophils originate in the bone marrow and circulate in the blood for approximately 10 hours before exiting into the tissues and onto the body surface. During their time in the blood stream, the neutrophils travel more slowly in the post capillary venules than the RBCs. Therefore, the neutrophils become more concentrated in the post capillary venules than in the rest of the circulation. Since all blood goes through the lungs, this means that up to 50% of the neutrophils are in the post capillary venules of the lung at any one time (Morris, 2007). This system efficiently clears bacteria from the blood as the bacteria pass through the dense concentration of neutrophils in the lungs, giving the neutrophils the opportunity to phagocytose and destroy the bacteria.

During a fever or an inflammation event (acute or chronic), there is more demand put on the immune system. Fever changes the dynamics of the circulation in some way so

that the neutrophils and the RBCs travel at the same rate and so the neutrophils are not concentrated in the post capillary venules but are more evenly spread within the blood (Morris, 2007). This is an explanation for why the white cell count rises during infection. However, this does mean that bacteria will not be cleared from the lungs/blood as efficiently. Thus, if bacteraemia is common then bacteria are likely to stay in the circulation longer in patients with fever. Furthermore, lung complaints and diseases and been linked as a major comorbidity in RA. In particular, interstitial lung disease (ILD). Bongartz *et al.* studied 1185 people with and without RA; 7.7% of the individuals with RA also had ILD, compared to 0.9% of the healthy controls (Bongartz, 2010).

8.2 Infection

8.2.1 Cause or Consequence?

For decades, it has been known that there is an increased risk of serious infection in people with RA. Observational studies have shown at least a 2-fold increased risk of serious infection in RA, subsequently supported by numerous cohort studies (Doran[b], 2002; Franklin, 2007; Smitten, 2008). The heightened susceptibility of patients with RA can be explained by the pathobiology of the disease itself, the impact of chronic comorbid conditions and the nature of the immunosuppressive treatments used (Listing, 2013). It is considered that premature ageing of the immune system in RA

contributes to weakened protection against infectious organisms. Additional chronic comorbidities and lifestyle factors increase the risk in individual patients.

Furthermore, there is an increased risk of infection in RA patients treated with drugs that inhibit TNF-α. For example, TNF inhibitors were already showing a strong association with an increased risk of TB in RA, soon after licensing (Keane, 2001; Mohan, 2004). However, there is relatively little known about the effect of TNF-α inhibitor therapy and the reactivation of latent infections (Strangfeld, 2009).

The inhibition of TNF-α in patients with active RA, has proved to be an effective treatment, particularly as a vital alternative for patients whose disease is insufficiently controlled by DMARDs. Similarly, patients with alkylosing spondylitis, psoriatic arthritis and inflammatory bowel disease are also seeing the benefit of treatments using new biologics, including TNF-α inhibitors. Due to the relatively recent introduction of biologic treatments, little is known about the long term effects. In time, this will be overcome by the biologics registers that have been set up to keep track of all patients treated using biologic drugs; for example, BSRBR-RA in the UK and RABBIT in Germany.

Immunosuppression and ageing both contribute to a decline in cellular immunity which has been linked to the reactivation of latent varicella zoster virus (VZV) (Thomas, 2004). Immunodeficiency in general has shown to have a strong link to an increase (Veenstra, 1995), recipients of bone transplants (Locksley, 1985) and children with leukaemia (Glynn, 1990; Kost, 1996).

Treatment using monoclonal anti-TNF-α antibodies (infliximab and adalimumab), has shown to increase the incidence of herpes zoster (Strangfeld, 2009). However, etanercept, a fusion protein, does not appear to give an increased risk of herpes zoster.

Herpes zoster is a neurocutaneous disease caused by VZV, which can be reactivated after the initial infection. The disease is characterized by a painful vesicular dermatomal rash and is one of the most common adverse events reported in clinical trials of anti–TNF-α biologics. Subsequent complications include bacterial superinfection.

The use of GCs as an immunosuppressant have been shown to increase the risk of infection 4-fold (in a dose dependent manner) (Listing, 2013). The use of GCs and TNF inhibitors together results in a significantly elevated risk of infection for the patient. This combination is discouraged, particularly in populations with additional risk factors such as age.

Listing *et al.* compared the rates of infections in patients treated with biologic agents with the rates in patients receiving conventional DMARDs. It was observed that there were considerably more infections in patients treated with etanercept or infliximab. In the control group however, the incidence of serious infections was 2.7–2.8 times lower than in patients treated using biologic agents. Their data suggest, as in many other studies, that the major contributor to the increased incidence of infection is probably caused by TNF inhibitors. In particular, there was the most concern for lower respiratory tract infections (especially pneumonia), bacterial skin infections (erysipelas), and bone and joint infections (Listing, 2005). In relation to this study, S.aureus is known to cause infections in all of these sites, therefore indicating that infection is a consequence of RA.

RA has been linked with a noticeable risk of infectious diseases, based on observational studies over the last 60 years (Cobb et al., 1953; Uddin et al., 1970). Controlled observational studies found that mortality in RA patients (age-adjusted)

was about 2-fold higher compared with the general population; where infectious disease proved to be one of the three leading causes of premature death - in the USA and in Europe (Wolfe, 1994; Perez-Sola, 2011; Mutru, 1985; Mikuls, 2002).

Transient, usually asymptomatic bacteraemia occurs in a wide variety of procedures and manipulations, particularly those associated with mucous membrane trauma (Everett, 1970). Interventions such as tonsillectomy, tracheal intubation, bronchoscopy, sigmoidoscopy, barium enema and liver biopsy can lead to bacteraemia. It is most likely that S.aureus gets into the body via the lungs, genitourinary tract, a break in the skin or through the mucosa (Sullivan, 1973; Everett, 1977; Aryee, 2016).

8.2.2 TNF-α

TNF plays a central role within a complicated network of cytokines. TNF is a pro-inflammatory cytokine and is involved in cellular homeostasis including cellular communication, differentiation and apoptotic death. Furthermore, TNF has been widely linked to the development of cancer and autoimmunity, due to its diverse functions and subsequent effects (Brenner, 2015). TNF was first associated with tumour regression and with cachexia accompanying chronic, invasive diseases (Keffer, 1991). TNF is mainly a product of macrophage activation and has been established as an immune regulator in both inflammatory and normal states (Beutler, 1989). A number of other cell types are known to produce TNF but the quantities are much less. Examples include activated T cells (Steffen, 1988; Kinkhabwala, 1990), natural killer cells (Peters, 1986) and mast cells (Gordon, 1990). Conversely, almost

every cell type in the human body is found to express a TNF specific plasma membrane receptor (Schall, 1990; Loetscher, 1990). Deregulation of its receptor, TNFR, and changes to the signalling pathway have been attributed to a number of inflammatory illnesses, including various types of arthritis. Deregulated production of TNF itself in humans is known to contribute to the development of diseases such as cancer-associated cachexia (Oliff, 1987), endotoxic shock (Beutler, 1985), graft versus host disease (Piguet, 1987), autoimmunity (Held, 1990) and RA (Saxne, 1988; Yocum, 1989).

TNF-α was first described, in relation to inflammatory disease, by George Kollias and colleagues at the Hellenic Pasteur Institute, Athens in 1991(Keffer, 1991). Using transgenic mouse models, it was demonstrated that TNF played a role in arthritis and that the blocking of TNF-associated signalling pathways using a monoclonal antibody to human TNF completely prevented disease development.

Inflammation, characteristic of RA, is localised in the synovial lining, a monolayer of synovial cells that lines diarthroidal joints. In a diseased state, the synovial lining becomes thickened due to synovial cell proliferation and infiltration by inflammatory cells, such as macrophages (Lubberts, 2000). The pannus is the subsequent proliferative mass that invades and destroys articular cartilage and bone, leading to irreversible destruction of joint structure and function and ultimately a significant amount of pain (Lubberts, 2000). Several recent studies have implicated cytokines in the pathogenesis of this disease. Elevated levels of TNF (Saxne, 1988; Di Giovine, 1988) as well as IL-1 (Nouri, 1984) and IL-6 (Hirano, 1988) are consistently found in the synovial fluid of RA patients. Additionally, *in vitro* models of human synovial fibroblast cell lines have been shown to proliferate when triggered by rTNF and rIL-1 (Gitter, 1989; Butler, 1988).

8.2.3 ACPA and RF

Mori *et al.* evaluated the utility of ACPA (second generation, anti-CCP2) as a diagnostic marker for RA in patients with active tuberculosis. Among patients with active tuberculosis, anti-CCP2 was detected in six (6.7%). The rate of anti-CCP2 in patients with newly diagnosed RA was 82.1% versus 0.4% in healthy controls. Additionally, IgM RF was detected in 18% of the tuberculosis group and 72.6% of the RA group (Mori, 2009).

8.2.4 Molecular Mimicry

There are a number of suggested mechanisms which infer that infections can initiate and/or exacerbate autoimmune diseases. One of the most discussed mechanisms is molecular mimicry. This hypothesis describes when a foreign protein/antigen shares sequence homology or structural similarities to the human self-antigens, thus mimicking the native protein (Fujinami, 1985).

It has been indicated that at most, there are 30,000 protein coding genes in the human genome (Baltimore, 2001); but they are thought to code for in excess of 250,000 proteins (Morris, 2012). By comparison, bacteria have 3000 to 5000 genes coding for approximately 3000 to 5000 proteins (Tlaskalova-Hogenova, 2011). There are approximately 400 different species of bacteria that can make up the commensal gut flora and any individual can carry about 40 at any one time (Moore, 1974; Moore, 1978). Therefore, the complexity of the proteome of the microbial flora is of the same

order of magnitude as that of the human body. Additionally, proteins that perform essential functions are conserved in evolution and since all forms of life are related, there will be considerable mimicry between human and bacterial proteins (Morris, 2007). Therefore, it may be assumed that for every protein within a human cell membrane, there will be a bacterial product capable of combining with it and switching the function it controls, on or off (Morris, 2004).

Molecular mimicry has typically been characterised by the cross-reaction of autoreactive B cells and/or T cells. However, it has been found that structural relatedness between pathogen and self-protein, does not account for T cell activation in a number of autoimmune diseases (Cusick, 2012).

Another proposed mechanism, possibly misinterpreted for molecular mimicry, is the expression of dual TCRs on a single T cell. Such T cells have dual reactivity to both foreign and self-antigens leaving the host vulnerable to foreign insults capable of triggering an autoimmune response (Cusick, 2012).

Morris *et al.* discussed molecular mimicry in irritable bowel syndrome (IBS), chronic fatigue syndrome (CFS) and anorexia nervosa (AN), which have a number of features in common, with not only each other but RA (Morris, 2016).

1. They are much more common in women than in men.

2. The onset of the disorder is most likely to occur in the middle years. The age incidence rises to a peak in the second, third or fourth decades, depending on the specific condition. The incidence then declines and onset in old age is uncommon.

3. There is some evidence that they are becoming more common with technological, social and economic progress in society.

4. There are suggestions that infection can precipitate and exacerbate the conditions but the links remain somewhat tenuous.

The immune system's job is complex. 20 amino acids can combine in ten trillion ways to form a polypeptide chain ten amino acids long ($20^{10} = 10^{13}$) (Morris, 2016). From the human and bacterial proteomes, there is probably fewer than 1 million proteins, which are on average, 300 amino acids in length. Therefore, the total number of polypeptides of length 10 amino acids is less than 10^9. T cell receptors are the main APCs and it is their job to efficiently differentiate similar to self from different than self (Morris, 2016).

8.2.5 Evasion of the Immune System

The evasion of pathogens from the immune system is not a new concept (Finlay, 2006). This phenomenon has been described in relation to viruses, bacteria and parasites; for example, HIV, TB and malaria (Morens, 2004; Fauci, 2005). *S.aureus* has also been described as having developed novel immune evasion phenotypes (Richards, 2015).

8.3 Questionnaires

There is no doubt that increased age plays a role in the increased incidence of infection (Strangfeld, 2011; Galloway, 2011; Laube, 2004).This is further exacerbated

by comorbidities which significantly increase the risk; for example, chronic obstructive pulmonary disease and other chronic lung diseases (Strangfeld, 2011; Dixon, 2006; Curtis, 2007; Greenberg, 2010), chronic kidney diseases (Strangfeld, 2011; Curtis, 2007) and diabetes mellitus (Curtis, 2007; Greenberg, 2010).

Smoking is the most potent lifestyle risk factor. It is linked to the pathogenesis of RA (Symmons, 1997; Klareskog, 2006) and at the same time is a risk factor for infectious diseases, not only in RA but in the general population (Stampfli, 2009). A complex immune defence system protects us against harmful agents and pathogens. Exposure to cigarette smoke directly or indirectly, distinctly affects the immune system; this compromises the host's ability to mount the appropriate immune and inflammatory response. These adverse effects on the human immune system not only occur in active smokers, but also in passive smokers. Such effects can persist for decades after exposure has ended (Stampfli, 2009). Quirke *et al.* have described bronchiectasis (BR) as a potent model for autoimmunity induced by chronic bacterial infection (Quirke, 2015). Subsequent periodontis and smoking-induced lung inflammation gives rise to increased levels of antibodies to un-citrullinated RA antigens and citrullinated proteins. The study authors suggest that the ACPA response is not citrulline specific throughout the initial stages of tolerance breakdown but that it becomes more specific in patients with BR in whom BR/RA develops (Quirke, 2015).

8.4 Conclusion

Overall, the presence of *S.aureus* in the urine of patients with RA or closed fractures, without any active infection, is clearly raised. The RA patients in particular showed an overwhelming presence of staphylococcal enterotoxins in their urine. The immune system of RA patients is known to be considerably suppressed by the current treatments available (Murphy, 2008), however both populations can be affected by the immunosuppressive effects of staphylococcal SAgs (Ferens, 1998). There is evidence that suggests that toxins produced by *S.aureus* are capable of initiating and maintaining an inflammatory event. Furthermore there is opportunity for the immune system to deregulate and lead to the development of autoimmune disease. An immune response instigated by staphylococcal toxins causes the release of a range of pro-inflammatory cytokines and their effects can be systemic. Additionally, inflammation may play a role in controlling the amount of toxin that enters the urine; especially if the toxins are part of larger immune complexes. Such immune complexes may be too large to be filtered into the urine by the kidneys under normal conditions.

The strength of this study lies in the novel techniques developed and the new, original datasets produced. Using a well-defined western blot protocol, it was possible to design a process that could screen each patient urine sample for all four staphylococcal enterotoxins. Subsequently, building on a protocol for the preparation of urine samples for analysis by mass spectrometry, a complex technique was developed to process the patient urine samples ready to be run through the LCMS-IT-TOF.

This is the first time that high levels of *S.aureus* toxins have been detected in RA patients. It is also the first time that the increased presence of *S.aureus* in RA patients has been described using a technique to analyse their urine. Additionally, the data collected from the fracture patient cohort has given us novel findings on the presence of *S.aureus* toxins in the urine of an out-patient population with closed fractures. Furthermore, a LCMS technique was developed that was able to detect proteotypic staphylococcal peptides in urine.

However, there were some limitations within this study:

- Samples were collected on different days and at varying times of the day.
- The fracture patients were, on average, slightly younger so may have more bacterial exposure.
- Similarly, the fracture patients, depending on what bone they had fractured, are probably more mobile than the RA patients – giving them the ability to be exposed to more pathogens.
- 'No infection' was based on the lack of symptoms confirmed by each individual's opinion of their own health status. We would only be able to confirm no infection for sure if we screened each patient but this would take a lot of time and be very expensive.

It is unlikely that *S.aureus* alone can be the cause of RA but there is sufficient evidence that suggests that it could be a contributor. The bacterium may be a factor alongside the other pathogens discussed: EBV, *P. gingivalis* or *P.mirabilis*; genetics and/ or lifestyle factors, in particular, smoking.

8.5 Future Work

Given more time, the MS method should be developed further. The initial work carried out, and data collected, has shown that the methodology works. With more tests and further changes of the parameters, the method could become automated and more sensitive.

It remains unclear as to whether *S.aureus* is a cause or consequence of RA but there is considerable evidence so far to support either. There needs to be substantially more research concerning the primary data produced by this study. In relation to the aspect of infection, it would be interesting if the positive/negative *S.aureus* results were compared with each patient's treatment at the time of sample collection. This would show if there is any increased incidence of infection in the individuals being treated using new biologics or biosimilars. Furthermore, comparison of the *S.aureus* presence/absence with RF positivity and the presence of anti-CCPs also may or may not show an association with infection. We used the father's occupation of each patient to try and anticipate the SES of their childhood. This may not have been very accurate and it may be useful to include the patient's work/career history to see if what they do day-to-day has any bearing on their RA status.

Now that there is a well-defined western blot protocol to look for these toxins in urine, samples from other cohorts could be analysed to produce a large dataset, to compare the presence of *S.aureus* amongst controlled populations. With more data will come more clarity as to whether it is more likely that *S.aureus* is a cause rather than a consequence.

Appendix A

Table A1 Table to show the corresponding three and one letter codes for each amino acid.

Amino Acid Name	Three Letter Code	One Letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ilu	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Appendix B

B1 Amino Acid Sequence of AH

MKTRIVSSVTTTLLLGSILMNPVAGAADSDINIKTGTTDIGSNTTVKTGDLVTY
DKENGMHKKVFYSFIDDKNHNKKLLVIRTKGTIAGQYRVYSEEGANKSGLA
WPSAFKVQLQLPDNEVAQISDYYPRNSIDTKEYMSTLTYGFNGNVTGDDTGK
IGGLIGANVSIGHTLKYVQPDFKTILESPTDKKVGWKVIFNNMVNQNWGPYD
RDSWNPVYGNQLFMKTRNGSMKAADNFLDPNKASSLLSSGFSPDFATVITMD
RKASKQQTNIDVIYERVRDDYQLHWTSTNWKGTNTKDKWTDRSSERYKIDW
EKEEMTN 319

B2 Amino Acid Sequence of SEB

MYKRLFISHVILIFALILVISTPNVLAESQPDPKPDELHKSSKFTGLMENMKVL
YDDNHVSAINVKSIDQFLYFDLIYSIKDTKLGNYDNVRVEFKNKDLADKYKD
KYVDVFGANYYYQCYFSKKTNDINSHQTDKRKTCMYGGVTEHNGNQLDKY
RSITVRVFEDGKNLLSFDVQTNKKKVTAQELDYLTRHYLVKNKKLYEFNNSP
YETGYIKFIENENSFWYDMMPAPGDKFDQSKYLMMYNDNKMVDSKDVKIE
VYLTTKKK 266

B3 Amino Acid Sequence of SEC1

MNKSRFISCVILIFALILVLFTPNVLAESQPDPTPDELHKASKFTGLMENMKVL
YDDHYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEGLAKKYK
DEVVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNL
QNVLIRVYENKRNTISFEVQTDKKSVTAQELDIKARNFLINKKNLYEFNSSPYE
TGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTVDSKSVKIEVH
LTTKNG 266

B4 Amino Acid Sequence of TSST-1

MNKKLLMNFFIVSPLLLATTATDFTPVPLSSNQIIKTAKASTNDNIKDLLDWYS
SGSDTFTNSEVLDNSLGSMRIKNTDGSISLIIFPSPYYSPAFTKGEKVDLNTKRT
KKSQHTSEGTYIHFQISGVTNTEKLPTPIELPLKVKVHGKDSPLKYGPKFDKKQ
LAISTLDFEIRHQLTQIHGLYRSSDKTGGYWKITMNDGSTYQSDLSKKFEYNT
EKPPINIDEIKTIEAEIN 234

B5 Amino Acid Sequence of SEC2

MNKSRFISCVILIFALILVLFTPNVLAESQPDPTPDELHKSSEFTGTMGNMKYL
YDDHYVSATKVMSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDLAKKYK

DEVVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNL
QNVLIRVYENKRNTISFEVQTDKKSVTAQELDIKARNFLINKKNLYEFNSSPYE
TGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTVDSKSVKIEVH
LTTKNG 266

B6 Amino Acid Sequence of SEC3

MYKRLFISRVILIFALILVISTPNVLAESQPDPMPDDLHKSSEFTGTMGNMKYL
YDDHYVSATKVKSVDKFLAHDLIYNISDKKLKNYDKVKTELLNEDLAKKYK
DEVVDVYGSNYYVNCYFSSKDNVGKVTGGKTCMYGGITKHEGNHFDNGNL
QNVLVRVYENKRNTISFEVQTDKKSVTAQELDIKARNFLINKKNLYEFNSSPY
ETGYIKFIENNGNTFWYDMMPAPGDKFDQSKYLMMYNDNKTVDSKSVKIEV
HLTTKNG 266

B7 Amino Acid Sequence of BSA

MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRFKDLGEEHFKGLVLIAFSQY
LQQCPFDEHVKLVNELTEFAKTCVADESHAGCEKSLHTLFGDELCKVASLRE
TYGDMADCCEKQEPERNECFLSHKDDSPDLPKLKPDPNTLCDEFKADEKKFW
GKYLYEIARRHPYFYAPELLYYANKYNGVFQECCQAEDKGACLLPKIETMRE
KVLASSARQRLRCASIQKFGERALKAWSVARLSQKFPKAEFVEVTKLVTDLT
KVHKECCHGDLLECADDRADLAKYICDNQDTISSKLKECCDKPLLEKSHCIAE
VEKDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGSFLYEYSRRHPEYAVS

VLLRLAKEYEATLEECCAKDDPHACYSTVFDKLKHLVDEPQNLIKQNCDQFE
KLGEYGFQNALIVRYTRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPCT
EDYLSLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPDETYVPKAFD
EKLFTFHADICTLPDTEKQIKKQTALVELLKHKPKATEEQLKTVMENFVAFVD
KCCAADDKEACFAVEGPKLVVSTQTALA 607

B8 Amino Acid Sequence of HA

MKWVTFISLLFLFSSAYSRGVFRRDAHKSEVAHRFKDLGEENFKALVLIAFAQ
YLQQCPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLR
ETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEE
TFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDEL
RDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLVT
DLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHC
IAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPD
YSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNC
ELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAK
RMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDET
YVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVM
DDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGL 600

Appendix C

C1 ExPASy: BSA & Trypsin

Position	Peptide Cleavage
45-65	GLVLIAFSQYLQQCPFDEHVK
03-19	WVTFISLLLLFSSAYSR
319-336	DAIPENLPPLTADFAEDK
169-183	HPYFYAPELLYYANK
529-544	LFTFHADICTLPDTEK
508-523	RPCFSALTPDETYVPK
469-482	MPCTEDYLSLILNR
184-197	YNGVFQECCQAEDK
267-280	ECCHGDLLECADDR
347-359	DAFLGSFLYEYSR
139-151	LKPDPNTLCDEFK
438-451	VPQVSTPTLVEVSR
387-399	DDPHACYSTVFDK
421-433	LGEYGFQNALIVR
569-580	TVMENFVAFVDK
375-386	EYEATLEECCAK
286-297	YICDNQDTISSK
106-117	ETYGDMADCCEK
89-100	SLHTLFGDELCK
76-88	TCVADESHAGCEK
402-412	HLVDEPQNLIK
361-371	HPEYAVSVLLR
300-309	ECCDKPLLEK
66-75	LVNELTEFAK
460-468	CCTKPESER
588-597	EACFAVEGPK
499-507	CCTESLVNR
310-318	SHCIAEVEK
549-557	QTALVELLK
413-420	QNCDQFEK
598-607	LVVSTQTALA
123-130	NECFLSHK
37-44	DLGEEHFK
161-167	YLYEIAR
249-256	AEFVEVTK
131-138	DDSPDLPK
483-489	LCVLHEK
562-568	ATEEQLK
257-263	LVTDLTK
341-346	NYQEAK
581-587	CCAADDK

29-34	SEIAHR
212-218	VLASSAR
198-204	GACLLPK
236-241	AWSVAR
490-495	TPVSEK
118-122	QEPER
205-209	IETMR
223-228	CASIQK
524-528	AFDEK
101-105	VASLR
157-160	FWGK
281-285	ADLAK
558-561	HKPK
229-232	FGER
25-28	DTHK

C2 ExPASy: HA & Trypsin

Position	**Peptide Cleavage**
311-337	SHCIAEVENDEMPADLPSLA ADFVESK
139-160	LVRPEVDVMCTAFHDNEETF LK
45-65	ALVLIAFAQYLQQCPFEDHV K
470-490	MPCAEDYLSVVLNQLCVLHE K
525-543	EFNAETFTFHADICTLSEK
397-413	VFDEFKPLVEEPQNLIK
43525.00	WVTFISLLFLFSSAYSR
265-281	VHTECCHGDLLECADDR
509-524	RPCFSALEVDETYVPK
170-183	HPYFYAPELLFFAK
348-360	DVFLGMFLYEYAR
414-426	QNCELFEQLGEYK
439-452	VPQVSTPTLVEVSR
287-298	YICENQDSISSK
76-88	TCVADESAENCDK
384-396	CCAAADPHECYAK
570-581	AVMDDFAAFVEK
106-117	ETYGEMADCCAK
362-372	HPDYSVVLLLR
187-198	AAFTECCQAADK
301-310	ECCEKPLLEK

66-75	LVNEVTEFAK
500-508	CCTESLVNR
123-130	NECFLQHK
89-97	SLHTLFGDK
599-609	LVAASQAALGL
589-597	ETCFAEEGK
550-558	QTALVELVK
376-383	TYETTLEK
427-434	FQNALLVR
37-44	DLGEENFK
131-138	DDNPNLPR
162-168	YLYEIAR
250-257	AEFAEVSK
98-105	LCTVATLR
258-264	LVTDLTK
199-205	AACLLPK
29-34	SEVAHR
342-347	NYAEAK
491-496	TPVSDR
237-242	AWAVAR
118-122	QEPER
224-229	CASLQK
206-210	LDELR
464-468	HPEAK
566-569	EQLK
282-286	ADLAK
559-562	HKPK
230-233	FGER
243-246	LSQR

Appendix D1 - RA Patient Questionnaire

'Identification of the foreign protein component of immune complexes in rheumatological disease'

Any information given in this in this questionnaire will only be used for purposes related to this research project. Questionnaires are anonymous and will not be traced back to you.

The aim of this questionnaire is to collect supplementary data on participants of this study. Current literature includes numerous theories and statistics. We would like to use the data from these questionnaires to corroborate or disprove previous studies.

- **Please select your gender:** Male ☐ Female ☐

- **What is your disease activity score (DAS28)?** _____

- **How old are you?**

25-34	☐	55-64	☐	85-94	☐
35-44	☐	65-74	☐	>94	☐
45-54	☐	75-84	☐		

- **At what age did your Rheumatoid Arthritis -related symptoms begin?**
- **At what age were you formally diagnosed with Rheumatoid Arthritis?**
- **How many years have you had Rheumatoid Arthritis (since diagnosis)?**

- **To which of the following ethnic groups do you belong?**

White	British ☐	Irish ☐	Other ☐					
Black or black British	Caribbean ☐	African ☐	Other ☐					
Asian or Asian British	Indian ☐	Pakistani ☐	Bangladeshi ☐	Other ☐				
Mixed	White and black Caribbean ☐	White and black African ☐	White and Asian ☐	Other ☐				
Chinese ☐								
Other ethnic group ☐								
Prefer not to say ☐								

- **Do you smoke?**

Never have	☐	<10/day	☐	20-30/day	☐
In the past	☐	10-20/day	☐	>30/day	☐

- **Has there been/are there any other cases of Rheumatoid Arthritis in your (blood-related) family?**

Grandmother	☐	Sister	☐	Auntie	☐
Mother	☐	Daughter	☐		
Grandfather	☐	Brother	☐	Uncle	☐
Father	☐	Son	☐		
No	☐	Other:	_____		

- **Where were you born?**

North West	☐	West Midlands	☐	Wales	☐
North East	☐	East Midlands	☐	Scotland	☐
Yorkshire	☐	South West	☐	N.Ireland	☐
		South East	☐	Outside the UK	☐

- **Amongst your siblings (if any), were you:**

1st Born	☐	4th Born	☐
2nd Born	☐	5th + Born	☐
3rd Born	☐		

- **As a child, did you share a bedroom?**

No ☐ Yes ☐

If Yes:

- At what age? _____
- For how many years? _____
- With how many people? _____

- **Do you currently have any active infections?** _____
- **Are you currently taking antibiotics?** _____

- **What was your mother's occupation during your childhood?** _____
- **What was your father's occupation during your childhood?** _____

- **In what type of area did you live for the majority of your early childhood?**

Urban ☐ Suburban ☐ Rural ☐

- **In what type of area do you currently live?**

Urban ☐ Suburban ☐ Rural ☐

- **Did you live or spend a lot of time on a farm during your early childhood?**

No ☐ Yes ☐

- **Which pets did you have during your childhood?** (select all applicable)

Dog ☐ Guinea Pig ☐

Cat ☐ Bird ☐

Rabbit ☐ None ☐

- **Does your Rheumatoid Arthritis appear to be worse in a particular season?** (select all applicable)

No difference ☐ Autumn ☐

Spring ☐ Winter ☐

Summer ☐

For Internal Use Only

Unique Identifier Number: _____

Appendix D2 - Control Patient Questionnaire

'Identification of the foreign protein component of immune complexes in rheumatological disease'

Any information given in this in this questionnaire will only be used for purposes related to this research project. Questionnaires are anonymous and will not be traced back to you.

The aim of this questionnaire is to collect supplementary data on participants of this study. Current literature includes numerous theories and statistics. We would like to use the data from these questionnaires to corroborate or disprove previous studies.

- **Please select your gender:** Male ☐ Female ☐

- **How old are you?**

25-34	☐	55-64	☐	85-94	☐
35-44	☐	65-74	☐	>94	☐
45-54	☐	75-84	☐		

- **To which of the following ethnic groups do you belong?**

White	British	☐	Irish	☐	Other	☐			
Black or black British	Caribbean	☐	African	☐	Other	☐			
Asian or Asian British	Indian	☐	Pakistani	☐	Bangladeshi	☐	Other	☐	
Mixed	White and black Caribbean	☐	White and black African	☐	White and Asian	☐	Other	☐	
Chinese	☐								
Other ethnic group	☐								
Prefer not to say	☐								

- **Do you smoke?**

Never have	☐	<10/day	☐	20-30/day	☐
In the past	☐	10-20/day	☐	>30/day	☐

- **Has there been/are there any cases of Rheumatoid Arthritis in your (blood-related) family?**

Grandmother	☐	Sister	☐	Auntie	☐
Mother	☐	Daughter	☐		
Grandfather	☐	Brother	☐	Uncle	☐
Father	☐	Son	☐		
No	☐	Other:	_____		

- **Where were you born?**

North West	☐	West Midlands	☐	Wales	☐
North East	☐	East Midlands	☐	Scotland	☐
Yorkshire	☐	South West	☐	N.Ireland	☐
		South East	☐	Outside the UK	☐

- **Amongst your siblings (if any), were you:**

1st Born	☐	4th Born	☐
2nd Born	☐	5th + Born	☐
3rd Born	☐		

- **As a child, did you share a bedroom?**

  No ☐ Yes ☐

  If Yes:

  - At what age? _____
  - For how many years? _____
  - With how many people? _____

- **Do you currently have any active infections?** _____
- **Are you currently taking antibiotics?** _____

- **What was your mother's occupation during your childhood?**_____
- **What was your father's occupation during your childhood?**_____

- **In what type of area did you live for the majority of your early childhood?**

  Urban ☐ Suburban ☐ Rural ☐

- **In what type of area do you currently live?**

  Urban ☐ Suburban ☐ Rural ☐

- **Did you live or spend a lot of time on a farm during your early childhood?**

  No ☐ Yes ☐

- **Which pets did you have during your childhood?** (select all applicable)

  Dog ☐ Guinea Pig ☐

  Cat ☐ Bird ☐

  Rabbit ☐ None ☐

For Internal Use Only
Unique Identifier Number: _____

Appendix E1 Alpha Haemolysin (33kDa)

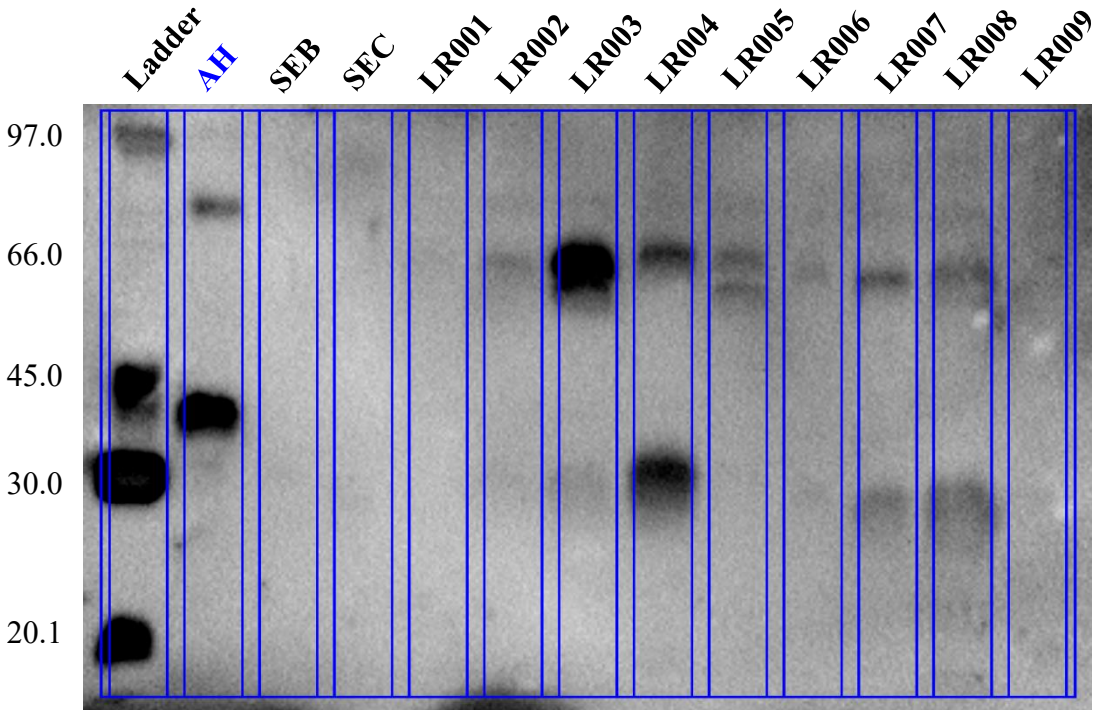


Figure E1 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR001-009 (28µl) (Lane 5-13).**

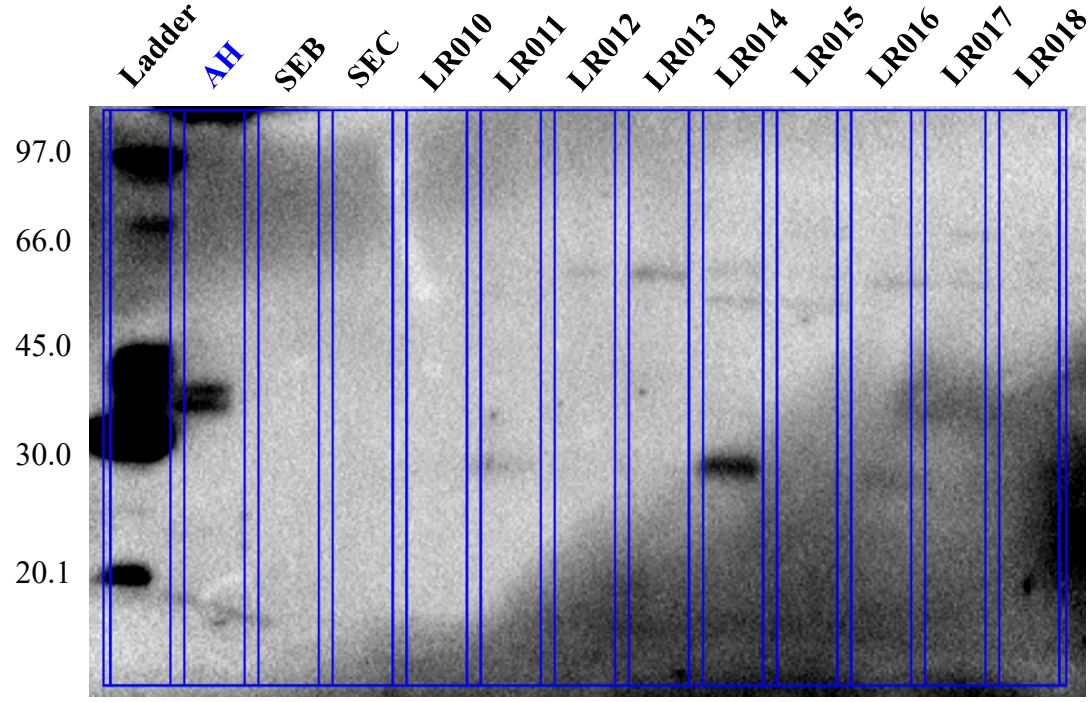


Figure E2 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR010-018 (28µl) (Lane 5-13).**

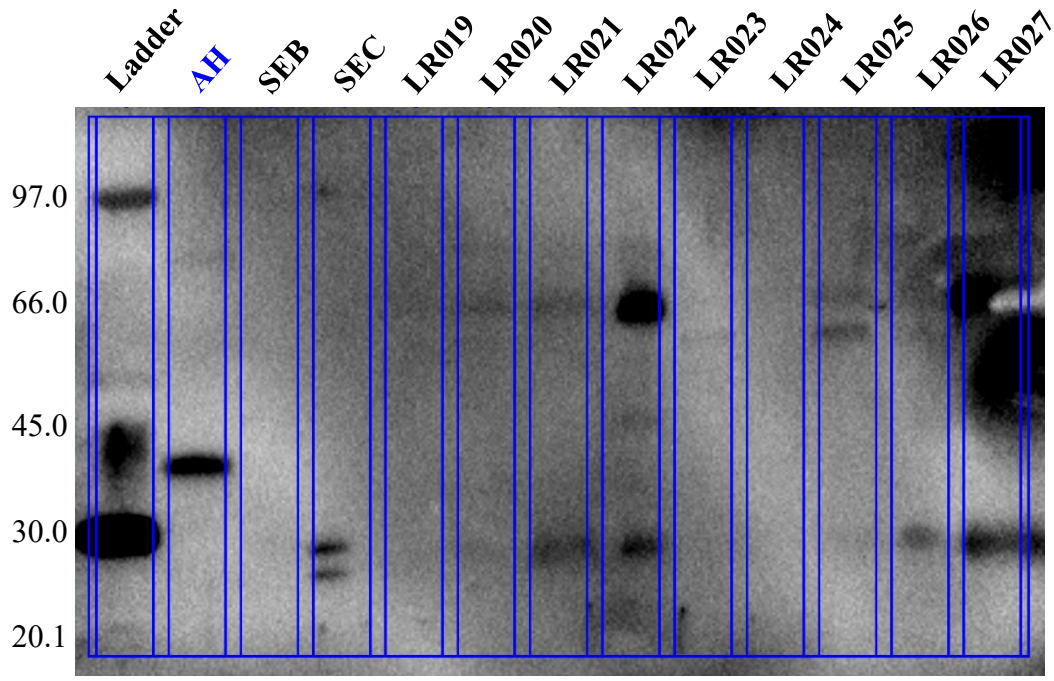


Figure E3 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR019-027 (28µl) (Lane 5-13).**

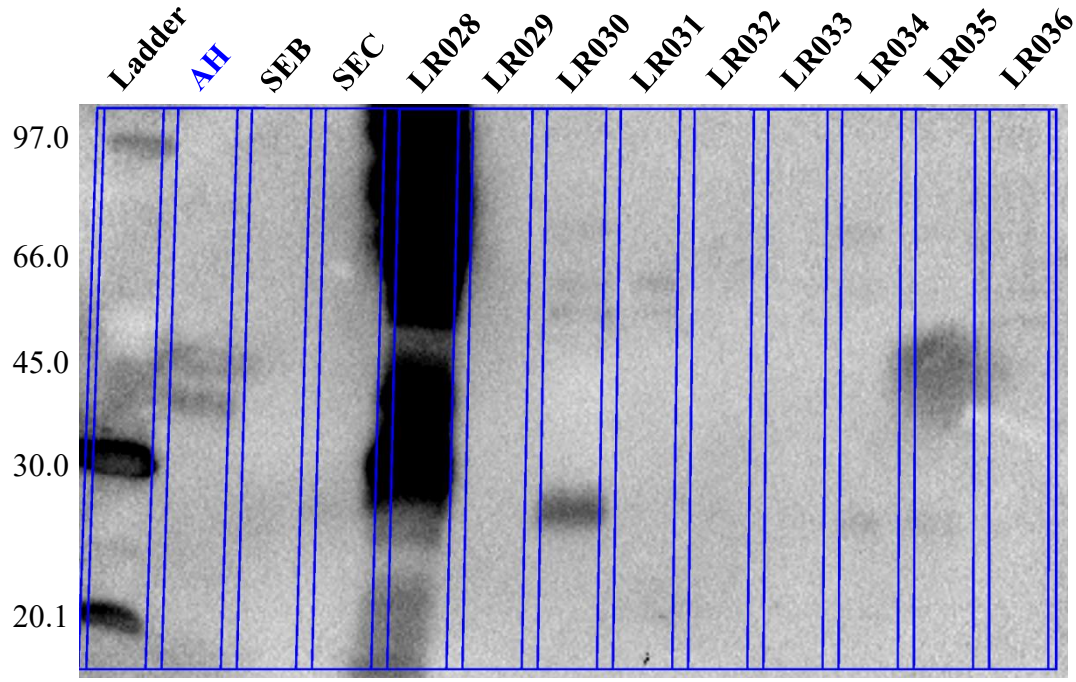


Figure E4 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR028-036 (28µl) (Lane 5-13).**

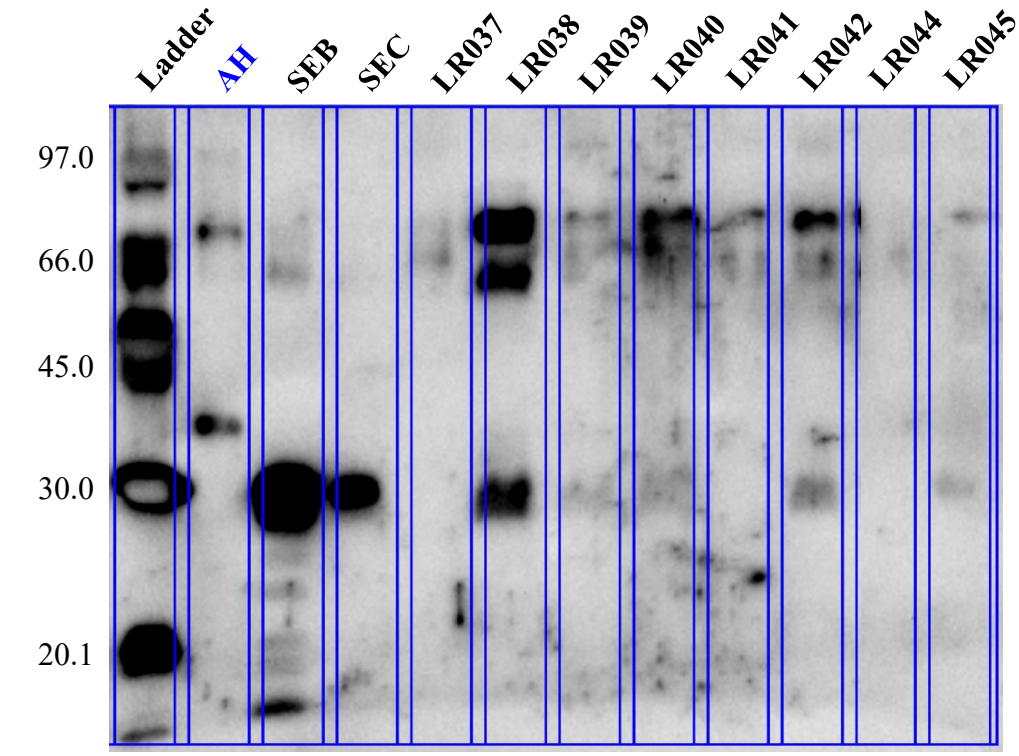


Figure E5 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR037-045 (28µl) (Lane 5-12).**

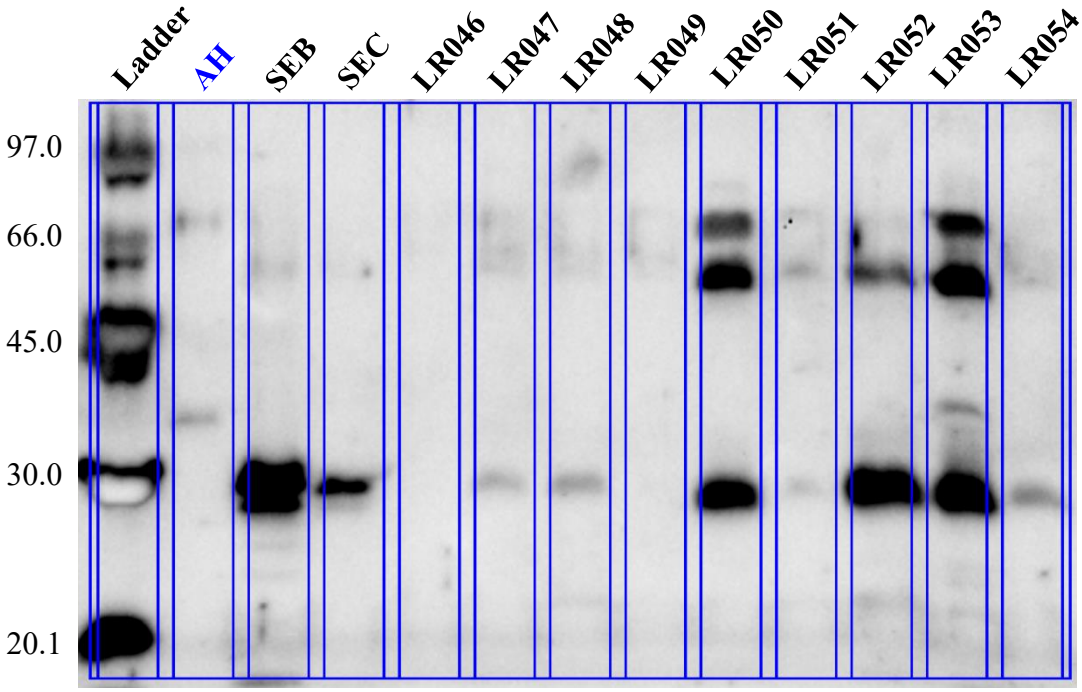


Figure E6 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR046-054 (28µl) (Lane 5-13).**

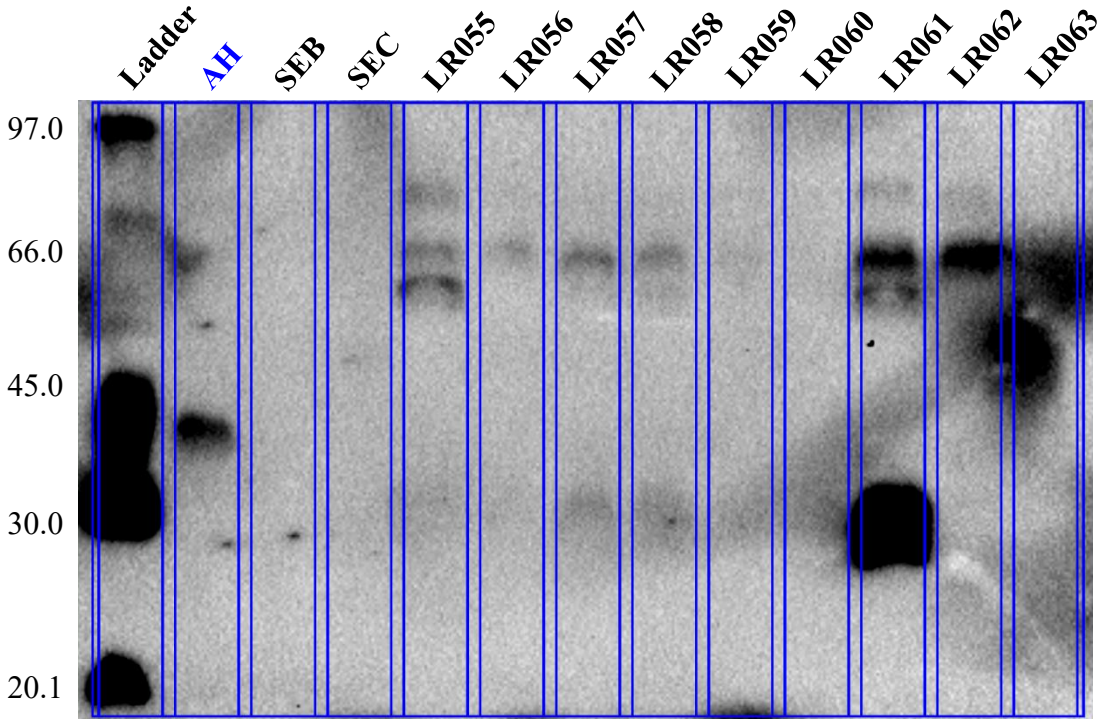


Figure E7 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR055-063 (28µl)(Lane 5-13).**

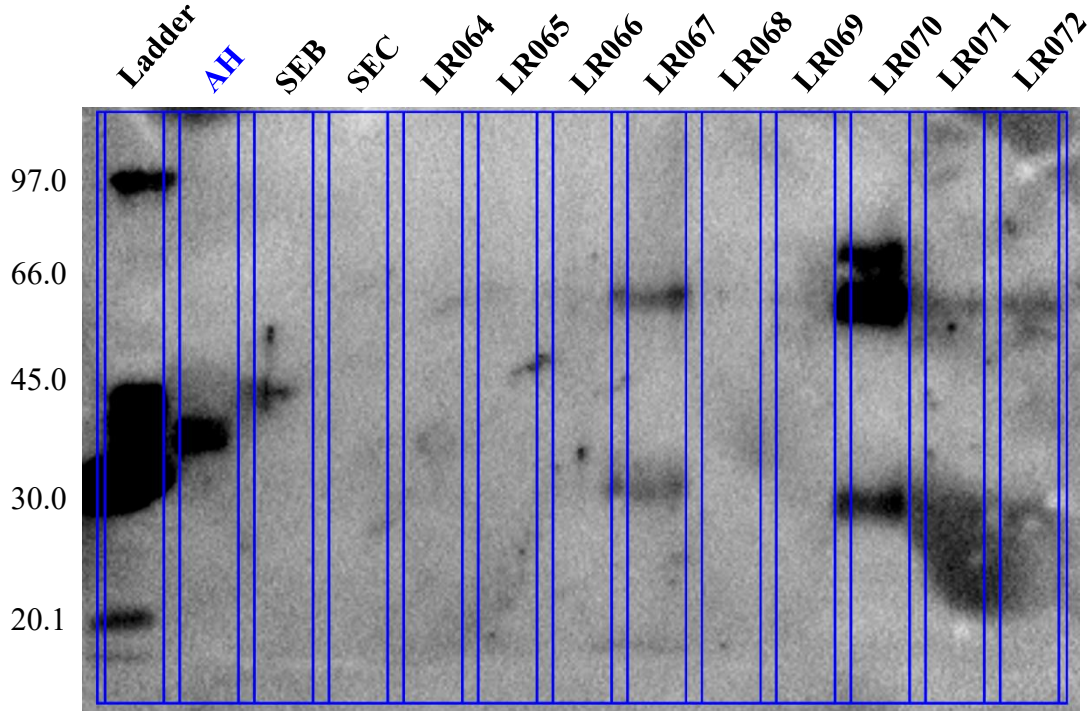


Figure E8 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR064-072 (28µl) (Lane 5-13).**

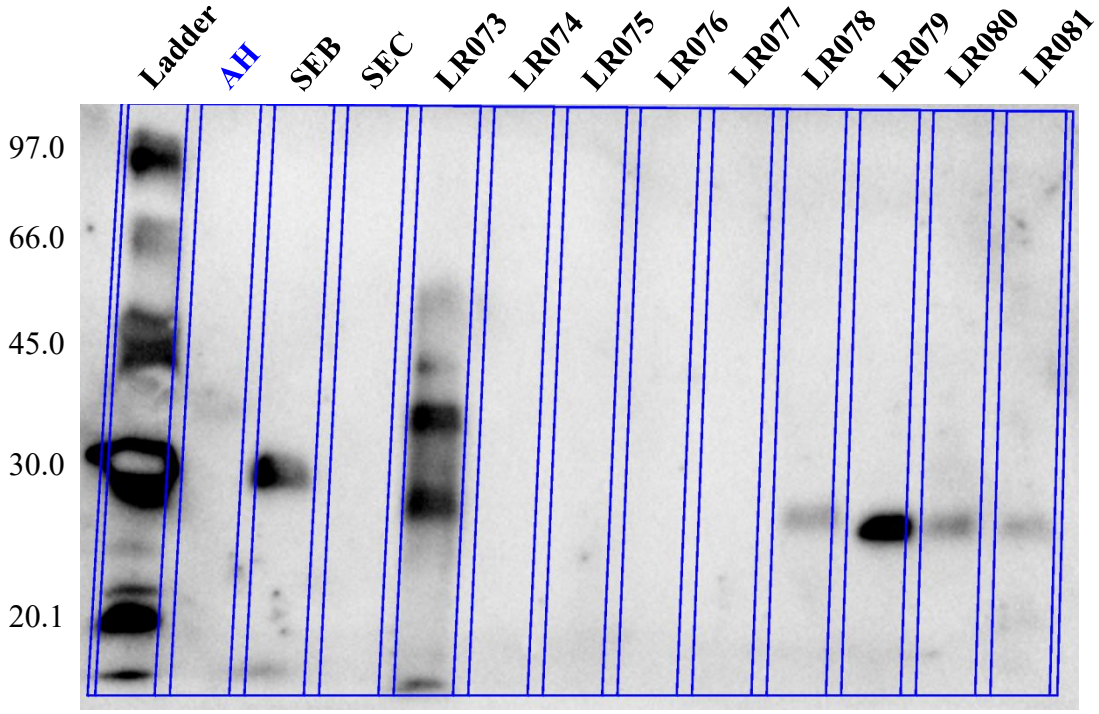


Figure E9 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR073-081 (28µl) (Lane 5-13).**

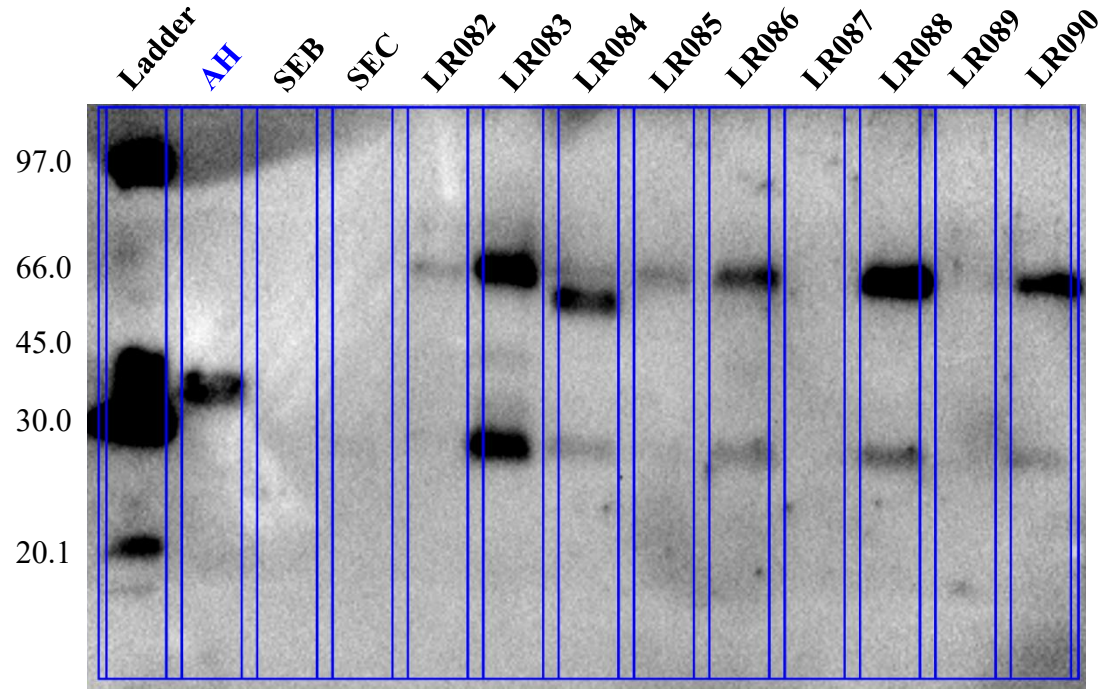


Figure E10 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR082-090 (28µl) (Lane 5-13).**

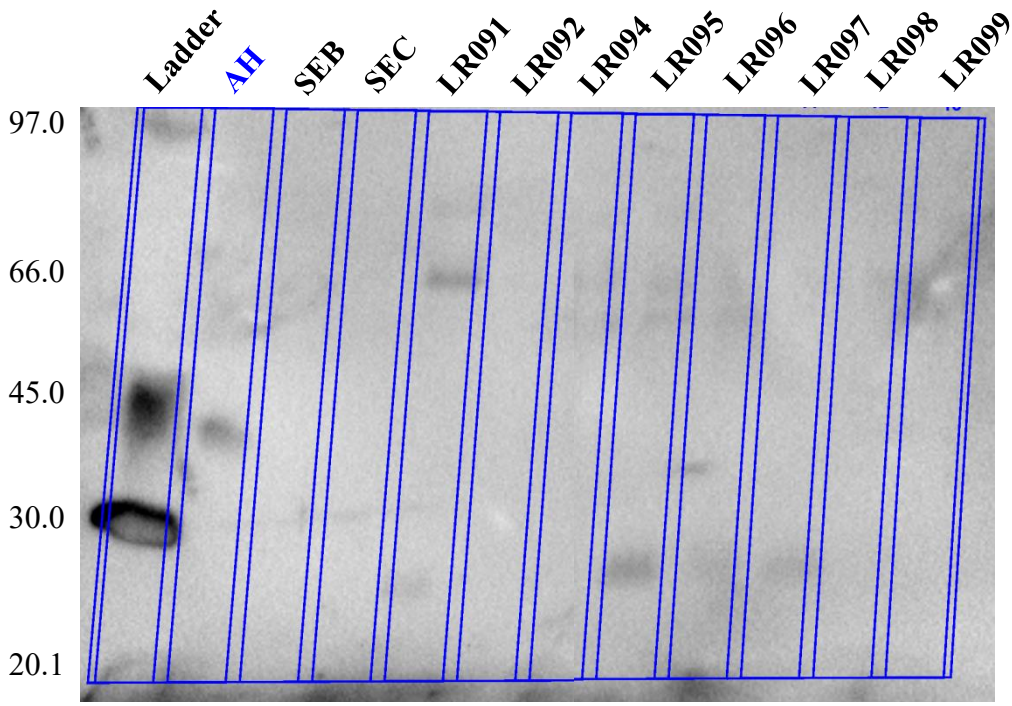


Figure E11 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR091-099 (28µl) (Lane 5-12).**

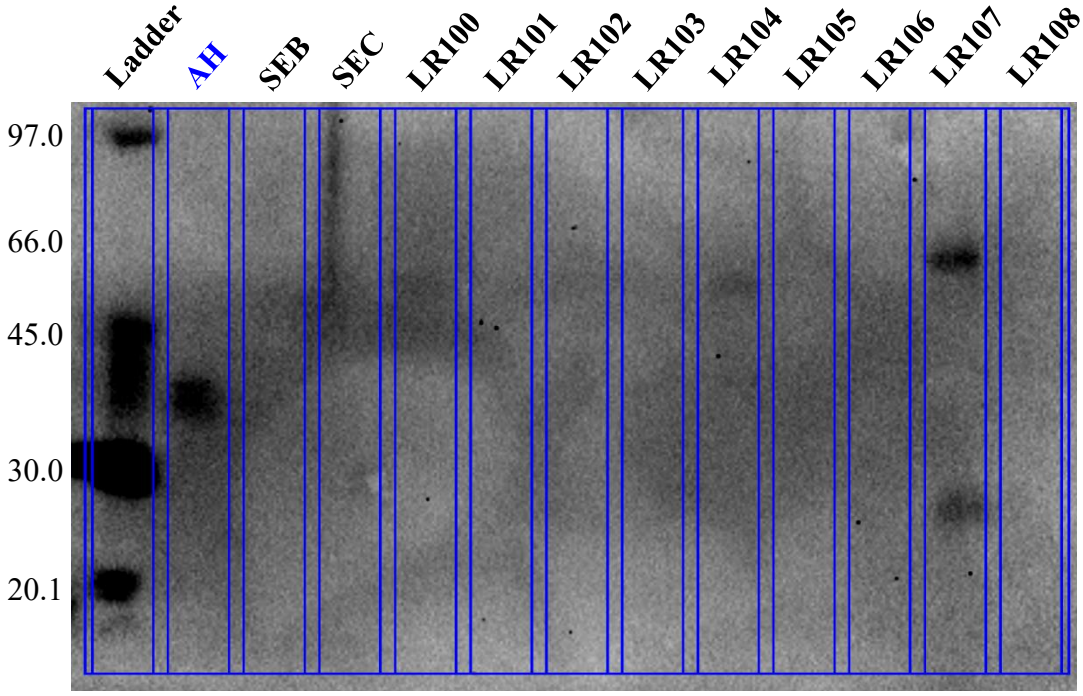


Figure E12 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR100-108 (28µl) (Lane 5-13).**

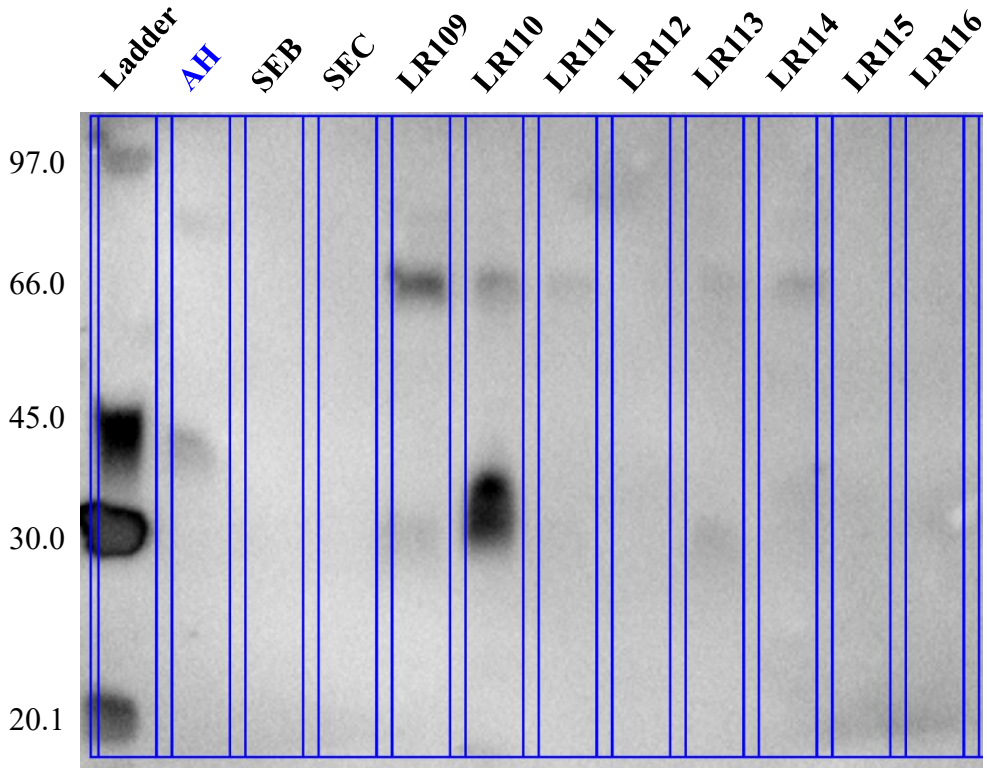


Figure E13 Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR109-117 (28µl) (Lane 5-12).

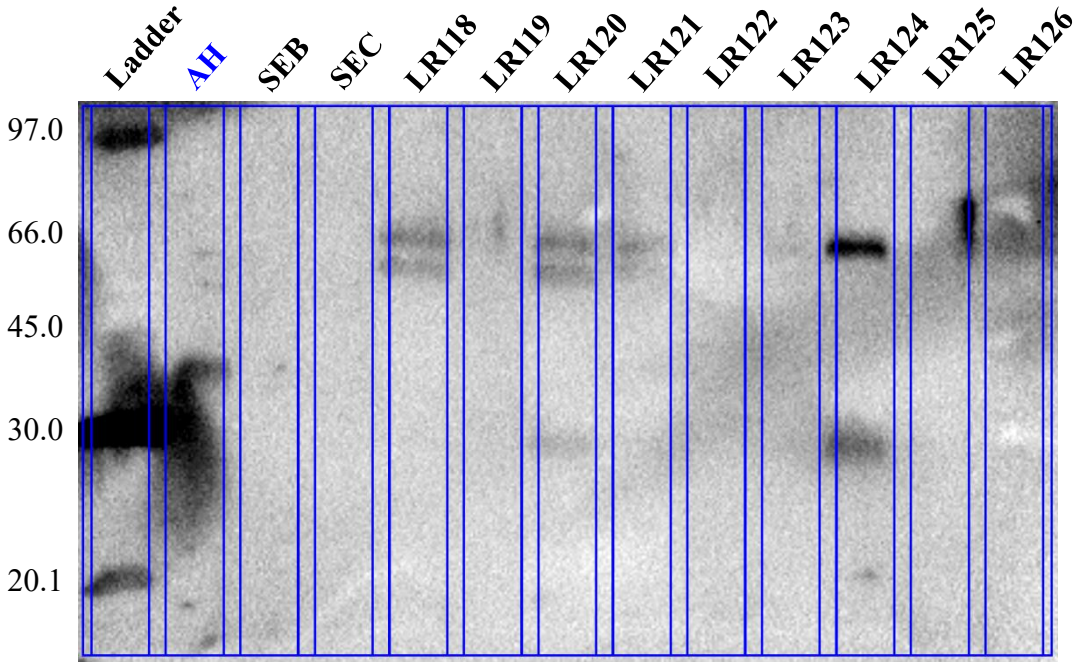


Figure E14 Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR118-126 (28µl) (Lane 5-13).

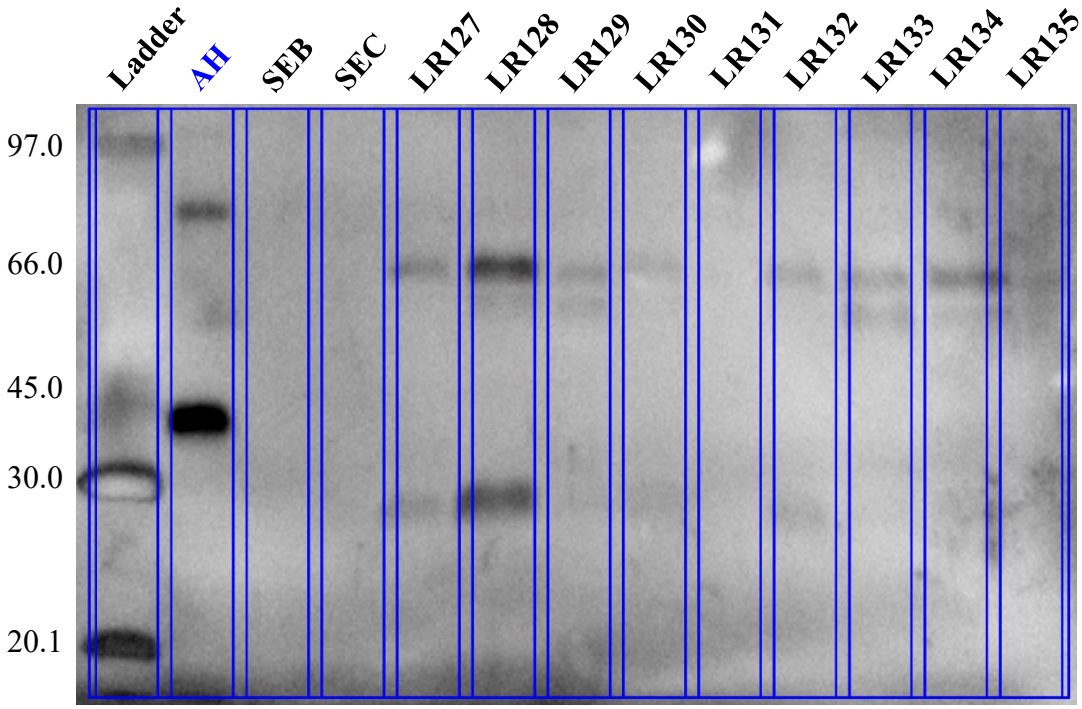


Figure E15 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR127-135 (28µl) (Lane 5-13).**

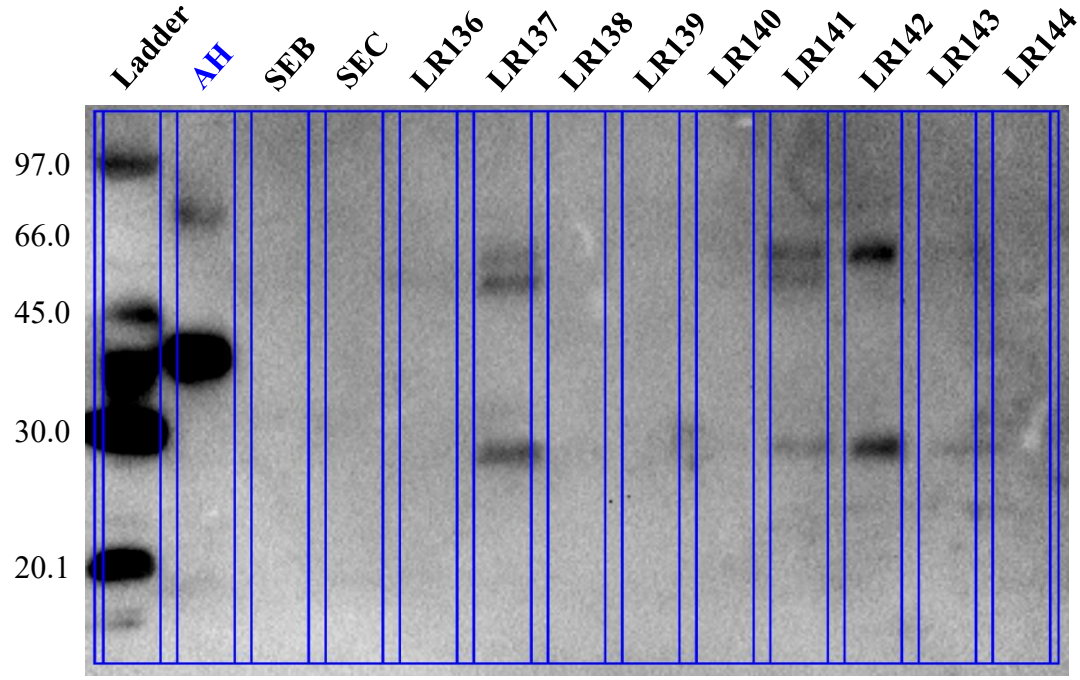


Figure E16 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR136-144 (28µl) (Lane 5-13).**

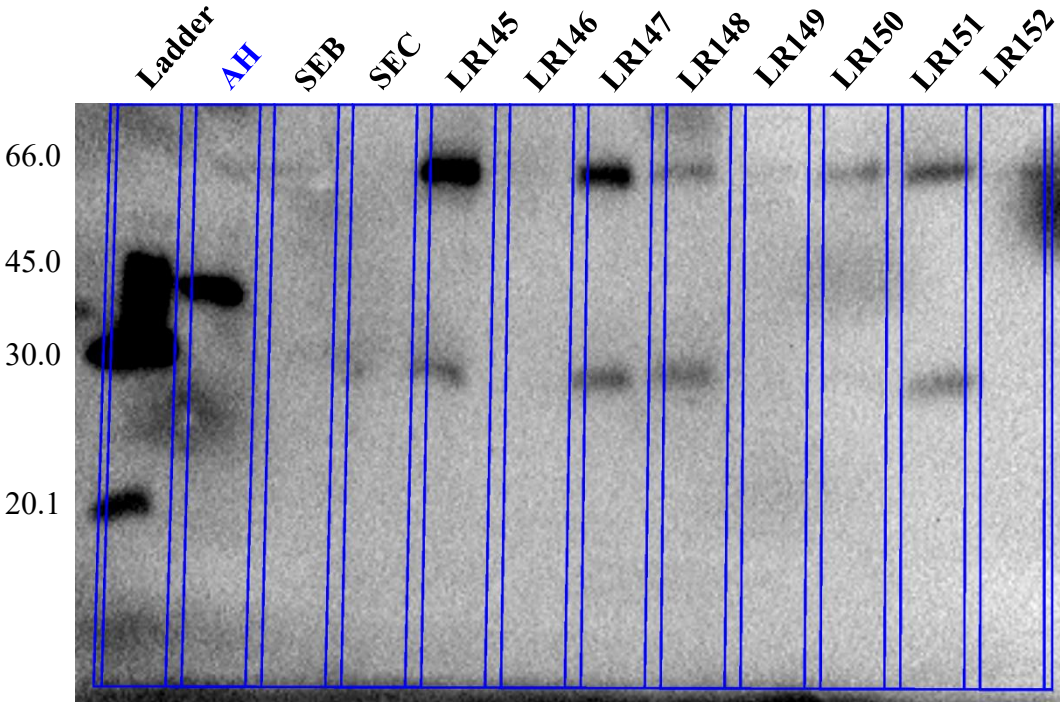


Figure E17 Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR145-152 (28µl) (Lane 5-13).

Appendix E2 Staphylococcal Enterotoxin B (28kDa)

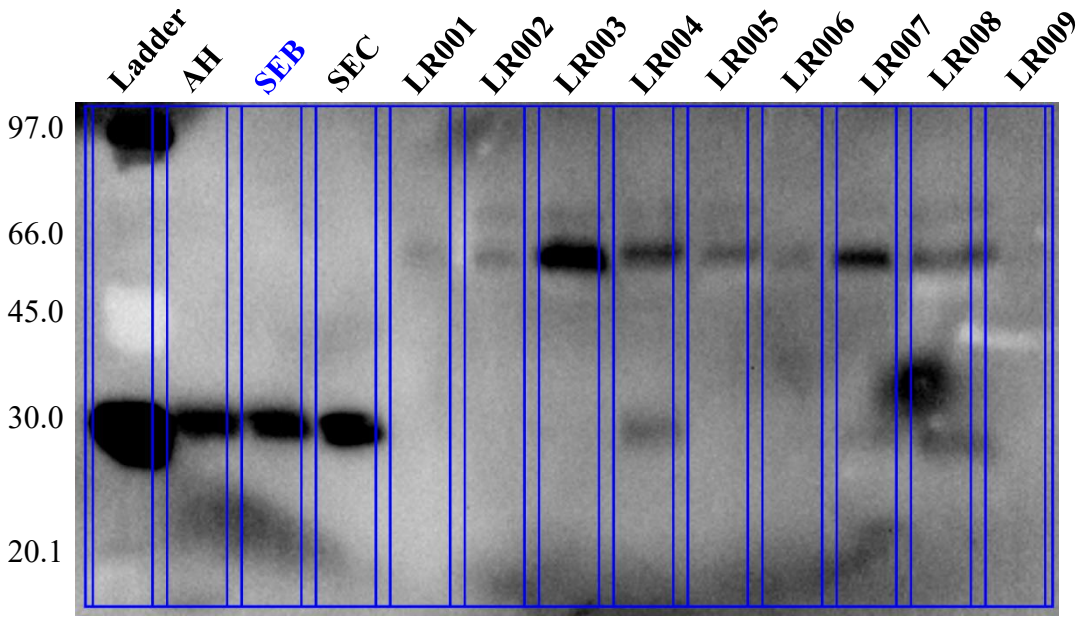


Figure E18 **Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR001-009 (28µl) (Lane 5-13).**

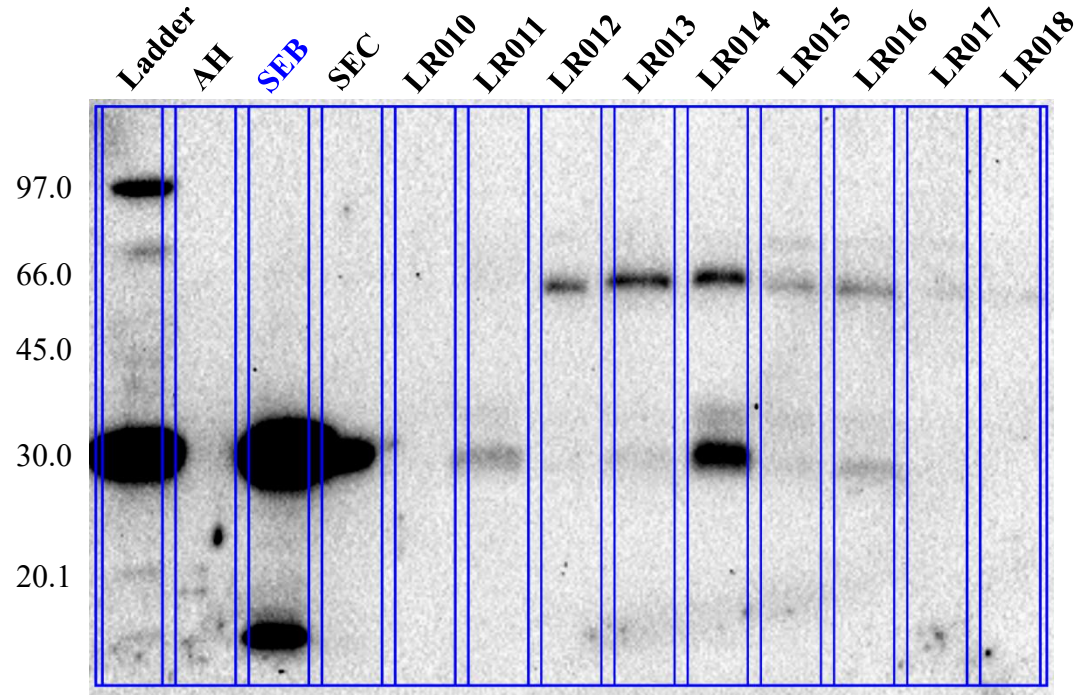


Figure E19 **Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR010-018 (28µl) (Lane 5-13).**

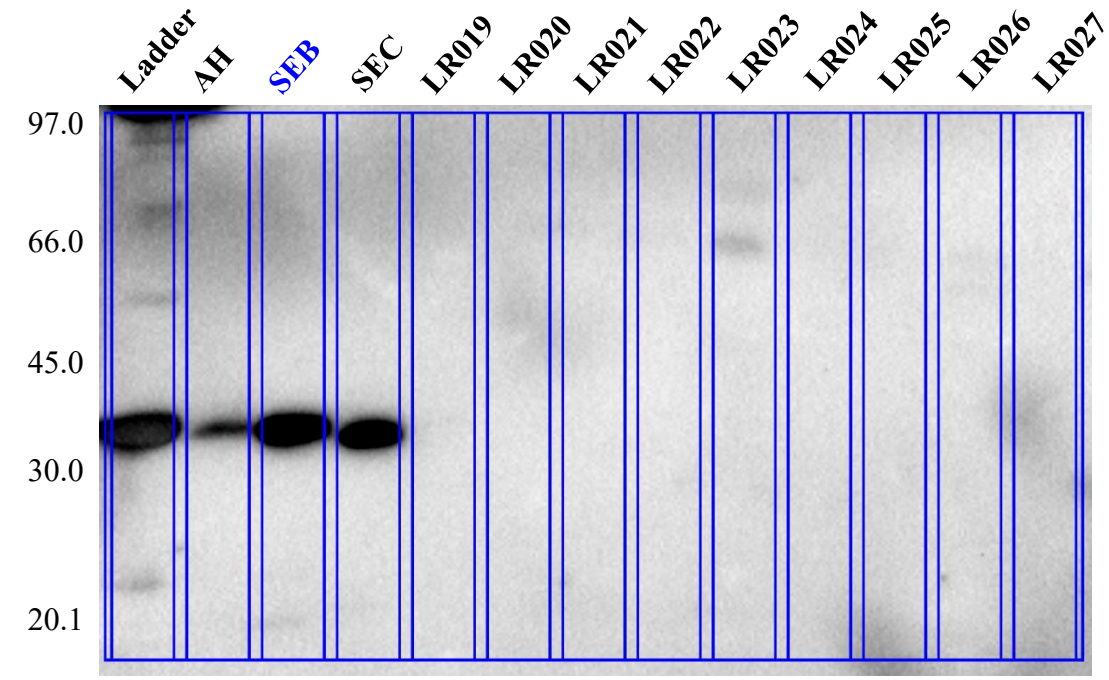


Figure E20 **Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR019-027 (28µl) (Lane 5-13).**

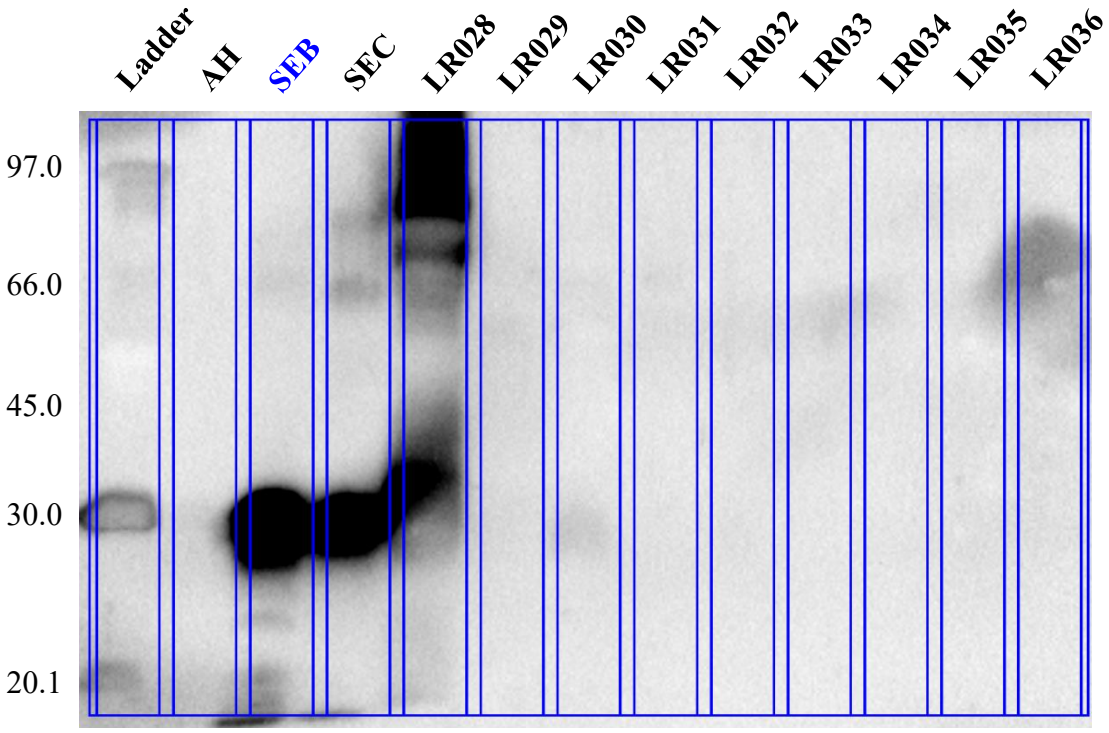


Figure E21 **Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR028-036 (28µl) (Lane 5-13).**

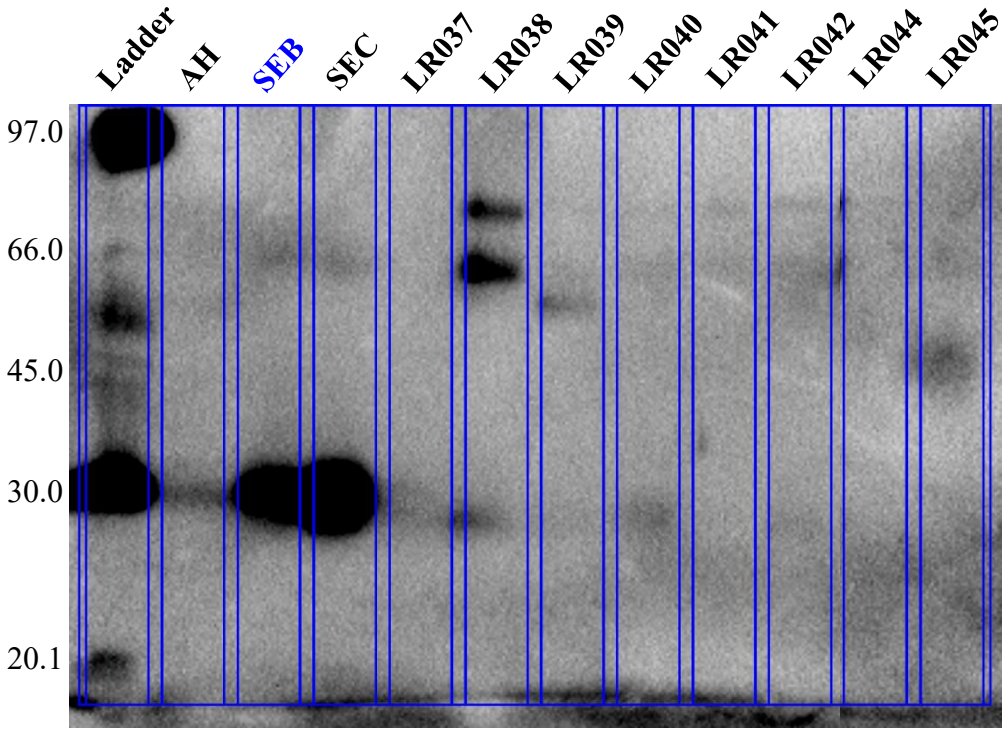


Figure E22 **Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR037-045 (28µl) (Lane 5-12).**

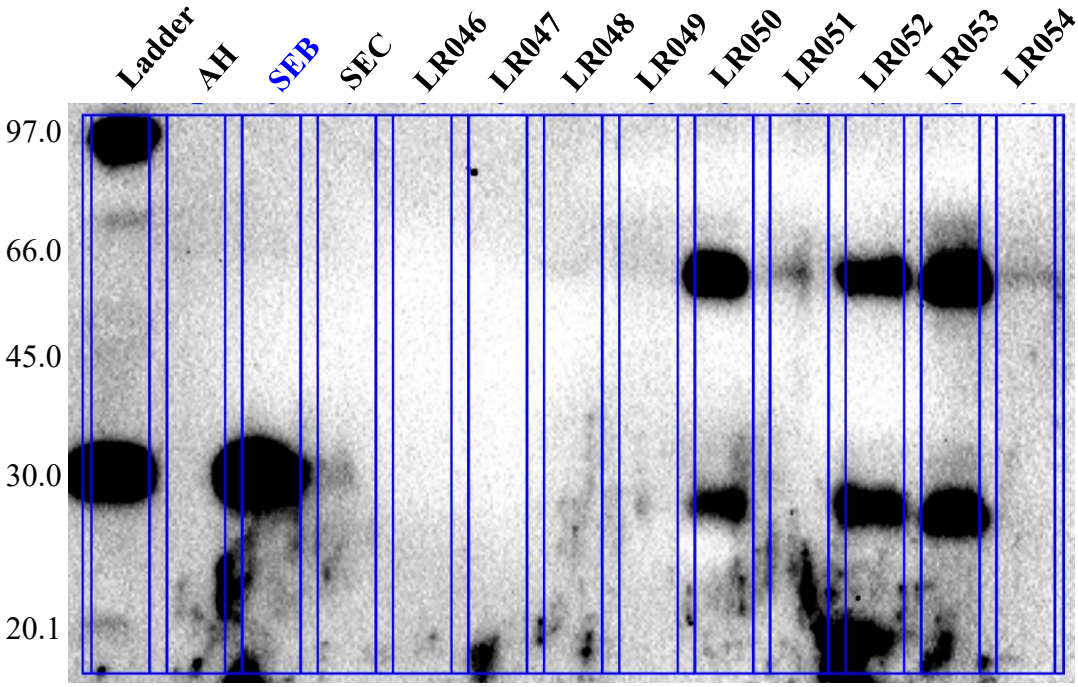


Figure E23 **Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR046-054 (28µl) (Lane 5-13).**

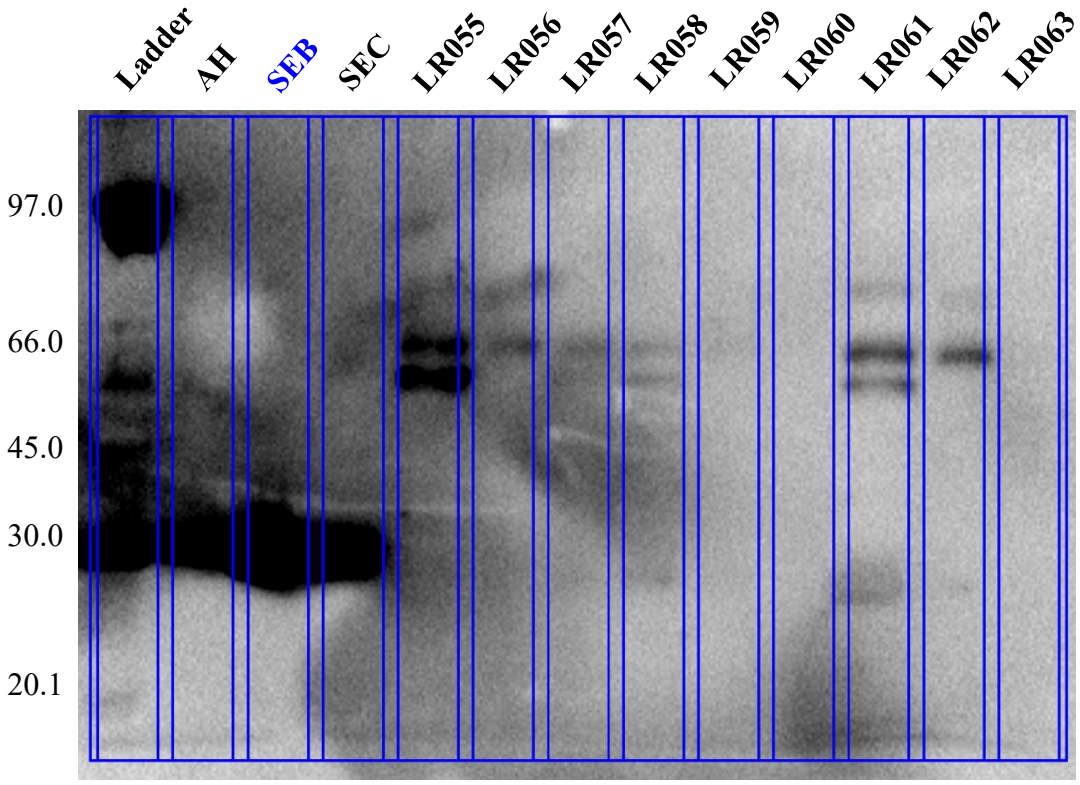


Figure E24 **Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR055-063 (28µl) (Lane 5-13).**

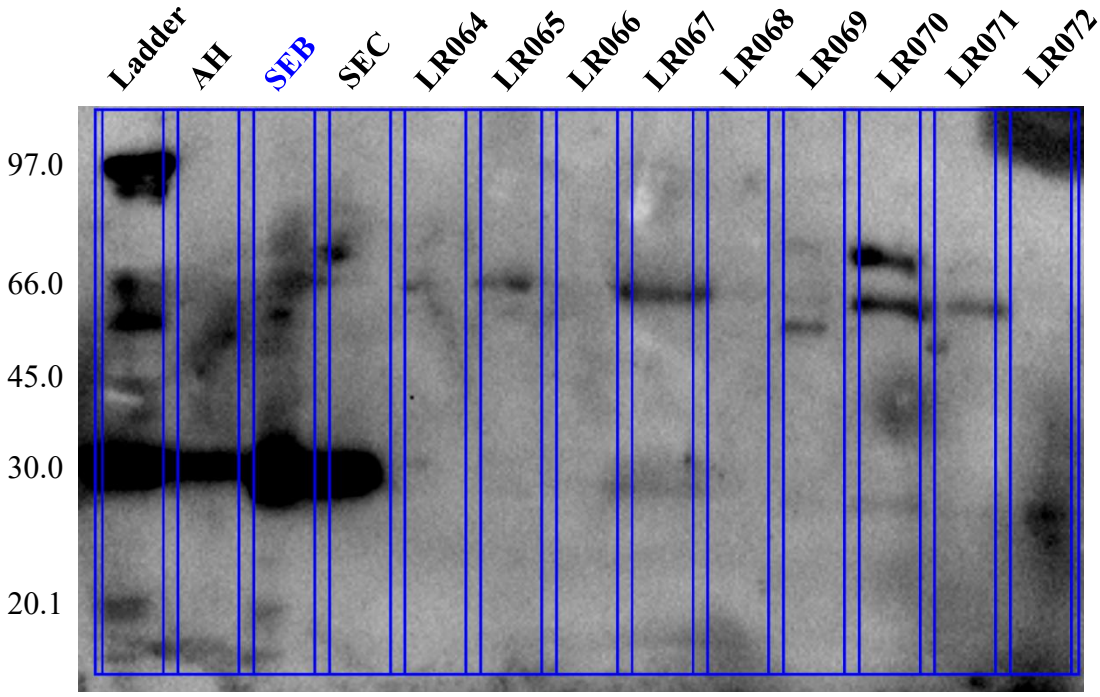


Figure E25 **Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR064-072 (28µl) (Lane 5-13).**

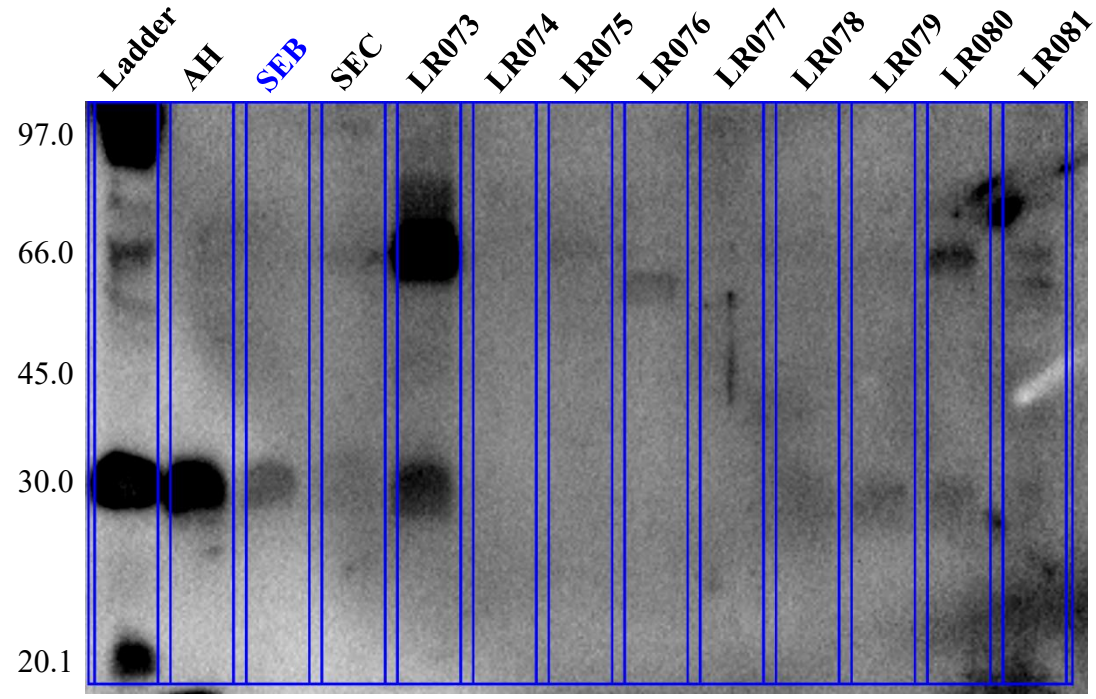


Figure E26 **Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR073-081 (28µl) (Lane 5-13).**

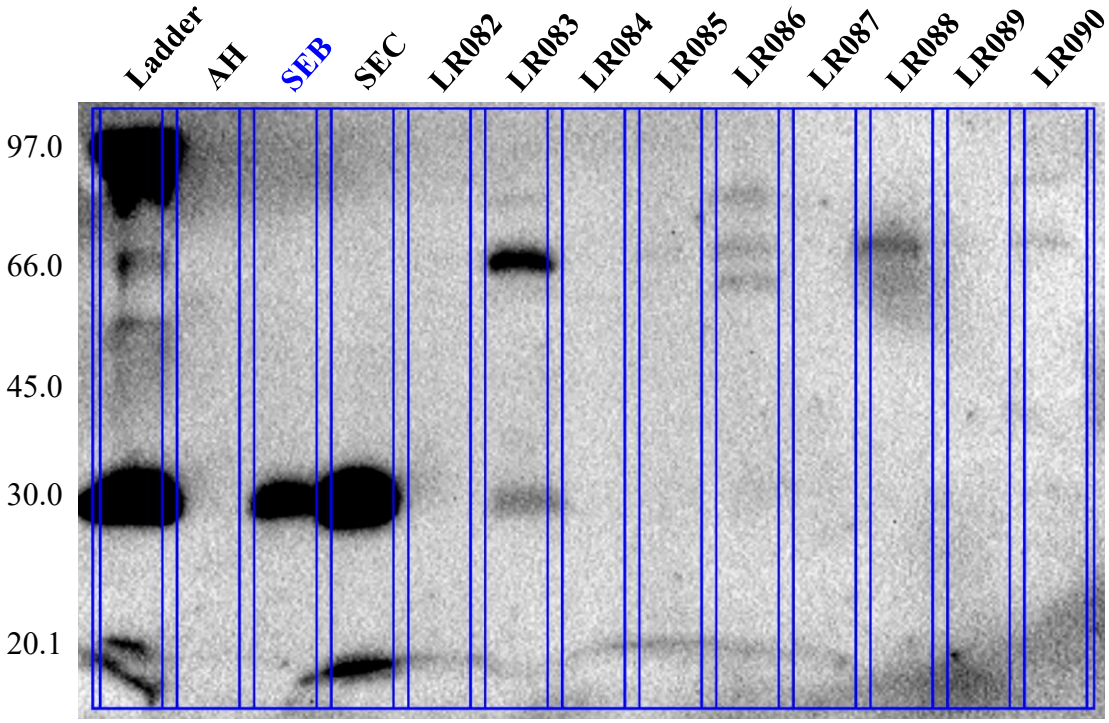


Figure E27 **Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR082-090 (28µl) (Lane 5-13).**

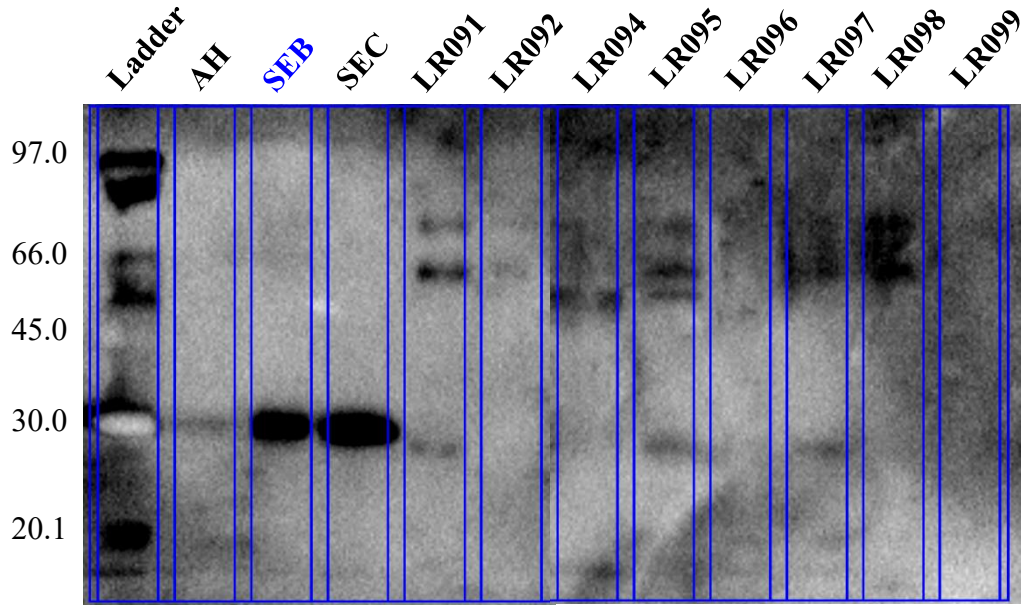


Figure E28 Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR091-099 (28µl) (Lane 5-12).

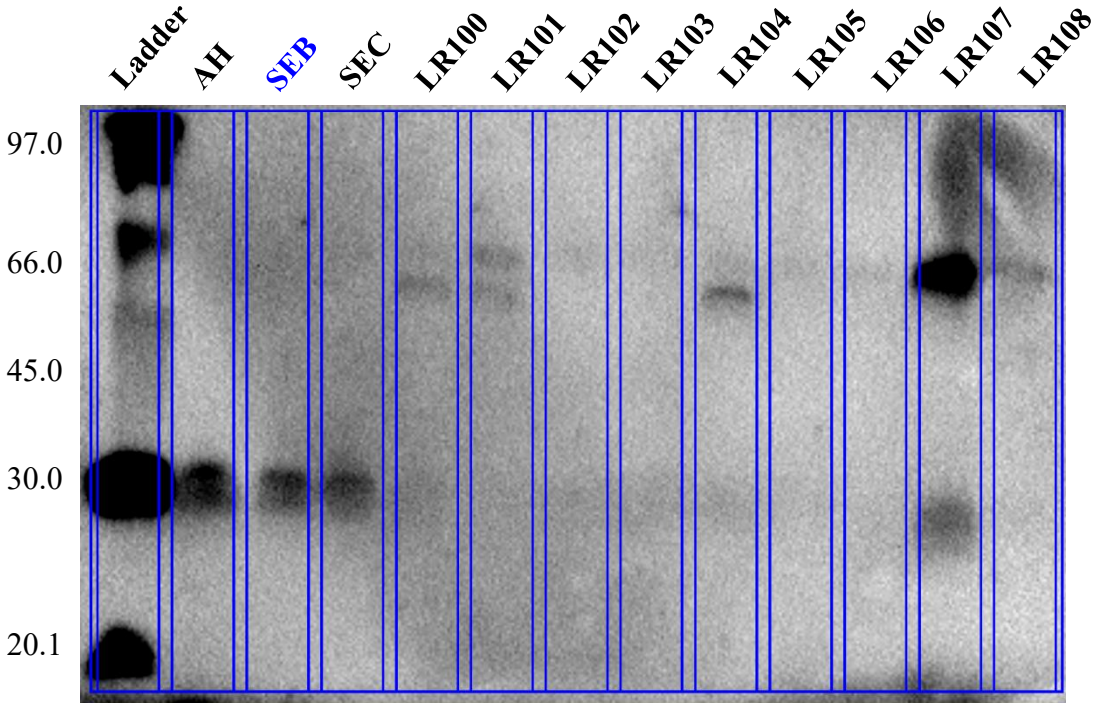


Figure E29 Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR100-108 (28µl) (Lane 5-13).

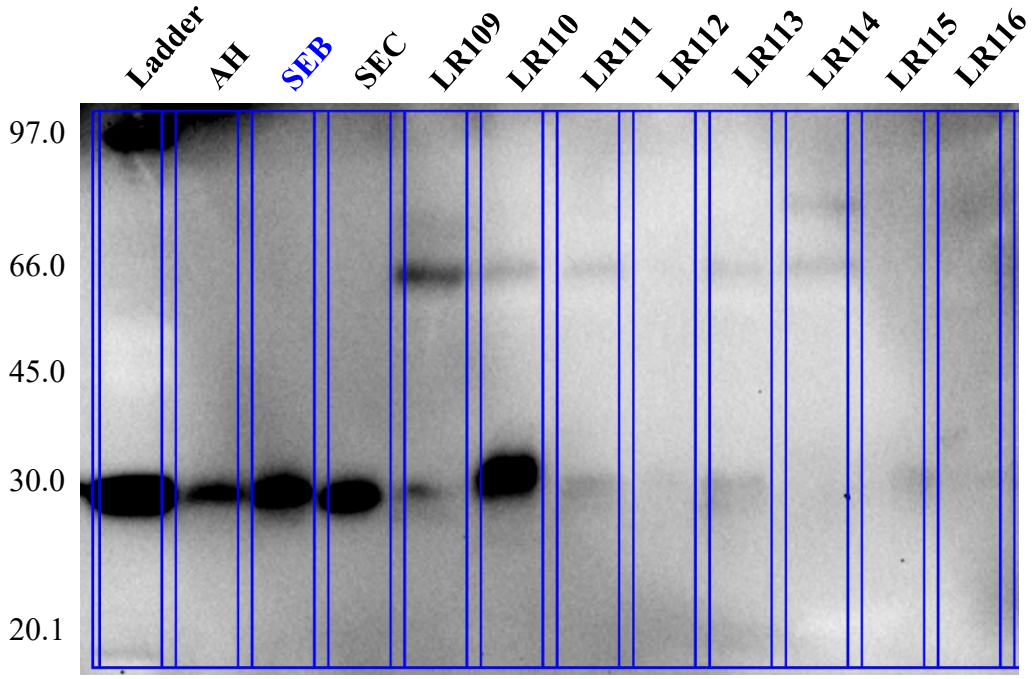


Figure E30 **Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR109-117 (28µl) (Lane 5-12).**

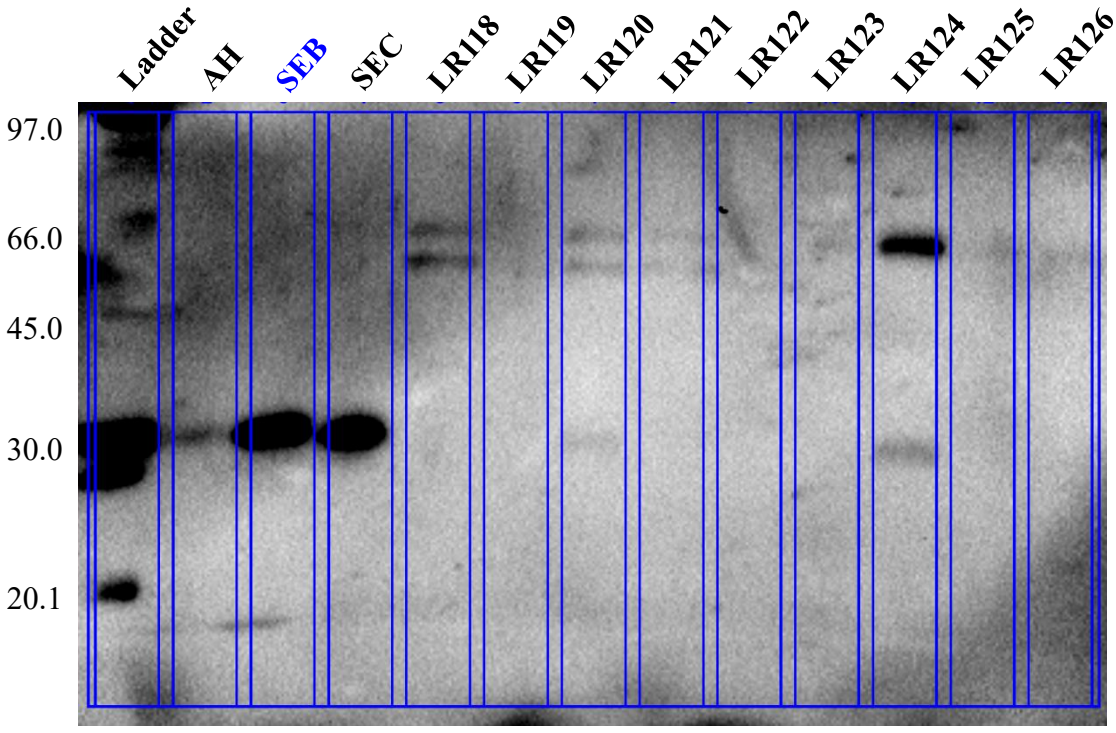


Figure E31 **Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR118-126 (28µl) (Lane 5-13).**

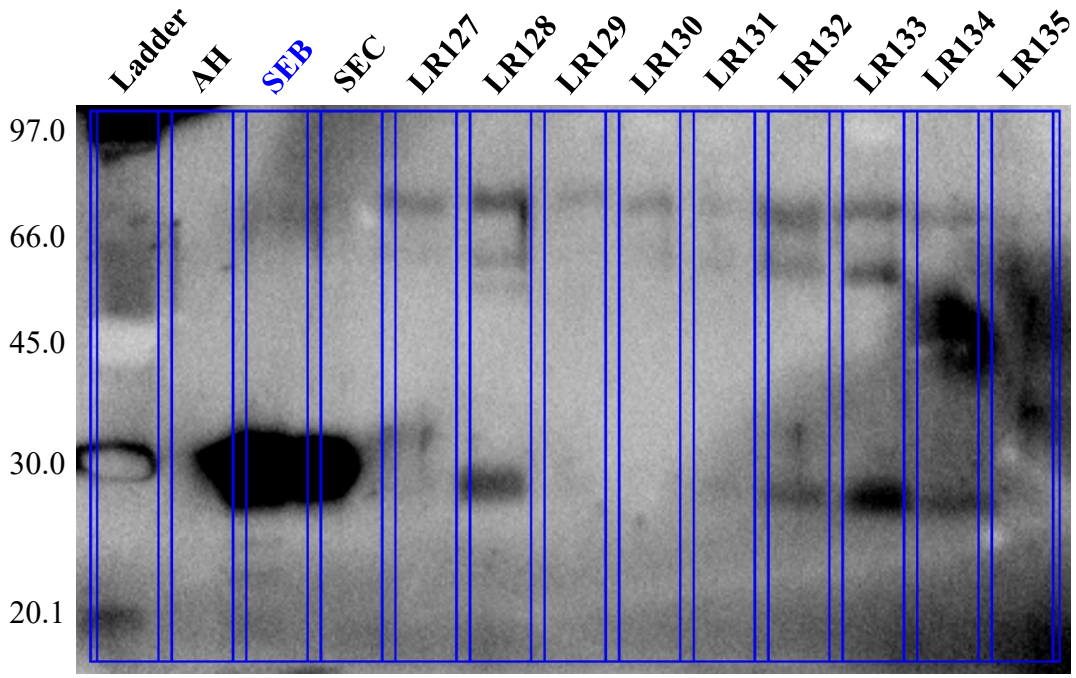


Figure E32 **Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR127-135 (28µl) (Lane 5-13).**

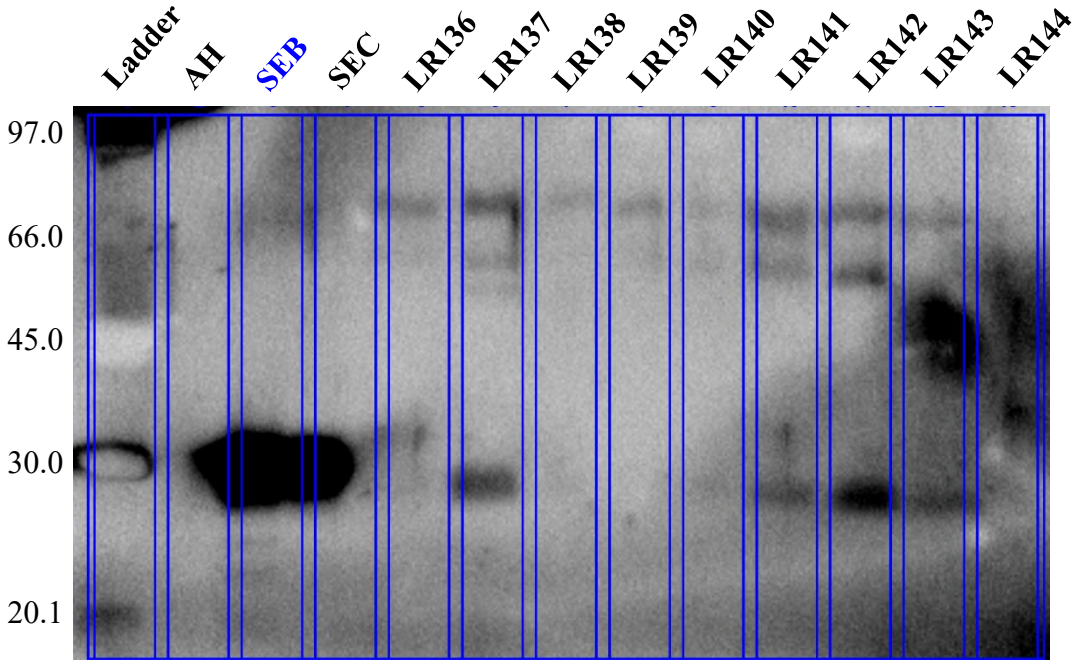


Figure E33 **Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR136-144 (28µl) (Lane 5-13).**

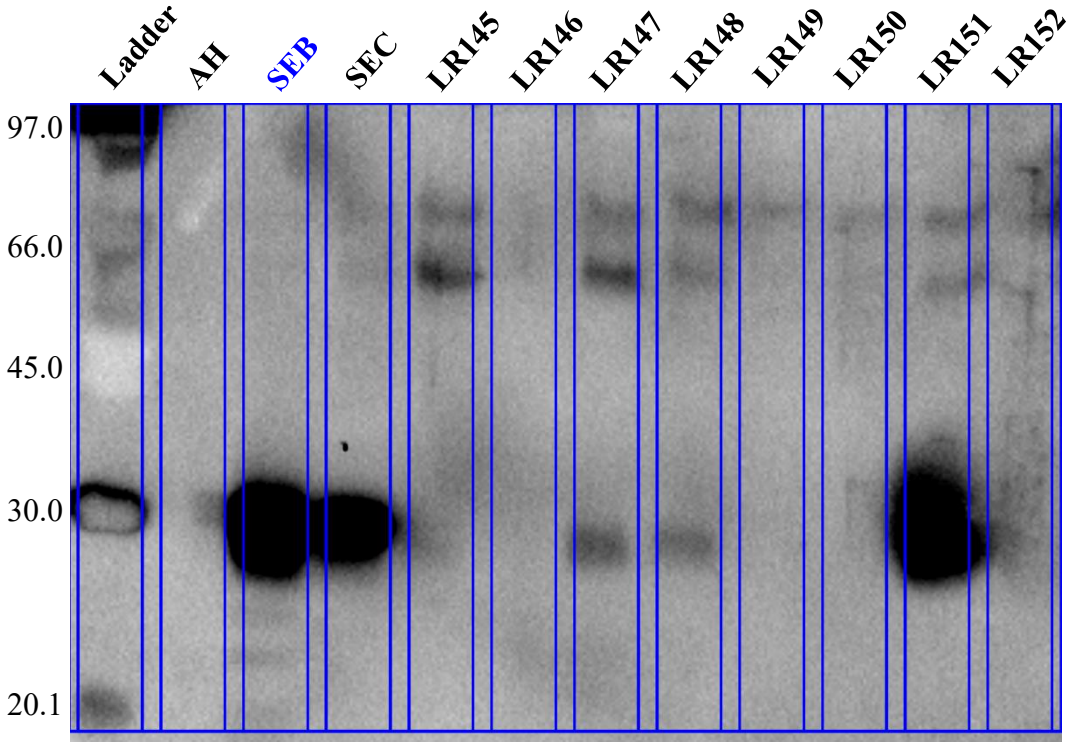


Figure E34 Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR145-152 (28µl) (Lane 5-13).

Appendix E3 Staphylococcal Enterotoxin C (30kDa)

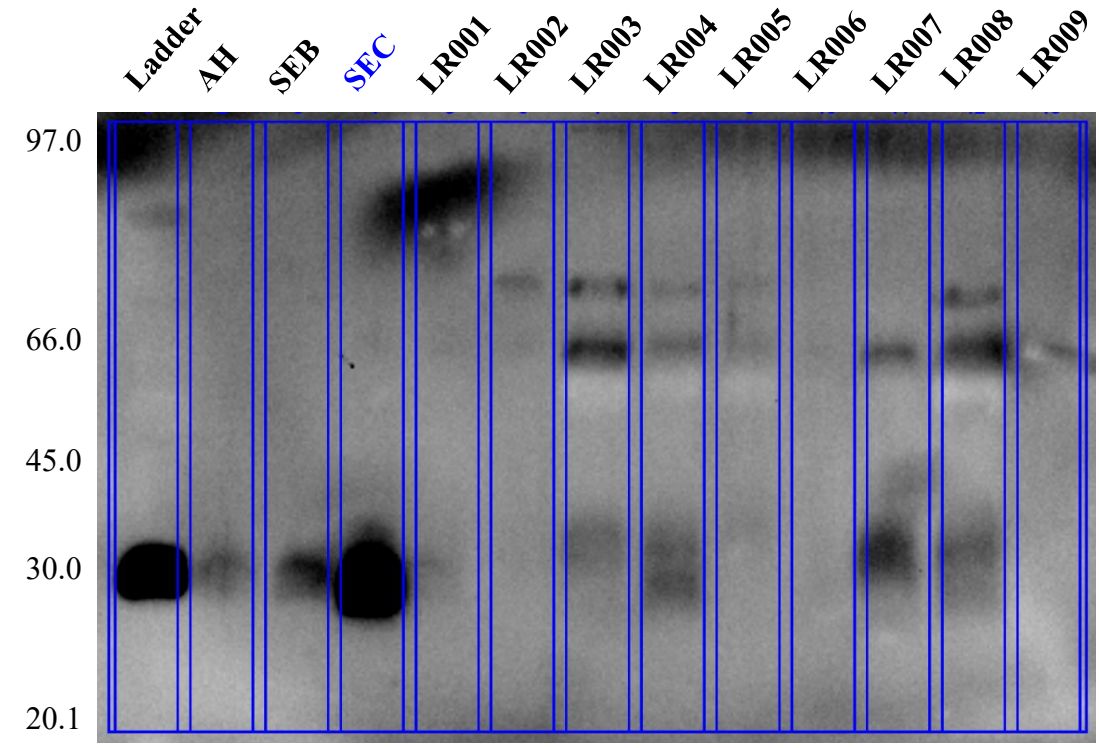


Figure E35 Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR001-009 (28µl) (Lane 5-13).

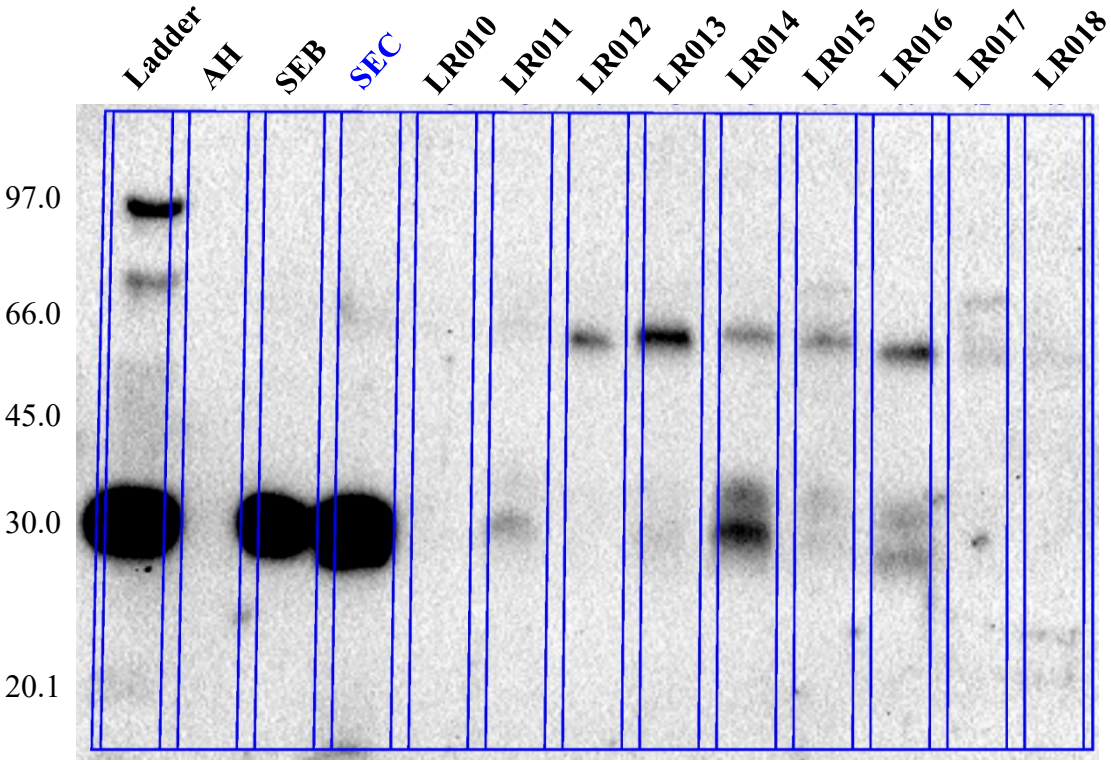


Figure E36 Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR010-018 (28µl) (Lane 5-13).

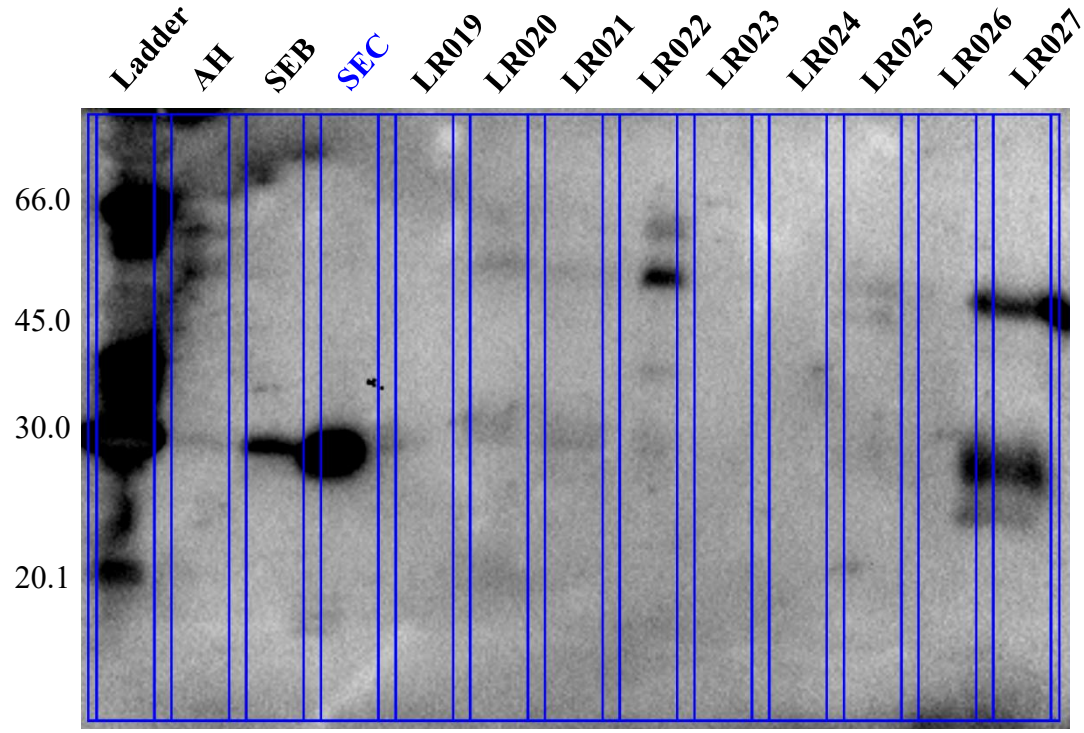


Figure E37 Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR018-027 (28µl) (Lane 5-13).

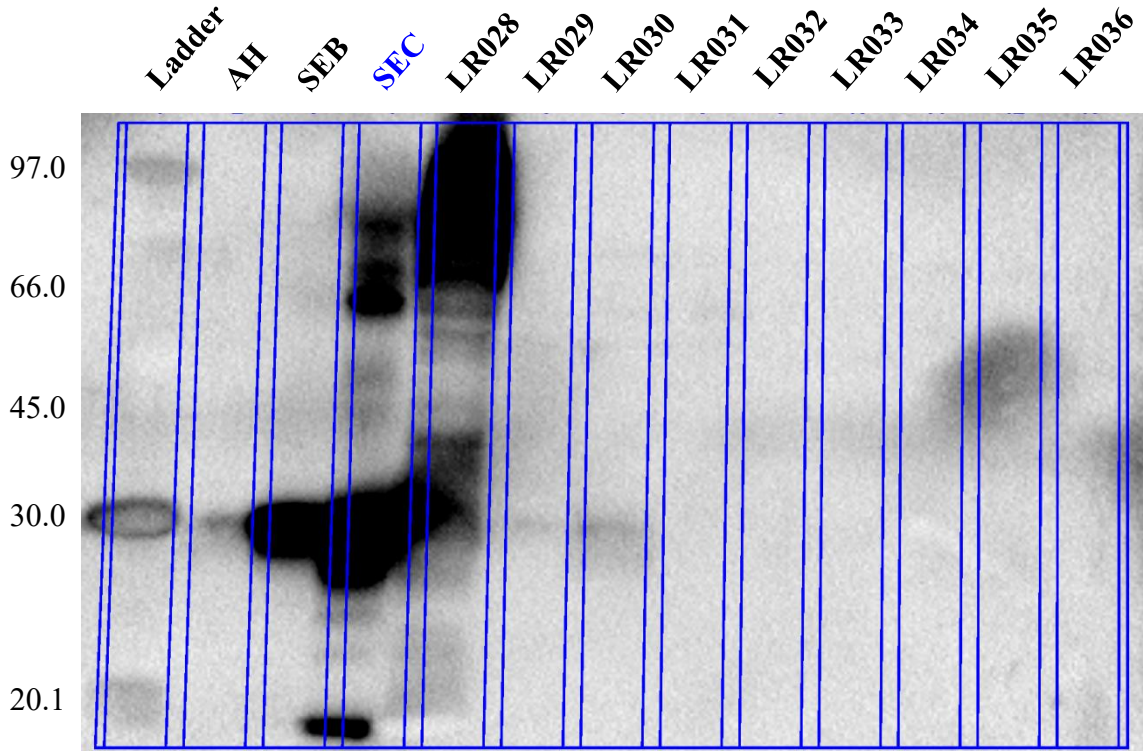


Figure E38 Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR028-036 (28µl) (Lane 5-13).

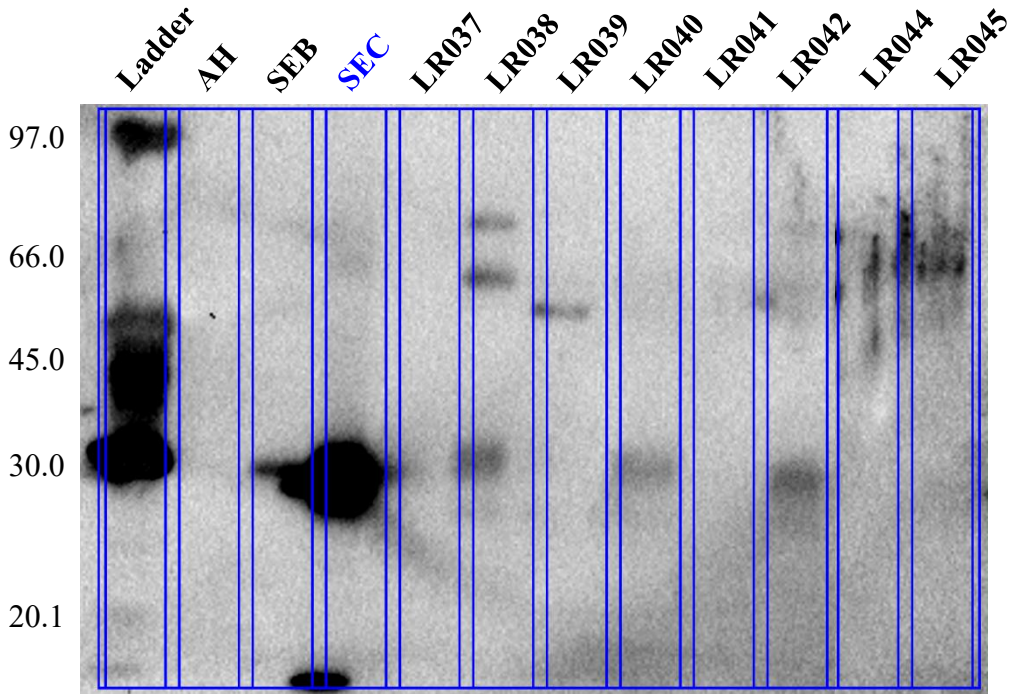


Figure E39 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR037-045 (28µl) (Lane 5-12).**

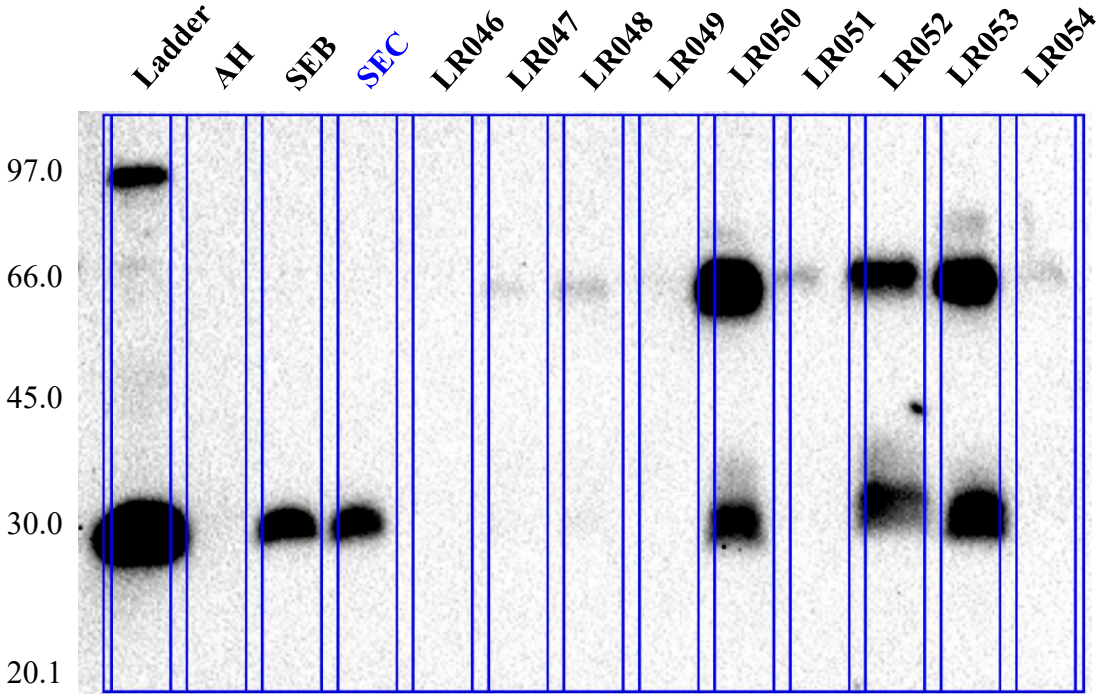


Figure E40 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR0046-054 (28µl) (Lane 5-13).**

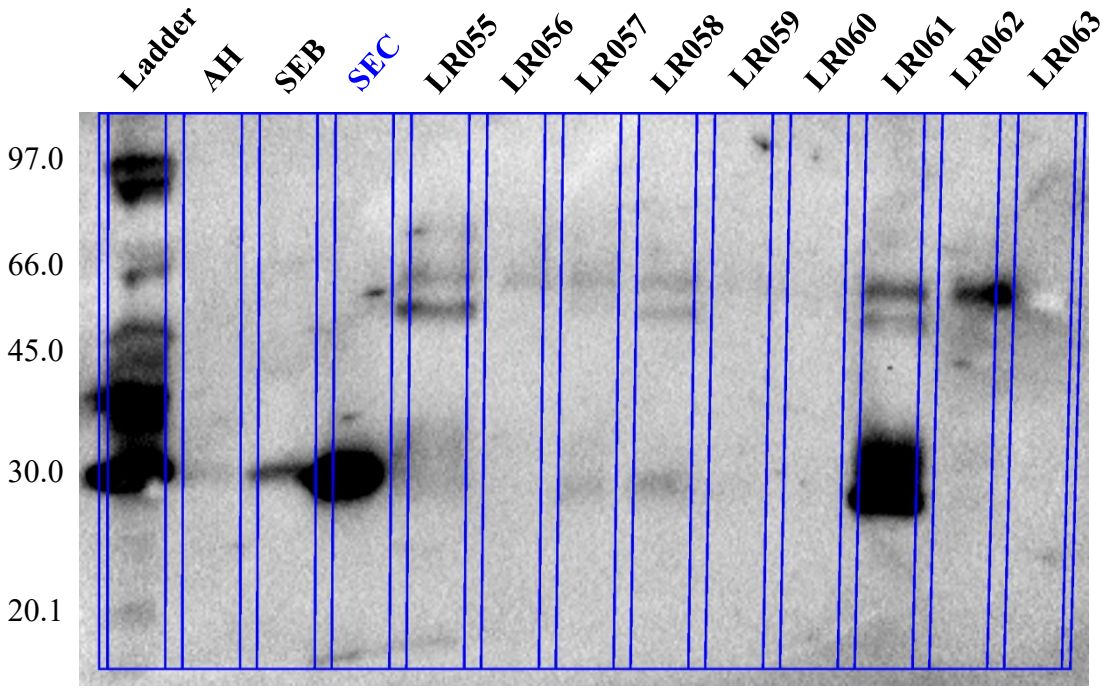


Figure E41 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR055-063 (28µl) (Lane 5-13).**

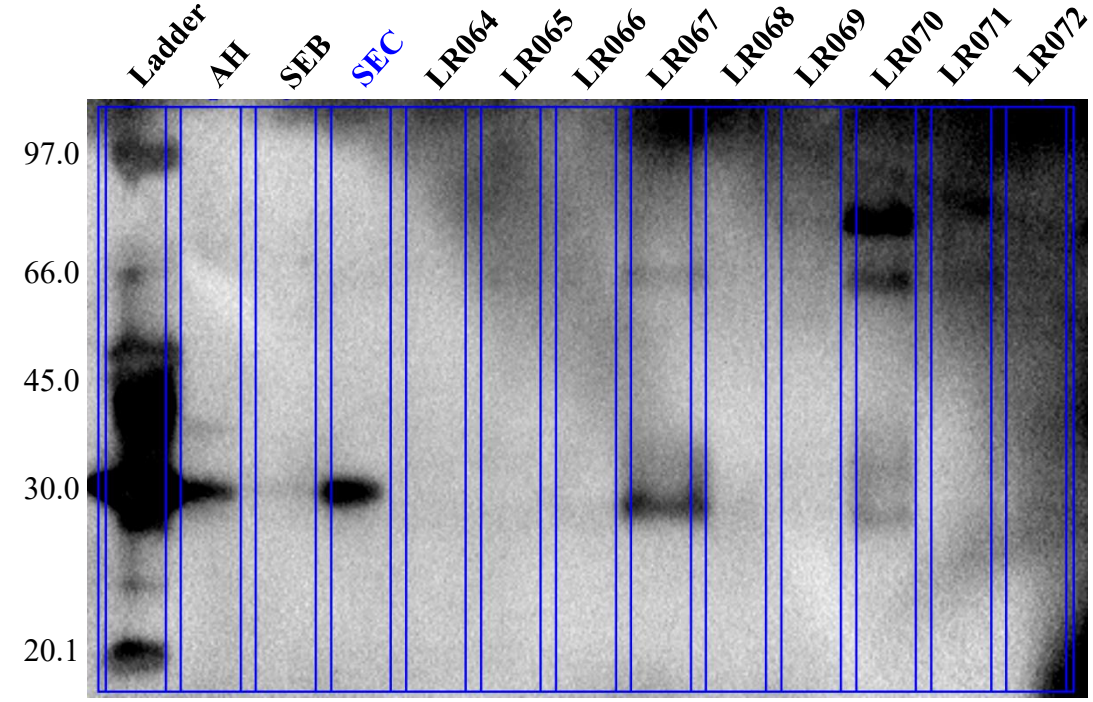


Figure E42 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR064-072 (28µl) (Lane 5-13).**

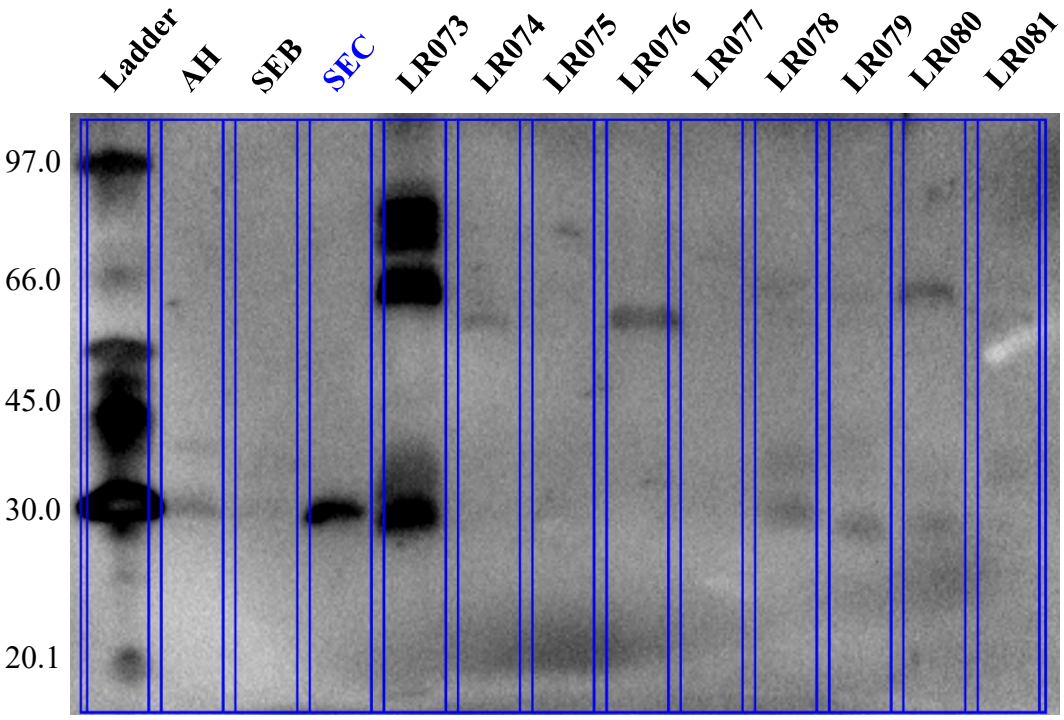


Figure E43 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR073-081 (28µl) (Lane 5-13).**

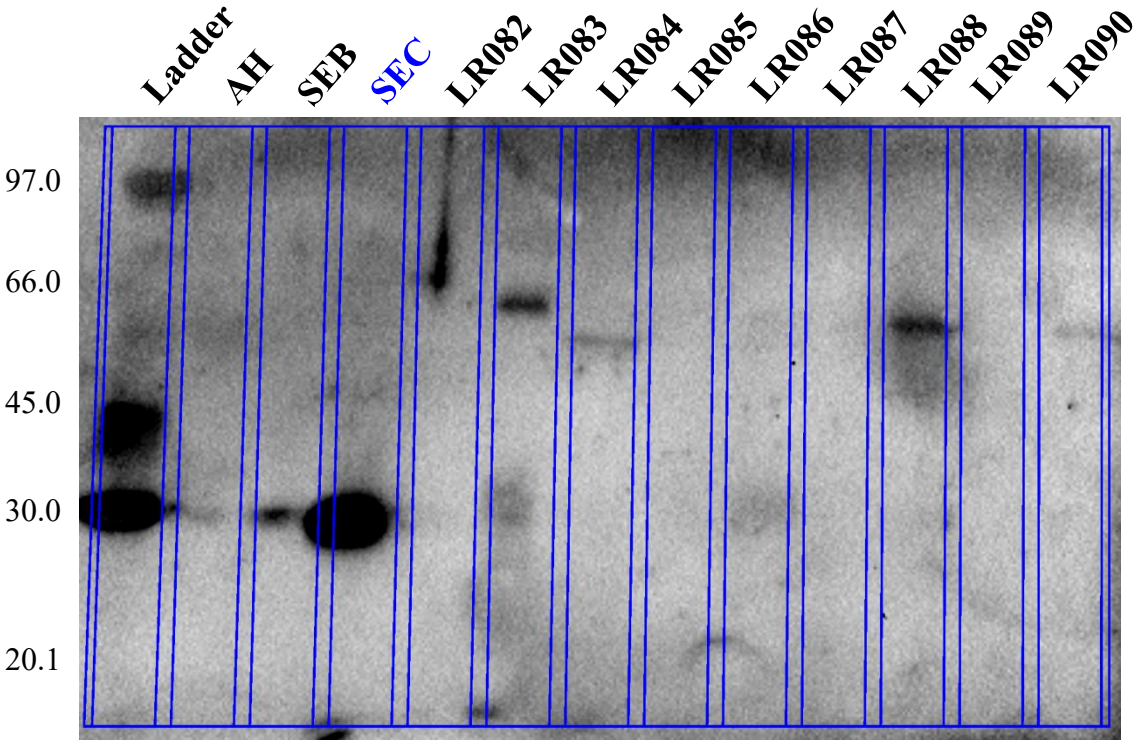


Figure E44 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR082-090 (28µl) (Lane 5-13).**

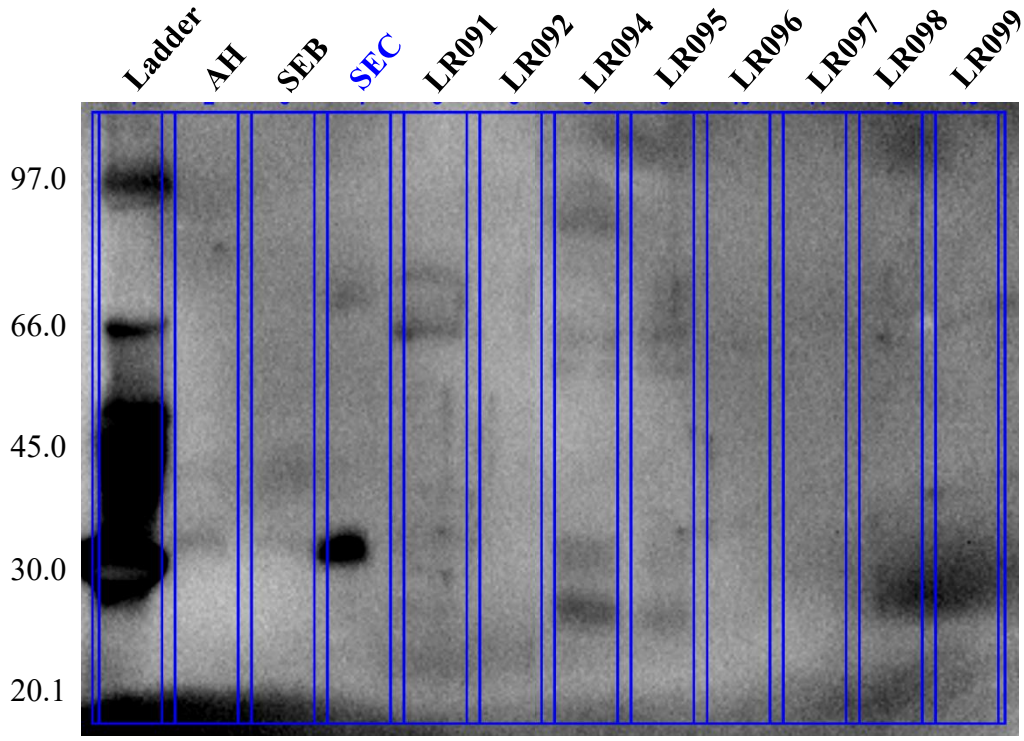


Figure E45 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR091-099 (28µl) (Lane 5-12).**

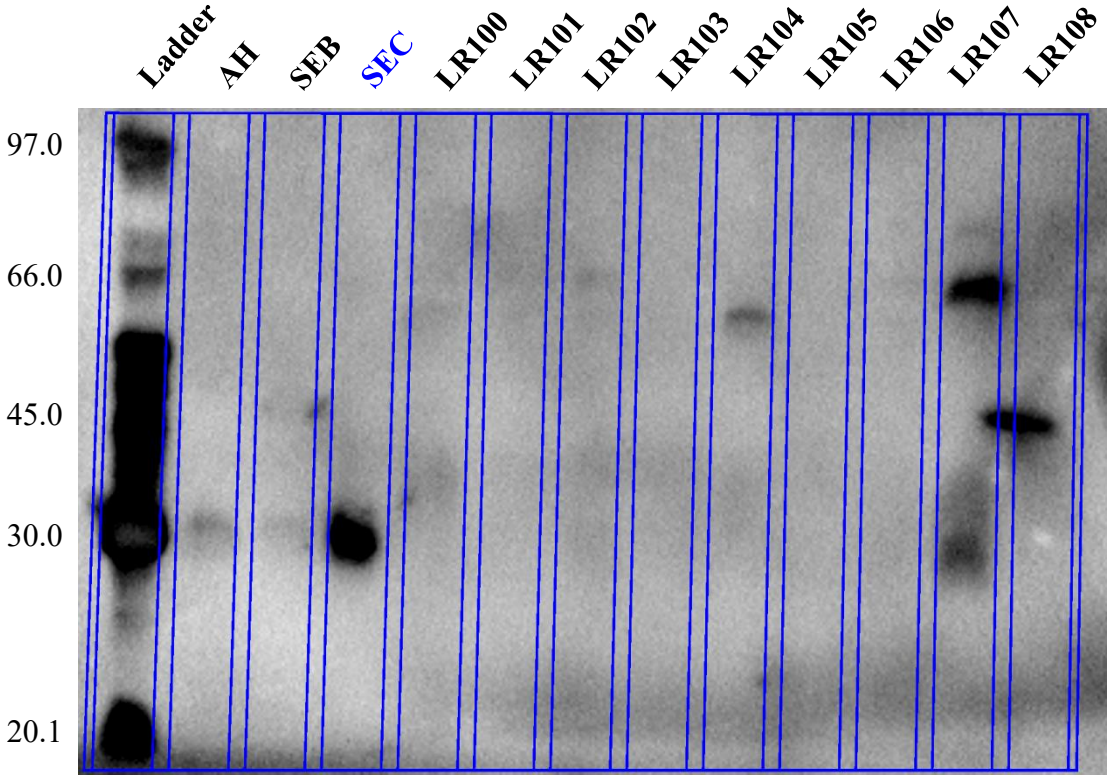


Figure E46 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR100-108 (28µl) (Lane 5-13).**

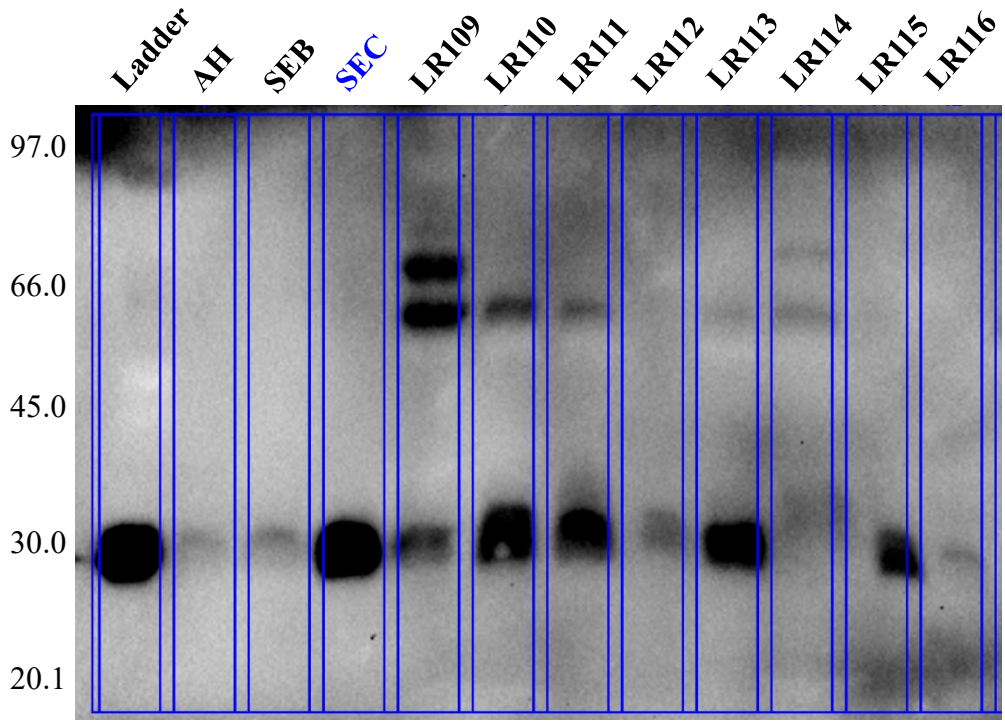


Figure E47 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR109-117 (28µl) (Lane 5-12).**

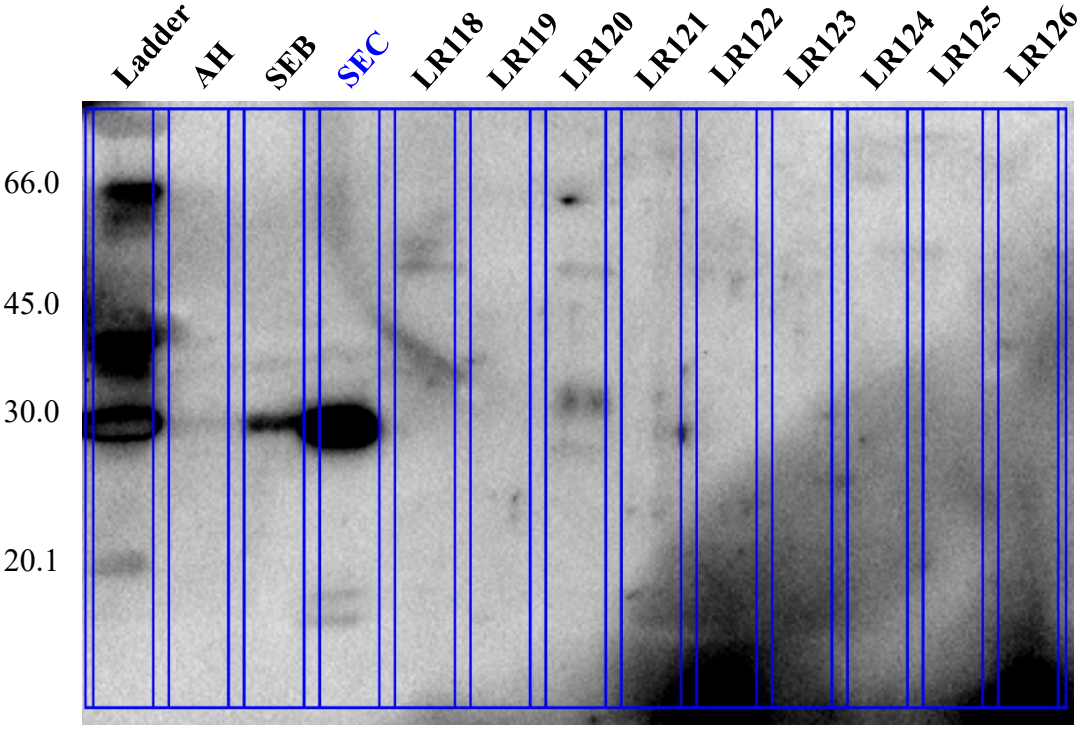


Figure E48 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR118-126 (28µl) (Lane 5-13).**

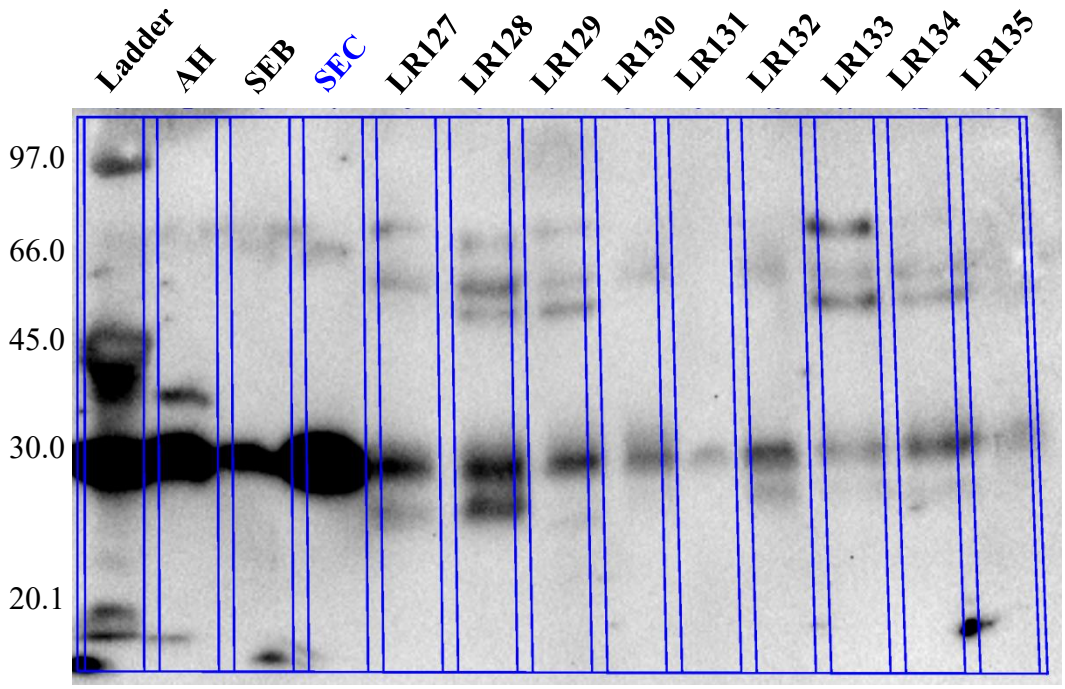


Figure E49 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR127-135 (28µl) (Lane 5-13).**

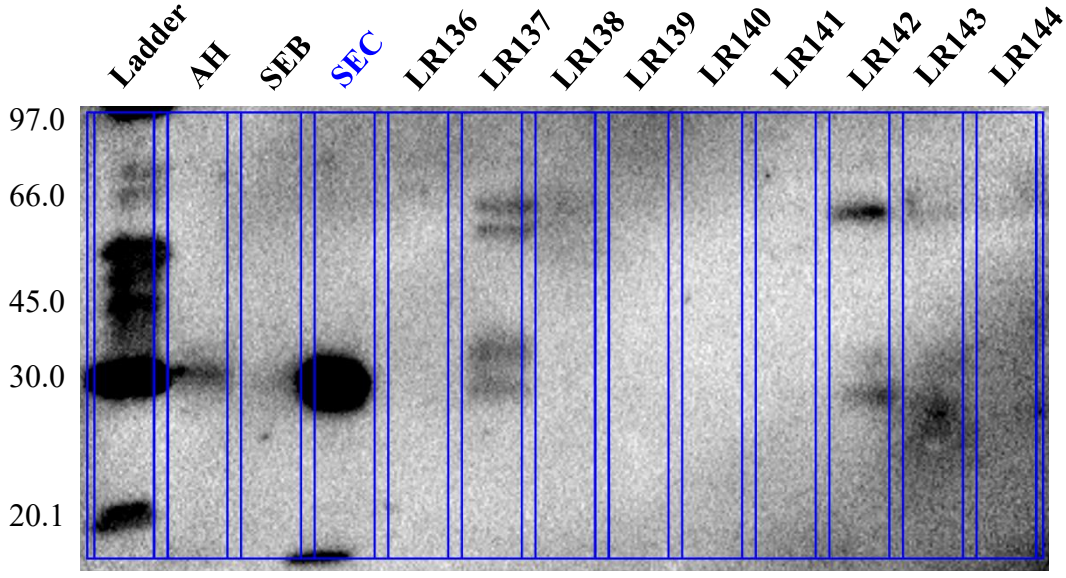


Figure E50 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR136-144 (28µl) (Lane 5-13).**

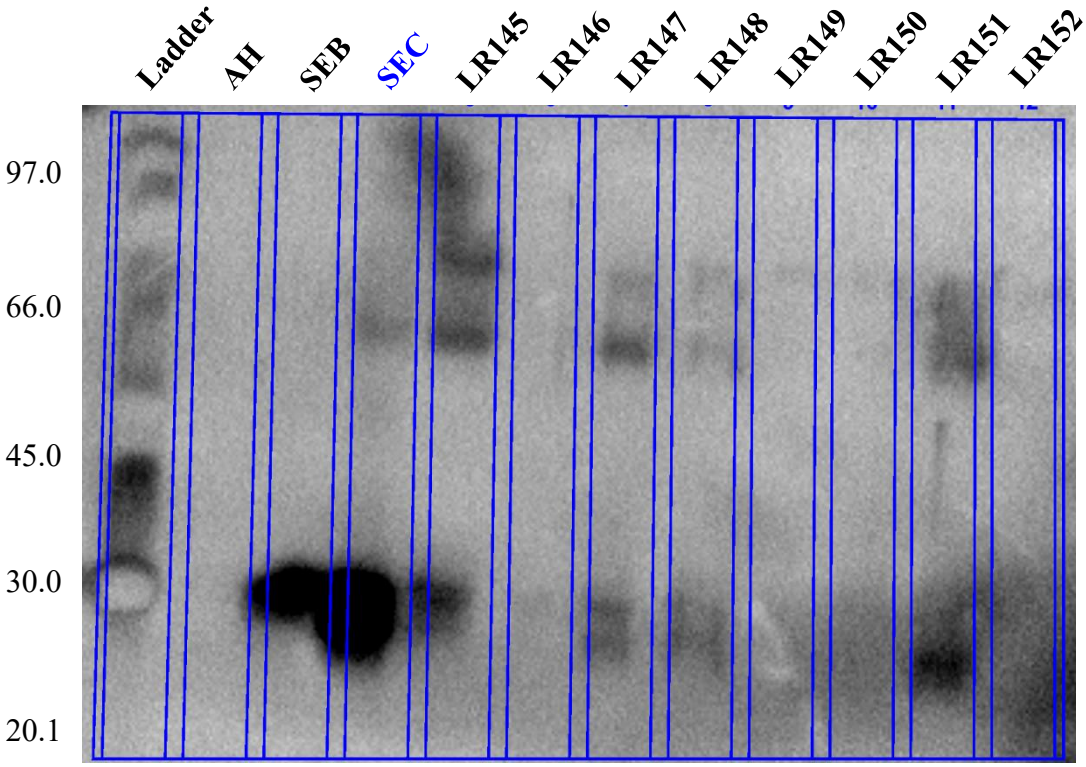


Figure E51 Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LR145-152 (28µl) (Lane 5-13).

Appendix E4 Toxic Shock Syndrome Toxin 1 (24kDa)

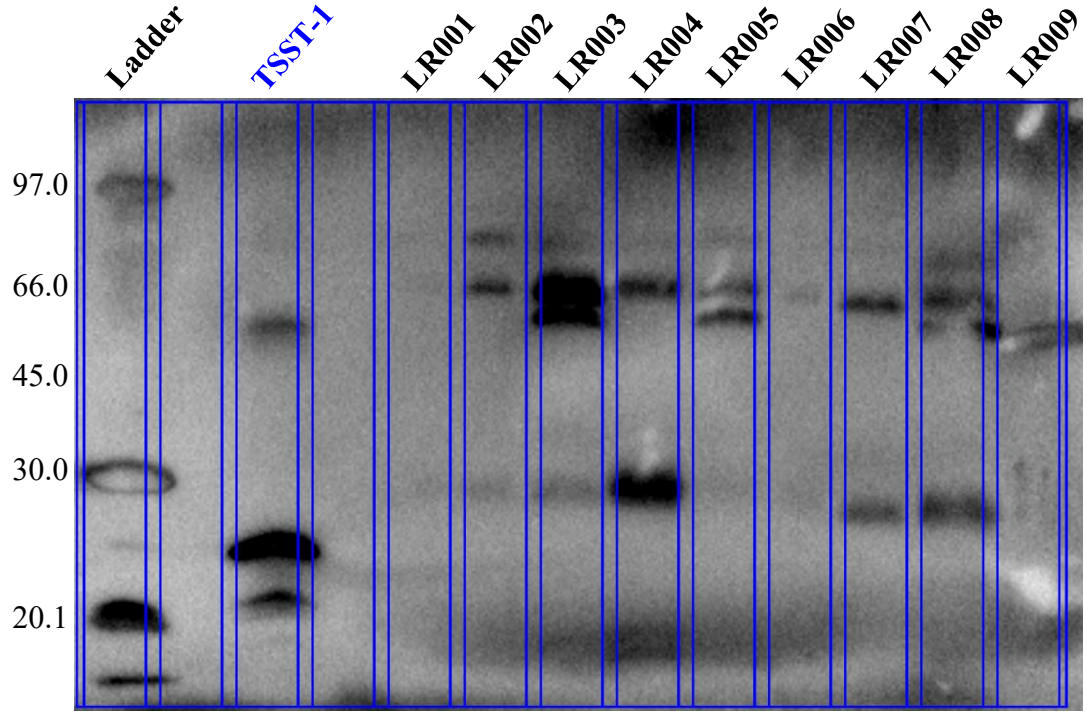


Figure E52 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LR001-009 (28µl) (Lane 5-13).**

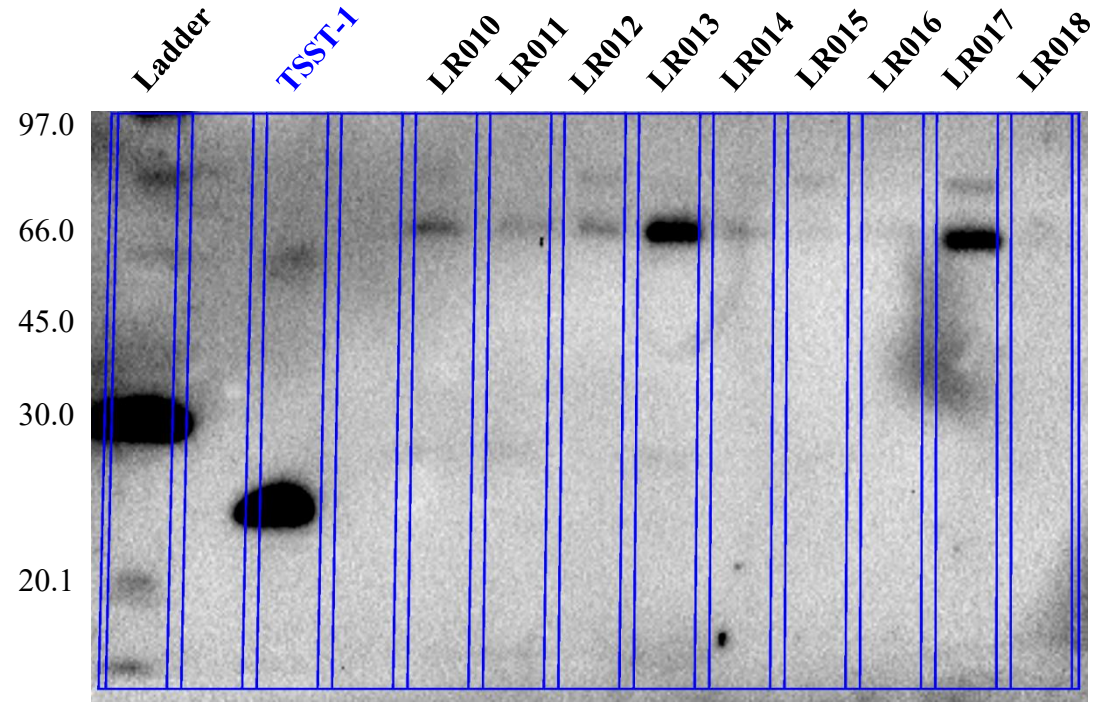


Figure E53 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LR010-018 (28µl) (Lane 5-13).**

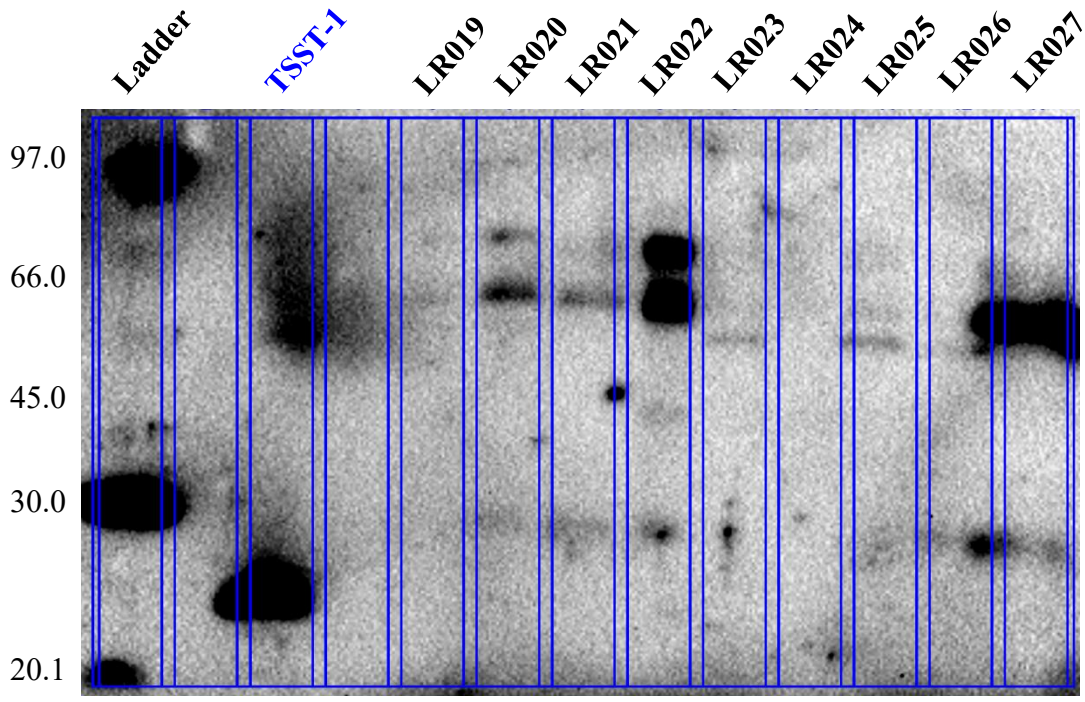


Figure E54 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LR019-027 (28µl) (Lane 5-13).**

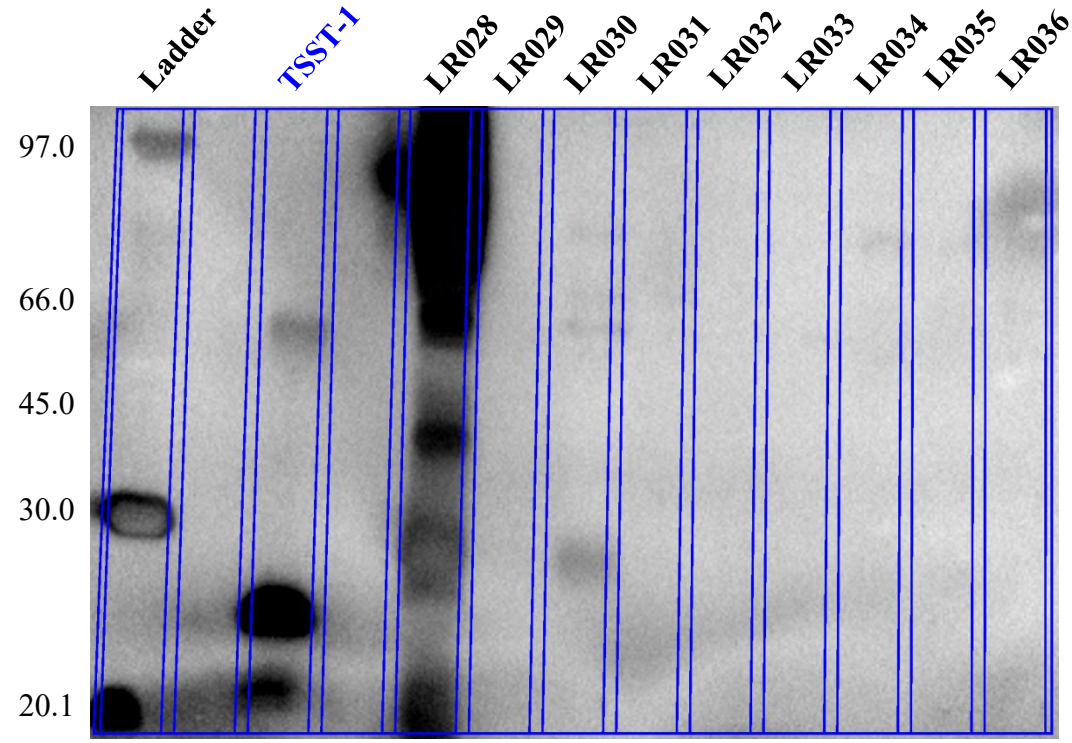


Figure E55 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LR028-036 (28µl) (Lane 5-13).**

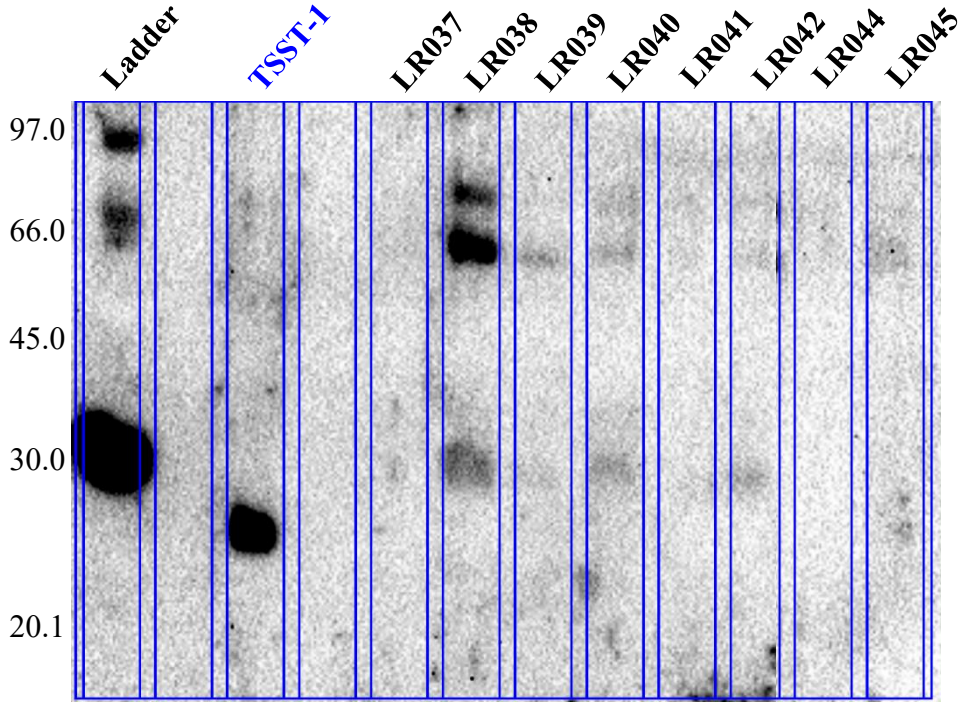


Figure E56 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LR037-045 (28µl) (Lane 5-12).**

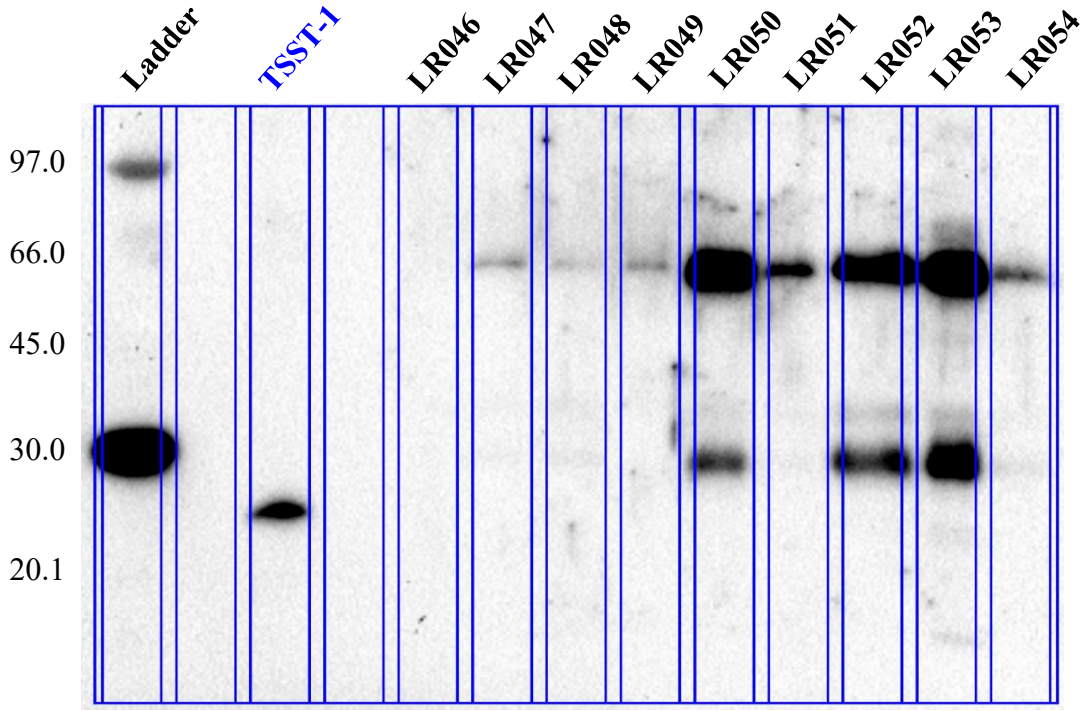


Figure E57 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LR046-054 (28µl) (Lane 5-13).**

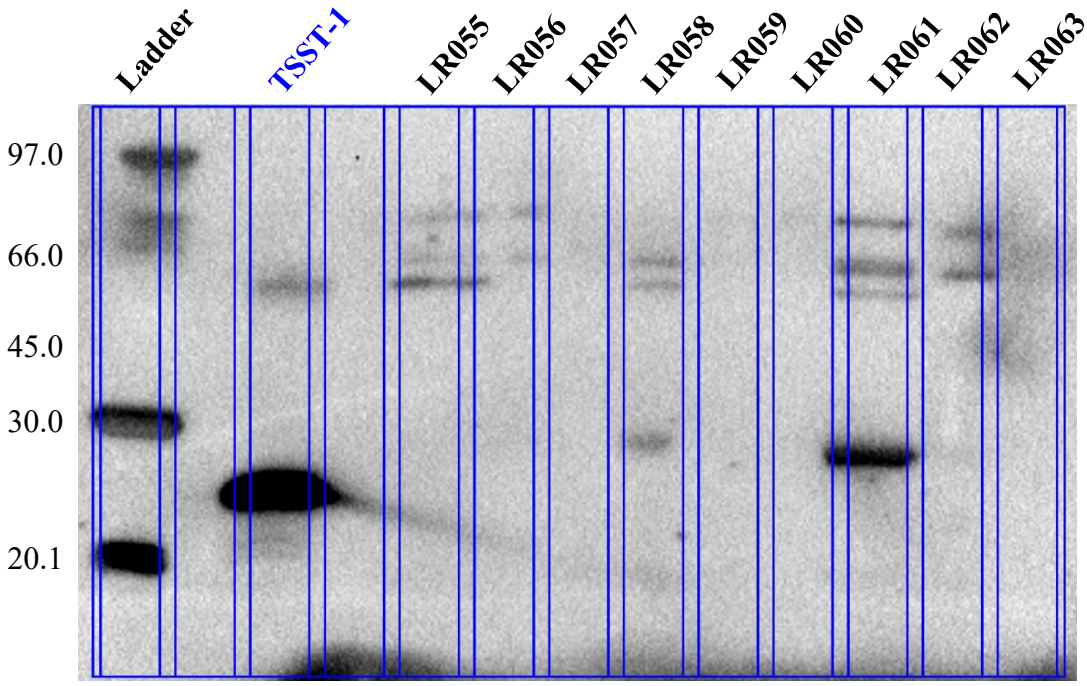


Figure E58 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LR055-063 (28µl) (Lane 5-13).**

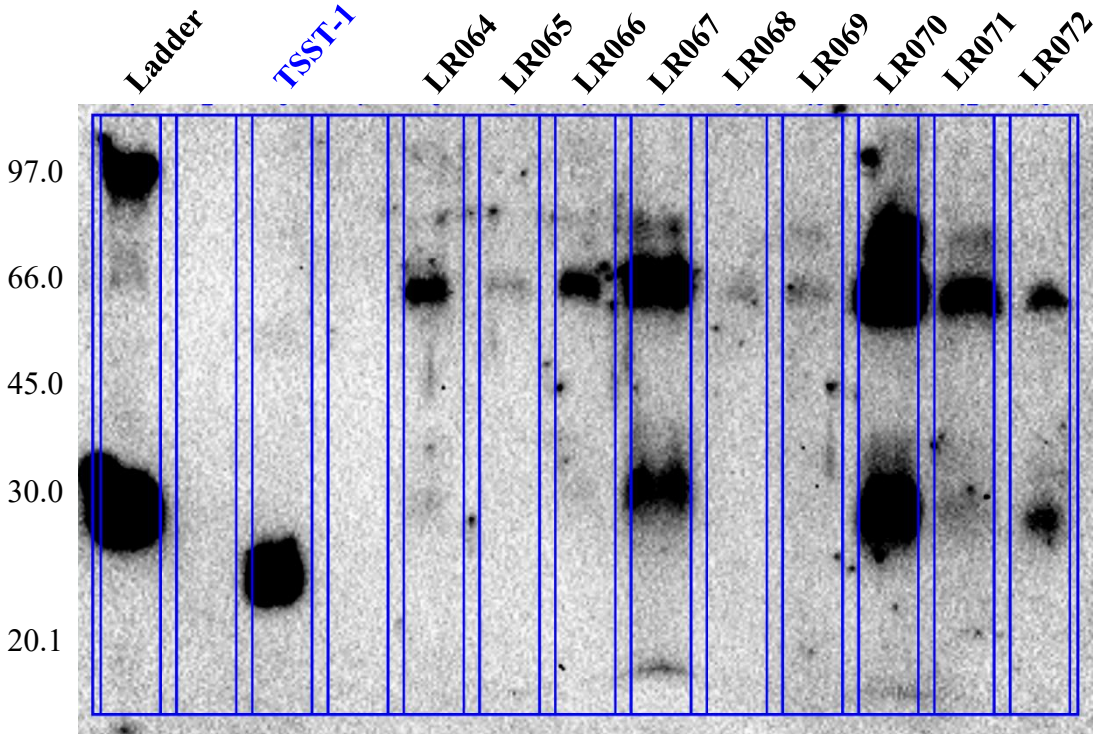


Figure E59 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LR064-072 (28µl) (Lane 5-13).**

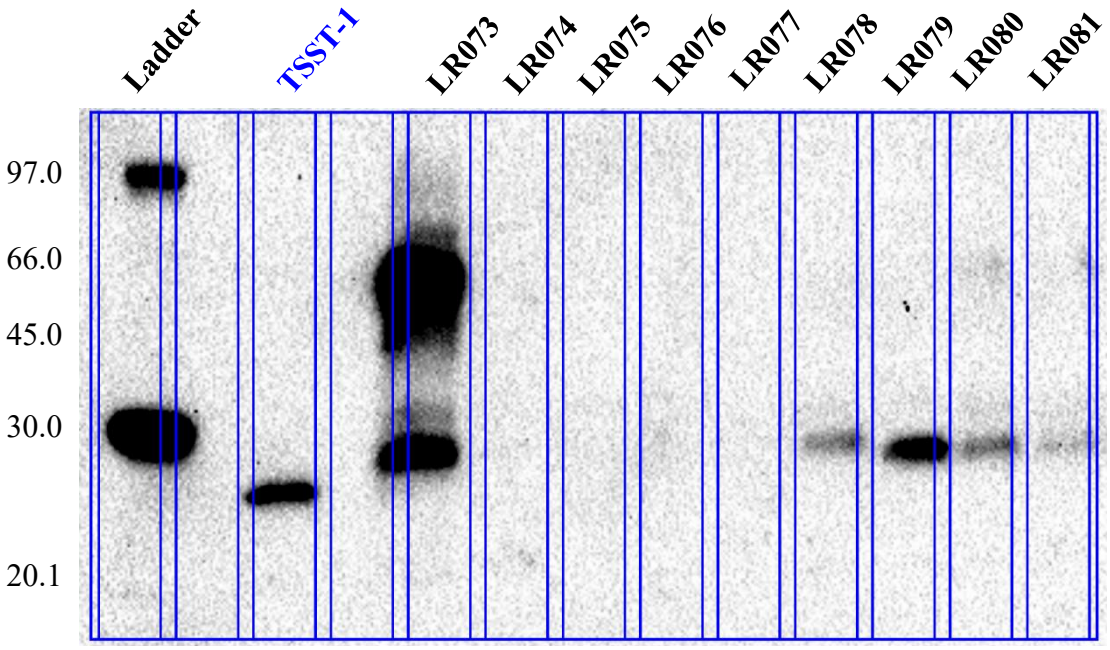


Figure E60 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LR073-081 (28µl) (Lane 5-13).**

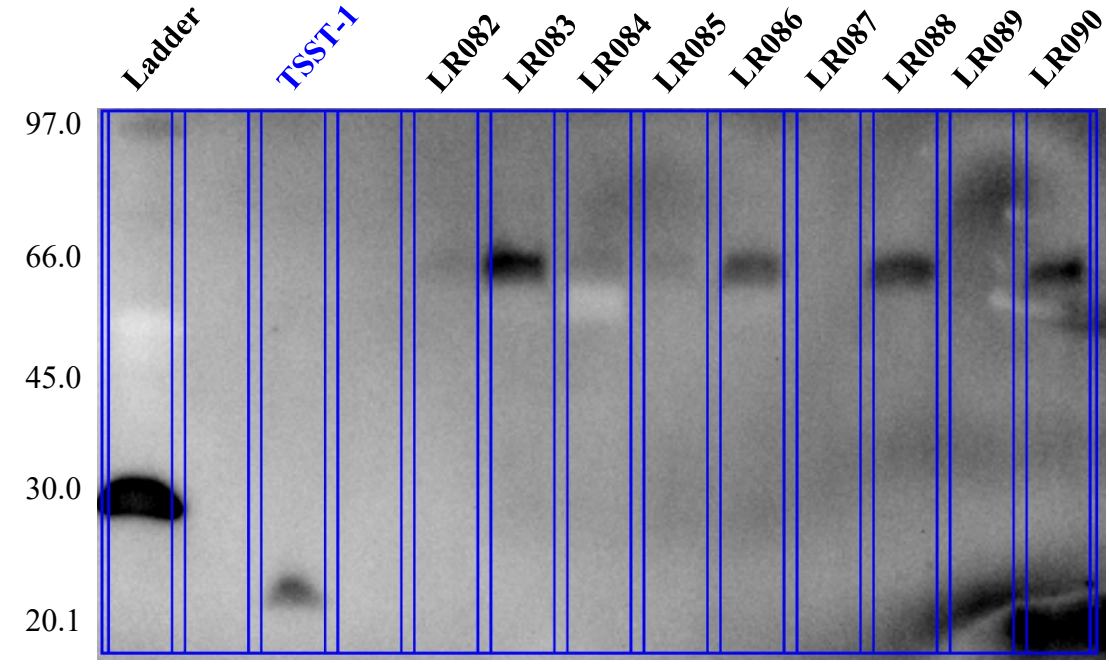


Figure E61 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LR082-090 (28µl) (Lane 5-13).**

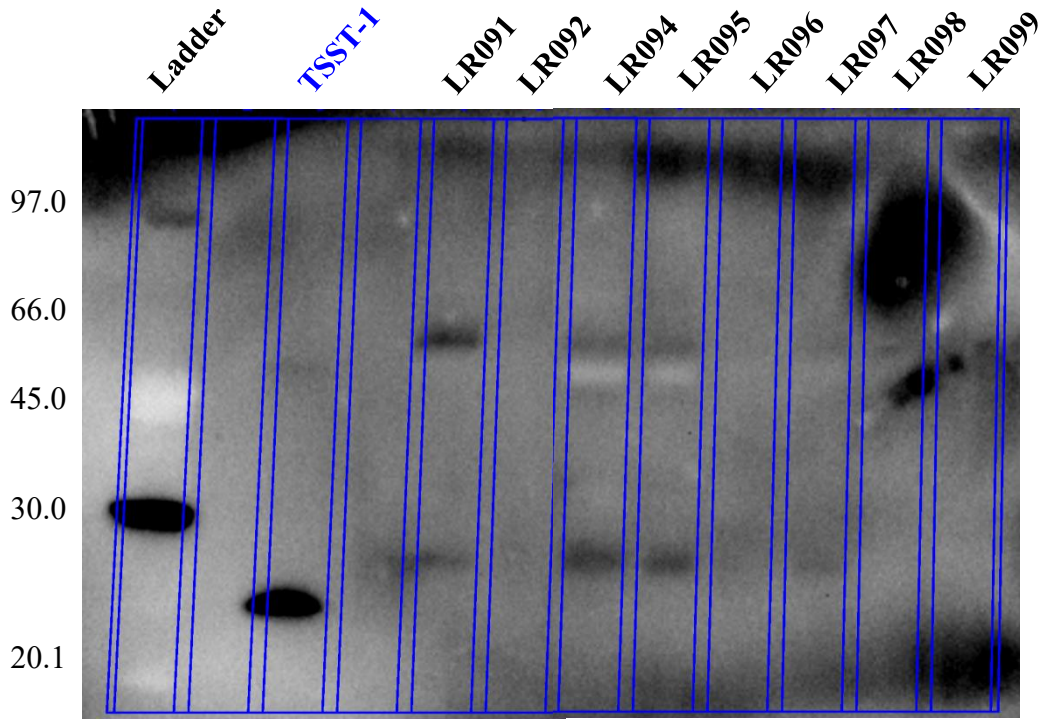


Figure E62 Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LR091-099 (28µl) (Lane 5-12).

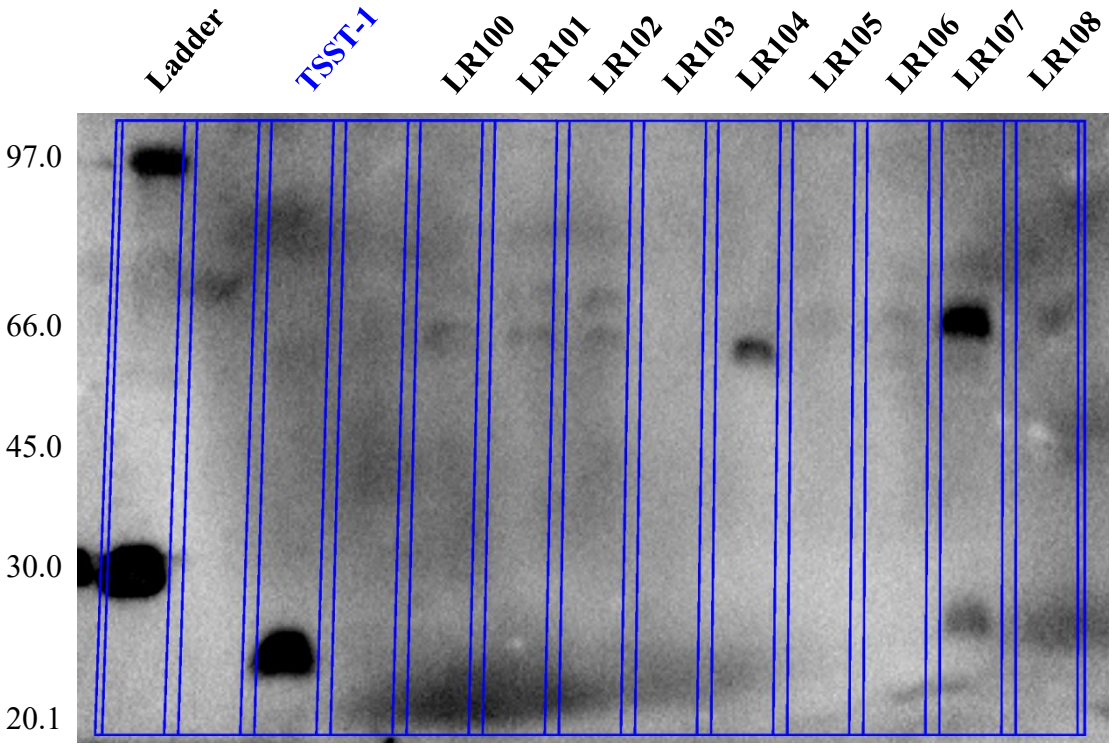


Figure E63 Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LR100-108 (28µl) (Lane 5-13).

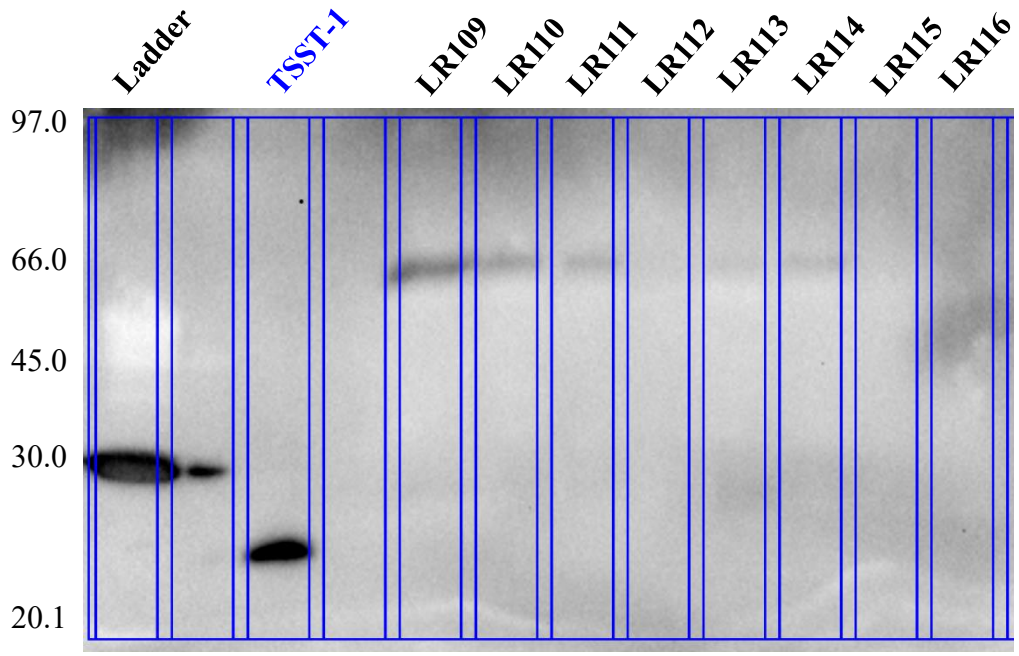


Figure E64 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LR109-117 (28µl) (Lane 5-12).**

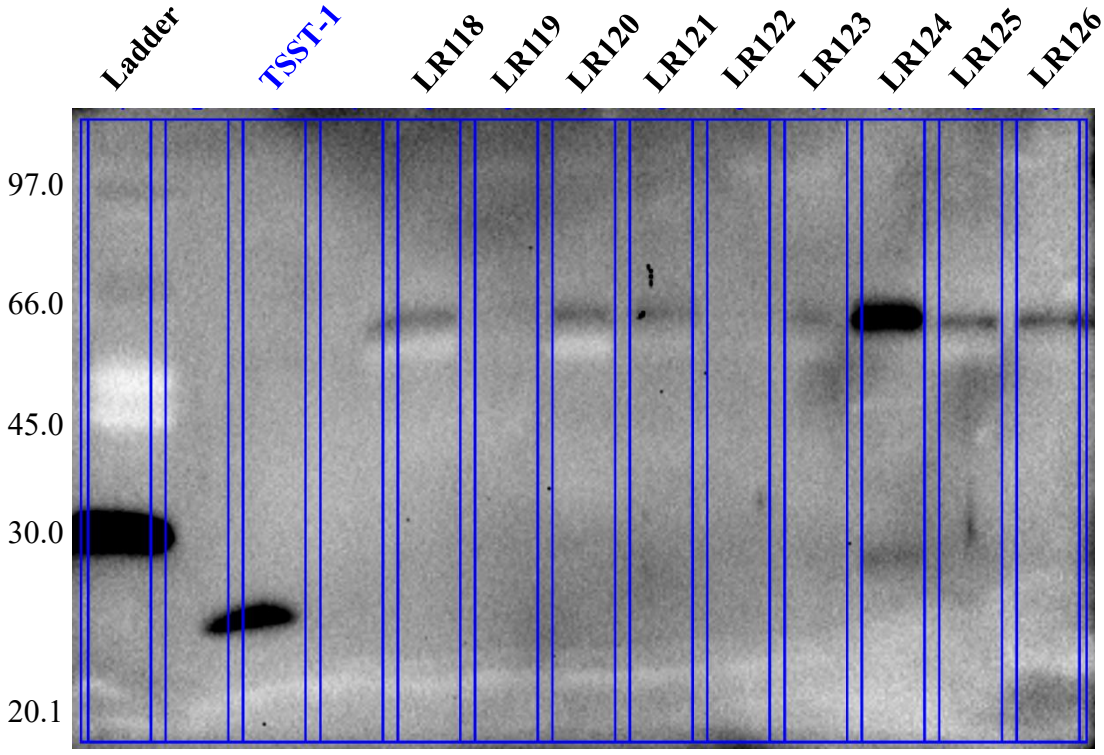


Figure E65 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LR118-126 (28µl) (Lane 5-13).**

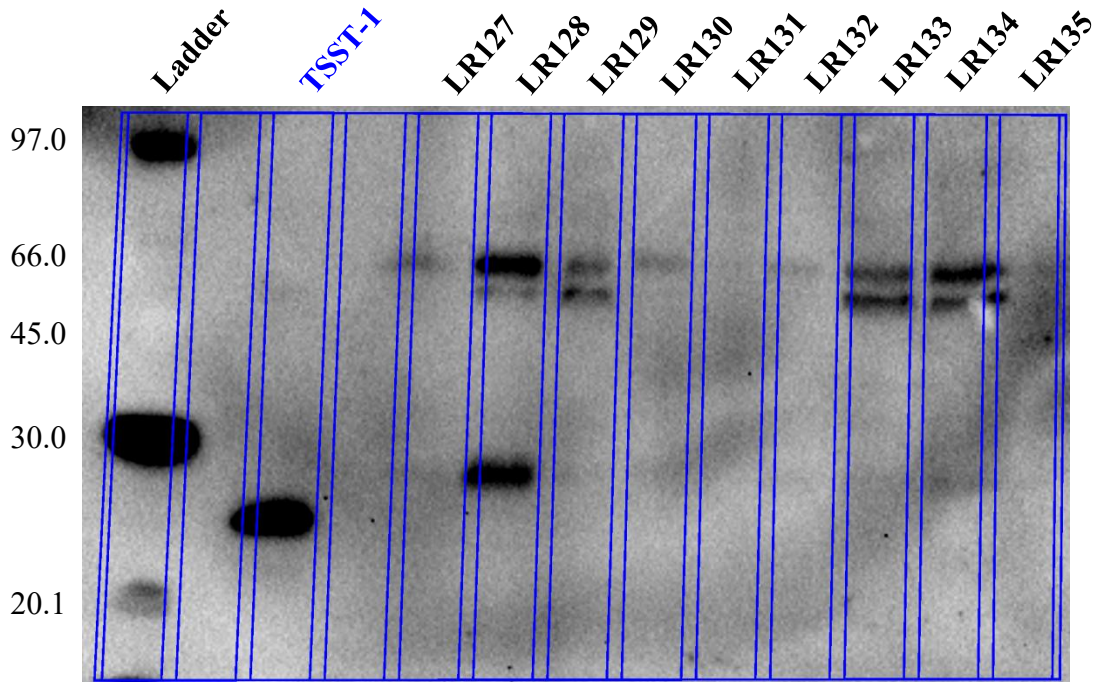


Figure E66 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LR127-135 (28µl) (Lane 5-13).**

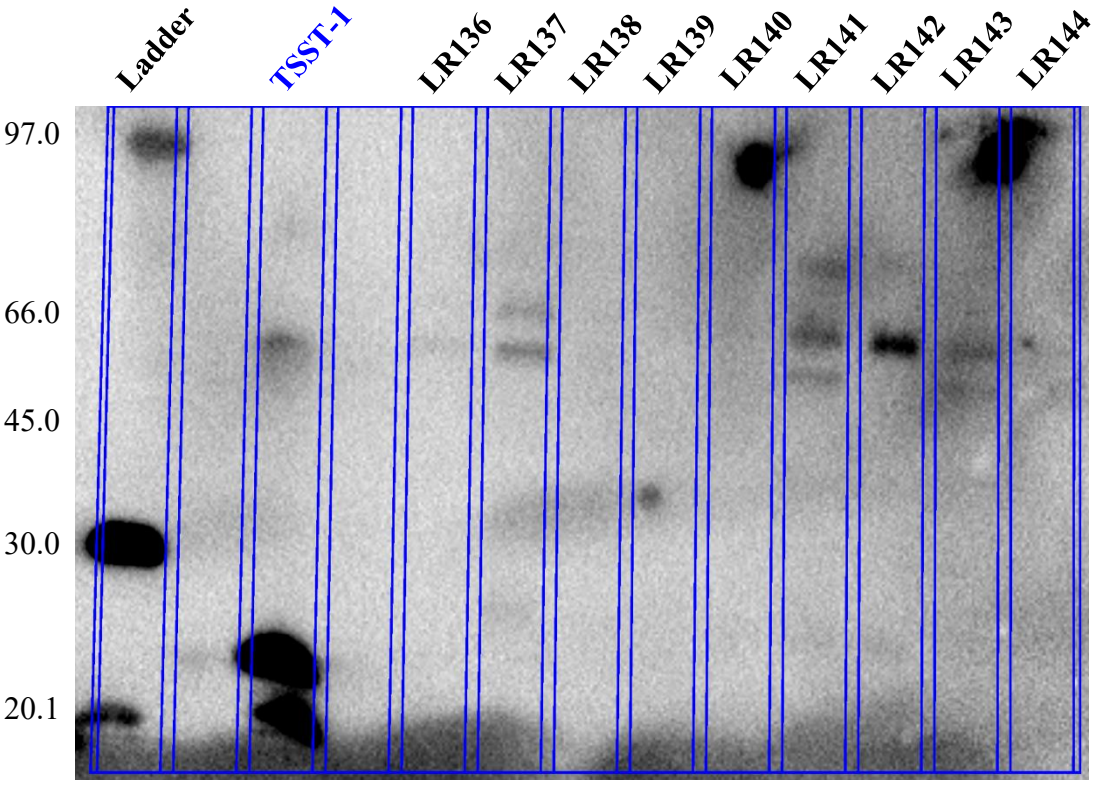


Figure E67 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LR136-144 (28µl) (Lane 5-13).**

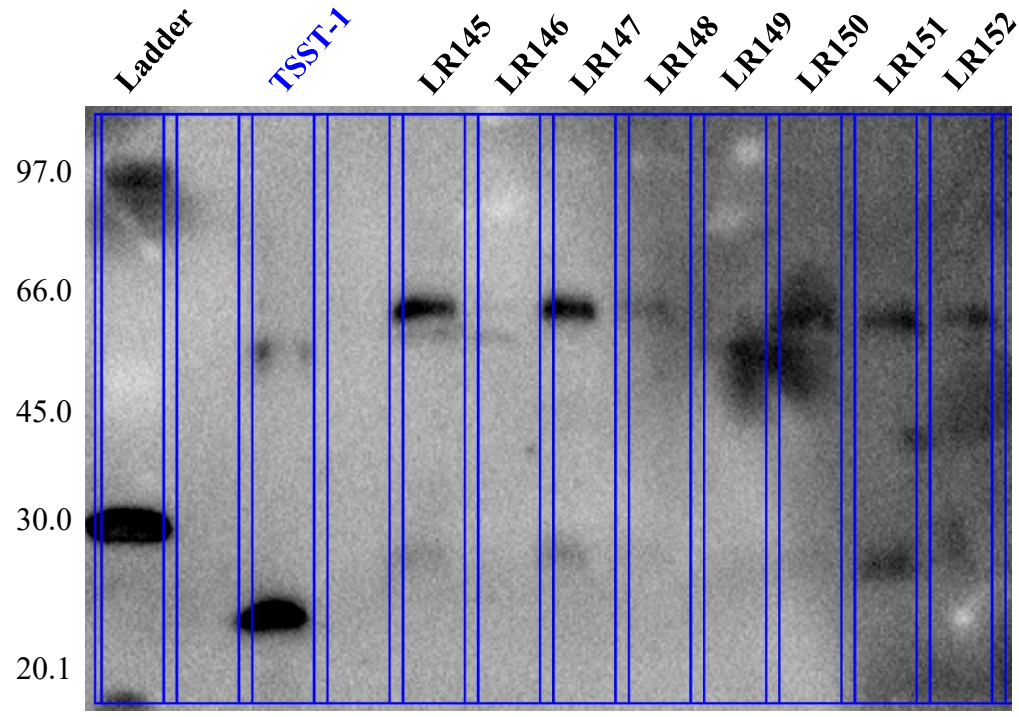


Figure E68 Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LR145-152 (28µl) (Lane 5-13).

Appendix F1 Alpha Haemolysin (33kDa)

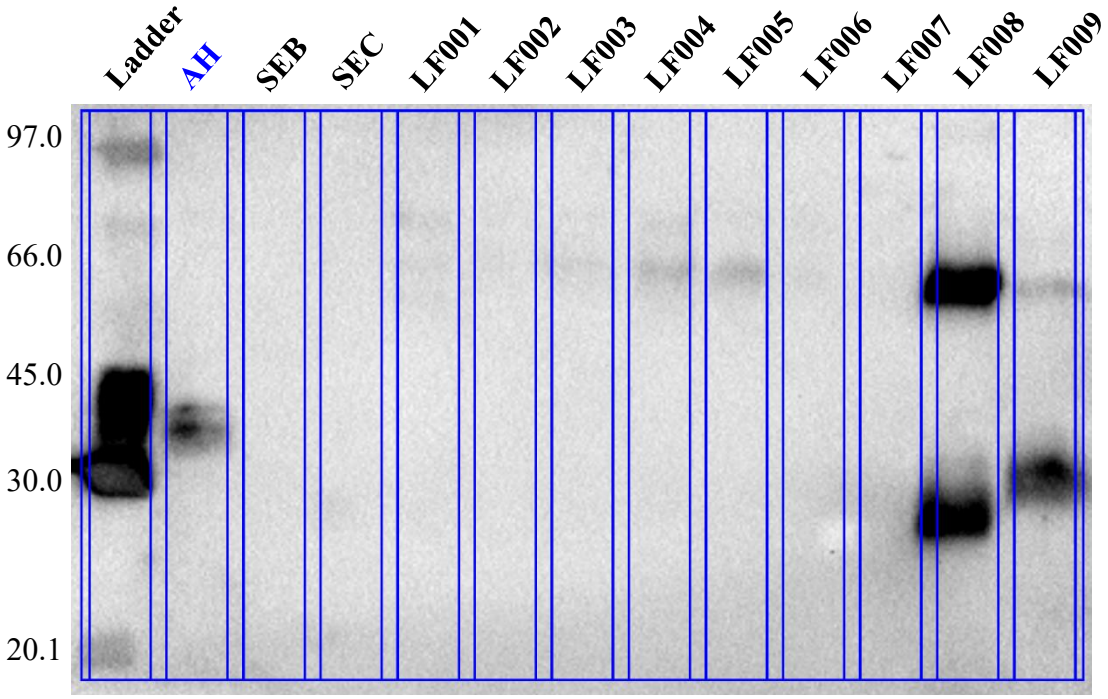


Figure F1 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF001-009 (28µl) (Lane 5-13).**

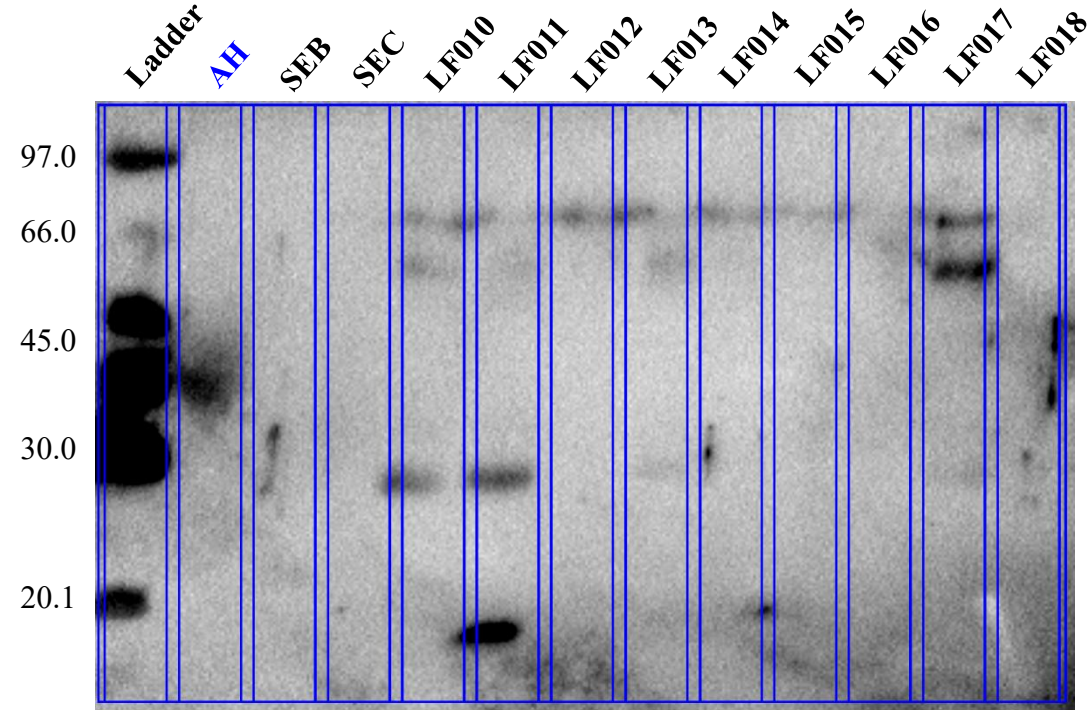


Figure F2 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF010-018 (28µl) (Lane 5-13).**

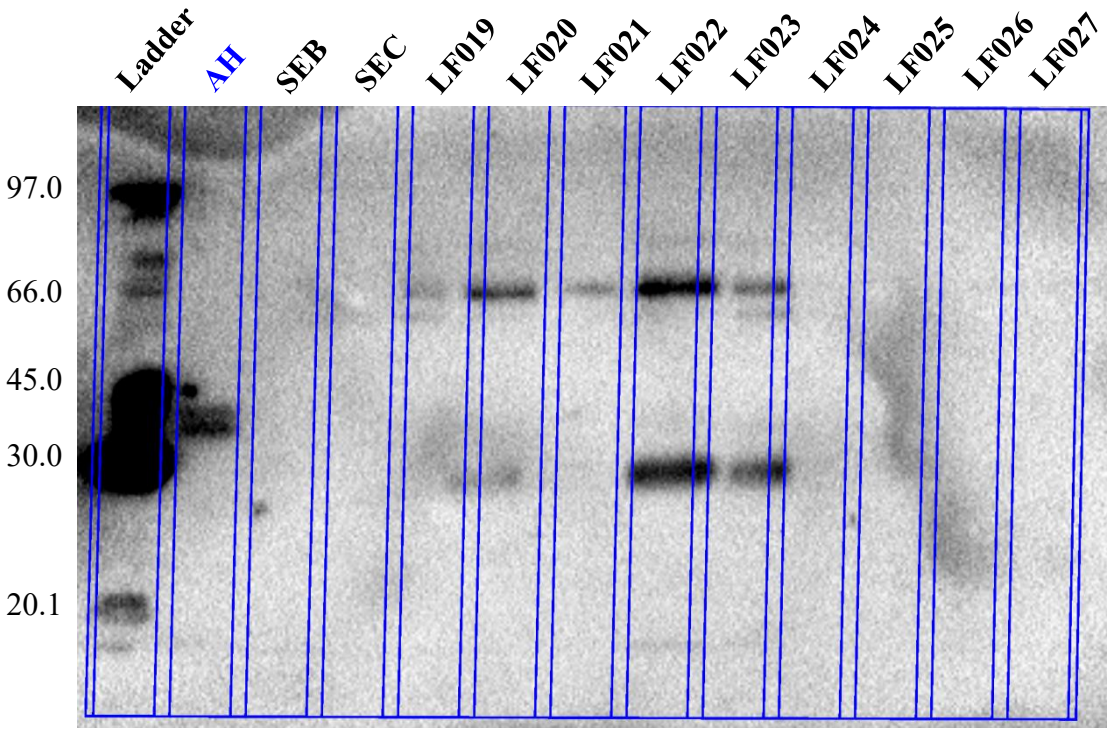


Figure F3 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF019-027 (28µl) (Lane 5-13).**

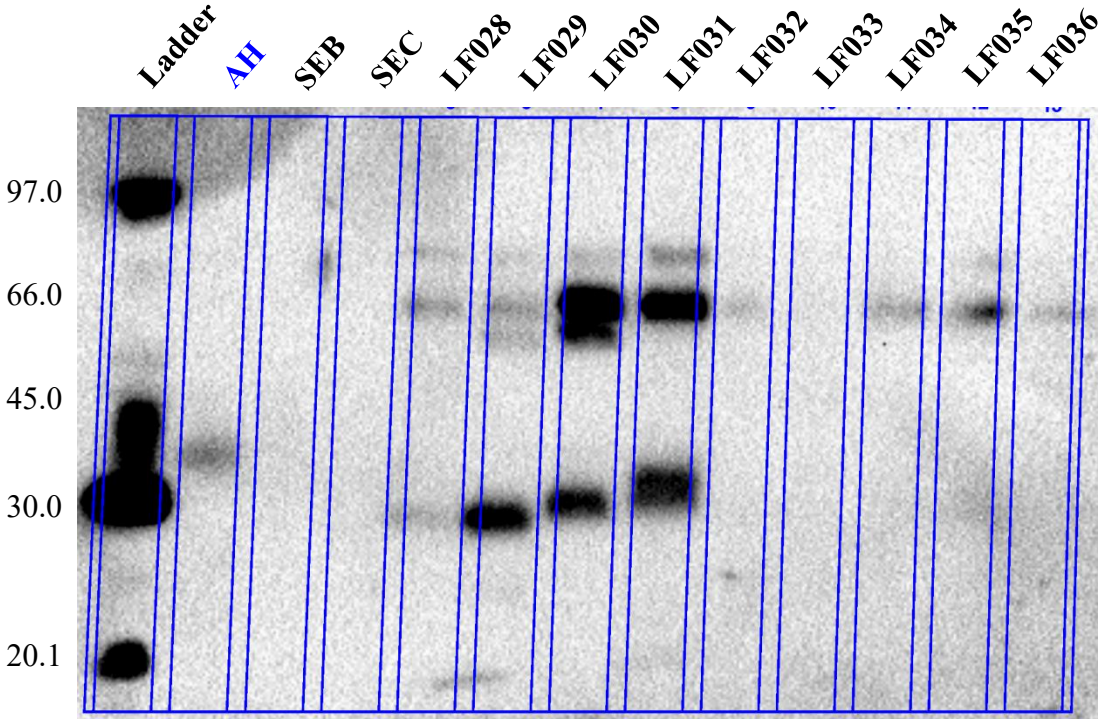


Figure F4 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF028-036 (28µl) (Lane 5-13).**

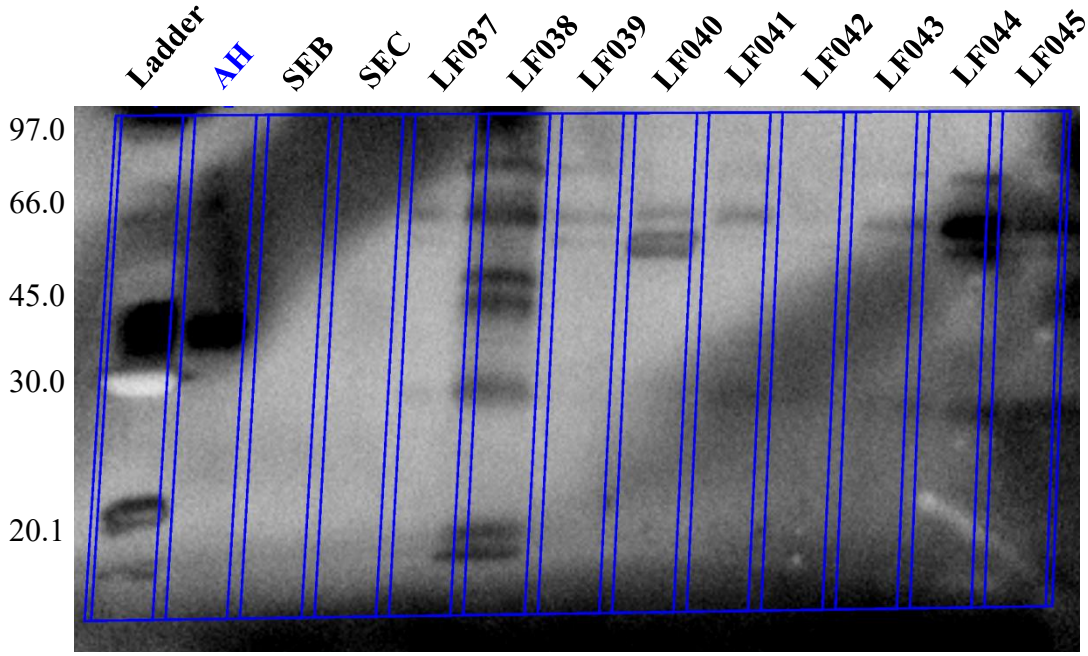


Figure F5 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF037-045 (28µl) (Lane 5-13).**

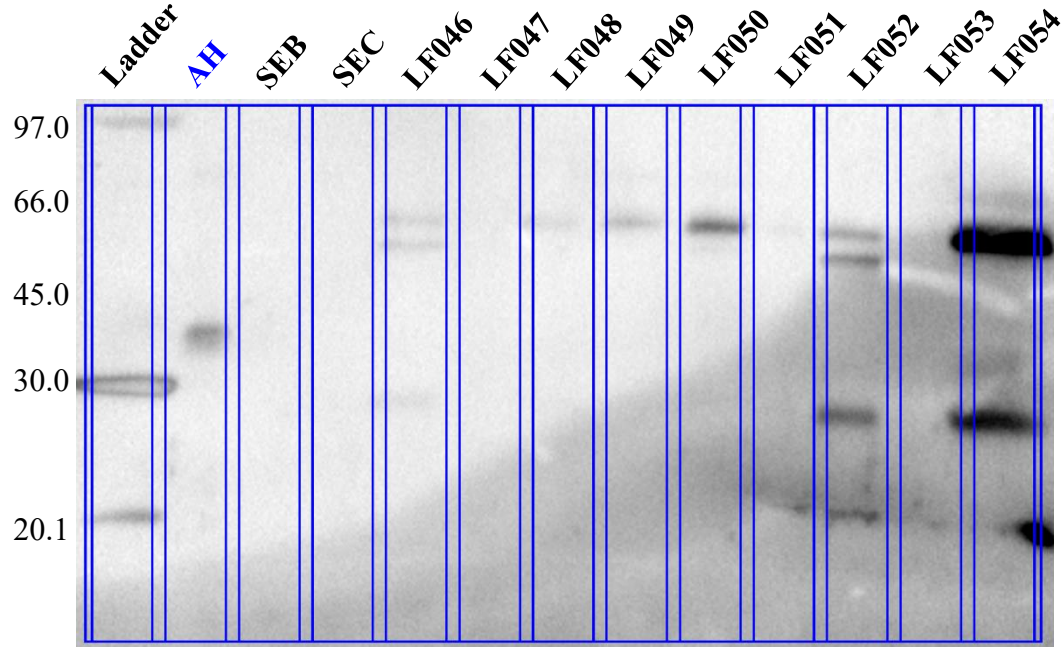


Figure F6 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF046-054 (28µl) (Lane 5-13).**

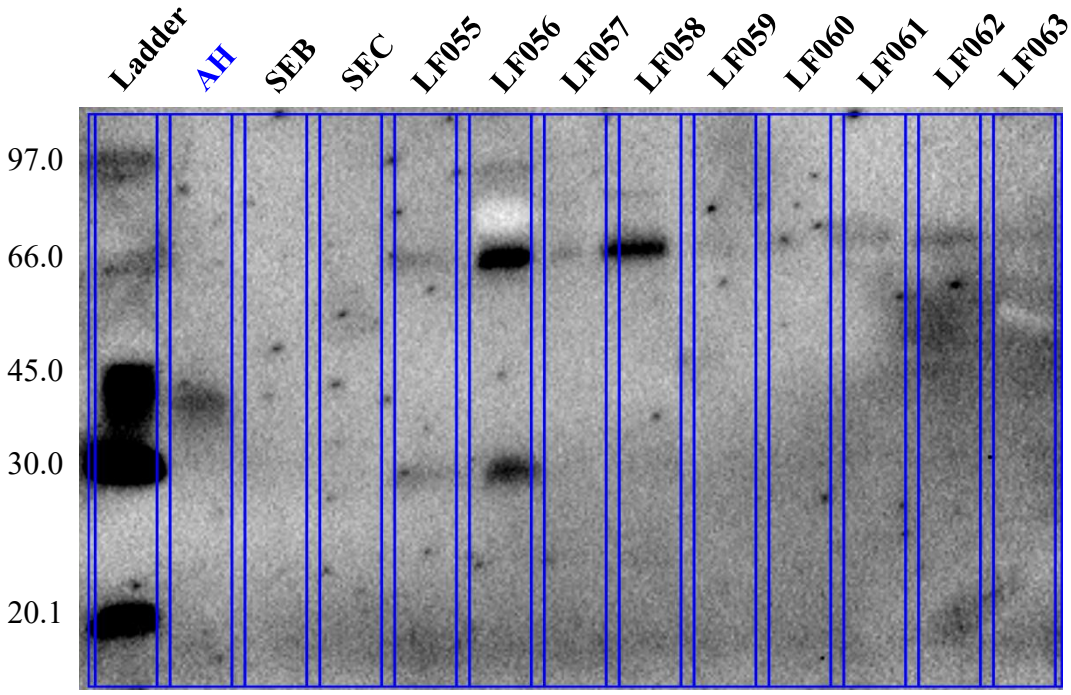


Figure F7 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF055-063 (28µl) (Lane 5-13).**

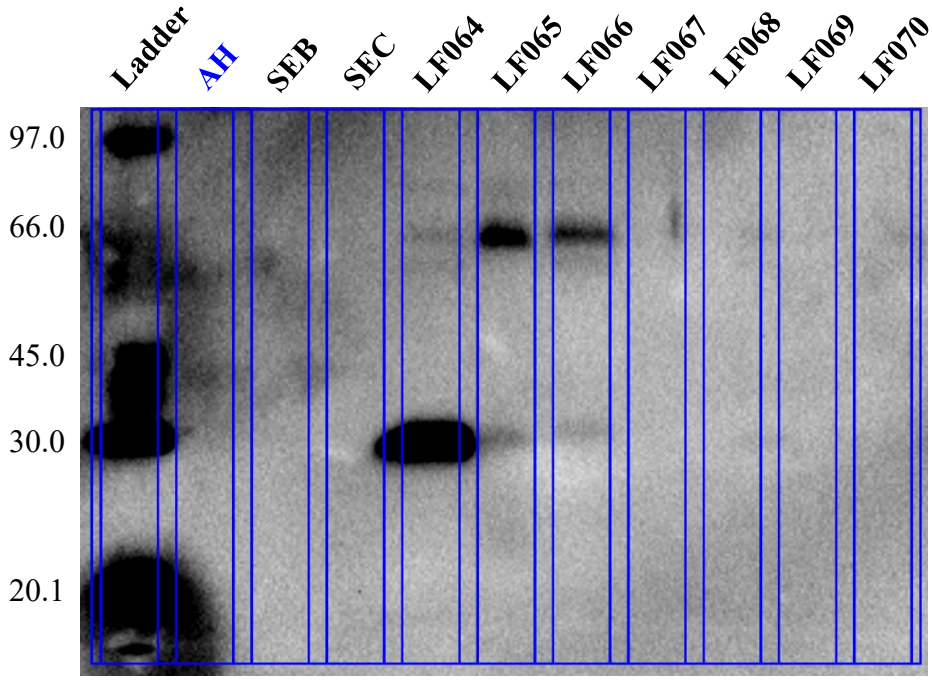


Figure F8 **Western blot membrane incubated with αAH primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF037-045 (28µl) (Lane 5-11).**

Appendix F2 Staphylococcal Enterotoxin B (28kDa)

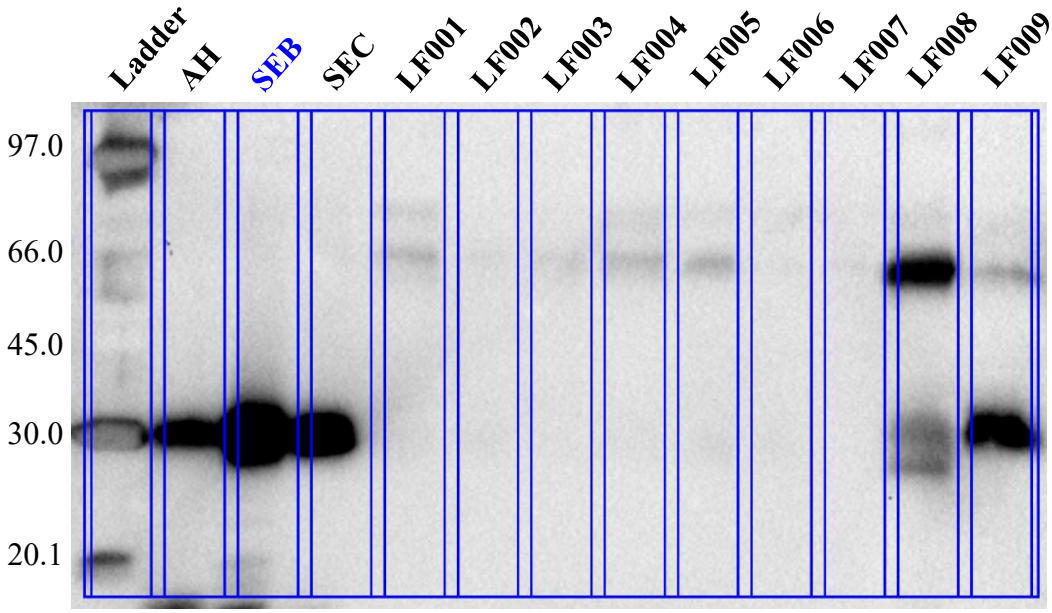


Figure F9 **Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF001-009 (28µl) (Lane 5-13).**

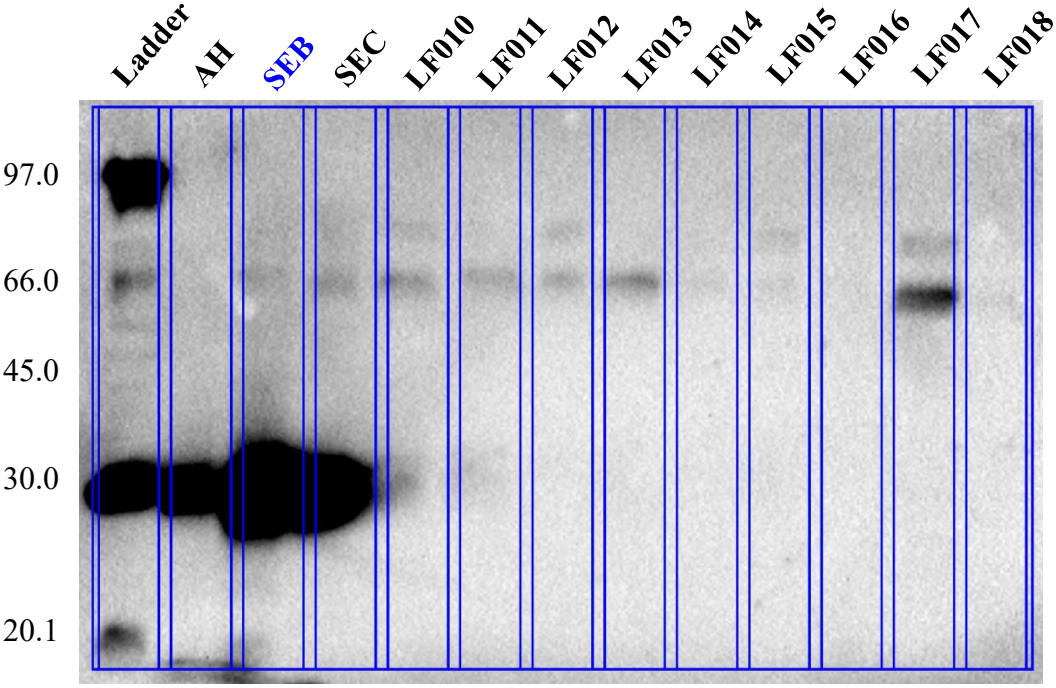


Figure F10 **Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF010-018 (28µl) (Lane 5-13).**

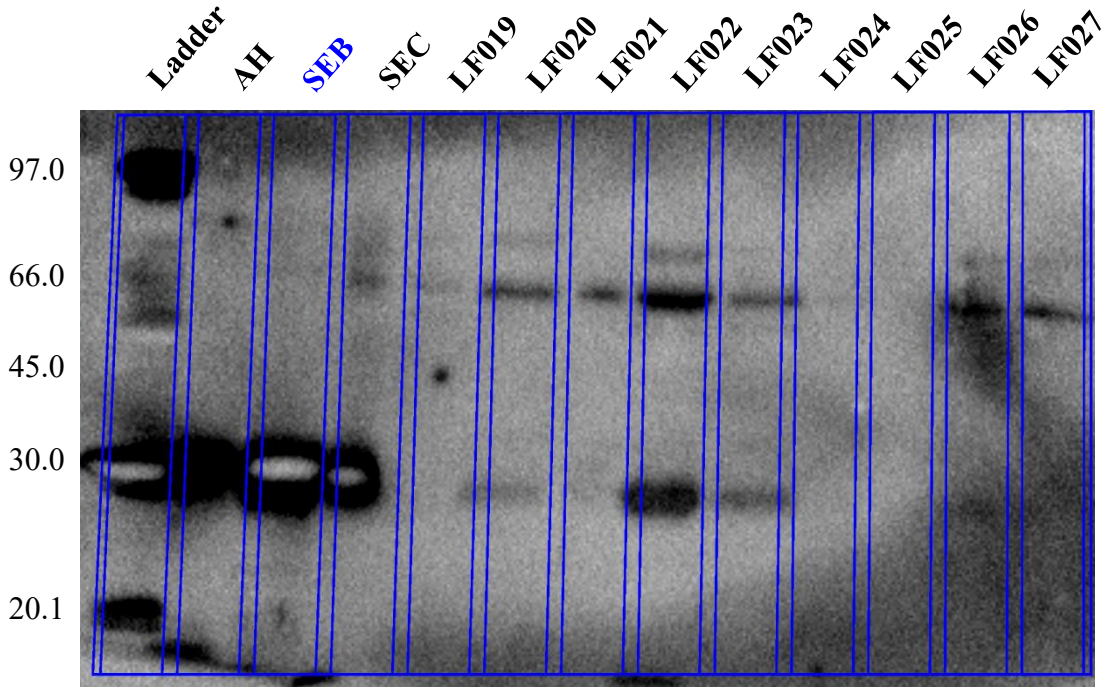


Figure F11 Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF019-027 (28µl) (Lane 5-13).

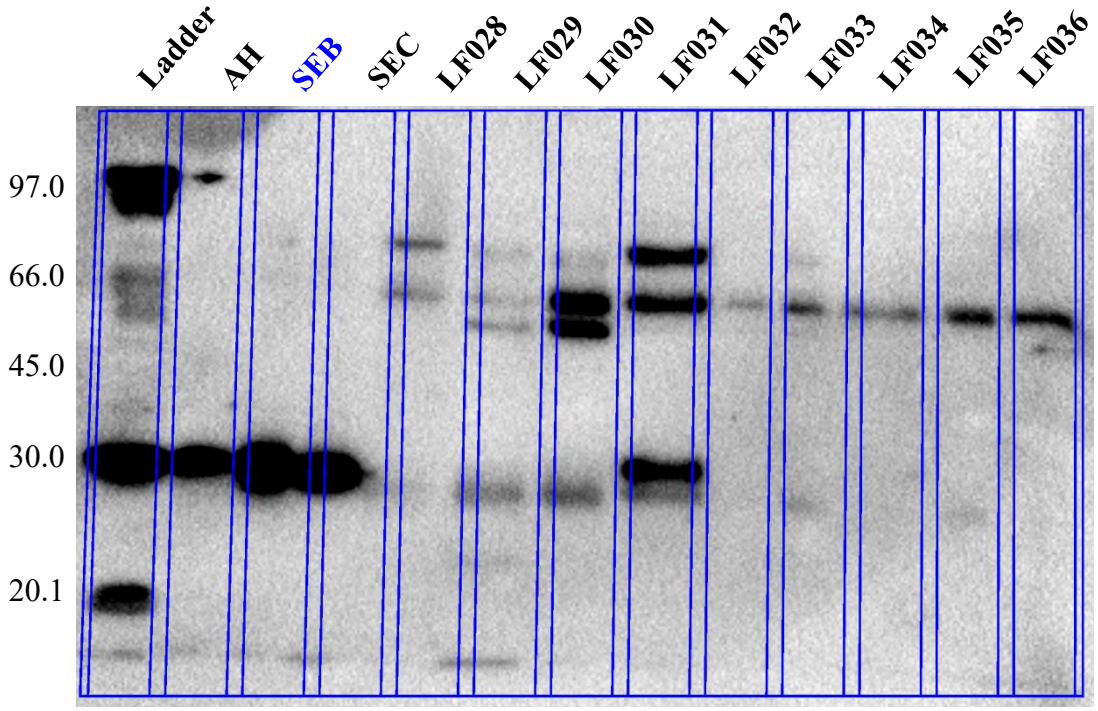


Figure F12 Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF028-036 (28µl) (Lane 5-13).

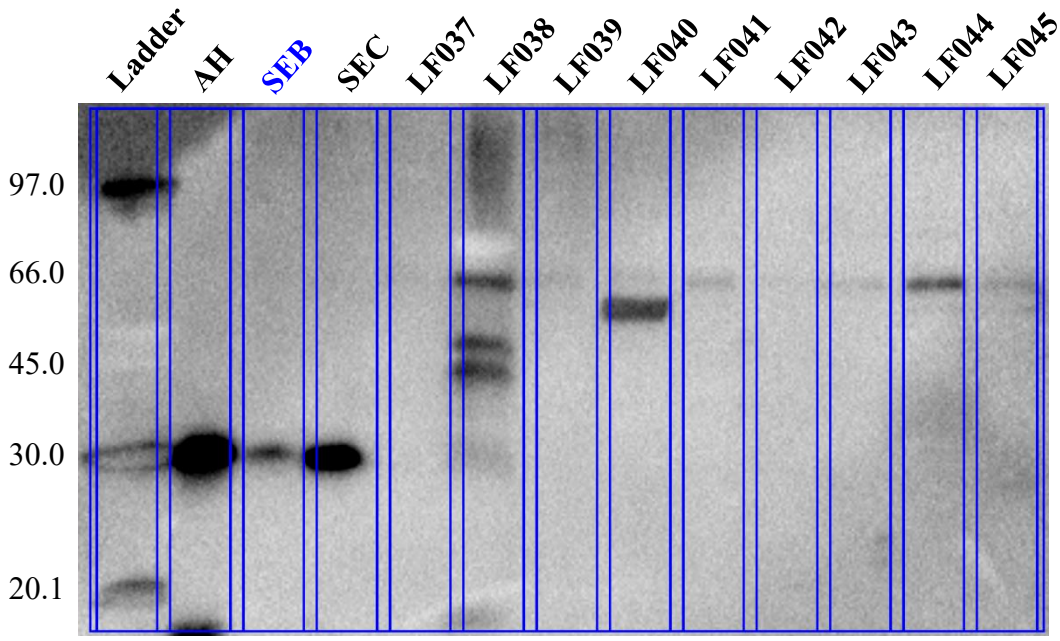


Figure F13 **Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF037-045 (28µl) (Lane 5-13).**

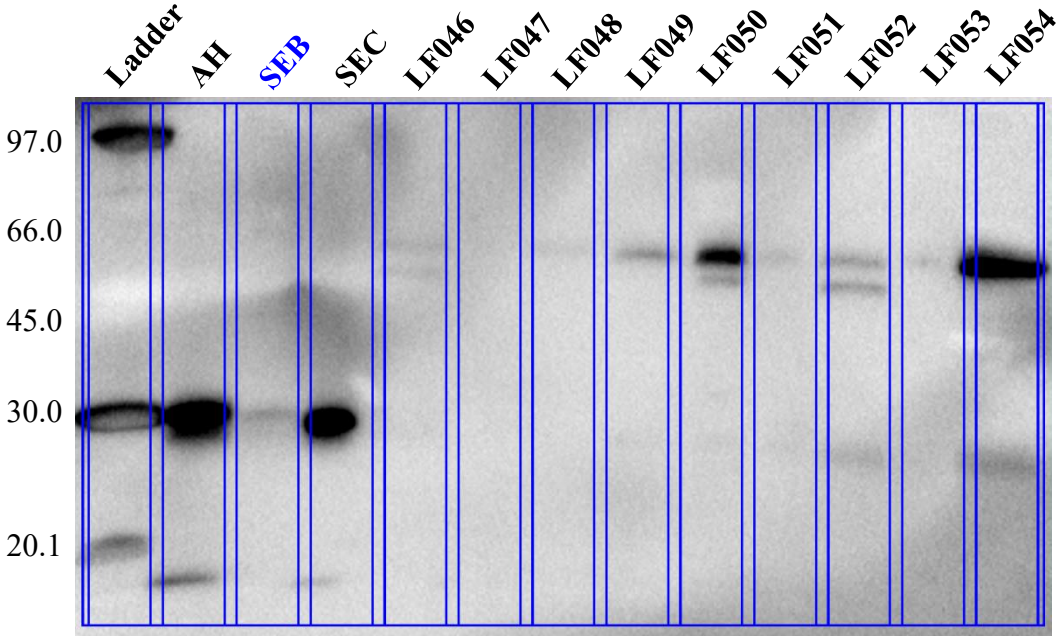


Figure F14 **Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF046-054 (28µl) (Lane 5-13).**

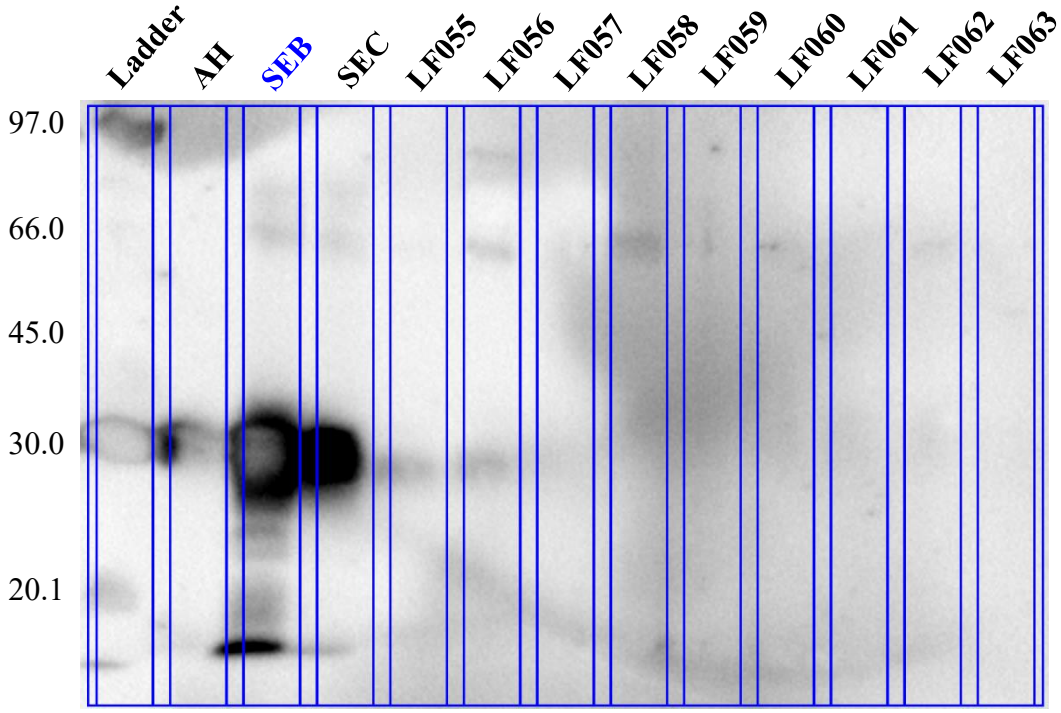


Figure F15 Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF055-063 (28µl) (Lane 5-13).

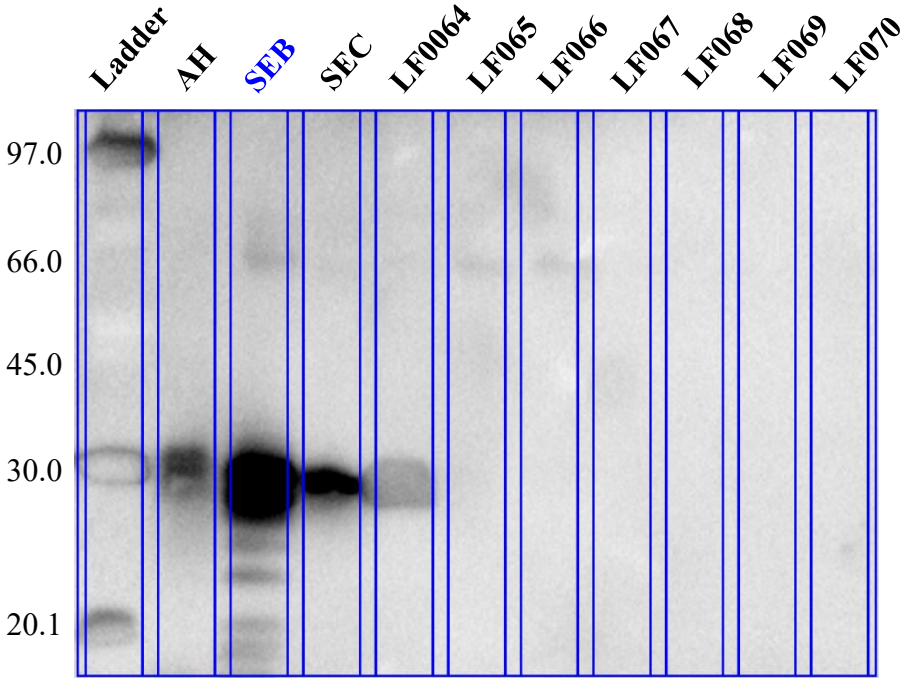


Figure F16 Western blot membrane incubated with αSEB primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF064-070 (28µl) (Lane 5-11).

Appendix F3 Staphylococcal Enterotoxin C (30kDa)

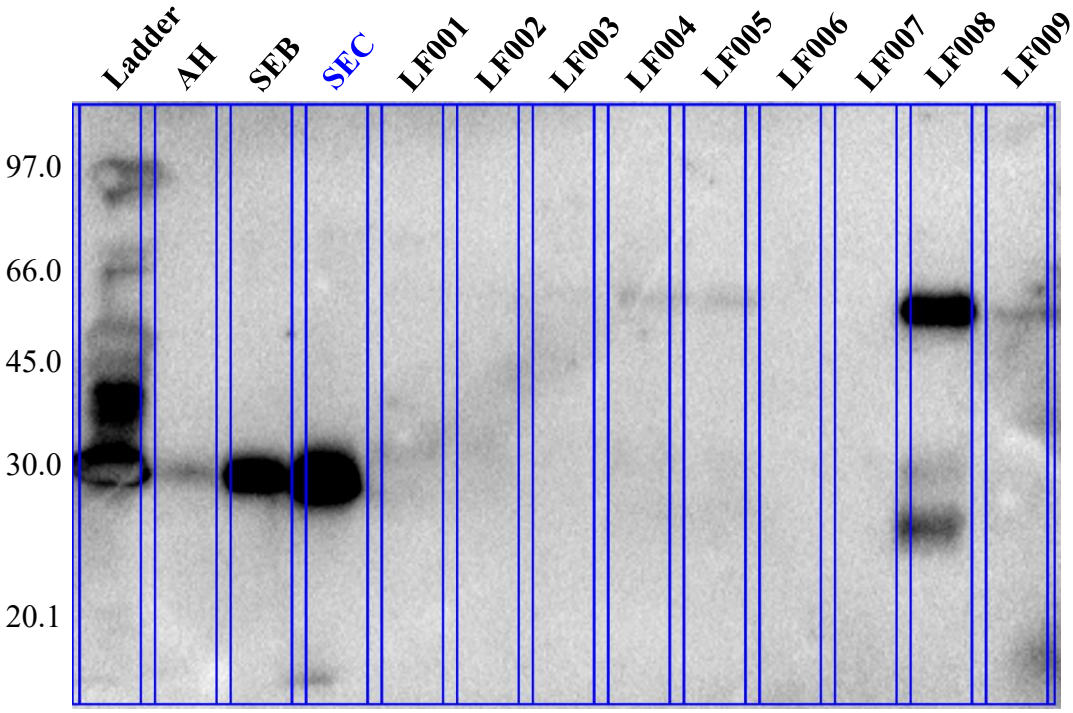


Figure F17 Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF001-009 (28µl) (Lane 5-13).

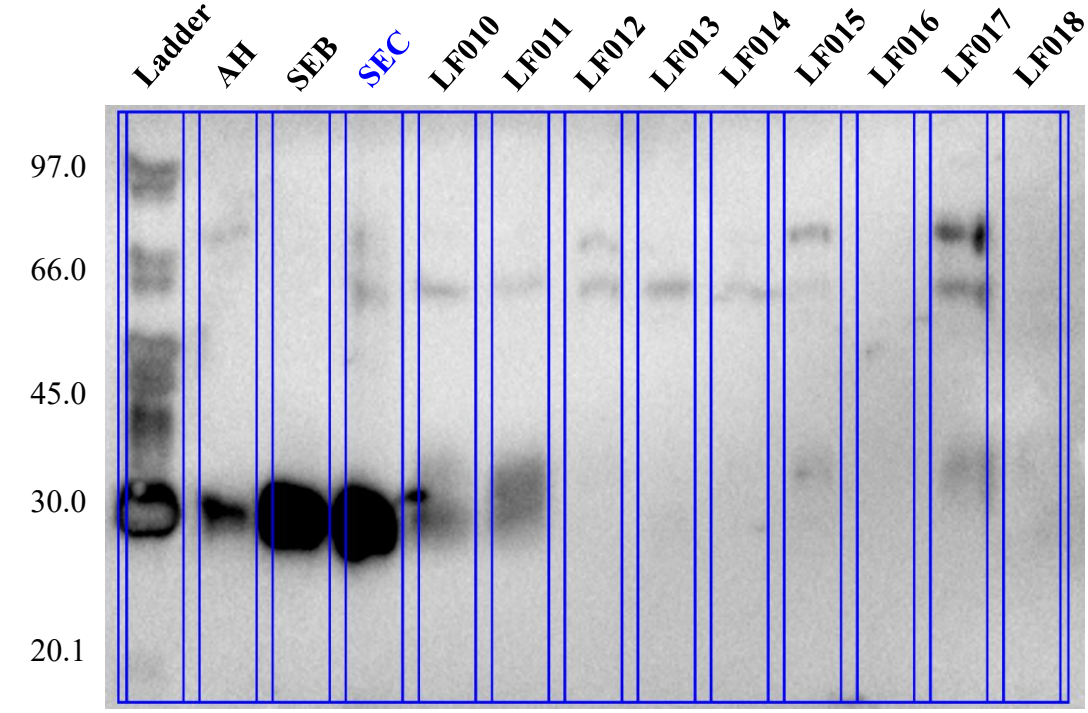


Figure F18 Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF010-018 (28µl) (Lane 5-13).

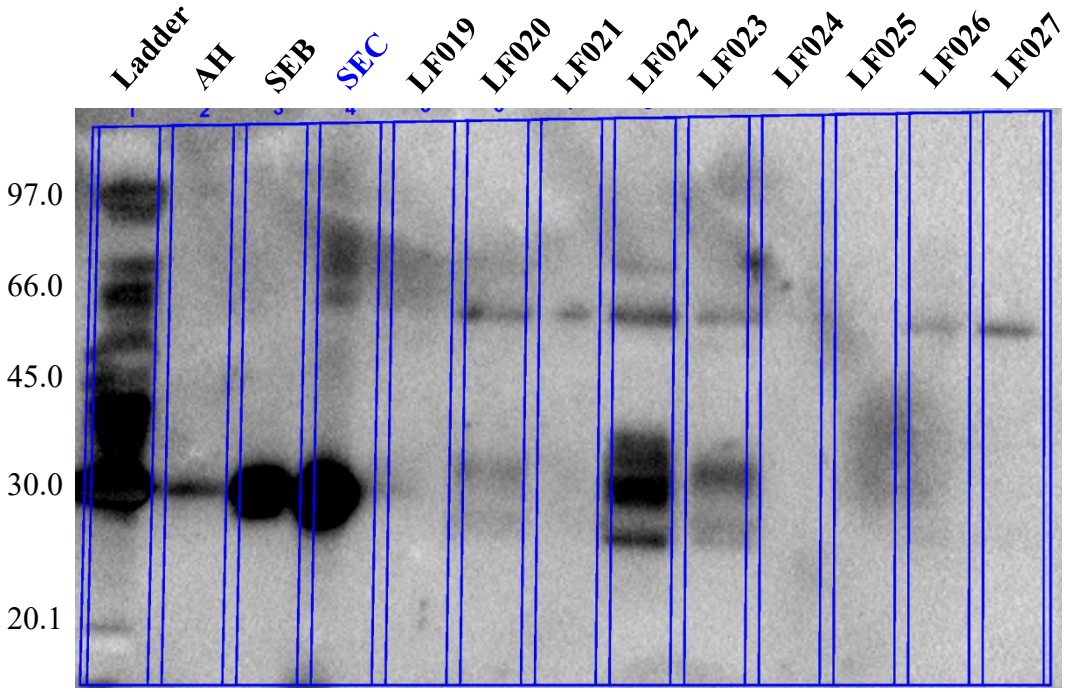


Figure F19 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF019-027 (28µl) (Lane 5-13).**

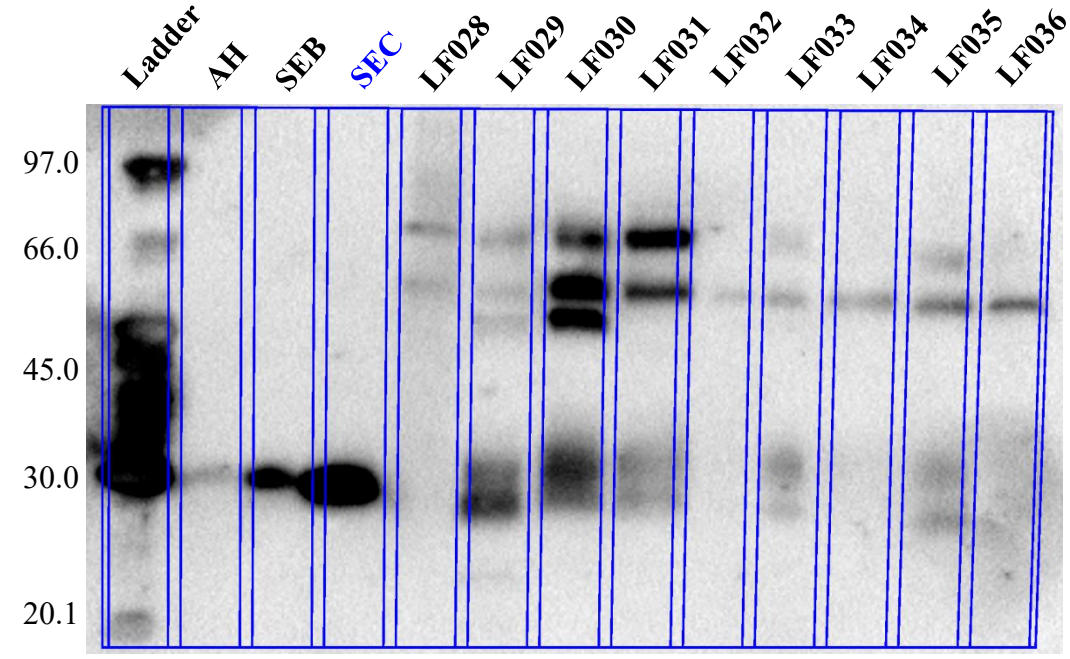


Figure F20 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF028-036 (28µl) (Lane 5-13).**

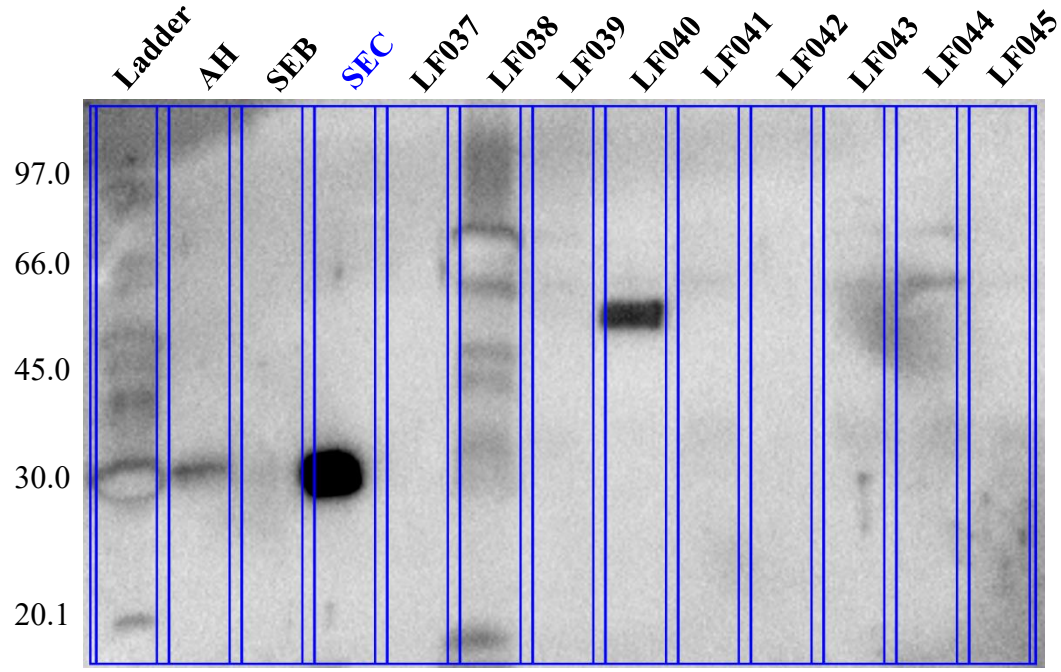


Figure F21 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF037-045 (28µl) (Lane 5-13).**

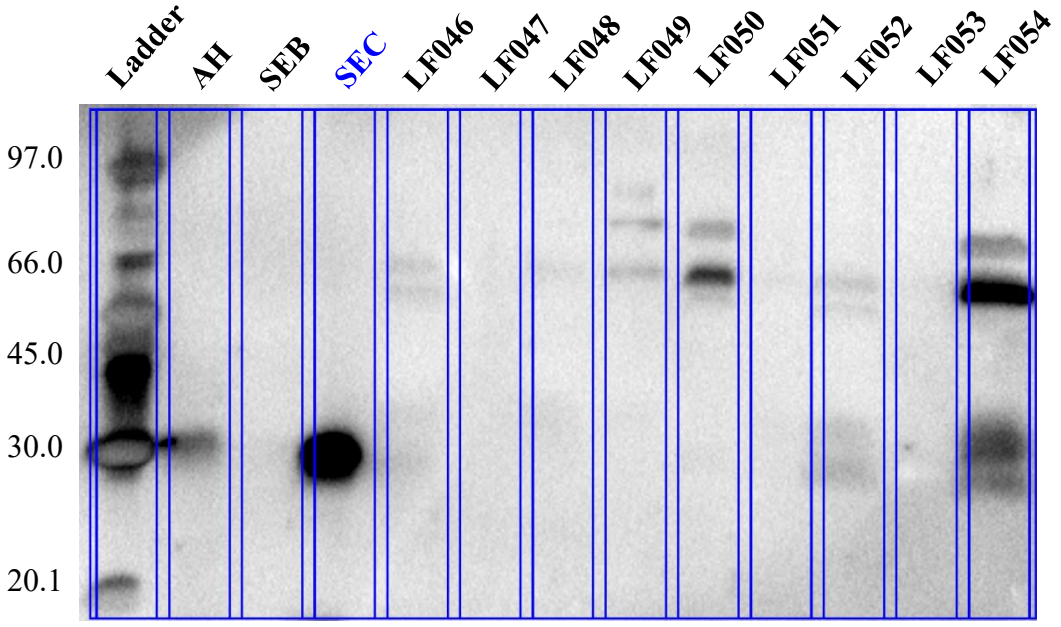


Figure F22 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF046-054 (28µl) (Lane 5-13).**

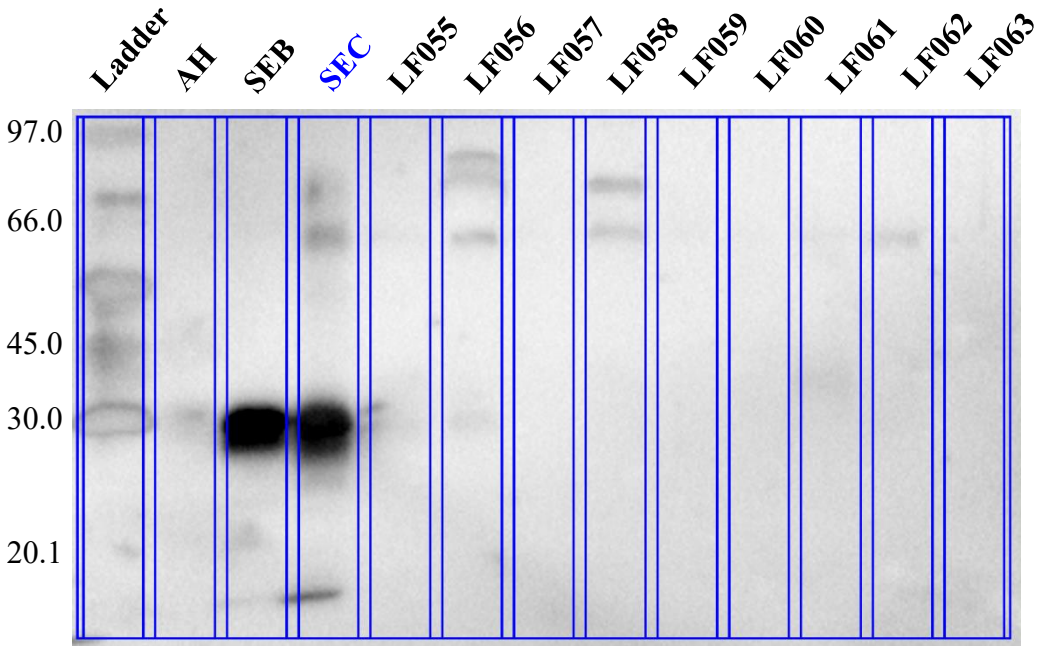


Figure F23 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF055-063 (28µl) (Lane 5-13).**

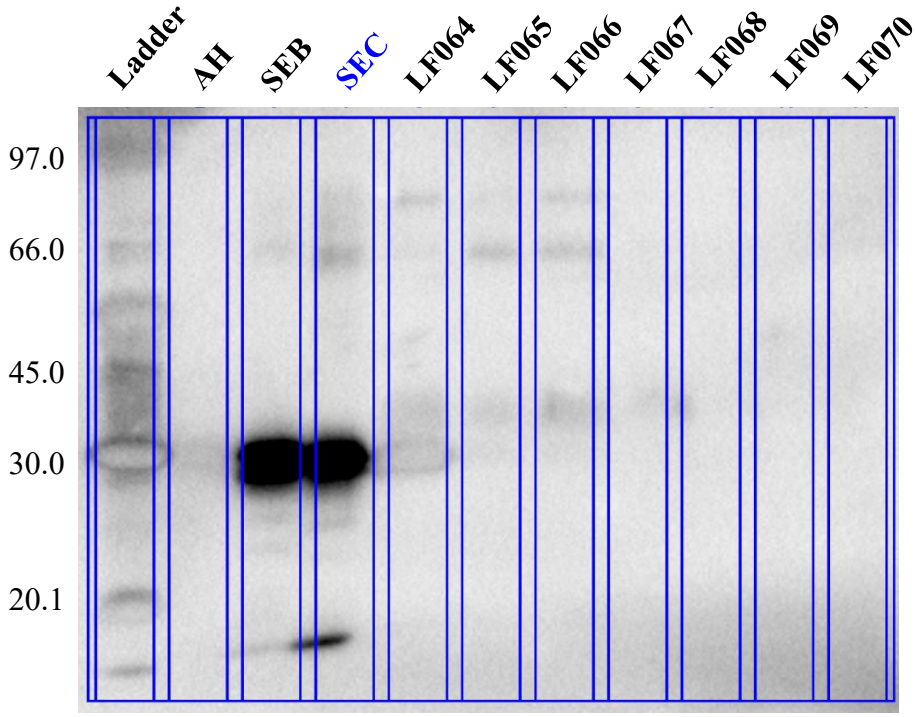


Figure F24 **Western blot membrane incubated with αSEC primary antibody (1:2000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control AH, SEB and SEC (1µg/ml) (Lane 2-4) and neat samples of urine from patients LF064-070 (28µl) (Lane 5-11).**

Appendix F4 Toxic Shock Syndrome Toxin 1 (24kDa)

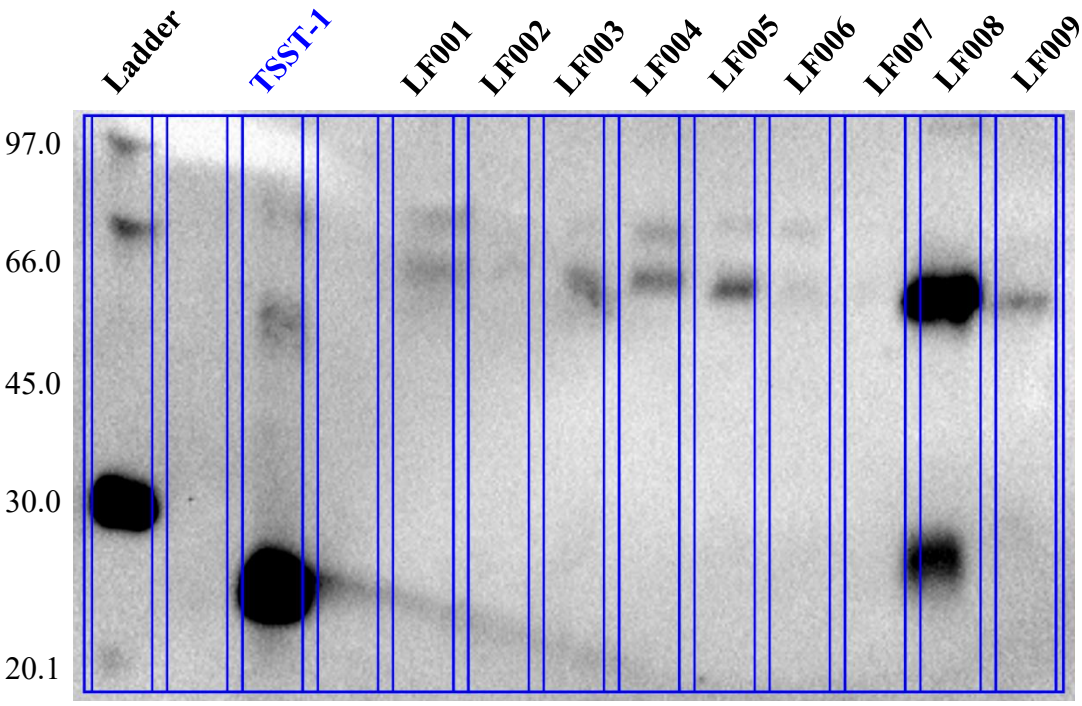


Figure F25 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LF001-009 (28µl) (Lane 5-13).**

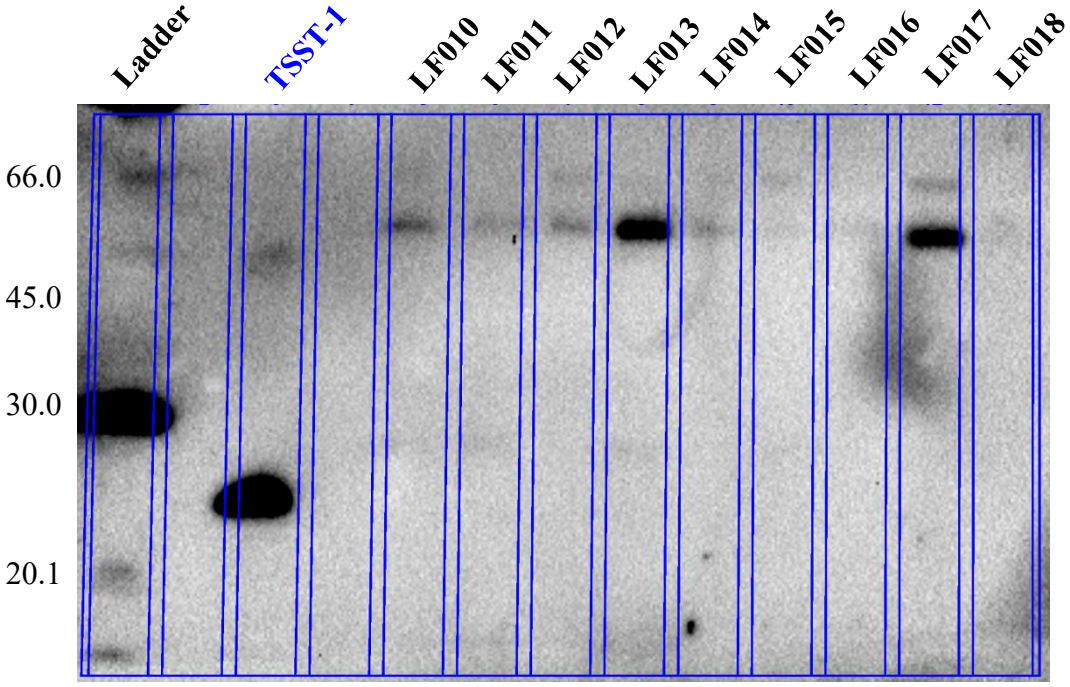


Figure F26 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LF010-018 (28µl) (Lane 5-13).**

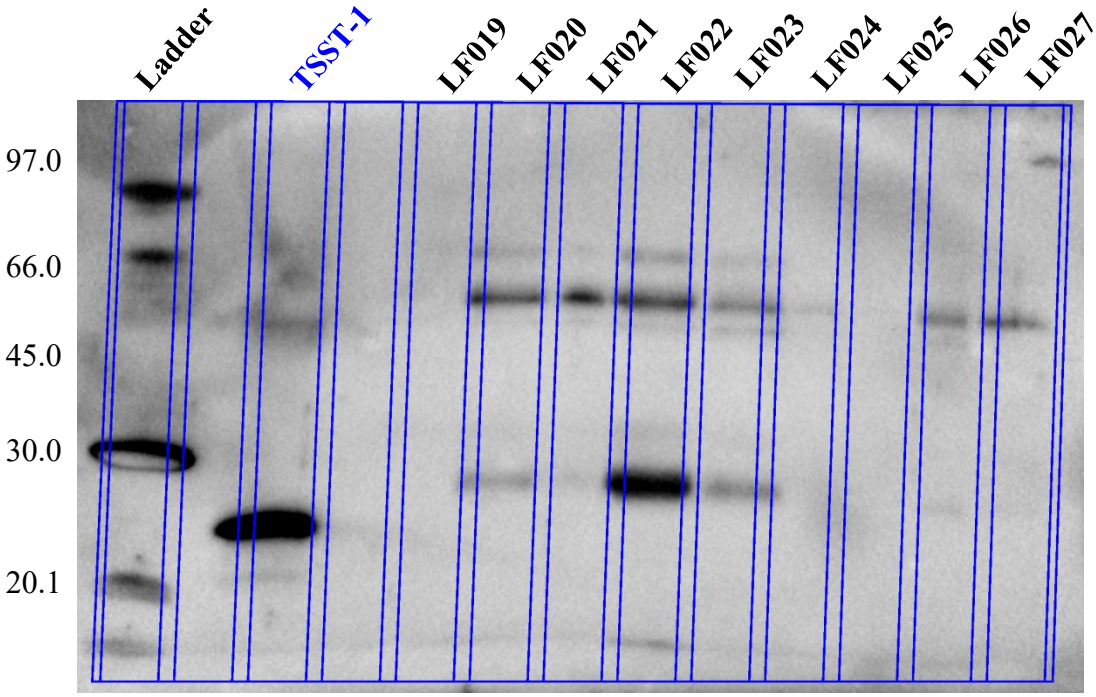


Figure F27 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LF019-027 (28µl) (Lane 5-13).**

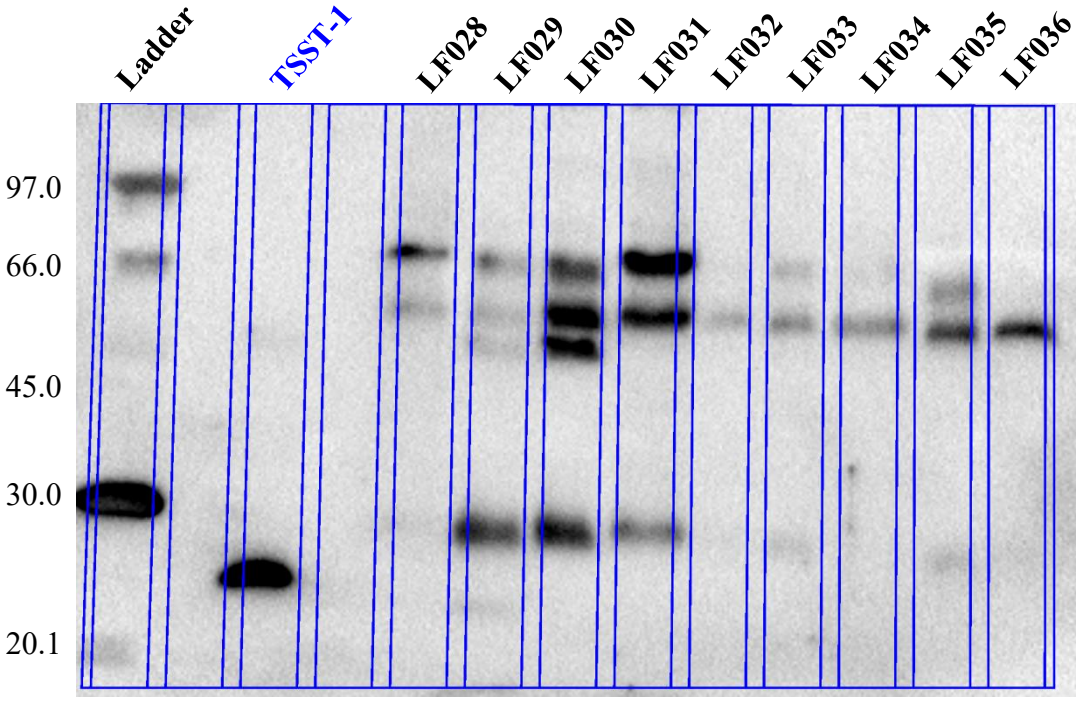


Figure F28 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LF028-036 (28µl) (Lane 5-13).**

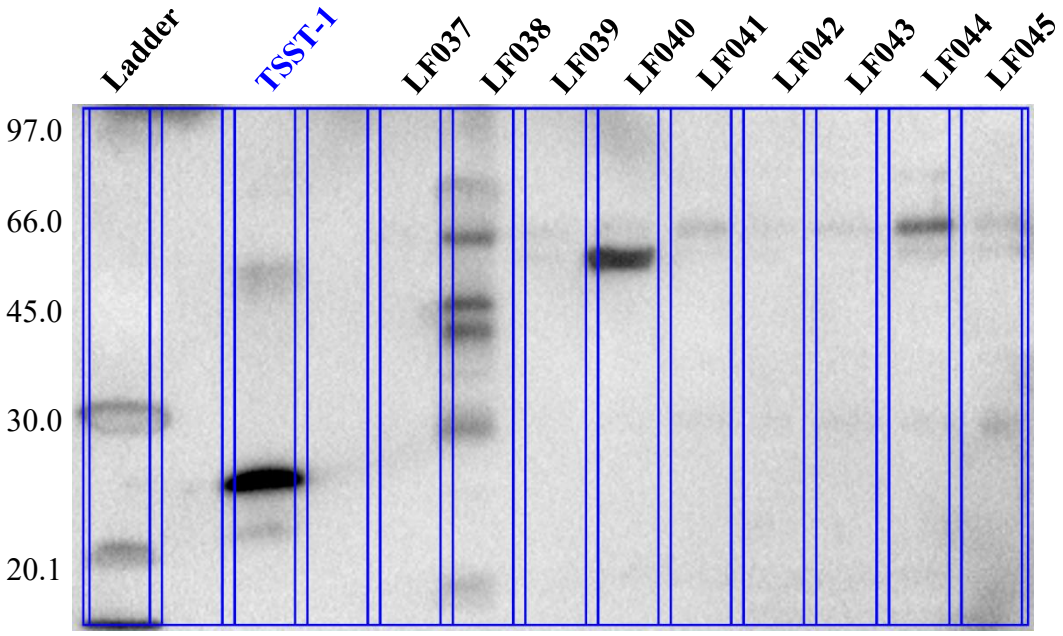


Figure F29 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LF037-045 (28µl) (Lane 5-13).**

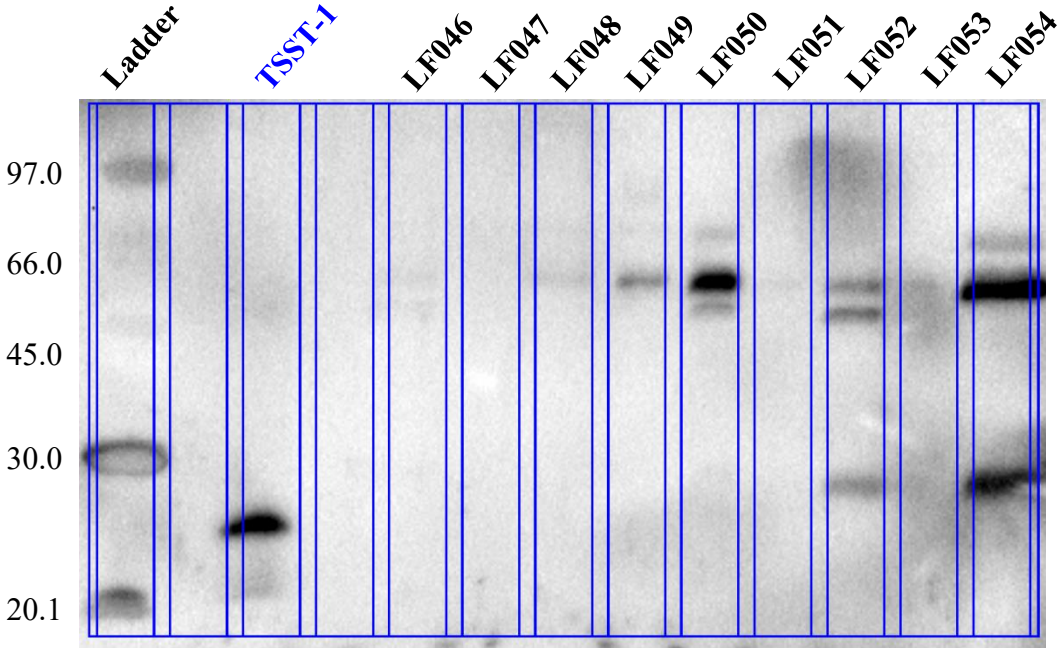


Figure F30 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LF046-054 (28µl) (Lane 5-13).**

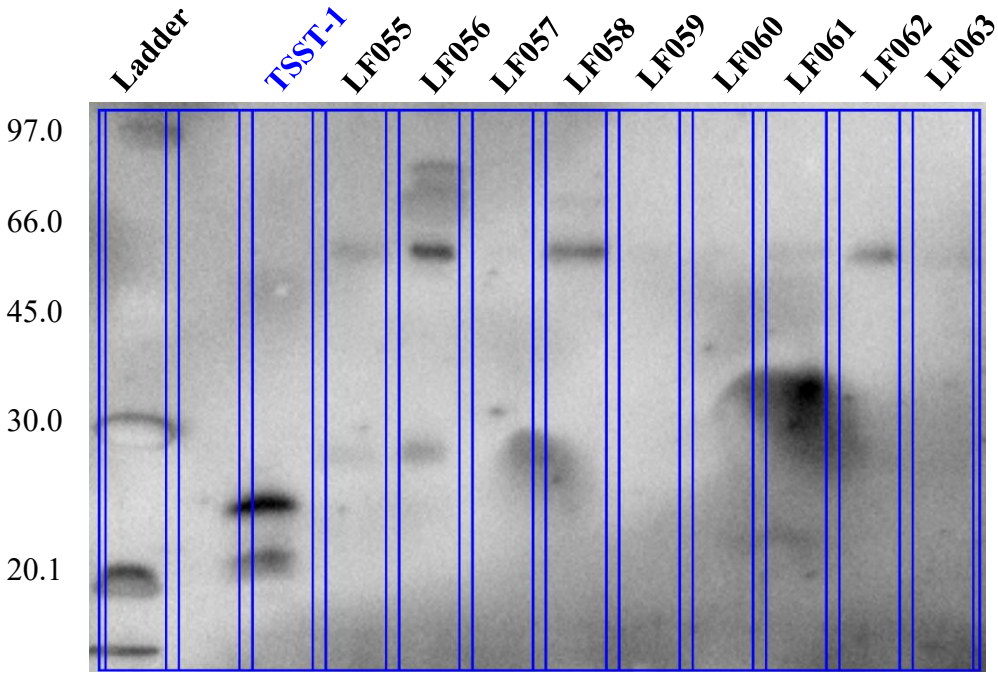


Figure F31 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LF055-063 (28µl) (Lane 4-12).**

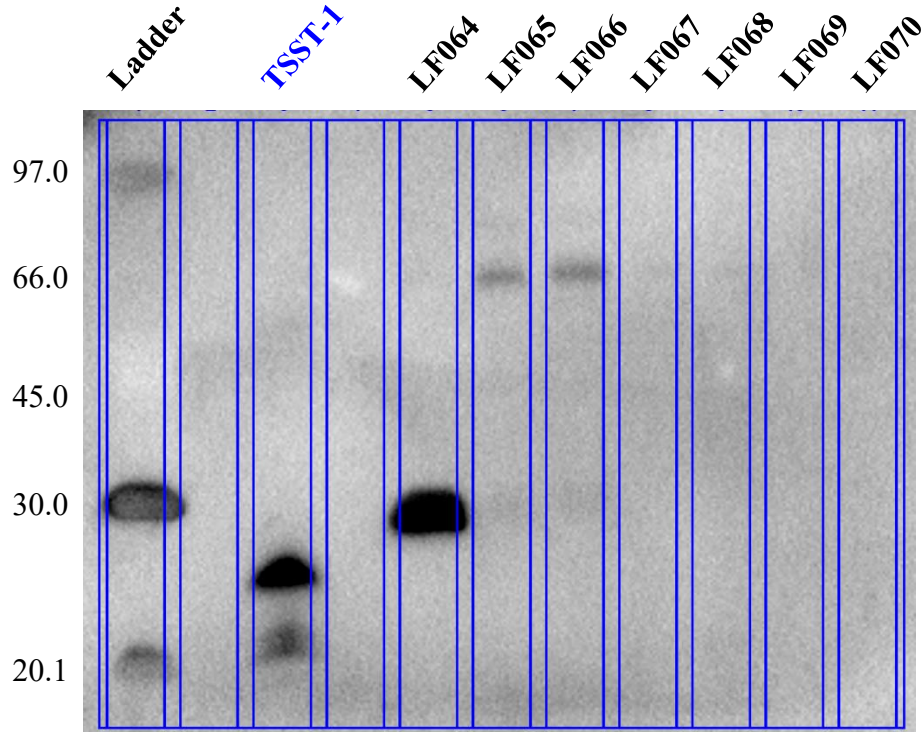


Figure F32 **Western blot membrane incubated with αTSST-1 primary antibody (1:1000). Low molecular weight ladder (kDa) (20µl) (Lane 1), Control TSST-1 (1µg/ml) (Lane 3) and neat samples of urine from patients LF064-070 (28µl) (Lane 5-11).**

Appendix G

Table G1 LCMS data for the most concentrated patient samples, as observed by Western Blot analysis. Observed masses for precursor and product ions are reported against the calculated theoretical, mass accuracy is reported in ppm.

Sample Name	Segment	Toxin Detected	Measured Precursor Mass [m/z]	Calc. Precursor [m/z]	Diff. [ppm]	Product Mass [m/z]	Calc. Product Mass [m/z]	Diff. [ppm]
LR014								
LR028	1	TSST-1	423.2532	423.2456	18.0	746.4261	746.4120	18.9
						631.3881	631.3820	9.7
						518.2990	518.3030	-7.7
						404.2601	404.2560	10.1
						316.1935	316.1940	-1.6
	5	TSST-1	789.3879	789.3988	-13.8	657.3407	657.3460	-8.1
						575.2603	575.2677	-12.9
						544.2590	544.2619	-5.3
	12	TSST-1	560.8444	560.8524	-14.3	910.5548	910.5614	-7.2
						809.5060	809.5137	-9.5
						712.4524	712.4609	-11.9
						599.3706	599.3769	-10.5
						551.8444	551.8478	-6.2
						470.3298	470.3343	-9.6
						455.7787	455.7846	-12.9
						405.2552	405.2608	-13.8
						357.2450	357.2502	-14.6
LR038	4	SEC1	746.8589	746.8493	12.9	Too Weak		
	12	TSST-1	560.8386	560.8524	-24.6	910.5390	910.5614	-24.6
						809.5036	809.5137	-12.5
LR050	12	TSST-1	*Too Weak*			910.5560	910.5614	-5.9
						809.5094	809.5137	-5.3
						712.4631	712.4609	3.1
LR052	12	TSST-1	560.8637	560.8524	20.1	809.5069	809.5137	-8.4
						599.3744	599.3769	-4.2
						405.2588	405.2608	-4.9
LR053	12	TSST-1	560.8425	560.8524	-17.7	910.5511	910.5614	-11.3
						809.5076	809.5137	-7.5
						712.4694	712.4609	11.9
						599.3742	599.3769	-4.5
						551.8356	551.8478	-22.1
						470.3264	470.3343	-16.8
						405.2574	405.2608	-8.4

						357.2468	357.2502	-9.5
LR061		None Detected						
LR073	1	TSST-1	423.2433	423.2456	-5.4	746.3989	746.4120	-17.6
						631.3864	631.3820	7.0
						518.2955	518.3030	-14.5
						404.2524	404.2560	-8.9
						316.1951	316.1940	3.5
	4	SEC1	746.8442	746.8493	-6.8	936.4681	936.4791	-11.7
						737.8385	737.8447	-8.4
						574.7752	574.7830	-13.6
	7	SEC1	552.2898	552.3008	-19.9	917.4899	917.4944	-4.9
						844.3956	844.4053	-11.5
						816.4355	816.4468	-13.8
						745.4160	745.4096	8.6
						617.3480	617.3511	-5.0
						543.2915	543.2961	-8.5
						459.2439	459.2512	-15.9
						375.2207	375.2244	-9.9
	12	TSST-1	560.8440	560.8524	-15.0	910.5646	910.5614	3.5
						809.5099	809.5137	-4.7
						712.4591	712.4609	-2.5
						599.3737	599.3769	-5.3
						551.8383	551.8478	-17.2
						470.3271	470.3343	-15.3
						455.7774	455.7846	-15.8
						405.2549	405.2608	-14.6
						357.2475	357.2502	-7.6
						312.1891	312.1924	-10.6
LR079		None Detected						
LR083		None Detected						
LR110		None Detected						
LR111		None Detected						
LR113		None Detected						
LR133		Signals Too Weak						
LR142		None Detected						
LR151		None Detected						
LF008		None Detected						

LF009	1	TSST-1		423.2456		746.4365	746.4120	32.8
						631.3639	631.3820	-28.7
						518.3013	518.3030	-3.3
						404.2692	404.2560	32.7
						316.1933	316.1940	-2.2
	4	SEC1	746.8394	746.8493	-13.3	936.4695	936.4791	-10.3
						737.8357	737.8447	-12.2
						574.7756	574.7830	-12.9
						468.7324	468.7435	-23.7
	5	TSST-1	789.3954	789.3988	-4.3			
	6	SEC1	705.3501	705.3672	-24.2	847.4348	847.4526	-21.0
						718.3975	718.4100	-17.4
	7	SEC1	552.2981	552.3008	-4.9	917.4911	917.4944	-3.6
						816.4410	816.4468	-7.1
						745.4055	745.4096	-5.5
						617.3444	617.3511	-10.9
						543.2873	543.2961	-16.2
						459.2457	459.2512	-12.0
						375.2178	375.2244	-17.6
	12	TSST-1	obscured			910.5495	910.5614	-13.1
						809.5137	809.5137	0.0
						712.4548	712.4609	-8.6
						599.3761	599.3769	-1.3
						470.3293	470.3343	-10.6
						405.2529	405.2608	-19.5
LF010	1	TSST-1	423.2364	423.2456	-21.7	746.4030	746.4120	-12.1
						631.3812	631.3820	-1.3
						518.3018	518.3030	-2.3
						404.2569	404.2560	2.2
						316.1935	316.1940	-1.6
	4	SEC1	746.8378	746.8493	-15.4	936.4733	936.4791	-6.2
						737.8367	737.8447	-10.8
						574.7748	574.7830	-14.3
						526.2881	526.2990	-20.7
						468.7349	468.7435	-18.3
	5	TSST-1	789.3825	789.3988	-20.6	657.3418	657.3460	-6.4
						544.2597	544.2619	-4.0
	6	SEC1	705.3585	705.3672	-12.3	847.4429	847.4526	-11.4
						718.4027	718.4100	-10.2
						597.8115	597.8225	-18.4
	7	SEC1	552.2954	552.3008	-9.8	917.4859	917.4944	-9.3
						816.4393	816.4468	-9.2
						617.3520	617.3511	1.5
						543.2944	543.2961	-3.1

						459.2470	459.2512	-9.1
						375.2187	375.2244	-15.2
	8	SEB	too weak			744.3764	744.3787	-3.1
						687.8303	687.8366	-9.2
						548.7933	548.7915	3.3
	12	TSST-1	560.8503	560.8524	-3.7	910.5534	910.5614	-8.8
						809.5026	809.5137	-13.7
						712.4535	712.4609	-10.4
						599.3660	599.3769	-18.2
						551.8270	551.8478	-37.7
						470.3288	470.3343	-11.7
						405.2566	405.2608	-10.4
LF011		None Detected						
LF022	1	TSST-1	too weak					
LF023	1	TSST-1	too weak					
LF029	1	TSST-1	too weak					
	7	SEC1	too weak					
LF030	1	TSST-1	too weak			631.3833	631.3820	2.1
						316.1917	316.1940	-7.3
LF031	1	TSST-1	too weak					
LF054	1	TSST-1	too weak			631.3926	631.3820	16.8
						518.2922	518.3030	-20.8
						316.1931	316.1940	-2.8
LF056	1	TSST-1	too weak			746.4208	746.4120	11.8
						631.3736	631.3820	-13.3
						518.2910	518.3030	-23.2
						316.1915	316.1940	-7.9
	4	SEC1	746.8380	746.8493	-15.1	936.4728	936.4791	-6.7
						737.8390	737.8447	-7.7
						574.7788	574.7830	-7.3
						468.7333	468.7435	-21.8
	5	TSST-1		789.3988		657.3390	657.3460	-10.6
	7	SEC1	552.2996	552.3008	-2.2	917.4909	917.4944	-3.8
						816.4468	816.4468	0.0
						745.4012	745.4096	-11.3
						617.3401	617.3511	-17.8
						543.2882	543.2961	-14.5
						488.3031	488.3085	-11.1
						459.2501	459.2512	-2.4
						375.2231	375.2244	-3.5
	10	SEB	654.8424	654.8433	-1.4	909.4537	909.4682	-15.9
						780.4164	780.4256	-11.8
						667.3367	667.3416	-7.3
						552.3111	552.3146	-6.3

						389.2379	389.2513	-34.4
	12	TSST-1	560.8402	560.8524	-21.8	910.5628	910.5614	1.5
						809.5091	809.5137	-5.7
						599.3621	599.3769	-24.7
						405.2596	405.2608	-3.0
LF064								

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