Secondary Bacterial Infection in Buruli Ulcer

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Dekan

Dedicated to the three parents I have been blessed to have

My dad Stephen (you are gone but your memory lives on)

My mum Ruth (without whom I would have given up)

My father Rev. Bempah (I couldn't have asked for a better father after dad)

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Summary

Buruli ulcer (BU) is a chronic debilitating disease of the skin and soft tissues caused by *Mycobacterium ulcerans*. It is one of the 17 neglected tropical diseases according to the World Health Organization and has been reported in over 30 countries with tropical and sub-tropical conditions globally. *M. ulcerans* is traditionally considered as an environmental pathogen and even though BU was discovered over half a century ago, the environmental reservoir and exact mode of transmission of this pathogen remain obscure. This makes it challenging to formulate strategies for its prevention. As such, control strategies geared towards the early detection and treatment of cases are vital to minimize morbidity, disability and the socio-economic burden associated with the disease. The introduction of antibiotic therapy for treatment in 2004 to replace surgery as first-line therapy has brought about an improvement in the management of the disease. However, despite reported successful outcomes with the antibiotic treatment, the healing process is still characterized by long hospitalizations as a result of delayed wound closure.

In this thesis, we explored the factors which could contribute to the observed delayed wound healing in two BU treatment centers in Ghana; the Ga-West Municipal Hospital and the Obom Health Center. Through a combination of clinical, microbiological and histopathological analysis, we identified secondary infection of BU lesions by other bacteria as a major cause of delayed healing. Through quantitative microbiological studies, we analysed the evolution of the bacterial burden and identified increased loads of bacteria post treatment which could negatively impact on the healing potential of the wounds. Furthermore, we explored co-infection with Human immunodeficiency virus (HIV) in the Ga-West Municipal Hospital as a challenge to the management of BU and described challenges associated with the management of this co-infection. Studying the isolated bacterial species through phenotypic, molecular and whole genome approaches helped to identify health-care associated transmission through health workers and equipment as well as self transmission as potential sources of wound infection within the health centers. With these results, we made recommendations for the improvement of wound management in the health centers and made a case for the need for wound management guidelines which were absent in the health centers. We followed this up with the development of local guidelines for wound care and the implementation of several interventions in the health centers. We also identified antibiotic resistance as an increasing problem and described in detail through whole genome sequencing, a recently emerged and rapidly spreading clone of community acquired methicillin resistant *Staphylococcus aureus* with sequence type 88 in Ghana which has the potential to become a serious public health threat with implications for healthcare. This alarming result

therefore calls for the urgent establishment of a surveillance system to monitor the use and distribution of antibiotics in Ghana and the emergence of antibiotic resistant pathogens.

Zusammenfassung

Das Buruli-Ulkus (BU) ist eine chronische, verheerende Erkrankung der Haut und des Weichgewebes, die durch *Mycobacterium ulcerans* hervorgerufen wird. Das BU gehört gemäss Weltgesundheitsorganisation zu den 17 vernachlässigten Tropenkrankheiten und das Auftreten der Krankheit wurde aus mehr als 30 tropischen und sub-tropischen Ländern weltweit gemeldet. Bisher wurde allgemein angenommen, dass *M. ulcerans* ein Umwelterreger ist, doch obwohl das BU seit über einem halben Jahrhundert bekannt ist, bleiben die Infektionsquellen und der genaue Übertragungsweg des Erregers unklar. Dadurch ist es schwierig Präventionsmassnahmen zu entwickeln. Strategien zur Kontrolle der Krankheit, die eine frühe Erkennung und Behandlung von Patienten anstreben, sind entscheidend, um die mit der Krankheit verbundene Morbidität, Invalidität und wirtschaftlich-soziale Belastung möglichst gering zu halten. Die Einführung der Antibiotika-Therapie für die Behandlung der Krankheit im Jahre 2004, die die operative Behandlung als Erstlinientherapie abgelöst hat, hat wesentlich zur Verbesserung des Krankheitsmanagements beigetragen. Obwohl mit der Antibiotikabehandlung Berichten zufolge erfolgreiche Ergebnisse erzielt werden, ist der Heilungsprozess infolge von verzögerter Wundheilung noch immer von langen Krankenhausaufenthalten geprägt.

In dieser Doktorarbeit haben wir in zwei Behandlungszentren für das BU in Ghana (das kommunale Ga-West Krankenhaus und das Gesundheitszentrum in Obom) die Faktoren erforscht, die zur beobachteten verzögerten Wundheilung beitragen könnten. Mit Hilfe einer Kombination aus klinischen, mikrobiologischen und histopathologischen Analysen, haben wir die Sekundärinfektion von BU Läsionen durch andere Bakterien als den Hauptgrund für die verzögerte Heilung bestimmt. Durch quantitative, mikrobiologische Analysen, haben wir die Entwicklung der bakteriellen Belastung untersucht und eine erhöhte Belastung durch Bakterien nach der Behandlung festgestellt, was einen negativen Einfluss auf das Heilungsvermögen der Wunden haben könnte. Des Weiteren haben wir im kommunalen Ga-West Krankenhaus erforscht, dass eine Ko-Infektion mit dem Humanen Immundefizienz-Virus (HIV) eine Herausforderung für die Bewältigung des BU darstellt und mit der Behandlung dieser Ko-Infektion verbundene Schwierigkeiten beschrieben. Die Analyse von isolierten Bakterienspezies mit Hilfe von phänotypischen, molekularen und genomumfassenden Ansätzen, hat dazu beigetragen sowohl die mit der Gesundheitspflege verbundene Übertragung durch das Gesundheitspersonal und die Geräte als auch die Selbstübertragung als mögliche Quelle für die Wundinfektion innerhalb der Gesundheitszentren zu identifizieren. Mit diesen Resultaten, konnten wir Empfehlungen für die Verbesserung der Wundversorgung in den Gesundheitszentren geben und für die Notwendigkeit

von Richtlinien für die Wundversorgung, die bisher in den Gesundheitszentren gefehlt haben, plädieren. Wir haben dies durch die Entwicklung von lokalen Richtlinien für die Wundbehandlung und die Durchführung von mehreren Massnahmen in den Gesundheitszentren weiterverfolgt. Zudem haben wir Antibiotikaresistenz als ein zunehmendes Problem identifiziert und mit Hilfe der Sequenzierung von Gesamtgenomen einen in Ghana unlängst aufgetretenen und sich rasch ausbreitenden Klon von im Krankenhaus erworbenen methicillinresistenten *Staphylococcus aureus* Bakterien mit dem Sequenztyp 88, der das Potential hat eine schwerwiegende Bedrohung für die öffentliche Gesundheit darzustellen, im Detail beschrieben. Dieses besorgniserregende Ergebnis macht daher deutlich, dass eine dringende Einrichtung eines Kontrollsystems notwendig ist, das den Gebrauch und die Verteilung von Antibiotika in Ghana und das Auftreten von Antibiotikaresistenzen überwacht.

Introduction

Buruli Ulcer Disease

Buruli ulcer (BU), a disease caused by the bacteria *Mycobacterium ulcerans* is known as one of the 17 neglected tropical diseases according to the World Health Organization (WHO) [1]. This disease was first clinically described in 1948 by MacCallum et al [2]; however earlier reports of the existence of a disease marked by large ulcerations in Africa and Australia had been made as far back as the second half of the nineteenth century [3-4]. It is the third most common mycobacterial disease of non-immunocompromised humans after tuberculosis and leprosy and the least understood [5]. Though it is thought to have a low mortality rate, it has great socioeconomic impact on the affected and their communities in terms of morbidity and its stigmatizing complications [6]. This prompted the WHO to name it as an emerging public health problem with the launch of a Global Buruli ulcer Initiative in 1998 [7] and the subsequent signing of the Yamoussoukro declaration on Buruli ulcer by representatives of more than 20 countries as a pledge to control the disease [8]. Since then, global efforts have intensified to improve control and accelerate research into the many unknowns of this disease.

Etiology and Clinical Features

The etiologic agent is a slow-growing acid fast bacillus similar to *Mycobacterium tuberculosis* (Mtb). In contrast to Mtb, it has an optimal growth temperature of 30-32^{0}C and is sensitive to temperatures of 37^{0}C or higher [9]. It also produces a necrotizing and immunosuppressive polyketide toxin called mycolactone as its virulence factor which plays an important role in its pathogenesis [10-12]. Genomic analysis of MU showed that it diverged from *Mycobacterium marinum* about a million years ago by horizontal gene transfer and reductive evolution [13]. While evolving, this bacterium acquired a 174-kb virulent plasmid pMUM001 and it has been hypothesized that this plasmid aided its adaptation to a new environment [14-17]. This plasmid carries a cluster of genes encoding giant polyketide synthases and polyketide-modifying enzymes responsible for the production of the macrolide toxin mycolactone. Mycolactone diffuses into subcutaneous tissues inducing necrosis and ulceration by its cytotoxic properties.

The disease presents with a spectrum of forms, which can be either non-ulcerative or ulcerative. The non-ulcerative forms are characterized by nodules, papules, plaques and oedema. The nodule is a painless, firm and palpable subcutaneous form frequently found in Africa (Figure 1a) while the

papule is a painless raised skin lesion with erythema in the surrounding skin observed mainly in Australia (Figure 1b). Disseminated forms of the disease may present as a plaque (Figure 1c), which is a hardened, raised and dry painless lesion usually covered with discoloured skin or an oedema characterized by a firm and diffuse non-pitting swelling with nonspecific edges (Figure 1d). If left untreated, these forms can evolve into severe large ulcerated lesions with rugged undermined, hyperpigmented edges and bases which contain a whitish necrotic slough (Figure 1e). The different presentations of the disease have also been classified into categories by the WHO, taking into account the size of the lesions, number of lesions and the site of lesion presentation. Thus, there are three categories: category I for single lesions less than 5cm in diameter, category II for lesions between 5-15cm in diameter and category III for single lesions above 15cm, multiple lesions, lesions found at critical sites and cases of osteomyelitis. In about 85% of cases [18], the disease presents mainly on the limbs but the infection can also traverse the deep fascia and affect muscles, blood vessels, bones and joints finally leading to dramatic functional limitations in the affected.

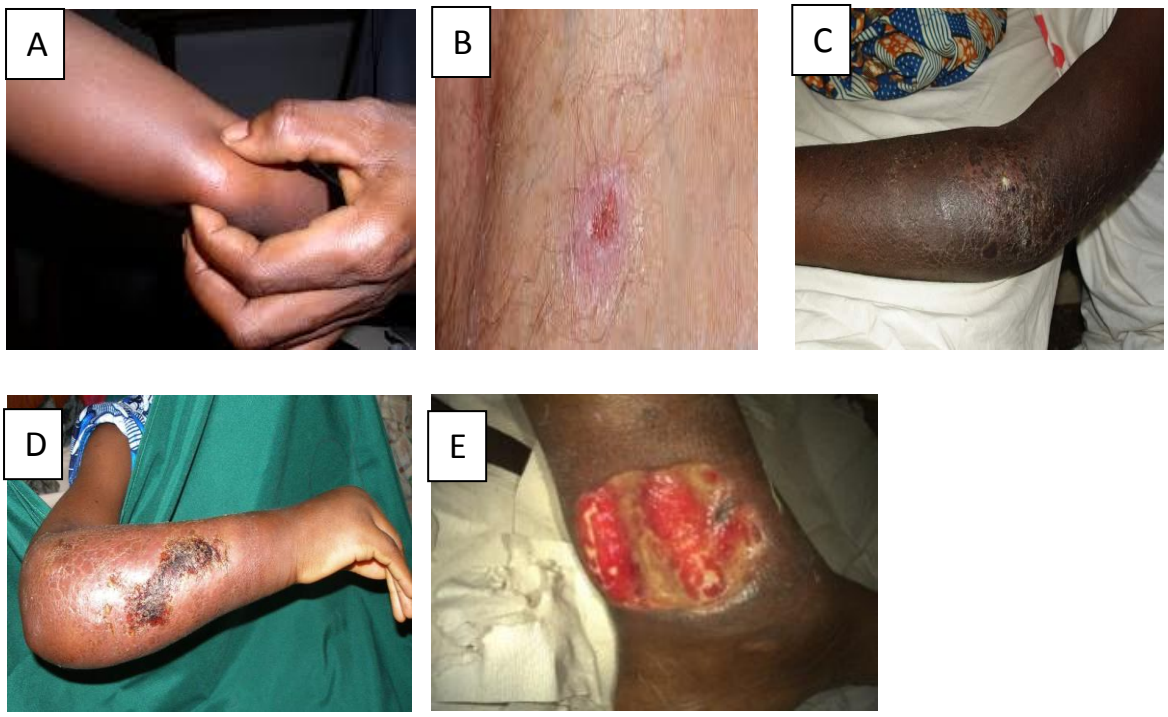

Figure 1: Clinical Presentations of Buruli ulcer disease, A= nodule, B=papule, C=plaque, D=oedema, E=ulcer

Epidemiology and Diagnosis

BU has been reported in 34 countries (Figure 2) globally with tropical, subtropical and temperate climates near areas of stagnant or slow flowing rivers and marshlands; an association that has been shown to be a risk factor for infection [19-20]. Geographically, the disease has been described in Africa, Asia, the Americas and Australia [21]. Africa is the worst affected continent with the highest incidence reported in Western African countries of Ghana, Cote d'Ivoire and Benin [22-27]. It is estimated that over 5000-6000 cases are reported yearly; though a decline has been seen recently with only 2,251 new cases reported in 2014 [28]. All age groups are affected by the disease but in Africa the greatest burden is in children below the age of 15 years [28].

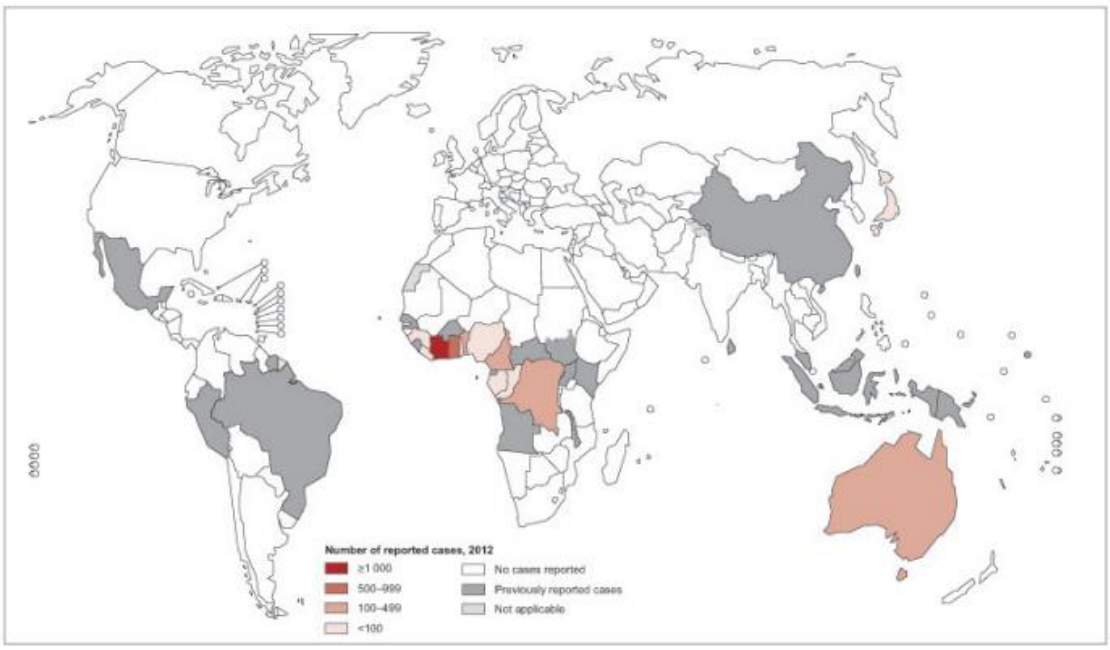

Figure 2: Global Distribution of Buruli ulcer, 2013

BU can be clinically diagnosed and experienced health workers in endemic areas may be able to make an accurate clinical diagnosis of the disease [29-30]. However, in practice, misdiagnosis is common [22, 31-32] with reported rates up to about 50% [33] and the proportion of false negatives is usually not accessed, since patients are sent away and not followed up. Clinical diagnosis should therefore be confirmed by laboratory tests, either by conventional or molecular methods. Misdiagnosis is stemming from the large number of organisms which also cause cutaneous infections and other non-infectious pathologies which may resemble BU [34]. Laboratory diagnosis also helps to confirm the accurate prevalence and incidence, helps to identify new foci, aids in the management of the disease and facilitates the differentiation of relapses from re-infection after treatment.

Tests available for the confirmation of BU diagnoses are; direct examination of acid fast bacilli in Ziehl-Neelson stained smears, in vitro culture, histopathology and the polymerase chain reaction (PCR) amplification of the insertion sequence 2404 elements (IS*2404*) found in multiple copies in the genome of *M. ulcerans.* Smear microscopy is the simplest and most widely used method which is also available in most endemic areas as a first line diagnostic test. It however has a low sensitivity with reported rates of about 50% even with strong clinical suspicion. Culture is not readily available in many endemic areas and is precluded as a rapid diagnostic method because of the slow-growing nature of the bacilli though sensitivity rates of up to 60% have been reported. Histopathological analysis is highly sensitive; confirming 90% of all cases but unavailable in many endemic settings. Molecular diagnostic methods are fast with a high sensitivity but unavailable in many endemic areas. PCR amplification of IS*2404* is considered the gold standard diagnostic method with sensitivity around 96% and WHO recommends that at least 70% of all clinically suspected cases be confirmed by a PCR positive result [35]. This test however presents technical difficulties with implementation in a field setting, is expensive and also requires a high level of technical expertise. It is therefore restricted to only a few well-equipped and specialized reference laboratories.

Treatment

Spontaneous healing of BU lesions has been reported by several research groups [36-38] especially for early non-ulcerative forms [37]. Historically however, surgery was the standard treatment of choice for management of the disease and involved the removal of dead tissues through debridement or excision, followed by skin grafting. With this method, wide margins including healthy tissue had to be excised to stop the infection, ensure the complete removal of all affected tissue and prevent recurrence or relapse at the affected site. This treatment modality however presented with a number of challenges including long hospital stays [6] by patients and its effect on the health centers which were ill-equipped and lacked the capacity to accommodate patients for long periods of time. Surgery was also accessible to only a fraction of affected patients and the dramatic nature of the procedure leading to wide and traumatizing excisions and skin grafting often involving multiple operations impacted poorly on control efforts as affected patients will be reluctant to report at health facilities for treatment for fear of surgery. Also, lesions would finally heal at the expense of more severe sequelae than would result from simple surgical interventions consequently resulting in more functional disabilities in the absence of physiotherapy. In addition, though there was success with this method, recurrence was not uncommon and rates ranged

between 16%-28% [39-40]. In a retrospective comparative study, 47% of patients from one treatment center had either ulcers that never healed or healed, but had a recurrence, supporting the hypothesis that wider surgical excision improves the chance of healing of BU[41].

To minimize or circumvent surgical interventions, several other approaches involving chemical and physical topical treatments have been explored and proposed. These include the use of 6% nitrogen oxides which was reported to kill *M. ulcerans* in vitro and also in a small randomized control trial [42-43], hyperbaric oxygen, phenytoin powder which appeared promising in accelerating healing and showed ulcer surface reduction of more than 50% in a randomised control trial [44-45] and clay which was shown to allow quick resolution of oedemas and vigorous debriding of ulcers [46]. Application of heat, taking advantage of the temperature sensitivity of *M. ulcerans* has also been documented with its efficacy demonstrated in several studies [9, 47-49]. Some of these other methods however have limited practicality in remote areas where most of the cases are reported from, because of the complicated nature of the devices and dressings needed for their implementation, high costs and the discomfort it brought for patients [5].

Finding a drug treatment regimen thus represented a major research priority for the WHO and with it the search for the appropriate antibiotic combination to combat this disease. Many antibiotics were found to show excellent results in vitro against *M. ulcerans* with various combinations inhibiting its growth in animal models [37, 50-56]. High sensitivity of *M. ulcerans* to rifampicin [50], aminoglycosides [51], macrolides [52] and quinolones [51] was reported and studies in animal models showed that monotherapies of rifampicin and aminoglycosides was highly efficacious by exerting a strong bactericidal effect on *M. ulcerans* [54-55]. Monotherapy was however discouraged because *M. ulcerans* could become resistant to rifampicin and resistant mutants had been observed after monotherapy in mice [57]. The aminoglycosides on the other hand have long term side effects as they are known to cause ototoxic damage, vestibule-toxic impairments, nephrotoxicity and encephalopathy [58-61]

Based on these results and clinical trials performed in Ghana [62], the WHO issued guidelines with a combination of rifampicin and streptomycin for 8 weeks as the treatment of choice for management of the disease with the hope of minimizing indications of surgery and hopefully also decrease relapse rates [63]. Evidence accumulating after the implementation of these guidelines shows treatment success with recurrence rates of less than 3% [64].

Wound healing

Wound healing is a complex process made up of four main stages, hemostasis, inflammation, proliferation and tissue remodeling or resolution. These phases are integrated and highly overlap. Interference with one or more of these phases leading to the inability of the wound to progress through the normal stages of healing results in the formation of a chronic wound. Such wounds would not respond normally to traditional wound management practices and standard protocols of care [65]. Factors that can affect wound healing could be either local or systemic. Local factors such as oxygenation, infection, venous sufficiency and foreign bodies affect the characteristics of the wound itself [66-67]. Systemic factors such as age, gender, sex hormones, stress, ischemia, diseases, medications, alcoholism and smoking, immunocompromised conditions and nutrition affect the overall health and disease state of the affected individual, consequently affecting the ability of the wound to heal [66].

Wound microbiology

The human body is estimated to contain 10^{14} microbial cells playing various roles in the maintenance of health. Some of these microbes however, have the potential to cause disease and this is seen under opportunistic circumstances such as a breach in skin integrity leading to an injury. When a wound occurs, microorganisms which are normally found at the skin surface gain access into the underlying tissues, contaminating the wound. Other sources of wound contaminants are the environment and endogenous sources involving mucous membranes of the gastrointestinal, oropharyngeal and the genitourinary mucosa [68]. The establishment of a wound microbiota goes through several stages called a wound infection continuum. First is the contamination stage characterized by the presence of non-replicating microorganisms [65, 67]. At this stage, the microorganisms are suppressed or regulated by appropriate host defenses if the affected individual is not immunocompromised or physiologically challenged. Next is a colonization stage consisting of reversible and irreversible adhesion stages and characterized by the presence of replicating microorganisms in the absence of tissue damage. In the reversible adhesion stage, microorganisms access the wound environment using chemical receptors and either attach to the wound surface or remain planktonic based on the environmental signals [65]. At this stage of colonization, they can be detached by the application of low levels of force and are sensitive to host defenses and antimicrobial agents. As this stage progresses, stronger surface interactions are created leading to the formation of an irreversible attachment, biofilms and the production of intracellular and extracellular substances for polymeric encasement. Multiplication begins and an inherent resistance to antimicrobial agents develops. Continuous multiplication leads to the creation of micro-colonies

and a complex environment is created leading to decreased oxygen availability and hypoxia at certain sites in the wound. This will encourage the proliferation of anaerobic organisms further complicating the microbial community and increasing microbial diversity. Subsequently, critical colonization occurs where the microorganisms may colonize and multiply within the wound, inducing a non-healing state without clinical signs of infection or a visible host reaction. A mature biofilm is created when the microbial ecosystem climaxes and stabilizes. Exponential multiplication of bacteria within this mature biofilm leads to local infection driven by the bacterial burden exceeding a certain level and inducing host immune responses. If local infection is not managed properly and microbial burdens are uncontrolled, systemic infection may develop where bacteria invade new tissues and can find their way into the bloodstream causing bacteremia leading to septicemia, organ failure and death in severe cases [65].

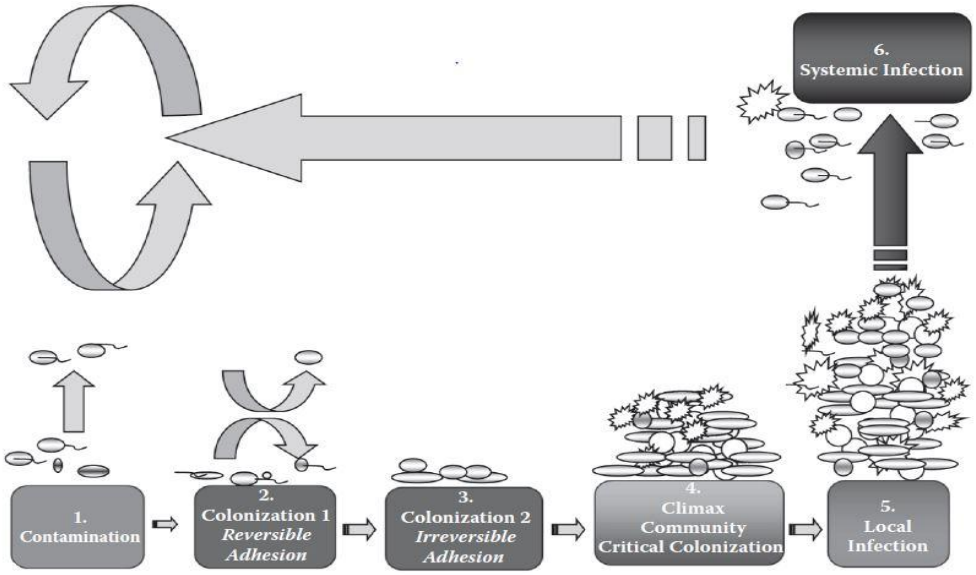

Figure 2: The wound infection continuum

Figure adapted from Percival et al [65]

The quantitative and qualitative microbiology of a wound is important as it impacts on wound healing. Quantitatively, the role of microorganisms in the clinical course of many diseases and infections has been found to be related to its load. Bendy et al [69] reported that wound healing in decubitus ulcers progressed only when the microbial load of wound fluid was below 10^6cfu/ml and Majewski [70] also demonstrated that patients with wound contamination $< 5 \times 10^4$ cfu/cm^2 had more successful skin grafts. Other studies also found that the risk of wound infection increased with bacterial loads above 10^5cfu/ml and this has led to the conclusion that the determination of bacterial loads could be useful in the prediction of wound healing and infection. One exception however is where a wound is contaminated with ß-haemolytic streptococci which have been found

to cause disease even at levels lower than 10^5cfu/g of tissue. Chronic wounds may have a polymicrobial etiology involving both aerobic and anaerobic microorganisms. Microbial pathogens frequently isolated from wounds include Enterococcus spp, *Staphylococcus aureus*, coagulase negative Staphylococci, *Escherichia coli*, *Pseudomonas aeruginosa*, Proteus sp, Enterobacter sp. However, *S. aureus*, *P. aeruginosa* and ß-haemolytic Streptococci have been commonly implicated in wound infection and delayed wound healing due to their ability to produce destructive virulence factors notably enzymes and toxins.

Wound Healing in Buruli ulcer disease

The evolution of BU disease varies with its severity which depends on the form, extent and localization. Early limited lesions and small ulcers have been shown to heal with antibiotic therapy alone without the need for surgery in 81% of cases [71]. In a proportion of affected patients, wound healing is delayed [72] and the end of antimycobacterial therapy is followed by a period of monitoring and wound management often ending with some form of surgical intervention if the lesions do not show signs of healing by secondary intention. Management of BU disease may be complicated by secondary bacterial infection [73] or Human immunodeficiency virus (HIV) co-infection [74-75] which affect the healing potential of wounds ultimately impacting treatment outcome.

Secondary Bacterial Infection

According to the World Health Organization (WHO) [76], secondary infection in BU should be suspected when a wound develops cellulitis or becomes painful. Secondary infection is not well characterized and recognized in BU disease as it is assumed to be uncommon [76]; therefore, only few studies have documented the occurrence of secondary infection in BU disease [73, 77-79]. It is assumed that secondary infections may result in severe complications such as sepsis, tetanus and death [77]. In the two studies that described the microbial flora of BU wounds, the isolated organisms included *S. aureus*, *P. aeruginosa*, *P. mirabilis*, Group A Streptococci, coagulase negative Staphylococci and several species of Enterobaceriaceae and other Gram negative pathogens [73, 79]. *S. aureus* and *P. aeruginosa* were the dominant isolates in both studies [73, 79]. These two organisms are well known as common causes of infection of other wound types [65-68], are frequently implicated in health-care associated infections and exhibit increased resistance to antimicrobials through both intrinsic and acquired mechanisms [80-81]. They also form biofilms contributing to antibiotic tolerance [80] and persistence and thus infection by either

of these organisms can result in worse patient outcomes [82]. Of concern in these studies was the high frequency of methicillin resistant *S. aureus* (MRSA) isolation. In the Ghanaian study, 33% of isolated *S. aureus* were MRSA while 38% MRSA was reported by the study in Benin. An MRSA frequency of 13% was also reported by a recent study in Ghana [83]. MRSA infection is one of the current global threats to public health. The organisms were first detected in hospitals but the recent emergence of MRSA strains with a community origin has created a greater challenge in the fight against antimicrobial resistant pathogens.

Though standard guidelines for the management of secondary infections in BU are currently unavailable, it behooves all stake holders in the health sector to optimize wound management practices and actively implement all strategies necessary to prevent or reduce the occurrence of secondary infection.

BU-HIV Co-infection

A high prevalence of HIV exists within areas of Africa burdened by BU, where adult HIV prevalences between 1-5% have been reported [84]. Thus, co-infection between HIV and BU has been increasingly recognized [84-85]. This co-infection presents an important challenge in the management of BU disease with studies suggesting that HIV may affect clinical presentation and result in multiple and larger lesions and also slower wound healing [74-75, 86]. Disseminated disease has been reported in individuals presenting with BU-HIV co-infection [86-89]. Though similar presentations were also reported in HIV negative BU cases [90], HIV infection was found to be a risk factor for disseminated disease and a significant predictor of bone involvement in the clinical course of the disease [91-92]. It is also thought to result in more severe BU [75, 84-85] and a study by Vincent *et al* reported that over 70% of HIV positive BU patients developed more severe disease in comparison with 50% of HIV negative patients [85]. HIV has been considered as a risk factor for BU disease [74-75] and its prevalence was reportedly three times higher among BU patients in an endemic area in Akonolinga, Cameroon compared with the regional prevalence [75]. Also, similar results have been obtained from Ghana and Benin where BU patients are 4 and 8 times more likely to have HIV than non-BU patients [74, 93]. BU-HIV co-infected patients often present with severe immunosuppression [75, 84]. In a study conducted in a BU endemic area in Cameroon, 70% of BU-HIV infected patients were found to have CD4 counts $\leq$ 500 cells/mm^3 at diagnosis, needing antiretroviral therapy [75]. Data from this study also found a higher mortality in BU-HIV co-infected individuals as compared with BU patients without HIV [75].

Preliminary guidelines have been issued by the WHO on the management of this co-infection [94]. Despite these guidelines, lack of information still exists with regards to the best way to manage HIV patients with active BU, optimal ART regimens, when to start ART, the impact of HIV on BU treatment outcomes, drug-drug interactions between BU treatment drugs and ART regimens and whether ART influences the occurrence of paradoxical reactions in BU patients and more scientific studies are needed to better understand the epidemiological, clinical and treatment implications of BU-HIV co-infection [84].

Goal

The goal of this PhD study was to gain an understanding into the evolution of BU wounds during the course of treatment and wound healing delay.

Objectives

1. Identify causes of wound healing delay in BU disease in treatment centers in the Ga-South and Ga-West municipalities in Ghana.
2. Explore challenges associated with the management of BU disease.
3. Gain an understanding into possible routes of secondary infection of BU lesions.
4. Provide information on the genetic background of *Staphylococcus aureus* species isolated from BU lesions.
5. Characterize the antibiotic resistance profiles of bacteria isolated from BU lesions.

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Results

Chapter 1

Secondary Bacterial Infections of Buruli Ulcer Lesions Before and After Chemotherapy with Streptomycin and Rifampicin

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Abstract

Buruli ulcer (BU), caused by *Mycobacterium ulcerans* is a chronic necrotizing skin disease. It usually starts with a subcutaneous nodule or plaque containing large clusters of extracellular acid-fast bacilli. Surrounding tissue is destroyed by the cytotoxic macrolide toxin mycolactone produced by microcolonies of *M. ulcerans*. Skin covering the destroyed subcutaneous fat and soft tissue may eventually break down leading to the formation of large ulcers that progress, if untreated, over months and years. Here we have analyzed the bacterial flora of BU lesions of three different groups of patients before, during and after daily treatment with streptomycin and rifampicin for eight weeks (SR8) and determined drug resistance of the bacteria isolated from the lesions. Before SR8 treatment, more than 60% of the examined BU lesions were infected with other bacteria, with *Staphylococcus aureus* and *Pseudomonas aeruginosa* being the most prominent ones. During treatment, 65% of all lesions were still infected, mainly with *P. aeruginosa*. After completion of SR8 treatment, still more than 75% of lesions clinically suspected to be infected were microbiologically confirmed as infected, mainly with *P. aeruginosa* or *Proteus mirabilis*. Drug susceptibility tests revealed especially for *S. aureus* a high frequency of resistance to the first line drugs used in Ghana. Our results show that secondary infection of BU lesions is common. This could lead to delayed healing and should therefore be further investigated.

Author Summary

Buruli ulcer (BU) can lead to large ulcerative lesions due to extensive skin loss caused by the necrotizing effect of the main virulence factor mycolactone. For a long time the general perception was that BU lesions are not infected by other bacteria because of a postulated antimicrobial effect of the macrolide toxin, mycolactone. In this study, we analyzed laboratory confirmed BU lesions before, during, and after streptomycin/rifampicin treatment. Contrary to popular belief, our findings show that BU lesions are frequently co-colonized with other potential bacterial pathogens before, during, and after antibiotic treatment. For example, 75% of cases that were clinically indicative of being infected after treatment were microbiologically confirmed as infected. Most microbiologically infected cases were also confirmed by histopathological analysis. The most prominent bacterial species isolated included *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Proteus mirabilis*. When we tested the isolates against first line drugs used in Ghana, the isolates were found to be resistant to most of these drugs. This study indicates that wound care practices need to be improved and that wound infection may be a common cause of wound healing delay in BU.

Introduction

Buruli ulcer (BU) caused by *Mycobacterium ulcerans* is a necrotizing skin disease that affects mainly impoverished communities in Western and Central Africa. It is the third most common mycobacterial disease of humans after tuberculosis and leprosy. BU lesions are characterized by extensive necrosis and minimal pain and inflammation [1,2]. The pathogenesis of the disease is believed to be initiated by the inoculation of *M. ulcerans* into the subcutaneous layer of the skin, which may be facilitated by trauma or an insect vector. Most BU lesions are found at the extremities and contain extracellular clusters of acid-fast bacilli (AFB) in the subcutaneous fat tissue. The incubation period seems to be highly variable, and has been estimated to range from two weeks to three years, with an average of two to three months [3]. The disease begins typically as a painless nodule under the skin and gradually enlarges and erodes through the skin surface, leaving a well-demarcated ulcer with a necrotic slough in the base and widely undermined edges [3 ,4].

Traditionally, the mainstay treatment of BU was surgical removal of infected tissues followed by skin grafting [1]. This led to long hospital stays with the accompanied social problems of losses of school time by children and a large economical burden directly and indirectly to the affected families. Since 2006, after a pilot study in Ghana, the first line treatment of BU is SR8 (eight weeks of streptomycin daily injections and oral therapy with rifampicin) [5–7]. This has reduced surgery to an adjunct procedure in BU management. The general perception is that this treatment modality will reduce the length of stay in health facilities, since it removes the fear of surgery and encourages early reporting to the formal health sector for treatment. SR8 makes a decentralization of treatment possible, since staff of peripheral health facilities can administer streptomycin injections.

The pathogenesis of BU is mediated mainly by a polyketide derived macrolide toxin, named mycolactone, with potent tissue necrotizing [8] and immunosuppressive activities [9,10]. Mycolactone produced by clusters of *M. ulcerans* leads to the destruction of the surrounding soft skin tissue and to the formation of devitalized, avascular tissue and ‘necrotic slough’ at the wound bed, which is very characteristic of BU [11]. The necrotic tissue could provide an ideal medium for bacterial growth and may disturb and delay wound healing. While there is a popular belief that secondary infections of BU lesions are rare, because mycolactone has antimicrobial activities, there is no published evidence base for this.

It is controversial, whether bacteria present in wounds contribute to delays in wound healing, because wounds generally harbor transient microorganisms (contamination) [12]. The surfaces of wounds have microbial populations at each stage of healing and some of the bacteria may be

involved in mutually beneficial relationships with the host preventing more virulent organism from infecting deeper tissues. Such beneficial organisms include coagulase negative Staphylococcus and Corynebaceria species [12–14]. These contaminating organisms are derived from the normal flora of the surrounding skin, mucous membranes or from external environmental sources. Usually the immune defense mechanisms of the host can contain these contaminants with no harm and negative consequence to wound healing. However, some of the contaminating organisms can also go on to colonize, massively multiply and delay wound healing. Only when a critical concentration of these microorganisms is reached, signs of infection including erythema, pain, increase in temperature, odor and discoloration of granulation tissue are observed. Therefore assessment of wound infection has to be based both on the density of microorganisms as well as on the presence of specific pathogenic species [15,16]. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and beta-hemolytic streptococci are regarded as primary indicators for a delayed healing and infection in both acute and chronic wounds. Bacterial loads exceeding 10^6 colony forming units (CFU)/g of tissue or tissue fluid, accumulations of pus cells and presence of specific pathogenic organisms are being used as indicators for wound infection in contrast to wound contamination [16–19]. Factors predisposing a wound to infection include the non-observance of principles of good hygienic procedures during dressing and the presence of necrotic tissue or slough within the wound margin [13], which is commonly found in BU lesions. The extent of secondary infections in BU and their contribution to frequently observed delays in healing has not been studied so far. Here we have analyzed BU lesions before, during and after antimicrobial treatment for the presence of secondary infection.

Materials and Methods

Study participants and sample collection

The participants involved in the study were recruited from the Amasaman District Hospital and the Obom Health Centre in the Ga-West and Ga South Municipality, respectively. The participants were all laboratory confirmed BU cases and the analyzed samples fall into three main categories: 1) samples from 53 BU patients recruited consecutively before treatment; 2) samples from 20 BU patients recruited consecutively between four and six weeks after start of SR8 and 3) samples from 31 BU patients whose lesions were clinically suspected of secondary infection after SR8 treatment. Some of the participants overlapped in some of the categories: 71 of the participants were sampled once for analysis, 12 twice and 3 thrice within the study period, thus in total 104 individual samples, 84 swabs and 20 tissue samples, from 86 participants were analyzed. The swabs were obtained from 52 cases before treatment, 20 cases during and 12 cases after treatment and analyzed microbiologically (Table S1). The tissue samples for histopathological analysis were obtained from one case before treatment and 20 cases after treatment. Except for one sample taken after treatment, all tissue samples were also analyzed microbiologically (Table S1).

A detailed questionnaire was used to obtain standard demographic data, document the clinical presentation of lesions and other lesion characteristics. Altogether the study involved 86 participants comprising 32 (37%) females and 54 (63%) males. The females' age ranged between two and 72 years and the males were between four months and 82 years. Median age for both groups was 33 years. Seventy-seven of the cases had lesions located on the limbs, three in the head and neck region, and one each located on the buttocks, armpit and back respectively; the lesion location of three participants was not documented.

Only 2/86 patients were pre-ulcerative. These lesions, one nodule and one plaque, were sampled later during surgery. The remaining 84 patients had ulcers; 78 of them had only ulcers, one had an ulcer and a nodule, three had ulcers with edema, and two had ulcers with osteomyelitis. Based on the judgment of the responsible clinician, surgical debridement was performed for 1 patient prior to treatment and for 20 patients after completion of SR8. Biopsy samples were collected in each instance for histopathological analysis (Figure 1).

Laboratory confirmation of BU disease was done by IS*2404* PCR and Ziehl-Neelsen microscopy as previously described [20,21]. Three swab samples were collected from clinically suspected ulcerative cases before treatment; one for IS*2404*-PCR based confirmation of BU, one for preparation of a direct smear for microscopic examination for the detection of bacteria and

neutrophils after Gram staining (Figure S1), and the third was inserted into a sterile tube containing 3 ml of PBS for enumeration of the bacterial burden and the isolation of specific bacterial species. All swab specimens were collected from the undermined edges of lesions by first moistening the swab with sterile PBS using the Levine method of collecting swab specimen [22]. This has been found to be the best method for taking swabs as it is more reflective of tissue bioburden as compared to other methods [23]. After cleaning the wound surface with normal saline, a swab was rotated over a 1 cm^2 area with sufficient pressure to collect the fluid from within the wound tissue.

From cases that were sampled during treatment and those that were clinically suspected of having a bacterial infection after completion of SR8, three swab specimens were collected before surgery, and treated as above, except for the procedures for the laboratory confirmation of BU disease by PCR, since all cases had been previously confirmed as BU within the framework of a bigger study. From SR8 treated patients that underwent surgical management, tissue sample were analyzed if there was clinical suspicion of a secondary bacterial infection. While one sample was aseptically transferred into a clean sterile tube for enumeration of the bacterial load and species identification, a second sample was directly transferred into 10% neutral buffered formalin for histopathological analysis.

The samples for bacteriological analysis were placed in an ice chest with ice packs to prevent bacterial multiplication and transported to the Bacteriology Department of the Noguchi Memorial Institute for Medical Research (NMIMR) for analysis, Tissue samples for were shipped to the Swiss Tropical and Public Health Institute for histopathological analysis.

Ethics statement

Ethical clearance was obtained from the institutional review board of the Noguchi Memorial Institute for Medical Research (Federal-wide Assurance number FWA00001824). The procedures for sampling in this study were essentially the same as those used in routine management of BU in Ghana. However, written informed consent was collected from all participants before study inclusion. In the case of children below sixteen years, written informed consent was collected from their parents or guardians. Patients were assured of the confidentiality of all information collected during the study.

Enumeration of the bacterial load and isolation of bacteria

When swab samples reached the microbiology laboratory, the volume of PBS was topped up to 5 ml and both the swab and the PBS were transferred into a sterile glass tissue culture tube containing glass beads. The tubes were vortexed for about two minutes to dislodge any particles

that were sticking to the swabs. Using the resulting stock suspension, serial dilutions from 10^{-2} to 10^{-6} were prepared. Hundred microlitres of serial dilutions of the swab or tissue suspensions were transferred into sterile Petri dishes and inoculated by the pour plate method using Plate Count Agar for total aerobic counts. The agar was left on the lab bench to set after which it was incubated at 37^{0}C for 18–24 hours. The remaining 10^{-1} dilution of the suspension was centrifuged at 8,000 g for 25 minutes and after decanting, the pellet was inoculated onto MacConkey, Blood and Chocolate agar and incubated under aerobic conditions. The aerobic agar plates were examined after 24 hours and growing colonies were subcultured on Blood and MacConkey agar plates to obtain pure cultures. After incubation, the plates were examined using a colony counting chamber (Gallenkamp, UK) and those with colony counts between 30 and 300 were selected for computing CFU/ml or CFU/g, respectively, by multiplying the counts by the dilution factors. The lesion from which the sample was taken was classified as clean, contaminated or infected as indicated in the data analysis section. For tissue specimen, one gram of sample was weighed in a sterile plastic stomacher bag. Nine milliliters of PBS were added, samples were macerated in a stomacher and the resulting suspension was transferred into a sterile test-tube. Using this stock suspension, serial dilutions were prepared and plated out.

Species identification of bacterial isolates

Distinct bacterial colonies from the Blood and MacConkey agar plates were purified on Nutrient agar plates for identification. Bacterial isolates were Gram stained [24] and identified by biochemical tests as well as by molecular methods. Gram negative rod isolates were characterized by cytochrome oxidase analysis, and with Analytical Profile Index (API 20E) strips (bio-Me´rieux SA, Marcy-l'E'toile, France) according to the manufacturer's instructions. Gram positive cocci were analyzed after Gram staining using the catalase test to differentiate between Staphylococcus spp. and Streptococcus spp. In order to further discriminate the catalase positive Gram positive cocci and especially to identify Staphylococcus spp., the Staphylase kit Prolex Latex Agglutination System (Pro-Lab Diagnostics) was used. Gram positive bacteria were further characterized using the Hain Lifescience Genotype Product series for Gram positive bacteria Genotype BC Gram positive version 3.0 and Genotype staphylococcus version 2 test kits (Hain Lifescience, Germany). Where species identification failed with the analytical profile index and the other biochemical assays, identification was achieved by MALDI-TOF mass spectrometry [25].

Drug susceptibility testing

Susceptibility of isolates to specific drugs was tested using the Kirby-Bauer disc diffusion method on Mueller Hinton agar [26]. Sensitivity was tested against antibiotics such as cotrimoxazole, ampicillin, tetracycline, ciprofloxacin, amikacin, gentamicin, penicillin, erythromycin, cefuroxime, cefixime, ceftriaxone, chloramphenicol and flucloxacillin. In addition, Gram positive cocci were tested against methicillin and vancomycin. The results of isolation and drug sensitivity tests were provided to the treating clinician at the collaborating health facility. Since the locally available disc systems varied in coverage, some antibiotics were only tested with a subset of isolates. One limitation of this study is that we did not test for susceptibility against streptomycin and rifampicin.

Histopathology

Histopathological analysis was done for all SR8 treated patients needing surgical management and presenting with a lesion clinically suspicious for secondary infection. Surgically excised tissue samples were immediately fixed after excision in 10% neutral-buffered formalin for 24 h at room temperature to maintain tissue structures. Afterwards samples were directly transferred to 70% ethanol for storage and transport. Tissue specimens were subsequently dehydrated, embedded into paraffin, and cut into 5 µm sections. After deparaffinization and rehydration, sections were stained with Ziehl-Neelsen/Methyleneblue (ZN) according to WHO standard protocols [3]. In this staining AFB appear pink and other bacteria are stained blue. Tissue sections were analyzed with a Leica DM2500 Microscope and pictures were either taken with a Leica DFC 420C camera or with an Aperio ScanScope XT.

Analysis of recycled bandages

Recycled bandages from fifteen confirmed BU cases were collected conveniently before wound dressing for microbiological analysis. Ten grams bandage was weighed, added to 90 ml of sterile PBS and macerated with a laboratory blender to give a 10^{-1} dilution. Using this suspension, serial dilutions from 10^{-2} to 10^{-6} were prepared. Hundred microlitres of these serially diluted suspensions were transferred into sterile Petri dishes and inoculated by the pour plate method using Plate Count Agar for total aerobic counts. Bacterial enumerations were performed as described above. In addition the left over suspension was centrifuged at 3,000 g for 20 mins and the resulting pellet was plated for bacterial isolation.

Data analysis

The values obtained from plate counts were computed into CFU/ml for wound exudates (swabs) or CFU/g for tissue sample. The antibiogram of each isolate was interpreted according to the manufacturer's specification as resistant, intermediate or susceptible. The percentages of cases in each category were then computed.

Classification of wounds

Lesions were classified microbiologically as clean if no bacteria were isolated, as contaminated if bacterial counts were $< 10^6$ CFU/g or ml and as infected if counts were $> 10^6$ CFU/g or ml of specimen. Lesions were clinically classified as infected based on the following criteria: 1. friable, bleeding granulation tissue despite appropriate care and management; 2. purulent discharge (yellow or green) from wound or drain placed in wound; 3. pain or tenderness, localized swelling (edema), or redness/heat; 4. tissue necrosis; 5. skin grafting failure; abnormal odor coming from the wound site; delayed healing not previously anticipated. Twenty-four of the patients clinically classified as infected were in-patients and seven were out-patients, who were reporting twice a week for wound dressing. During wound dressing, the wounds were cleaned with normal saline to wash away debris. Wounds that appeared necrotic or had an offensive odor were cleaned again with vinegar and dressed with povidine iodine.

Results

Bacterial infection of lesions from PCR-confirmed BU patients before and during SR8 treatment

Swab samples of 52 consecutively recruited IS*2404* PCR confirmed BU cases with ulcerative lesions were sampled before the commencement of SR8 treatment. Samples from three participants (5.7%) did not yield any aerobic growth on plate count agar (Table 1). Seventeen (32.1%) of the lesions with total CFU counts of 1.7×10^3 to 9.0×10^5 CFU/ml (average 3.2×10^5 CFU/ml) were microbiologically classified as contaminated. Microbiologically Infected lesions were observed in 33/52 patients (63.5%); aerobic counts from this group ranged between 1.0×10^6 to 3.5×10^9 CFU/ml with an average value of 1.1×10^9 CFU/ml. The most frequently identified bacterial species from the infected lesions prior to start of treatment (Table 1) were *S. aureus* (n = 9; 21.4%), *P. aeruginosa* (n = 7; 16.7%) and *P. mirabilis* (n = 6; 14.3%).

The responsible clinician decided to perform wound debridement of one of the lesions prior to SR8 initiation, since it showed clinical signs of a strong secondary infection (Figure 1D). A biopsy specimen was taken and the histopathological analysis of the tissue sample (Figure 1A–C) revealed, typical hallmarks of BU, such as fat cell ghosts, tissue necrosis and epidermal hyperplasia (Figure 1A). In addition, clusters of cocci were observed in the subcutaneous tissue between the fat cells (Figure 1A box, B, C). This area probably represents the tissue base of the undermined edges. These findings correlated well with the microbiological analysis, since *S. aureus* was isolated in large numbers from the lesion (1.2×10^9 CFU/g).

Twenty laboratory-confirmed BU cases were consecutively sampled between four and six weeks after start of SR8 treatment and analyzed for infection of the lesions. Of these lesions, 7/20 (35.0%) and 13/20 (65.0%) were microbiologically classified as contaminated or infected, respectively; clean wounds were not observed (Table 1). The aerobic bacterial load ranged between 1.5×10^6 and 3.5×10^9 CFU/ml, with an average value of 5.6×10^8 CFU/ml for the microbiologically infected lesions. The contaminated lesions had counts between 5.2×10^3 and 7.3×10^5 CFU/ml (average 3.3×10^5 CFU/ml). Also here *P. aeruginosa* (n = 6; 35.3%) and *P. mirabilis* (n = 2; 11.8%), but not *S. aureus* (n = 0), were the most frequently identified bacterial species isolated from the infected lesions (Table 1).

Bacterial infection of BU lesions with clinical signs of infection after completion of SR8 treatment

Thirty-one BU lesions with clinical signs of secondary bacterial infection after completion of SR8 treatment were sampled for laboratory investigation. Clinical signs indicative for secondary

infection were documented for 28 of them and included: localized pain (28/28), viscous/purulent discharge (28/28), edema (5/28) and localized heat (4/28). In addition, delayed healing not previously anticipated (17/28), offensive odor (15/28) and discoloration of tissues both within and at the wound margins (3/28) were regarded as signs of secondary infection (Table 2). The time at which infection was detected ranged from a few weeks to fifteen months after completion of SR8.

Seven (22.6%) of the 31 lesions clinically suspected to be infected were not confirmed microbiologically by aerobic bacterial count analysis, as the total plate count ranged only between 1.3×10^3 and 8.9×10^5 CFU/ml (average 2.7×10^5 CFU/ml). The remaining twenty-four (77.4%) lesions that were microbiologically confirmed as infected had plate counts ranging between 1.2×10^6 and 3.5×10^9 CFU/ml (average value of 1.2×10^9). *P. aeruginosa* (n = 8; 32%), *P. mirabilis* (n = 5; 20%) and *S. aureus* (n = 3; 12%) dominated among the isolates.

The bacterial load observed in cases analyzed within four weeks post SR8 ranged between 1.3×10^3 and 4.0×10^9 CFU/ml; that between five and 12 weeks was between 9.3×10^4 and 1.2×10^9 CFU/ml; and that between 9 and 15 months post SR8 ranged between 2.7×10^6 and 1.8×10^9 CFU/ml. Nineteen tissue samples and 12 swab samples were analyzed (Table S1) and the bacterial load ranged between 1.3×10^3 and 4.0×10^9 CFU/ml for tissues and between 5.2×10^7 and 2.1×10^9 for swabs.

Tissue samples from 20/31 of the microbiologically analyzed lesions showing clinical signs of secondary infection after completion of SR8 were also analyzed by histopathology, since the responsible clinicians decided to perform a wound debridement. Microbiological analysis had categorized 16 of these lesions as infected and four as contaminated. None of the microbiologically contaminated wounds presented in the histopathological analysis with a detectable secondary infection. In contrast 12/16 (75%) of the lesions classified microbiologically as infected presented with an infection either with cocci, rods or both (Table 2). Infection was mainly observed in the stratum corneum (6/12; 50%) or on the open ulcer surface (3/12; 25%) and only rarely (3/12; 25%) deeper inside the excised tissue (Table 2). Histopathological analysis of specimen from patient 9 (Figure 2 A– D) revealed a layer of densely packed rods at the open ulcer surface visible already at low magnification as an intensely blue stained band (Figure 2B) At higher magnification, clusters of rod shaped bacteria were observed (Figure 2 C,D). Microbiological analysis confirmed the presence of *P. aeruginosa*. Tissue excised from patient 16 (Figure 2E–H) showed a double infection: cocci being present inside the stratum corneum (data not shown) as well as an extensive infection of the dermal and subcutaneous tissue with rods (Figure F–H). Microbiological analysis isolated *S. aureus* as well as Gram-negative rods. In most of our analysis, histopathological and microbiological results showed a good correlation for most of the patients (Table 2).

Drug susceptibility pattern of bacterial isolates

Using the disc diffusion assay, a total of 98 Gram-negative rods and Gram-positive cocci obtained from BU wounds were tested for resistance against antibiotics commonly used in Ghana. None of the isolates tested was sensitive to all drugs included in the analysis (Table 3). Five Gram-negative rods were resistant to all tested drugs. More than 70% of the 18 *S. aureus* isolates obtained from infected (n = 12) or contaminated (n = 6) lesions were resistant to flucloxacillin, ampicillin and penicillin. In contrast, 15/18 (83%) were susceptible to gentamicin. The prevalence of *S. aureus* isolates resistant to methicillin (MRSA) and vancomycin (VRSA) was 33% and 17%, respectively. Likewise most of the *P. aeruginosa* strains were resistant to most of the tested drugs. However, most isolates (18/22; 82%) were susceptible to gentamicin. Results for the other Gram-negative and -positive bacteria are provided in Table 3.

Microbiological analysis of recycled bandages

When monitoring wound management procedures, it was realized that patients and care-givers were instructed by health workers to wash and recycle dressing bandages. We therefore conveniently sampled dressings that have been used and washed for the next dressing. Seventeen bandages from fifteen BU cases were analyzed and as shown in Table 4, all of them had some bacterial contamination with total aerobic plate counts ranging between 2.2×10^3 and 3.2×10^8 CFU/g with an average count of 2.8×10^7 and a median value of 1.2×10^5 CFU/g. While bacterial species identified included commensals such as staphylase negative Staphylococcus spp., also potential pathogens including *S. aureus, P. aeruginosa, Flavibacterium oryzihabitans, Enterobacter agglomerans* and *Enterobacter cloacae* were isolated. The drug susceptibility patterns of isolates are indicated in Table 4. Similar isolates were also isolated from patients' wounds.

Discussion

Mycolactone, the cytotoxic macrolide toxin of *M. ulcerans* plays a key role in the pathology of BU. It causes apoptosis of mammalian cells [8,27] and has immunomodulatory activity [28,29]. Since a number of macrolides have antibiotic activity against a broad spectrum of bacteria, including streptococci, pneumococci, staphylococci, enterococci, mycoplasma, mycobacteria, rickettsia, and chlamydia [30], it has been speculated that mycolactone secreted by *M. ulcerans* during active disease prevents secondary bacterial infections of BU lesions. The goal of this study was to find out whether ulcerative BU lesions are indeed rarely colonized or infected by other bacterial species. To address this, BU wounds were characterized before SR8 treatment by both direct smear microscopic analysis for the presence of bacteria and neutrophils [20] and by pour plate determination of aerobic CFU counts. More than 60% of the lesions tested before treatment had bacterial counts $\geq 10^6$ CFU/ml and direct smear examination frequently showed large numbers of bacteria and neutrophils (Figure S1). A broad spectrum of bacterial species was isolated from the lesions with *S. aureus, P. aeruginosa* and *P. mirabilis* being the most frequently found species. This suggests that *M. ulcerans* infection and mycolactone secretion does not prevent secondary bacterial infections.

Chronic wounds often have a bacterial burden that is massively exceeding levels used to define lower limits for the definition of infection in acute surgical wounds (i.e. 10^6 CFU/g of tissue). However, many chronic wounds go on to closure despite levels of infecting microorganisms $\geq 10^8$ CFU/g of tissue, with infection by Group B streptococci being one exception to this rule [12,13,16]. Because of the intrinsic differences in the way acute and chronic wounds respond to the burden of microorganisms, emphasis is currently being placed on holistic assessments, with clinical signs and symptoms playing key roles in the diagnosis of chronic wound infection. Clinical signs usually employed for diagnosis include erythema, edema, heat, purulent exudates with concurrent inflammation, pain, delayed healing, discoloration of granulation tissue, friable granulation tissue, pocketing at the base of the wound, foul odor, and wound breakdown [13,14,17]. In particular increasing pain and wound breakdown have been shown to be good predictors of infection in chronic wounds. In this study we combined clinical, histopathological, qualitative and quantitative microbiological methods to analyze BU lesions for the presence of infections after completion of SR8 treatment. Lesions from 28 patients showing clinical signs of infection were included in this analysis. 75% of these lesions yielded CFU counts $>10^6$ CFU/ml (average value of 1.2×10^9) and frequently species with pathogenic potential, such as *S. aureus, P. aeruginosa, S. haemolyticus, E. cloacae and K. pneumonia* were isolated. Pain and yellow discharge turned out to be highly predictive clinical indicators for infection. For the patients that had clinical signs of infection after

SR8, culture and drug susceptibility testing results were submitted to the treating officer. However documentation of the treatment and subsequent follow-up of patients was beyond the scope of this study.

A study analyzing the microbial flora of healing and non-healing decubitus ulcers [31] found *S. aureus*, Streptococcus spp., *E. coli*, Klebsiella spp., Proteus spp. and *P. aeruginosa* as the main organisms that caused infection of the ulcers. Chronic venous ulcers have been found to be infected with *S. aureus, P. aeruginosa*, Coagulase-negative staphylococci, Proteus spp. and anaerobic bacteria [32]. Thus most of the organisms isolated in this study from BU lesions have also been found associated with infection of other types of wounds. Similar to what has been reported in other studies [33], lesions were in many cases infected with more than one bacterial species (Table 2). Our data on the microflora of lesions upon admission indicate that BU lesions may be contaminated from the communities as a result of improper wound care practices by the patients in their quest to treat the infection either on their own or with the help of traditional healers or herbalists. There is major concern about subsequent acquisition of antibiotic resistant organisms from the hospital settings. After the present pilot study demonstrating colonization and infection during and after SR8 treatment, we plan to perform longitudinal studies with patient cohorts to study the influence of BU wound management practices on secondary bacterial infections.

The method used for collecting wound specimens can influence the data obtained from microbiological culturing. Currently, collection of a biopsy specimen is the gold standard for determining the presence and identity of microorganisms within the wound bed tissue [12,16,34–37]. However, there are limitations as to which healthcare providers can collect biopsies, the availability of laboratories offering microbiological culture testing on biopsies, the expenses involved with the performance of these tests, and the potential for further tissue damage and delay of wound healing when biopsies are taken. In the present study we employed swabbing [22,36] as the main sampling procedure and performed histopathological studies with tissue specimen only from 20 cases that underwent surgical intervention. The histopathological analysis detected bacterial populations in 75% (12/16) of the analyzed lesions classified as infected and in none (0/4) of the lesions classified as contaminated. This strong correlation between results obtained with tissue and swab samples confirms results of previous studies [23] indicating that microbiological swabbing is a good sampling procedure for the determination of infection of wounds. Histopathological analysis detected infecting bacteria populations only rarely deeper inside the excised tissue and mainly in the stratum corneum or on the open ulcer surface, where bacteria are accessible for the swabs.

Contamination of BU lesions prior to SR8 treatment may be a result of wound care practices by the

patients. Also during SR8 treatment a range of bacterial species, with Gram-negative rods dominating, were isolated from the lesions. This indicates that SR8 does not necessarily eliminate contamination or secondary infection of lesions. Bacterial species, such as *P. aeruginosa, K. pneumoniae* and *S. aureus* isolated from infected lesions after completion of SR8 treatment, may however also have been acquired from the hospital setting. A detailed characterization of isolates is required to address this important issue further. Both mono and multiple antibiotic resistant strains were isolated with high frequency from the BU lesions. For example all the tested *S. aureus* strains were resistant to penicillin, 22% were methicillin resistant and 17% vancomycin resistant. Dependent on the setting, both lower (Nigeria, [38]) or higher (South Africa, [39]) frequencies have been reported in Africa. Most worrying in this context is the high (83%) level of resistance of *S. aureus* isolates to flucloxacillin, which is in Ghana the main antibiotic in use for treating skin infections such as boils and cellulitis. In addition, we acknowledge that true VRSA is rare, and that the occurrence of apparent VRSA is being followed up through referral of isolates to an international reference laboratory.

Postoperative infections of wounds represent the commonest surgical complication causing substantial increases in the duration and costs of hospital stays [40]. Our pilot study involving BU patients at different time points of SR8 treatment indicates that secondary bacterial infection may be a prominent cause for delays in wound healing and skin grafting failures. These findings call for an optimization of BU wound management and hygiene procedures to better control secondary infections. Also the choice of treatment of secondary infections with locally available antimicrobial agents requires a better understanding of the infecting flora and of drug susceptibility patterns. Our study did not follow the same patients from beginning of treatment till they were healed and this has limited the ability to determine causes and consequences of wound infection. More studies are required to ascertain the impact and source of wound infection in SR8 treatment of BU and to support development of guidelines for wound care in BU case management. In addition to wounds we also analyzed bandages that have been washed by the patients themselves to be re-used for wound dressing. From these bandages we isolated potential wound pathogens including *S. aureus, P. aeruginosa*, *Flavibacterium oryzihabitans, Enterobacter agglomerans* and *Enterobacter cloacae*; thus the bacteria profile of the wound samples was comparable to that of the bandages. These findings indicate that the recycling of bandages may not be a good practice as it may be one of the sources of wound infection. We recommend that if for economical reasons bandages need to be recycled, they must be washed well with an appropriate disinfectant.

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Author Contributions

Conceived and designed the experiments: DYM GP MN. Performed the experiments: DYM GSK MTR KAA KQF EOM AP. Analyzed the data: DYM GSK MTR GP. Contributed reagents/materials/analysis tools: AP BA IL CKM MN. Wrote the paper: DYM MTR GP.

Figures

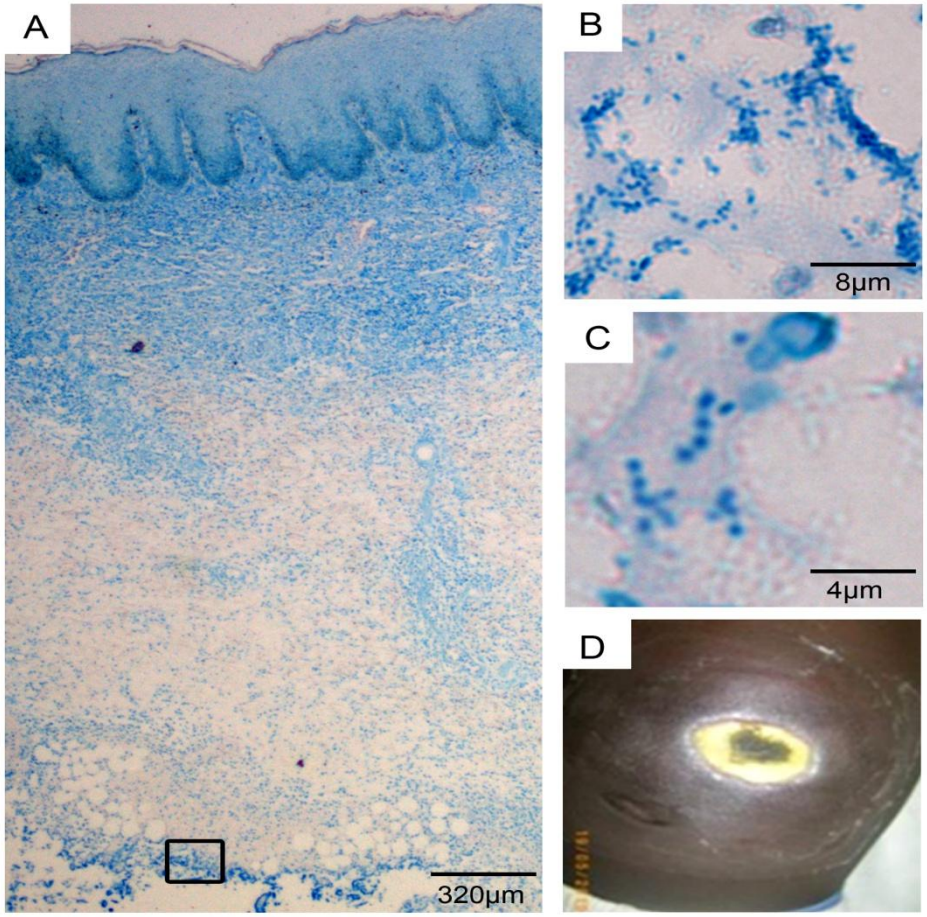

Figure 1: Histopathological analysis of tissue excised before start of SR8 treatment.

Histological sections were stained with Ziehl-Neelsen (acid fast bacteria) and methylene blue (DNA, secondary infection). A: Overview over excised tissue specimen revealing infection at the lower end of the specimen (box), as well as BU characteristic histopathological features, including fat cell ghosts, necrosis and epidermal hyperplasia. B/C: higher magnification revealing the presence of cocci. D: clinical presentation of the lesion on the belly.

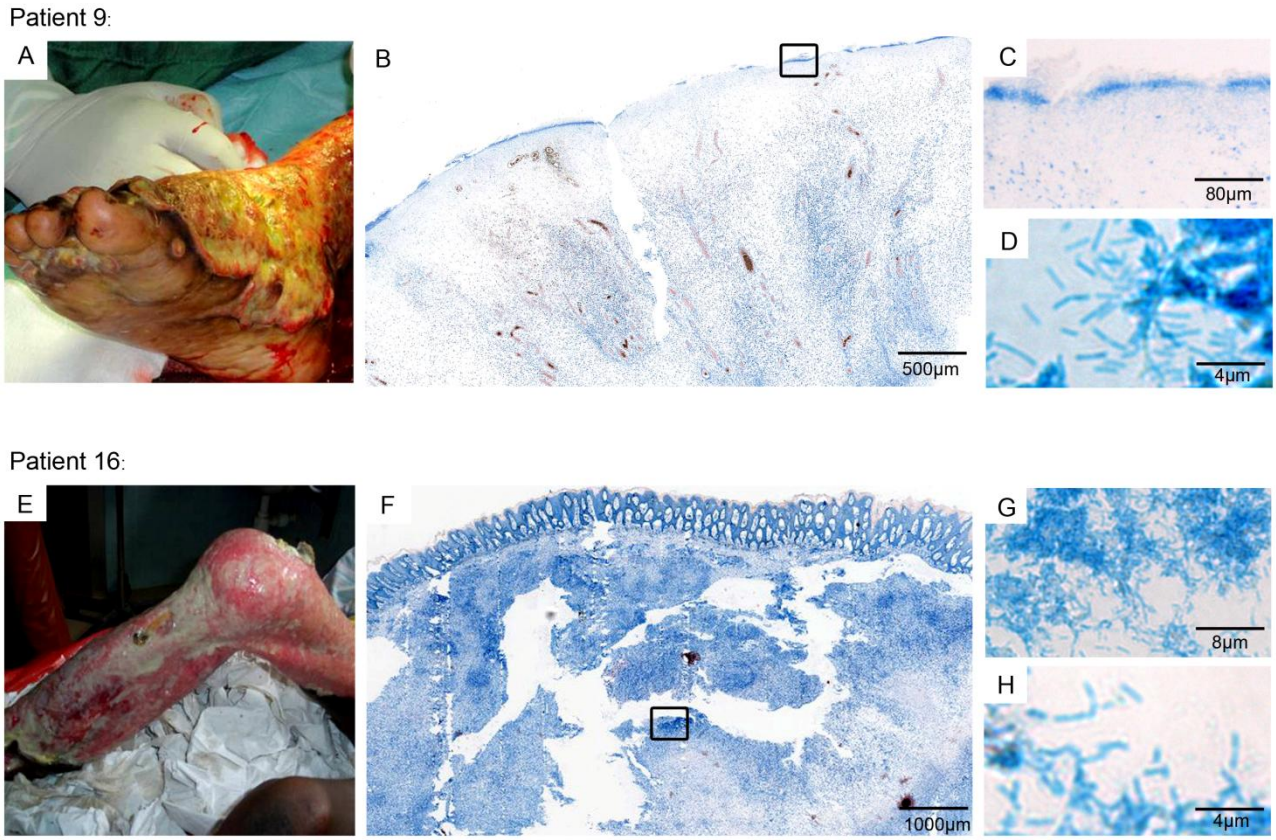

Figure 2: Histopathological analysis of tissue from two patients excised weeks after SR8 treatment respectively.

Histological sections were stained with Ziehl-Neelsen (acid fast bacteria) and methylene blue (DNA, secondary infection). A: clinical presentation of patient 9 presenting with a large lesion on the right foot. B: overview over excised tissue specimen (open ulcer surface) revealing the presence of an infection (blue band, box). C/D: higher magnification confirming the presence of densely packed rods. E: clinical presentation of patient 16 presenting with a large lesion covering the left leg. F: overview over excised tissue specimen revealing an epidermal hyperplasia as well as a strong edema. G/H: secondary infection with rods of the dermal and subcutaneous tissue.

Tables

Table 1: Spectrum of bacterial species isolated from BU lesions before, during or after SR8 treatment.

Time of sampling	Clean wounds	Contaminated wounds	Infected wounds	Spectrum of bacteria isolates from infected cases n (%)		
Before SR8 Treatment (n = 53)	3 (6%)	17 (32%)	33 (62%)	9	(22%)	*Staphylococcus aureus*
				7	(17%)	*Pseudomonas aeruginosa*
				6	(15%)	*Proteus mirabilis*
				3	(7%)	Coagulase negative Staph.
				3	(7%)	*Chryseomonas luteola*
				2	(5%)	*Enterobacter cloacae*
				2	(5%)	*Klebsiella pneumonia*
				2	(5%)	*Escherichia coli*
				1	(2%)	*Streptococcus dysgalactia*
				1	(2%)	*Providencia stuartii*
				1	(2%)	*Staphylococcus haemolyticus*
				1	(2%)	*Morganella morganii*
				1	(2%)	*Streptococcus agalactia*
				1	(2%)	*Staphylococcus warneri*
				1	(2%)	*Proteus vulgaris*
During SR8 Treatment (n = 20)	0 (0%)	7 (35%)	13(65%)	6	(38%)	*Pseudomonas aeruginosa*
				2	(13%)	*Proteus mirabilis*
				1	(6%)	*Staphylococcus warneri*
				1	(6%)	Coagulase negative Staph.
				1	(6%)	*Enterobacter cloacae*
				1	(6%)	*Providencia stuartii*
				1	(6%)	*Staphylococcus haemolyticus*
				1	(6%)	*Enterococcus gallinum*
				1	(6%)	*Flavibacterium oryzihabitans*
				1	(6%)	*Chryseomonas luteola*
After SR8 Treatment (n = 31; clinically diagnosed for secondary infection)	0 (0%)	7 (23%)	24(77%)	8	(32%)	*Pseudomonas aeruginosa*
				5	(20%)	*Proteus mirabilis*
				3	(12%)	*Staphylococcus aureus*
				2	(8%)	*Escherichia coli*
				2	(8%)	*Providencia stuartii*
				2	(8%)	*Klebsiella pneumoniae*
				1	(4%)	Coagulase negative Staph.
				1	(4%)	*Alcaligenes faecalis*
				1	(4%)	Acinetobacter sp

Table 2: Presentation of wounds that were clinically infected after SR8 compared to microbiology and histology findings.

BU Case	Clinical Presentation									Microbiological category	Species	Histopathology	Location (in the tissue)
	Odor	Pain	Green discharge	Yellow discharge	Necrotic tissue	Bloody discharge	WHD[1]	Edema	SGF[2]				
01	Yes	Yes	No	Yes	Yes	No	Yes	No	Yes	Infected	Mixed growth	Rods/Cocci	Stratum corneum
02	Yes	Yes	No	Yes	Yes	No	Yes	No	Yes	Infected	*Providencia stuartii*	None	
03	No	Yes	No	Yes	Yes	No	Yes	Yes	No	Infected	*Klebsiella pneumoniae*	Rods	Stratum corneum
04	No	Yes	No	Yes	No	No	No	No	No	Infected	Mixed growth/*S. aureus*	Rods/Cocci	Stratum corneum
05	No	Yes	No	Yes	Yes	No	No	No	No	Infected	*Pseudomonas aeruginosa*	n/d	
06	Yes	Yes	No	Yes	Yes	No	Yes	No	No	Infected	*Klebsiella pneumoniae*, Coagulase negative Staphylococcus species	n/d	
07	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Infected	*E. coli, Pseudomonas aeruginosa, S. aureus*	n/d	
08	No	Yes	No	Yes	Yes	No	No	No	No	Contaminated	*Pseudomonas aeruginosa*	None	
09	Yes	Yes	Yes	Yes	Yes	No	Yes	No	No	Infected	*Pseudomonas aeruginosa*	Rods	Ulcer surface
10	Yes	Yes	No	Yes	Yes	No	No	No	No	Infected	*Proteus mirabilis*	None	
11	Yes	Yes	No	Yes	Yes	No	Yes	Yes	No	Infected	*Proteus mirabilis, Enterobacter cloaccae*	n/d	
12	Yes	Yes	No	Yes	Yes	No	Yes	No	No	Infected	*Proteus mirabilis*, mixed growth	None	
13	No	Yes	No	Yes	Yes	No	Yes	No	No	Infected	*Proteus mirabilis*	n/d	
14	Yes	Yes	No	Yes	Yes	Yes	Yes	No	No	Infected	*Enterobacter cloacae*, Gram positive cocci	Rods/Cocci	Ulcer surface
15	Yes	Yes	No	Yes	Yes	No	Yes	No	No	Infected	*S. aureus, Pseudomonas aeruginosa*	Rods	Ulcer surface
16	Yes	Yes	Yes	Yes	Yes	No	Yes	No	No	Infected	*S. aureus*, gram negative rods	Rods/Cocci	Stratum corneum (cocci), Dermis, Subcutis (rods)
17	No	Yes	No	Yes	Yes	No	No	No	Yes	Infected	Gram negative rods	Rods	Dermis, subcutis
18	Yes	Yes	No	Yes	Yes	No	Yes	No	No	Infected	*Pseudomonas aeruginosa*	Rods/Cocci	Stratum corneum
19	No	Yes	No	Yes	Yes	No	No	No	Yes	Infected	Mixed growth	Rods/Cocci	Stratum corneum
20	Yes	Yes	No	Yes	Yes	Yes	No	No	No	Contaminated	Gram negative rods	None	
21	No	Yes	No	Yes	Yes	No	Yes	No	No	Contaminated	Candida sp, *Klebsiella*	None	

											pneumonia		
22	No	Yes	No	Yes	Yes	No	No	No	No	Contaminated	*Pseudomonas aeruginosa*	n/d	
23	No	Yes	No	Yes	No	No	Yes	No	No	Contaminated	Coagulase negative Staphylococcus species	n/d	
24	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Infected	*Acinetobacter sp.*	Rods/Cocci	Subcutis
25	Yes	Yes	No	Yes	Yes	No	Yes	No	No	Infected	*S. aureus*	Rods	Subcutis
26	No	Yes	No	Yes	No	No	No	No	Yes	Infected	*Pseudomonas aeruginosa*	None	
27	No	Yes	No	Yes	No	Yes	No	Yes	No	Contaminated	*Providencia stuartii*	None	
28	No	Yes	No	Yes	No	No	No	No	No	Contaminated	*Enterobacter cloacae*	n/d	

We compared the clinical presentation to microbiological categorization based on quantification and histological findings. Lesions with a bacterial load less than 10^6 CFU/ml (or CFU/g) were categorized as contaminated, while lesions with bacterial loads above were considered as infected.

[1]WHD = Wound healing delay.

[2]SGF = Skin grafting failure. n/d =

not done.

Table 3: Antibiotic susceptibility pattern of different bacterial species isolated from BU wounds.

Pathogen	Drug Tested	Number Tested	Susceptible, n(%)	Int. Resistant, n(%)	Resistant n(%)
Pseudomonas aeruginosa	Gentamicin	22	18(81.9)	1(4.5)	3(13.6)
	Ceftriaxone	13	3(23.1)	7(53.8)	3(23.1)
	Cefotaxime	20	1(5.0)	1(5.0)	18(90)
	Ampicillin	22	0(0)	0(0)	22(100)
	Tetracycline	22	3(13.6)	1(4.5)	18(81.9)
	Cotrimoxazole	22	3(13.6)	2(0)	17(77.3)
	Cefuroxime	22	0(0)	0(0)	22(100)
	Chloramphenicol	21	2(9.5)	2(9.5)	17(81)
Staphylococcus aureus	Tetracycline	18	12(66.7)	0(0)	6(33.3)
	Cotrimoxazole	18	16(88.9)	0(0)	2(11.1)
	Erythromycin	18	9(50)	9(50)	0(0)
	Ampicillin	18	2(11.1)	1(5.6)	15(83.3)
	Flucloxacillin	18	3(16.7)	0(0)	15(83.3)
	Cefuroxime	18	9(50)	1(5.6)	8(44.4)
	Gentamicin	18	15(83.3)	0(0)	3(16.7)
	Methicillin	18	12(66.7)	0(0)	6(33.3)
	Vancomycin	18	15(83.3)	0(0)	3(16.7)
	Penicillin	18	0(0)	0(0)	18(100)
Other gram positive	Tetracycline	13	6(46.2)	0(0)	7(53.8)
	Cotrimoxazole	13	7(53.8)	0(0)	6(46.2)
	Erythromycin	13	6(46.2)	3(23.1)	4(30.7)
	Ampicillin	13	3(23.1)	1(7.7)	9(69.2)
	Flucloxacillin	13	2(15.4)	0(0)	11(84.6)
	Cefuroxime	13	6(46.2)	0(0)	7(53.8)
	Gentamicin	13	11(84.6)	0(0)	2(15.3)
	Penicillin	13	2(15.4)	0(0)	11(84.6)
Other gram negatives	Gentamicin	45	37(82.2)	1(2.2)	7(15.6)
	Ceftriaxone	17	10(58.8)	2(11.8)	5(29.4)
	Cefotaxime	39	16(41.0)	4(10.3)	19(48.7)
	Ampicillin	45	0(0)	1(2.2)	44(97.8)
	Tetracycline	45	1(2.3)	0(0)	44(97.8)
	Cotrimoxazole	45	7(15.6)	0(0)	38(84.4)
	Cefuroxime	45	5(11.1)	10(22.2)	30(66.7)
	Chloramphenicol	39	6(15.4)	2(5.1)	31(79.5)

Table 4: Microbiological analysis of recycled bandages

CASE	BACTERIAL LOAD (CFU/g)	ORGANISM ISOLATED	ANTIBIOTIC SUSCEPTIBILITY		
			SENSITIVE	INTERMEDIATE	RESISTANT
CASE 1	9.5610^{7}	*Enterobacter agglomerans*	CTX, TET, AMK, COT, GEN, CHL		AMP, CRX
CASE 2	5.3610^{7}	*Staphylococcus warneri*	COT, CRX, GEN	ERY	PEN, AMP, FLX, TET
CASE 3	5.5610^{4}	Staphylase negative Staphylococcus	TET, COT, CRX, GEN	ERY	PEN, AMP, FLX
CASE 4	1.10610^{6}	N/D			
CASE 5	3.2610^{8}	Staphylase positive Staphylococcus	TET, COT, CRX, GEN		PEN, AMP, FLX, TET
CASE 6	1.22610^{5}	N/D			
CASE 7	1.67610^{6}	*Flavibacterium oryzihabitans*	TET, AMK, GEN	CRX, CTX	AMP, COT, CHL
CASE 8	8.6610^{5}	Staphylase negative Staphylococcus	GEN		PEN, AMP, FLX, ERY, TET, COT, CRX
		Pseudomonas sp	CTX, TET, COT, CHL		AMP, CRX, AMK,GEN
CASE 9	4.1610^{3}	Staphylase negative Staphylococcus	TET, COT, CRX, GEN		PEN, AMP, FLX, ERY
CASE 10	3.3610^{5}	Staphylase negative Staphylococcus	GEN	CRX	PEN, AMP, FLX, ERY, TET, COT
CASE 11	3.1610^{3}	Staphylase negative Staphylococcus	TET, GEN	CRX	PEN, AMP, FLX, ERY, COT
CASE 12	6.3610^{4}	Staphylase negative Staphylococcus	TET, COT, CRX, GEN	ERY	PEN, AMP, FLX
CASE 13	1.65610^{5}	*Enterobacter cloacae*	CTX, TET, COT, GEN, CHL	AMK	AMP, CRX
CASE 14A	4.4610^{4}	Staphylase positive Staphylococcus	TET, GEN	CRX	PEN, AMP, FLX, ERY, COT
CASE 14B	5.3610^{3}	Staphylase negative Staphylococcus	GEN		PEN, AMP, FLX, ERY, TET, COT, CRX
CASE 15A	NEGLIGIBLE	Staphylase negative Staphylococcus	GEN	ERY, CRX	PEN, AMP, FLX, TET, COT
CASE 15B	NEGLIGIBLE	N/D			

AMP = Ampicillin, CXM = Cefixime, CXC = Cloxacillin, COT = Cotrimoxazole, ERY = Erythromycin, GEN = Gentamicin, TET = Tetracycline, PEN = Penicillin, CRX = Cefuroxime, CHL = Chloramphenicol, CTR = Ceftriaxone, CTX = Cefotaxime.

Supplementary materials

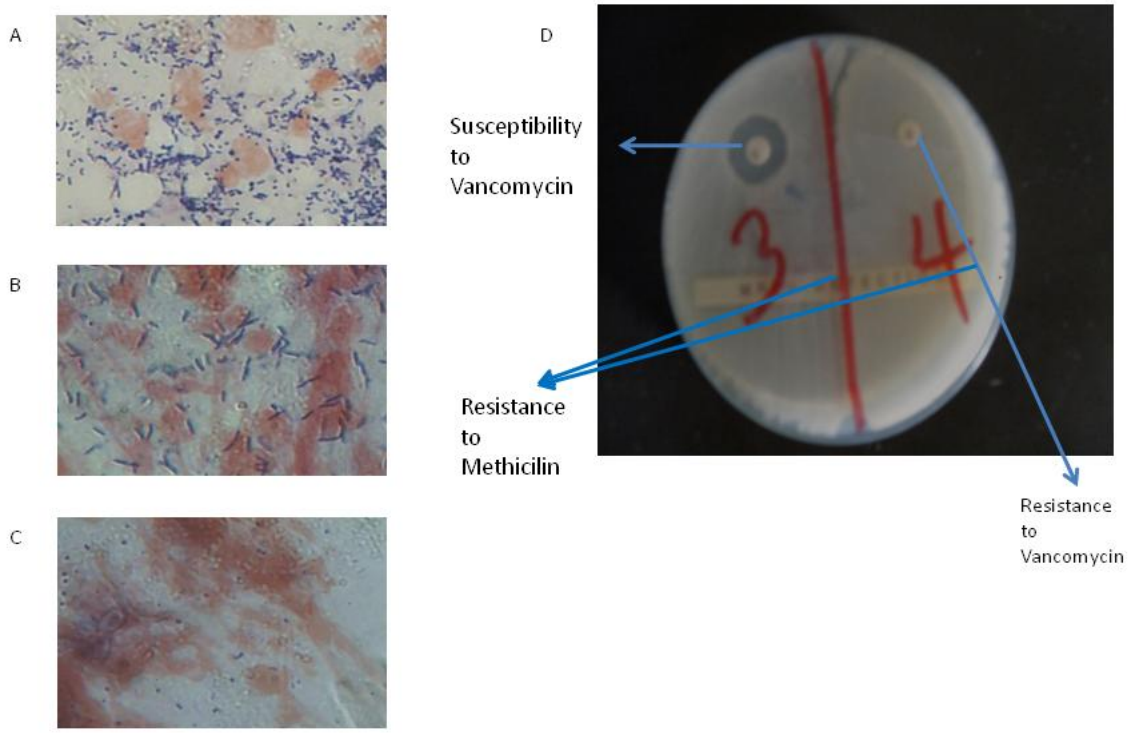

Figure S1: Direct smear examination of infected wounds and Kirby-Bauer plate of a VRSA isolate.

Exudates from infected wounds were smeared directly over clean microscopic slides. The slides were then stained by the Gram procedure and viewed under oil immersion. While the exudate on Plate A is derived from the lesion of the patient whose biopsy was analyzed by histopathology before SR8 (Figure 1), the smears on plate B and C were taken from cases after SR8 treatment. Plate D depicts the drug susceptibility result of two *S. aureus* isolates. While one strain is both methicillin and vancomycin resistant, the other is methicillin resistant, but vancomycin susceptible.

Table S1: Samples taken and types of analysis conducted at various stages of treatment

Types of samples and analysis performed	**Before treatment**	**During treatment**	**After treatment**
Swab samples used for microbiology	52	20	12
Biopsy samples used both for histopathology and microbiology	1	0	19
Biopsy samples used only for histopathology	0	0	1

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Chapter 2

Longitudinal assessment of the bacterial burden of Buruli ulcer wounds during treatment

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Abstract

Introduction: Bacterial contamination is common to all wounds. The bacterial burden of wounds has been found to have an inverse relationship with chronic wound healing. In seeking to develop a better understanding of the evolution of Buruli ulcer (BU) wounds, we performed a longitudinal study to quantify the bacterial burden of BU wounds during the course of Streptomycin/Rifampicin (SR) treatment.

Materials and Methods: Twenty-one IS*2404* PCR confirmed patients were longitudinally followed during the course of their treatment. Swab or tissue samples obtained from the lesions were quantitatively analysed to determine the bacterial burden pre, during and post SR treatment. Also the species of bacterial isolates obtained at these time-points were identified.

Results: Based on the determination of the bacterial burden18/22 (81.8%) pre-treatment, 15/25 (57.7%) during treatment and 36/48 (75.0%) post-treatment samples were classified as super-infected, respectively. Thirty distinct bacterial species including two species of anaerobic Clostridia (*Clostridium perfringens and Clostridium sporogenes*) were identified among 114 isolates. While *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Chryseomonas luteola* dominated pre-treatment, *P. aeruginosa* dominated during and post treatment.

Conclusion: Most BU patients presented with lesions with a high bacterial load which increased significantly post treatment. Therefore good wound care is necessary to control the microbial burden of BU wounds especially post treatment to minimise complications.

Introduction

Buruli ulcer (BU), a chronic debilitating disease caused by *Mycobacterium ulcerans* (Mu) affects mainly the soft tissues of the skin. If non-ulcerative stages of the disease (nodules, papules, oedema or plaques) are not treated, extensive tissue destruction by the macrolide toxin mycolactone can lead to large rugged ulcers, which are the hallmark of BU [1]. The first line treatment for BU; daily administration of oral rifampicin (10 mg/kg) and intramuscular streptomycin (15 mg/kg) for 8 weeks (SR8) was introduced by the World Health Organisation (WHO) in 2004 [2-3]. Early stages, especially nodules and papules, heal in most cases shortly after completion of the SR8 treatment without the need for adjunct surgical interventions. Large ulcerative lesions, however can take as long as a year or more to heal and bacterial superinfection may occur if wound care is not optimal.

Localized wound infection has been identified as a significant cause of impaired healing and wound chronicity [4]. Wound infection occurs when the growth of microorganisms within the wound is uncontrolled by host defence mechanisms and can lead to deeper and more severe pathology and sepsis. Through biofilm formation, the pathogenic effects of bacteria may be increased. Endotoxin release by Gram-negative bacteria in wounds leads to elevated levels of proinflammatory cytokines (IL-1 and tumour necrosis factor). Additionally, factors including the release of free radicals, degradation of growth factors, production of metabolic products, consumption of local oxygen and interference with collagen formation may result in a non-conducive wound environment. With high bacterial loads, the effects of these mechanisms will be increased, thereby leading to impaired wound healing.

Diagnosing wound infection is very challenging and optimally requires clinical signs and symptoms as well as quantitative and qualitative microbiological investigations involving direct microscopy and cultures. Bacterial loads above 10^5cfu/g are the accepted gold standard in diagnosing localised infection [5-7]. The quantity of bacteria in wounds has been found to have an inverse relationship with the healing of chronic wounds [5, 8-10] with studies showing that wound healing progresses only when bacterial counts are below 10^6cfu/ml [11-13].

In a previous cross sectional study, we identified wound infection as a probable cause of healing delay [14]. The study reported a good correlation between clinically suspected infected lesions and results of microbiological cultures. In seeking to have a better understanding of the evolution of the wounds we conducted a longitudinal study to quantify the bacterial burden of BU wounds during the course of SR8 treatment.

Methodology

Ethics Statement

Ethical clearance was obtained from the Institutional Review Board of the Noguchi Memorial Institute (Federal-wide Assurance number FWA00001824). Informed consent was obtained from the patients at their first hospital attendance after the objectives of the project had been explained to them in a language they understand. Sampling and confirmation of BU followed the approved WHO recommendations [15].

Study participants and clinical presentation of lesions

The study was conducted at the Ga West Municipal Hospital (GWMH) in the Greater Accra Region of Ghana; one of the main BU treatment facilities in the country. Participants were recruited into the study after the clinical BU diagnosis had been confirmed by at least IS*2404* PCR before commencement of SR8 treatment. Twenty-one (21) patients were recruited into the study out of which 17 were in-patients while four were outpatients who took the treatment at other health centers but reported at the GWMH periodically for review. The male to female ratio was 8:13. Their ages ranged from 5 to 69 years with a mean of 35 ± 18, and their weights ranged from 18-115kg. At their first hospital attendance, 14 patients presented with single lesions and seven presented with multiple lesions out of which three presented with an oedema and an ulcer, two presented with two ulcers and two others presented with a nodule, plaque and ulcer. The lesions of two were classified as category I lesions (size of <5 cm at the widest diameter), four as category II lesions (size between 5 and 15 cm at the widest diameter) and fifteen as category III lesions (size >15 cm at the widest diameter or multiple lesions). Nineteen of the lesions were found on the lower limbs and two on the upper limbs. Of the lesions on the lower limbs, 12 were found on the leg, four on the ankle and three on the foot; whilst on the upper limb, one each was found on the arm and the elbow. Three patients had other co-morbidities; HIV, hypertension and diabetes mellitus. Out of the 21 cases, 17 took the SR treatment for 8 weeks and three took the treatment for 12 weeks because the treating clinician suspected the lesions to still be active after 8 weeks of treatment. One patient who had previously had BU and had undergone the 8 weeks of treatment a year before was prescribed a four-week treatment course after a new lesion

appeared at another location. During the course of treatment, one patient, who was positive for HIV, developed disseminated lesions on other parts of the lower limb.

Clinical assessment

The wounds were clinically accessed for signs of infection using a wound assessment chart. The clinical features assessed were the appearance of the wound (necrotic, granulation, epithelialisation, and slough), the wound exudate level (low, high, medium, serous, serosanguineous, purulent, malodour) and the appearance of the surrounding skin (macerated, oedematous, erythematous, indurated, dry scaling, healthy/intact). Category II and III lesions were assessed at two week intervals whilst category I lesions were assessed at weekly intervals.

Sample collection

Samples were taken from the lesions of patients for microbiological analysis to determine the bacterial burden and were also cultured to isolate and identify infecting bacteria, as previously described [14]. During the course of SR treatment, the bacterial burden of lesions was investigated bi-weekly. Post SR treatment, wound cultures were done upon advice from the responsible clinician when the lesion was suspected to be infected. Microbiological assessments were also made when patients underwent excision and debridement. A total of ninety-six (96) samples were collected from the patients. These included 85 swabs and 11 tissue samples. Swab samples were collected by the Levine method [16] pre, during and post treatment from the undermined edges of lesions whilst tissue samples were collected post treatment after patients had undergone surgical procedures. Two swabs were taken from each lesion; one was placed in 5ml Phosphate buffered saline (PBS) for enumeration of the bacterial burden and isolation of aerobic infecting bacteria and the second was inserted into Robertson's Cooked meat media for the isolation of anaerobic bacteria. Two tissue samples were taken from patients and one was aseptically transferred into Robertson's Cooked meat media while the second was transferred into sterile transport containers. The samples were transported cold from the hospital to the laboratory at the Noguchi Memorial Institute for Medical Research and processed there.

Quantitative and qualitative cultures

Quantitative cultures were carried out by the pour plate method, as previously described [14]. Values obtained from the enumeration of bacterial colonies were computed as colony forming units per ml (cfu/ml) for swabs and colony forming units per gram (cfu/g) for tissues. Wounds were classified as infected, if bacterial counts of $>10^6$cfu/g or ml were obtained, contaminated if bacterial counts were $<10^6$cfu/g or ml and clean if no bacterial colonies grew on the agar plates. Comparison between bacterial loads was done by *t* tests and one-way ANOVA calculations in SPSS v 24.0 [17]

For the isolation of infecting bacterial species, 10^{-1} dilutions of the sample suspensions were pelleted and the sediments cultured on Blood, MacConkey and Mannitol Salt agars (Oxoid Ltd, Basingstoke, UK). Biochemical oxidase, catalase and coagulase tests were performed to presumptively identify bacteria species. Further characterization of Gram-negative rods was done using Analytical profile index (API20E) strips (bio-Merieux SA, Marcy-l'E'toile, France). The Staphylase Kit, Prolex latex agglutination system (Pro-Lab Diagnostics) was used to differentiate the catalase positive Gram positive bacteria, *Staphylococcus aureus* from other Staphylococcus species.

The Robertson's cooked meat medium containing the sample was incubated anaerobically in a glass jar with an anaerobic gas pack at 37°C for 48-73 hours. Tubes with signs of growth were subcultured on blood agar and incubated anaerobically with a metronidazole antibiotic disc to check for resistance. The Gram reaction of positive cultures was determined and identification of isolated microorganisms by MALDI-TOF mass spectrometry was outsourced to Mabritec AG, Riehen, Switzerland.

Results

Clinical assessment of wounds

Pre treatment, the majority of the lesions had clinical indications of infection, seemed to be in the proliferative stage of wound healing by having granulation tissue and epithelialisation, had undermined edges, and high wound exudates (Table 1). Changes in clinical presentation during treatment are shown in Table 1. Post treatment, all 15 assessed lesions presented with some granulation tissue and slough, 4/15 (26.6%) had necrotic tissues, 3/15 (20%) presented with epithelialisation whilst the edges of 5/15 (33.3%) were still undermined (Table 1).

Bacterial burden and assessment of infection

The bacterial burden of 22 lesions from 20 patients was determined pre SR. The bacterial load ranged from 0 to 3.00 x 10^9cfu/ml, with a mean of 2.79 x 10^9cfu/ml and a median of 8.05 x 10^6cfu/ml. Two (9.1%), two (9.1%) and eighteen (81.8%) samples were classified microbiologically as clean, contaminated and infected, respectively (Figure 1).

During treatment, 26 samples were collected from the lesions of 20 patients (four patients were sampled twice and two patients had two lesions each). The bacterial load of the samples ranged from 0 to 2.06 x 10^9cfu/ml, with a mean of 1.5 x 10^8cfu/ml and a median of 1.13 x 10^6cfu/ml. Two (7.7%) samples were clean, eight (30.8%) were contaminated and fifteen (57.7%) were infected (Figure 1). The load of one of the samples could not be determined as the culture got contaminated.

Post treatment, 48 samples were collected between weeks 8 and 75 from 25 lesions of 20 patients. Ten lesions were sampled once, nine lesions twice, four lesions thrice and two lesions four times. The bacterial load of the samples ranged from 0 to 3.00 x 10^9 cfu/ml, with a mean of 3.49 x 10^8 cfu and a median of 1.69 x 10^8cfu/ml. One (2.1%), eleven (22.9%), and thirty-six (75.0%) samples were classified as clean, contaminated and infected respectively (Figure 1).

Bacterial loads were obtained for 17 patients (18 lesions) at all three stages. During treatment the bacterial load of 12 lesions decreased and that of six lesions increased compared to the values obtained pre-treatment. Post treatment, the loads of 16 lesions increased from the values obtained during treatment while that of two lesions decreased. Overall, most patients

thus presented with bacterial loads which reduced during treatment but increased dramatically post treatment (Figure 2). The difference between the bacterial loads at all three time points was significant ($p<0.05$). Pairwise comparison of the bacterial loads however showed that a statistically significant difference existed only between the loads during and post treatment.

Isolated bacterial flora

One hundred and fourteen bacterial isolates were recovered from qualitative cultures made up of 28 different species of aerobic bacteria and two species of Clostridia (*Clostridium sporogenes* and *Clostridium perfringens*). At pre-treatment, 14 distinct species were identified among 28 isolates, dominated by *Enterococcus faecalis* (5/28, 17.8%), *Pseudomonas aeruginosa* (4/28, 14.3%) and *Chryseomonas luteola* (4/28, 14.3%) (Table 2, S1 Table). Twelve species were identified among 29 isolates recovered during treatment and the main species was *P. aeruginosa* (12/29, 41.4%). Fifty-seven bacterial isolates were recovered post treatment out of which 20 distinct bacterial species, dominated by *P. aeruginosa* (16/57, 28.1%), were identified (Table 2, S1 Table).

Post SR8 treatment follow-up

After antibiotic treatment, twelve of the patients underwent surgical interventions. Three of them had excisions only, whilst nine patients underwent excision and skin grafting. The grafts of three of the patients failed and two had a second skin grafting. The lesions of thirteen patients got healed with an average healing time of 27-28 weeks post treatment. The rest of the patients were still dressing their wounds at the time of preparation of this manuscript and the time of dressing ranged at that time between 4 weeks to 75 weeks post treatment.

Discussion

We investigated the bacterial burden of PCR confirmed BU wounds to gain an understanding into the evolution of microbial flora in BU wounds during treatment, which could have implications on the healing process. Our findings show that bacterial loads and bacterial diversity are high prior to S/R treatment, decrease during treatment and increase dramatically post treatment. About thirty distinct bacterial species were found colonising BU wounds with gram-negative rods dominating.

We previously established that BU wounds could be infected by bacterial pathogens contrary to formerly held beliefs that they were sterile as a result of the presence of mycolactone [14]. However, it has recently been demonstrated that mycolactone is inactive against the bacterial species *Streptococcus pneumoniae, Neisseria meningitidis, Escherichia coli* as well as the yeast *Saccharomyces cerevisae* and amoeba *Dictyostelium discoideum* [18]. The results obtained from this study confirms our previous finding and findings from others [19-20] on secondary infection of BU wounds.

A decrease in bacterial load during treatment compared to pre and post treatment was observed. This appears to be due to administration of the broad spectrum antibiotics within that time period. A study by Gardener [21] also reported decreased bacterial loads in study subjects who were on systemic antibiotics during the time of study, compared to those not on antibiotics. This result was also in good agreement with observed clinical signs, such as fewer lesions presenting with slough, necrosis and high wound exudate production.

The bacterial species isolated from the BU wounds are representative of the spectrum of bacterial species usually isolated from chronic wounds [22]. Colonisation of most wounds by Gram negative bacteria indicates that they were at an advanced stage of bacterial colonisation, since early colonising bacteria in chronic wounds are mainly Gram positive organisms, notably Staphylococci and Beta-haemolytic Streptococci, which are replaced by Gram negative organisms as the wound microbiota becomes more complex [23]. Many BU patients report late to the formal health centers for treatment [24]. The period between the time of ulceration and the time of first presentation thus provides contaminating bacteria with adequate time to multiply and establish a complex microbiota accounting for the high number of Gram negatives isolated from the lesions.

P. aeruginosa was dominant at all time points. This organism is detected in wounds at its later natural history when a complex microbial flora is being formed [25] and is also frequently cited as a source of wound healing delay and wound infection [9, 26-27]. Two anaerobes were isolated from the lesions of two patients' pre and during treatment. The presence of anaerobic bacteria indicates that the wounds are at a stage of irreversible adhesive colonization, as anaerobes are only known to colonise lesions after a hypoxic state has been created due to increased multiplication of the wound microflora [23]. Bacteria at this stage also develop inherent resistance to antibiotics which could impact on wound healing [23]. Previous work we did at the study health center has shown high levels of antibiotic resistance among bacteria isolated from lesions of hospitalized BU patients, including MRSA and MDR *P. aeruginosa* [14, 28]. Most of the isolated bacteria are known nosocomial pathogens and since BU treatment is associated with long hospital stays [29], BU patients are at high risk for the acquisition of these pathogens in the health centers.

A potential limitation of this study is that it did not formally assess the impact of the bacterial burden on wound healing outcome. However, various studies have shown that an increased bacterial load negatively impacts wound healing and the presence of bacteria in wounds even in the absence of obvious clinical signs can inhibit the normal wound healing process. Therefore BU wound management guidelines should consider the bacterial load and entail strategies for decreasing bacterial load at all time points during the course of the infection.

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Figures

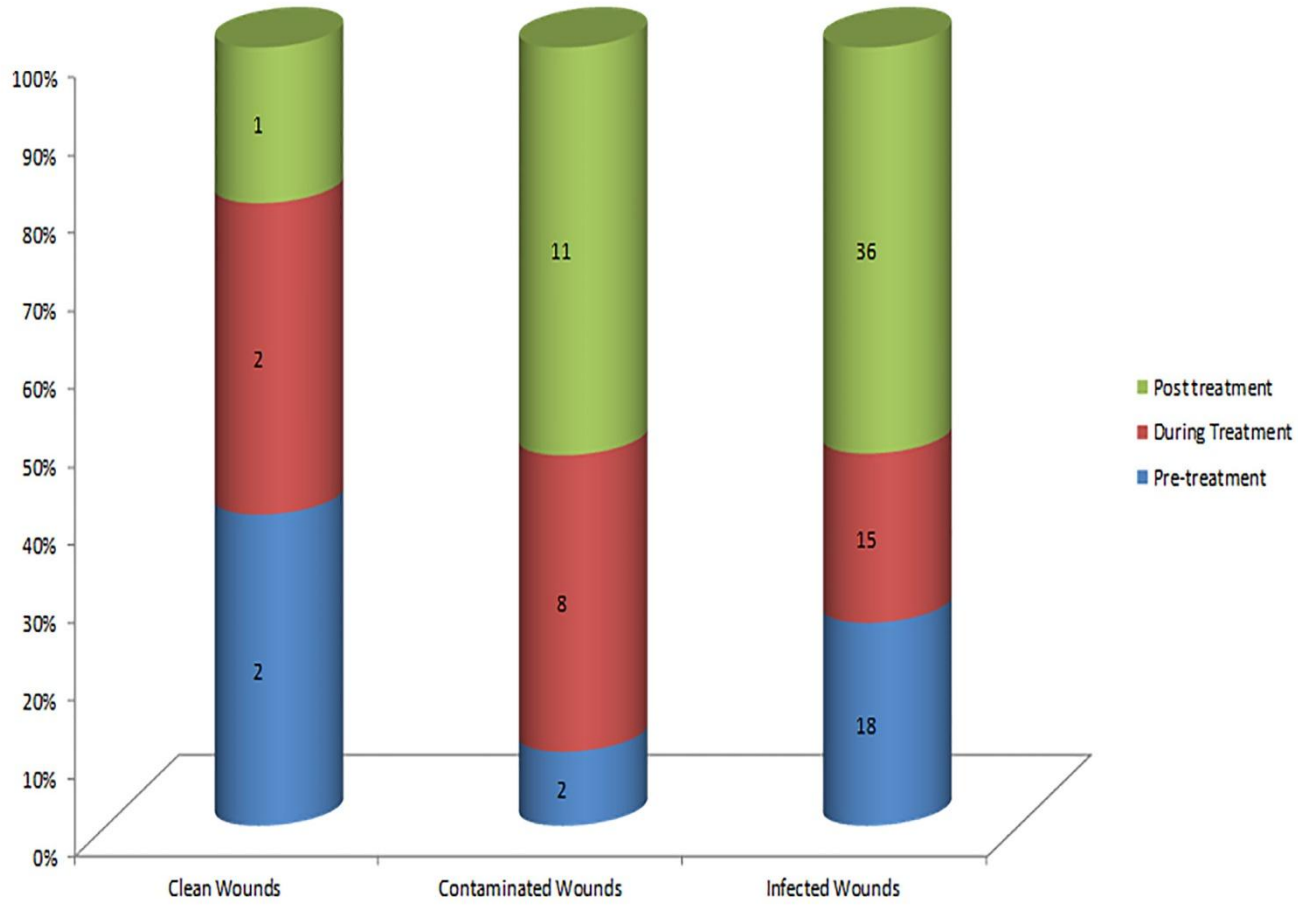

Figure 1: Bacterial loads at different time points

Bacterial loads obtained at different stages of treatment classified as clean, contaminated and infeccted. Blue coloured: loads obtained pre-treatment, red coloured: loads obtained during treatment, green coloured: loads obtained post treatment.

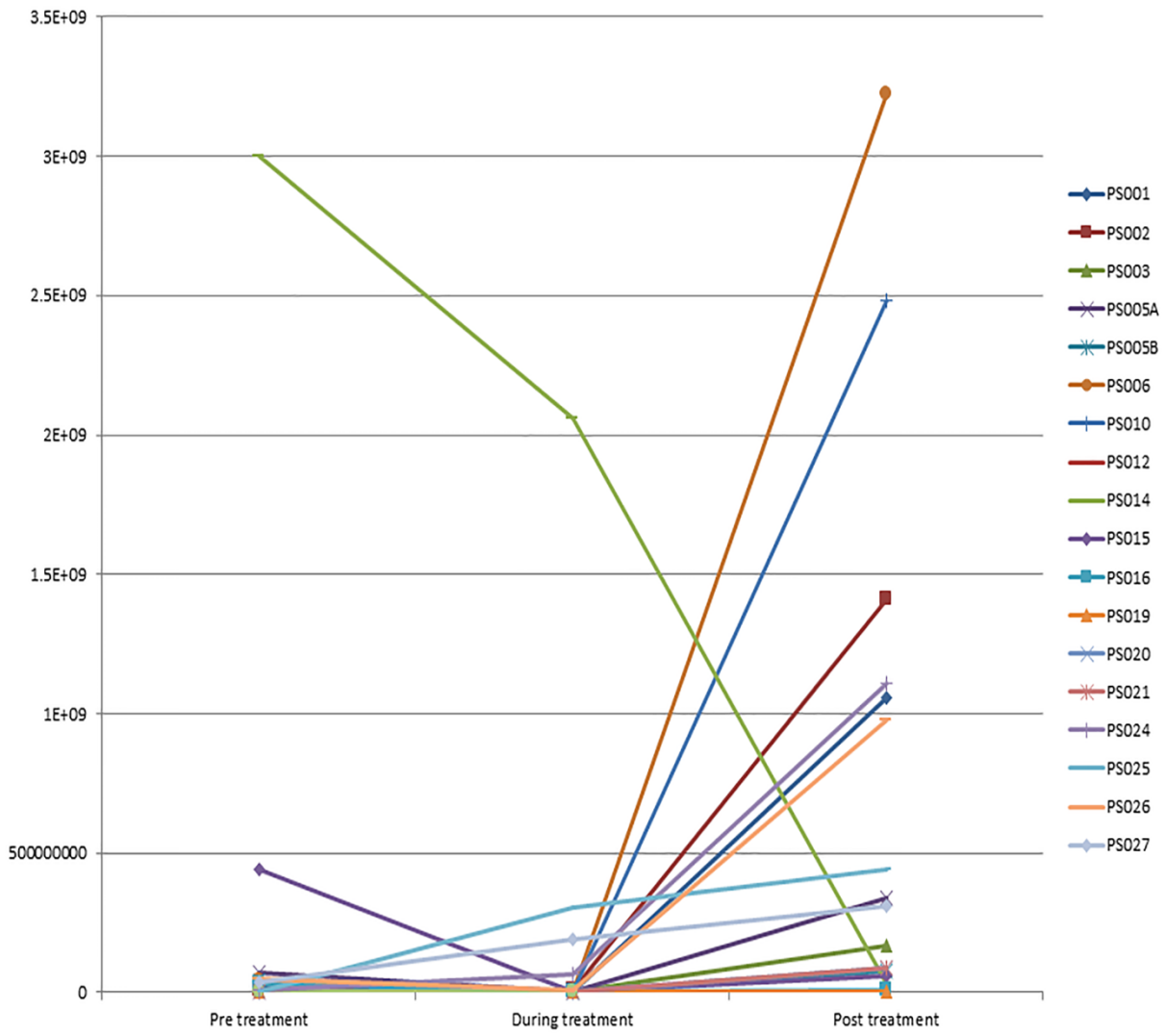

Figure 2: Bacterial loads of individual patients

Bacterial loads of 17 patients at all three stages showing the evolution of the bacterial burden.

Tables

Table 1- Clinical presentations of wounds

Description / Presentations	Week 0	Week 2	Week 4	Week 8	Post treatment
Number of patients assessed	**21**	**4**	**19**	**19**	**15**
Tissue type on wound bed					
Granulation tissue	11	3	15	14	15
Slough	14	2	16	12	15
Necrotic tissue	2	1	3	1	4
Epithelialisation	2	1	3	5	3
Undermining	13	2	5	6	5
Wound exudates and type					
Low	None	None	4	8	None
High	16	1	3	5	12
Medium	3	2	10	3	3
Serous	10	1	14	10	7
Serosanguineous	3	1	1	1	3
sanguineous	None	None	None	None	None
Purulent (green/brown)	4	1	2	4	7
malodor	16	1	7	5	9
Surrounding skin					
Macerated	None	None	3	2	5
Oedematous	11	1	4	5	7
Erythema	None	None	None	None	None
Indurated	5	1	5	2	3
Dry and scaling	3	1	5	2	5
Healthy /intact	1	1	6	7	2

Table 2: Bacterial species isolated

Time Point	Bacteria isolated	Number isolated n (%)
Pre-treatment	*Pseudomonas aeruginosa*	4 (14.3)
	Staphylococcus aureus	3 (10.7)
	Chryseomonas lutoela	4 (14.3)
	Proteus mirabilis	2 (7.1)
	Enterobacter cloacae	2 (7.1)
	Aeromonas sobria	1 (3.6)
	Escherichia coli	1 (3.6)
	Flavibacterium oryzihabitans	1 (3.6)
	Micrococcus luteus	1 (3.6)
	Acinetobacter baumanii	1 (3.6)
	Enterococcus faecalis	5 (17.8)
	Morganella morganii	1 (3.6)
	Enterobacter sakazakii	1 (3.6)
	Clostridium sporogenes	1 (3.6)
	Total bacterial isolates	28 (100)
During treatment	*Pseudomonas aeruginosa*	12 (41.4)
	Proteus mirabilis	4 (13.8)
	Chryseomonas luteola	2 (6.9)
	Coagulase Negative Staphylococcus	2 (6.9)
	Enterobacter cloacae	1 (9.1)
	Pseudomonas fluorescence	1 (3.4)
	Citrobacter diversus	1(3.4)
	Serratia marcescens	1 (3.4)
	Enterobacter sp	1 (3.4)
	Enterococcus faecalis	1 (3.4)
	Providencia stuartii	1 (3.4)
	Streptococcus anginosus_milleri_group	1 (3.4)
	Clostridium perfringens	1 (3.4)
	Total bacterial isolates	29 (100)
Post treatment	*Pseudomonas aeruginosa*	16 (28.1)
	Enterobacter cloacae	6 (10.5)
	Staphylococcus aureus	5 (8.8)
	Pseudomonas fluorescence	4 (7.0)
	Proteus mirabilis	5 (8.8)
	Chryseomonas luteola	3 (5.3)
	Aeromonas sobria	2 (3.5)
	Enterobacter sakazakii	2 (3.5)
	Leclercia adecarboxylata	2 (3.5)
	Citrobacter fruendii	1 (1.8)
	Flavimonas oryzihabitans	1 (1.8)

Tatumella ptyseos	1 (1.8)
Klebsiella pneumoniae	1 (1.8)
Enterobacter sp	1 (1.8)
Coagulase Negative Staphylococcus	1 (1.8)
Staphylococcus epidermidis	1 (1.8)
Alcaligenes faecalis	1 (1.8)
Serratia marcescens	1 (1.8)
Aeromonas salmonicida	1 (1.8)
Enterococcus faecalis	1 (1.8)
Providencia rettgeri	1 (1.8)
Total bacterial isolates	57 (100)

Supplementary Material

S1 Table: Bacteria isolated from individual patients at different time points

Patient ID	Time of sample collection	Sample type	Organism (s) isolated
PS001	week 0	swab	*Enterobacter cloacae, Morganella morganii*
	week 4	swab	*Pseudomonas aeruginosa, Providencia stuartii*
	week 8	swab	*Enterobacter sakazakii, Enterococcus faecalis*
	9 weeks post SR8	tissue	*Pseudomonas fluorescence, Pseudomonas aeruginosa, Alcaligens faecalis, Enterobacter cloacae*
PS002	week 0	swab	*Flavibacterium oryzihabitans, Enterococcus faecalis*
	week 4	swab	*Pseudomonas aeruginosa, Clostridium perfringens*
	week 8	swab	*Chryseomonas luteola*
	4 weeks post SR8	tissue	*Chryseomonas luteola, Proteus mirabilis*
	59 weeks post SR8	swab	*Enterobacter cloacae*
	75 weeks post SR8	swab	*Pseudomonas aeruginosa, Tatumella ptyseos, Enterobacter cloacae, Pseudomonas fluorescence*
PS003	week 0	swab	*Pseudomonas aeruginosa, Clostridium sporogenes*
	week 4	swab	*Pseudomonas aeruginosa, Serratia marcescens*
	week 8	swab	*Aeromonas sobria, Serratia marcescens*
PS005A	week 0	swab	*Enterobacter sakazakii, Enterococcus faecalis*
	week 4	swab	*Proteus mirabilis*
	week 8	swab	*Pseudomonas aeruginosa, Providencia rettgeri*
PS005B	week 0	swab	*Proteus mirabilis*
	week 4	swab	*Pseudomonas aeruginosa, Proteus mirabilis*
	week 8	swab	*Pseudomonas aeruginosa*
PS006	week 0	swab	*E. coli*
	week 4	swab	*Chryseomonas luteola*
	week 8	swab	*Aeromonas sobria, Proteus mirabilis, Leclercia adecarboxylata*
	11 weeks post SR8	tissue	*Enterobacter sakazakii, Leclercia adecarboxylata*

PS010	week 0	swab	*Pseudomonas aeruginosa, Enterococcus faecalis*
	week 4	swab	*Enterobacter sp, Enterococcus faecalis*
	week 8	swab	*Proteus mirabilis, Chryseomonas luteola*
	53 weeks post SR12	swab	*Flavimonas oryzihabitans*
PS012	week 0	swab	*Micrococcus luteus*
	week 4	swab	*Enterobacter cloacae*
	week 8	swab	*Pseudomonas aeruginosa*
PS014	week 0	swab	*Chryseomonas luteola, Enterococcus faecalis*
	week 4	swab	*Pseudomonas fluorescence, Streptococcus anginosus_milleri_group*
	week 8	swab	*Pseudomonas fluorescence, Enterobacter cloacae, Staphylococcus epidermidis*
	27 weeks post SR8, SGF	swab	Gram positive rods
	60 weeks post SR8	swab	*Pseudomonas aeruginosa*
PS015	week 0	swab	*Pseudomonas aeruginosa, Enterococcus faecalis*
	week 4	swab	*Pseudomonas aeruginosa*
	week 8	swab	*Pseudomonas aeruginosa, Proteus mirabilis*
	29 weeks post SR8, SGF	swab	*Pseudomonas fluorescence*
PS016	week 0	swab	*Staphylococcus aureus, Acinetobacter baumanii, Enterobacter cloacae*
	week 4	swab	*Pseudomonas aeruginosa*
	week 8	swab	*Pseudomonas aeruginosa*
	18 weeks post SR8	tissue	No growth
	4 weeks post SR8	tissue	*Pseudomonas aeruginosa*
PS017A	week 0	swab	*Chryseomonas luteola*
	post SR8	swab	*Staphylococcus aureus*
	post SR8	swab	*Enterobacter cloacae*
PS017B	week 0	swab	*Chryseomonas luteola*
	post SR8	swab	*Staph aureus*
	post SR8	swab	*Aeromonas salmonicida*
PS019	week 0	swab	Yeast
	week 4	swab	Yeast
	week 8	swab	Gram negative rods (unidentified)

PS020	week 0	swab	*Chryseomonas luteola*
	week 4	swab	*Chryseomonas luteola*
	week 8	swab	Gram negative rods (unidentified)
PS021	week 0	swab	Gram negative rods (unidentified)
	week 2	swab	*Pseudomonas aeruginosa*
	week 4	swab	*Proteus mirabilis*
	week 8	swab	Gram positive rods
	post SR8 lesion B	tissue	*Enterobacter cloacae*
	post SR8 lesion A	swab	*Pseudomonas aeruginosa*
	post SR8 lesion B	swab	*Pseudomonas aeruginosa*
	post SR8 lesion C	swab	Gram positive rods
	post SR8 lesion A	swab	*Staph aureus*
	post SR8 lesion B	swab	*Enterobacter sp*
	post SR8 lesion C	swab	*Staph aureus*
	post SR8 lesion D	swab	*Staph aureus*
PS023	week 0	swab	No growth
	week 6	swab	*Coagulase negative Staphylococci*
PS024	week 0	swab	*Staphylococcus aureus*
	week 4	sswab	*Citrobacter diversus*
	week 8	swab	*Pseudomonas aeruginosa*
	post SR8	tissue	*Citrobacter fruendii*
	post SR8	swab	*Pseudomonas aeruginosa*
	post SR8	swab	*Pseudomonas aeruginosa*
PS025	week 0	swab	*Aeromonas sobria*
	week 2	swab	Gram positive rods
	week 4	swab	Gram positive rods
	week 8	swab	*Coagulase negative Staphylococci*
PS026	week 0	swab	*Proteus mirabilis*
	week 2	swab	*Pseudomonas aeruginosa*
	week 4	swab	*Proteus mirabilis, Coagulase Negative Staphylococcus*

	week 8	swab	*Proteus mirabilis*
	post SR8	tissue	*Klebsiella pneumoniae*
PS027	week 0	swab	*Pseudomonas aeruginosa*
	week 2	swab	*Pseudomonas aeruginosa*
	week 4	swab	*Pseudomonas aeruginosa*
	week 8	tissue	*Pseudomonas aeruginosa*
	post SR8	swab	*Pseudomonas aeruginosa*
PS028	week 4	swab	*Pseudomonas aeruginosa*
	week 6	swab	*Pseudomonas aeruginosa*
	week 8	swab	Gram positive rods
PS029	week 0	swab	*Staphylococcus aureus*
	week 4	swab	No growth

SGF: skin graft failure

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Chapter 3

Challenges Associated with Management of Buruli Ulcer/Human Immunodeficiency Virus Co-infection in a Treatment Center in Ghana: A Case Series Study

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Abstract

The synergy between *Mycobacterium tuberculosis* infection and human immunodeficiency virus (HIV)/ acquired immunodeficiency syndrome is well established but not so in Buruli ulcer (BU). We screened confirmed BU cases for HIV infection and followed seven BU/HIV-coinfected patients. Management of BU/HIV was based on the World Health Organization guidelines and patient condition. The HIV positivity among BU patients (8.2%; 11/134) was higher compared with that of general patients attending the facility (4.8%; 718/14,863; P = 0.07) and that of pregnant women alone (2.5%; 279/11,125; P = 0.001). All seven BU/HIV-coinfected cases enrolled in the study presented with very large (category III) lesions with four having multiple lesions compared with 54.5% of category III lesions among HIV-negative BU patients. During the recommended BU treatment with streptomycin and rifampicin (SR) all patients developed immune infiltrates including CD4 T cells in their lesions. However, one patient who received antiretroviral therapy (ART) 1 week after beginning SR treatment developed four additional lesions during antibiotic treatment, while two out of the four who did not receive ART died. Further evidence is required to ascertain the most appropriate time to commence ART in relation to SR treatment to minimize paradoxical reactions.

Introduction

Buruli ulcer (BU), a disease caused by *Mycobacterium ulcerans*, leads mainly to the destruction of skin tissues [1]. The disease is rarely fatal, but delayed treatment often results in contracture deformities because of the massive skin destruction by the cytotoxic macrolide, mycolactone [2,3]. The disease presents in two main active clinical forms: non-ulcerative (papule, nodule, plaque, and edema) and ulcerative. Severe forms include osteomyelitis and disseminated (or multifocal) lesions [1,4]. BU lesions are categorized based on World Health Organization (WHO) classification into category I, which consist of lesions with a size of < 5 cm at the widest diameter; category II, which consist of lesions with a size between 5 and 15 cm at the widest diameter; and category III, which consist of lesions with a size > 15 cm at the widest diameter, lesions at critical sites and multiple lesions [1]. The WHO recommended first-line treatment of BU is daily injection with streptomycin (SR) and oral rifampicin for 8 weeks, and if necessary, surgery either to improve healing and/or to correct deformities [1,5,6]. The efficacy of this treatment regimen has been confirmed in several studies [7-9]. Active lesions present with large focal clusters of extracellular acid-fast bacilli (AFB) and only minor leukocyte infiltration [10,11]. Antimicrobial therapy leads to massive leukocyte infiltration, which culminates in the development of ectopic lymphoid structures in the lesions [12]. Some studies have reported paradoxical reactions in BU patients, which is defined as an increase in lesion size of > 100% after initial improvement, and/or the appearance of a new lesion(s) following or during antimycobacterial treatment [13,14]. Currently, it is not clear whether immune reconstitution inflammatory syndrome (IRIS)–like mechanisms, secondary infections [15], or other mechanisms are primarily responsible for impaired wound healing and deterioration of lesions during and after SR treatment in some of the BU patients. A retrospective study conducted in Cameroon revealed that human immunodeficiency virus (HIV) infection may affect the clinical presentation and severity of BU disease with a reported increased incidence of multiple, larger, and ulcerated BU lesions [16-18]. Data available on the absorption of antituberculosis medications in tuberculosis (TB)/HIV coinfection compared with HIV-negative patients showed conflicting results [19,20]. Therefore, whether antimycobacterial combination treatment is less efficacious in persons with HIV infection is unknown and needs to be systematically studied.

The associations between HIV infection and some other infectious diseases are clearly defined,

but that between BU and HIV is not fully understood [21]. It is well known that HIV infection is fueling the global TB epidemic and the convergence of the TB and the HIV epidemics pose new public health challenges [22]. The interaction between HIV and TB in coinfected persons is bidirectional and synergistic; on one hand, HIV infection predisposes to the development of active TB, on the other hand the course of HIV-related immunodeficiency is worsened by active TB infection [22,23]. Although it is clearly known that HIV/acquired immunodeficiency syndrome (AIDS), which leads to reduced CD4 helper T-cell activity, is a risk factor for TB, immune protection mechanisms in BU disease are not fully understood [24]. However, like in other mycobacterial diseases, adaptive immune responses championed by $CD4^+$ activation of macrophages are presumably also crucial for protection against BU. Although large clusters of toxin-producing *M. ulcerans* bacteria are found in established BU lesions, there are indications that multiplication of the pathogen in phagocytes plays a role in the early steps of the infection [25,26]. Furthermore, BU disease leads to reduced interferon (IFN)-g release [27].

Management of HIV/AIDS over the years has seen progressive improvement in drug therapy and clearer guidelines, which has dramatically decreased mortality and incidence of AIDS-defining opportunistic infections [28,29]. Despite this breakthrough in management, there is still the challenge of a paradoxical IRIS in HIV patients on antiretroviral therapy (ART), which is a phenomenon that has been defined to be a new type B or C AIDS-defining condition or emergence of a range of mucocutaneous or autoimmune conditions diagnosed within 180 days of starting ART with a corresponding CD4 response [30]. In the presence of a treated or ongoing opportunistic infection such as TB, such paradoxical IRIS could occur in which case it would be defined as 1) a new, worsening or recurrent sign or symptom consistent with an exaggerated or atypical inflammatory reaction to the previously diagnosed opportunistic infection, 2) the exclusion of medication toxicity or other disease processes as the cause of the abnormal event, and 3) a supportive evidence be it by some specialized imaging or histopathology [31]. The combined effect of the IRIS associated with HIV patients on ART [30] and the paradoxical reaction experienced in BU patients on antimicrobial treatment [13,14] could possibly pose a challenge to the management of BU/HIV coinfection.

Anemia is common among sub-Saharan Africans with BU [32]. Susceptibility to BU is associated with polymorphism in the gene for the iron transporter protein NRAMP1 [33]. Models exist to explain iron deficiency anemia in mycobacterial diseases such as BU. Notable among them suggests sequestration of Fe^{2+} from the body into phagosomes and the lack of NRAMP1 to

export the iron back, as the possible cause of the anemia [34], which could be worsened with an HIV coinfection depending on the clinical stage and state of immunity [35,36].

This study compares the prevalence of HIV infection among confirmed BU patients at a district hospital in Ghana with the general population of patients in that same facility and also describes BU/HIV coinfection cases highlighting the challenges associated with the mana gement of BU/HIV coinfection.

Materials and Methods

Ethical statement

Ethical clearance for the study was obtained from the institutional review board of the Noguchi Memorial Institute for Medical Research (NMIMR) (Federal-wide Assurance number FWA00001824). All study participants were well informed of the study objectives and written informed consent was obtained either from the patient or from the guardian of the patient. Sampling and laboratory confirmation for both *M. ulcerans* and HIV infection followed the national approved procedure. All confirmed cases were referred for appropriate treatment of BU and HIV/AIDS.

Study participants

The participants involved in the study were passively recruited from the Ga West Municipal Hospital in Amasaman, Ghana, from October 2009 to March 2013; one clinician at the health facility who made the final clinical diagnostic decision reviewed all patients in this study. A participant was included in this study if he/she met the WHO clinical definition for the different BU lesions and was positive for at least IS*2404* polymerase chain reaction (PCR) as previously described [37]. Basic demographic data and clinical history of cases were recorded by adapting the BU01 form of the WHO. Lesions were categorized according to the WHO classification as previously described [1]. In addition lesions were classified either as ulcer, edema, nodule, or papule as well as single or multiple lesions [2]. Patients were screened for HIV infection by collecting blood samples after counseling and consent has been sought. Two immunochromatographic (lateral flow) strip–based rapid test kits, OraQuick (OraSure,Bethlehem, PA) and/or First Response HIV 1-2.0 Card Test (PMC Medical Pvt. Ltd., Daman, India), were used for initial screening, and samples that tested positive were confirmed by the Inno-Lia HIV I/II immunoblot assay (Immunogenetics, Gent, Belgium) [38].

BU confirmation

Swabs were collected from the undermined edges of ulcerative lesions and fine needle aspirates were collected from cases with non-ulcerative lesions for bacteriological confirmation of BU disease [39,40]. All samples were analyzed by IS*2404* quantitative PCR, microscopy and culture. For those patients presenting with multiple lesions, samples were collected from each lesion. For

patients who underwent surgery, tissue biopsies were taken for further microbiological analyses at NMIMR and histopathology at Swiss Tropical and Public Health Institute.

Treatment and monitoring

The patients were treated with daily SR (15 mg/kg/day intramuscular injection) and rifampicin (10 mg/kg/day orally) according to the WHO protocol for duration of 8 weeks [8]. Patients were questioned about side effects from the antibiotic treatment at each biweekly clinical assessment and asked to report any problems to the health center between periodic reviews. Surgical debridement was done 4 weeks after antibiotic treatment of BU for some of the lesions. Biopsy samples from the wound edges were sent for culture and histopathology. Any septic wound diagnosed clinically was confirmed by culture and sensitivity testing using swab samples and was treated accordingly. Biweekly wound assessment was done involving wound measurement and photography using a wound imaging, measurement, and documentation device, ARANZ Medical Silhouette Mobile™ (ARANZ Medical, Christchurch, New Zealand). Standard moist wound management practices were conducted, which involved saline dressing and covering of the floor of the ulcers with Vaseline gauze [41]. Frequency of dressing change was individualized according to the characteristics of the ulcer. Following initial wound excision after antibiotic treatment, surgical debridement and skin grafting subsequently done was tailored to the need of each patient and on the discretion of the attending surgeon. New lesions occurring during the course of therapy or follow-up period were closely examined and analyzed by culture and in certain cases by histopathology [13,14].

Laboratory and radiological investigations

Baseline complete blood count, liver function test, blood urea electrolytes and creatinine, erythrocyte sedimentation rate, fasting blood sugar and sickling test with/without Hb electrophoresis as indicated were done for all patients. Unless clinically indicated earlier for a patient, complete blood count, liver function test, and blood urea electrolytes and creatinine tests were routinely repeated 8 weeks to ascertain any renal or hepatic toxicity and to assess the hemoglobin levels. Baseline CD4 count was done and repeated at 6-month intervals for only two patients until the wound was completely healed and patient discharged. X-rays of the limbs with

the ulcers were done for all patients as part of baseline investigations to rule out osteomyelitis associated with BU/HIV coinfection. TB was ruled out in all the patients clinically and by means of normal chest X-rays. Computerized tomography scan of the head was done for one patient who developed some neurological deficits during wound management to rule out any space occupying lesions most especially cerebral toxoplasmosis.

Histopathology

Histopathological analysis was done for all surgical debridement and excisions. Surgically excised tissue samples were immediately fixed in 10% neutral-buffered formalin for 24 hours at room temperature to maintain tissue structures. Afterward samples were directly transferred to 70% ethanol for storage and transport. Tissue specimens were subsequently dehydrated, embedded into paraffin, and cut into 5 mm sections. After deparaffinization and rehydration, sections were stained with Ziehl–Neelsen (ZN)/methylene blue, hematoxylin or by immunohistochemistry according to WHO standard protocols [42]. The following antibodies were used for T-cell staining (CD3, Dako; CD4, cell marque; CD8, Serotec) and B-cell staining (CD20, Dako). Tissue sections were analyzed with a Leica DM2500 Microscope (Wetzlar, Germany). Pictures were either taken with a Leica DFC 420C camera or with an Aperio ScanScope XT.

Data analysis

All data were entered into Microsoft Excel package and verified before exporting into the statistical package Stata (Release 12; Stata Corporation, College Station, TX) that was used to perform all the statistical analyses. The c^2 tests at 95% confidence (CI) level was used to compare HIV prevalence among BU patients and HIV in the general hospital attendants and also among pregnant women screened at the same health facility.

Results

Study participants and prevalence of HIV/BU coinfection

All PCR-confirmed BU patients (67 males and females each) were screened for HIV at the Ga West Municipal Hospital. Their age range was between 3 and 86 years with a mean age of 33.8 years (standard deviation [SD]: 21.6); 36 (26.8%) were ≤ 15 years. Eleven (8.2%) of the cases (5 females and 6 males) were HIV positive. During the period, 14,863 individuals were tested for HIV infection in the same facility and the positivity rate was 4.8% (718/14,863) though lower was not significant, P = 0.070. On the other hand prevalence was significantly lower (P value = 0.001) among pregnant women using the preventing mother to child transmission (PMTCT) facility (2.5%; 279/11,125). The age range of the HIV-positive BU cases was between 12 and 65 years with a mean age of 37.1 years (SD: 13.1). Only one of the 11 HIV-positive BU cases was a child (aged 12), the remaining 10, were between 29 and 65 years of age. Seven out of the 11 HIV/BU coinfected cases were followed during treatment and Table 1 shows the baseline characteristics of these seven study participants. The other four patients were lost to follow-up. All the seven patients went through some form of traditional or herbal treatment of the ulcers with duration of ulcers before antibiotic therapy ranging from 4 to 24 months. Four of them were males and three were females. The ages were from 12–46 years with a mean age of 33.1 years (SD: 11.7 years). All seven patients presented with category III lesions with six presenting with lower limb lesions and one presenting with an upper limb lesion. In comparison, 67/123 (54.47%) HIV-negative BU patients presented with category III lesions. One of the BU/HIV coinfected patients presented with an ulcer and a plaque not yet ulcerated at presentation, whereas the others had ulcerated forms only at presentation. Four had multiple lesions at presentation whereas three had single lesions. *M. ulcerans* infection of all seven patients was reconfirmed by IS*2404* PCR. In addition, 6/7 were reconfirmed by microscopic detection of AFBs after ZN staining and 3/7 were confirmed by positive *M. ulcerans* culture.

The mean hemoglobin level (Table 2) at baseline was 8.7 g/dL (SD: 2.5). One of the patients had a normal hemoglobin level at presentation but worsening anemia with new lesions formed. Median CD4 counts at baseline (Table 2) were 318 cells/mm^3 (interquartile range [IQR]: 265–750 cells/mm^3). Two patients who were at WHO clinical stage 2 of HIV infection at baseline had 6 monthly CD4 count repeated until study end point. Of these two patients, one started ART 1 week after beginning of SR treatment and the other started ART 1 week after completing SR

treatment. The CD4 counts (Table 2) measured after 6 months dropped to 185 cells/mm^3 from a baseline of 298 cells/mm^3 (for patient starting ART 1 week after SR started), which then improved to 586 cells/mm^3 at 1 year, whereas the second patient showed a steady rise in the CD4 count from baseline.

Duration of SR treatment

The duration of SR treatment was 8 weeks according to WHO protocol. However, three patients had treatment extended to 12 weeks upon the discretion of the clinician, whereas one patient had SR changed after 26 days to rifampicin–amikacin for an additional 30 days because of suspected adverse reaction to SR. One patient (case 5) died after 40 days on SR treatment. Time of starting ART in relation to SR treatment. Of the three patients (cases 1, 2, and 3) who took ART during wound management, one (case 2) commenced 1 week after starting SR, another (case 3) started 1 week after completing SR, and the other (case 1) started 13 weeks after completing SR. The type of ART combination given is as shown in Table 3. Two (cases 4 and 5) of the remaining four patients died and the other two (cases 6 and 7) were referred to ART centers away from the center of the study after wounds had healed with no recurrence after 6 months of follow-up, because no ART center was established at the district hospital at the time of their management. Figure 1 shows immune infiltrates observed in one patient (case 2) who developed four new lesions with excessive exudates at different sites of the same limb. One at the anteromedial aspect of the proximal third of the right limb, the other on the right lateral aspect of the thigh, and the other two developed on the medial aspect of the knee and the dorsum of the right foot. The onset of the infiltration started about 2 weeks after starting the SR treatment. The other lesions developed following the administration of additional SR treatment after completion of the 8 weeks' standard regimen. The new lesions were managed by saline dressing and required no further antibiotic treatment.

Wound healing rate

We designated a wound healed when complete (100%) epithelialization of the ulcer had occurred. The study adopted an endpoint to healing at 52 weeks at which point all ulcers that were not 100% epithelialized were censored or classified as failing to heal [43]. The healing rate at the study endpoint was 42.9% (3/7) with a median time to healing of 37 weeks (IQR: 36–37)

(Table 3). Two patients (cases 4 and 5) died within the study period and two (cases 2 and 3) had still not healed completely 56 and 64 weeks after start of treatment, respectively. The three patients (cases 1, 6, and 7) that healed had excision and skin grafting done. One of the nonhealing wounds (case 3) had the graft breaking down leading to a recurrent ulcer after 40 weeks of complete epithelialization and discharge from the hospital. *M. ulcerans* culture of tissue samples was negative ruling out relapse and the reopening was probably due to poor scar care.

Development of immune infiltrates

For histopathological analysis, tissue samples were collected after completion of at least the standard 8 weeks of SR treatment. All samples still presented with BU typical histopathological features, such as epidermal hyperplasia, fat cell ghosts, and some remaining tissue necrosis (Figure 2A, B, and E). In addition, all samples showed immune infiltration, blood vessel and granuloma formation (Figure 2A, C, and D), as is commonly observed in antibiotic-treated lesions of HIV-negative BU patients.[12] Mixed infiltrates containing large numbers of CD3 positive T cells were found mainly in the dermis and subcutaneous tissue layer. Granulomas and clusters of CD20 positive B cells were embedded in the mixed unstructured infiltrates. Granulomas were mainly formed by macrophages, giant cells, T cells, and B cells (Figure 2F–I). In all lesions the infiltration contained CD4 positive T cells, even when CD4 blood counts (Table 2) were low. However, in 4/6 patients analyzed, $CD8^+$ T cells outnumbered $CD4^+$ cells. Larger numbers of N-elastase-positive neutrophils were only observed in association with secondary bacterial infections. AFBs were detected in only 2/6 analyzed tissue samples as scattered beaded bacteria.

Discussion

Little is known about the impact of HIV infection on susceptibility to *M. ulcerans* infection and BU treatment outcomes such as cure, recurrence, long-term disability, and the incidence of paradoxical reactions secondary to antibiotic treatment. In a study conducted in 426 BU patients and 613 controls in southern Benin [44], a significantly higher HIV prevalence was observed among BU patients than in controls (2.6% versus 0.3%; P = 0.003). Although in this study, the HIV prevalence among BU patients (8.2%) was higher than that (4.8%) among general patients attending the same health facility, this difference was not statistically significant (P value = 0.070). In contrast, a significant difference (P value = 0.001) was found with the HIV prevalence of 2.5% among pregnant women attending the same health facility during the same period. The cases that were followed had varying disease presentation, responses to treatment as well as treatment outcomes. All seven BU/HIV coinfected cases included in the study presented with category III lesions as compared with 54.47% of category III lesions in HIV-negative BU cases. Severity of BU disease did not necessarily reflect the level of underlying immune suppression especially when using CD4 as the marker, as a case with CD4 counts below 300 had no multifocal disease, while another case with CD4 counts above 500 developed multiple lesions. One of the BU/HIV coinfected patients developed chronic osteomyelitis (case 4). This patient was severely immunosuppressed, as evidenced by a CD4 count of 37 and clinical stage 4 HIV disease. Since osteomyelitis is also occurring in HIV-negative patients, further studies are required to establish whether a severe immune suppressed state increases the risk for developing osteomyelitis. Although CD4 blood counts were reduced in the six patients analyzed by histopathology (unfortunately no tissue sample became available from the patient with the very low CD4 blood count of 37), substantial numbers of CD4 positive T cells were found in mixed infiltrates and granulomas emerging in the treated BU lesions. However, CD8 T cells outnumbered CD4 T cells in 4/6 patients (Figure 2H and I).

The HIV coinfected BU cases presented here showed mild to moderate anemia at admission but severe anemia concomitant with new lesions in one patient (case 2). Anemia has been found to be present at varying degrees in HIV patients, correlating with the clinical stage and state of immunity of the patient [35,36]. However within the cases followed, we found severe anemia as defined by the CDC even though the CD4 count of the patient was above 500. Literature also supports that some mycobacteria such as *M. tuberculosis* and *M. avium* complex can cause peripheral blood cytopenia in HIV patients but no such documentation with *M. ulcerans* is

currently available [45]. Persistent anemia resulting from a BU/HIV coinfection could account for the delayed healing of wounds (median time to wound healing being 37 weeks) of the cases presented here.

One of the BU-HIV coinfected cases (case 2) developed new lesions 2 weeks after starting SR treatment, coinciding with 1 week after starting ART. Whether this occurrence was a result of the relative early onset of the ART with SR treatment is unclear. The appropriate time to start ART in HIV patients with opportunistic infection has always been a dilemma to clinicians since ART can trigger severe IRIS-like reactions when it is commenced early. On the other hand, delaying treatment could similarly lead to worsening of the disease condition and prognoses. With TB/HIV coinfection, it is recommended that ART should be started 2 weeks into antimicrobial treatment [46]; however, based on a large randomized controlled trial it is recommended to delay ART till 5 weeks into antimicrobial treatment when managing HIV/ Cryptococcus coinfection [47]. Hence, the optimal time to starting ART may depend on the specific coinfection and known interactions of some ART with antimicrobial agents. Studies have shown that taking ART containing nevirapine alongside SR treatment leads to a decreased C_{max} and C_{min} of nevirapine due to interaction with rifampicin [48-50]. This notwithstanding, early onset of ART and co-trimoxazole prophylaxis is highly recommended by WHO preliminary guidelines for management of HIV/BU coinfection [51] to build up immunity and to fight opportunistic infections associated with HIV as these could worsen the prognosis of the condition. Here 4/7 BU/HIV coinfected patients did not start with ART treatment within the study period because of unavailability of an ART center within or close to the study center. Since HIV testing has become a standard element in BU management, access to an ART center should be secured as a part of BU care. The 2/7 study participants who died within the study period as a result of worsening disease were not started on ART. Possibly, such mortality could have been averted by early onset of ART.

The duration of SR treatment of 3/7 of the HIV/BU coinfected cases studied here was extended based on the judgment of the responsible clinicians to 12 weeks, since deterioration of the lesions suggested *M. ulcerans* ongoing disease activity after completion of the standard 8 weeks of treatment. However, no laboratory confirmation for the presence of viable *M. ulcerans* bacteria before the extension of the antibiotic treatment was available.

Conclusion

Despite the limitations of the small sample size, the difficulties in assessing the immunological statuses of some participants, as well as ascertaining the viral loads of the patients, results of this study indicate that HIV coinfection could predispose BU patients to the development of more severe clinical forms (large and multiple lesions) and delayed wound healing. Although early onset of ART in BU/HIV coinfection is recommended, systematic studies are required to develop detailed guidelines for the management of BU/HIV coinfected patients as there are for TB and Cryptococcus/HIV coinfections. Further studies would be required to determine the cumulative effect of the IRIS and paradoxical reactions in BU/HIV coinfected patients on ART and SR treatments.

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Figures

Figure 1. Emergence of new lesions during streptomycin (SR) treatment in one of the enrolled coinfected patients (case 2).

(A) Features of the first lesion before the start of SR treatment, (B) appearance of a new lesion after 2 weeks of antibiotic treatment, (C) appearance of a third lesion after 4 weeks of SR treatment, (D) appearance of a fourth lesion after 6 weeks of SR treatment, and (E) increase in wound sizes after surgical excision and appearance of a fifth lesion after start of the SR treatment.

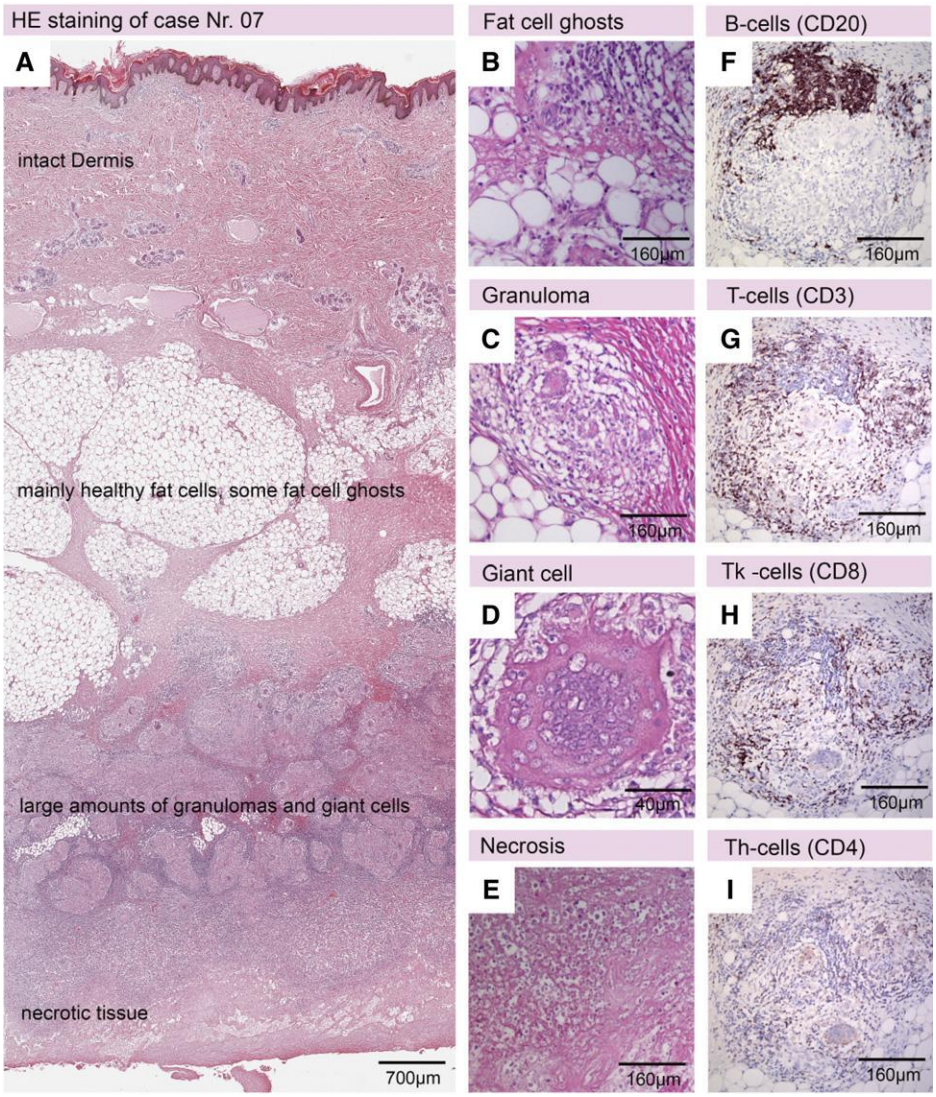

Figure 2. Emergence of immune infiltrates in the Buruli ulcer (BU) lesion during SR treatment in a representative patient (case 7).

Histological sections were stained either with hematoxylin–eosin (A–E), or with antibodies against CD20 (F), CD3 (G), CD8 (H), or CD4 (I) (counterstain hematoxylin). (A) cross section through a BU lesion presenting with epidermal hyperplasia, an intact dermis, healthy fat cells as well as fat cell ghosts, a layer of numerous well-defined granulomas, and a necrotic area. Fat cell ghosts (B), granulomas (C), giant cells (D), and necrosis (E) are shown at higher magnification. Immunohistochemical analysis of the granulomas showed the presence of B-cell clusters (F) as well as the presence of large numbers of CD3, CD4, and CD8 positive T cells (G, H, and I).

Tables

Table 1: Demographics, clinical features and diagnostic data of study participants with BU and HIV coinfection

Patients parameters	**Values**
Total no.	7
Sex(no. of males/females	4/3
Median(range) age yr.	33.7(12-46)
No.(%) with indicated site of lesion	
Upper limb	1(14.2)
Lower limb	6(85.7)
No. (%) with indicated clinical form of lesion	
Ulcer	6(85.7)
Plaque/ulcer	1(14.2)
No. (%) with indicated category of lesion	
III	7(100)
No.(%) with indicated number of lesion	
Single	3(42.8)
Multiple	4(57.1)
No. with indicated laboratory confirmation results	
M. ulcerans culture (+/-)	3/4
PCR for IS*2404* (+/-)	7

Table 2: CD4 counts and Hb level monitoring of the seven enrolled patients at 8-weeks intervals

BU case	WK0		WK 8		WK16		WK24		WK 32		WK 40		WK48	
	CD4	HB	CD4	HB	CD4	HB	CD4	HB	CD4	HB	CD4	HB	CD4	HB
1	318	8.8g/dl	-	8.2 g/dl	-	10.5 g/dl	-	-	-	-	-	-	-	-
2	298	11.4g/dl	-	7.9g/dl	-	7.0g/dl	185	8.2g/dl	-	8.2g/dl	-	9.9g/dl	558	11g/dl
3	265	7g/dl	-	11.8g/dl	-	10.2g/dl	394	8.0g/dl	-	7.2g/dl	-	9.2g/dl	455	8.7g/dl
4	37	9.5g/dl	-	8.2g/dl	-	-	-	-	-	-	-	-	-	-
5	791	5.0g/dl	-	6.4g/dl	-	6.2g/dl	-	8.2g/dl	-	-	-	-	-	-
6	730	12.g/dl	-	11.8g/dl	-	12.1g/dl	-	-	-	-	-	-	-	-
7	751	7.1g/dl	-	10.7g/dl	-	9.6g/dl	-	-	-	-	-	-	-	-

Table 3: Laboratory confirmation, HIV clinical stage, and treatment characteristics of the seven patients enrolled

BU Case	BU confirmatio	Retro serotype/HIV clinical stage	SR treatment	HIV treatment	Treatment outcome	Time to complet wound healing
1	PCR	HIV 1, Stage 2	SR8	Tenofovir, lamivudine, efavirenz + co-trimoxazole	Woun healed with skin grafting	37 weeks
2	PCR, ZN	HIV 1, Stage 2	SR12	Tenofovir, lamivudine, nevirapine + co-trimoxazole	Still dressing wound at 64 weeks of treatment	Failed to heal
3	PCR, ZN	HIV 1, Stage 2	SR12	Tenofovir, lamivudine, efavirenz + co-trimoxazole	Wound re-opened after 40 weeks of discharge. Still dressing wound at 52 weeks	Re-opened leg ulcer
4	PCR, ZN	HIV 1, Stage 4	SR8	Co-trimoxazole	Patient died of deterioration of disease	Censored
5	PCR, ZN	HIV 1, Stage 4	SR8	Co-trimoxazole	Patient died of sever Staphylococcus aureus septic and anemia 26 weeks after completion of treatment	Censored
6	PCR, ZN	HIV 1, Stage 2	SR8	Co-trimoxazole	Wound healed after skin grafting	37 weeks
7	PCR, ZN	HIV 1, Stage 2	SR12	Co-trimoxazole	Wound healed after skin grafting	36 weeks

BU = Buruli ulcer; HIV = human immunodeficiency virus; PCR = polymerase chain reaction; SR = streptomycin/rifampicin; ZN = Ziehl–Neelsen.

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Chapter 4

Possible health-care associated transmission as a cause of secondary infection and population structure of *Staphylococcus aureus* isolates from two wound treatment centers in Ghana

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Abstract

We have previously shown that secondary infection of Buruli ulcer (BU) wounds were frequently caused by *Staphylococcus aureus.* Such complications may lead to significant healing delays. To gain understanding into possible routes of secondary infection, we characterised *S. aureus* isolates from patient lesions and surrounding environments across two Ghanaian health centres. One hundred and one *S. aureus* isolates were isolated from wounds (n=93, 92.1%) and the hospital environment (n=8, 7.9%) by microbiological culture and characterised by the *spa* gene, *mecA* and the Pantone Valentine Leukocidin (*PVL*) toxin followed by *spa* sequencing and whole genome sequencing (WGS) of a subset of 49 isolates. Susceptibility testing of the isolates to commonly prescribed antibiotics was performed. *Spa* typing and sequencing of the *spa* gene from 91 isolates identified 29 different *spa* types with t355 (ST152), t186 (ST88), and t346 dominating. While many distinct strains were isolated from both health centers, genotype clustering was also identified within centers. These clusters were confirmed by phylogenomic analysis. Twenty-four (22.8%) isolates were identified as methicillin-resistant *S. aureus* (MRSA) and *lukFS* genes encoding PVL were identified in 67 (63.8%) of the isolates. Phenotype screening showed widespread resistance to tetracycline, erythromycin, rifampicin, amikacin and streptomycin. Genomics confirmed the widespread presence of antibiotic resistance genes to ß-lactams, chloramphenicol, trimethoprim, quinolone, streptomycin and tetracycline. Our findings indicate that the health-care environment likely contributes to the superinfection of BU wounds and calls for improved training in wound management and infection control techniques.

Introduction

Microbial contamination and colonization of wounds is common to all wounds healing by secondary intention and has been proposed as a precondition to the formation of granulation tissue and stimulation of wound healing [1]. Wound microflora is usually polymicrobial [2] comprising organisms such as staphylococci, enterococci, streptococci, facultative Gram-negative bacteria and anaerobic bacteria [3]. When wound colonization is not managed properly and host defences fail to control the multiplication of microorganisms, localized wound infection occurs, which can progress to severe and deep-seated infections. Infection of a wound may lead to delayed healing, an increase in treatment costs and increased trauma to the affected patients [3] .

Wounds can be infected through three main sources; the surrounding skin, endogenous sources such as the nasal mucosa, gastrointestinal tract and genitourinary tract and the wider environment. Within a health care facility, the sources of contamination and subsequent infection of a wound may include health care workers (HCW), patients and the inanimate environment. Direct contact of a patient with an infected HCW during general care or medical treatment can result in the transmission of microorganisms. Indirectly, an infected patient or HCW could touch and contaminate an object, instrument or surface and subsequent contact between the item and a second patient is likely to contaminate the patient leading to infection.

Staphylococcus aureus is a notorious opportunistic nosocomial pathogen, and one of the main organisms involved in infection of chronic wounds [4]. It may be carried asymptomatically by a carrier and transferred from patient to patient. It is estimated that about 25% of the normal population may be carriers, with higher carriage rates around 50% in insulin dependent diabetics, intravenous drug users and dialysis patients [5].

In a previous study, we identified infection of Buruli ulcer (BU) wounds by bacterial pathogens as a possible cause of wound healing delay among study participants [6]. Several bacterial species were isolated with *S. aureus* and *Pseudomonas aeruginosa* dominating. Despite the finding that majority of the organisms identified were nosocomial pathogens, the possible routes of infection of the lesions were not examined in this study. In seeking to have a better understanding of the possible routes of infection of the lesions, we initiated the current study to characterize *S. aureus* isolated from different sources in two health facilities in Ghana treating BU.

Materials and Methods

Study sites, participants and sample collection

The study was carried out at the two main BU treatment centers in the Ga West and Ga South districts of the Greater Accra Region of Ghana, designated facility A and B respectively. The study involved the analysis of *S. aureus* isolates from wounds of microbiologically confirmed BU (BU) and non-BU (NBU) patients (initially suspected BU cases that were not confirmed by any of the three confirmation methods (Ziehl-Neelson microscopy, IS2404 PCR and culture) who received treatment either as in-patients on admission or out-patients at one of the two health centers from October 2010 to February 2014. Lesions of patients were swabbed and the samples analyzed at the Bacteriology Department of the Noguchi Memorial Institute for Medical Research. A total of 173 samples were collected from 162 patients and *S. aureus* was isolated from 88 samples originating from 76 patients, 61 of whom were BU and 15 NBU patients. Of the 61 BU patients, 56 were outpatients and five were inpatients at the time of sampling, while all the NBU patients were outpatients. The five inpatients had been on admission for four weeks or more and therefore isolates from these patients could be classified as potentially hospital acquired. To elucidate the sources of infection, between August 2013 and February 2014 hands of health care workers, dressing rooms and wards of the health centers, surfaces of dressing tables, door handles, instruments and equipment as well as dressing solutions and materials were sampled for microbiological analysis. Swab samples were also taken from the palms of nurses and their gloved hands in between dressing of patients and the lesions of the patients they dressed before and after dressing. Swab samples were transported in phosphate buffered saline at 4^0C to the Noguchi Memorial Institute for Medical Research. Eighty-six samples were collected and analyzed and *S. aureus* was isolated from 13 of them (see web only appendix, Figure A1).

Microbiological methods

The samples were processed and inoculated on Blood and Mannitol salt agars and incubated for 18-24 hours at 37^0C after which they were examined. Identification of Staphylococcus species was by colony and microscopic morphology, catalase reaction and the coagulase biochemical test (BD). The Staphylase Kit, BD BBL™ Staphyloslide Latex Test (Becton, Dickinson and Company) was used to differentiate the catalase positive Gram positive bacteria, *S. aureus* from other Staphylococcus species.

Antibiogram of isolated bacteria

Antibiotic susceptibility testing was determined by the Kirby-Bauer disc diffusion method according to CLSI guidelines [7]. Susceptibility was determined for the antibiotics; amikacin (30µg), sulphamethoxazole-trimethoprim (23.75µg/1.25µg), ampicillin (10µg), tetracycline (30µg), gentamicin (10µg), erythromycin (15µg), cefuroxime (30µg), ceftriaxone (30µg), chloramphenicol (30µg), cefoxitin (30µg), rifampicin (5µg), streptomycin (10µg), vancomycin (30µg), clindamycin (2µg) and cefotaxime (30µg). Cartridges of antibiotics were obtained from Oxoid and Beckton Dickinson. *S. aureus* ATCC 25923 was used as reference strain.

Genotyping

Traditional Molecular typing

Crude DNA extracts were prepared by boiling and used as template in the PCR reaction. Genetic relatedness of the isolates was determined through amplification of the polymorphic X region of the protein A (*spa*) gene using the primer pairs spa-1113F and spa-1514R (see web only appendix, Table A1) [8] and DNA from *S. aureus* ATCC® 25923 as positive control. A total of 95 isolates out of the 101 were typed using these primers. A number of suspected *S. aureus* strains could not be typed by these primers and were further typed using another set of primers spaT3-F and spa-1517R (see web only appendix, Table A1). These primers were previously described to detect *S. aureus* strains which may have rearrangements in the IgG-binding region of the gene where the forward primer is located, making them untypeable by the original *spa* primers [9]. Sequencing of the *spa* gene from positive isolates was outsourced to Macrogen (Netherlands, Europe) and sequences analyzed and assigned to *spa* types using DNAGear [10]. The relationship between strains was investigated using the Staden package [11] and a maximum likelihood phylogenetic tree produced in Mega 5.05 (www.megasoft.net) and visualised in FigTree v1.4.2. (http://tree.bio.ed.ac.uk/software/figtree/).

Genes for *mecA* and PVL were sought using the primers mecA P4, mecA P7 and pvl-F, pvl-R (see web only appendix, Table A1) [12-13] . A mecA and PVL positive isolate *S. aureus* 282-101 from the Statens Serum Institute (SSI, Denmark) kindly provided by Dr Beverly Egyir,

was used as positive control. Agr types were determined using the primers pan agr F, agr 1-R, agr 2-R, agr 3-R and agr 4-R (see web only appendix, Table A1) [14]. PCR reactions contained 12.5 µl HotStarTaq Mastermix (Qiagen), 5 µl nuclease free water, 2 µl of each primer and 3.5 µl of DNA. Results of *mecA*, *agr* and PVL typing were analyzed using Microsoft Excel.

Whole genome sequencing and analysis

From the 101 isolates, a subset of 70 isolates were selected for WGS made up of 31 MRSA isolates and 39 other MSSA isolates spanning the clusters observed from the phylogenetic tree obtained from sequencing of the *spa* gene. Genomic DNA was extracted with the Qiagen DNeasy Blood and tissue kit (Qiagen) according to the manufacturer's instruction and sequenced using the Illumina MiSeq platform (2x 250bp sequencing by synthesis chemistry using DNA libraries prepared using Nextera XT (Illumina, San Diego, California, USA). Resulting sequence reads were processed using a custom bioinformatics analysis pipeline Nullarbor (nullarbor.pl 0.6, https://github.com/tseemann/nullabor) to *de novo* assemble and also align read data against the Sa_aus0325 reference genome (S. Giulieri et al., unpublished). Nullarbor uses a BLAST-based method to screen contigs from *de novo* assemblies for the presence of antibiotic resistance genes. Pairwise alignments of core genome SNPs were used with FastTree [15] to infer maximum likelihood phylogenetic trees using the general time reversible model of nucleotide substitution. Resulting trees were visualized in FigTree v1.4.2. The topologies of rooted phylogenetic trees obtained from both *spa* typing and WGS using *Staphylococcus simiae* CCM7213 as an outgroup were compared by tanglegram in Dendroscope (v3.4.4) [16].

Results

Bacterial isolates

One hundred and one (101) *S. aureus* isolates were identified from the sources sampled. Eighty-eight isolates were recovered from 76 patients; 72 (81.6%) were from 61 BU patients and 15 from NBU wounds. One isolate each was identified from 66 patients and multiple isolates from the other 10 patients. Five patients had two isolates from a single lesion collected at one time point, one patient had two isolates from two lesions, three isolates were identified from a patient with three lesions (patient A, one from each), two patients had two isolates sampled at two different time points during the course of treatment and one patient with three isolates sampled thrice at different time points during the course of treatment.

S. aureus was isolated from 13 (15.1%) of the samples collected during sampling of HCWs and the patients they attended to as well as the environment and equipment in the health facilities.. The isolates were made up of five from patient lesions, six from equipment (forceps) and one each from the hand of a healthcare worker and a table used for dressing wounds. All these isolates were from Facility B. In total, 53 isolates were from Facility A while 48 isolates were from Facility B.

Population structure of *S. aureus* and epidemiological association

Spa typing identified 29 different *spa* types including 15 (14.9%) singles. The three dominant *spa* types (t355, t186 and t346) were found in seven (6.93%) isolates each (see web only appendix, Table A2). Thirty-five isolates could not be assigned a *spa* type. Phylogenetic analysis identified ten different clusters, where a cluster was defined as a group of two or more isolates found on the same branch of the tree (Clusters A-H, Figure 1), which consisted of health center specific clusters, and common clusters. Clusters A, C, D, H and J were made up of strains from both health centers (Figure 1), clusters B, G, and I made up of mainly strains from Facility A while clusters E and F were made up mainly of strains from Facility B (Figure 1). Within cluster F were isolates cultured from samples taken on the same day from the hand of a health care worker and patients dressed consecutively by this worker, isolates from samples taken on a different day from one equipment (forceps) after it had been used on four patients consecutively and two isolates recovered from a patient at two different time points (weeks 2 and 8) during treatment (Figure 1). This finding led us to conclude that

transmission events were ongoing within this health center. Cluster G was made up mainly of MRSA isolates (Figure 1) while cluster I was made up of isolates from three different lesions of one patient which had the same *spa* type (t2500) (Figure 1), suggesting that the three lesions were infected from a common source, possibly from the patients' microbiota.

Detection of *mecA* and PVL genes and Agr type

PCR screening for the *mecA* gene identified 31 isolates as MRSA. The genotypes of 24 of these isolates correlated with their antibiogram results; however *mecA* was also detected in seven isolates showing susceptible phenotypes. Twenty-one of these were from BU patients while eight were from NBU patients and two from the environment. Four of the MRSA were isolated from in-patients. The PVL gene was detected in 66 isolates; 51 from BU patients, 12 from NBU patients and three from the environment. Both *mecA* and PVL genes were detected in 20 isolates made up of 14 from BU patients, five from NBU patients and one from the environment. Nineteen (18.8%) isolates belonged to agr type 1, 23 (22.8%) isolates to agr type 2, 30 (29.7%) isolates to agr type 3, three (2.9%) isolates to agr type 4, and four (3.9%) isolates showed bands for both agr types 2 and 3, while 22 (21.8%) isolates were non typable and possibly agr defective mutants.

Antibiogram of isolates

Over 70% of the isolates were susceptible to amikacin (89, 88%) and gentamicin (89, 88%). Resistance rates above 50% were recorded against the antibiotics ampicillin (89, 88%), tetracycline (57, 56.4%) and chloramphenicol (67, 66%). Lower resistance rates of 25%, 26% and 37% were also recorded against the cephalosporins; ceftriaxone, cefotaxime and cefuroxime, respectively while 26% of the isolates showed reduced susceptibility to vancomycin (Table 1). Comparing the antibiograms of MRSA and MSSA isolates, a significant proportion of MRSA isolates were resistant to the antibiotics cefotaxime, tetracycline, ceftriaxone and cefoxitin ($p < 0.05$) (Table 2). Resistance to chloramphenicol and ampicillin was equally high among MRSA and MSSA.

Whole Genome Sequencing Analysis

During the course of this project we had the opportunity to investigate some of the isolates further by WGS. From an initial 70 isolates subjected to WGS, 21 were of low read-coverage and excluded from analysis. We first inferred multilocus sequence typing data (MLST) from the WGS data. The 49 isolates belonged to 12 different sequence types (ST) with ST15 (13 isolates) and ST88 (11 isolates) dominating. The remainder belonged to ST 1(1 isolate), ST 5 (6 isolates), ST 6 (1 isolate), ST 72 (2 isolates), ST 121 (2 isolates), ST 152 (3 isolates), ST395 (1 isolate), ST 707 (1 isolate), ST 2434 (1 isolate) and ST 3248 (3 isolates)(see web only appendix, supplementary data B1). Four isolates represented new STs. The 11 ST88 isolates were all MRSA and will be described elsewhere (Kpeli et al, manuscript in preparation).

Read-mapping of the 49 genomes against the Sa_aus0325 reference sequence produced a 2.2Mbp core genome with 100,361 single nucleotide polymorphisms (SNPs). A maximum likelihood phylogeny was inferred from pairwise comparisons of these SNPs (Figure 2). Among the 13 ST15 isolates, 10 were isolated from one health center, including from the hand of a health care worker, patients and equipment, corresponding with results from *spa* typing and giving support to the conclusion that transmission events were ongoing within this health center. The three isolates from patient A were of the same ST type (ST 3248) and between them had SNP differences of 29 bp, 51 bp and 34 bp (see web only appendix, supplementary data B2) also corresponding with the *spa* typing results discussed above. This small number of SNPs differences points to the isolates spreading from a common source.

We then inferred the resistome of each isolate from the WGS data (see methods). Antibiotic resistance genes coding for resistance to ß-lactams (*blaZ*), chloramphenicol (*cat* and *catpC221*), trimethoprim (*dfrG*), methicillin (*mecA*), quinolone (*norA*), streptomycin (*str*) and tetracycline (*tetK, tetL* and *tetM*), were identified in 48 (98%), one (2%), 22 (45%), 13 (25.5%), 13 (25.5%), 49 (100%), 12 (24.5%), eight (16.3%), 12 (24.5%) and 12 (24.5%) of the 49 isolates, respectively (see web only appendix, supplementary data B1). We further investigated the *rpoB* gene of rifampicin resistant strains and identified two known amino acid substitutions H481N and I527M implicated in rifampicin resistance in seven and one isolate respectively(see web only appendix, supplementary data B1). Other mutations were also found within the rifampin resistance-determining (Rif) region of the rifampicin resistant isolates but further studies will need to be done to ascertain if these mutations contribute to rifampicin resistance.

Analysis of the topologies of the phylogenies produced by *spa* typing and WGS shows lots of agreement but also some differences (Figure 3). Clustering of MRSA, isolates from patient A, and isolates from facility B from a HCW, patients dressed by this worker and an equipment were both predicted by the two methods. However, though spa typing predicted the clustering of isolate SA_NOG-W15 which is an MRSA with other MRSA isolates, this cluster was not confirmed by WGS which predicted it to cluster with isolate SA_NOG-W28 an MSSA.

Discussion

This study confirmed healthcare associated infection (HAI) as a source of wound infection within our study health centers. Our analysis shows that *spa* typing is useful for predicting transmission patterns in resource limited settings but that there is also a need for access to low-cost microbial genomics in developing countries. Genome analysis rapidly revealed widespread antibiotic resistance among the isolates and clearly identified likely transmission clusters.

In previous work [6], we found that wound infection may be a source of healing delay. The findings from our current study implicate the health care environment (including health care personnel) as possible sources of *S.aureus* infection. From our cluster analysis using *spa* typing, we inferred three modes of wound infection; two health facility related sources through a HCW and the inanimate environment (Figure 1), and the third source through self infection (Figure 1). This result corresponds with that of previous studies which have implicated HCW, patients and the inanimate environment in the transmission and subsequent acquisition of *S. aureus* in health care settings [17]. HAIs are known to negatively impact health care delivery around the world. Effective infection prevention and control (IPC) practices especially compliance with hand hygiene recommendations will lead to significant reduction in the rate of HAIs. Ghana has a policy document to aid the training of HCW in IPC (www.tbghana.gov.gh). However, a monitoring survey in selected health facilities within the Greater Accra region showed that the compliance level of HCW to these guidelines is low (Ghana Health Service 2011, *Infection Prevention and Control. A survey in Greater Accra.* unpublished) with rates below the 70% recommended by the World Health Organization (apps.searo.who.int>PDS_DOCS). Adherence to strict policies supported by periodic training and monitoring of HCWs are required to ensure compliance with existing IPC guidelines and to decrease the frequency of HAIs.

With regard to the patient-specific clusters, Yeboah-Manu and colleagues [6] reported that some patients recycle bandages used in wound dressing due to inadequate financial support during their treatment period, and this could result in the transfer of pathogens from one lesion to another. Additionally, wound management in Ghana is influenced to a high degree by local traditional beliefs and practices. Beliefs revolving around the category of people deemed qualified to manage wounds affect the behaviour of patients. A recent study from our team (Koka et al manuscript in preparation) reports that in many communities, pregnant women and nursing mothers are seen as unqualified to manage wounds and in cases where

HCWs fall into this category, patients resort to the redressing of their wounds after the HCW has dressed them. This could also lead to the transfer of pathogenic organisms into the lesions as patients do not observe proper aseptic procedures during wound redressing. Thus, there is a need to counsel patients to adhere to the biomedical wound care and management practices to reduce or avoid self infection of their lesions.

This study was limited by not performing a thorough investigation of other body sites of the patients where *S. aureus* is known to exist as a normal flora. An exhaustive investigation should have included culture of samples from other areas of the patients such as the skin, hand and anterior nares of the nasal cavity to compare between strains from these sites and the wounds. The study may also have been affected by the Hawthorne effect at Facility A and this could account for no *S. aureus* isolates from sampling the environment and HCWs at this facility.

Two methods were employed to arrive at our conclusion of HAIs; the single gene locus DNA sequence-based marker *spa* typing and WGS. As revealed from our analysis, phylogenies from both methods predicted similar clusters. While WGS reveals variability across the whole genome and is able to discriminate down to single nucleotide differences, *spa* typing looks at genetic variability at a single locus between 200-600 bp in length. *Spa* typing is less expensive and demanding in terms of infrastructure and expertise and has a shorter turnaround time compared to WGS. Our results show that the level of discrimination of *spa* typing is adequate to guide infection control and also supports its use in epidemiological studies. However, the lack of congruence between the two methods is likely indicating that spa typing lacks resolution to be able to differentiate between highly genetically related isolates. This partial sequencing technique also cannot reveal the finer genetic details that accumulate during the evolution of bacterial populations. Therefore, although *spa* typing is useful for the prediction of transmission events in resource limited settings, where access to the newer, more expensive and more advanced WGS technologies is limited, we should be looking for ways to implement low cost microbial genomics in these countries because the rich data obtained from pathogen genomes can be used to make well-informed decisions to control the spread of disease.

Another important finding from this study is the high genetic diversity and PVL-positivity among the isolates. This agrees with existing knowledge on the genetic diversity and PVL prevalence among African *S. aureus* isolates [18-20]. It also supports the assertion that Africa is a PVL endemic continent with high prevalence of PVL being a distinguishing trait of

African *S. aureus* isolates [19] compared with Asia, Europe and the USA [21]. Additionally, we identified t355 as one of the most prevalent *spa* types. This correlates with studies in Ghana and other African countries which identified this *spa* type as one of the most widespread, hence suggesting it to be widely established and distributed in Africa [18, 22-23].

Clinical *S. aureus* isolates are known to be *agr* positive [24] and this is also evidenced from our results with 79 (78.2%) isolates having this locus. However 22 (21.8%) isolates were defective for *agr* function. Previous research suggests that *agr* defective mutants can interact with *agr* positive variants during clinical infection [24]. These *agr* defective mutants play an important role in persistent infection by forming thicker biofilms as compared to *agr* positive isolates [25]. This phenomenon might be playing a role in delayed wound healing, which we observed among patients within our study health centers [6].

Antibiotic resistance rates from both phenotypic and resistome investigations confirms the increasing prevalence of drug resistance in Ghana [26]. Thirty-one isolates (30.7%) were confirmed as MRSA. This data is consistent with the recognized fact that Africa has an intermediate prevalence of MRSA, usually between 25-50% [27]. Out of the thirty-one isolates, seven showed susceptible phenotypes though the *mecA* gene was detected in molecular analysis. Phenotypically susceptible *mecA* positive clones have been reported by various research groups [28-29]. The MRSA phenotype is regulated by two genes *mecI-mecR1*. The induction of MRSA expression by this system is however very slow and may render some isolates with the *mecA* gene phenotypically susceptible. The existence of such strains represents a hidden reservoir for transmission of the methicillin resistance gene in any environment. In many resource limited settings, clinicians mostly rely on the results of culture and drug susceptibility testing to guide treatment of patients and most laboratories are also not equipped for molecular testing of organisms. As these clones can only be detected through molecular analysis, they will be reported as susceptible organisms which will lead to treatment failure.

Conclusion

Our findings indicate that health-care associated transmission contributes to wound infection and calls for periodic training in IPC practices to prevent the occurrence of epidemics of nosocomial MRSA.

Acknowledgements

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Transparency declarations

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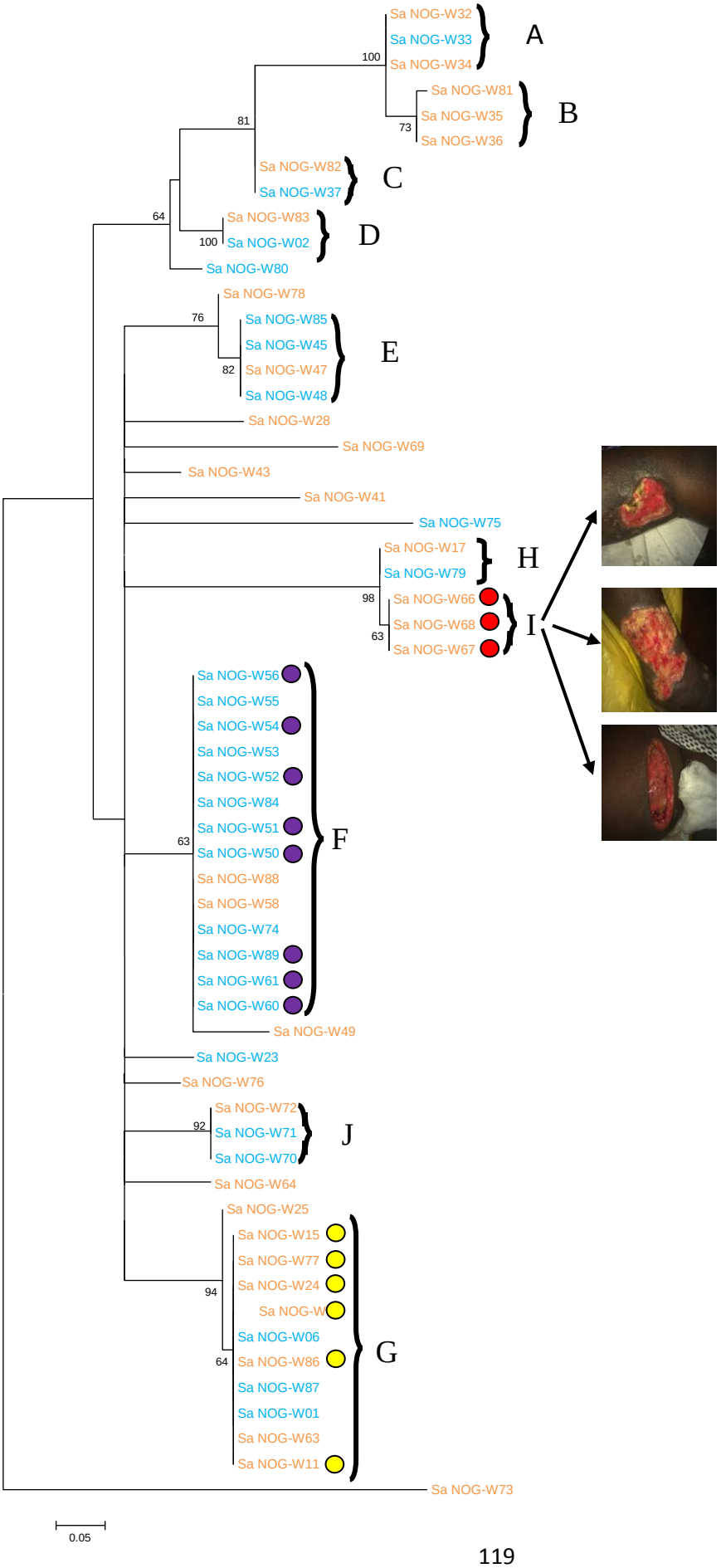
Figures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81
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C
64
Sa NOG-W83
100
Sa NOG-W02
D
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Sa NOG-W78
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82
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Sa NOG-W63
Sa NOG-W11
G
Sa NOG-W73
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Figure1: ***Spa*** **phylogeny showing clusters and relationships between isolates:**

Maximum likelihood phylogeny of *spa* gene. The tree was rooted in the midpoint. Numbers in nodes indicate support values in the form of proportions of bootstrap pseudoreplicates. Branches with support values higher than 55% are collapsed. A-J = the clusters identified. The green colored strains are from Health center A, and the blue ones Health center B. The yellow coloured circles represent MRSA and the violet circles show isolates from a HCW, patients and equipment in health center B.

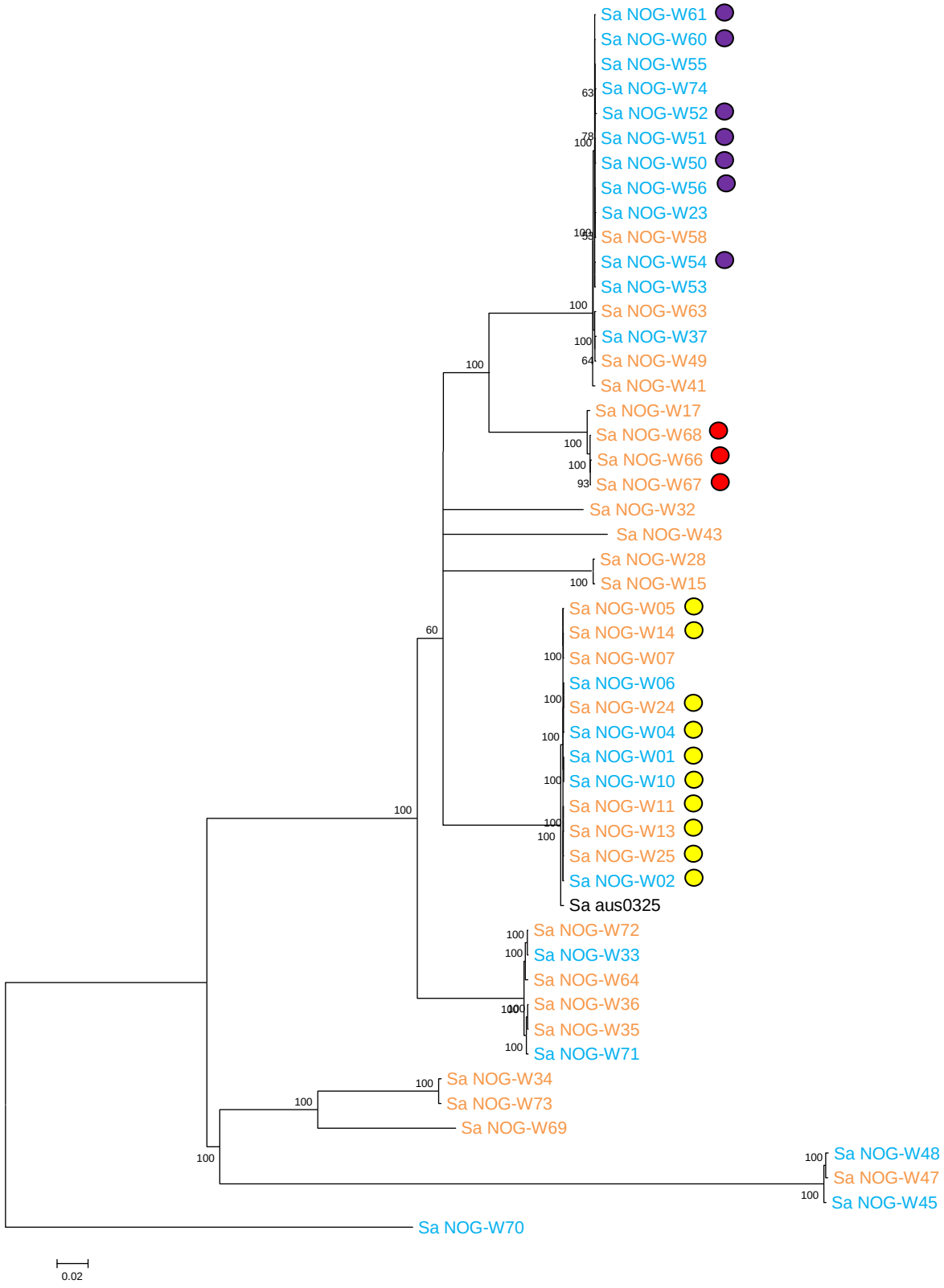
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Figure2: Whole genome phylogeny of sequenced isolates

Maximum likelihood phlogeny of WGS isolates. The tree was rooted in the midpoint. Numbers in nodes indicate support values in the form of proportions of bootstrap pseudoreplicates. Branches with support values higher than 55% are collapsed. Green colored strains are from Health center A and the blue ones Health center B. The red coloured circles represent isolates from patient A, yellow coloured circles represent MRSA and the violet circles show isolates from a HCW, patients and equipment in health center B.

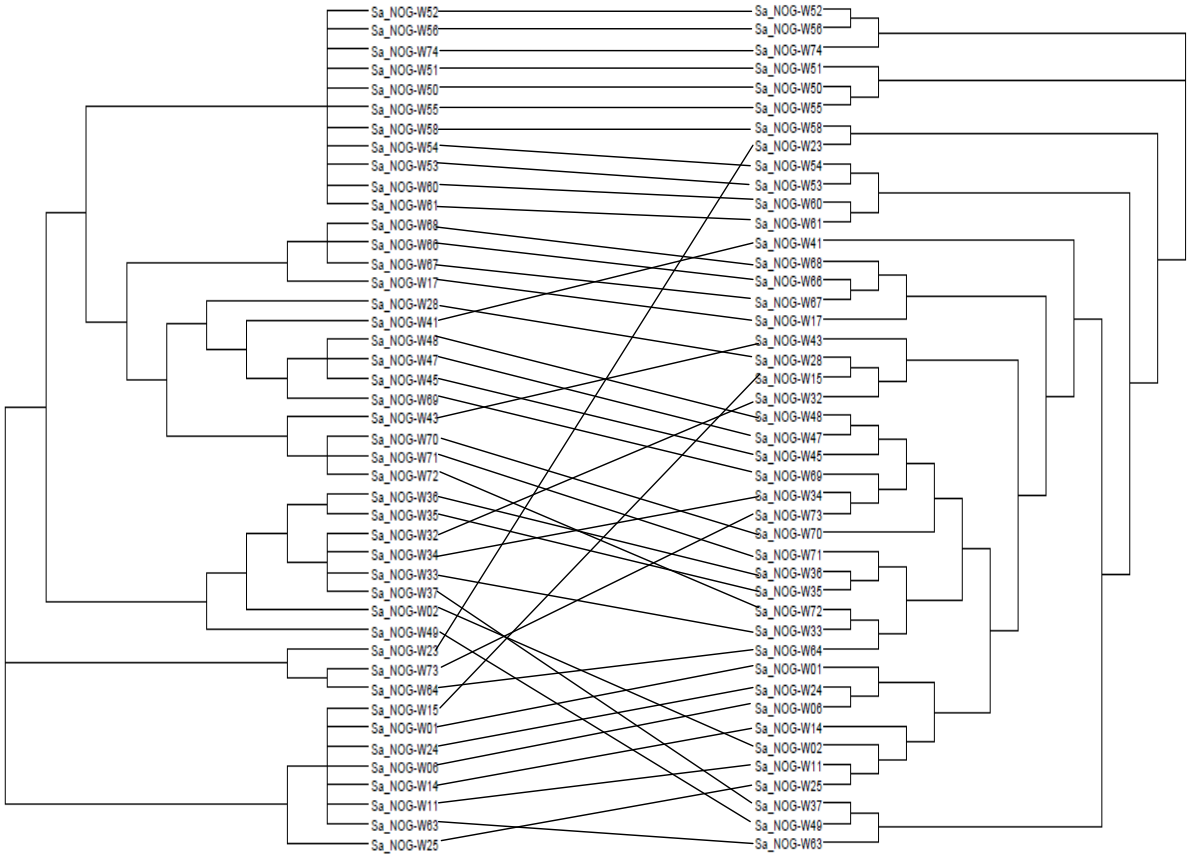

Figure 3: Tanglegram of *spa* and whole genome phylogenies

Tanglegram of *spa* (a) and whole genome sequencing (b) phylogenies produced in dendroscope. Topologies show some agreement between phylogenies but an overall lack of congruence.

Tables

Table 1: Antibiogram of isolates

Antibiotics	Antibiogram Sensitive n(%)	Intermediate n(%)	Resistant n(%)
Amikacin	89 (88)	7 (7)	5 (5)
Cefotaxime	56 (55)	19 (19)	26 (26)
Gentamicin	89 (88)	4 (4)	8 (8)
Tetracycline	39 (38.6)	5 (5)	57 (56.4)
Chloramphenicol	26 (26)	8 (8)	67 (66)
Ceftriaxone	60 (59)	16 (16)	25 (25)
Cotrimoxazole	65 (64.3)	4 (4)	32 (31.6)
Cefuroxime	64 (63)	0	37 (37)
Ampicillin	8 (8)	4 (4)	89 (88)
Clindamycin	52 (51.4)	18 (18)	31 (30.6)
Cefoxitin	66 (65)	5 (5)	30 (30)
Erythromycin	41 (41)	37 (36)	23 (23)
Rifampicin	46 (45.5)	9 (9)	46 (45.5)
Streptomycin	66 (65.3)	9 (9)	26 (25.7)
Vancomycin	75 (74)		26 (26)

Table 2: Comparison of antibiotic resistance between MRSA and MSSA

Antibiotics	Resistance rates		p-value
	MRSA, N=31 n(%)	MSSA, N=70 n(%)	
Amikacin	3 (9.6)	2 (2.8)	0.167
Cefotaxime	17 (54.8)	9 (12.9)	0.000
Gentamicin	3 (9.6)	5 (7.1)	0.698
Tetracycline	24 (77.4)	33 (47.1)	0.005
Chloramphenicol	24 (77.4)	43 (61.4)	0.170
Ceftriaxone	17 (54.8)	8 (11.4)	0.000
Cotrimoxazole	10 (32.3)	22 (31.4)	1.000
Cefuroxime	9 (29.0)	28 (40.0)	0.372
Ampicillin	29 (93.5)	60 (85.7)	0.335
Clindamycin	6 (19.4)	25 (35.7)	0.110
Cefoxitin	19 (61.3)	11 (15.7)	0.000
Erythromycin	8 (25.8)	15 (21.4)	0.617
Rifampicin	11 (35.5)	35 (50.0)	0.200
Streptomycin	5 (16.1)	21 (30.0)	0.217
Vancomycin	11 (35.5)	15 (21.4)	0.147

Supplementary Materials

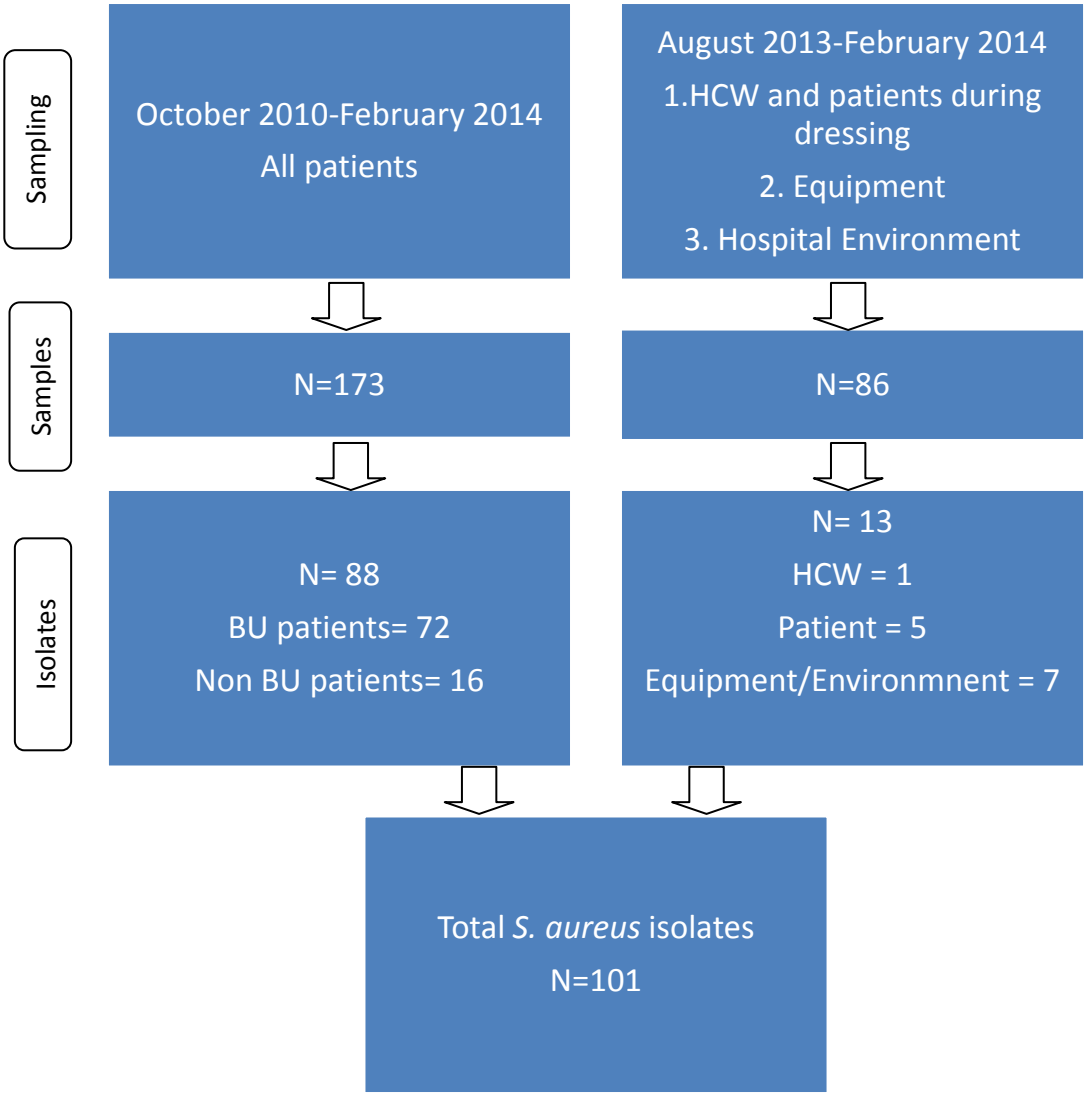

Figure A1: Flowchart of study design

Table A1: Primer sequences

Primer Name	Primer Sequence 5′--3′
spa-1113F	TAAAGACGATCCTTCGGTGAGC
spa-1514R	CAGCAGTAGTGCCGTTTGCTT
spaT3-F	CAACGCAATGGTTTCATCCA
spa-1517R	GCTTTTGCAATGTCATTTACTG
mecA P4	TCCAGATTACAACTTCACCAGG
mecA P7	CCACTTCATATCTTGTAACG
pvl-F	GCTGGACAAAACTTCTTGGAATAT
pvl-R	GATAGGACACCAATAAATTCTGGATTG
pan agr F	ATGCACATGGTGCACATGC
agr 1-R	GTCACAAGTACTATAAGCTGCGAT
agr 2-R	GTATTACTAATTGAAAAGTGCCATAGC
agr 3-R	CTGTTGAAAAAGTCAACTAAAAGCTC
agr 4-R	CGATAATGCCGTAATACCCG

Table A2: Spa types of isolated *Staphylococcus aureus* strains

Spa type	Frequency
t161	1
t 210	1
t 460	1
t 002	1
t 008	1
t 085	1
t 1123	1
t 127	2
t 1458	1
t 186	7
t 208	1
t 2235	1
t 2304	3
t 2422	2
t 2500	3
t 2649	1
t 311	5
t 314	2
t 335	2
t 346	7
t 355	7
t 448	2
t 537	1
t 591	1
t 769	3
t 786	3
t 803	1
t 939	3
t 948	1
unknown	35

Supplementary Data B1

		Antibiotic resistance genes identified										
Isolate ID	**ST**	***blaZ***	***cat***	***cat(pC221)***	***dfrG***	***mecA***	***norA***	***str***	***tet(K)***	***tet(L)***	***tet(M)***	***rpoB* mutation**
Sa_NOG-W01	88	√	.	.	.	√	√	.	.	√	√	.
Sa_NOG-W02	88	√	.	√	.	√	√	.	.	√	√	H481N
Sa_NOG-W04	88	√	.	.	.	√	√	.	.	√	√	.
Sa_NOG-W05	-	√	.	√	√	√	√	.	.	√	√	H481N
Sa_NOG-W06	88	√	.	.	.	√	√	.	.	√	√	.
Sa_NOG-W07	88	√	.	√	.	√	√	.	.	√	√	H481N
Sa_NOG-W10	88	√	.	.	.	√	√	.	.	√	√	.
Sa_NOG-W11	88	√	.	.	√	√	√	.	.	√	√	H481N
Sa_NOG-W13	88	√	.	√	√	√	√	.	.	√	√	.
Sa_NOG-W14	88	√	.	√	.	√	√	.	.	√	√	H481N
Sa_NOG-W15	72	√	.	√	.	√	√	.	√	.	.	.
Sa_NOG-W17	1	√	.	.	√	.	√	.	.	.	.	.
Sa_NOG-W23	15	√	.	√	.	.	√	√	.	.	.	.
Sa_NOG-W24	88	√	.	.	.	√	√	.	.	√	√	.
Sa_NOG-W25	88	√	.	√	√	√	√	.	.	√	√	.
Sa_NOG-W28	72	√	.	.	.	.	√	.	.	.	.	.
Sa_NOG-W32	6	√	.	.	.	.	√	.	.	.	.	.
Sa_NOG-W33	5	√	.	.	.	.	√	.	.	.	.	.
Sa_NOG-W34	121	√	.	.	√	.	√	.	.	.	.	.
Sa_NOG-W35	5	√	.	.	.	.	√	.	.	.	.	H481N
Sa_NOG-W36	5	√	.	.	.	.	√	.	.	.	.	H481N
Sa_NOG-W37	-	√	.	.	√	.	√	.	.	.	.	.
Sa_NOG-W41	2434	√	√	.	√	.	√	.	.	.	.	.
Sa_NOG-W43	-	√	.	.	√	.	√	.	.	.	.	.
Sa_NOG-W45	152	√	.	.	.	.	√	.	.	.	.	I527T
Sa_NOG-W47	152	.	.	.	.	.	√	.	.	.	.	.
Sa_NOG-W48	152	√	.	√	.	.	√	.	.	.	.	.
Sa_NOG-W49	15	√	.	√	√	.	√	.	√	.	.	.
Sa_NOG-W50	15	√	.	√	.	.	√	√	.	.	.	.
Sa_NOG-W51	15	√	.	√	.	.	√	√	.	.	.	.
Sa_NOG-W52	15	√	.	√	.	.	√	√	.	.	.	.
Sa_NOG-W53	-	√	.	√	.	.	√	√	.	.	.	.
Sa_NOG-W54	15	√	.	√	.	.	√	√	.	.	.	.
Sa_NOG-W55	15	√	.	√	.	.	√	√	.	.	.	.
Sa_NOG-W56	15	√	.	√	.	.	√	√	.	.	.	.
Sa_NOG-W58	15	√	.	√	.	.	√	√	.	.	.	.
Sa_NOG-W60	15	√	.	√	.	.	√	√	.	.	.	.
Sa_NOG-W61	15	√	.	√	.	.	√	√	.	.	.	.
Sa_NOG-W63	15	√	.	.	√	.	√	.	√	.	.	.
Sa_NOG-W64	5	√	.	.	√	.	√	.	.	.	.	.
Sa_NOG-W66	3248	√	.	.	.	.	√	.	√	.	.	.
Sa_NOG-W67	3248	√	.	.	.	.	√	.	√	.	.	.
Sa_NOG-W68	3248	√	.	.	.	.	√	.	√	.	.	.

Sa_NOG-W69	707	√	.	.	.	.	√	.	.	.	.	.
Sa_NOG-W70	395	√	.	√	.	.	√	.	√	.	.	.
Sa_NOG-W71	5	√	.	.	.	.	√	.	.	.	.	.
Sa_NOG-W72	5	√	.	.	.	.	√	.	.	.	.	.
Sa_NOG-W73	121	√	.	.	√	.	√	.	√	.	.	.
Sa_NOG-W74	15	√	.	√	.	.	√	√	.	.	.	.

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ID	Reference	Sa_NOG-W01	Sa_NOG-W02	Sa_NOG-W04	Sa_NOG-W05	Sa_NOG-W06	Sa_NOG-W07	Sa_NOG-W10	Sa_NOG-W11	Sa_NOG-W13	Sa_NOG-W14	Sa_NOG-W15	Sa_NOG-W17	Sa_NOG-W23	Sa_NOG-W24	Sa_NOG-W25	Sa_NOG-W28
Reference	0	382	395	395	379	382	374	382	375	380	361	13166	13358	13740	378	386	13149
Sa_NOG-W01	382	0	111	107	95	94	90	0	91	96	77	13153	13343	13726	90	102	13135
Sa_NOG-W02	395	111	0	124	108	111	103	111	90	95	90	13168	13357	13739	107	101	13150
Sa_NOG-W04	395	107	124	0	108	49	103	107	104	109	90	13165	13360	13737	49	115	13148
Sa_NOG-W05	379	95	108	108	0	95	87	95	88	93	74	13152	13344	13725	91	99	13134
Sa_NOG-W06	382	94	111	49	95	0	90	94	91	96	77	13153	13347	13724	24	102	13135
Sa_NOG-W07	374	90	103	103	87	90	0	90	83	88	49	13145	13338	13720	86	94	13127
Sa_NOG-W10	382	0	111	107	95	94	90	0	91	96	77	13153	13343	13726	90	102	13135
Sa_NOG-W11	375	91	90	104	88	91	83	91	0	47	70	13146	13339	13719	87	53	13128
Sa_NOG-W13	380	96	95	109	93	96	88	96	47	0	75	13151	13344	13722	92	58	13133
Sa_NOG-W14	361	77	90	90	74	77	49	77	70	75	0	13134	13326	13707	73	81	13116
Sa_NOG-W15	13166	13153	13168	13165	13152	13153	13145	13153	13146	13151	13134	0	12505	12858	13149	13157	165
Sa_NOG-W17	13358	13343	13357	13360	13344	13347	13338	13343	13339	13344	13326	12505	0	12114	13343	13350	12516
Sa_NOG-W23	13740	13726	13739	13737	13725	13724	13720	13726	13719	13722	13707	12858	12114	0	13722	13730	12849
Sa_NOG-W24	378	90	107	49	91	24	86	90	87	92	73	13149	13343	13722	0	98	13131
Sa_NOG-W25	386	102	101	115	99	102	94	102	53	58	81	13157	13350	13730	98	0	13139
Sa_NOG-W28	13149	13135	13150	13148	13134	13135	13127	13135	13128	13133	13116	165	12516	12849	13131	13139	0
Sa_NOG-W32	13438	13418	13431	13432	13416	13419	13411	13418	13410	13415	13398	12350	12647	12971	13415	13419	12334
Sa_NOG-W33	13914	13902	13918	13919	13904	13907	13899	13902	13899	13904	13886	13674	14207	13769	13903	13910	13684
Sa_NOG-W34	29191	29177	29196	29195	29179	29183	29174	29177	29172	29180	29162	28926	28858	29369	29179	29186	28921
Sa_NOG-W35	13936	13924	13940	13941	13926	13929	13921	13924	13921	13926	13908	13703	14223	13782	13925	13932	13707
Sa_NOG-W36	13942	13930	13946	13947	13932	13935	13927	13930	13927	13932	13914	13709	14231	13792	13931	13938	13709
Sa_NOG-W37	13723	13709	13722	13724	13708	13711	13703	13709	13702	13707	13690	12866	12107	237	13707	13713	12855
Sa_NOG-W41	13741	13727	13740	13742	13726	13729	13721	13727	13720	13725	13708	12881	12121	267	13725	13731	12874
Sa_NOG-W43	13997	13982	13996	13998	13982	13985	13975	13982	13976	13981	13963	12870	13300	13550	13981	13987	12850
Sa_NOG-W45	42551	42533	42551	42550	42536	42538	42532	42533	42531	42538	42518	42455	42465	42667	42534	42541	42435

Sa_NOG-W47	42577	42559	42577	42576	42562	42564	42558	42559	42557	42564	42544	42506	42490	42696	42560	42567	42486
Sa_NOG-W48	42565	42549	42567	42566	42552	42554	42548	42549	42547	42554	42534	42476	42501	42688	42550	42557	42456
Sa_NOG-W49	13754	13740	13753	13755	13739	13742	13734	13740	13733	13738	13721	12872	12124	232	13738	13744	12863
Sa_NOG-W50	13755	13741	13754	13752	13740	13739	13735	13741	13734	13737	13722	12873	12125	39	13737	13745	12858
Sa_NOG-W51	13755	13741	13754	13752	13740	13739	13735	13741	13734	13737	13722	12863	12129	43	13737	13745	12858
Sa_NOG-W52	13736	13722	13735	13733	13721	13720	13716	13722	13715	13718	13703	12876	12134	70	13718	13726	12865
Sa_NOG-W53	13744	13730	13743	13741	13729	13728	13724	13730	13723	13726	13711	12865	12112	50	13726	13734	12854
Sa_NOG-W54	13754	13740	13753	13751	13739	13738	13734	13740	13733	13736	13721	12867	12116	52	13736	13744	12856
Sa_NOG-W55	13769	13755	13768	13766	13754	13753	13749	13755	13748	13751	13736	12873	12123	43	13751	13759	12862
Sa_NOG-W56	13756	13742	13755	13753	13741	13740	13736	13742	13735	13738	13723	12870	12118	40	13738	13746	12861
Sa_NOG-W58	13752	13738	13751	13749	13737	13736	13732	13738	13731	13734	13719	12864	12116	32	13734	13742	12857
Sa_NOG-W60	13750	13736	13749	13747	13735	13734	13730	13736	13729	13732	13717	12863	12120	44	13732	13740	12856
Sa_NOG-W61	13749	13735	13748	13746	13734	13733	13729	13735	13728	13731	13716	12864	12115	41	13731	13739	12857
Sa_NOG-W63	13751	13737	13750	13752	13736	13739	13731	13737	13730	13735	13718	12874	12124	235	13735	13741	12867
Sa_NOG-W64	13936	13924	13940	13941	13926	13929	13921	13924	13921	13926	13908	13688	14215	13781	13925	13932	13694
Sa_NOG-W66	13365	13354	13368	13371	13355	13358	13349	13354	13350	13355	13337	12527	340	12156	13354	13361	12537
Sa_NOG-W67	13370	13355	13369	13372	13356	13359	13350	13355	13351	13356	13338	12528	335	12151	13355	13362	12535
Sa_NOG-W68	13354	13339	13353	13356	13340	13343	13334	13339	13335	13340	13322	12530	339	12135	13339	13346	12521
Sa_NOG-W69	29775	29756	29773	29769	29754	29758	29752	29756	29752	29758	29740	29632	29554	30079	29756	29764	29627
Sa_NOG-W70	43058	43039	43056	43057	43037	43044	43036	43039	43034	43040	43024	42713	42751	43174	43040	43045	42731
Sa_NOG-W71	13945	13933	13949	13950	13935	13938	13930	13933	13930	13935	13917	13702	14229	13788	13934	13941	13706
Sa_NOG-W72	13911	13897	13913	13914	13899	13902	13894	13897	13894	13899	13881	13668	14196	13749	13898	13905	13675
Sa_NOG-W73	29101	29087	29106	29105	29089	29093	29084	29087	29082	29090	29072	28868	28790	29315	29089	29096	28857
Sa_NOG-W74	13742	13728	13741	13739	13727	13726	13722	13728	13721	13724	13709	12868	12122	54	13724	13732	12859

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ID	Sa_NOG-W32	Sa_NOG-W33	Sa_NOG-W34	Sa_NOG-W35	Sa_NOG-W36	Sa_NOG-W37	Sa_NOG-W41	Sa_NOG-W43	Sa_NOG-W45	Sa_NOG-W47	Sa_NOG-W48	Sa_NOG-W49	Sa_NOG-W50	Sa_NOG-W51	Sa_NOG-W52	Sa_NOG-W53	Sa_NOG-W54
Reference	13438	13914	29191	13936	13942	13723	13741	13997	42551	42577	42565	13754	13755	13755	13736	13744	13754
Sa_NOG-W01	13418	13902	29177	13924	13930	13709	13727	13982	42533	42559	42549	13740	13741	13741	13722	13730	13740
Sa_NOG-W02	13431	13918	29196	13940	13946	13722	13740	13996	42551	42577	42567	13753	13754	13754	13735	13743	13753
Sa_NOG-W04	13432	13919	29195	13941	13947	13724	13742	13998	42550	42576	42566	13755	13752	13752	13733	13741	13751
Sa_NOG-W05	13416	13904	29179	13926	13932	13708	13726	13982	42536	42562	42552	13739	13740	13740	13721	13729	13739
Sa_NOG-W06	13419	13907	29183	13929	13935	13711	13729	13985	42538	42564	42554	13742	13739	13739	13720	13728	13738
Sa_NOG-W07	13411	13899	29174	13921	13927	13703	13721	13975	42532	42558	42548	13734	13735	13735	13716	13724	13734
Sa_NOG-W10	13418	13902	29177	13924	13930	13709	13727	13982	42533	42559	42549	13740	13741	13741	13722	13730	13740
Sa_NOG-W11	13410	13899	29172	13921	13927	13702	13720	13976	42531	42557	42547	13733	13734	13734	13715	13723	13733
Sa_NOG-W13	13415	13904	29180	13926	13932	13707	13725	13981	42538	42564	42554	13738	13737	13737	13718	13726	13736
Sa_NOG-W14	13398	13886	29162	13908	13914	13690	13708	13963	42518	42544	42534	13721	13722	13722	13703	13711	13721
Sa_NOG-W15	12350	13674	28926	13703	13709	12866	12881	12870	42455	42506	42476	12872	12873	12863	12876	12865	12867
Sa_NOG-W17	12647	14207	28858	14223	14231	12107	12121	13300	42465	42490	42501	12124	12125	12129	12134	12112	12116
Sa_NOG-W23	12971	13769	29369	13782	13792	237	267	13550	42667	42696	42688	232	39	43	70	50	52
Sa_NOG-W24	13415	13903	29179	13925	13931	13707	13725	13981	42534	42560	42550	13738	13737	13737	13718	13726	13736
Sa_NOG-W25	13419	13910	29186	13932	13938	13713	13731	13987	42541	42567	42557	13744	13745	13745	13726	13734	13744
Sa_NOG-W28	12334	13684	28921	13707	13709	12855	12874	12850	42435	42486	42456	12863	12858	12858	12865	12854	12856
Sa_NOG-W32	0	13689	28949	13707	13713	12962	12995	13076	42403	42436	42443	12977	12983	12983	13002	12974	12978
Sa_NOG-W33	13689	0	29222	422	434	13761	13783	14343	42442	42462	42467	13766	13772	13766	13777	13757	13771
Sa_NOG-W34	28949	29222	0	29253	29248	29369	29393	29165	38693	38710	38718	29368	29386	29382	29384	29375	29381
Sa_NOG-W35	13707	422	29253	0	42	13775	13796	14380	42478	42494	42501	13776	13785	13781	13790	13770	13786
Sa_NOG-W36	13713	434	29248	42	0	13787	13806	14376	42475	42491	42498	13786	13797	13791	13800	13780	13796
Sa_NOG-W37	12962	13761	29369	13775	13787	0	269	13555	42663	42692	42684	93	228	232	247	225	227
Sa_NOG-W41	12995	13783	29393	13796	13806	269	0	13575	42693	42722	42714	258	266	264	293	259	255
Sa_NOG-W43	13076	14343	29165	14380	14376	13555	13575	0	42616	42655	42657	13566	13567	13571	13561	13552	13565
Sa_NOG-W45	42403	42442	38693	42478	42475	42663	42693	42616	0	276	304	42676	42683	42677	42687	42672	42675

Sa_NOG-W47	42436	42462	38710	42494	42491	42692	42722	42655	276	0	214	42707	42714	42708	42708	42701	42706
Sa_NOG-W48	42443	42467	38718	42501	42498	42684	42714	42657	304	214	0	42697	42704	42698	42708	42693	42696
Sa_NOG-W49	12977	13766	29368	13776	13786	93	258	13566	42676	42707	42697	0	221	221	246	222	220
Sa_NOG-W50	12983	13772	29386	13785	13797	228	266	13567	42683	42714	42704	221	0	26	53	35	35
Sa_NOG-W51	12983	13766	29382	13781	13791	232	264	13571	42677	42708	42698	221	26	0	53	37	39
Sa_NOG-W52	13002	13777	29384	13790	13800	247	293	13561	42687	42708	42708	246	53	53	0	58	62
Sa_NOG-W53	12974	13757	29375	13770	13780	225	259	13552	42672	42701	42693	222	35	37	58	0	28
Sa_NOG-W54	12978	13771	29381	13786	13796	227	255	13565	42675	42706	42696	220	35	39	62	28	0
Sa_NOG-W55	12985	13780	29388	13793	13803	228	264	13567	42683	42712	42704	223	24	28	51	31	27
Sa_NOG-W56	12984	13775	29377	13788	13798	221	257	13558	42676	42705	42697	220	29	25	46	30	36
Sa_NOG-W58	12980	13765	29383	13778	13788	227	259	13560	42672	42701	42693	216	25	25	56	32	34
Sa_NOG-W60	12980	13763	29373	13778	13788	221	257	13556	42666	42697	42687	214	35	35	56	30	30
Sa_NOG-W61	12977	13764	29370	13777	13787	216	244	13553	42669	42698	42690	207	30	28	53	21	25
Sa_NOG-W63	12979	13766	29373	13780	13790	100	259	13567	42669	42700	42690	87	222	218	251	223	221
Sa_NOG-W64	13705	283	29237	440	446	13773	13795	14359	42446	42468	42471	13784	13782	13780	13793	13771	13785
Sa_NOG-W66	12669	14230	28865	14247	14249	12145	12156	13328	42481	42510	42517	12161	12163	12167	12181	12148	12152
Sa_NOG-W67	12657	14231	28861	14248	14242	12140	12149	13321	42478	42507	42516	12156	12160	12164	12176	12143	12149
Sa_NOG-W68	12646	14241	28863	14258	14258	12128	12133	13305	42458	42487	42494	12140	12148	12148	12164	12131	12135
Sa_NOG-W69	29728	30002	14952	30031	30025	30082	30108	29867	39511	39522	39533	30089	30096	30090	30096	30085	30085
Sa_NOG-W70	42845	42715	39358	42732	42733	43169	43197	42560	51743	51763	51772	43177	43185	43185	43188	43176	43174
Sa_NOG-W71	13715	434	29255	193	195	13779	13805	14364	42474	42492	42497	13790	13789	13787	13800	13778	13792
Sa_NOG-W72	13684	37	29220	439	453	13741	13763	14330	42436	42456	42461	13754	13750	13750	13761	13739	13753
Sa_NOG-W73	28876	29162	254	29192	29188	29313	29336	29086	38653	38672	38696	29312	29332	29326	29328	29319	29325
Sa_NOG-W74	12982	13773	29377	13788	13798	241	279	13564	42674	42705	42693	226	43	39	64	50	50

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ID	Sa_NOG-W55	Sa_NOG-W56	Sa_NOG-W58	Sa_NOG-W60	Sa_NOG-W61	Sa_NOG-W63	Sa_NOG-W64	Sa_NOG-W66	Sa_NOG-W67	Sa_NOG-W68	Sa_NOG-W69	Sa_NOG-W70	Sa_NOG-W71	Sa_NOG-W72	Sa_NOG-W73	Sa_NOG-W74
Reference	13769	13756	13752	13750	13749	13751	13936	13365	13370	13354	29775	43058	13945	13911	29101	13742
Sa_NOG-W01	13755	13742	13738	13736	13735	13737	13924	13354	13355	13339	29756	43039	13933	13897	29087	13728
Sa_NOG-W02	13768	13755	13751	13749	13748	13750	13940	13368	13369	13353	29773	43056	13949	13913	29106	13741
Sa_NOG-W04	13766	13753	13749	13747	13746	13752	13941	13371	13372	13356	29769	43057	13950	13914	29105	13739
Sa_NOG-W05	13754	13741	13737	13735	13734	13736	13926	13355	13356	13340	29754	43037	13935	13899	29089	13727
Sa_NOG-W06	13753	13740	13736	13734	13733	13739	13929	13358	13359	13343	29758	43044	13938	13902	29093	13726
Sa_NOG-W07	13749	13736	13732	13730	13729	13731	13921	13349	13350	13334	29752	43036	13930	13894	29084	13722
Sa_NOG-W10	13755	13742	13738	13736	13735	13737	13924	13354	13355	13339	29756	43039	13933	13897	29087	13728
Sa_NOG-W11	13748	13735	13731	13729	13728	13730	13921	13350	13351	13335	29752	43034	13930	13894	29082	13721
Sa_NOG-W13	13751	13738	13734	13732	13731	13735	13926	13355	13356	13340	29758	43040	13935	13899	29090	13724
Sa_NOG-W14	13736	13723	13719	13717	13716	13718	13908	13337	13338	13322	29740	43024	13917	13881	29072	13709
Sa_NOG-W15	12873	12870	12864	12863	12864	12874	13688	12527	12528	12530	29632	42713	13702	13668	28868	12868
Sa_NOG-W17	12123	12118	12116	12120	12115	12124	14215	340	335	339	29554	42751	14229	14196	28790	12122
Sa_NOG-W23	43	40	32	44	41	235	13781	12156	12151	12135	30079	43174	13788	13749	29315	54
Sa_NOG-W24	13751	13738	13734	13732	13731	13735	13925	13354	13355	13339	29756	43040	13934	13898	29089	13724
Sa_NOG-W25	13759	13746	13742	13740	13739	13741	13932	13361	13362	13346	29764	43045	13941	13905	29096	13732
Sa_NOG-W28	12862	12861	12857	12856	12857	12867	13694	12537	12535	12521	29627	42731	13706	13675	28857	12859
Sa_NOG-W32	12985	12984	12980	12980	12977	12979	13705	12669	12657	12646	29728	42845	13715	13684	28876	12982
Sa_NOG-W33	13780	13775	13765	13763	13764	13766	283	14230	14231	14241	30002	42715	434	37	29162	13773
Sa_NOG-W34	29388	29377	29383	29373	29370	29373	29237	28865	28861	28863	14952	39358	29255	29220	254	29377
Sa_NOG-W35	13793	13788	13778	13778	13777	13780	440	14247	14248	14258	30031	42732	193	439	29192	13788
Sa_NOG-W36	13803	13798	13788	13788	13787	13790	446	14249	14242	14258	30025	42733	195	453	29188	13798
Sa_NOG-W37	228	221	227	221	216	100	13773	12145	12140	12128	30082	43169	13779	13741	29313	241
Sa_NOG-W41	264	257	259	257	244	259	13795	12156	12149	12133	30108	43197	13805	13763	29336	279
Sa_NOG-W43	13567	13558	13560	13556	13553	13567	14359	13328	13321	13305	29867	42560	14364	14330	29086	13564
Sa_NOG-W45	42683	42676	42672	42666	42669	42669	42446	42481	42478	42458	39511	51743	42474	42436	38653	42674

Sa_NOG-W47	42712	42705	42701	42697	42698	42700	42468	42510	42507	42487	39522	51763	42492	42456	38672	42705
Sa_NOG-W48	42704	42697	42693	42687	42690	42690	42471	42517	42516	42494	39533	51772	42497	42461	38696	42693
Sa_NOG-W49	223	220	216	214	207	87	13784	12161	12156	12140	30089	43177	13790	13754	29312	226
Sa_NOG-W50	24	29	25	35	30	222	13782	12163	12160	12148	30096	43185	13789	13750	29332	43
Sa_NOG-W51	28	25	25	35	28	218	13780	12167	12164	12148	30090	43185	13787	13750	29326	39
Sa_NOG-W52	51	46	56	56	53	251	13793	12181	12176	12164	30096	43188	13800	13761	29328	64
Sa_NOG-W53	31	30	32	30	21	223	13771	12148	12143	12131	30085	43176	13778	13739	29319	50
Sa_NOG-W54	27	36	34	30	25	221	13785	12152	12149	12135	30085	43174	13792	13753	29325	50
Sa_NOG-W55	0	23	29	33	24	222	13792	12165	12160	12146	30096	43189	13799	13760	29332	41
Sa_NOG-W56	23	0	26	34	19	219	13789	12160	12153	12141	30087	43182	13796	13757	29321	32
Sa_NOG-W58	29	26	0	28	25	217	13779	12156	12151	12135	30091	43188	13786	13749	29327	36
Sa_NOG-W60	33	34	28	0	17	211	13775	12159	12154	12138	30081	43173	13782	13743	29317	42
Sa_NOG-W61	24	19	25	17	0	208	13776	12153	12146	12130	30078	43173	13783	13744	29314	39
Sa_NOG-W63	222	219	217	211	208	0	13784	12159	12156	12140	30089	43180	13790	13754	29317	225
Sa_NOG-W64	13792	13789	13779	13775	13776	13784	0	14234	14233	14249	30012	42716	430	292	29175	13789
Sa_NOG-W66	12165	12160	12156	12159	12153	12159	14234	0	29	51	29563	42762	14248	14221	28798	12162
Sa_NOG-W67	12160	12153	12151	12154	12146	12156	14233	29	0	34	29560	42763	14247	14220	28796	12157
Sa_NOG-W68	12146	12141	12135	12138	12130	12140	14249	51	34	0	29559	42765	14263	14230	28788	12141
Sa_NOG-W69	30096	30087	30091	30081	30078	30089	30012	29563	29560	29559	0	39809	30027	29990	14974	30087
Sa_NOG-W70	43189	43182	43188	43173	43173	43180	42716	42762	42763	42765	39809	0	42744	42698	39346	43170
Sa_NOG-W71	13799	13796	13786	13782	13783	13790	430	14248	14247	14263	30027	42744	0	437	29190	13796
Sa_NOG-W72	13760	13757	13749	13743	13744	13754	292	14221	14220	14230	29990	42698	437	0	29154	13759
Sa_NOG-W73	29332	29321	29327	29317	29314	29317	29175	28798	28796	28788	14974	39346	29190	29154	0	29313
Sa_NOG-W74	41	32	36	42	39	225	13789	12162	12157	12141	30087	43170	13796	13759	29313	0

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Chapter 5

Genomic analysis of ST88 Community-Acquired methicillin resistant *Staphylococcus aureus* in Ghana

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Genomic analysis of ST88 Community-Acquired methicillin resistant *Staphylococcus aureus* in Ghana

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Abstract

Background. The emergence and evolution of community-acquired methicillin resistant *Staphylococcus aureus* (CA-MRSA) strains in Africa is poorly understood. However, one particular MRSA lineage called ST88, appears to be rapidly establishing itself as an ``African´´ CA-MRSA clone. In this study, we employed whole genome sequencing to provide more information on the genetic background of ST88 CA-MRSA isolates from Ghana and to describe in detail ST88 CA-MRSA isolates in comparison with other MRSA lineages worldwide.

Methods. We first established a complete ST88 reference genome (AUS0325) using PacBio SMRT sequencing. We then used comparative genomics to assess relatedness among 17 ST88 CA-MRSA isolates recovered from patients attending Buruli ulcer treatment centers in Ghana, three non-African ST88s and 15 other MRSA lineages.

Results. We show that Ghanaian ST88 forms a discrete MRSA lineage (harbouring SCC*mec*-IV [2B]). Gene content analysis identified five distinct genomic regions enriched among ST88 isolates compared with the other *S. aureus* lineages. The Ghanaian ST88 isolates had only 658 core genome SNPs and there was no correlation between phylogeny and geography, suggesting the recent spread of this clone. The lineage was also resistant to multiple classes of antibiotics including ß-lactams, tetracycline and chloramphenicol.

Discussion. This study reveals that *S. aureus* ST88-IV is a recently emerging and rapidly spreading CA-MRSA clone in Ghana. The study highlights the capacity of small snapshot genomic studies to provide actionable public health information in resource limited settings. To our knowledge this is the first genomic assessment of the ST88 CA-MRSA clone.

Introduction

Since the 1990s, community acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections have been increasing worldwide [1-2]. CA-MRSA clones are known to be more virulent than hospital-acquired MRSA, with infections linked to significant mortality and morbidity [3-8]. First reported in Australia and the United States, CA-MRSA occurrence has been increasing, with epidemics due to clones such as ST8 USA300 in the United States [9], ST93 and ST1 in Australia [10], ST80 in Europe [11], ST59 in China and Taiwan [12], ST772 in India [13-16] and ST72 in South Korea [17]. Other identified CA-MRSA clones belong to ST30 (South West Pacific clone) [18], ST45 (Berlin clone) [19], ST1 (USA400) [16] and ST78 (Western Australian MRSA-2) [18]. In Africa, the distribution of MRSA clones in general is not well understood [20]. A recent review on MRSA in Africa with data from 15 of the 54 countries identified community clones of ST8-IV [2B] (USA300) and ST88-IV [2B] "West Australia MRSA-2 clone" in both community and health care associated infections in seven countries and a "Brazilian/Hungarian clone" ST239-III [3A] in hospital acquired infections in nine countries [20]. The European ST80-IV [2B] clone was limited to Algeria, Egypt and Tunisia while clonal types ST22-IV [2B], ST36-II [2A], and ST612-IV [2B] have only been reported so far in South Africa [20]. Among the two CA-MRSA clones, the ST8IV [2B] clone is an internationally disseminated clone recognized in every continent except Antarctica [21]. The ST88-IV [2B] CA-MRSA clone however is predominant in Sub-Saharan Africa (West, Central and East Africa) with reported rates of 24.2-83.3% of all MRSA isolates [22]. Studies from Angola [23], Cameroon [24], Gabon [25-26], Ghana [27-29], Madagascar [24], Niger [24], Nigeria [30-32] and Senegal [24] have identified it as a major circulating clone within both hospital and community settings. It was also detected in children from West Africa who underwent surgery in Switzerland but had been hospitalized in their home countries prior to surgical treatment [33]. Globally, this clone has been identified in China [34] and Japan [35] in lower proportion (5.3-12.5%) than in Africa and sporadically in Belgium [36], Portugal [37] and Sweden [38].

Control of MRSA infections is assisted by a thorough knowledge of the epidemiology and dissemination of specific clones. To this end we employed whole genome sequencing and comparative genomics to describe in detail ST88 CA-MRSA isolates in comparison to other MRSA lineages worldwide.

Materials & Methods

Bacterial isolates and antibiogram analysis

The 17 ST88 *S. aureus* isolates analyzed from Ghana are listed in Table 1 and comprised five strains isolated in the Akwapim South District (Eastern Region) of Ghana with previously published genome data (GenBank accession numbers LFNJ00000000, LFNI00000000, LFNH00000000, LFMH00000000, LFMG00000000) [29] and 12 isolates recovered from wounds of 11 patients attending Buruli ulcer (BU) treatment centers in the Ga-South and Ga-West municipalities (Greater Accra Region) of Ghana with two isolates from one patient; one a PVL positive isolate and the other PVL negative. Patients were outpatients, nine of whom had laboratory confirmed BU. Initial isolate identification was made using colony and microscopic morphology, catalase and coagulase biochemical reactions and a Staphylase kit BD BBL^{TM} Staphyloslide Latex Test (Becton, Dickinson and Company) for further confirmation. Antibiograms were determined using the Kirby Bauer disc diffusion method according to CLSI guidelines [39] and PCR targeting the *mecA* gene [40] for identification of MRSA. Ethical clearance was obtained from the institutional review board of the Noguchi Memorial Institute for Medical Research (NMIMR) (Federal-wide Assurance number FWA00001824). All study participants were well informed of the study objectives and written informed consent was obtained either from the patient or from the guardian of the patient.

DNA Extraction, Whole Genome Sequencing and Analysis

Genomic DNA was extracted from isolates using the Qiagen DNeasy kit and protocol (Qiagen, Hilden, Germany). DNA libraries were prepared using Nextera XT (Illumina, San Diego, California, USA) and whole genome sequencing was performed using the Illumina MiSeq with 2 x 300 bp chemistry. Small Molecular Real Time sequencing was performed on the RS-II (Pacific Biosciences) using P6-C4 chemistry, and reference genome assembly was performed as described [41].

Read mapping, variant calling and phylogenomic analysis

The sequence reads were processed using *Nullarbor* (nullarbor.pl 0.6, https://github.com/tseemann/nullarbor), a recently developed bioinformatics pipeline for public health microbial genomics as described previously [42]. *S. aureus* ST88 raw sequence reads with accession numbers ERS1354589-600 have been deposited in the European Nucleotide Archive (ENA), Project PRJEB15428 (url - http://www.ebi.ac.uk/ena/data/view/PRJEB15428. Ortholog clustering was performed using Roary [43] and was visualized with Fripan (http://drpowell.github.io/FriPan/). Recombination within the core genome was inferred using ClonalFrameML v1.7 [44] with the whole genome alignment generated by Nullarbor. Using FastTree v2.1.8 [45], a ML tree was generated and used as a guide tree for ClonalFrameML. Positions in the reference genome that were not present in at least one genome (non-core) were omitted from the analysis using the "ignore_incomplete_sites true" option and providing ClonalFrameML with a list of all non-core positions. Maximum likelihood trees were constructed using a recombination free SNP alignment using FastTree. Bootstrap support was derived from comparisons between the original tree against 1000 trees that were built upon pseudo-alignments (sampled from the original alignment with replacement).

Results and Discussion

ST88 complete reference genome

A prerequisite for high-resolution comparative genomics by read-mapping is a high-quality, complete reference genome, closely related to the bacterial population under investigation [42]. There were no fully-assembled ST88 *S. aureus* genomes publicly available, so to address this issue, we selected the methicillin-susceptible, penicillin-resistant ST88 *S. aureus* isolate AUS0325. This clinical isolate was obtained in 2013 from a patient in Melbourne, Australia who had a persistent infection of a prosthetic joint, and was part of a separate, unpublished study. The AUS0325 genome comprised a 2,771,577 bp circular chromosome with 32.9% GC content. There were no plasmids; the beta-lactamase operon (*bla*) was carried by the Tn*552* transposon and integrated into the chromosome. The overall chromosome architecture of AUS0325 was like representative *S. aureus* genomes from other community-associated lineages (ST1, ST8 and ST93) but with five distinct regions of difference, discussed in more detail below (Fig. 1A). We took advantage of the PacBio data to define the Sa_aus0325 methylome. Motif analysis and inspection of the AUS0325 annotation identified two active type I restriction modification *hsdMS* loci. Protein alignment of the two *hsdS* alleles with previously characterised *hsdS* proteins allowed the attribution of target recognition sequences to either allele [46] (Table 2). The first *hsdS* recognized a motif not previously described, while the second *hsdS* contained an identical sequence to the target recognition domain-2 of CC30-2 and ST93-2, which recognises TCG (Table 2).

ST88 population structure

To understand the genomic diversity and evolutionary origin of the ST88 isolates, a core genome phylogeny was inferred by mapping reads from the 17 ST88 isolates (Table 1, Fig. 1B), two published ST88 MRSA genomes from Lebanon and USA and 15 other geographically and genetically distinct *S. aureus* clones to AUS0325 (Table 3, Fig. 1B). To assess the clonal ancestry, SNPs within inferred regions of recombination (71,862 clonal SNPs; 26,570 recombinogenic SNPs) (Fig. S1) were removed and a maximum likelihood phylogenomic tree was established using the clonal core SNP alignment (71,862 SNPs). All 20 ST88 genomes formed a discrete, closely related lineage, defined by only 1,759 core genome SNPs, compared with 71,862 SNPs among all 35 *S. aureus* genomes (Fig. 1B, Fig.

2A). The global tree was rooted using *Bacillus_subtilis*_B4068 (GenBank ID: JXHK00000000) [47] as an outgroup and this phylogeny indicated ST88 shares a most recent common ancestor (MRCA) with ST72 (Fig. 1A).

Five distinct genomic regions were identified by ortholog comparisons, enriched among the ST88 genomes compared to the 15 other diverse *S. aureus* genomes. These regions included νSAα (GI-3, Fig. 1) that harboured 10 staphylococcal superantigen-like (*ssl*) genes, of which four were uniquely present in the ST88 isolates. Upregulation of SSLs has been reported in some CA-MRSA strains and may be involved in neutrophil and complement activation [48-49]. GI-3 also harboured the first of the two functional type I restriction modification *hsdMS* loci (see above, Table 2). GI-1 and GI-4 may be mobile integrative elements of unknown function with the presence of putative integrases and four and 12 CDS respectively, all encoding hypothetical proteins. GI-1 also harbours elements of a putative restriction modification system (Fig. 1, Table S1). GI-2 contains 13 CDS, most of unknown function although three CDS may encode membrane proteins (Fig. 1, Table S1). GI-5 had 14 CDS, that included the second of the type I restriction modification *hsdMS* loci and seven CDS encoding putative proteases (Table 2, Table S1).

Evolution and molecular epidemiology of ST88 in Ghana

To assess the evolutionary relationships among the ST88 genomes, a phylogenomic tree comprised exclusively of ST88 genomes was established using clonal, core SNPs (1,759 clonal SNPs; 207 recombinogenic SNPs) (Fig. S2, Fig. 2A). The tree was rooted using an ST93 genome (Sa_JKD6159) as an outgroup. The phylogeny and the restricted genomic diversity (658 core SNPs) suggests that the spread of ST88 MRSA in Ghana is a recent phenomenon, with isolates from the United States, Australia and Lebanon ancestral to the spread of these isolates in Ghana. Five specific clusters of CDS were also found to be exclusively present with the African ST88 genomes (Fig. 2C). These CDS were different to the five genomic regions identified in all ST88 relative to other *S. aureus* clones (Fig. 1B) suggesting that they were horizontally acquired by an ST88 MRCA that has since spread in Ghana, (although a significantly larger sampling effort would be required to confirm this hypothesis). These regions harbour CDS suggestive of plasmid, phage and other mobile DNA elements (Table S2). We conducted a phylogeographic analysis to formally assess the relationship between the Ghanaian ST88 phylogeny and the specific geographic origin of the

isolates, based on patient villages. However, there was no correlation between geography and phylogeny, suggesting again that the spread of ST88 in Ghana has been recent and rapid (Fig. 3).

Phenotypic and genotypic antibiotic resistance

All 17 Ghanaian ST88 isolates harboured a SCC*mec*-IV [2B] cassette, and displayed phenotypic resistance to β-lactams, tetracycline, and chloramphenicol (Table 1). Isolates were variably resistant to erythromycin, clindamycin, trimethoprim, amikacin and streptomycin (Table 1). There was agreement between phenotypic and inferred genotypic resistance (Fig. 1B). For the four genes (*blaZ, mecA, tetL, tetM*) detected in all 12 ST88 isolates from the Greater Accra Region, resistance correlated with phenotypic resistance to all β-lactams and tetracyclines. Six isolates showed phenotypic and genotypic resistance to chloramphenicol (Table 1, Fig. 1B). Five of these isolates were from the same health center, however the time of isolation and the geographic origins of the patients were different, suggesting that these isolates are spread across the region and were not acquired from a common source.

Conclusions

The analysis presented here suggests that *S. aureus* ST88-IV is an emerging CA-MRSA clone in Ghana. This has the potential to become a serious public health threat, with implications for the treatment of *S. aureus* infections in Ghana, where there is no developed surveillance infrastructure to monitor antibiotic resistance. The abuse and misuse of antibiotics by health care givers and patients in Ghana is extensive [50]. The development of resistance is furthermore encouraged by the widespread availability of higher classes of antibiotics to lower level health centers from regional medical stores, in addition to the unrestricted sale of these medicines to over-the-counter medicine sellers by pharmaceutical wholesalers- even though existing laws are supposed to limit the scope of these facilities to handle such medicines. Also implicated and widely documented are the prescribing practices of clinicians; with over-reliance on presumptive treatment and haphazardly prescribing antibiotics without recourse to due laboratory investigation. CA-MRSA has undergone rapid evolution and expansion worldwide. Because of its epidemic potential and limited treatment options, vigilance and antibiotic stewardship programmes need to be put in place to prevent further spread.

Acknowledgements

We are grateful to the health care workers and patients at the Ga West and Ga South Municipalities and to Miss Nana Ama Amissah for giving us access to genome data.

Data Summary

1. Strain data protocol has been deposited in Figshare; DOI: 10.6084/m9.figshare.3863475 (url - https://figshare.com/articles/Table_S1/3863475)
2. *S. aureus* ST88 raw sequence reads have been deposited in ENA, Project PRJEB15428 (url - http://www.ebi.ac.uk/ena/data/view/ PRJEB15428)
3. AUS0325 Chromosome Assembly has been deposited in ENA, LT615218 (url - http://www.ebi.ac.uk/ena/data/view/LT615218)
4. AUS0325 PacBio raw sequence reads have been deposited in ENA, ERS1354601 (url -http://www.ebi.ac.uk/ena/data/view/ERS1354601)

Figures

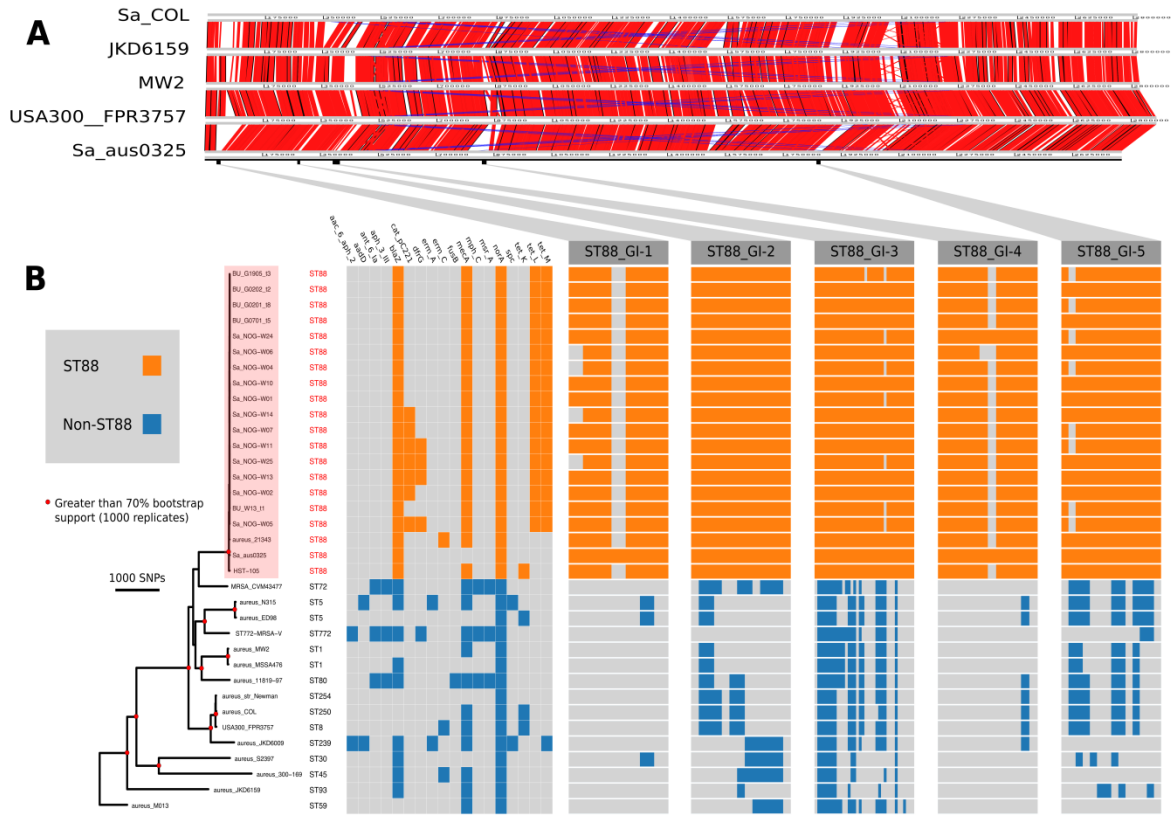

Figure. 1: Comparative genomic analysis of *S. aureus* ST88. (a) DNA-DNA comparisons visualized using the Artemis Comparison Tool of three CA-MRSA representative chromosomes and S. aureus COL against the complete chromosome of ST88 isolate AUS0325. (b) Core genome phylogeny and accessory genome elements identified among ST88 isolates. The phylogeny was based on an alignment of 71,862 non-recombinogenic core genome SNPs (indels excluded) and inferred using FastTree. Nodes with greater than 70% bootstrap support (1000 replicates) are labelled with red dots. Antibiotic resistance genes were identified using Abricate (https://github.com/tseemann/abricate) and genomic islands (GIs) enriched among ST88 isolates were identified by ortholog comparisons using Roary and visualized using FriPan. CDS present in specific GI are listed in Table S1.

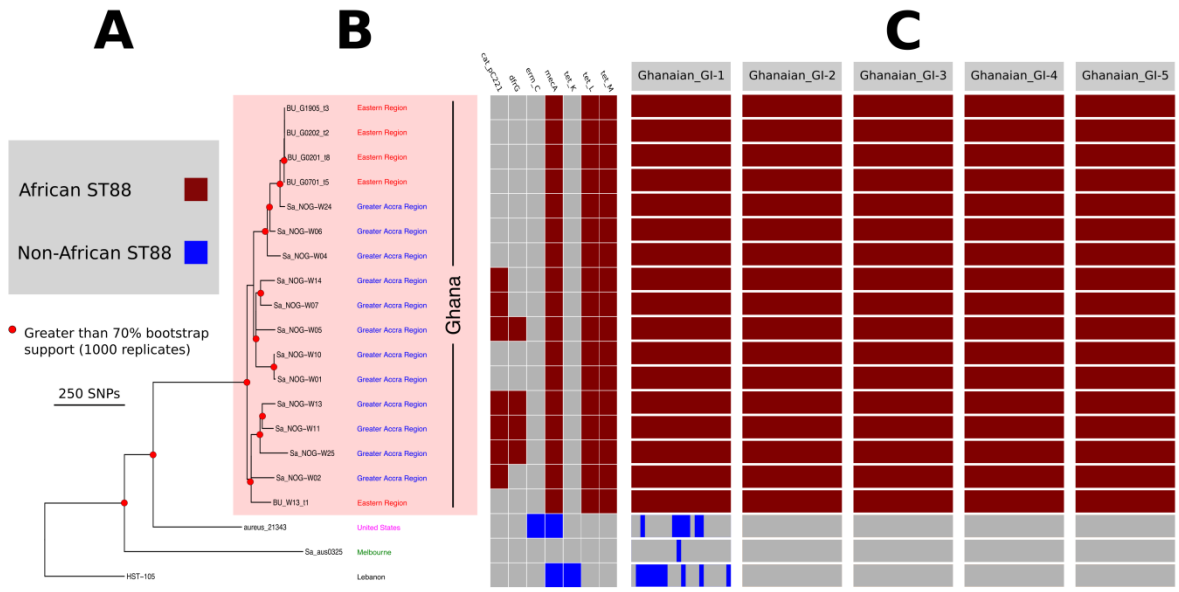

Figure. 2: High resolution ST88 phylogeny and accessory genome analysis. (a). Phylogeny inferred by read-mapping and variant identification among only ST88 genomes. Tree was produced using FastTree based on a pairwise alignment of 1,759 non-recombinogenic core genome SNPs among the 20 ST88 genomes. All major nodes in the tree (red circles) had greater than 70% bootstrap support (1000 replicates). (b) Accessory gene content variation among the 20 ST88 genomes as assessed by ortholog comparisons using Roary. (c) Distinct genomic islands (GI) identified in Ghanaian isolates.

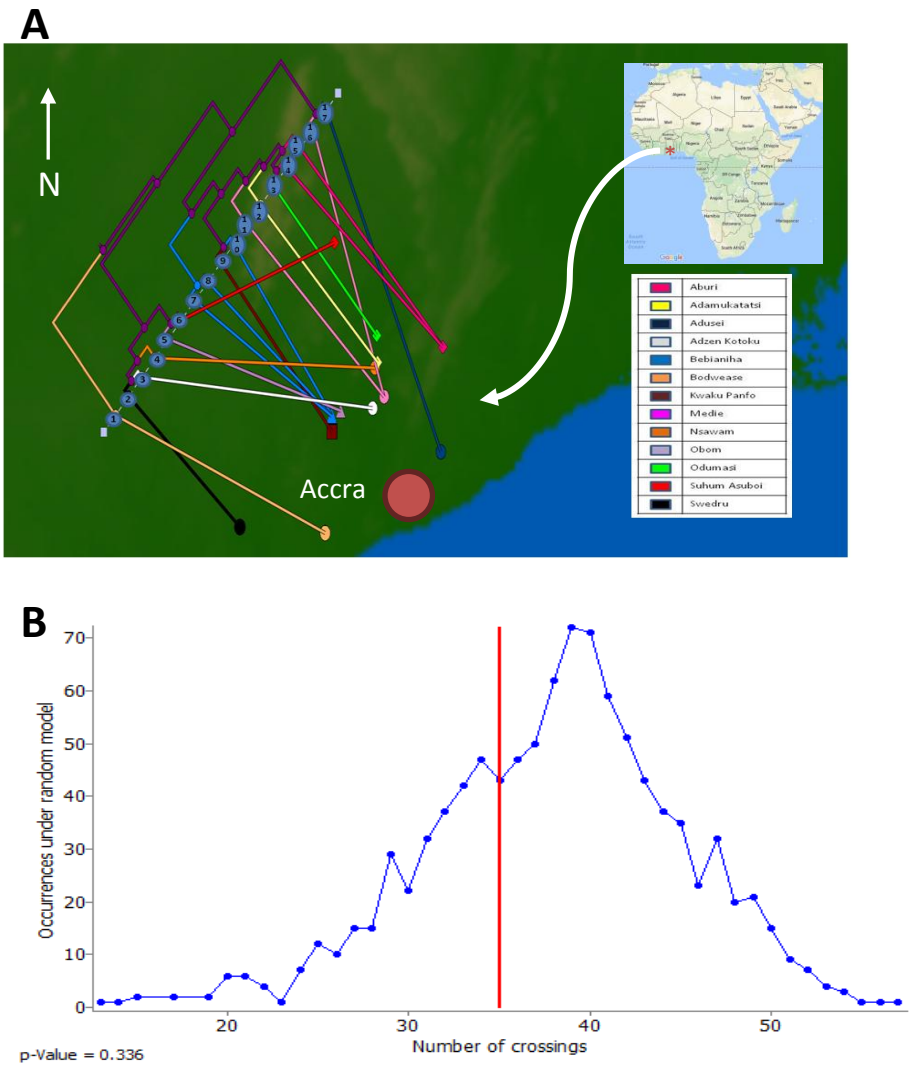

Figure 3: Relationship between phylogeny of Ghanaian ST88 and their geographic origin. (a) Phylogeographic alignment of phylogeny against isolate origin geography performed with GenGIS software and (b) Monte-Carlo analysis following 1000 permutations of tree tips and geography of originating villages. The arrangement derived from the data was not significantly different to that which is expected by chance alone (p value >0.05), indicating a lack of geographical structure among the ST88 genomes.

Tables

Table 1: *S. aureus* ST88 isolates tested in this study

Isolate ID	Origin (Ghana)	Phenotypic resistance*	Genotype (*spa*, *agr*, PVL)	Reference
Sa_NOG-W02	Greater Accra Region	cld, tet, amp, ery, fox, ctx, chl, cro	t939, agr-3, PVL +	This study
Sa_NOG-W25	Greater Accra Region	gen, amk, cld, str, amp, tet, sxt, cfx, ctx, chl, cro	t448, agr-3, PVL -	This study
Sa_NOG-W11	Greater Accra Region	str, amk, gen, sxt, cfx, cld, fox, ctx, tet, chl, cro, amp, ery	t186, agr-3, PVL +	This study
Sa_NOG-W13	Greater Accra Region	gen, str, amk, ctx, tet, chl, cro, sxt, cfx, amp, cld, fox	07-12-12-118-13-13, agr-3, PVL +	This study
Sa_NOG-W01	Greater Accra Region	amk, cfx, tet, ctx, chl, cro, fox	t186, agr-3, PVL +	This study
Sa_NOG-W10	Greater Accra Region	sxt, ery, gen, str, amk, cld, amp, cfx, tet, fox, ctx, chl, cro	t186, agr-3, PVL -	This study
Sa_NOG-W07	Greater Accra Region	gen, str, amp, tet, sxt, cfx, chl, cro, ctx, fox, cld, ery,	t448, agr-3, PVL -	This study
Sa_NOG-W14	Greater Accra Region	gen, ery, sxt, amk, cld, str, tet, amp, cfx, ctx, chl, cro, fox,	t2649, agr-3, PVL +	This study
Sa_NOG-W04	Greater Accra Region	sxt, ery, gen, str, amk, amp, cfx, tet, fox, ctx, chl, cro	07-12-21-17-13-13-34-34-33-34-34, agr-3, PVL -	This study

Sa_NOG-W06	Greater Accra Region	sxt, gen, amk, cld, amp, tet, cfx, fox, chl, cro	t786, agr-3, PVL -	This study
Sa_NOG-W24	Greater Accra Region	gen, sxt , amk, str, amp, tet, cfx, ctx, chl, cro, fox,	t786, agr-3, PVL +	This study
Sa_NOG-W05	Greater Accra Region	ery, amk, str, amp, cfx, tet, sxt, cld,	t186, agr-3, PVL -	This study
BU_G0701_t5	Eastern Region	fox, ben, oxa, tet, chl	t786, agr-3, PVL -	(Amissah et al., 2015b)
BU_G0201_t8	Eastern Region	fox, ben, oxa, tet, chl	t786, agr-3, PVL -	(Amissah et al., 2015b)
BU_G0202_t2	Eastern Region	fox, ben, oxa, tet, chl	t786, agr-3, PVL -	(Amissah et al., 2015b)
BU_G1905_t3	Eastern Region	fox, ben, oxa, tet, chl	t786, agr-3, PVL -	(Amissah et al., 2015b)
BU_W13_11	Eastern Region	fox, ben, oxa, tet, chl	t186, agr-3, PVL -	(Amissah et al., 2015b)

Notes: *oxacillin=oxa, cefoxitin=fox, tetracycline=tet, chloramphenicol=chl, cefuroxime=cfx, erythromycin=ery, clindamycin=cld, sulphamethxazole-trimethoprim=sxt, amikacin=amk, streptomycin=str, ampicillin=amp, cefotaxime=ctx, ceftriaxone=cro, gentamicin=gen, benzylpenicillin=ben, *spa=Staphylococcus aureus* Protein A, *agr*=Accesory Gene regulator, PVL=Pantone Valentine Leukocidin toxin

Table 2: Sa_aus0325 methylome analysis

HsdS (nucleotide position)	**TRD1**	**N**	**TRD2**
397,724 -> 399,280	ACC	5	RTGT
1,849,852 <- 1,851,408	GAG	6	TCG

Table 3: Comparator Reference Genomes

Sequence type	Region/Country of origin	MSSA/MRSA	Reference Strain	Assembly/Accesssion number
ST8	USA/Canada	CA-MRSA	*Staphylococcus aureus* subsp. *aureus* USA 300 FPR 3757	NC_007793.1
ST 1	USA/Canada	CA-MRSA	*Staphylococcus aureus* subsp. *aureus* MW2	NC_003923.1
ST 80	Europe	CA-MRSA	*Staphylococcus aureus* 11819-97	NC_017351.1
ST45	Europe/ USA/Canada	CA-MRSA	*Staphylococcus aureus* subsp. *aureus* 300-169	GCA_000534855.1
ST 30	Europe/ Australia/Asia	CA-MRSA	*Staphylococcus aureus* subsp. *aureus*_S2397	GCA_000577595.1
ST 72	Asia	CA-MRSA	*Staphylococcus aureus* MRSA_CVM43477	GCA_000830555.1
ST 59	Asia	CA-MRSA	*Staphylococcus aureus* subsp. *aureus* M013	NC_016928.1
ST93	Australia	CA-MRSA	*Staphylococcus aureus* subsp. *aureus* JKD 6159	NC_017338.1
ST 250	England	HA-MRSA	*Staphylococcus aureus* subsp. *aureus* COL	NC_002951.2
ST254	Japan	MSSA	*Staphylococcus aureus* subsp. *aureus* Newman	NC_009641.1
ST1	United Kingdom	MSSA	*Staphylococcus aureus* subsp. *aureus* MSSA476	NC_002953.3
ST5	Ireland	MSSA	*Staphylococcus aureus* subsp. *aureus* ED98	NC_013450.1
ST5	Japan	MRSA	*Staphylococcus aureus* subsp. *aureus* N315	NC_002745.2
ST 239	Australia	MRSA	*Staphylococcus aureus* subsp. *aureus* JKD 6008	NC_017341.1

ST772	India	MRSA	*Staphylococcus aureus* subsp. *aureus*_ST772-MRSA	GC_000516935.1
ST 88	Lebanon	MRSA	HST-105	GCA_000564895.1
ST 88	United States	MSSA	*Staphylococcus aureus* subsp *aureus*_21343	GCA_000245595.2

Supplementary Materials

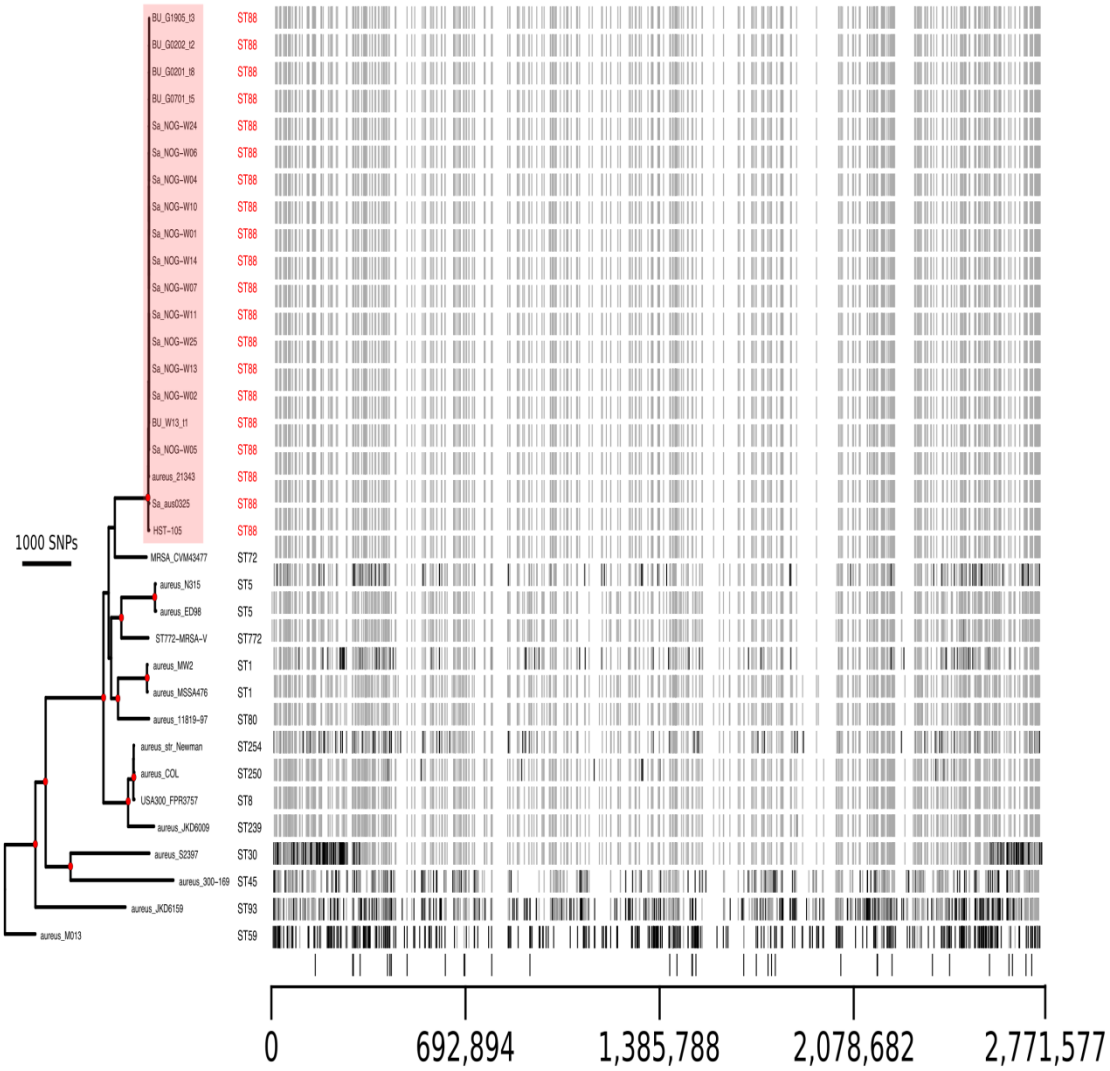

Figure. S1: Recombination analysis among the 20 ST88 and 15 non-ST88 genomes. Light grey and black blocks denote recombination regions detected in ancestral nodes and the sampled genomes, respectively. In total there were 98,432 core SNPs, 26,570 of which were located within inferred regions of recombination.

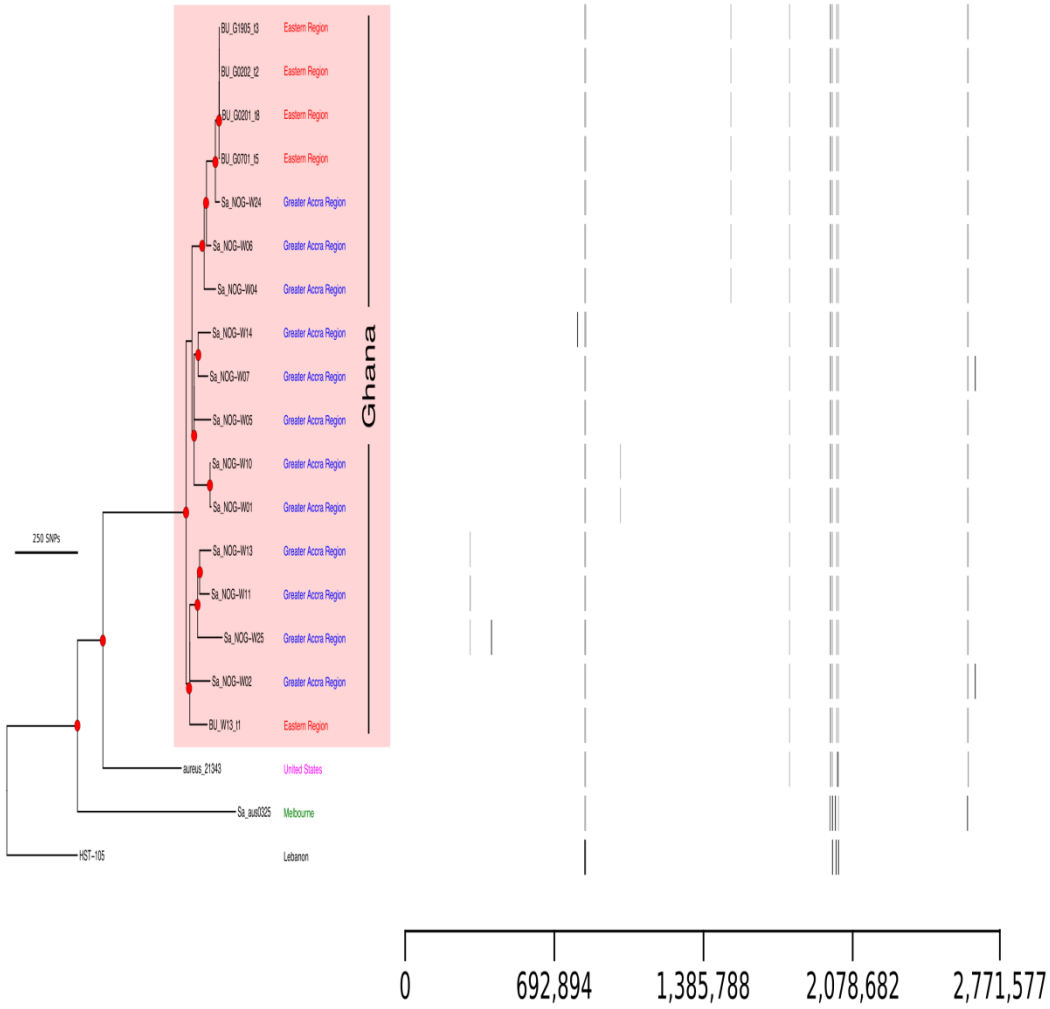

Figure. S2: Recombination analysis among the 20 ST88 genomes. Light grey and black blocks denote recombination regions detected in ancestral nodes and the sampled genomes, respectively. In total there were 1,966 core SNPs, 207 of which were located within inferred regions of recombination.

Table S1: Genomic regions enriched in *S. aureus* MRSA ST88

Feature	Gene	Annotation	No. isolates	No. sequences	Avg sequences per isolate	Genome Fragment	Order within Fragment	Accessory Fragment	Accessory Order with Fragment	Min group size nuc	Max group size nuc	Avg group size nuc
ST88_GI-1	group_572	hypothetical protein	16	16	1	1	4209	1	1497	737	1730	1667
ST88_GI-1	group_2165	putative restriction enzyme	20	20	1	1	4206	1	1494	1904	1904	1904
ST88_GI-1	group_399	hypothetical protein	20	20	1	1	4205	1	1493	290	917	808
ST88_GI-1	group_2873	hypothetical protein	1	1	1	1	4863	1	129	284	284	284
ST88_GI-1	group_1298	hypothetical protein	20	20	1	1	4864	1	1492	182	182	182
ST88_GI-1	group_403	Integrase core domain protein	23	23	1	1	4865	1	1490	422	806	451
ST88_GI-1	yjaB	putative N-acetyltransferase YjaB	20	20	1	1	4860	1	1488	458	458	458
ST88_GI-2	group_2187	hypothetical protein	20	20	1	1	4534	2	17	491	491	491
ST88_GI-2	group_1491	hypothetical protein	29	29	1	1	4535	2	30	413	683	673
ST88_GI-2	group_1492	hypothetical protein	29	29	1	1	4531	2	32	611	611	611
ST88_GI-2	group_939	hypothetical protein	24	24	1	1	4528	2	42	500	500	500
ST88_GI-2	group_1493	hypothetical protein	20	20	1	1	4521	2	6	269	482	471
ST88_GI-2	group_940	hypothetical protein	24	24	1	1	4522	2	50	491	491	491
ST88_GI-2	group_2188	hypothetical protein	27	27	1	1	4520	2	65	683	683	683
ST88_GI-2	group_419	hypothetical protein	24	24	1	1	4517	2	70	272	491	472
ST88_GI-2	group_941	hypothetical membrane protein	24	24	1	1	4515	2	71	302	473	330
ST88_GI-2	group_942	hypothetical protein	25	25	1	1	4513	2	72	374	374	374
ST88_GI-2	group_943	putative membrane protein	25	25	1	1	4512	2	73	389	389	389
ST88_GI-2	group_597	putative membrane protein	25	25	1	1	4511	2	81	392	392	392
ST88_GI-2	group_2189	hypothetical protein	20	20	1	1	4508	2	80	500	500	500
ST88_GI-3	group_960	sodium/dicarboxylare symporter	20	20	1	1	602	233	1	209	209	209
ST88_GI-3	group_610	hypothetical protein	35	35	1	1	603			944	956	955
ST88_GI-3	group_4341	hypothetical protein	35	35	1	1	604			662	662	662
ST88_GI-3	yflT	putative heat induced stress protein	35	35	1	1	605			407	440	408

ST88_GI-3	xprT	putative xanthine phosphoribosyltransferase	35	35	1	1	606			578	578	578
ST88_GI-3	pbuX	NCS2 family nucleobase:cation symporter-2	35	35	1	1	607			1211	1268	1264
ST88_GI-3	guaB	putative inosine-5'-monophosphate dehydrogenase	35	35	1	1	608			1466	1466	1466
ST88_GI-3	guaA	bifunctional GMP synthase/glutamine amidotransferase protein	35	35	1	1	614			1541	1541	1541
ST88_GI-3	group_2197	abortive infection bacteriophage resistance protein	26	26	1	1	528	1	989	1106	1106	1106
ST88_GI-3	group_2198	hypothetical protein	26	26	1	1	527	1	990	392	392	392
ST88_GI-3	group_961	hypothetical protein	24	24	1	1	525	1	991	221	479	253
ST88_GI-3	group_1509	hypothetical protein	22	22	1	1	514	1	992	185	584	547
ST88_GI-3	group_612	hypothetical protein	33	33	1	1	650	1	993	152	152	152
ST88_GI-3	group_613	hypothetical protein	33	33	1	1	656	1	978	359	359	359
ST88_GI-3	group_614	cobalt (Co2) ABC superfamily ATP binding cassette transporter, membrane protein	34	34	1	1	660	1	976	845	845	845
ST88_GI-3	set6	superantigen-like protein	20	20	1	1	661	1	963	413	680	666
ST88_GI-3	set7	superantigen-like protein	32	32	1	1	666	1	957	695	695	695
ST88_GI-3	set8	superantigen-like protein	28	28	1	1	669	1	954	1058	1070	1068
ST88_GI-3	group_2199	superantigen-like protein	19	19	1	1	667	1	890	992	992	992
ST88_GI-3	set10	superantigen-like protein 5	20	20	1	1	678	1	906	704	704	704
ST88_GI-3	group_2201	superantigen-like protein	20	20	1	1	679	1	907	698	698	698
ST88_GI-3	set11	superantigen-like protein 7	20	20	1	1	680	1	908	695	695	695
ST88_GI-3	set12	superantigen-like protein	31	31	1	1	692	1	949	698	698	698
ST88_GI-3	set13	superantigen-like protein, exotoxin 13	33	33	1	1	697	1	946	311	698	686
ST88_GI-3	set14	superantigen-like protein, exotoxin 14	32	32	1	1	698	1	940	683	683	683
ST88_GI-3	hsdM_1	Type I restriction-modification system methyltransferase subunit	26	26	1	1	716	1	939	1556	1556	1556

ST88_GI-3	hsdS_1	restriction endonuclease S subunit	20	20	1	1	701	1	923	1187	1187	1187
ST88_GI-3	set15	superantigen-like protein	20	20	1	1	700	1	922	692	692	692
ST88_GI-3	group_2205	hypothetical protein	20	20	1	1	699	1	921	1502	1502	1502
ST88_GI-3	group_4807	hypothetical protein	35	35	1	1	724			308	308	308
ST88_GI-3	lpl3	staphylococcal tandem lipoprotein	20	20	1	1	725	5	10	809	809	809
ST88_GI-3	lpl8	tandem lipoprotein	20	20	1	1	726	5	11	752	776	771
ST88_GI-3	lpl2	staphylococcal tandem lipoprotein	21	21	1	1	727	5	63	815	815	815
ST88_GI-3	group_2207	putative membrane protein	20	20	1	1	728	5	64	1322	1322	1322
ST88_GI-3	group_2208	hypothetical protein	20	20	1	1	729	5	65	323	323	323
ST88_GI-3	group_2209	hypothetical protein	20	20	1	1	730	5	1	296	296	296
ST88_GI-4	group_2126	hypothetical protein	20	20	1	1	2877	1	1144	152	152	152
ST88_GI-4	group_2127	hypothetical protein	20	20	1	1	2876	1	1143	242	242	242
ST88_GI-4	group_2128	Integrase core domain protein	20	20	1	1	2875	1	1142	491	491	491
ST88_GI-4	group_2129	hypothetical protein	20	20	1	1	2874	1	1141	647	647	647
ST88_GI-4	group_2130	hypothetical protein	20	20	1	1	2873	1	1140	407	407	407
ST88_GI-4	group_373	hypothetical protein	19	19	1	1	2872	1	1139	251	764	723
ST88_GI-4	group_374	hypothetical protein	2	2	1	1	2871	1	1138	191	380	285
ST88_GI-4	group_2131	hypothetical protein	20	20	1	1	388	1	1137	392	392	392
ST88_GI-4	group_851	hypothetical protein	20	20	1	1	389	1	1136	512	512	512
ST88_GI-4	group_2132	hypothetical protein	20	20	1	1	390	1	1135	608	608	608
ST88_GI-4	group_2133	hypothetical protein	27	27	1	1	1200	1	1134	323	323	323
ST88_GI-4	group_2134	hypothetical protein	20	20	1	1	387	1	1133	248	248	248
ST88_GI-5	hsdS_2	restriction endonuclease S subunit	20	20	1	1	4869	1	138	1235	1238	1235
ST88_GI-5	hsdM_2	Type I restriction-modification system methyltransferase subunit	21	21	1	1	4868	1	188	1556	1556	1556
ST88_GI-5	group_1075	glutamyl endopeptidase	30	30	1	1	3330	1	183	224	719	694
ST88_GI-5	group_1723	glutamyl endopeptidase	27	27	1	1	3329	1	182	719	719	719
ST88_GI-5	group_1722	glutamyl endopeptidase	21	21	1	1	3328	1	141	716	716	716

ST88_GI-5	group_1721	glutamyl endopeptidase	21	21	1	1	3326	1	143	719	719	719
ST88_GI-5	group_1720	glutamyl endopeptidase	21	21	1	1	2633	1	144	722	722	722
ST88_GI-5	splB	glutamyl endopeptidase	30	30	1	1	3323	1	180	164	722	703
ST88_GI-5	group_688	glutamyl endopeptidase	30	30	1	1	3322	1	179	329	716	695
ST88_GI-5	group_2297	hypothetical protein	20	20	1	1	3317	1	175	581	581	581
ST88_GI-5	group_1074	hypothetical protein	29	29	1	1	3315	1	177	218	698	317
ST88_GI-5	group_2296	hypothetical protein	24	24	1	1	3314	1	176	473	473	473
ST88_GI-5	group_1073	hypothetical protein	25	25	1	1	3293	1	165	791	791	791
ST88_GI-5	group_2295	hypothetical protein	20	20	1	1	3292	1	161	221	221	221

Table S1 cont'

	ST88	NON-ST88	NON-ST88	NON-ST88	NON-ST88	NON-ST88	NON-ST88	NON-ST88	NON-ST88	NON-ST88	NON-ST88	NON-ST88
Feature	**COUNTER**	**aureus_11819-97**	**aureus_300-169**	**aureus_COL**	**aureus_ED98**	**aureus_JKD6009**	**aureus_JKD6159**	**aureus_M013**	**aureus_MSSA476**	**aureus_MW2**	**aureus_N315**	**aureus_S2397**
ST88_GI-1	_2560_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-1	_2465_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-1	_2488_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-1	_4262_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-1	_2443_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-1	_2384_	*	*	*	aureus_ED98_01750	*	*	*	*	*	aureus_N315_01678	aureus_S2397_00184
ST88_GI-1	_2466_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-2	_2467_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-2	_2205_	aureus_11819-97_00278	*	aureus_COL_00266	aureus_ED98_00256	*	*	*	aureus_MSSA476_00265	aureus_MW2_00268	aureus_N315_00285	*
ST88_GI-2	_2206_	aureus_11819-97_00279	*	aureus_COL_00267	aureus_ED98_00257	*	*	*	aureus_MSSA476_00266	aureus_MW2_00269	aureus_N315_00286	*
ST88_GI-2	_2360_	*	*	aureus_COL_00269	*	*	*	*	*	*	*	*
ST88_GI-2	_2447_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-2	_2361_	aureus_11819-97_00282	*	aureus_COL_00273	*	*	*	*	*	*	*	*
ST88_GI-2	_2265_	aureus_11819-97_00283	aureus_300-169_02192	aureus_COL_00274	*	*	aureus_JKD6159_00277	*	*	*	*	*
ST88_GI-2	_2358_	*	aureus_300-169_02201	*	*	aureus_JKD6009_00450	*	*	*	*	*	aureus_S2397_01480
ST88_GI-2	_2362_	*	aureus_300-169_02195	*	*	aureus_JKD6009_00444	*	aureus_M013_00273	*	*	*	aureus_S2397_02248
ST88_GI-2	_2349_	*	aureus_300-169_02196	*	*	aureus_JKD6009_00445	*	aureus_M013_00274	*	*	*	aureus_S2397_02247
ST88_GI-2	_2350_	*	aureus_300-169_02197	*	*	aureus_JKD6009_00446	*	aureus_M013_00275	*	*	*	aureus_S2397_02246
ST88_GI-2	_2342_	*	aureus_300-169_02198	*	*	aureus_JKD6009_00447	*	aureus_M013_00276	*	*	*	aureus_S2397_02245
ST88_GI-2	_2468_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-3	_2491_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-3	_1724_	aureus_11819-97_00365	aureus_300-169_02282	aureus_COL_00432	aureus_ED98_00343	aureus_JKD6009_01296	aureus_JKD6159_00359	aureus_M013_00359	aureus_MSSA476_00353	aureus_MW2_00356	aureus_N315_00374	aureus_S2397_01807
ST88_GI-3	_614_	aureus_11819-97_00366	aureus_300-169_02283	aureus_COL_00433	aureus_ED98_00344	aureus_JKD6009_01297	aureus_JKD6159_00360	aureus_M013_00360	aureus_MSSA476_00354	aureus_MW2_00357	aureus_N315_00375	aureus_S2397_01808
ST88_GI-3	_1725_	aureus_11819-97_00367	aureus_300-169_02284	aureus_COL_00434	aureus_ED98_00345	aureus_JKD6009_01298	aureus_JKD6159_00361	aureus_M013_00361	aureus_MSSA476_00355	aureus_MW2_00358	aureus_N315_00376	aureus_S2397_01809
ST88_GI-3	_1493_	aureus_11819-97_00368	aureus_300-169_02285	aureus_COL_00435	aureus_ED98_00346	aureus_JKD6009_01299	aureus_JKD6159_00362	aureus_M013_00362	aureus_MSSA476_00356	aureus_MW2_00359	aureus_N315_00377	aureus_S2397_01810

ST88_GI-3	_224_	aureus_11819-97_00369	aureus_300-169_02286	aureus_COL_00436	aureus_ED98_00347	aureus_JKD6009_01300	aureus_JKD6159_00363	aureus_M013_00363	aureus_MSSA476_00357	aureus_MW2_00360	aureus_N315_00378	aureus_S2397_01811
ST88_GI-3	_1066_	aureus_11819-97_00370	aureus_300-169_02287	aureus_COL_00437	aureus_ED98_00348	aureus_JKD6009_01301	aureus_JKD6159_00364	aureus_M013_00364	aureus_MSSA476_00358	aureus_MW2_00361	aureus_N315_00379	aureus_S2397_01812
ST88_GI-3	_941_	aureus_11819-97_00371	aureus_300-169_02288	aureus_COL_00438	aureus_ED98_00349	aureus_JKD6009_01302	aureus_JKD6159_00365	aureus_M013_00365	aureus_MSSA476_00359	aureus_MW2_00362	aureus_N315_00380	aureus_S2397_01813
ST88_GI-3	_2305_	aureus_11819-97_00372	*	*	*	*	*	aureus_M013_00366	aureus_MSSA476_00360	aureus_MW2_00363	*	*
ST88_GI-3	_2306_	aureus_11819-97_00373	*	*	*	*	*	aureus_M013_00367	aureus_MSSA476_00361	aureus_MW2_00364	*	*
ST88_GI-3	_2363_	aureus_11819-97_00374	*	*	*	*	*	*	aureus_MSSA476_00362	aureus_MW2_00365	*	*
ST88_GI-3	_2395_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-3	_2072_	aureus_11819-97_00381	*	aureus_COL_00442	aureus_ED98_00353	aureus_JKD6009_01334	aureus_JKD6159_00370	aureus_M013_00372	aureus_MSSA476_00368	aureus_MW2_00371	aureus_N315_00384	*
ST88_GI-3	_2073_	aureus_11819-97_00385	aureus_300-169_02302	aureus_COL_00443	aureus_ED98_00354	aureus_JKD6009_01335	*	aureus_M013_00376	aureus_MSSA476_00372	aureus_MW2_00375	aureus_N315_00385	aureus_S2397_02120
ST88_GI-3	_1993_	aureus_11819-97_00386	aureus_300-169_02303	aureus_COL_00444	aureus_ED98_00355	aureus_JKD6009_01336	*	aureus_M013_00377	aureus_MSSA476_00373	aureus_MW2_00376	aureus_N315_00386	aureus_S2397_02119
ST88_GI-3	_2449_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-3	_2137_	aureus_11819-97_00388	*	aureus_COL_00446	aureus_ED98_00357	aureus_JKD6009_01338	*	aureus_M013_00380	aureus_MSSA476_00375	aureus_MW2_00378	aureus_N315_00388	*
ST88_GI-3	_2244_	aureus_11819-97_00389	*	aureus_COL_00447	aureus_ED98_00358	*	*	*	aureus_MSSA476_00376	aureus_MW2_00379	aureus_N315_00389	*
ST88_GI-3	_2498_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-3	_2470_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-3	_2471_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-3	_2472_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-3	_2143_	aureus_11819-97_00393	*	*	aureus_ED98_00362	aureus_JKD6009_00989	*	aureus_M013_00385	aureus_MSSA476_00381	aureus_MW2_00384	aureus_N315_00393	*
ST88_GI-3	_2056_	aureus_11819-97_00394	*	aureus_COL_00449	aureus_ED98_00363	aureus_JKD6009_00990	aureus_JKD6159_00385	aureus_M013_00386	aureus_MSSA476_00382	aureus_MW2_00385	aureus_N315_00394	*
ST88_GI-3	_2138_	aureus_11819-97_00395	*	aureus_COL_00450	aureus_ED98_00364	aureus_JKD6009_00991	*	aureus_M013_00387	aureus_MSSA476_00383	aureus_MW2_00386	aureus_N315_00395	*
ST88_GI-3	_2319_	aureus_11819-97_00396	aureus_300-169_02312	aureus_COL_00451	aureus_ED98_00365	aureus_JKD6009_00992	*	aureus_M013_00388	aureus_MSSA476_00384	aureus_MW2_00387	aureus_N315_00396	*
ST88_GI-3	_2473_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-3	_2474_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-3	_2475_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-3	_1082_	aureus_11819-97_00400	aureus_300-169_02316	aureus_COL_00455	aureus_ED98_00369	aureus_JKD6009_00996	aureus_JKD6159_00391	aureus_M013_00392	aureus_MSSA476_00388	aureus_MW2_00391	aureus_N315_00400	aureus_S2397_00998
ST88_GI-3	_2476_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-3	_2450_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-3	_2420_	*	*	*	*	*	*	aureus_M013_00403	*	*	*	*
ST88_GI-3	_2477_	*	*	*	*	*	*	*	*	*	*	*

ST88_GI-3	_2478_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-3	_2479_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-4	_2455_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-4	_2456_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-4	_2457_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-4	_2458_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-4	_2459_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-4	_2500_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-4	_3712_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-4	_2460_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-4	_2490_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-4	_2461_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-4	_2264_	aureus_11819-97_00824	*	aureus_COL_00817	aureus_ED98_00729	aureus_JKD6009_00481	*	*	*	*	aureus_N315_00762	*
ST88_GI-4	_2462_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-5	_2486_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-5	_2439_	aureus_11819-97_01791	*	aureus_COL_01778	aureus_ED98_01754	*	*	*	aureus_MSSA476_01723	aureus_MW2_01744	aureus_N315_01682	*
ST88_GI-5	_2171_	aureus_11819-97_01792	*	aureus_COL_01779	aureus_ED98_01755	*	*	*	aureus_MSSA476_01724	aureus_MW2_01745	aureus_N315_01683	aureus_S2397_01407
ST88_GI-5	_2260_	aureus_11819-97_01793	*	aureus_COL_01781	aureus_ED98_01756	*	*	*	*	*	aureus_N315_01684	*
ST88_GI-5	_2426_	*	*	*	*	*	*	*	*	*	*	aureus_S2397_01405
ST88_GI-5	_2425_	*	*	*	*	*	aureus_JKD6159_01752	*	*	*	*	*
ST88_GI-5	_2424_	*	*	*	*	*	aureus_JKD6159_01753	*	*	*	*	*
ST88_GI-5	_2180_	aureus_11819-97_01796	*	aureus_COL_01783	aureus_ED98_01758	*	*	*	aureus_MSSA476_01726	aureus_MW2_01747	aureus_N315_01686	aureus_S2397_01402
ST88_GI-5	_2197_	aureus_11819-97_01797	*	aureus_COL_01784	aureus_ED98_01759	*	aureus_JKD6159_01754	*	aureus_MSSA476_01727	aureus_MW2_01748	aureus_N315_01687	*
ST88_GI-5	_2483_	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-5	_2203_	aureus_11819-97_01799	*	aureus_COL_01786	aureus_ED98_01761	*	*	*	aureus_MSSA476_01729	aureus_MW2_01750	aureus_N315_01689	*
ST88_GI-5	_2357_	*	*	*	aureus_ED98_01762	*	*	*	*	*	aureus_N315_01690	*
ST88_GI-5	_2325_	*	*	*	aureus_ED98_01763	*	aureus_JKD6159_01758	*	*	*	aureus_N315_01691	*
ST88_GI-5	_2482_	*	*	*	*	*	*	*	*	*	*	*

Table S1 cont'

	NON-ST88	NON-ST88	NON-ST88	NON-ST88	ST88	ST88	ST88	ST88	ST88
Feature	**aureus_str._Newman**	**MRSA_CVM43477**	**ST772-MRSA-V**	**USA300_FPR3757**	**aureus_21343**	**BU_G0201_t8**	**BU_G0202_t2**	**BU_G0701_t5**	**BU_G1905_t3**
ST88_GI-1	*	*	*	*	aureus_21343_02477	BU_G0201_t8_01011	BU_G0202_t2_00597	BU_G0701_t5_00943	BU_G1905_t3_00972
ST88_GI-1	*	*	*	*	aureus_21343_02478	BU_G0201_t8_01012	BU_G0202_t2_00596	BU_G0701_t5_00942	BU_G1905_t3_00971
ST88_GI-1	*	*	*	*	aureus_21343_02479	BU_G0201_t8_01013	BU_G0202_t2_00595	BU_G0701_t5_00941	BU_G1905_t3_00970
ST88_GI-1	*	*	*	*	*	*	*	*	*
ST88_GI-1	*	*	*	*	aureus_21343_02480	BU_G0201_t8_01014	BU_G0202_t2_00594	BU_G0701_t5_00940	BU_G1905_t3_00969
ST88_GI-1	*	*	*	*	aureus_21343_02481	BU_G0201_t8_01015	BU_G0202_t2_00593	BU_G0701_t5_00939	BU_G1905_t3_00968
ST88_GI-1	*	*	*	*	aureus_21343_02482	BU_G0201_t8_01016	BU_G0202_t2_00592	BU_G0701_t5_00938	BU_G1905_t3_00967
ST88_GI-2	*	*	*	*	aureus_21343_00775	BU_G0201_t8_01403	BU_G0202_t2_00940	BU_G0701_t5_01548	BU_G1905_t3_01577
ST88_GI-2	aureus_str._Newman_00237	MRSA_CVM43477_00130	*	USA300_FPR3757_00292	aureus_21343_00774	BU_G0201_t8_01404	BU_G0202_t2_00941	BU_G0701_t5_01549	BU_G1905_t3_01578
ST88_GI-2	aureus_str._Newman_00238	MRSA_CVM43477_00129	*	USA300_FPR3757_00293	aureus_21343_00773	BU_G0201_t8_01405	BU_G0202_t2_00942	BU_G0701_t5_01550	BU_G1905_t3_01579
ST88_GI-2	aureus_str._Newman_00240	MRSA_CVM43477_00128	*	USA300_FPR3757_00295	aureus_21343_00772	BU_G0201_t8_01406	BU_G0202_t2_00943	BU_G0701_t5_01551	BU_G1905_t3_01580
ST88_GI-2	*	*	*	*	aureus_21343_00771	BU_G0201_t8_01407	BU_G0202_t2_00944	BU_G0701_t5_01552	BU_G1905_t3_01581
ST88_GI-2	aureus_str._Newman_00244	*	*	USA300_FPR3757_00299	aureus_21343_00770	BU_G0201_t8_01408	BU_G0202_t2_00945	BU_G0701_t5_01553	BU_G1905_t3_01582
ST88_GI-2	aureus_str._Newman_00245	MRSA_CVM43477_02294	*	USA300_FPR3757_00300	aureus_21343_00769	BU_G0201_t8_01409	BU_G0202_t2_00946	BU_G0701_t5_01554	BU_G1905_t3_01583
ST88_GI-2	*	MRSA_CVM43477_02285	*	*	aureus_21343_00768	BU_G0201_t8_01410	BU_G0202_t2_00947	BU_G0701_t5_01555	BU_G1905_t3_01584
ST88_GI-2	*	*	*	*	aureus_21343_00766	BU_G0201_t8_01412	BU_G0202_t2_00949	BU_G0701_t5_01557	BU_G1905_t3_01586
ST88_GI-2	*	MRSA_CVM43477_02289	*	*	aureus_21343_00765	BU_G0201_t8_01413	BU_G0202_t2_00950	BU_G0701_t5_01558	BU_G1905_t3_01587
ST88_GI-2	*	MRSA_CVM43477_02288	*	*	aureus_21343_00764	BU_G0201_t8_01414	BU_G0202_t2_00951	BU_G0701_t5_01559	BU_G1905_t3_01588
ST88_GI-2	*	MRSA_CVM43477_02287	*	*	aureus_21343_00763	BU_G0201_t8_01415	BU_G0202_t2_00952	BU_G0701_t5_01560	BU_G1905_t3_01589
ST88_GI-2	*	*	*	*	aureus_21343_00762	BU_G0201_t8_01416	BU_G0202_t2_00953	BU_G0701_t5_01561	BU_G1905_t3_01590
ST88_GI-3	*	*	*	*	aureus_21343_02563	BU_G0201_t8_01502	BU_G0202_t2_01039	BU_G0701_t5_01647	BU_G1905_t3_01676
ST88_GI-3	aureus_str._Newman_00401	MRSA_CVM43477_00245	ST772-MRSA-V_00357	USA300_FPR3757_00385	aureus_21343_02562	BU_G0201_t8_01503	BU_G0202_t2_01040	BU_G0701_t5_01648	BU_G1905_t3_01677
ST88_GI-3	aureus_str._Newman_00402	MRSA_CVM43477_00244	ST772-MRSA-V_00358	USA300_FPR3757_00386	aureus_21343_02561	BU_G0201_t8_01504	BU_G0202_t2_01041	BU_G0701_t5_01649	BU_G1905_t3_01678
ST88_GI-3	aureus_str._Newman_00403	MRSA_CVM43477_00243	ST772-MRSA-V_00359	USA300_FPR3757_00387	aureus_21343_02560	BU_G0201_t8_01505	BU_G0202_t2_01042	BU_G0701_t5_01650	BU_G1905_t3_01679

ST88_GI-3	aureus_str._Newman_00404	MRSA_CVM43477_00242	ST772-MRSA-V_00360	USA300_FPR3757_00388	aureus_21343_02559	BU_G0201_t8_01506	BU_G0202_t2_01043	BU_G0701_t5_01651	BU_G1905_t3_01680
ST88_GI-3	aureus_str._Newman_00405	MRSA_CVM43477_00241	ST772-MRSA-V_00361	USA300_FPR3757_00389	aureus_21343_02558	BU_G0201_t8_01507	BU_G0202_t2_01044	BU_G0701_t5_01652	BU_G1905_t3_01681
ST88_GI-3	aureus_str._Newman_00406	MRSA_CVM43477_00240	ST772-MRSA-V_00362	USA300_FPR3757_00390	aureus_21343_02557	BU_G0201_t8_01508	BU_G0202_t2_01045	BU_G0701_t5_01653	BU_G1905_t3_01682
ST88_GI-3	aureus_str._Newman_00407	MRSA_CVM43477_00239	ST772-MRSA-V_00363	USA300_FPR3757_00391	aureus_21343_02556	BU_G0201_t8_01509	BU_G0202_t2_01046	BU_G0701_t5_01654	BU_G1905_t3_01683
ST88_GI-3	*	MRSA_CVM43477_00238	ST772-MRSA-V_00364	*	aureus_21343_02555	BU_G0201_t8_01510	BU_G0202_t2_01047	BU_G0701_t5_01655	BU_G1905_t3_01684
ST88_GI-3	*	MRSA_CVM43477_00237	ST772-MRSA-V_00365	*	aureus_21343_02554	BU_G0201_t8_01511	BU_G0202_t2_01048	BU_G0701_t5_01656	BU_G1905_t3_01685
ST88_GI-3	*	*	ST772-MRSA-V_00366	*	aureus_21343_02553	BU_G0201_t8_01512	BU_G0202_t2_01049	BU_G0701_t5_01657	BU_G1905_t3_01686
ST88_GI-3	*	MRSA_CVM43477_00230	ST772-MRSA-V_00372	*	aureus_21343_02552	BU_G0201_t8_01513	BU_G0202_t2_01050	BU_G0701_t5_01658	BU_G1905_t3_01687
ST88_GI-3	aureus_str._Newman_00411	MRSA_CVM43477_00229	ST772-MRSA-V_00373	USA300_FPR3757_00395	aureus_21343_02551	BU_G0201_t8_01514	BU_G0202_t2_01051	BU_G0701_t5_01659	BU_G1905_t3_01688
ST88_GI-3	aureus_str._Newman_00412	*	ST772-MRSA-V_00374	USA300_FPR3757_00396	aureus_21343_02550	BU_G0201_t8_01515	BU_G0202_t2_01052	BU_G0701_t5_01660	BU_G1905_t3_01689
ST88_GI-3	aureus_str._Newman_00413	MRSA_CVM43477_01399	ST772-MRSA-V_00375	USA300_FPR3757_00397	aureus_21343_02549	BU_G0201_t8_01516	BU_G0202_t2_01053	BU_G0701_t5_01661	BU_G1905_t3_01690
ST88_GI-3	*	*	*	*	aureus_21343_02548	BU_G0201_t8_01517	BU_G0202_t2_01054	BU_G0701_t5_01662	BU_G1905_t3_01691
ST88_GI-3	aureus_str._Newman_00415	MRSA_CVM43477_01397	ST772-MRSA-V_00377	USA300_FPR3757_00399	aureus_21343_02547	BU_G0201_t8_01518	BU_G0202_t2_01055	BU_G0701_t5_01663	BU_G1905_t3_01692
ST88_GI-3	aureus_str._Newman_00416	*	*	USA300_FPR3757_00400	aureus_21343_02546	BU_G0201_t8_01519	BU_G0202_t2_01056	BU_G0701_t5_01664	BU_G1905_t3_01693
ST88_GI-3	*	*	*	*	aureus_21343_02545	BU_G0201_t8_01520	BU_G0202_t2_01057	BU_G0701_t5_01665	*
ST88_GI-3	*	*	*	*	aureus_21343_02544	BU_G0201_t8_01521	BU_G0202_t2_01058	BU_G0701_t5_01666	BU_G1905_t3_01695
ST88_GI-3	*	*	*	*	aureus_21343_02543	BU_G0201_t8_01522	BU_G0202_t2_01059	BU_G0701_t5_01667	BU_G1905_t3_01696
ST88_GI-3	*	*	*	*	aureus_21343_02542	BU_G0201_t8_01523	BU_G0202_t2_01060	BU_G0701_t5_01668	BU_G1905_t3_01697
ST88_GI-3	aureus_str._Newman_00421	MRSA_CVM43477_01392	ST772-MRSA-V_00382	USA300_FPR3757_00405	aureus_21343_02541	BU_G0201_t8_01524	BU_G0202_t2_01061	BU_G0701_t5_01669	BU_G1905_t3_01698
ST88_GI-3	aureus_str._Newman_00422	MRSA_CVM43477_01391	ST772-MRSA-V_00383	USA300_FPR3757_00406	aureus_21343_02540	BU_G0201_t8_01525	BU_G0202_t2_01062	BU_G0701_t5_01670	BU_G1905_t3_01699
ST88_GI-3	aureus_str._Newman_00423	MRSA_CVM43477_01390	ST772-MRSA-V_00384	USA300_FPR3757_00407	aureus_21343_02539	BU_G0201_t8_01526	BU_G0202_t2_01063	BU_G0701_t5_01671	BU_G1905_t3_01700
ST88_GI-3	aureus_str._Newman_00424	MRSA_CVM43477_01389	ST772-MRSA-V_00385	USA300_FPR3757_00408	aureus_21343_02538	BU_G0201_t8_01527	BU_G0202_t2_01064	BU_G0701_t5_01672	*
ST88_GI-3	*	*	*	*	aureus_21343_02537	BU_G0201_t8_01528	BU_G0202_t2_01065	BU_G0701_t5_01673	BU_G1905_t3_02504
ST88_GI-3	*	*	*	*	aureus_21343_02536	BU_G0201_t8_01529	BU_G0202_t2_01066	BU_G0701_t5_01674	BU_G1905_t3_02503
ST88_GI-3	*	*	*	*	aureus_21343_02535	BU_G0201_t8_01530	BU_G0202_t2_01067	BU_G0701_t5_01675	BU_G1905_t3_02502
ST88_GI-3	aureus_str._Newman_00428	MRSA_CVM43477_01385	ST772-MRSA-V_00389	USA300_FPR3757_00412	aureus_21343_02534	BU_G0201_t8_01531	BU_G0202_t2_01068	BU_G0701_t5_01676	BU_G1905_t3_02501
ST88_GI-3	*	*	*	*	aureus_21343_02533	BU_G0201_t8_01532	BU_G0202_t2_01069	BU_G0701_t5_01677	BU_G1905_t3_02500
ST88_GI-3	*	*	*	*	aureus_21343_02532	BU_G0201_t8_01533	BU_G0202_t2_01070	BU_G0701_t5_01678	BU_G1905_t3_02499
ST88_GI-3	*	*	*	*	aureus_21343_02531	BU_G0201_t8_01534	BU_G0202_t2_01071	BU_G0701_t5_01679	BU_G1905_t3_02498

ST88_GI-3	*	*	*	*	aureus_21343_02530	BU_G0201_t8_01535	BU_G0202_t2_01072	BU_G0701_t5_01680	BU_G1905_t3_02497
ST88_GI-3	*	*	*	*	aureus_21343_02529	BU_G0201_t8_01536	BU_G0202_t2_01073	BU_G0701_t5_01681	BU_G1905_t3_02496
ST88_GI-3	*	*	*	*	aureus_21343_02528	BU_G0201_t8_01537	BU_G0202_t2_01074	BU_G0701_t5_01682	BU_G1905_t3_02495
ST88_GI-4	*	*	*	*	aureus_21343_00931	BU_G0201_t8_00649	BU_G0202_t2_00275	BU_G0701_t5_00276	BU_G1905_t3_00273
ST88_GI-4	*	*	*	*	aureus_21343_00930	BU_G0201_t8_00650	BU_G0202_t2_00274	BU_G0701_t5_00275	BU_G1905_t3_00274
ST88_GI-4	*	*	*	*	aureus_21343_00929	BU_G0201_t8_00651	BU_G0202_t2_00273	BU_G0701_t5_00274	BU_G1905_t3_00275
ST88_GI-4	*	*	*	*	aureus_21343_00928	BU_G0201_t8_00652	BU_G0202_t2_00272	BU_G0701_t5_00273	BU_G1905_t3_00276
ST88_GI-4	*	*	*	*	aureus_21343_00927	BU_G0201_t8_00653	BU_G0202_t2_00271	BU_G0701_t5_00272	BU_G1905_t3_00277
ST88_GI-4	*	*	*	*	aureus_21343_00926	BU_G0201_t8_00654	BU_G0202_t2_00270	BU_G0701_t5_00271	BU_G1905_t3_00278
ST88_GI-4	*	*	*	*	*	*	*	*	*
ST88_GI-4	*	*	*	*	aureus_21343_00925	BU_G0201_t8_00655	BU_G0202_t2_00269	BU_G0701_t5_00270	BU_G1905_t3_00279
ST88_GI-4	*	*	*	*	aureus_21343_00924	BU_G0201_t8_00656	BU_G0202_t2_00268	BU_G0701_t5_00269	BU_G1905_t3_00280
ST88_GI-4	*	*	*	*	aureus_21343_01877	BU_G0201_t8_00657	BU_G0202_t2_00267	BU_G0701_t5_00268	BU_G1905_t3_00281
ST88_GI-4	aureus_str._Newman_00796	*	*	USA300_FPR3757_00775	aureus_21343_01876	BU_G0201_t8_00658	BU_G0202_t2_00266	BU_G0701_t5_00267	BU_G1905_t3_00282
ST88_GI-4	*	*	*	*	aureus_21343_01875	BU_G0201_t8_00659	BU_G0202_t2_00265	BU_G0701_t5_00266	BU_G1905_t3_00283
ST88_GI-5	*	*	*	*	aureus_21343_01106	BU_G0201_t8_02668	BU_G0202_t2_02575	BU_G0701_t5_02313	BU_G1905_t3_02645
ST88_GI-5	aureus_str._Newman_01780	MRSA_CVM43477_01485	*	USA300_FPR3757_01793	aureus_21343_01107	*	BU_G0202_t2_02574	BU_G0701_t5_02312	*
ST88_GI-5	aureus_str._Newman_01781	MRSA_CVM43477_01486	*	USA300_FPR3757_01794	aureus_21343_01108	BU_G0201_t8_02548	BU_G0202_t2_02573	BU_G0701_t5_02311	BU_G1905_t3_02464
ST88_GI-5	aureus_str._Newman_01783	MRSA_CVM43477_01488	*	USA300_FPR3757_01796	aureus_21343_01592	BU_G0201_t8_02547	BU_G0202_t2_02572	BU_G0701_t5_02310	BU_G1905_t3_02463
ST88_GI-5	*	*	*	*	aureus_21343_01593	BU_G0201_t8_02546	BU_G0202_t2_02571	BU_G0701_t5_02309	BU_G1905_t3_02462
ST88_GI-5	*	*	*	*	aureus_21343_01595	BU_G0201_t8_02544	BU_G0202_t2_02569	BU_G0701_t5_02307	BU_G1905_t3_02460
ST88_GI-5	*	*	*	*	aureus_21343_01596	BU_G0201_t8_02543	BU_G0202_t2_02568	BU_G0701_t5_02306	BU_G1905_t3_02459
ST88_GI-5	aureus_str._Newman_01785	MRSA_CVM43477_01490	*	USA300_FPR3757_01798	aureus_21343_01597	BU_G0201_t8_02542	BU_G0202_t2_02567	BU_G0701_t5_02305	BU_G1905_t3_02458
ST88_GI-5	aureus_str._Newman_01786	MRSA_CVM43477_01491	*	USA300_FPR3757_01799	aureus_21343_01598	BU_G0201_t8_02541	BU_G0202_t2_02566	BU_G0701_t5_02304	BU_G1905_t3_02457
ST88_GI-5	*	*	*	*	aureus_21343_01599	BU_G0201_t8_02540	BU_G0202_t2_02565	BU_G0701_t5_02303	BU_G1905_t3_02456
ST88_GI-5	aureus_str._Newman_01788	MRSA_CVM43477_01493	*	USA300_FPR3757_01801	aureus_21343_01600	BU_G0201_t8_02539	BU_G0202_t2_02564	BU_G0701_t5_02302	BU_G1905_t3_02455
ST88_GI-5	*	MRSA_CVM43477_01494	ST772-MRSA-V_01864	*	aureus_21343_01601	BU_G0201_t8_02538	BU_G0202_t2_02563	BU_G0701_t5_02301	BU_G1905_t3_02454
ST88_GI-5	*	MRSA_CVM43477_01495	ST772-MRSA-V_01865	*	aureus_21343_01602	BU_G0201_t8_02537	BU_G0202_t2_02562	BU_G0701_t5_02300	BU_G1905_t3_02453
ST88_GI-5	*	*	*	*	aureus_21343_01603	BU_G0201_t8_02536	BU_G0202_t2_02561	BU_G0701_t5_02299	BU_G1905_t3_02452

Table S1 cont'

	ST88	ST88	ST88	ST88	ST88	ST88	ST88	ST88	ST88	ST88	ST88	ST88	ST88	ST88	ST88
Feature	**BU_W13_t1**	**HST-105**	**Sa_aus0325**	**Sa_NOG-W01**	**Sa_NOG-W02**	**Sa_NOG-W04**	**Sa_NOG-W05**	**Sa_NOG-W06**	**Sa_NOG-W07**	**Sa_NOG-W10**	**Sa_NOG-W11**	**Sa_NOG-W13**	**Sa_NOG-W14**	**Sa_NOG-**	**Sa_NOG-W25**
ST88_GI-1	BU_W13_t1_01354	HST-105_02277	Sa_aus0325_00029	Sa_NOG-W01_00264	Sa_NOG-W02_01656	*	Sa_NOG-W05_00596	*	Sa_NOG-W07_00525	Sa_NOG-W10_01168	Sa_NOG-W11_00597	Sa_NOG-W13_00296	*	Sa_NOG-01424	*
ST88_GI-1	BU_W13_t1_01355	HST-105_02276	Sa_aus0325_00030	Sa_NOG-W01_00265	Sa_NOG-W02_01655	Sa_NOG-W04_01082	Sa_NOG-W05_00595	Sa_NOG-W06_00441	Sa_NOG-W07_00526	Sa_NOG-W10_01169	Sa_NOG-W11_00596	Sa_NOG-W13_00297	Sa_NOG-W14_01014	Sa_NOG-01423	Sa_NOG-W25_02365
ST88_GI-1	BU_W13_t1_01356	HST-105_02275	Sa_aus0325_00031	Sa_NOG-W01_00266	Sa_NOG-W02_01654	Sa_NOG-W04_01083	Sa_NOG-W05_00594	Sa_NOG-W06_00440	Sa_NOG-W07_00527	Sa_NOG-W10_01170	Sa_NOG-W11_00595	Sa_NOG-W13_00298	Sa_NOG-W14_01015	Sa_NOG-01422	Sa_NOG-W25_02364
ST88_GI-1	*	*	Sa_aus0325_00032	*	*	*	*	*	*	*	*	*	*	*	*
ST88_GI-1	BU_W13_t1_01357	HST-105_02273	Sa_aus0325_00033	Sa_NOG-W01_00267	Sa_NOG-W02_01653	Sa_NOG-W04_02297	Sa_NOG-W05_00593	Sa_NOG-W06_02268	Sa_NOG-W07_00528	Sa_NOG-W10_01171	Sa_NOG-W11_00594	Sa_NOG-W13_00299	Sa_NOG-W14_02313	Sa_NOG-01421	Sa_NOG-W25_02535
ST88_GI-1	BU_W13_t1_01358	HST-105_02272	Sa_aus0325_00034	Sa_NOG-W01_00268	Sa_NOG-W02_01652	Sa_NOG-W04_02296	Sa_NOG-W05_00592	Sa_NOG-W06_02267	Sa_NOG-W07_00529	Sa_NOG-W10_01172	Sa_NOG-W11_00593	Sa_NOG-W13_00300	Sa_NOG-W14_02312	Sa_NOG-01420	Sa_NOG-W25_02534
ST88_GI-1	BU_W13_t1_01359	HST-105_02271	Sa_aus0325_00035	Sa_NOG-W01_00269	Sa_NOG-W02_01651	Sa_NOG-W04_02295	Sa_NOG-W05_00591	Sa_NOG-W06_02266	Sa_NOG-W07_00530	Sa_NOG-W10_01173	Sa_NOG-W11_00592	Sa_NOG-W13_00301	Sa_NOG-W14_02311	Sa_NOG-01419	Sa_NOG-W25_02533
ST88_GI-2	BU_W13_t1_01484	HST-105_01286	Sa_aus0325_00229	Sa_NOG-W01_01752	Sa_NOG-W02_01815	Sa_NOG-W04_01424	Sa_NOG-W05_01462	Sa_NOG-W06_01324	Sa_NOG-W07_01218	Sa_NOG-W10_01556	Sa_NOG-W11_01727	Sa_NOG-W13_01320	Sa_NOG-W14_01354	Sa_NOG-00892	Sa_NOG-W25_00917
ST88_GI-2	BU_W13_t1_01485	HST-105_02651	Sa_aus0325_00230	Sa_NOG-W01_01751	Sa_NOG-W02_01816	Sa_NOG-W04_01425	Sa_NOG-W05_01463	Sa_NOG-W06_01325	Sa_NOG-W07_01219	Sa_NOG-W10_01557	Sa_NOG-W11_01728	Sa_NOG-W13_01321	Sa_NOG-W14_01355	Sa_NOG-00893	Sa_NOG-W25_00918
ST88_GI-2	BU_W13_t1_01486	HST-105_02652	Sa_aus0325_00231	Sa_NOG-W01_01750	Sa_NOG-W02_01817	Sa_NOG-W04_01426	Sa_NOG-W05_01464	Sa_NOG-W06_01326	Sa_NOG-W07_01220	Sa_NOG-W10_01558	Sa_NOG-W11_01729	Sa_NOG-W13_01322	Sa_NOG-W14_01356	Sa_NOG-00894	Sa_NOG-W25_00919
ST88_GI-2	BU_W13_t1_01487	HST-105_02653	Sa_aus0325_00232	Sa_NOG-W01_01749	Sa_NOG-W02_01818	Sa_NOG-W04_01427	Sa_NOG-W05_01465	Sa_NOG-W06_01327	Sa_NOG-W07_01221	Sa_NOG-W10_01559	Sa_NOG-W11_01730	Sa_NOG-W13_01323	Sa_NOG-W14_01357	Sa_NOG-00895	Sa_NOG-W25_00920
ST88_GI-2	BU_W13_t1_01488	HST-105_02640	Sa_aus0325_00233	Sa_NOG-W01_01748	Sa_NOG-W02_01819	Sa_NOG-W04_01428	Sa_NOG-W05_01466	Sa_NOG-W06_01328	Sa_NOG-W07_01222	Sa_NOG-W10_01560	Sa_NOG-W11_01731	Sa_NOG-W13_01324	Sa_NOG-W14_01358	Sa_NOG-00896	Sa_NOG-W25_00921
ST88_GI-2	BU_W13_t1_01489	HST-105_02639	Sa_aus0325_00234	Sa_NOG-W01_01747	Sa_NOG-W02_01820	Sa_NOG-W04_01429	Sa_NOG-W05_01467	Sa_NOG-W06_01329	Sa_NOG-W07_01223	Sa_NOG-W10_01561	Sa_NOG-W11_01732	Sa_NOG-W13_01325	Sa_NOG-W14_01359	Sa_NOG-00897	Sa_NOG-W25_00922
ST88_GI-2	BU_W13_t1_01490	HST-105_02638	Sa_aus0325_00235	Sa_NOG-W01_01746	Sa_NOG-W02_01821	Sa_NOG-W04_01430	Sa_NOG-W05_01468	Sa_NOG-W06_01330	Sa_NOG-W07_01224	Sa_NOG-W10_01562	Sa_NOG-W11_01733	Sa_NOG-W13_01326	Sa_NOG-W14_01360	Sa_NOG-00898	Sa_NOG-W25_00923
ST88_GI-2	BU_W13_t1_01491	HST-105_02637	Sa_aus0325_00236	Sa_NOG-W01_01745	Sa_NOG-W02_01822	Sa_NOG-W04_01431	Sa_NOG-W05_01469	Sa_NOG-W06_01331	Sa_NOG-W07_01225	Sa_NOG-W10_01563	Sa_NOG-W11_01734	Sa_NOG-W13_01327	Sa_NOG-W14_01361	Sa_NOG-00899	Sa_NOG-W25_00924
ST88_GI-2	BU_W13_t1_01493	HST-105_02635	Sa_aus0325_00238	Sa_NOG-W01_01743	Sa_NOG-W02_01824	Sa_NOG-W04_01433	Sa_NOG-W05_01471	Sa_NOG-W06_01333	Sa_NOG-W07_01227	Sa_NOG-W10_01565	Sa_NOG-W11_01736	Sa_NOG-W13_01329	Sa_NOG-W14_01363	Sa_NOG-00901	Sa_NOG-W25_00926
ST88_GI-2	BU_W13_t1_01494	HST-105_02634	Sa_aus0325_00239	Sa_NOG-W01_01742	Sa_NOG-W02_01825	Sa_NOG-W04_01434	Sa_NOG-W05_01472	Sa_NOG-W06_01334	Sa_NOG-W07_01228	Sa_NOG-W10_01566	Sa_NOG-W11_01737	Sa_NOG-W13_01330	Sa_NOG-W14_01364	Sa_NOG-00902	Sa_NOG-W25_00927
ST88_GI-2	BU_W13_t1_01495	HST-105_02633	Sa_aus0325_00240	Sa_NOG-W01_01741	Sa_NOG-W02_01826	Sa_NOG-W04_01435	Sa_NOG-W05_01473	Sa_NOG-W06_01335	Sa_NOG-W07_01229	Sa_NOG-W10_01567	Sa_NOG-W11_01738	Sa_NOG-W13_01331	Sa_NOG-W14_01365	Sa_NOG-00903	Sa_NOG-W25_00928
ST88_GI-2	BU_W13_t1_01496	HST-105_02632	Sa_aus0325_00241	Sa_NOG-W01_01740	Sa_NOG-W02_01827	Sa_NOG-W04_01436	Sa_NOG-W05_01474	Sa_NOG-W06_01336	Sa_NOG-W07_01230	Sa_NOG-W10_01568	Sa_NOG-W11_01739	Sa_NOG-W13_01332	Sa_NOG-W14_01366	Sa_NOG-00904	Sa_NOG-W25_00929
ST88_GI-2	BU_W13_t1_01497	HST-105_02631	Sa_aus0325_00242	Sa_NOG-W01_01739	Sa_NOG-W02_01828	Sa_NOG-W04_01437	Sa_NOG-W05_01475	Sa_NOG-W06_01337	Sa_NOG-W07_01231	Sa_NOG-W10_01569	Sa_NOG-W11_01740	Sa_NOG-W13_01333	Sa_NOG-W14_01367	Sa_NOG-00905	Sa_NOG-W25_00930
ST88_GI-3	BU_W13_t1_01587	HST-105_01550	Sa_aus0325_00327	Sa_NOG-W01_01654	Sa_NOG-W02_01341	Sa_NOG-W04_01523	Sa_NOG-W05_01561	Sa_NOG-W06_01423	Sa_NOG-W07_01317	Sa_NOG-W10_01655	Sa_NOG-W11_01824	Sa_NOG-W13_01419	Sa_NOG-W14_01453	Sa_NOG-00991	Sa_NOG-W25_01015
ST88_GI-3	BU_W13_t1_01588	HST-105_01551	Sa_aus0325_00328	Sa_NOG-W01_01653	Sa_NOG-W02_01340	Sa_NOG-W04_01524	Sa_NOG-W05_01562	Sa_NOG-W06_01424	Sa_NOG-W07_01318	Sa_NOG-W10_01656	Sa_NOG-W11_01825	Sa_NOG-W13_01420	Sa_NOG-W14_01454	Sa_NOG-00992	Sa_NOG-W25_01016
ST88_GI-3	BU_W13_t1_01589	HST-105_01552	Sa_aus0325_00329	Sa_NOG-W01_01652	Sa_NOG-W02_01339	Sa_NOG-W04_01525	Sa_NOG-W05_01563	Sa_NOG-W06_01425	Sa_NOG-W07_01319	Sa_NOG-W10_01657	Sa_NOG-W11_01826	Sa_NOG-W13_01421	Sa_NOG-W14_01455	Sa_NOG-00993	Sa_NOG-W25_01017
ST88_GI-3	BU_W13_t1_01590	HST-105_01553	Sa_aus0325_00330	Sa_NOG-W01_01651	Sa_NOG-W02_01338	Sa_NOG-W04_01526	Sa_NOG-W05_01564	Sa_NOG-W06_01426	Sa_NOG-W07_01320	Sa_NOG-W10_01658	Sa_NOG-W11_01827	Sa_NOG-W13_01422	Sa_NOG-W14_01456	Sa_NOG-00994	Sa_NOG-W25_01018

ST88_GI-3	BU_W13_t1_01591	HST-105_01554	Sa_aus0325_00331	Sa_NOG-W01_01650	Sa_NOG-W02_01337	Sa_NOG-W04_01527	Sa_NOG-W05_01565	Sa_NOG-W06_01427	Sa_NOG-W07_01321	Sa_NOG-W10_01659	Sa_NOG-W11_01828	Sa_NOG-W13_01423	Sa_NOG-W14_01457	Sa_NOG-00995	Sa_NOG-W25_01019
ST88_GI-3	BU_W13_t1_01592	HST-105_01555	Sa_aus0325_00332	Sa_NOG-W01_01649	Sa_NOG-W02_01336	Sa_NOG-W04_01528	Sa_NOG-W05_01566	Sa_NOG-W06_01428	Sa_NOG-W07_01322	Sa_NOG-W10_01660	Sa_NOG-W11_01829	Sa_NOG-W13_01424	Sa_NOG-W14_01458	Sa_NOG-00996	Sa_NOG-W25_01020
ST88_GI-3	BU_W13_t1_01593	HST-105_01556	Sa_aus0325_00333	Sa_NOG-W01_01648	Sa_NOG-W02_01335	Sa_NOG-W04_01529	Sa_NOG-W05_01567	Sa_NOG-W06_01429	Sa_NOG-W07_01323	Sa_NOG-W10_01661	Sa_NOG-W11_01830	Sa_NOG-W13_01425	Sa_NOG-W14_01459	Sa_NOG-00997	Sa_NOG-W25_01021
ST88_GI-3	BU_W13_t1_01594	HST-105_01557	Sa_aus0325_00334	Sa_NOG-W01_01647	Sa_NOG-W02_01334	Sa_NOG-W04_01530	Sa_NOG-W05_01568	Sa_NOG-W06_01430	Sa_NOG-W07_01324	Sa_NOG-W10_01662	Sa_NOG-W11_01831	Sa_NOG-W13_01426	Sa_NOG-W14_01460	Sa_NOG-00998	Sa_NOG-W25_01022
ST88_GI-3	BU_W13_t1_01595	HST-105_01558	Sa_aus0325_00335	Sa_NOG-W01_01646	Sa_NOG-W02_01333	Sa_NOG-W04_01531	Sa_NOG-W05_01569	Sa_NOG-W06_01431	Sa_NOG-W07_01342	Sa_NOG-W10_01663	Sa_NOG-W11_01832	Sa_NOG-W13_01427	Sa_NOG-W14_01461	Sa_NOG-00999	Sa_NOG-W25_01023
ST88_GI-3	BU_W13_t1_01596	HST-105_01559	Sa_aus0325_00336	Sa_NOG-W01_01645	Sa_NOG-W02_01332	Sa_NOG-W04_01532	Sa_NOG-W05_01570	Sa_NOG-W06_01432	Sa_NOG-W07_01343	Sa_NOG-W10_01664	Sa_NOG-W11_01833	Sa_NOG-W13_01428	Sa_NOG-W14_01462	Sa_NOG-01000	Sa_NOG-W25_01024
ST88_GI-3	BU_W13_t1_01597	HST-105_01560	Sa_aus0325_00337	Sa_NOG-W01_01644	Sa_NOG-W02_01331	Sa_NOG-W04_01533	Sa_NOG-W05_01571	Sa_NOG-W06_01433	Sa_NOG-W07_01344	Sa_NOG-W10_01665	Sa_NOG-W11_01834	Sa_NOG-W13_01429	Sa_NOG-W14_01463	Sa_NOG-01001	Sa_NOG-W25_01025
ST88_GI-3	BU_W13_t1_01598	HST-105_01561	Sa_aus0325_00338	Sa_NOG-W01_01643	Sa_NOG-W02_01330	Sa_NOG-W04_01534	Sa_NOG-W05_01572	Sa_NOG-W06_01434	Sa_NOG-W07_01345	Sa_NOG-W10_01666	Sa_NOG-W11_01835	Sa_NOG-W13_01430	Sa_NOG-W14_01464	Sa_NOG-01002	Sa_NOG-W25_01026
ST88_GI-3	BU_W13_t1_01599	HST-105_01562	Sa_aus0325_00339	Sa_NOG-W01_01642	Sa_NOG-W02_01329	Sa_NOG-W04_01535	Sa_NOG-W05_01573	Sa_NOG-W06_01435	Sa_NOG-W07_01346	Sa_NOG-W10_01667	Sa_NOG-W11_01836	Sa_NOG-W13_01431	Sa_NOG-W14_01465	Sa_NOG-01003	Sa_NOG-W25_01027
ST88_GI-3	BU_W13_t1_01600	HST-105_01563	Sa_aus0325_00340	Sa_NOG-W01_01641	Sa_NOG-W02_01328	Sa_NOG-W04_01536	Sa_NOG-W05_01574	Sa_NOG-W06_01436	Sa_NOG-W07_01347	Sa_NOG-W10_01668	Sa_NOG-W11_01837	Sa_NOG-W13_01432	Sa_NOG-W14_01466	Sa_NOG-01004	Sa_NOG-W25_01028
ST88_GI-3	BU_W13_t1_01601	HST-105_01564	Sa_aus0325_00341	Sa_NOG-W01_01640	Sa_NOG-W02_01327	Sa_NOG-W04_01537	Sa_NOG-W05_01575	Sa_NOG-W06_01437	Sa_NOG-W07_01348	Sa_NOG-W10_01669	Sa_NOG-W11_01838	Sa_NOG-W13_01433	Sa_NOG-W14_01467	Sa_NOG-01005	Sa_NOG-W25_01029
ST88_GI-3	BU_W13_t1_01602	HST-105_01565	Sa_aus0325_00342	Sa_NOG-W01_01639	Sa_NOG-W02_01326	Sa_NOG-W04_01538	Sa_NOG-W05_01576	Sa_NOG-W06_01438	Sa_NOG-W07_01349	Sa_NOG-W10_01670	Sa_NOG-W11_01839	Sa_NOG-W13_01434	Sa_NOG-W14_01468	Sa_NOG-01006	Sa_NOG-W25_01030
ST88_GI-3	BU_W13_t1_01603	HST-105_01566	Sa_aus0325_00343	Sa_NOG-W01_01638	Sa_NOG-W02_01325	Sa_NOG-W04_01539	Sa_NOG-W05_01577	Sa_NOG-W06_01439	Sa_NOG-W07_01350	Sa_NOG-W10_01671	Sa_NOG-W11_01840	Sa_NOG-W13_01435	Sa_NOG-W14_01469	Sa_NOG-01007	Sa_NOG-W25_01031
ST88_GI-3	BU_W13_t1_01604	HST-105_01567	Sa_aus0325_00344	Sa_NOG-W01_01637	Sa_NOG-W02_01324	Sa_NOG-W04_01540	Sa_NOG-W05_01578	Sa_NOG-W06_01440	Sa_NOG-W07_01351	Sa_NOG-W10_01672	Sa_NOG-W11_01841	Sa_NOG-W13_01436	Sa_NOG-W14_01470	Sa_NOG-01008	Sa_NOG-W25_01032
ST88_GI-3	BU_W13_t1_01605	HST-105_01568	Sa_aus0325_00345	Sa_NOG-W01_01636	Sa_NOG-W02_01323	Sa_NOG-W04_01541	Sa_NOG-W05_01579	Sa_NOG-W06_01441	Sa_NOG-W07_01352	Sa_NOG-W10_01673	Sa_NOG-W11_01842	Sa_NOG-W13_01437	Sa_NOG-W14_01471	Sa_NOG-01009	Sa_NOG-W25_01033
ST88_GI-3	BU_W13_t1_01606	HST-105_01569	Sa_aus0325_00346	Sa_NOG-W01_01635	Sa_NOG-W02_01322	Sa_NOG-W04_01542	Sa_NOG-W05_01580	Sa_NOG-W06_01442	Sa_NOG-W07_01353	Sa_NOG-W10_01674	Sa_NOG-W11_01843	Sa_NOG-W13_01438	Sa_NOG-W14_01472	Sa_NOG-01010	Sa_NOG-W25_01034
ST88_GI-3	BU_W13_t1_01607	HST-105_01570	Sa_aus0325_00347	Sa_NOG-W01_01634	Sa_NOG-W02_01321	Sa_NOG-W04_01543	Sa_NOG-W05_01581	Sa_NOG-W06_01443	Sa_NOG-W07_01354	Sa_NOG-W10_01675	Sa_NOG-W11_01844	Sa_NOG-W13_01439	Sa_NOG-W14_01473	Sa_NOG-01011	Sa_NOG-W25_01035
ST88_GI-3	BU_W13_t1_01608	HST-105_01571	Sa_aus0325_00348	Sa_NOG-W01_01633	Sa_NOG-W02_01320	Sa_NOG-W04_01544	Sa_NOG-W05_01582	Sa_NOG-W06_01444	Sa_NOG-W07_01355	Sa_NOG-W10_01676	Sa_NOG-W11_01845	Sa_NOG-W13_01440	Sa_NOG-W14_01474	Sa_NOG-01012	Sa_NOG-W25_01036
ST88_GI-3	BU_W13_t1_01609	HST-105_01572	Sa_aus0325_00349	Sa_NOG-W01_01632	Sa_NOG-W02_01319	Sa_NOG-W04_01545	Sa_NOG-W05_01583	Sa_NOG-W06_01445	Sa_NOG-W07_01356	Sa_NOG-W10_01677	Sa_NOG-W11_01846	Sa_NOG-W13_01441	Sa_NOG-W14_01475	Sa_NOG-01013	Sa_NOG-W25_01037
ST88_GI-3	BU_W13_t1_01610	HST-105_01573	Sa_aus0325_00350	Sa_NOG-W01_02682	Sa_NOG-W02_01318	Sa_NOG-W04_01546	Sa_NOG-W05_01584	Sa_NOG-W06_01446	Sa_NOG-W07_01357	Sa_NOG-W10_01678	Sa_NOG-W11_01847	Sa_NOG-W13_01442	Sa_NOG-W14_01476	Sa_NOG-01014	Sa_NOG-W25_01038
ST88_GI-3	BU_W13_t1_01611	HST-105_01574	Sa_aus0325_00351	Sa_NOG-W01_02681	Sa_NOG-W02_01317	Sa_NOG-W04_01547	Sa_NOG-W05_01585	Sa_NOG-W06_01447	Sa_NOG-W07_01358	Sa_NOG-W10_01679	Sa_NOG-W11_01848	Sa_NOG-W13_01443	Sa_NOG-W14_01477	Sa_NOG-01015	Sa_NOG-W25_01039
ST88_GI-3	*	HST-105_01575	Sa_aus0325_00352	*	Sa_NOG-W02_01316	*	*	Sa_NOG-W06_01448	Sa_NOG-W07_01359	Sa_NOG-W10_01680	Sa_NOG-W11_01849	Sa_NOG-W13_01444	Sa_NOG-W14_01478	*	*
ST88_GI-3	BU_W13_t1_02323	HST-105_01576	Sa_aus0325_00353	Sa_NOG-W01_02549	Sa_NOG-W02_01315	Sa_NOG-W04_02379	Sa_NOG-W05_02514	Sa_NOG-W06_01449	Sa_NOG-W07_01360	Sa_NOG-W10_01681	Sa_NOG-W11_01850	Sa_NOG-W13_01445	Sa_NOG-W14_01479	Sa_NOG-02449	Sa_NOG-W25_02623
ST88_GI-3	BU_W13_t1_02324	HST-105_01577	Sa_aus0325_00354	Sa_NOG-W01_02548	Sa_NOG-W02_01314	Sa_NOG-W04_02380	Sa_NOG-W05_02513	Sa_NOG-W06_01450	Sa_NOG-W07_01361	Sa_NOG-W10_01682	Sa_NOG-W11_01851	Sa_NOG-W13_01446	Sa_NOG-W14_01480	Sa_NOG-02450	Sa_NOG-W25_02622
ST88_GI-3	BU_W13_t1_02325	HST-105_01578	Sa_aus0325_00355	Sa_NOG-W01_02547	Sa_NOG-W02_01313	Sa_NOG-W04_02381	Sa_NOG-W05_02512	Sa_NOG-W06_01451	Sa_NOG-W07_01362	Sa_NOG-W10_01683	Sa_NOG-W11_01852	Sa_NOG-W13_01447	Sa_NOG-W14_01481	Sa_NOG-02451	Sa_NOG-W25_02621
ST88_GI-3	BU_W13_t1_02326	HST-105_01579	Sa_aus0325_00356	Sa_NOG-W01_02546	Sa_NOG-W02_01312	Sa_NOG-W04_02382	Sa_NOG-W05_02511	Sa_NOG-W06_01452	Sa_NOG-W07_01363	Sa_NOG-W10_01684	Sa_NOG-W11_01853	Sa_NOG-W13_01448	Sa_NOG-W14_01482	Sa_NOG-02452	Sa_NOG-W25_02620
ST88_GI-3	BU_W13_t1_02327	HST-105_01580	Sa_aus0325_00357	Sa_NOG-W01_02545	Sa_NOG-W02_01311	Sa_NOG-W04_02383	Sa_NOG-W05_02510	Sa_NOG-W06_01453	Sa_NOG-W07_02483	Sa_NOG-W10_01685	Sa_NOG-W11_01854	Sa_NOG-W13_01449	Sa_NOG-W14_01483	Sa_NOG-02453	Sa_NOG-W25_02619
ST88_GI-3	BU_W13_t1_02328	HST-105_01581	Sa_aus0325_00358	Sa_NOG-W01_02544	Sa_NOG-W02_01310	Sa_NOG-W04_02384	Sa_NOG-W05_02509	Sa_NOG-W06_01454	Sa_NOG-W07_02484	Sa_NOG-W10_01686	Sa_NOG-W11_01855	Sa_NOG-W13_01450	Sa_NOG-W14_01484	Sa_NOG-02454	Sa_NOG-W25_02618
ST88_GI-3	BU_W13_t1_02329	HST-105_01582	Sa_aus0325_00359	Sa_NOG-W01_02543	Sa_NOG-W02_01309	Sa_NOG-W04_02385	Sa_NOG-W05_02508	Sa_NOG-W06_01455	Sa_NOG-W07_02485	Sa_NOG-W10_01687	Sa_NOG-W11_01856	Sa_NOG-W13_01451	Sa_NOG-W14_01485	Sa_NOG-02455	Sa_NOG-W25_02617

ST88_GI-3	BU_W13_t1_02330	HST-105_01583	Sa_aus0325_00360	Sa_NOG-W01_02542	Sa_NOG-W02_01308	Sa_NOG-W04_02386	Sa_NOG-W05_02507	Sa_NOG-W06_01456	Sa_NOG-W07_02486	Sa_NOG-W10_01688	Sa_NOG-W11_01857	Sa_NOG-W13_01452	Sa_NOG-W14_01486	Sa_NOG-02456	Sa_NOG-W25_02616
ST88_GI-3	BU_W13_t1_02331	HST-105_01584	Sa_aus0325_00361	Sa_NOG-W01_02541	Sa_NOG-W02_01307	Sa_NOG-W04_02387	Sa_NOG-W05_02506	Sa_NOG-W06_01457	Sa_NOG-W07_02487	Sa_NOG-W10_01689	Sa_NOG-W11_01858	Sa_NOG-W13_01453	Sa_NOG-W14_01487	Sa_NOG-02457	Sa_NOG-W25_02615
ST88_GI-3	BU_W13_t1_02332	HST-105_01585	Sa_aus0325_00362	Sa_NOG-W01_02540	Sa_NOG-W02_01306	Sa_NOG-W04_02388	Sa_NOG-W05_02505	Sa_NOG-W06_01458	Sa_NOG-W07_02488	Sa_NOG-W10_01690	Sa_NOG-W11_01859	Sa_NOG-W13_01454	Sa_NOG-W14_01488	Sa_NOG-02458	Sa_NOG-W25_02614
ST88_GI-4	BU_W13_t1_00816	HST-105_00744	Sa_aus0325_00793	Sa_NOG-W01_00676	Sa_NOG-W02_00272	Sa_NOG-W04_00275	Sa_NOG-W05_00275	Sa_NOG-W06_00971	Sa_NOG-W07_00780	Sa_NOG-W10_00275	Sa_NOG-W11_00275	Sa_NOG-W13_00445	Sa_NOG-W14_00475	Sa_NOG-00267	Sa_NOG-W25_00100
ST88_GI-4	BU_W13_t1_00817	HST-105_00743	Sa_aus0325_00794	Sa_NOG-W01_00675	Sa_NOG-W02_00273	Sa_NOG-W04_00274	Sa_NOG-W05_00274	Sa_NOG-W06_00970	Sa_NOG-W07_00781	Sa_NOG-W10_00274	Sa_NOG-W11_00274	Sa_NOG-W13_00444	Sa_NOG-W14_00474	Sa_NOG-00266	Sa_NOG-W25_00099
ST88_GI-4	BU_W13_t1_00818	HST-105_00742	Sa_aus0325_00795	Sa_NOG-W01_00674	Sa_NOG-W02_00274	Sa_NOG-W04_00273	Sa_NOG-W05_00273	Sa_NOG-W06_00969	Sa_NOG-W07_00782	Sa_NOG-W10_00273	Sa_NOG-W11_00273	Sa_NOG-W13_00443	Sa_NOG-W14_00473	Sa_NOG-00265	Sa_NOG-W25_00098
ST88_GI-4	BU_W13_t1_00819	HST-105_00741	Sa_aus0325_00796	Sa_NOG-W01_00673	Sa_NOG-W02_00275	Sa_NOG-W04_00272	Sa_NOG-W05_00272	Sa_NOG-W06_00968	Sa_NOG-W07_00783	Sa_NOG-W10_00272	Sa_NOG-W11_00272	Sa_NOG-W13_00442	Sa_NOG-W14_00472	Sa_NOG-00264	Sa_NOG-W25_00097
ST88_GI-4	BU_W13_t1_00820	HST-105_00740	Sa_aus0325_00797	Sa_NOG-W01_00672	Sa_NOG-W02_00276	Sa_NOG-W04_00271	Sa_NOG-W05_00271	Sa_NOG-W06_00967	Sa_NOG-W07_00784	Sa_NOG-W10_00271	Sa_NOG-W11_00271	Sa_NOG-W13_00441	Sa_NOG-W14_00471	Sa_NOG-00263	Sa_NOG-W25_00096
ST88_GI-4	BU_W13_t1_00821	HST-105_00739	Sa_aus0325_00798	Sa_NOG-W01_00671	Sa_NOG-W02_00277	Sa_NOG-W04_00270	Sa_NOG-W05_00270	*	Sa_NOG-W07_00785	Sa_NOG-W10_00270	Sa_NOG-W11_00270	Sa_NOG-W13_00440	Sa_NOG-W14_00470	Sa_NOG-00262	Sa_NOG-W25_00095
ST88_GI-4	*	*	Sa_aus0325_00799	*	*	*	*	*	*	*	*	*	*	Sa_NOG-00811	*
ST88_GI-4	BU_W13_t1_00822	HST-105_00738	Sa_aus0325_00800	Sa_NOG-W01_00670	Sa_NOG-W02_00278	Sa_NOG-W04_00269	Sa_NOG-W05_00269	Sa_NOG-W06_00171	Sa_NOG-W07_00786	Sa_NOG-W10_00269	Sa_NOG-W11_00269	Sa_NOG-W13_00439	Sa_NOG-W14_00469	Sa_NOG-00810	Sa_NOG-W25_00094
ST88_GI-4	BU_W13_t1_00823	HST-105_00737	Sa_aus0325_00801	Sa_NOG-W01_00669	Sa_NOG-W02_00279	Sa_NOG-W04_00268	Sa_NOG-W05_00268	Sa_NOG-W06_00172	Sa_NOG-W07_00787	Sa_NOG-W10_00268	Sa_NOG-W11_00268	Sa_NOG-W13_00438	Sa_NOG-W14_00468	Sa_NOG-00809	Sa_NOG-W25_00093
ST88_GI-4	BU_W13_t1_00824	HST-105_00736	Sa_aus0325_00802	Sa_NOG-W01_00668	Sa_NOG-W02_00280	Sa_NOG-W04_00267	Sa_NOG-W05_00267	Sa_NOG-W06_00173	Sa_NOG-W07_00788	Sa_NOG-W10_00267	Sa_NOG-W11_00267	Sa_NOG-W13_00437	Sa_NOG-W14_00467	Sa_NOG-00808	Sa_NOG-W25_00092
ST88_GI-4	BU_W13_t1_00825	HST-105_00735	Sa_aus0325_00803	Sa_NOG-W01_00667	Sa_NOG-W02_00281	Sa_NOG-W04_00266	Sa_NOG-W05_00266	Sa_NOG-W06_00174	Sa_NOG-W07_00789	Sa_NOG-W10_00266	Sa_NOG-W11_00266	Sa_NOG-W13_00436	Sa_NOG-W14_00466	Sa_NOG-00807	Sa_NOG-W25_00091
ST88_GI-4	BU_W13_t1_00826	HST-105_00734	Sa_aus0325_00804	Sa_NOG-W01_00666	Sa_NOG-W02_00282	Sa_NOG-W04_00265	Sa_NOG-W05_00265	Sa_NOG-W06_00175	Sa_NOG-W07_00790	Sa_NOG-W10_00265	Sa_NOG-W11_00265	Sa_NOG-W13_00435	Sa_NOG-W14_00465	Sa_NOG-00806	Sa_NOG-W25_00090
ST88_GI-5	BU_W13_t1_02586	HST-105_02561	Sa_aus0325_01715	Sa_NOG-W01_02274	Sa_NOG-W02_02431	Sa_NOG-W04_02527	Sa_NOG-W05_02614	Sa_NOG-W06_02350	Sa_NOG-W07_02629	Sa_NOG-W10_02058	Sa_NOG-W11_02557	Sa_NOG-W13_02618	Sa_NOG-W14_01854	Sa_NOG-02532	Sa_NOG-W25_02265
ST88_GI-5	*	HST-105_02560	Sa_aus0325_01716	Sa_NOG-W01_02273	Sa_NOG-W02_02430	*	*	Sa_NOG-W06_02349	*	Sa_NOG-W10_02057	*	Sa_NOG-W13_02617	Sa_NOG-W14_01853	*	Sa_NOG-W25_02264
ST88_GI-5	BU_W13_t1_02691	HST-105_02559	Sa_aus0325_01717	Sa_NOG-W01_02272	Sa_NOG-W02_02429	Sa_NOG-W04_02377	Sa_NOG-W05_02474	Sa_NOG-W06_02348	Sa_NOG-W07_02419	Sa_NOG-W10_02056	Sa_NOG-W11_02448	Sa_NOG-W13_02616	Sa_NOG-W14_01852	Sa_NOG-02447	Sa_NOG-W25_02263
ST88_GI-5	BU_W13_t1_02690	HST-105_02329	Sa_aus0325_01718	Sa_NOG-W01_02271	Sa_NOG-W02_02428	Sa_NOG-W04_02376	Sa_NOG-W05_02473	Sa_NOG-W06_02347	Sa_NOG-W07_02418	Sa_NOG-W10_02055	Sa_NOG-W11_02447	Sa_NOG-W13_02615	Sa_NOG-W14_01851	Sa_NOG-02446	Sa_NOG-W25_02262
ST88_GI-5	BU_W13_t1_02689	HST-105_02330	Sa_aus0325_01719	Sa_NOG-W01_02270	Sa_NOG-W02_02427	Sa_NOG-W04_02375	Sa_NOG-W05_02472	Sa_NOG-W06_02346	Sa_NOG-W07_02417	Sa_NOG-W10_02054	Sa_NOG-W11_02446	Sa_NOG-W13_02614	Sa_NOG-W14_01850	Sa_NOG-02445	Sa_NOG-W25_02261
ST88_GI-5	BU_W13_t1_02687	HST-105_02332	Sa_aus0325_01720	Sa_NOG-W01_02268	Sa_NOG-W02_02425	Sa_NOG-W04_02373	Sa_NOG-W05_02470	Sa_NOG-W06_02344	Sa_NOG-W07_02415	Sa_NOG-W10_02052	Sa_NOG-W11_02444	Sa_NOG-W13_02612	Sa_NOG-W14_01848	Sa_NOG-02443	Sa_NOG-W25_02259
ST88_GI-5	BU_W13_t1_02686	HST-105_02333	Sa_aus0325_01721	Sa_NOG-W01_02267	Sa_NOG-W02_02424	Sa_NOG-W04_02372	Sa_NOG-W05_02469	Sa_NOG-W06_02343	Sa_NOG-W07_02414	Sa_NOG-W10_02051	Sa_NOG-W11_02443	Sa_NOG-W13_02611	Sa_NOG-W14_01847	Sa_NOG-02442	Sa_NOG-W25_02258
ST88_GI-5	BU_W13_t1_02685	HST-105_02334	Sa_aus0325_01722	Sa_NOG-W01_02266	Sa_NOG-W02_02423	Sa_NOG-W04_02371	Sa_NOG-W05_02468	Sa_NOG-W06_02342	Sa_NOG-W07_02413	Sa_NOG-W10_02050	Sa_NOG-W11_02442	Sa_NOG-W13_02610	Sa_NOG-W14_01846	Sa_NOG-02441	Sa_NOG-W25_02257
ST88_GI-5	BU_W13_t1_02684	HST-105_02335	Sa_aus0325_01723	Sa_NOG-W01_02265	Sa_NOG-W02_02422	Sa_NOG-W04_02370	Sa_NOG-W05_02467	Sa_NOG-W06_02341	Sa_NOG-W07_02412	Sa_NOG-W10_02049	Sa_NOG-W11_02441	Sa_NOG-W13_02609	Sa_NOG-W14_01845	Sa_NOG-02440	Sa_NOG-W25_02256
ST88_GI-5	BU_W13_t1_02683	HST-105_02336	Sa_aus0325_01724	Sa_NOG-W01_02264	Sa_NOG-W02_02421	Sa_NOG-W04_02369	Sa_NOG-W05_02466	Sa_NOG-W06_02340	Sa_NOG-W07_02411	Sa_NOG-W10_02048	Sa_NOG-W11_02440	Sa_NOG-W13_02608	Sa_NOG-W14_01844	Sa_NOG-02439	Sa_NOG-W25_02255
ST88_GI-5	BU_W13_t1_02475	HST-105_02337	Sa_aus0325_01725	Sa_NOG-W01_02263	Sa_NOG-W02_02420	Sa_NOG-W04_02368	Sa_NOG-W05_02465	Sa_NOG-W06_02339	Sa_NOG-W07_02410	Sa_NOG-W10_02047	Sa_NOG-W11_02439	Sa_NOG-W13_02532	Sa_NOG-W14_01843	Sa_NOG-02438	Sa_NOG-W25_02254
ST88_GI-5	BU_W13_t1_02474	HST-105_02338	Sa_aus0325_01726	Sa_NOG-W01_02262	Sa_NOG-W02_02419	Sa_NOG-W04_02367	Sa_NOG-W05_02464	Sa_NOG-W06_02338	Sa_NOG-W07_02409	Sa_NOG-W10_02046	Sa_NOG-W11_02438	Sa_NOG-W13_02531	Sa_NOG-W14_01842	Sa_NOG-02437	Sa_NOG-W25_02253
ST88_GI-5	BU_W13_t1_02473	HST-105_02339	Sa_aus0325_01727	Sa_NOG-W01_02261	Sa_NOG-W02_02418	Sa_NOG-W04_02366	Sa_NOG-W05_02463	Sa_NOG-W06_02337	Sa_NOG-W07_02408	Sa_NOG-W10_02045	Sa_NOG-W11_02437	Sa_NOG-W13_02530	Sa_NOG-W14_01841	Sa_NOG-02436	Sa_NOG-W25_02252
ST88_GI-5	BU_W13_t1_02472	HST-105_02340	Sa_aus0325_01728	Sa_NOG-W01_02260	Sa_NOG-W02_02417	Sa_NOG-W04_02365	Sa_NOG-W05_02462	Sa_NOG-W06_02336	Sa_NOG-W07_02407	Sa_NOG-W10_02044	Sa_NOG-W11_02436	Sa_NOG-W13_02529	Sa_NOG-W14_01840	Sa_NOG-02435	Sa_NOG-W25_02251

Table S2: Genomic regions enriched in *S. aureus*MRSA ST88 from Ghana

Feature	Annotation
Ghaniaian_GI-1	Replication protein
Ghaniaian_GI-1	putative tetracycline resistance protein
Ghaniaian_GI-1	tetracycline resistance protein TetM
Ghaniaian_GI-1	hypothetical protein
Ghaniaian_GI-1	hypothetical protein
Ghaniaian_GI-1	hypothetical protein
Ghaniaian_GI-1	putative ATP/GTP-binding protein
Ghaniaian_GI-1	putative antirestriction protein
Ghaniaian_GI-1	hypothetical protein
Ghaniaian_GI-1	replication initiation factor
Ghaniaian_GI-1	DNA segregation ATPase FtsK/SpoIIIE
Ghaniaian_GI-1	hypothetical protein
Ghaniaian_GI-1	hypothetical protein
Ghaniaian_GI-2	AP2 domain protein
Ghaniaian_GI-2	phage tape measure protein
Ghaniaian_GI-2	phage protein
Ghaniaian_GI-2	transcriptional activator
Ghaniaian_GI-2	hypothetical protein
Ghaniaian_GI-2	hypothetical protein
Ghaniaian_GI-2	hypothetical protein
Ghaniaian_GI-2	hypothetical protein
Ghaniaian_GI-2	putative phage protein
Ghaniaian_GI-2	acetyltransferase (GNAT) family protein
Ghaniaian_GI-2	phage protein
Ghaniaian_GI-2	phage protein
Ghaniaian_GI-2	phage protein
Ghaniaian_GI-2	phage protein
Ghaniaian_GI-2	hypothetical protein
Ghaniaian_GI-2	phage protein
Ghaniaian_GI-3	hypothetical protein
Ghaniaian_GI-3	hypothetical protein
Ghaniaian_GI-3	hypothetical protein
Ghaniaian_GI-3	hypothetical protein
Ghaniaian_GI-3	hypothetical protein
Ghaniaian_GI-3	hypothetical protein
Ghaniaian_GI-3	Phage regulatory protein Rha (Phage_pRha)
Ghaniaian_GI-3	hypothetical protein
Ghaniaian_GI-4	pathogenicity island protein integrase
Ghaniaian_GI-4	Excisionase from transposon Tn916
Ghaniaian_GI-4	Helix-turn-helix domain protein
Ghaniaian_GI-4	Sigma-70 Region 4 protein

Ghaniaian_GI-4	putative transcriptional regulator
Ghaniaian_GI-4	cassette chromosome recombinase B
Ghaniaian_GI-5	hypothetical protein
Ghaniaian_GI-5	recombinase/resolvase
Ghaniaian_GI-5	transposase for transposon Tn552
Ghaniaian_GI-5	major facilitator superfamily multidrug :cation symporter
Ghaniaian_GI-5	Gram positive anchor
Ghaniaian_GI-5	Initiator Replication protein
Ghaniaian_GI-5	putative 27.7 kDa protein
Ghaniaian_GI-5	Relaxase/Mobilisation nuclease domain protein
Ghaniaian_GI-5	Bacterial mobilisation protein (MobC)
Ghaniaian_GI-5	hypothetical protein
Ghaniaian_GI-5	ABC superfamily ATP binding cassette transporter ABC protein
Ghaniaian_GI-5	hypothetical protein
Ghaniaian_GI-5	hypothetical protein
Ghaniaian_GI-5	hypothetical protein
Ghaniaian_GI-5	hypothetical protein
Ghaniaian_GI-5	hypothetical protein
Ghaniaian_GI-5	putative transcriptional regulator
Ghaniaian_GI-5	fibrinogen-binding protein
Ghaniaian_GI-5	hypothetical protein
Ghaniaian_GI-5	hypothetical protein
Ghaniaian_GI-5	fibronectin-binding protein A
Ghaniaian_GI-5	hypothetical protein
Ghaniaian_GI-5	Replication protein
Ghaniaian_GI-5	hypothetical protein
Ghaniaian_GI-5	hypothetical protein
Ghaniaian_GI-5	Replication protein
Ghaniaian_GI-5	hypothetical protein
Ghaniaian_GI-5	phage transcriptional regulator
Ghaniaian_GI-5	hypothetical protein
Ghaniaian_GI-5	Transposase IS116/IS110/IS902 family protein
Ghaniaian_GI-5	hypothetical protein
Ghaniaian_GI-5	putative transposase for
Ghaniaian_GI-5	transposase for IS431mec
Ghaniaian_GI-5	putative deoxyribose-phosphate aldolase
Ghaniaian_GI-5	hypothetical protein

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Chapter 6

Antimicrobial drug susceptibility patterns of clinical isolates of Gram-negative bacteria from two primary health care facilities in Ghana

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Abstract

Objectives: The upsurge of antibiotic resistant organisms is of concern to global health as it is making treatable infections difficult to treat. We characterized the resistant profiles of bacteria isolated from wounds of patients attending two health facilities in Ghana.

Methods: Phenotypic antibiotic susceptibility testing of *Pseudomonas aeruginosa* (n=89), *Escherichia coli* (n=23), *Enterobacter cloacae* (n=26), *Klebsiella pneumonia* (n=25), and *Proteus mirabilis* (n=37) clinical isolates was performed by the Kirby Bauer disc diffusion and multidrug-resistance *P. aeruginosa* identified. Isolates were further investigated for Extended Spectrum ß-Lactamase (ESBL) production and metallo ß-lactamase (MBL) production. Resistance conferring genes $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, $bla_{\text{IMP-1}}$, $bla_{\text{VIM-1}}$, $bla_{\text{OXA-2}}$, $bla_{\text{OXA-48}}$, bla_{NDM}, bla_{KPC} and bla_{SPM} were analysed by PCR.

Results: High frequency of resistance (>50%) to common first line antibiotics and a lower frequency (<50%) to aminoglycosides, ciprofloxacin, aztreonam, ceftazidime, imipenem and colistin was observed. ESBL production was confirmed in 26 isolates phenotypically while 50% of the isolates harboured at least one ESBL-conferring gene. Carbapenem encoding genes were detected in 41% of isolates. Eight antibiotic conferring genes were detected.

Conclusions: The observed frequency of ESBL and carbapenem resistance indicates the need to set up surveillance networks and to strictly enforce policies which guide rational use of antibiotics in Ghana.

Introduction

Gram-negative bacteria (GNB) are important causes of both nosocomial and community acquired infections such as pneumonia, bloodstream infections, wound and surgical site infections and meningitis. ß-lactam antibiotics, which include the penicillins, cephalosporins, carbapenems and monobactams, are commonly prescribed for treatment of infections due to GNB. Extensive use of these antibiotics has led to an increase in resistance mediated by a variety of mechanisms [1]; the most common and clinically significant being the production of β-lactamase enzymes [2]. These enzymes are usually encoded by genes present on plasmids, which have the potential of being horizontally transferred among strains and species. These plasmids also frequently carry genes that confer resistance to other classes of antibiotics such as aminoglycosides and quinolones, further limiting therapeutic options [3-4].

As resistance increased, many new antibiotics termed as 'β-lactamase-stable β-lactams' which include the oxyimino cephalosporins, cephamycins, aztreonam, temocillin and carbapenems were introduced in the 1970's [5]. The oxyimino cephalosporins gained widespread use and with it came the emergence of resistance due to extended spectrum β-lactamases (ESBL's). The first ESBL-producing organisms were detected in Europe in 1983 [6]. Since then, over 1000 ESBL's have been described worldwide, found in many different genera of *Enterobacteriaceae* and *Pseudomonas aeruginosa* [7]. ESBL enzymes are commonly encoded by TEM, SHV and CTX-M plasmid types and occasionally by OXA, PER, VEB, GES, BES, TLA, SFO and IBC types [3]. The treatment of choice for severe infections due to ESBL-producing organisms is the carbapenems. However, carbapenem resistance has been detected recently, mediated by class A carbapenemases (eg KPC, GES), class B metallo-β-lactamases (eg VIM, IMP, NDM, SPM, IMI types) and class D carbapenemases (OXA-23, -48, -51, -58 and -143) [8-9].

Also on the increase is the prevalence of *P. aeruginosa* isolates with a multidrug-resistant (MDR) phenotype. These organisms have been implicated in 13% of all healthcare-associated *P. aeruginosa* infections in the United States and over 400 deaths yearly, leading to their classification as a serious threat by the Center for Disease Control (CDC) (www.cdc.gov).

Surveillance of antimicrobial resistance is crucial to help in the formulation of policies, put in place proper interventions to curb the spread of resistant pathogens [10], as well as to guide clinicians on retreatment regimens. However, in many African countries, surveillance

systems to monitor the occurrence and spread of drug resistant pathogens are lacking [11]. Studies on ESBL-producing bacteria and other antibiotic resistant pathogens in Africa have been performed in the Northern and Southern regions of the continent [11]. The few studies conducted in Ghana have shown high rate of antibiotic resistance, which may be fuelled by the misuse and abuse of drugs by both patients and healthcare professionals through indiscriminate, partial and improper use [12-15]. Routine detection of ESBLs in primary and regional health facilities is absent in Ghana due to lack of both infrastructural capacity and expertise to detect these resistant carrying organisms [14]. This poses a great threat to patient care and infection control measures as most of the ESBLs are easily transmitted within the hospital setting between patients and health care workers [3]. While few studies have been done to assess this problem, the available information is scanty. Most of the studies focused on the phenotypic detection of ESBLs and the very few which incorporated molecular detection, limited their research to the detection of the most common ESBL conferring genes $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{SHV} [16-18]. Furthermore, no study in Ghana has investigated carbapenem resistance.

This study was initiated to assess antimicrobial resistance among GNB isolated from wounds of patients and the environment of two primary health care centers and provides baseline data on a broad spectrum of resistance genes for Ghana.

Materials and Methods

Bacterial strains

Two hundred (n=200) Gram-negative bacterial isolates from wounds of patients attending two health centers in the Ga district of the Greater Accra region of Ghana between May 2010 and January 2014 were used in the study. The isolates consisted of 81 *P. aeruginosa*, 37 *Proteus mirabilis*, 26 *Enterobacter cloacae*, 25 *Klebsiella pneumonia*e and 23 *Escherichia coli* isolates. The isolates were cultured aerobically from swab samples collected from the undermined edges of the wounds and inoculated on MacConkey (Oxoid Ltd, Basingstoke, UK) and Blood agar (Oxoid blood agar base with 5% sheep blood, Oxoid Ltd Basingstoke, UK). Bacterial isolates were identified presumptively based on morphology and oxidase reaction and further confirmation was done using the Analytical Profile Index 20E (API20E) strips (bio-Mérieux SA, Marcy-l'É'toile, France).

Antibiotic susceptibility testing

Antibiotic susceptibility of the isolates to the following antibiotics: amikacin (30µg), gentamicin (10µg), tobramycin (10µg), ceftriaxone (30µg), cefuroxime (30µg), cefotaxime (30µg), ceftazidime (30µg), sulphamethoxazole-trimethoprim (23.75µg/1.25µg), ciprofloxacin (5µg), aztreonam (30µg), ticarcillin-clavulanic acid (75µg/10µg), ampicillin (10µg), chloramphenicol (30µg), colistin (10µg) and imipenem (10µg) was determined by the Kirby-Bauer disc diffusion method according to CLSI standards [19]. Additionally, *P. aeruginosa* isolates were tested against streptomycin (10µg) and rifampicin (5µg). The antibiotic discs were purchased from Oxoid (Oxoid Ltd, Basingstoke, UK) and Becton Dickinson (Becton, Dickinson and Company, New Jersey, USA). MDR in *P. aeruginosa* was defined as a resistance of $\geq$ 1 antimicrobial agent in $\geq$3 antimicrobial classes of aminoglycosides (gentamicin, tobramycin, amikacin), antipseudomonal carbapenems (imipenem, meropenem doripenem), antipseudomonal cephalosporins (ceftazidime, cefepime), antipseudomonal penicillins and β-lactamase inhibitors (ticarcillin-clavulanic acid, piperacillin-tazobactam), monobactams (aztreonam) and polymyxins (colistin, polymyxin B).

Phenotypic detection of ESBL

Each bacterial isolate was first screened for ESBL production by the disc diffusion method using the antibiotics ceftriaxone (30µg), cefotaxime (30µg), ceftazidime (30µg) and aztreonam (30µg) according to CLSI guidelines [19]. An isolate with reduced susceptibility to any of the four antibiotics was subjected to confirmatory testing.

ESBL confirmation was done in a stepwise manner involving three methods; the recommended CLSI double disc confirmatory method [19], the modified CLSI confirmatory test [20] and a combination disc method using discs of cefepime alone and in combination with clavulanic acid [21]. All screen-positive isolates were first tested with the CLSI confirmatory test using discs of ceftazidime and cefotaxime alone and in combination with clavulanic acid. Isolates not confirmed by the CLSI method were further tested by the modified CLSI ESBL confirmatory test. Furthermore, *E. cloacae* and *P. aeruginosa* isolates with known chromosomal AmpC's, which tested negative with both methods were further tested with a combination of cefepime (30µg) with and without clavulanic acid (10µg). *E.coli* ATCC 25922 and *K. pneumonia* ATCC 700603 were used as negative and positive controls respectively.

In addition, isolates of *P. mirabilis*, *K pneumonia*e and *E. coli* were investigated for other mechanisms which could contribute to the negative ESBL confirmatory test such as AmpC. Discs of cefoxitin (30µg) and an AmpC detection set (D69C, MastGroup Ltd) were employed for the confirmation of chromosomal or plasmid acquired AmpC within these isolates. Isolates resistant to cefoxitin were suspected of having an AmpC and confirmation was done using the AmpC detection test kit. The kit contained three discs of combinations of a cephalosporin (cefpodoxime 10µg) with an AmpC inducer (disc A), an AmpC inducer and an ESBL inhibitor (disc B) and an AmpC inducer, an ESBL inhibitor and an AmpC inhibitor (disc C). An organism was classified AmpC positive if zone sizes of C-A and C-B were ≥5mm and negative if all zone sizes were within 3mm.

Phenotypic detection of MBL producers

The isolates were first screened for MBL production by the disc diffusion method using discs of imipenem (10 µg). Isolates with a reduced susceptibility to imipenem were confirmed for MBL production using the double disc potentiation method [22]. Briefly, Mueller Hinton agar plates were inoculated with 0.5 McFarland bacterial suspensions by streaking evenly.

Two imipenem (10 µg) discs were then placed 10mm apart on the plate after which 10µl of 0.5M EDTA was added to one of the imipenem discs to obtain a concentration of 750µg and the plates incubated at 37^0C overnight. Isolates with an increase in zone size of ≥ 7mm between the imipenem only and imipenem EDTA discs were confirmed as MBL producers.

Detection of ESBL and carbapenem encoding genes

Crude genomic DNA from isolates was extracted by boiling a loopfull of overnight cultures in 200µl sterile distilled water for 20 minutes. The following genes *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{IMP-1}}$, *bla*$_{\text{VIM-1}}$, *bla*$_{\text{OXA-2}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{NDM}}$, *bla*$_{KPC}$ and *bla*$_{\text{SPM}}$ were sought using the primers and cycling conditions listed in table 1[23-30]. The PCR reaction mixture contained 4µl 5x FIREPol® Mastermix (containing FIREPol® DNA Polymerase, 5x Reaction buffer, 12.5mM $MgCl_2$ and 1mM of each dNTPs), 0.6 µl 10mM concentration of each primer, 12.8µl nuclease free water and 2µl of template DNA. PCR products (8 µl) were electrophoresed in 1% agarose gel, stained in a gel red bath and detected by ultraviolet (UV) transillumination. Amplicons were sized by comparing with a 1kb ladder (Promega Corporation, Madison, WI). The *bla*$_{\text{CTX-M}}$ positive isolates were further sub-classified into *bla*$_{\text{CTX-M1}}$, *bla*$_{\text{CTX-M2}}$ and *bla*$_{\text{CTX-M9}}$ groups.

Results

Antibiograms of bacterial species

Among the Enterobacteriaceae tested, resistance to sulphamethoxazole-trimethoprim (82/111, 73.8%), chloramphenicol (83/99, 84%), cefuroxime (70/111, 63.0%), ticarcillin-clavulanic acid (79/111, 71.2%) and ampicillin (57/60, 95.0%) were recorded at high frequency (Table 2). Resistance rates to gentamicin (22/111, 19.8%), amikacin (28/111, 25.2%) tobramycin (40/111, 36.0%) as well as ciprofloxacin (26/111, 23.4%), colistin (36/79, 45.6%) and imipenem (6/111, 5.4%) were lower (Table 2).

The resistance rate for *P. aeruginosa* to ceftriaxone (70/89, 79.0%), cefotaxime (80/89, 90.0), ticarcillin clavulanic acid (61/89, 68.5) and streptomycin (49/89, 55.0) was high and that against gentamicin (19/89, 21.3%), amikacin (18/, 20.2%), tobramycin (5/89, 5.6%), ciprofloxacin (9/89, 10.1%), aztreonam (30/89, 34.0), colistin (8/89, 8.9) and imipenem (2/89, 2.2%) was relatively lower. Twenty four isolates of *P. aeruginosa* were identified as MDR.

Resistance rates of the Enterobacteriaceae to the third generation cephalosporins; ceftriaxone and cefotaxime were lower in contrast to higher rates observed among the *P. aeruginosa* isolates (Table 2). However, resistance to ceftazidime was low among all the bacterial groups.

ESBL and MBL strains

A total of one hundred and forty-nine (149/200) isolates were screen-positive for ESBL comprising 84/89 *P. aeruginosa*, 21/37 *P. mirabilis*, 15/25 *K. pneumonia*, 6/23 *E. coli* and 23/26 *E. cloacae*. Out of these, 12 (7.8%) were confirmed as ESBL by the recommended CLSI confirmatory test while 98 (65.7%) isolates mostly *P. aeruginosa* (n=65, 66.3%) showed extended-spectrum cephalosporin (ESC) resistance but no synergy between the clavulanate and the ESC.

Further testing of these 98 isolates by other methods confirmed 14 (14.3%) as ESBL producers (Table 3) with eight (8.2%) confirmed by the modified CLSI method and 6 (6.1%) by the cefepime/cefepime clavulanic acid double disc combination test. Hence out of the 149 isolates, 26 (17.4%) were confirmed as ESBL producers by the three methods.

Among the ESC-resistant isolates of *K. pneumoniae, P. mirabilis,* and *E. coli,* AmpC's were detected in 8 including *K. pneumoniae* (3), *P. mirabilis* (4) and *E. coli* (1) by the Mast Group AmpC detection test kit which could account for the negative confirmatory test in these isolates (Table 3). MBL production was confirmed in three isolates comprising *P. aeruginosa* (2) and one *K. pneumoniae* (1) (Table 3).

ESBL and carbapenemase encoding genes

Among the ESBL encoding genes tested, $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV} and $bla_{\text{OXA-2}}$ were detected in 36 (24.2%), 33 (22.1%), 14 (10.1%) and 23 (15.4%) of the isolates, respectively. One ESBL gene was identified in 44 isolates, two genes in 27 isolates, and all three genes in three *K. pneumoniae* isolates (Table 4). Further classification of CTX-M genes identified Group 1 (n=7) and Group 9 (n=2). All together, 50% of the analyzed isolates harboured at least one ESBL encoding gene.

The carbapenemase encoding genes detected were $bla_{\text{VIM-1}}$ (34, 17%), bla_{NDM} (50, 25%), $bla_{\text{IMP-1}}$ (2, 0.5%) and bla_{KPC} (20, 10%) while no isolate harboured bla_{SPM} and $bla_{\text{OXA-48}}$ genes. We did not identify any carbapenemase encoding genes in 118 isolates, one gene only was detected in 60 isolates, 21 isolates harboured 2 genes each (Table 5) and three genes (bla_{NDM}+ $bla_{\text{VIM-1}}$+ bla_{KPC}) were identified in 1 *K. pneumoniae* isolate. In total, 82 isolates making 41% of total isolates harboured ß-lactam genes coding for carbapenemases.

Discussion

Antibiotics are an essential part of health care in Ghana where the burden of infectious diseases requiring antibiotic treatment is very high. This study aimed to 1) determine the phenotypic antimicrobial resistance profile of GNB and 2) identify some genes mediating resistance to ESBLs and carbapenems. Our analysis revealed that 1) the GNB studied have a high rate of resistance to the common first-line drugs and relatively lower rates of resistance to the injectables gentamicin, amikacin, tobramycin and carbapenem antibiotic imipenem, 2) there is a high level ß-lactam resistance mediated by a wide range of extended spectrum ß-lactamase and carbapenemase genes 3) phenotypic detection of ESBL's may be challenging and require more than one method for confirmation.

The high resistance to ampicillin, chloramphenicol, cefuroxime and sulphamethoxazole-trimethoprim has also been reported by a nationwide surveillance of antimicrobial resistance in Ghana recently published by Opintan [31] and studies from Uganda [32], Nigeria [33] and Tanzania [34]. These antibiotics are old, cheap and commonly prescribed as first line treatment drugs for many bacterial infections. They are also easily acquired over-the-counter and without prescription which has contributed to their abuse and misuse due to the non-existence of a developed regulatory machinery to enforce the National Drug Policy [35]. They are also commonly used in animal husbandry as growth supplements to prevent infection and increase yield; thus residues are easily passed on to humans when they ingest food [36-37]. These results therefore call for the urgent set up of a surveillance system to monitor antibiotic use and also the strict enforcement of rules that govern the use of antibiotics in the country.

We however found comparably low resistance to the injectables gentamicin, amikacin and tobramycin and this correlates with findings from Opintan [31]. The low rates may be attributed to the limited use of these antibiotics due to their mode of administration, high cost which discourages self medication and also the fact that they are only prescribed for serious and life threatening infections. In contrast, streptomycin, which is also an injectable aminoglycoside, recorded high resistance. This finding is not surprising as high resistance of different pathogens to streptomycin (>20%) including *Mycobacterium tuberculosis* (Mtb) [38] and *E. coli* [39] from both human and animal sources have been recorded in Ghana. Streptomycin, which until recently was used as a first-line treatment drug for tuberculosis (TB) has been widely used in Ghana where TB prevalence is 282 per 100,000 in the population (http://www.who.int/tb/country/data/profiles/en/). It is also widely used in animal

husbandry for treatment and disease prevention [40]. Thus the observed high resistance is evidence of the selection pressure provided by years of streptomycin use in both human and veterinary medicine. Our findings also suggest that there is very little or no possible cross resistance among the aminoglycosides analysed in the study.

Carbapenem antibiotics are the last choice in treatment of infections due to ESBL producing organisms; the low resistance to imipenem detected in this study correlates with findings by Hackman [41] and Adu-Sarkodie [42]. Interestingly meropenem, the only carbapenem approved for use in Ghana has also recorded no resistance so far [15] even though it has been on the market since 2002. Thus carbapenems may currently be the best choice for treatment of infections, however caution should be taken in their use, as prolonged systemic therapy would select for resistant strains.

The proportion of MDR *P. aeruginosa* isolates detected in this study was high (27.0%) compared to 9.5% in an Egyptian study [43]. Infection with MDR *P. aeruginosa* has been associated with adverse clinical outcomes such as increased morbidity and mortality, prolonged hospitalization and the need for surgical interventions. Due to the limited therapeutic options available in treating infections by MDR strains of *P. aeruginosa*, it is important to optimize infection control and stewardship strategies especially in heath care settings where such strains are commonly found.

Genes implicated in conferring ESBL (50%) and carbapenem resistance (41%) were frequently detected in the study. The most prevalent ESBL type in this study was the TEM type, which is similar to findings by Feglo [17] in Kumasi, Ghana but in contrast to research by Hackman [41] in Accra, Ghana who detected CTX-M as the most predominant type. Though CTX-M types are known as the most prevalent ESBL type globally [16-17, 44-50], the percentage within our study was very low, which could be explained as due to the low levels of the main ESBL producers *K. pneumoniae* and *E. coli* [3] (23.9%) in this study. No SHV-type ESBLs were identified among the *P. aeruginosa* isolates and research has reported only rare isolates of *P. aeruginosa* harbouring these genes [51-52]. TEM genes were detected in 50% of *P. mirabilis* isolates which is consistent with studies that found these bacteria to be associated with the production of TEM-type enzymes [53-57].

The carbapenemase encoding genes detected in this study were IMP, VIM-1, NDM and KPC with NDM being the most prevalent. Of particular concern is the presence of NDM and KPC genes, which have become major foci of worldwide attention as the acquisition of KPC is

characterized by multi drug resistance with very limited therapeutic options, thus associated with significant morbidity and mortality. Its worldwide emergence and the difficulty in identifying them in routine susceptibility screening tests poses an infection control challenge to the health care system [58]. The NDM gene on the other hand is associated with non clonally related isolates and species. The gene encoding this enzyme is also found on plasmids of different sizes with significant mobility and could be present in different GNB from one patient [59]. It has also been identified as a source of community acquired infection [60]. Carbapenemase production is neither associated with the expression of specific virulence factors nor specific clinical infection but to difficult-to-treat infections. Due to their rapid and global expansion, the epidemic potentials of *Enterobacteriaceae* producing such enzymes cannot be downplayed and more collaboration is needed between clinicians and reference or public health laboratories in identifying and preventing infections due to carbapenem producers.

In identifying carbapenemase producers, the molecular detection of carbapenem encoding genes was used as the primary result in this study. Though potential carbapenem producers could be identified by phenotypic susceptibility testing, many carbapenemase producers do not confer obvious resistance levels to carbapenems [61]. Also, MBL detection methods which are based on ß-lactam-chelator combinations have been shown to work well for *K. pneumoniae* and *E. coli* isolates but have not been thoroughly tested for other Enterobacteria [62]. In addition, the development of phenotypic tests for the detection of OXA-type carbapenemases has been hindered due to their enzymatic properties which prevents their inhibition by clavulanic acid, tazobactam, sulbactam or other zinc chelators [62]. Thus the molecular method is considered the reference standard and was applied in this study as such.

The study also highlights the challenges encountered with the phenotypic detection of ESBLs. Three methods were combined to confirm ESBL production in 26 isolates. ESBLs occur in many *Enterobacteriaceae* but the current confirmatory tests which rely on the synergy between the extended-spectrum cephalosporin and clavulanate are recommended only for the detection and confirmation of ESBLs in *K. pneumoniae* spp, *E. coli* and *P. Mirabilis* [19]. This will lead to non-detection of many producers as no guidelines currently exist for the detection and reporting of ESBLs in other members of the *Enterobacteriaceae*.

Additionally, the co-existence of ESBLs with other ß-lactamase types (KPC and MBL) and other mechanisms of ß-lactam resistance (AmpC, carbapenemases and overproduction of cephalosporinases) further complicate their phenotypic detection [20, 63-65]. MBL and KPC

hydrolyze ESBL's while the existence of the other mechanisms masks their presence leading to underestimation of their true prevalence in phenotypic screening tests. Though molecular assays may provide accurate results in the detection of ESBL genes, they are inaccessible to many laboratories and are also expensive. The confirmatory tests used in this study in addition to the standard CLSI method have been proposed by other research groups to improve the detection of ESBLs in the presence of other mechanisms and we were able to confirm 14 other isolates as ESBLs. Our findings therefore support the suggestion that the current CLSI confirmatory method is inadequate in detecting ESBLs in the presence of other mechanisms [20, 64, 66-67]. As a single ESBL-producing isolate may have multiple mechanisms of resistance, it is important to have an accurate phenotypic detection assay capable of detecting ESBLs regardless of the underlying resistant mechanism. In the absence of such an assay, we propose the harmonization of different methods to improve ESBL detection in the clinical laboratory.

Conclusions

Based on our findings, Ghana needs to put in place mechanisms to regulate the sale of antibiotics, as many are easily accessible over the counter. Antimicrobial surveillance systems to monitor drug resistance within the country also need to be set up and policies, which guide the rationale use of antibiotics, must be strictly enforced. The challenges associated with the phenotypic detection and confirmation of ESBL requires a combination of methods to confirm the different mechanisms.

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Ethics Approval and Consent to participate

Ethical clearance for the study was obtained from the institutional review board of the Noguchi Memorial Institute for Medical Research (Federal-wide Assurance number FWA00001824). All study participants were well informed of the study objectives and written informed consent was obtained.

TABLE 1: Primers for detection of ESBL and Carbapenem genes

Primer	Sequence 5'- 3'	Band size bp	PCR Conditions	References
TEM-1 F	ATG AGT ATT CAA CAT TTC CG	867	95^0C–5min,30(95^0C-30sec,50^0C1.30min, 72^0C-	[29]
TEM-1 R	CTG ACA GTT ACC AAT GCT TA		1.30min), 72^0C-10min, 4^0C-∞	
CTX-F	CGCTTTGCGATGTGCAG	550	95^0C–5min, 30(95^0C-30sec,50^0C-1.30min,72^0C-	[23]
CTX-R	ACCGCGATATCGTTGGT		1.30min), 72^0C-10min, 4^0C-∞	
SHV 1	GGG TTA TTC TTA TTT GTC GC	900	95^0C–5min, 30(95^0C-30sec,56^0C-1.00min,72^0C-	[29]
SHV 2	TTA GCG TTG CCA GTG CTC		1.30min), 72^0C-10min, 4^0C-∞	
OXA II F	AAG AAA CGC TAC TCG CCT GC	478	95^0C–5min, 30(95^0C-30sec,62^0C-1.00min,72^0C-	[28]
OXA II R	CCA CTC AAC CCA TCC TAC CC		1.00min), 72^0C-10min, 4^0C-∞	
NDM-F	ACCGCCTGGACCGATGACCA	263	95^0C–5min, 30(95^0C-30sec,62^0C-1.00min,72^0C-	[25]
NDM-R	GCCAAAGTTGGGCGCGGTTG		1.00min), 72^0C-10min, 4^0C-∞	
VIM 1-F	AGTGGTGAGTATCCGACA	261	95^0C–5min, 30(95^0C-30sec,54^0C-40sec,72^0C-	[26]
VIM 1-R	ATGAAAGTGCGTGGAGAC		60sec), 72^0C-10min, 4^0C-∞	
KPC-F	CTTGCTGCCGCTGTGCTG	489	95^0C–5min, 30(95^0C-30sec,58^0C-1.00min,72^0C-	[24]
KPC-R	GCAGGTTCCGGTTTTGTCTC		1.30min), 72^0C-10min, 4^0C-∞	
IMP 1-F	ACCGCAGCAGAGTCTTTGCC	587	95^0C–5min, 30(95^0C-30sec,58^0C-1.00min,72^0C-	[26]
IMP 1-R	ACAACCAGTTTTGCCTTACC		1.30min), 72^0C-10min, 4^0C-∞	
OXA 48-F	TTGGTGGCATCGATTATCGG	743	95^0C–5min, 30(95^0C-30sec,58^0C-1.00min,72^0C-	[27]

OXA 48-R	GAGCACTTCTTTTGTGATGGC		1.30min), 72^{0}C-10min, 4^{0}C-∞	
SPM –F	GCGTTTTGTTTGTTGCTC	786	95^{0}C–5min, 30(95^{0}C-30sec,51^{0}C-60sec,72^{0}C-	[26]
SPM –R	TTGGGGATGTGAGACTAC		1.30min), 72^{0}C-10min, 4^{0}C-∞	
CTX-M1F	GCGTGATACCACTTCACCTC	260	95^{0}C–5min, 30(95^{0}C-30sec,53^{0}C-1.00min,72^{0}C-	[30]
CTX-M1R	TGAAGTAAGTGACCAGAATC		1.00min), 72^{0}C-10min, 4^{0}C-∞	
CTX-M2F	TGATACCACCACGCCGCTC	341	95^{0}C–5min, 30(95^{0}C-30sec,50^{0}C-1.30min,72^{0}C-	[30]
CTX-M2R	TATTGCATCAGAAACCGTGGG		1.30min), 72^{0}C-10min, 4^{0}C-∞	
CTX-M9F	ATCAAGCCTGCCGATCTGGTTA	293	95^{0}C–5min, 30(95^{0}C-30sec,61^{0}C-1.00min,72^{0}C-	[30]
CTX-M9-R	GTAAGCTGACGCAACGTCTGC		1.00min), 72^{0}C-10min, 4^{0}C-∞	

TABLE 2 Antibiograms of bacterial isolates

Antibiotics	***Pseudomonas aeruginosa N=89 n ,%***		**Enterobacteriaceae**									
			Klebsiella pneumoniae N= 25 n ,%		***Proteus mirabilis N= 37 n ,%***		***E. coli N= 23 n ,%***		***Enterobacter cloacae N= 26 n ,%***		**Total n ,%**	
	NT[a]	R[b]	NT	R	NT	R	NT	R	NT	R	NT	R
Ceftriaxone	89	70, 79.0	25	10, 40.0	37	7, 18.9	23	5, 21.7	26	7, 27	111	29, 26.1
Gentamicin	89	19, 21.3	25	6, 24.0	37	4, 10.8	23	7, 30.4	26	5,19.2	111	22, 19.8
Tobramycin	89	5, 5.6	25	9, 36.0	37	8, 21.6	23	9, 39.1	26	14, 53.8	111	40, 36.0
Cefuroxime	ND[c]		25	12, 48.0	37	21, 57.0	23	18, 78.3	26	19, 73	111	70, 63.0
Sulphamethoxazole-Trimethoprim	ND		25	15, 60.0	37	30, 81.0	23	19, 82.6	26	18, 69.2	111	82, 73.8
Cefotaxime	89	80, 90.0	25	15, 60.0	37	11, 30.0	23	5, 21.7	26	15, 57.7	111	46, 41.4
Ciprofloxacin	89	9, 10.1	25	4, 16.0	37	8, 21.6	23	8, 34.7	26	6, 23.1	111	26, 23.4
Aztreonam	89	30, 34.0	25	5, 20.0	37	17, 46.0	23	6, 26.1	26	13, 50	111	71, 34.0
Ticarcillin-clavulanic acid	89	61, 68.5	25	24, 96.0	37	10, 27.0	23	21, 91.3	26	24, 92.3	111	79,71.2
Ceftazidime	89	10, 11.2	25	6, 24.0	37	6, 16.2	23	6, 26.1	26	11,42.3	111	29, 26.1
Imipenem	89	2, 2.2	25	1, 4.0	37	4, 10.8	23	1, 4.3	26	0	111	6, 5.4
Amikacin	89	18, 20.2	25	8, 32.0	37	6, 16.2	23	1, 4.3	26	13, 50	111	28, 25.2
Colistin	89	8, 8.9	14	6, 43.0	35	22, 62.8	11	4, 36.4	19	4, 21.0	79	36, 45.6
Chloramphenicol	ND		20	15, 75.0	36	35, 97.2	20	17, 85.0	23	16, 69.5	99	83, 84.0
Ampicillin	ND		ND		37	34, 91.8	23	20, 86.9	ND		60	57, 95.0
Streptomycin	89	49, 55.0										

a (NT)= number tested, *b* (R) =resistant, *c* (ND) =Not done; isolates are intrinsically resistant to antibiotics

TABLE 3: Phenotypic detection of ESBL, MBL and MDR strains

Bacterial species	**No of isolates**	**ESBL screening positive n (%)**	**ESBL confirmation n (%)**				**AmpC n (%)**	**MBL n (%)**	**MDR n (%)**	**ESBL+ MDR n (%)**	**ESBL+ MDR+ MBL n (%)**
			[a]CLSI test	Modified CLSI	[b]Cef/[c]Cef Clav	Total					
Pseudomonas aeruginosa	89	84 (94.5)	4/84 (4.7)	4/65 (6.2)	3/65 (4.6)	11/84 (13.1)	N/AP	2(2.2)	24(27.0)	24 (27.0)	2 (2.2)
Proteus mirabilis	37	21 (55.0)	1/21(4.8)	2/13 (15.4)	N/AP[d]	3/21 (14.3)	4 (28.6)	0	N/AP	N/AP	N/AP
Klebsiella pneumoniae	25	15 (57.6)	2/15(13.3)	0	N/AP	2/15 (13.3)	3 (42.9)	1 (3.8)	N/AP	N/AP	N/AP
Enterobacter cloacae	26	23 (88.5)	3/23(13)	2/12 (16.7)	3/12 (25)	8/23 (34.8)	N/AP	0	N/AP	N/AP	NA
Escherichia coli	23	6 (26.1)	2/6(33.3)	0	N/AP	2/6 (33.3)	1 (100)	0	N/AP	N/AP	N/AP
Total	200	149 (74.5)	12/149 (8.0)	8 (8.2)	6 (7.8)	26/149(17.4)	8 (38.1)	3 (2.6)	24(27.0)	24(27.0)	2 (2.2)

a CLSI = Clinical Laboratory Standards Institute, *b* Cef =Cefepime, *c* Cef/Clav= Cefepime-Clavulanic acid, *d* (N/AP)= not applicable

TABLE 4: ESBL genes detected in isolates

Bacterial species	N	Isolates with one gene only n (%)					Isolates with two genes identified n (%)						
		CTX-M	TEM-1	SHV	OXA-2	Total	CTX+TEM-1	CTX+SHV	CTX+OXA-2	TEM-1+SHV	TEM-1+OXA-2	SHV+OXA-2	Total
Pseudomonas aeruginosa	84	12 (63.2)	1 (5.3)	0	6 (31.6)	19 (22.6)	5 (33.3)	0	8 (53.3)	0	2 (13.3)	0	15 (18)
Klebsiella pneumoniae	15	0	1 (25)	3 (75)	0	4 (26.7)	0	1 (14.3)	1 (14.3)	4 (57.1)	0	1(14.3)	7 (46.7)
Proteus mirabilis	21	0	9 (100)	0	0	9 (43)	0	0	0	0	0	0	0
E. coli	6	3 (75)	1 (25)	0	0	4 (66.7)	0	0	0	0	0	0	0
Enterobacter cloacae	23	0	5 (62.5)	0	3 (37.5)	8 (34.8)	0	1 (20)	2 (40)	2 (40)	0	0	5 (33.3)
Total	149	15 (10.1)	17 (11.4)	3 (2)	9 (6)	44 (29.5)	5(3.4)	2 (1.3)	11 (7.4)	6 (4.02)	2 (1.3)	1 (0.7)	27 (18.1)

TABLE 5: Carbapenem genes detected in isolates

Bacterial species		Isolates with one gene only n (%)					Isolates with two genes identified n (%)			
	N	NDM	VIM-1	KPC	IMP-1	Total	NDM + VIM-1	NDM+KPC	VIM 1+KPC	Total
Pseudomonas aeruginosa	89	23 (72)	1 (3.1)	8 (25)	0	32 (36)	5 (45.5)	5 (45.5)	1 (9.1)	11 (12.4)
Klebsiella pneumoniae	25	2 (16.7)	7 (58.3)	2(16.7)	1 (8.3)	12 (48)	3 (75)	0	1 (25)	4 (16)
Proteus mirabilis	37	1 (33.3)	2 (66.7)	0	0	3 (8.1)	0	0	0	0
E. coli	23	0	2 (100)	0	0	2 (8.7)	0	0	0	0
Enterobacter cloacae	26	4(36.4)	5 (45.5)	2 (18.2)	0	11 (42.3)	6 (100)	0	0	6 (100)
Total	200	30 (15)	17 (8.5)	12 (6)	1 (0.5)	60 (30)	14 (7)	5 (2.5)	2 (1)	15

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Discussion

The first diagnosis of Buruli ulcer in Ghana was made in the Greater Accra Region in 1969 in a 7 year old girl admitted at the Korle-Bu Teaching Hospital [1]. Following from this, several other cases were identified in people who used the same water supply (River Densu and along its tributaries) as the infected patient. Years later, BU has been reported in all 10 regions of Ghana in over 400 communities with the identification of numerous endemic foci [2]. A national crude prevalence of 20.7/100,000 in 1998 makes Ghana the second most endemic country reporting BU disease after Cote d'Ivoire [2] and makes BU the second most prevalent mycobacterial disease after tuberculosis. A nationwide case study identified ten districts in Ghana with the most caseloads of the disease and the highest and lowest prevalence of 150.8/100,000 and 57.0/100,000 in the Amansie West District in the Ashanti Region and the Kwawu South District of the Eastern Region respectively [2]. BU is found in many impoverished communities with low income and very limited access to resources. This, coupled with the fact that the treatment of the disease required surgery negatively impacted the health seeking behavior of BU patients for many years as the affected and their families were unable to shoulder the financial and economic pressure that came with treatment. The introduction of antibiotic treatment for BU therefore brought a welcome relief to many affected people as the fear of surgery was reduced and patients were more willing to seek treatment at the biomedical facilities. However, observations made across several health centers showed that despite antibiotic therapy, the healing process was often still associated with long hospital stays before wound closure was achieved. Healing delay, wound deterioration and skin graft failure was frequently observed among affected patients and the quest to understand these observations led to the initiation of the current studies. Within the scope of this PhD thesis, we have identified secondary infection of BU lesions by other bacteria (Chapter 2) as a possible contributing factor to delayed wound healing in BU disease. We also discovered other factors which could challenge the management of the disease such as the evolution of the bacterial burden (Chapter 3) and HIV co-infection (Chapter 4). Studying the bacteria isolated from the lesions through phenotypic, molecular and whole genome sequencing approaches has helped us to gain an understanding of the source and diversity of the bacteria causing secondary infection. These analyses revealed widespread antibiotic resistance as a serious problem in Ghana which could have major detrimental consequences for health care if not addressed promptly (Chapters 5, 6 and 7).

Early case detection to achieve fast healing of BU lesions

The Greater Accra Region where this study was conducted is the third most prevalent BU reporting region (18.5/100,000) and the former Ga District in this region was the fifth most prevalent BU reporting district (87.7/100,000) in Ghana till it was split into the Ga-South and Ga-West Municipalities in 2009. The study was carried out in two main health centers, the Obom Health Center in the Obom sub-district of the Ga-South Municipality and the Ga-West Municipal Hospital. The most severe forms of BU disease in Ghana is currently reported in these two municipalities [3]. With the introduction of antibiotic therapy (SR8) for the treatment of BU and the various successes reported by this treatment regimen, we observed in these health centers that a number of cases still stayed post SR8 for extended periods of time - at times over two years - before complete wound closure was obtained. Clinical observations led us to investigate if secondary infection by other bacteria could contribute to the frequently observed healing delay. Analysis of BU lesions before, during and after SR8 revealed that secondary infection is common in BU disease (Results, Chapter 2) despite the belief that it was unlikely because of the presence of mycolactone which was thought to exert a sterilizing effect on the lesions [4]. However, no antimicrobial activities have been found in studies with synthetic mycolactone [5].

Our study revealed that most cases present at health centers with lesions that are already clinically and microbiologically infected (Results chapter 2). Late reporting of cases is frequently observed in BU disease with patients presenting with large ulcers [6-7]. Studies have revealed that biomedical health facilities are often not the first point of call for BU patients [6, 8-10]. The health seeking behaviour of BU affected patients is deeply rooted in their socio-cultural beliefs and practices. In many communities, an unhealing wound is thought to be caused by spiritual forces, charms, witches, ancestral spirits or even the gods of the land [8]. As a result, traditional healers and witch doctors are first consulted to deal with the spiritual forces suspected to cause the disease. Some patients will also practice self-treatment at home. Because the early clinical manifestations of the disease which could be a painless nodule, plaque or a small ulcer, will not be perceived as serious, patients will attempt to administer ‘first aid’ at home. Thus cases may only report to the biomedical health centers after all efforts have been exhausted by these various approaches and no improvement has been seen. Large wounds are therefore presented which may have been treated over long periods of time with various concoctions usually not prepared under aseptic conditions. Secondary infections may therefore contribute to healing delays in such cases. Currently,

various strategies including active case search and the engagement of former patients and traditional healers in referring cases is being practiced in both Ghana and Cameroon [3, 11] to ensure early detection and timely healing.

Wound bacterial load and HIV can complicate management of BU lesions

Wound infection is not only dependent on the presence of infecting bacterial species but also on their numbers. Various studies on wounds with other etiology have demonstrated that levels of bacteria above 10^5cfu/ml are a critical level in the prediction of wound infection and wound healing outcomes [12-16]. Above this level, negative outcomes such as wound healing impairment and breaking of grafts has been reported. We quantified the bacterial load of 86 BU patients through a cross-sectional study and found that the majority of BU wounds before (62%), during (65%) and post treatment (77%) had bacterial levels above this limit (Chapter 2). We further investigated the evolution of the bacterial load at all three stages in longitudinally analysed patients (Chapter 3) and found that the bacterial load was higher pre and post treatment when no antibiotic therapy was being taken, but lowered during antibiotic treatment. While the Wound Healing Society views the microbial load and the presence of beta-hemolytic streptococci as the best predictors of wound infection [17], clinicians usually rely on clinical signs and symptoms in their diagnosis of wound infection. However, clinical signs are not always expressed in chronic disease states; for example in diabetic foot ulcers, inflammatory responses to infection is often impeded by peripheral vascular disease, hyperglycemia, oxygenation, neuropathy and anti-inflammatory drug use among others [18-19]. The immunosuppressive property of mycolactone in BU disease also has the potential to dampen first-line inflammatory responses to wound infection [20]. Therefore determining the bacterial load appears to be a valuable component of wound management even in the absence of overt clinical signs and guidelines for wound care should include practices aimed at reducing the bacterial load.

Apart from the bacterial load, HIV co-infection can also complicate the management of BU (Chapter 4). HIV infection and BU epidemiologically overlap in Sub-Saharan Africa which accounts for more than 80% of globally reported HIV infections [21]. We reported a higher prevalence of HIV among BU patients compared to that of other patients attending the health facility and this has also been reported in Cameroon and Benin [22-23]. While it is clearly known that HIV/AIDS which leads to reduced CD4 helper T cell activity is a risk factor for tuberculosis, immune protection mechanisms in BU disease are not fully understood. There is

growing evidence, that HIV infection is affecting the clinical presentation, disease severity and mortality of BU cases, but the actual impact of HIV/AIDS on BU disease is not fully characterized [21, 23-26]. In this context, we also reported a case of disseminated disease in a BU patient co-infected with HIV (Chapter 4). Even though preliminary guidelines exist in the management of BU-HIV co-infection [27], there is a lack of information on the impact of antiretroviral therapy (ART) on the incidence and severity of paradoxical reactions which have been reported in HIV cases on ART co-infected with other microorganisms such as TB [28], Cryptococcus [29] and *Mycobacterium avium* complex [30]. More research is therefore needed to fill the knowledge gaps that exist in the interaction between BU and HIV and the effect of both ART and SR8 on treatment outcomes. HIV testing is currently standard in BU management; therefore integration of HIV treatment centers with BU treatment centers should jointly facilitate timely management and the provision of best models of care.

Decentralized treatment of uncomplicated cases to avoid nosocomial infections

Many different infecting bacterial species which could negatively impact on the healing potential of the wounds were isolated from the lesions of our study patients within the course of the study. Some of these bacterial species are known nosocomial pathogens and led us to investigate the source of these pathogens into the wounds. Through a combination of phenotypic, molecular (*spa* typing) and whole genome sequencing approaches, we identified health-center and patient sources of infection 8 (Chapter 5).

Most health centers in BU endemic areas are low resourced and not adequately equipped to manage the wounds presented or the number of patients reporting at a time. The pressure placed on the limited available resources leads to compromises in good wound care practices ultimately affecting the management of cases. The benefit of improved infection prevention and control practices (IPC) in reducing health-care associated infections cannot be over-emphasized. Though IPC guidelines exist in many health centers, research has shown that compliance among health care workers to these guidelines is low. One reason for the non-compliance is understaffing at health facilities. The doctor-patient ratio in the Greater Accra and Northern regions of Ghana has been reported to be 1:3,712 and 1:21,751, respectively. The physician density per 1000 population is 0.13 and 0.04 in urban and rural areas of Ghana, respectively, while the density of nurses is 0.60 (urban) and 0.20 (rural) per 1000 population. Thus health centers are widely understaffed and this imbalance in health worker to patient

ratios impacts on access to good health care. There is the need for more health care workers to be recruited to health facilities and for adequate materials and equipment to be provided for health centers to reduce the occurrence of health-care associated infection of wounds. Providing incentives and improving remuneration of health workers will attract more people into the sector and greatly help to reduce the problem of understaffing that currently exists. Additionally, treatment of BU cases can be decentralized to reduce the pressure usually placed on secondary health facilities and health workers in these facilities. Small uncomplicated BU cases can be treated at peripheral health centers and CHPs compounds instead of the secondary health centers which should only be used as reference centers for complicated cases. This will go a long way to reduce pressure on the health workers and resources and the fewer patients will receive better care and attention.

Apart from deficiencies in health care provision, also patient behavior was found to have negative effects on wound healing. Patient adherence to biomedical treatment protocols is largely influenced by their financial status and their socio-cultural beliefs. Leaders of BU endemic countries agreed in the Yamoussoukro declaration that treatment of BU should be free. This however, is not entirely the case as the burden of wound management usually falls on the patients, leading to considerable costs which they are often unable to shoulder over longer periods of time. This has led to practices such as the recycling of bandages used in wound dressing. Sampling of some of these bandages showed that they were contaminated with the same type of bacterial pathogens as isolated from patient lesions indicating that they were not being washed properly before reuse and may be a source of re-infection. Consequently, we also identified the same bacterial species in multiple lesions of a patient pointing to a common source of infection. Apart from the recycling of bandages, some patients also redressed their wounds after health care workers had dressed them. This practice is largely influenced by patient's beliefs and perceptions regarding the management of wounds. Koka's study revealed that many patients know that wound care is delicate; however their response to wound management is deeply rooted in community perceptions of who qualifies to handle a wound. Pregnant women, breastfeeding mothers, women in their menstrual period and promiscuous young women are some of the people believed to be unqualified to manage wounds. It is believed that wounds dressed by any of these people will not heal or heal fast. Thus patients resorted to redressing their wounds if a health care worker fell into this category and this could lead to the infection of wounds through transfer from other body sites. To reduce the occurrence of self-infection, significant investment must be

made into wound care. Patients also need to be educated on the need to adhere to the biomedical treatment provided at the health centers to ensure faster healing of lesions.

The study also revealed the urgent need for proper wound management guidelines for BU. Apart from antibiotic therapy, wound care is very important in the management of affected cases; however no clear guidelines exist to guide wound management. The management of wounds is therefore left to the discretion of health care workers whose practices may not be optimal. This led us to make recommendations for proper wound management. In line with this, a wound care manual has been developed to educate health care workers on proper ways of managing wounds. Accordingly, training programs for health care workers within these two districts have been organized with the assistance of the National Buruli Ulcer Control Programme and experts from the Plastics and Burns unit of the Korle-Bu Teaching Hospital. However, constant refresher courses and monitoring is needed to ensure that these guidelines are adhered to.

Preference of good wound management over extensive use of additional antibiotics

No guidelines currently exist for the management of BU wounds secondarily infected with other bacterial pathogens. Specific guidelines are needed to know whether infected wounds should be treated with antimicrobial agents, the type of antimicrobial agents to use and whether topical or systemic antimicrobials should be administered. The definition of infection in many cases is subjective. Thus clinicians are unsure of whether antibiotic therapy should be administered especially in cases without evident clinical signs and symptoms. However, the prescription of antibiotics for secondary infection is widespread among BU patients with non-healing wounds [31]. Studies have found no evidence to support the use of systemic antibiotics in wound healing and evidence is also lacking on the required duration of treatment [32-34]. Therefore the use of antibiotics needs to be minimized as indiscriminate antibiotic use will contribute to the increasing problem of drug resistance.

Guidelines for the management of diabetic foot ulcers specified by the Wound Healing Society indicates that a topical antimicrobial agent should be used on wounds with microbial loads > 1 x 10^6 cfu/ml or any level of beta-hemolytic streptococci following adequate debridement to decrease the bacterial load as they will be more effective than systemic antimicrobials [17]. This treatment is however to be discontinued once the wound reaches bacterial balance to minimize cytotoxicity due to the antimicrobial agents and the development of resistance to the agent. The therapeutic efficacy of topical treatments is

compromised by the presence of necrotic or ischemic tissue. In such cases, surgical debridement can be employed and this will also lead to a significant reduction in the microbial load and expose healthy tissues needed for healing. This treatment protocol could be adapted for the management of BU wounds in the absence of definitive guidelines.

In the absence of wound infection however, good wound care is adequate to ensure wound healing. The choice of the correct type of dressing materials, keeping the wound bed moist at all times, irrigating the wound with physiological saline solution during cleansing, managing pain and working under aseptic conditions are some of the practices that will promote timely wound healing. In addition, adequate nutrition addressing protein-calorie malnutrition and deficiencies in minerals and vitamins also helps in wound healing. Over the course of this project, to aid in the provision of good wound care in the treatment centers, we provided equipment, refurbished a dressing room and aided in feeding of patients by providing breakfast after their antibiotic therapy. The impact of these interventions has been seen in a reduction of the length of stay in the health centers and an increase in the number of wounds healing without surgical interventions (Yeboah-Manu, personal communication)

Monitoring of the antibiotic resistance scenario in Ghana to avoid use of 'useless' antibiotics

The discovery of antibiotics is one of the greatest medical achievements of the 20th century which brought transformation to modern medicine and prevented many deaths. Before the antibiotic era, wound infections were treated by amputation and an estimated 70% of amputations performed during World War 1 were indicated as a result of infection [35]. Also, high fatality rates were reported for pneumonia (40%) [36], *S. aureus* bacteremia (80%) [37] and endocarditis (97%) [38]. After the discovery of penicillin in 1928, its introduction into the healthcare system in the 1940's and the subsequent introduction of many new antibiotics from the late 60's to the 80's fostered the belief that finally all bacterial infections could be treated. This victory over bacterial infections however was not to last with the emergence of resistant bacterial strains barely a decade after penicillin had been introduced. Bacteria posses a countless number of genetic elements for resistance to antimicrobials, which have evolved over millions of years. The inheritance of resistance genes, the acquisition of resistance elements on mobile genetic elements, horizontal transfer of resistance elements between species and the spontaneous occurrence of mutations leading to resistance are a few of the

ways by which bacteria develop resistance. Within our study, we reported wide-spread resistance among the isolated bacteria to commonly prescribed antibiotics and also identified multi-drug resistant strains of *S. aureus* and *P. aeruginosa* (Chapters 5, 6 and 7).

The burden of infectious diseases in Africa is high. As such, consumption of antibiotics is also on the increase. In many African countries, a high level of antibiotic resistance has been reported to commonly prescribed antibiotics [39]. A direct relationship exists between the use of antibiotics and the emergence and dissemination of resistance. Increased consumption of antibiotics leads to selection pressure on bacterial populations to survive where the fittest are selected for; which in many cases are the resistant strains. In Ghana, antibiotics are prescribed for many infections. Antibiotic use however is largely empirical, as many health facilities lack laboratory facilities for appropriate diagnostic guidance. Doctors also easily prescribe broad spectrum antibiotics for patients they assume cannot wait for diagnosis or who will be unwilling to return because of distance and travel costs. Additionally, patients exert pressure on medical practitioners to prescribe antibiotics for them as there is a general belief that they have only been attended to properly, if antibiotics are prescribed. Thus antibiotics are frequently inappropriately prescribed or over-prescribed. The abuse of antibiotics through self medication and inappropriate use is also common. Access to antibiotics is largely unregulated and many antibiotics are cheaply and easily available over-the-counter without prescription (Figure 1).

Figure 1: Easy access to antibiotics in Ghana

Antibiotics are easily obtainable in market places from unlicensed chemical sellers or freelance medecine men. Figure adapted from Asare [40]

In many communities, access to antibiotics is through the activities of chemists, roadside stalls and freelance medicine men who gain access to higher classes of antibiotics due to unregulation of their activities. Antibiotic resistance in Ghana is also driven by the availability of poor quality antibiotics on the open market. The prevalence of counterfeit, substandard and degraded antimicrobials is a persistent occurrence in Ghana and even though various efforts have been made by authorities to cleanse the markets of such products, they still find their way back after a while. Antibiotics are also widely used in agriculture to increase production and act as supplements for livestock. These are then ingested by humans when they consume food products treated with antibiotics which results in the transfer of resistant bacteria from livestock to humans. The effect of this unrestricted use of antibiotics has been the emergence of multi-drug resistant organisms which have become the current threat to health care worldwide. The past 50 years has seen an accelerated increase in the evolution and dissemination of multi-drug resistant organisms [41] and whole genome analysis of methicillin resistant *S. aureus* (MRSA) isolates revealed the recent emergence and rapid spread of a community-acquired MRSA clone (ST88-IV) in Ghana (Chapter 6).

The negative effects of antibiotic resistance are extensive and do not impact the affected patients alone but stretch to the healthcare system and the society. In patients, it reflects in treatment failure, increase in mortality and longer hospital stays. In addition, it also affects empiric treatment options for patients who have not been infected with resistant organisms who will be treated with broader spectrum antibiotics as a result of pathogen resistance to the narrower spectrum antibiotics. These drugs could be more expensive, toxic and sometimes less efficacious [41-42]. Within the healthcare system, antimicrobial resistance limits therapeutic options, threatens the safety and efficacy of surgeries and immunosuppressive procedures, interrupts hospital activity in cases where sections of hospitals have to be closed down to contain an outbreak among others [41]. The societal impact occurs when considerable financial investments have to be made to the health sector leading to diminished resources to other sectors of society. To curb this menace, surveillance programs need to be put in place to monitor the use of antibiotics as no active surveillance system currently exists in Ghana. Laws governing the distribution of antibiotics within the country need to be tightened and the activities of lower level health facilities and over-the-counter medicine sellers need to be regulated. Prescribing practices of doctors and healthcare practitioners also need to be monitored.

Conclusion

Antibiotic therapy has been successful in the treatment of early limited cases of BU. The median time to healing in large ulcers and those that may require some surgical interventions is not very well investigated, but is expected to be longer. Consequently, wound care is very vital in BU management.

In this thesis, we investigated the factors that could contribute to delayed wound healing observed in the BU treatment centers Obom Health Center and Ga-West Municipal Hospital and identified secondary wound infection, HIV co-infection and poor wound care practices as critical features. Our findings demonstrate the need for clear guidelines on wound care in BU case management, as we observed a general need for improvement in nursing practices. Consequently, with the help of the national BU control programme and other collaborators, several interventions were put in place including: the development of a wound management protocol and the design and distribution of wound dressing posters and assessment charts, equipping the health centers with needed equipment, refurbishment of a new dressing room at Ga West Municipal Hospital and providing wound care training for health workers in the facilities. Preliminary results from these interventions show an improvement in time to healing and a decrease in the duration of hospital stays by patients. Studying the bacteria isolated from the lesions of the patients helped us to identify the sources of infection of the lesions. The work of this thesis also contributes valuable data to the increasing global problem of drug resistance. The identification and characterization of an ST88 CA-MRSA clone presents the first genomic description of this 'African' MRSA clone and calls for vigilance and antibiotic stewardship programs to be put in place to prevent epidemics.

Overall, our study demonstrated that with coordinated efforts between researchers, clinicians and stakeholders in health care, BU disease can be effectively managed.

Future perspectives

1. Management of BU wounds needs to be improved.
2. Major investment in BU management is needed. While we provided several interventions for the management of the disease during the course of this study, the activities were grant funded and these interventions will therefore not be sustainable

in the long term. Other means of financing are therefore needed to maintain the standards currently laid down in the BU treatment centers.

3. Health care should be decentralized to reduce the health-worker to patient ratio at the secondary health centers and ensure that patients have access to proper care.
4. Antibiotic resistance is a problem that needs to be tackled. An active surveillance system urgently needs to be put in place to monitor drug use and spread of resistant pathogens in the country.

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