Rheumatic Heart Disease and Beta-hemolytic Streptococci in Salvador, Brazil: A Study of Slum Health

by

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Abstract

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Despite the near disappearance of rheumatic heart disease (RHD) in wealthier nations of the world, this disease continues to cause substantial morbidity and mortality in poor countries worldwide. The burden is projected to be particularly important among residents of urban slums in poor countries. However, the epidemiologic features of RHD in developing countries are poorly understood. RHD is characterized by damage to cardiac valves that is the long-term consequence of an immune process initiated by infection with *Streptococcus pyogenes* (Group A *Streptococcus;* GAS). Progression to RHD often takes decades; it would require decades-long prospective studies to observe associations between GAS infections and the outcome of RHD in the same subjects. Therefore, in this dissertation we first attempted to assess the current burden of RHD in Salvador (Chapter 2), a city where more than half of the population is living in slums, by conducting a population-based study of operations performed for cardiac valve disease. We found that a large proportion of valvular surgeries performed in Salvador from 2002-2005 was for RHD. We then wished to investigate possible reasons for the large burden of this disease. It is known that RHD is influenced by biological factors of beta-hemolytic *Streptococcus* as well as health-care seeking behavior and treatment of streptococcal infections. Therefore, in the third and fourth chapters of this dissertation we focused on the biological factors of *Streptococcus*, focusing our work to address current hypotheses regarding RHD pathogenesis as found in the literature, such as: 1) the association of infection or colonization with certain streptococcal strain types and clinical outcome; 2) the increased risk of RHD with repeated infections with a diverse set of GAS strains, and 3) the possible association of RHD with *Streptococcus spp.* other than GAS.

In Chapter 3, we compared the genotypes of GAS strains recovered from children aged 3-15 years of age who live in slum versus non-slum communities. This was done to determine if there are differences in the strain genotype distributions (as measured by genotype diversity) in these two populations. Detection of differences in genotype distributions by community (estimating slum communities to have higher diversity of genotypes circulating in the population compared to non-slum populations), would provide preliminary data to support the hypothesis that high GAS genotype diversity in

slums may be associated with the observation of high prevalence of RHD in slum populations compared to the low prevalence of RHD among non-slum populations. Furthermore, we investigated two additional species of beta-hemolytic streptococci from slum and non-slum communities as well, and found an unexpected finding that colonization with *Streptococcus dysgalactiae equisimilis* was associated with lower odds of sore throat in children (Chapter 4). We discuss possible explanations for this finding, including biological plausibility as well as alternate explanations. While our observational studies can not define causal associations between epidemiologic features of beta-hemolytic *Streptococcus* and the outcome of RHD, they provide preliminary data that the epidemiologic features of GAS and non-GAS infections in urban slums of Salvador may be distinct from that in non-slum populations.

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Chapter 1: Introduction

A. OBJECTIVES AND SPECIFIC AIMS

1.1 Burden of Rheumatic Heart Disease in Salvador

The objective of this study was to investigate the burden of end-stage rheumatic heart disease (RHD) in Salvador, Brazil. Salvador is a large city (population 3 million) in a middle income country where a large proportion of the population lives in slums (*favelas*) [1]. It is known that living in a slum is a risk factor for RHD, and that the risk associated with residence in a slum is above that seen in the rural poor [21-24]. Therefore, we hypothesized that RHD is an under-recognized and substantial disease burden for residents of Salvador. We sought to investigate this hypothesis with the following specific aims:

a. To describe the epidemiological characteristics and etiologies of valvular damage requiring surgery in residents of Salvador from 2002-2005, in order to determine what proportion of valve surgeries was due to RHD.

b. To identify risk factors for death among RHD valve surgery patients, as well as risk factors for death among all valve surgery patients.

1.2 Outpatient Clinic Study: *Streptococcus spp*. associated with pharyngitis

The progression of acute rheumatic fever (ARF) to RHD is thought to be influenced by bacterial strain factors. Therefore, in the third chapter of this dissertation, we studied the early event, pharyngitis caused by *Streptococcus pyogenes* (GAS), which is known to be associated with the eventual outcome of RHD. In the fourth chapter we studied group G streptococci (GGS) and group C streptococci (GCS) which also colonize the throat and have been hypothesized to be associated with both sore throat and RHD. In these projects, we evaluated the species composition and genotypes of *Streptococcus* isolates comparing children from slum and non-slum communities. We characterized patients by whether they had sore throat or not, and whether the genotypes of *Streptococcus* isolates recovered from theses patients differ between these communities with the following specific aims:

a. To compare the strain diversity and clonal composition of *Streptococcus pyogenes* (GAS) isolates from children with sore throat and children without sore throat from slum and non-slum populations in Salvador, Brazil.

b. To evaluate the species composition of GGS and GCS from children with sore throat and children without sore throat from slum and non-slum populations in Salvador, Brazil.

c. To determine if presence of GGS and GCS (*Streptococcus dysgalactiae equisimilis* or *Streptococcus anginosus*) is associated with higher or lower odds of sore throat in children in slum and non-slum populations in Salvador, Brazil.

B. RATIONALE

1.3 Slums in the 21st Century

In 2008, for the first time in human history, the majority of the world's population lived in cities [2]. The population growth within urban centers is not occurring at the same rate worldwide. In fact, more than 90% of the world's urban population growth by 2030 will be in less developed regions. In these parts of the world, a large proportion of the population will be living in slums. By 2030, assuming that the proportion of slum dwellers within the total urban population does not increase, close to 1.7 billion of the expected 3.93 billion urban residents in low-income and middle income countries will be living in slums [3, 4]. Clearly, improving health standards in slum populations will be paramount for the global health agenda of the 21st century. Public health efforts to address the urban condition will be facilitated with a better understanding of disease burden in slum populations.

In 2003, the United Nations Human Settlements Program (UN-Habitat) published the first comprehensive report providing a global description of slum communities. In this document, a slum is operationally defined as a human settlement that has one or more of the following characteristics: 1) inadequate access to safe water; 2) inadequate access to sanitation and other infrastructure; 3) poor structural quality of housing; 4) overcrowding; and 5) insecure residential status [5]. The report does not address disease burden in these communities.

Those living in slums have been shown to be at high risk for a variety of adverse health conditions [6]. However, specific risk factors that contribute to disease in slums are not well identified. In these communities, diseases are more likely to receive attention only after they become severe. Therefore, opportunities to prevent disease progression in the early stages of illness are lost. Rheumatic heart disease (RHD) is one example of a severe outcome of an easily treatable disease—pharyngitis due to *Streptococcus pyogenes*.

Recent studies suggest there have been epidemiological shifts in RHD — much of what is described in older literature may not apply to regions of the world where this disease is now prevalent. Whether the new findings in the literature reflect differences in host characteristics, the environment, or the genetics of the etiologic agent is unknown. Therefore, re-evaluation of the epidemiologic features of RHD and the causes of RHD is warranted for these environments [7].

1.4 Acute rheumatic fever/rheumatic heart disease in the 21st century

A great deal remains to be understood about acute rheumatic fever (ARF) and its chronic sequela, RHD. The majority of the literature on these diseases was created primarily in the first half of the 20th century, and these early works largely constitute the body of literature that exists today. New research on ARF and RHD has declined considerably in the past 50 years; available data are therefore from studies conducted primarily in the specific environments of the northern hemisphere countries. In some ways, this waning interest indirectly celebrates the near disappearance of these sequelae in affluent countries where research pursuits have been diverted to other public health concerns. However, for the majority of the world's population living in poverty where the incidence of death and disability due to RHD remains high, the need for further investigation is as pertinent as it ever was.

C. BACKGROUND

1.5 Global burden of group A streptococcal disease

Group A *Streptococcus* (*Streptococcus pyogenes*; GAS) causes a wide spectrum of diseases, ranging from relatively benign conditions such as pharyngitis and pyoderma to more severe diseases such as toxic shock syndrome and necrotizing fasciitis. RHD, ARF, and glomerulonephritis are immunologically-mediated complications of GAS infections [8]. Children are the major reservoir of GAS. Humans are the only recognized natural host of GAS [9].

A recent review estimated that over 500,000 deaths per year worldwide can be attributed to GAS, placing GAS among the major human pathogens [10]. This rate of death due to GAS is comparable to that caused by rotavirus, measles, *Haemophilus influenzae* type b, and hepatitis B; it is exceeded only by HIV, *Mycobacterium tuberculosis*, *Plasmodium falciparum*, and *Streptococcus pneumoniae*.

The greatest burden of GAS-related mortality and morbidity as measured by disability-adjusted life years (DALYs) is due to RHD. In this auto-immune disease, heart valves are permanently damaged, and the condition may progress to heart failure, atrial fibrillation and embolic stroke. In 2005, the global number of cases of RHD was estimated to be 15.6 – 19.6 million with 282,000 new cases and 233,000 deaths each year [10]. It is estimated that 79% of these RHD cases are in developing countries. In 2000, it was estimated that 6.6 million DALYs are lost per year due to RHD.

1.6 Clinical manifestations of rheumatic heart disease

RHD is most commonly diagnosed by Doppler echocardiography. The diagnosis of RHD is typically made only when an individual is found to have a new heart murmur or

undergoes echocardiography after becoming symptomatic (e.g., with congestive heart failure or chest pain). Both the echocardiography equipment and the specialized training required for echocardiography reading require resources which are often lacking where RHD is most prevalent.

Those who have progressed to end-stage RHD require surgical procedures including heart valve repair or replacement. A great deal of the DALYs associated with RHD is related to the young age at which the disease manifests. RHD often manifests in individuals in the late second to the fourth decades of life [11]. Often, corrective surgery is needed in the years following appearance or diagnosis of disease—among young adults at their most productive stages in life. These procedures are followed by long-term, if not life-long secondary antibiotic prophylaxis and anti-coagulation treatment. Heart valve replacements often need to be repeated or repaired, and are associated with increased risk for death and complications such as endocarditis [10, 12-14]. In addition, it has been shown that children diagnosed with RHD have a considerably greater chance of dropping out of school [15].

RHD remains a prominent cause of cardiac disease requiring admission to a hospital in many parts of the world [16, 17]. In less developed countries it is the most commonly acquired heart disease in children, adolescents, and young adults hospitalized or seen by specialist cardiology services [10]. Furthermore, RHD continues to be the cause of heart failure and death in pregnant women in less developed countries [18, 19].

Technological advancements in portable echocardiography have facilitated important new estimates of the burden of RHD. A seminal study by Marijon et al. reported prevalence estimates of RHD in randomly selected children aged 6 – 17 years that were substantially higher than what had been previously reported [20]. Using echocardiographic screening, they detected 21.5 RHD cases per 1000 in Cambodian children and 30.4 cases per 1000 in children in Mozambique. Furthermore, they reported a great discrepancy using two different diagnostic strategies to detect RHD. They found that systematic screening with echocardiography revealed a prevalence of RHD that was approximately ten times higher than what was detected with clinical screening (clinical examination to detect suspected RHD and confirmed by echocardiography).

1.7 Rheumatic heart disease in slum versus non-slum communities

The majority of studies have found that residents of rural areas are at higher risk of RHD than urban residents, with the exception of those living in slums; slum residents have a higher risk of RHD than the rural poor [21-24]. Recognized risk factors for RHD include low socioeconomic status, low maternal education, malnutrition, overcrowding, and restricted access to health care [25, 26].

A cross-sectional study from 1997 measuring the prevalence of RHD in children aged 5-16 years in Kinshasa, Democratic Republic of the Congo and adjoining slums in a semi-urban area of Kinshasa found that risk factors for RHD included: attending a slum school

and, for those in slum areas, birth in the rainy season, low birth-weight, low socio-economic status, malnutrition, crowding, and migrant status [27]. Surveys of school-age children in areas such as Agra, India and Sahafa Town, Sudan have found that the prevalence of ARF and RHD is significantly higher in those who reside in slum settlements [21, 28].

1.8 Cost of rheumatic heart disease in Brazil

An assessment of the costs of ARF (the acute autoimmune response preceding RHD) was conducted at a tertiary center in Sao Paulo, Brazil in 2001. Using data from both the public and private medical sectors, the study estimated that the direct and indirect annual cost of ARF in Brazil is $51,144,347.00 [15]. This sum also suggests a potentially larger cost: that of RHD. The annual expense attributable to RHD likely exceeds that of ARF. The surgical costs associated with RHD such as in-patient hospital care, the cost of heart valves and post-surgical immunosuppressive treatments greatly exceed the costs associated with treating ARF.

It is likely that ARF is greatly underreported. The diagnosis of ARF is primarily made using a symptom-based diagnostic algorithm known as the Jones Criteria, and is supported by laboratory findings when possible [29]. However, the limitations of these criteria are well documented. Many of the symptoms which constitute the Jones Criteria, such as fever, arthritis, arthralgia, and rash are associated with many differential diagnoses which can lead to misdiagnosis. [30]. People with limited access to health care may not seek care for these symptoms. The latent period between the onset of streptococcal pharyngitis and ARF is approximately 18 days, and it is often difficult to isolate the organism at the time that ARF symptoms develop. Consequently, under-diagnosis of ARF continues to be a serious problem [24, 31-33].

1.9 Health Care in Brazil

Brazil has a markedly uneven income distribution [34]. The wide (and growing disparities) in wealth in Brazil are associated with unequal access to medical care [35]. Although the federal constitution guarantees universal health care to all Brazilians through the Sistema Único de Saúde (SUS) program, the delivery of this care is limited by insufficient government funding [36]. Brazilian health care is funded by private medical insurance in addition to SUS, and discrepancies in level of care and mortality rates have been observed between these health insurance plans, even after adjusting for other factors [36, 37].

1.10 Streptococcal Pharyngitis

GAS pharyngitis is the acute infectious disease that precedes RHD. A properly performed and interpreted throat culture remains the diagnostic gold standard to identify GAS pharyngitis compared to other diagnostic techniques [38].

A recent comprehensive analysis of the global burden of GAS pharyngitis estimated that approximately 616 million cases of symptomatic GAS pharyngitis occur annually among people aged over 4 years, and that over 550 million of these illnesses occur in less developed countries [10]. The cost of GAS pharyngitis is substantial, from school absenteeism and loss of earnings in parents, to the cost of treatment and the risk of autoimmune sequelae.

In low-income regions, there is often no laboratory capacity for microbiologic diagnosis of GAS pharyngitis and it is difficult to measure incidence data for these regions. Presumptive diagnosis relies on clinical signs only, although the positive predictive value of clinical signs and symptoms has been shown to be extremely low. An evaluation of the World Health Organization clinical decision rule for streptococcal pharyngitis in developing countries revealed sensitivity estimates that did not exceed 9% [39, 40]. Clinicians often empirically treat all pharyngitis episodes with antibiotics, particularly when microbiologic testing is not possible. Bacterial culture and rapid tests for GAS are not routinely available for testing children with pharyngitis in public clinics of many developing and middle-income countries, including Brazil [41].

1.11 Microbiology and typing of pharyngeal streptococcal species

In addition to *Streptococcus pyogenes*, *Streptococcus dysgalactiae equisimilis* and *Streptococcus anginosus* commonly colonize the human pharynx. *Streptococcus dysgalactiae equisimilis* and *Streptoccus anginosus* can have Lancefield group C ß-hemolytic streptococcal (GCS) or group G ß-hemolytic streptococcal (GGS)–specific polysaccharide.

When Group A, C, and G *Streptococcus* are cultured on a blood agar plate, they exhibit characteristic beta-hemolysis. GAS can be subtyped into more than 150 types based on the antigenic property of a cell wall protein called the M protein and the sequence of the gene (*emm*) that encodes the M protein [42]. Other methods for genotyping streptococci include multilocus sequence typing (MLST) and restriction endonuclease fingerprinting of chromosomal DNA [43].

Streptococcus dysgalactiae equisimilis is closely related to GAS and is often termed ‘pyogenes-like’, because it shares virulence factors, including haemolysins, extracellular enzymes, and M-proteins, with GAS [44-46]. The *emm* genes of *Streptococcus dysgalactiae equisimilis* also display sequence heterogeneity at their 5’ ends, giving rise to at least 30 distinct *emm* sequence types.

The great majority of human GGS and GCS infections throughout the body are due to strains of *Streptococcus dysgalactiae equisimilis*. These organisms are generally considered commensal organisms. However, some strains are capable of causing classic streptococcal diseases and their sequelae [47]. These infections are often the same as those seen with GAS infection, and include pharyngitits, bacteremia, endocarditis, meningitis, septic arthritis, toxic shock syndrome, and infections of the respiratory tract

and skin [48-52]. GGS and GCS bacteremia and serious skin and soft tissue infections are often associated with underlying serious disease [52]. The incidence of invasive GGS and GCS infections have been increasing since the 1980's [53]. Certain *emm* types have been associated with invasive disease for GGS and GCS [53].

In some parts of the world, the prevalence of asymptomatic throat carriage of GCS and GGS is substantial. Studies conducted in aboriginal populations in Australia and children in India have found that the prevalence of pharyngeal carriage of group C and group G streptococci is higher than the prevalence of GAS [54, 55]. Yet, the reported incidence rates of ARF and RHD in the aboriginal population are some of the highest reported rates in the literature [56].

Like GAS, GCS and GGS have recently been suggested to have the potential to elicit an autoimmune response that may trigger ARF [57]. A study of GCS and GGS isolates from an aboriginal population in Australia demonstrated that antibodies against three GCS and two GGS strains reacted with human cardiac myosin, the target protein of the autoimmune response in RHD.

1.12 Genetic recombination between streptococcal species

GGS and GCS commonly colonize the same tissue sites (i.e. pharynx and skin) as GAS. This proximity facilitates gene exchange between the species. Genes which are exchanged include virulence factors as well as housekeeping genes. It was recently shown that mobile genetic elements, such as phages and transposons, play an important role in the ongoing inter-species transfers of genetic traits between GGS/GCS and GAS in the community [58].

Virulence genes that are shared between GGS and GAS include genes encoding the M protein, superantigenic exotoxins, fibronectin binding proteins, and C5a peptidase [44-46, 59, 60]. Evidence of lateral gene transfer between GAS and GGS has also been observed for housekeeping genes, where transfer was demonstrated to be predominantly unidirectional (GAS to GGS) [61]. The substantial overlap in the disease spectrum caused by GAS and GGS and strong evidence for cross-species acquisition of virulence traits suggests that particularly virulent GGS clones may have arisen by inter-species recombination [58, 61].

1.13 Pathogenesis of rheumatic heart disease

The pathogenesis of RHD is believed to be related to humoral and cell-mediated immune responses against host tissue antigens triggered by GAS after symptomatic pharyngitis (molecular mimicry hypothesis). CD4+ T cells that recognize both heart tissue and streptococcal M protein have been described [62, 63]. Certain *emm* types have been suggested to be rheumatogenic (i.e. 1, 3, 5, 6, 14, 18, 24, 27 and 29) [64, 65]. In the United States, the decline in the incidence of ARF has been hypothesized to be related to

the replacement of rheumatogenic *emm* types by non-rheumatogenic types in cases of acute streptococcal pharyngitis [66]. Conversely, transient or regional increases in ARF cases have been attributed to the introduction of new rheumatogenic types [65]. However, recent studies from highly endemic regions for ARF/RHD demonstrate that *emm* types previously suggested to be associated with ARF in the United States are not present [7, 67, 68].

1.14 Diversity of *emm*-types of group A *Streptococcus*

In populations with high prevalence of GAS, high diversity in the distribution of *emm* types is often observed. For example, a study conducted in Ethiopia found that 82 carriage isolates represented 43 sequence types [67]. A study in India found that 59 isolates represented 33 isolates, where many of the isolates represented novel sequence types [69]. In comparison, a study conducted in Rome found that 114 isolates represented 22 *emm* types. In some settings, a single *emm* type can constitute the majority observed isolates. A community study in South Korea examining community-wide GAS strain diversity found that *emm* 78 and *emm* 23 accounted for 69% of GAS isolates in one region, whereas in another region, 4 types accounted for 52% of isolates [70]. In Japan, only 29 *emm* types were detected among 906 clinical isolates [71]. And in the US, six serotypes accounted for 60% of all isolates recovered from patients with uncomplicated pharyngitis [72].

The generation of diverse *emm*-types may be due to overcrowding and lack of treatment, which increase opportunity for intra-species and inter-species transfer of genes and consequent genotypic and phenotypic diversity [73]. It has been hypothesized that exposure to a wide variety of *emm*-types may contribute to high diversity of anti-M protein antibodies, increasing the risk for an autoimmune response [41].

GAS *emm* type diversity and the predominance of certain *emm*- types may influence the relevance of new vaccines based on M protein strain type, including a 26-valent vaccine that has completed phase II trials [74-76]. The strain types selected for the vaccine were based on findings in the U.S. of GAS types associated with pharyngitis, necrotizing fasciitis, and other invasive streptococcal infections. In areas with diverse *emm* type profiles circulating in the community, vaccine efficacy against GAS infections may be substantially diminished.

1.15 Conclusions

It is known that RHD is the disabling consequence of an infectious disease which can be treated with antibiotics. However, very little else is known about the epidemiology of RHD in the populations that now suffer the most from this disease, namely those living in urban slums of developing countries. There are opportunities to develop a new body of knowledge for this poorly understood but important disease of some of the world's poorer populations.

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Chapter 2: Burden of rheumatic heart disease in Salvador, Brazil

A. INTRODUCTION

Valvular heart diseases are an important cause of morbidity and mortality worldwide. In the industrialized world, valve disease is primarily degenerative and predominantly affects those aged 65 and older [1]. In developing countries, valve disease is due largely to rheumatic heart disease (RHD), where prevalence peaks in those aged 30-40 years of age [2]. It is estimated that 15.6 – 19.6 million people are living with RHD worldwide. Of these, 79% are in less developed countries, where it is the most common cause of acquired heart disease in children, adolescents, and young adults hospitalized or seen by cardiology specialty services [3].

Brazil has an emerging economy and its medical infrastructure has undergone vast improvements in the past 20 years. However, economic disparities in Brazil persist and are substantial. The inequality between the rich and poor mirrors that seen comparing countries with contrasting levels of economic development [4]. In this context, in Brazil, valve disease may manifest the characteristics of both developed as well as developing countries. It is difficult to efficiently allocate resources for preventative measures without knowledge of the etiologies of valve disease. Therefore, we conducted a population-based study to estimate and describe the demographic and etiologic characteristics associated with heart valve disease surgery in the population of Salvador. A further aim was to identify risk factors for death in those undergoing valve surgery.

B. METHODS

2.1 Study Design

This was a population-based cross-sectional study. All hospitals and cardiac surgery teams (five teams) in Salvador performing cardiac surgery between January 2002 and December 2005 were identified and recruited into the study. The study consisted of two stages of data collection. The first stage was a review of registries of all cardiac surgery procedures to identify valve surgeries that were performed during the study period. The second stage consisted of detailed medical chart review of patients identified in the first stage of the study who were residents of Salvador at the time of valve surgery.

Salvador is located in the state of Bahia and is the only major city in the region with medical facilities conducting cardiac surgery. The five identified hospitals performed all cardiac surgeries in this city during the study period.

2.2 Data Collection

Review of Registries

All surgery teams recruited into this study maintained registries that recorded basic data on all cardiac surgery procedures and patients during the study period. The registries of all teams were reviewed by a Brazilian medical student and an additional member of the research team. Review of registries occurred from 21 June 2006 - January 18, 2007.

Patients undergoing valve (aortic, mitral, tricuspid) surgery during the study period were identified and data were collected for these patients. Data were not collected for surgeries conducted for non-valve related surgeries. For valve surgery patients, data were collected from the registries on date of surgery, age at surgery, sex, health insurance payment plan, procedure (prosthesis or repair), the valve(s) operated on, the city of residence of the patient at the time of surgery, and outcome (lived versus died). Data on outcome were available for only four of five hospitals.

Review of Medical Charts

A list of patients who were identified during the review of the registries and who were residents of Salvador at the time of surgery was generated. The medical charts of patients on this list were solicited from the five hospitals for review. Medical chart reviews were conducted by medical residents specializing in cardiology using a standardized data collection form.

The data collection form for medical chart review recorded information on age, sex, health insurance payment plan and race; diagnosis of valve involvement, valve surgery procedure (repair or prosthesis), type of prosthesis, echocardiographic findings of location and severity of valve lesion; specific echocardiographic findings, such as thickening of the mitral and aortic leaflets, fusion of the commissures, thickening of the aortic and mitral tendons, calcifications of the aortic and mitral leaflets, and anular dilation of the aortic mitral and tricuspid valves; and time (in days) spent in intensive care unit (ICU), total time hospitalized, previous surgeries, post-operative complications, co-morbidities, and outcome. Data on previous acute rheumatic fever (ARF), age of first attack of ARF, and number of ARF attacks were collected as well.

2.3 Eligibility Criteria

All patients who underwent valve surgery in Salvador from January 2002-December 2005 were eligible for inclusion into the study. Eligibility into the medical chart review portion of the study included residency in Salvador at the time of surgery.

2.4 Exclusion Criteria

Patients who underwent non-valvular cardiac surgery (i.e. pacemaker, stent, etc.) were not eligible for review. All patients who lived outside of Salvador at the time of surgery were not eligible for medical chart review.

2.5 Study Definitions

All valve disease etiologies (RHD, degenerative, endocarditis, other) were defined based on pathological, echocardiographic and other documentation in the medical record.

Six categories describing the underlying etiology for valvular disease were created: 1.) Rheumatic heart disease (RHD), 2.) Endocarditis associated with RHD, 3.) Endocarditis of unknown etiology, 4.) Degenerative disease, 5.) Other (includes surgeries due to tendinous cord rupture, congenital valve disease, Chagas disease, aortic aneurism, Marfan's syndrome, trauma, alcoholic myocardiopathy, Wegener's granulomatosis, coronary insufficiency, systemic lupus erythematosus, aortic dissection), and 6.) Unknown etiology.

Valve repair included annuloplasty and ring annuloplasty. Days of hospitalization were calculated from the time of admission to the time of discharge from the hospital. Data on outcome consisted of occurrence of death during surgery or while in the hospital following surgery. Post-operative cardiac insufficiency was defined as: ejection fraction <40% or dyspnoea, hepatomegaly, or edema of the legs.

2.6 Ethical Approval

Institutional Review Board (IRB) approval was obtained from all hospitals, the Comissão Nacional de Ética em Pesquisa (*Conep*) (National Bioethics Commission of Brazil), the Comitê de Ética em Pesquisa-Centro de Pesquisa Gonçalo Moniz – Fiocruz (Ethics Committee for Research – Fiocruz), and the University of California, Berkeley Committee for the Protection of Human Subjects.

2.7 Statistical Analysis

Analyses were conducted using STATA 11.0 (Stata Inc., College Station, Texas). Categorical variables were compared using the chi-square test or the two-tailed Fisher's exact test. Student's t-test was used to compare means. Statistical analysis was carried out by computing 95% confidence intervals and two tailed p-values. Two tailed p-values less than 0.05 were considered statistically significant. Selection of variables into the multivariable model was done by backward stepwise logistic regression with a cut-off of p=0.20. We used the multivariable models to evaluate risk factors for death while

controlling for covariates. Effect modification between covariates was evaluated by the Mantel-Haenszel test of homogeneity following bivariable stratification and by cross-product terms in the multivariable model. The age and etiology-specific rates of incident surgeries per year were calculated using the 2000 Instituto Brasileiro de Geografia e Estatística (IBGE) census data for Salvador, adjusted for the four year study period.

C. RESULTS

2.8 Characteristics of population identified in surgery registries

From January 1, 2002 to December 31, 2005, 1,320 valve surgeries (includes surgeries with ≥ 1 valve, including mitral, aortic and tricuspid repair and implant of prostheses; patients could undergo more than one surgery during the study period) were conducted at six hospitals in Salvador, Brazil. If residents of Bahia from outside Salvador are predominantly seeking care within the state, this count (1,320) approximates the number of valve surgeries in the whole state of Bahia as well. Census data from the IBGE reported 13,070,250 residents in the state of Bahia in 2000. Using the 2000 census data to estimate the population of Bahia from 2002-2005, it can be estimated that during this four year period, the mean annual incidence of valve surgery in Bahia was 2.5 per 100,000 residents.

Six hospitals performed cardiac surgery in Salvador from 2002-2005. The majority of all surgeries were conducted in Hospital 1 (n=737; 55.8%), followed by Hospital 5 (n=222; 16.8%) and Hospital 6 (n=178; 13.5%). The other three hospitals combined conduced 13.9% of the surgeries, with one hospital (Hospital 4) performing only two surgeries (0.15%). Five surgical teams (A, B, C, F, G) conducted valve surgery in these hospitals over this period, performing 19.1%, 20.9%, 31.6%, 13.5%, and 14.9% of surgeries, respectively.

Demographic Data

Data on age were available for 1256 (95.2%) patients. The mean age of these patients was 43.9 years (range 1-86 years, median 42 years). Sex was evenly distributed; 650 (49.2%) patients were male. Information on health insurance plan was available for 1310 (99.2%) patients. The federal public health insurance plan (SUS-Sistema Único de Saúde) was the most frequent payment source (n=880; 66.7%); private health insurance plans covered 399 (30.2%) surgeries, and 31 (2.4%) surgeries were paid for using cash.

Valve Procedures

The most frequent valve surgery procedures were mitral valve replacement (n=754; 1.442 per 100,000/year), aortic valve replacement (n=495; 0.947 per 100,000/year), and mitral valve repair (n=140; 0.268 per 100,000/year) (Table 1). The least performed surgery was tricuspid valve replacement (n=11, 0.021 per 100,000/year).

Outcome

Mortality data (measured as death occurring either during surgery or during the hospitalization) were available from five of six hospitals (Hospitals 1-5; n=1,140). During the study period, 109 (9.6%) patients died while in the hospital. Case-fatality ratios within hospitals ranged from 7.7% to 14.5% of procedures. The mean age of patients who died was 50.3 years. The only procedure significantly associated with death was tricuspid repair (p=0.005).

Residents of Salvador vs. Non-Residents

Residence data were available for 1,224 of 1,320 surgeries (Figure 1). Of these, 540 (40.9%) were conducted in residents of Salvador at the time of surgery and 684 lived outside of Salvador. We were not able to determine location of residence of 96 patients. Patients from outside of Salvador were younger (mean age: 41.9 years) than those from Salvador (mean age: 46.3 years)(p<.001). Among both residents and non-residents of Salvador, approximately half of the surgeries were conducted in male patients. Those from outside of Salvador were more likely to have SUS as their source of payment than those from Salvador (74.8% vs. 58.0%)(p<0.001).

The proportion of the different valve procedures did not differ between Salvador and non-Salvador residents (p > 0.1 for all procedures). Furthermore, outcome did not differ between Salvador and non-Salvador residents (9.1% died versus 9.5% died; p=0.84). However, those who died and were from outside of Salvador were younger (45.5 years) than those who died and were from Salvador (57.2 years)(p=0.006).

Incidence of Valve Surgery: Salvador Residents

Using the census estimate for Salvador for 2000 (2,443,107 residents), we estimate the mean annual incidence of valve surgery in Salvador during the study period to be 5.5 per 100,000 (n=540). For non-residents (those living in Bahia but outside of Salvador), the mean annual incidence of valve surgery was estimated to be 1.6 per 100,000 (n=684).

Data on age were available for 525 surgeries conducted in Salvador residents. The mean annual incidence of valve surgery increased with increasing decade of age (Table 2). The incidence peaked in the 70-79 age group (n=64; 32.1 per 100,000). For those in younger age group, the incidence was 3.7 per 100,000 for those aged 20-29 years, and 5.6 per 100,000 for those aged 30-39 years (Table 2).

2.9 Characteristics of population identified in medical chart review

Etiology of Valve Disease and Incidence Estimates

We were able to obtain the medical charts of 491 (90.7%) of the 540 cardiac surgery patients who were residents of Salvador during the study period. Of these 491 patients, the most common indication for surgery was RHD, which comprised 56.4% of all surgeries in our study population. In the population of Salvador, the mean annual incidence of surgery for RHD was 2.8 per 100,000. This incidence was 3.7 times higher

than the second most frequent etiology, degenerative valve disease, at 0.77 per 100,000. Degenerative disease comprised 15.3% of all surgeries in the study population (Table 3). Endocarditis (due to RHD and other causes) was the underlying etiology of 8.4% of all surgeries, with a mean annual incidence in Salvador of 0.42 per 100,000.

Age and Etiology of Valve Disease

Altogether, 273 (55.8%) surgeries were conducted in those under the age of 50 years. In this age group, 215 (78.7%) procedures were for valvular disease caused by RHD. In the total population, the age decade of 30-39 (n=85, 17.4%) had more surgeries than any other age category. In those aged 30-39 years, 69 (81.2%) of surgeries were for valvular disease caused by RHD. Of the patients with surgery due to RHD during the study period (n=277), more than one-quarter (28.1%) had a history of previous surgery due to RHD.

Of those aged 60-69 years (n=79), 23 (29.1%) of surgeries were for valvular disease caused by degenerative disease, 24 (30.4%) were for disease caused by RHD, and 22 (27.8%) were for disease with unknown etiology.

The mean age of those undergoing surgery for degenerative disease (n=75) was 67.9 years. In comparison, the mean ages of those having surgery for RHD or for endocarditis due to RHD, were 37.8 years and 31.5 years, respectively (Table 3). The mean age of surgery patients whose condition was due to endocarditis not associated with RHD (n=25) was 48.7 years.

Sex

Males had approximately half the odds of surgery due to RHD compared with females (OR=0.55, p=0.001). The mean age of females undergoing surgery for RHD (n=159) was higher than males (n=118) (39.6 vs. 35.3; p=0.03). The incidence of RHD surgery was higher for females than males as well (3.1 vs. 2.6 per 100,000). The annual incidence of surgery caused by complications of degenerative disease was higher for males than females (1.0 vs. 0.6 per 100,000). Surgery for endocarditis not associated with RHD occurred more frequently in males (0.4 vs. 0.2 per 100,000 per year), whereas surgery for disease caused by endocarditis associated with RHD was evenly distributed by sex (0.2 per 100,000 in males vs. 0.2 in females).

Race and Health Insurance

Race was associated with type of health insurance plan: 30 (76.9%) of 39 black patients used SUS to pay for their surgery, 128 (66.3%) of 193 mixed race patients used SUS to pay for surgery, and 83 (45.4%) of 183 white patients used SUS as a payment source ($p<0.001$).

Source of payment was associated with etiology ($p<0.001$). The majority (n=190, 69.6%) of operations for RHD (n=273) and endocarditis associated with RHD (15 of 16, 93.8%) were paid for by SUS. In comparison, 6 (24.0%) and 23 (31.5%) of procedures for valve disease caused by endocarditis (not associated with RHD) and degenerative disease, respectively, were paid for by SUS.

Length of Hospitalization

Mean total days in the hospital was longer for non-white (n=235) than white (n=256) patients (23.0 days vs. 18.3 days; p=0.004). Valve disease caused by endocarditis, both due to RHD and due to other causes, was associated with the highest overall number of days hospitalized (51.3 days and 28.3 days, respectively) (Table 3). Surgery for RHD was associated with the least amount of time hospitalized (17.7 days).

Degenerative disease and endocarditis due to RHD were the etiologies of valve disease associated with the highest number of days (6.5 days, both) in the intensive care unit (ICU). RHD was associated with the least amount of time in the ICU (4.2 days).

Surgical Procedure

Data were collected on type of prosthetic valve (biologic vs. metallic), and valve repair procedures. Implant of a biologic valve (porcine) was the most frequent procedure, with 208 (50.5%) of 412 patients with data on procedure undergoing this surgery. Implant of a metallic prosthesis followed in frequency, with 144 (34.9%) of patients receiving metallic valves. Repair procedures were conducted in 70 (17.0%) of patients. Of 207 patients receiving biologic prostheses and who have data on age, 87 (42.0%) were implanted in patients 50 years or younger. In those less than 30 years of age (n=90), 41 (45.6%) received biologic prostheses.

Multiple Valve Involvement

Multiple valve involvement (measured as moderate or severe disease in $\geq$2 valves) was diagnosed in 159 of 465 patients with data available for this variable. Multiple valve involvement was associated with RHD. Those with RHD had 1.5 times the odds of multiple valve involvement compared with those having surgery for other etiologies; this finding approached statistical significance (p=0.055). Multiple valve involvement was not associated with degenerative disease or endocarditis. Surgeries which were paid for with SUS had twice the odds of having multiple valve disease (OR=1.96, p=0.001) compared with those with private health insurance or out-of-pocket payment. Multiple valve disease was not associated with race, age, or death in bivariable analyses.

Outcome

A total of 58 (11.9%) surgeries of 489 with available outcome data resulted in death. The mean annual incidence of death due to valve surgery increased with increasing age, such that the age group 70-79 had the highest incidence of death (6.0 per 100,000), and those aged 20-29 had the lowest incidence of death (0.05 per 100,00). Of those undergoing valvular surgery, 27.8% of those aged 80-89 died, 22.2% of those aged 70-79, 17.7% of those aged 60-69, 12.8% of those aged 10-19 died, and 11.8% of those 40-49 died. Females were not more likely than males to die (p=0.63). Race and hospital were not associated with death in bivariable analyses. For every additional day spent in the ICU, the odds of death increased by 10% (p<0.001).

Death due to surgery for valve disease caused by RHD had the highest mean annual incidence overall, and was nearly twice as frequent as death due to degenerative disease

(0.3 per 100,000 vs. 0.1 per 100,000). Of 277 surgeries for valve disease caused by RHD, 25 (9%) patients died. A record of at least one previous valve surgery for complications of RHD was associated with death in RHD patients (p=0.001). Of 198 patients with no previous surgery, 12 (6.1%) patients died, compared to the death of 8 (12.7%) patients with one previous surgery, 5 (33.3%) of 15 patients with two previous surgeries. However, comparing all RHD to the other etiologies, those with RHD were least likely to die (OR=0.54, p=0.03).

The odds of death associated with specific etiology was the highest for those with endocarditis not associated with RHD compared with other etiologies (OR=3.15, p=0.01), among whom 7 of 25 (28.0%) died.

Of 76 patients with moderate or severe tricuspid insufficiency, 15 (19.7%) died (OR=2.2, p=0.014). Of patients with moderate or severe tricuspid insufficiency, 98.7% were patients with multiple valve involvement. The majority of patients (n=56, 73.7%) with moderate or severe tricuspid insufficiency had surgery due to RHD, and had higher odds of paying with SUS (OR=2.3; p=0.002). These associations remained significant following multivariable adjustment for the two variables (RHD OR=1.85, p=0.036; SUS OR=2.00, p=0.020).

The post-operative complications of endocarditis (n=12), arrhythmia (n=129), cardiac insufficiency (n=46), and bleeding associated with anti-coagulation (n=62) were all associated with death while in the hospital (p<0.05, all). Arrhythmia was associated with being older (OR per year=1.29, p<0.001), having a source of payment other than SUS (OR=0.65, p=0.040) with surgery for valve disease caused by degenerative disease (OR=1.68, p=0.051). Post-operative endocarditis was associated with indication for surgery due to endocarditis (associated with RHD, and not associated with RHD)(OR= 6.92, p=0.019; OR=6.68, p=0.007, respectively). Furthermore, post-operative endocarditis was associated with implant of biological valve (OR=9.09, p=0.012), and moderate or severe aortic insufficiency (OR=4.08, p=0.019). Bleeding associated with anticoagulation was associated with tricuspid insufficiency (OR=1.9, p=0.032), and SUS (OR=1.76, p=0.029).

Risk Factors for Death—multivariable analysis

Several variables were associated with death in multivariable analyses (Table 4). Diagnosis of moderate or severe tricuspid insufficiency was associated with 2.52 higher odds of death (p=0.022). Each additional day spent in the ICU was associated with a 7% higher odds of dying (p=0.008) and each additional year of age was associated with a 3% higher odds of dying (p=0.006). Three post-operative complications were associated with death, including endocarditis (OR=4.92; p=0.031), cardiac insufficiency (OR=3.43, p=0.006), and bleeding associated with anti-coagulation (OR=3.14, p=0.006). Multiple valve involvement was not significant in the multivariable model.

Risk Factors for Death among RHD patients

In multivariable analyses restricted to RHD patients, post operative endocarditis remained associated with a markedly increased odds of death (OR=18.37, p=0.004)

(Table 5). Furthermore, every additional previous surgery for RHD was associated with three times the odds of death (OR=3.03, p=0.001). Surgery at hospital 3 was associated with 16 times the odds of death (OR=16.00, p=0.009), and payment for surgery by SUS was associated with almost five times the odds of death (OR=4.67, p=0.060) when adjusted for the other variables in the model. The association between SUS as the source of payment and odds of death approached statistical significance.

D. DISCUSSION

In this study, we found that RHD was the most common reason for cardiac valvular surgery in Salvador, Brazil, and that the population undergoing valve surgery was quite young (mean: 43.9 years). Concurrently, we found a substantial burden of degenerative disease in older populations. The young, non-white, poorer populations suffered substantially from RHD, while the older, white population with private health insurance suffered the burden of degenerative disease.

An important finding was that the severe consequences of RHD manifest throughout the life course. In those aged ≥ 60 years, 21% of all valvular surgeries were due to RHD. We note that it is important to consider the morbidity and mortality due to RHD in later life in some populations [5].

Overall, the pattern of valve disease in Salvador more closely resembles what is observed in the developing world. In developed countries, 60–70% of surgical repairs of mitral regurgitation are due to degenerative disease, and only 2-5% are due to RHD [6-10]. In Salvador, 58% of mitral insufficiency was due to RHD, and 11% was due to degenerative disease. Additionally, from 1997-1999, RHD was the leading cause of valvular damage leading to surgery in China, where 77-81% of valve surgeries from 1997-1999 were due to RHD [13].

If we apply the 9.6% case-fatality ratio which was calculated from the surgery registry data to Hospital 6 (for which mortality data was not available), we can estimate 17 deaths would have occurred in Hospital 6 over the four year period. With this value, it can be estimated that a total of 126 deaths would have occurred due to valve surgery during the study period. Using Bahia census data, we can estimate the mean annual incidence of valve surgery mortality from 2002-2005 to be 0.24 per 100,000 for the state.

The overall incidence measure for valve surgery as well as the measure of overall mortality was approximately three and a half times as high for Salvador as what was calculated for the rest of Bahia. In Salvador, the estimated annual incidence of valve surgery was 5.5 per 100,000 from 2002-2005, and 1.6 per 100,000 for Bahia (not including Salvador). The annual mortality rate for Salvador was 0.59 per 100,000, while that for Bahia (not including Salvador) was estimated to be 0.16 per 100,000. It has been shown that those living in slums such as the *favelas* in Salvador, suffer higher rates of RHD than those in rural areas [15-17]. This may be reflected in our data as well. However, this discrepancy is also likely to reflect health care seeking behavior and obstacles of seeking specialized health care for those from the rural interior of the state.

We found a high case fatality ratio in our study. Overall, 12% of patients identified in the medical chart review, died. This finding is particularly striking as this mortality measure does not include death following discharge from the hospital. This proportion is higher than the 4-7.5% range which is documented in several large studies in the United States measuring mortality during hospitalization following mitral and aortic valve surgery [18-20]. Remarkably, the mean age of patients in these studies from the United States ranged from 61.3 to 71.6 years. The case fatality ratio among this much older population was approximately half that of our study population which on average was 44 years old; we detected an unusually high proportion of surgeries ending in death, particularly for a young population undergoing surgery.

In the literature, hospitalization case fatality ratios for valve surgery restricted to RHD patients are generally lower than those for all valve surgeries combined. A study conducted in Toronto of RHD patients (n=573, mean age= 54 years) reported 4.2% mortality during hospitalization [21], and a study conducted in France of 951 RHD surgery patients (mean age 25.8 years) undergoing valve repair reported a hospital case fatality ratio of 2% over 30 days post-procedure [22]. In our study, 9% of RHD patients with a mean age of 37.8 years, died. In the multivariable model, payment by SUS and re-operation were associated with higher odds of death. These factors may reflect a population which is poorer and suffering from early and more severe valvular disease.

The appropriateness of valve repair for patients with rheumatic mitral valve disease remains controversial. Valve repair is often preferred over valve replacement for mitral insufficiency because of lower operative mortality, better late survival, and less risk of thromoembolic complications [9, 21, 23]. However, valve repair is associated with a higher early re-operation rate than mitral valve replacement. In environments where the patient population has limited access to heath care, physicians may choose the solution which will require the least future medical visits, i.e. replacement [21]. Furthermore, rheumatic heart disease can cause severe valvular deformities requiring the surgical implantation of prostheses [24]. In this study, valve replacement was more than five times as frequent as mitral valve repair. In those less than 40 years old, mitral prostheses were approximately four times as frequent.

The choice between implanting mechanical or biologic prostheses in young patients is also controversial. Mechanical valves require ongoing anticoagulation treatment, and inadequate monitoring of treatment increases risk for thromboembolic events and haemorrhage [25]. Transportation difficulties, cultural factors, and distance from appropriate health services can contribute to poor anticoagulant adherence. However, biologic valves are associated with rapid deterioration in young patients; the American Heart Association (AHA) recommends the use of mechanical prostheses in adolescent and young patients for that reason [26]. Biologic valves typically require re-operations to replace the valve in the 10-15 years following implantation, and re-operation is an important risk factor for death in valve surgery. However, if a doctor feels that adequate monitoring can not be achieved, the doctor will choose to implant a biological prosthesis even in young patients, such as those 18 years old or younger [24]. In our study, 45.6% of

those less than 30 years of age received biologic prostheses. These patients will need at least one and likely several re-operative procedures over their lifetime. In our multivariable model, each re-operation increased the odds of death by a factor of three in RHD patients.

In our study, tricuspid insufficiency was independently associated with death in the multivariable model. Tricuspid insufficiency frequently follows mitral valve surgery, both in the long and the short term, and is associated with poor outcome [27-29]. In our study, 16% of those presenting with tricuspid disease also had previous surgery to treat mitral disease, and a third of these patients died.

The population-based design we used strengthened this study. By capturing all cases of valve surgery throughout the city, we avoided referral bias and the influence that is inherent in observing a study population from a select group of hospitals. The different hospitals in Salvador treat patient populations with different age structures and of different socioeconomic status. Therefore, the profile of disease etiologies, the overall severity of disease, and the outcomes in institutions can vary greatly.

However, there were several limitations to this study. Our study population represents only the most severe cases of valve disease, and does not provide an understanding of the overall burden of valve disease in Salvador. This population does not include what is likely to be a substantial number of individuals with moderate and undiagnosed valvular disease; the study patients only represent those who arrive at specialist care. This population may be more likely to represent those with greater access to care, and/or under-represent those who have already developed end-stage severe disease and are not able to be treated before death occurs. It is probable that those of lower socioeconomic status are disproportionately less likely to seek care for their disease, and as our study shows, they are more likely to present with RHD. By collecting data only on incident clinical cases receiving surgery, we cannot know the proportion of cases that are missed. A survey of the community using echocardiography would be the most effective way to measure RHD prevalence in this population.

During medical chart review, we found that specific echocardiographic findings (such as calcification, thickening of the leaflets, and fusion of commissures) as well as data on co-morbidities, were not complete or consistent in the medical charts across the hospitals. Therefore, we were not able to include these data in the analyses. These data would have offered further insight into our findings, but are not likely to have influenced the direction or strength of the findings which are presented here. Finally, RHD continues to be an important cause of heart failure and death in pregnant women in less developed countries, but data on pregnancy status were not collected for this study [30, 31].

Our categorization of severity and etiology of valve disease were based on clinician report, and the original echocardiographic reports were not reviewed or validated for this study. Differences in echocardiographic interpretations could have been possible for different physicians or between hospitals. Furthermore, assessment of the severity of disease may not have been uniform between clinicians within one center as well. Finally,

we had many patients (n=65) for whom the etiology of their valvular disease was not known.

In conclusion, we report the first population-based findings on the etiology and outcome of valve surgery in Salvador. In this population, the overall case fatality ratio was high. RHD and degenerative valvular disease caused the majority of surgeries, and RHD was almost four times more common than degenerative valvular disease. In addition to substantial morbidity and mortality, RHD imposes a large monetary cost to society. It has been estimated that the annual cost of ARF in Brazil is $51,144,347.00; the cost of RHD is likely to be much higher [32]. Unlike degenerative valvular disease, RHD can be prevented through appropriate antibiotic prophylactic strategies. When implemented effectively, the burden of end-stage disease and the associated costs can be significantly reduced through register-based secondary prophylaxis programs. Further investment in planning and resources for secondary prophylaxis would be a cost and life-saving strategy for Brazilians [33-36].

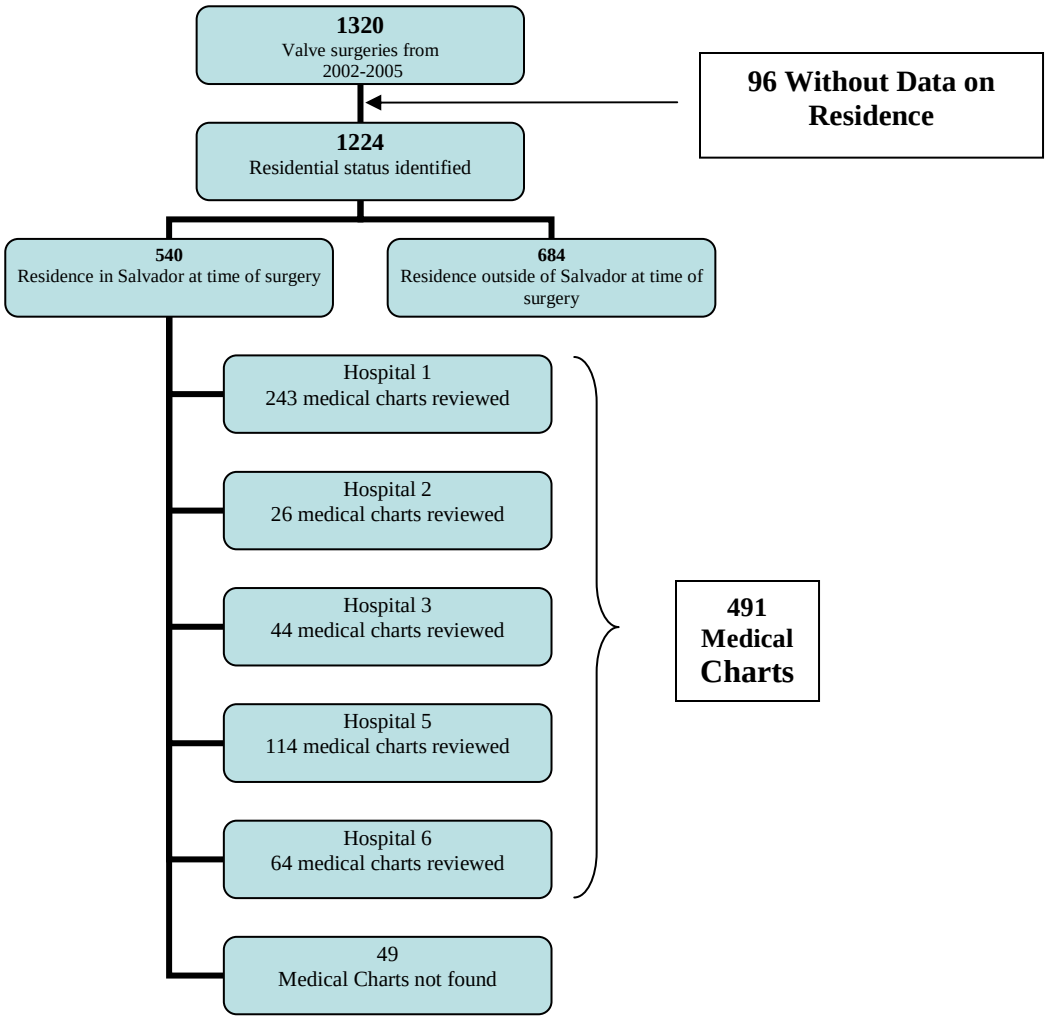

Figure 1. Diagram of valve surgeries included in study as identified from surgical registries, and distribution of valve surgeries by hospital for operations investigated by medical chart review

Table 1. Estimated mean annual incidence of valve surgery procedure and mean age at surgery in the state of Bahia, 2002-2005.

	Total # of Surgeries (1320) N (%)	Mean Age (SD)	Mean Annual Incidence per 100,000*
Prosthesis			
Mitral	754 (57)	43.0 (18.1)	1.442
Aortic	495 (37)	47.0 (20.6)	0.947
Tricuspid	11 (1)	32.2 (19.7)	0.021
Repair			
Mitral	140 (11)	32.4 (19.5)	0.268
Aortic	20 (2)	37.4 (26.4)	0.038
Tricuspid	86 (7)	37.3 (18.6)	0.164

*Estimated population: 13,070,250

Table 2. Estimated mean annual incidence of valve surgery procedures per decade of age, Salvador, 2002-2005.

Age Category in years	Count 2000 Census	Total # Valve Surgeries (2002-2005)*	Mean Annual Incidence per 100,000
0-9	414,730	6	0.362
10-19	505,684	44	2.175
20-29	503,201	75	3.726
30-39	399,208	90	5.636
40-49	292184	82	7.016
50-59	163064	63	9.659
60-69	93847	81	21.578
70-79	49888	64	32.072
80-89	21301	20	23.473
Total	2,443,107	540	5.526

*15 subjects with missing data on age

Table 3. Characteristics of patients and valve surgeries according to valvular disease etiology, Salvador, Brazil, 2002-2005.

Characteristics	RHD (n=277)	Endocarditis associated with RHD (n=16)	Endocarditis of unknown etiology (n=25)	Degenerative valve disease (n=75)	Other valve disease[1] (n=33)	Valve disease of unknown etiology (n=65)	Total (n=491)
	Number (%) or mean (SD)[2]						
Demographic							
Mean Age (years)	37.8 (15.9)	31.5 (13.7)	48.7 (18.7)	67.9 (12.2)	53.0 (18.8)	58.6 (13.9)	46.5 (19.5)
Male Sex	118 (43)	8 (50)	17 (68)	44 (59)	16 (48)	37 (57)	241 (49)
Race							
White	93 (40)	3 (23)	14 (64)	37 (57)	13 (45)	24 (43)	184 (44)
Mixed	119 (51)	9 (69)	5 (23)	19 (29)	12 (41)	30 (54)	194 (46)
Black	22 (9)	1 (8)	3 (14)	9 (14)	4 (14)	2 (4)	41 (10)
Payment Source							
Private	2 (1)	0 (0)	1 (4)	0 (0)	0 (0)	2 (3)	5 (1)
Health insurance	81 (30)	1 (6)	18 (72)	50 (68)	16 (48)	39 (61)	205 (42)
Public health system	190 (70)	15 (94)	6 (24)	23 (31)	17 (51)	23 (36)	274 (57)
Valve lesion[3]							
Mitral stenosis	114 (44)	2 (12)	0 (0)	5 (7)	2 (6)	4 (8)	127 (28)
Mitral insufficiency	148 (57)	10 (62)	12 (50)	29 (40)	21 (66)	36 (72)	256 (56)
Aortic stenosis	43 (17)	0 (0)	3 (13)	33 (47)	2 (6)	12 (23)	93 (21)
Aortic insufficiency	56 (21)	5 (33)	6 (26)	10 (14)	8 (26)	7 (14)	92 (20)
Tricuspid stenosis	2 (1)	0 (0)	1 (4)	1 (1)	0 (0)	0 (0)	4 (1)
Tricuspid insufficiency	56 (22)	1 (6)	3 (13)	6 (9)	2 (6)	8 (16)	76 (17)
≥2 valve lesions	100 (38)	6 (37)	8 (33)	19 (26)	6 (19)	20 (36)	159 (34)
Surgical procedure[4]							
Metal Prosthesis	101 (43)	3 (30)	7 (35)	8 (12)	7 (24)	18 (33)	144 (35)
Biological Prosthesis	100 (43)	5 (50)	8 (40)	48 (72)	19 (66)	28 (52)	208 (50)
Surgical repair	39 (17)	2 (20)	5 (25)	11 (16)	3 (10)	10 (19)	70 (17)
Outcome							
Hospital death[5]	25 (9)	1 (7)	7 (28)	13 (17)	4 (12)	8 (12)	58 (12)

Intra-operative death	6 (2)	0 (0)	0 (0)	2 (3)	0 (0)	1 (2)	9 (2)
Post-operative death	19 (7)	1 (7)	7 (28)	10 (13)	3 (9)	7 (11)	47 (10)
Days of ICU	4.2 (4.0)	6.5 (10.8)	5.3 (5.3)	6.5 (7.9)	5.3 (5.7)	4.6 (2.9)	4.8 (5.2)
Days of hospitalization	17.7 (14.1)	51.3 (29.3)	28.3 (21.5)	22.1 (19.7)	21.7 (14.9)	20.0 (18.6)	20.5 (17.8)

Note. RHD, rheumatic heart disease. ICU, intensive care unit.

[1] Other valve disease included patients with tendinous cord rupture (n=11), congenital valve disease (n=7), Chagas disease (n=4), aortic aneurism (n=4), Marfan's syndrome (n=1), trauma (n=1), alcoholic myocardiopathy (n=1), Wegener's granulomatosis (n=1), coronary insufficiency (n=1), systemic lupus erythematosus (n=1) and aortic dissection (n=1).

[2] Sum of percents may be different than 100% due to rounding.

[3] Includes moderate to severe valve lesions as described by echocardiography. Sum is greater than 100% because patients may have more than one valve lesion.

[4] Sum may be greater than 100% because patients may have undergone more than one procedure.

[5] Includes only within hospitalization deaths.

Figure 2. Mean annual incidence of valve surgery due to rheumatic heart disease, degenerative disease, and endocarditis by decade of age in Salvador, Brazil 2002-2005

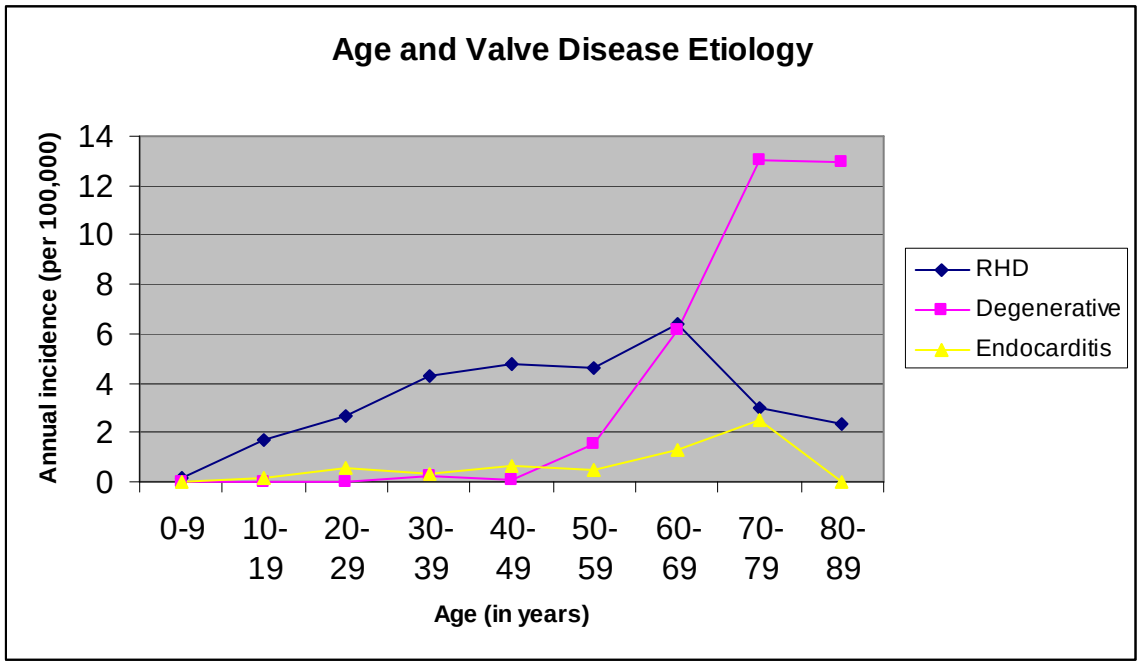

Table 4. Risk factors for death in cardiac valve surgery patients in Salvador, Brazil, 2002-2005

Covariate	Adjusted Odds Ratio (95% Confidence Interval)	P-value
Days in Intensive Care Unit	1.07 (1.02-1.12)	0.008
Age (per year)	1.03 (1.01-1.05)	0.006
Moderate or Severe Tricuspid Insufficiency	2.52 (1.14-5.54)	0.022
Post-operative Endocarditis	4.92 (1.15-20.99)	0.031
Post-operative Cardiac Insufficiency	3.43 (1.42-8.25)	0.006
Post-operative Bleeding Associated with Anti-Coagulation	3.14 (1.40-7.04)	0.006

Table 5. Risk factors for death in rheumatic heart disease patients undergoing cardiac valve surgery in Salvador, Brazil, 2002-2005

Covariate	Adjusted Odds Ratio (95% Confidence Interval)	P-value
Post-operative endocarditis	18.37 (2.51-134.66)	0.004
Number of previous surgeries for RHD	3.03 (1.55-5.92)	0.001
Surgery at hospital 3	16.00 (2.02-126.66)	0.009
SUS as the source of payment	4.67 (0.93-23.36)	0.060

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Chapter 3: Factors associated with Group A *Streptococcus emm* type diversity in a large urban setting

A. INTRODUCTION

Group A *Streptococcus* (*Streptococcus pyogenes;* GAS) causes a wide spectrum of diseases, ranging from pharyngitis and pyoderma to more severe diseases, such as toxic shock syndrome, necrotizing fasciitis, glomerulonephritis and rheumatic heart disease (RHD) [1]. Children are the major reservoir of GAS [2]. The highest prevalences of GAS infections and their complications are found in developing countries [3].

GAS strain typing is frequently used to characterize the epidemiology and pathogenesis of GAS infections. The most common target of typing methods is the M protein, which is a cell surface virulence factor serving as a target of the immune response to GAS that confers type-specific resistance. In 1996, a sequence-based typing system called *emm* sequence typing, which is based on the N-terminus hypervariable region (5') of the M protein gene, was described [4]. Many studies have been conducted using *emm* typing to show associations of specific strain types with disease outcomes [5-9]. In addition, information about geographic *emm* type distribution can be used to assess and predict potential candidate vaccine efficacy [10, 11], including that of a 26-valent vaccine that has recently completed phase II trials [12].

Epidemiologic studies have revealed that developing countries have high *emm* type diversity [13, 14], while industrialized countries are more likely to have a limited number of *emm* types [15-18]. This pattern was clearly demonstrated in a recent systematic review of the global distribution of GAS *emm* types [19]. However, comparisons that are made across continents cannot evaluate the impact of local factors on genotype distribution. In this study, we compared the *emm* types of GAS isolates obtained from children in slum and wealthy neighborhoods in the same city, Salvador, Brazil, to examine whether GAS *emm* type diversity differs in communities with contrasting environmental, demographic, and socioeconomic factors in the same urban center. A finding of distinct *emm* type diversity indices between communities in the same city would support the hypothesis that local epidemiologic features may play an important role in the generation of diversity of *emm* types in a population. Furthermore, we collected isolates from children with and without sore throat to identify associations of certain *emm* types with this clinical outcome.

B. METHODS

3.1 Study sites

This study was conducted at three pediatric outpatient urgent care clinics (clinics A, B, and C). Clinical services at Clinic A and Clinic B are offered free to patients through the publicly funded Unified Health System (SUS). These clinics serve primarily low-income patients. The socioeconomic status and demographic characteristics (household density, income, and education level of mother and father) of patients seeking care at Clinic A and Clinic B are similar. Clinic C serves wealthier clientele and only those with private insurance.

3.2 Patient recruitment

Patients aged 3-15 years were consecutively recruited from Clinics A-C from April 17, 2008 to October 31, 2008. Recruitment occurred while patients waited for their medical evaluation or immediately following their appointment. Parents/guardians of children were approached by a research team member for recruitment and consent to participate in the study. A brief description of the study, risks, benefits, and issues of confidentiality was provided. Following consent from parents/guardians and verbal assent from minors, a trained member of the research team administered a standardized questionnaire and collected a throat swab sample from the study participant. All members of the research team were trained in standardized technique for both procedures. Institutional Review Board (IRB) approval was obtained from all hospitals, the Comissão Nacional de Ética em Pesquisa (*Conep*) (National Bioethics Commission of Brazil), the Comitê de Ética em Pesquisa-Centro de Pesquisa Gonçalo Moniz – Fiocruz (Ethics Committee for Research – Fiocruz), and the University of California, Berkeley Committee for the Protection of Human Subjects.

Definitions: Cases were defined as those children whose chief complaint was sore throat. GAS culture-positive sore throat was defined as a child with a sore throat in whom GAS was isolated from the throat swab. Controls were defined as those visiting the clinics for other reasons (Appendix 1). Exclusion criteria included use of antibiotics in the past two weeks or any illness requiring inpatient hospitalization on the day of recruitment.

3.3 Data collection

The following variables were recorded: reason for visit to the hospital, date of birth of patient, sex of patient, household income, home address, whether in school and where, whether in daycare and where, total number of people living in house, number of children 15 years or younger in household, whether the case had sore throat in past six months, level of education of mother, and level of education of father.

3.4 Isolation and genotyping of streptococci

Swab cultures were obtained from the pharynx of the study children following a standard protocol. All study technicians were observed periodically at the clinic sites for proper and consistent swabbing technique. A sterile cotton swab tip was applied to the posterior pharynx and tonsils, as recommended by the Infectious Disease Society of America (IDSA) [20]. The swabs were immediately placed in Stuart transport medium, transported to the laboratory and plated the same day on 5% sheep blood agar. The plates were incubated at 37ºC for 24 - 48h with 5% CO_2. Streptococci were phenotypically identified by beta-hemolysis, colony morphology and the catalase test. Carbohydrate group identification (Groups A, B, C, F, G) was performed by positive latex agglutination (Remel PathoDx Strep Grouping Latex Test Kit, Remel, Lenexa, KS, USA). Pure culture samples were stored in 5% glycerol at -80ºC.

emm typing: *emm*-typing of all isolates were performed as described by the Centers for Disease Control and Prevention (CDC) protocol (http://www.cdc.gov/ncidod/biotech/strep/protocol_emm-type.htm).

3.5 Statistical analysis

Analyses were conducted using STATA 11.0 (Stata Inc., College Station, Texas). Categorical variables were compared using the chi-square test. Student's t-test and ANOVA were used to compare means, and multivariable logistic regression was used to evaluate the association between specific *emm* types and case status while controlling for covariates. Only those children with culture-positive GAS were included in the logistic regression model. Simpson's index of diversity was used to calculate the variation of the number of *emm* types of GAS isolates by clinic or by case status [21]. Higher index measures represent greater diversity of *emm* types, as the method calculates the probability that any two randomly selected isolates from the same population will be of different *emm* types. Confidence intervals (CI's, 95%) for the diversity index measures were calculated as previously described [22].

C. RESULTS

3.6 Demographic and clinical characteristics of study population

Between April 17, 2008 and October 31, 2008, 2194 children aged 3-15 years (759 in Clinic A, 518 in Clinic B, 917 in Clinic C), who met the eligibility criteria, were identified from the three study clinics. Of 2181 children with data on case status, 624 (28.6%) came with a complaint of sore throat (cases), and 1557 (71.4 %) came for other illnesses. The distribution of reasons for visit among the controls was comparable across the three hospitals.

The mean age of all the study children differed between slum (defined as patients from Clinics A and B combined, Appendix 2) and non-slum residents (defined as patients from Clinic C)(7.2 years vs. 7.8 years, respectively, $p < 0.001$)(Table 1). The sex distribution did not differ between slum and non-slum populations. One minimal monthly salary (MS) or less (equivalent to $246.10 USD as of April 2008) was reported by 648 (53.8%) of 1205 slum households, and 37 (4.3%) of 870 non-slum households as the household income ($p<0.001$). The mean number of members per household in the slum population (4.5) was greater than that in the non-slum population (4.0) ($p<.001$). The mean number of children <15 years per household was greater in the slum population (2.0 persons) than that in the non-slum population (1.6 persons) ($p <.001$).

The differences in mean age between cases (7.4 years) and controls (7.5 years) or between patients who tested positive for GAS (7.6 years) and negative for GAS (7.5) were not significant ($p>0.1$), either in the total population, or when stratified by slum status.

3.7 Microbiologic studies

In total, 529 *Streptococcus* isolates (groups A-G) from 2194 children were obtained (Table 1). Of these, 254 (48%) were GAS (Table 2). Of 253 GAS isolates (1 isolate missing case/control status), 125 (8%) were from controls and 128 (20.5%) were obtained from cases ($p<.001$). The proportion of cases who tested culture positive for GAS differed by slum (23.1%) vs. non-slum clinic subjects (17.4%), which approached statistical significance ($p= 0.08$). The proportion of controls that tested positive for GAS did not differ between slum vs. non-slum children (7.8% vs. 8.2%).

3.8 Diversity of *emm* types of group A *Streptococcus*

Of 254 GAS isolates, 238 yielded interpretable *emm* sequences. These 238 isolates represented 61 unique *emm* types. In the non-slum population, 94 isolates comprised 36 distinct *emm* types (38.3%). In the slum population, 144 isolates comprised 53 distinct *emm* types (36.8%). Between these two groups, the proportion of unique *emm* types did not differ ($p=0.81$). The proportion of unique *emm* types was higher for carriage isolates than for sore throat cases in the slum population, but this finding did not reach statistical significance ($p=0.11$) (Table 3).

Simpson's diversity index for the overall *emm* types was 96% (94%-97%). The index was 92% (89%-96%) for the non-slum population, and 97% (96%-98%) for slum children. Significantly, the CI's for slum vs. non-slum only overlap at the lower bound estimate for slum, and the upper bound estimate of non-slum. For both slum and non-slum populations, the diversity index was lower for cases than for controls [non-slum: 90% vs. 93%; slum 96% vs. 98%] (Table 3).

In both slum and non-slum populations, *emm*12.0 was the predominant type, followed by *emm*1.0 (Figure 1). However, in the non-slum population, 20 (21.3%) of 94 isolates were *emm*12.0, whereas in the slum population, 18 (12.5%) of 144 isolates were *emm*12.0 (p=0.07). *emm*1.0 was the second most prevalent *emm* type in both populations, and also constituted a larger proportion of non-slum isolates. In the non-slum population, 14 (14.9%) of 94 isolates were *emm*1.0, and in the slum population, *emm*1.0 constituted 9 (6.3%) of 144 isolates (p=0.03). The three most predominant *emm* types constituted a much larger proportion of isolates in the non-slum population as compared with the slum population (41.5% vs. 24.3%)(p=0.005), suggesting that GAS diversity was greater in the slum population.

emm types 12.0 (n=38), 1.0 (n=23), st2904.1 (n=13), 66.0 (n=12), 87.0 (n=9), 49.3 (n=8), and 27G.0 (n=8) were the most common types encountered in the study, accounting for 46.6% of the total. There were 22 *emm* types that were represented by only a single isolate. In total, 25 *emm* types were detected only in slum children, compared with only 8 *emm* types which were found only in non-slum children. The proportion of all *emm*-typable GAS isolates which were subtypes (allelic variants) did not differ between slum and non-slum populations, where 14 (9.7%) of 144 isolates from the slum population were subtypes, and 9 (9.6%) of 94 isolates from the non-slum population were subtypes (p=0.98).

emm types which were represented by only one isolate were more likely to be found in children 10 years or older, than in those 9 and younger, which approached statistical significance (p=0.08).

3.9 Group A *Streptococcus emm* type and case status

Three *emm* types were significantly associated with sore throat, conferring either increased or decreased odds of reporting sore throat. Of those who tested positive for GAS, those with *emm*12.0 (n=38) had 2.2 times the odds of having sore throat compared with those with a different *emm* type (p=0.04), after adjusting for age, income and number of children less than 15 years of age in the household (Table 4). For those patients with *emm*66.0 (n=12), the odds of sore throat were 8.7 times that of sore throat with other *emm* types in the multivariable model (p=0.04). Interestingly, a lower odds of reporting sore throat was seen for *emm*27G.0 (n=8), where those with this *emm* type had 0.1 times the odds of sore throat compared with other *emm* types in the multivariable model (p=0.07); this finding approached statistical significance. None of the associations between *emm* type and sore throat remain significant following adjustment with Bonferroni correction for multiple comparisons (tests of significance at the p=0.007 level).

3.10 Estimated coverage of 26-valent group A *Streptococcus* vaccine

In this study, 100 (42.0%) of the 238 *emm* typed isolates, and 15 (24.6%) of the 61 *emm* types would be covered by the 26-valent M-protein-based GAS vaccine, assuming cross-immunity between type 1.2 and subtype 1.25, between type 101 and subtype 101.1, and between type 33.0 and subtype 33.1 [12]. Stratifying by populations, 45 (47.9%) of 94 isolates and 10 (27.8%) of 36 *emm* types from non-slum children would be covered by the vaccine. In the slum, the coverage for isolates would be 52 (36.1%) of 144 isolates, and 11 (20.8%) of 53 *emm* types.

In children presenting with sore throat, 55 (44.7%) of 123 *emm* typed isolates, and 13 (30.2%) of 43 *emm* types would be covered by the current 26-valent M-protein-based GAS vaccine. In the non-slum population, 25 (51.0%) of 49 *emm* typed isolates, and 8 (33.3%) of 24 *emm* types would be covered. In the slum population, 32 (43.2%) of 74 *emm* typed isolates, and 10 (29.4%) of 34 *emm* types would be covered by the vaccine.

D. DISCUSSION

In Salvador, Brazil we found significant differences in *emm* type diversity in GAS isolates obtained from different populations in the same city. The diversity index was significantly higher among GAS isolates from children residing in slum communities (97%) compared to those living in wealthier neighborhoods (92%). In fact, the diversity index of the non-slum GAS isolates was closer to that of *emm* types identified from high income countries (92%) than to those found in the slum populations of Salvador [19]. Though this study evaluated only two communities, this study lends preliminary data to the hypothesis that *emm* diversity may be influenced by local factors, such as crowding and lack of access to health care which are more prevalent in slum communities. Crowding may facilitate increased transmission opportunities, and lower likelihood of antibiotic treatment for sore throat. Our data found significant differences in household density, type of health insurance plan, and income in slum versus non-slum communities.

In addition to *emm* type differences in GAS strains across high-income vs. low-income populations in the same city, we found certain *emm* types to be over or under-represented among children with sore throat [*emm*66.0 (OR = 8.7), *emm*12.0 (OR=2.2), *emm*27G.0 (OR=0.1)]. Although none of these associations remain significant following statistical adjustment for multiple comparisons (Bonferroni correction), the crude associations provide exploratory data which can be further investigated in the laboratory, comparing the presence of virulence factors in these strains compared with strains which were not associated with sore throat, or in future field trials of *emm* types and association with clinical outcome.

A vaccine against GAS will have substantial benefits worldwide. However, the impact on disease reduction could vary by region depending on the vaccine composition. Currently, the only vaccine to complete phase I/II trials is a 26-valent recombinant M protein

vaccine [12, 23]. While it has been postulated that this vaccine would offer substantial protection against invasive disease morbidity and mortality in the United States, studies from developing countries suggest that the current formulation would provide limited coverage in those areas [9, 13, 14]. In our study, only 42% of the total isolates, and 44.7% of isolates from cases, would be covered by the 26-valent M-protein-based GAS vaccine. This level of coverage is lower than what was recently estimated for Africa, Asia, Latin America, Middle East, and high income countries [19]. Additionally, our comparison of 26-valent vaccine coverage in slum (36%) versus non-slum (48%) overall clinic samples, and in samples found only in children with sore throat (slum: 43.2%; non-slum: 51.0%), revealed that coverage would not be equal even within the same city.

In conclusion, we hypothesize that local demographic and socioeconomic factors may contribute to the diversification of GAS *emm* types. While our analyses are based in a study of limited size, our finding of distinct diversity profiles of bacterial populations in different neighborhoods in the same city lends preliminary support of the proposed hypothesis. We also hypothesize that this distinction in *emm* type diversity might be particularly pronounced in cities with slums. The environmental and demographic conditions which characterize slums, such as crowding and lack of access to health care, may contribute to increased transmission of GAS, increased opportunities for transfer of genetic material between organisms, and consequent increased genotypic diversity. The opportunities for horizontal gene transfer may not be as frequent in wealthy communities; this may be reflected in lower diversity of genotypes. Further studies comparing genotype diversity indices in a larger number of communities of contrasting economic and demographic conditions would help elucidate the validity of this hypothesis. We note that it is not simply poverty itself that determines this difference [24, 25]. As the world expands toward the projected population size of two billion slum residents in less than 30 years, it will be essential to better understand the slum structural dynamics which may contribute to major differences in disease outcomes and vaccine efficacy [26].

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Table 1. Demographic characteristics and beta-hemolytic streptococcal groups isolated in children attending slum and non-slum clinics in Salvador 2002-2005

	Total Population (n=2194) N (%)	Non-slum[a] (n=917) N(%)	Slum[b] (n=1277) N(%)	
				p-value
Cases	624 (28.6)	287 (31.4)	337 (26.6)	--
Controls	1557	628	929	--
Mean Age in years (95%CI)	7.5 (7.3-7.6)	7.8 (7.6-8.1)	7.2 (7.1-7.4)	<.001
Sex				
Female	1060 (48.3)	443 (48.3)	617 (48.3)	1.0
Male	1134	474	660	
Monthly salary (in reais)[c]				
≤415	685 (33.0)	37 (4.3)	648 (53.8)	<.001
416-830	550 (26.5)	161 (18.5)	389 (32.3)	
831-1660	336 (16.2)	204 (23.4)	132 (11.0)	
1661-2490	195 (9.4)	173 (19.9)	22 (1.8)	
≥2491	309 (14.9)	295 (33.9)	14 (1.2)	
Mean # people /house (95%CI)	4.3 (4.2-4.3)	4.0 (3.9-4.1)	4.5 (4.4-4.6)	<.001
Mean # people ≤ 15 yrs. / house (95%CI)	1.8 (1.8-1.9)	1.6 (1.5-1.6)	2.0 (2.0-2.1)	<.001
Group A *Streptococcus*	254 (48.0)	99 (44.4)	155 (50.7)	0.33
Group B *Streptococcus*	34 (6.4)	24 (10.8)	10 (3.3)	.001
Group C *Streptococcus*	57 (10.8)	30 (13.5)	27 (8.8)	0.09
Group F *Streptococcus*	51 (9.6)	24 (10.8)	27 (8.8)	0.44
Group G *Streptococcus*	133 (25.1)	46 (20.6)	87 (28.4)	0.08

[a]Includes all children from Clinic C
[b]Includes all children from Clinics A and B
[c]119 missing data on income

Table 2. Demographic characteristics and beta-hemolytic streptococcal groups isolated in slum versus non-slum children in Salvador 2002-2005, stratified by sore throat (case) and carriage (control)

	Non-slum (n=915) [a]			Slum (n=1266) [b]		
	Case (%)	Control (%)	p-value	Case (%)	Control (%)	p-value
	287 (31.4)	628 (68.6)		337 (26.6)	929 (73.4)	
Mean Age (95% CI)	7.8 (7.3-8.2)	7.9 (7.6-8.1)	0.67	7.2 (6.8-7.5)	7.3 (7.1-7.5)	0.57
Male	139 (48.4)	335 (53.3)	0.17	146 (43.3)	508 (54.7)	<.001
Monthly Salary (in reais)	N=274	N=594		N=312	N=882	
≤415	11 (4.0)	26 (4.4)	0.02	178 (57.1)	468 (53.1)	0.75
416-830	38 (13.9)	123 (20.7)		91 (29.2)	291 (33.0)	
831-1660	58 (21.2)	145 (24.4)		34 (10.9)	96 (10.9)	
1661-2490	55 (20.1)	117 (19.7)		5 (1.6)	17 (1.9)	
≥2491	112 (40.9)	183 (30.8)		4 (1.3)	10 (1.1)	
Mean # persons per household (95% CI)	4.0 (3.9-4.2)	4.0 (3.9-4.1)	0.96	4.5 (4.3-4.8)	4.4 (4.3-4.5)	0.27
Mean # < 15 yrs. per household (95% CI)	1.5 (1.5-1.6)	1.6 (1.6-1.7)	0.16	2.1 (2.0-2.3)	2.0 (1.9-2.1)	0.06
Group A *Streptococcus*	50 (17.4)	49 (7.8)	<.001	78 (23.1)	76 (8.2)	<.001
Group B *Streptococcus*	7 (2.4)	17 (2.7)	0.81	4 (1.1)	6 (0.6)	0.34
Group C *Streptococcus*	8 (2.8)	22 (3.5)	0.57	6 (1.8)	21 (2.3)	0.60
Group F *Streptococcus*	6 (2.1)	18 (2.9)	0.50	7 (2.1)	20 (2.2)	0.93
Group G *Streptococcus*	8 (2.8)	38 (6.1)	0.04	17 (5.0)	70 (7.5)	0.12
No isolate	208 (72.5)	475 (75.6)	0.17	225 (66.8)	736 (79.2)	<.001

[a]Includes all patients from Clinic C. Case/control status missing for 2 study participants
[b]Includes all patients from Clinics A and B. Case/control status missing for 11 study participants

Table 3. Diversity of *Streptococcus pyogenes* (GAS) *emm* types in non-slum versus slum populations

	Simpson's Diversity Index	CI	Number of Unique *emm* Types	Number of Isolates	Proportion Unique
All	0.95	(0.94-0.97)	61	238	26.1%
Non-Slum	0.92	(0.89-0.96)	36	94	38.3%
Case	0.90	(0.84-0.97)	24	49	49.0%
Carriage	0.93	(0.88-0.97)	21	45	46.7%
Slum	0.97	(0.96-0.98)	53	144	36.8%
Case	0.96	(0.94-0.98)	34	74	45.9%
Carriage	0.98	(0.97-0.99)	41	69	59.4%

Table 4. Evaluation of the most common *emm* types and their association with sore throat or carriage, among all GAS culture-positive (n=253) patients

Emm Type	N (%)	Case (n=128)	Control (n=125)	Crude OR	p-value	95% CI	OR [a]	p-value	95% CI	Multivariable OR[b]	p-value	95% CI
12.0	38 (15.0)	25	13	2.09	0.05	1.0-4.3	2.12	0.04	1.0-4.4	2.21	0.04	1.1-4.7
1.0	23 (9.1)	11	12	0.89	0.78	0.4-2.1	0.88	0.77	0.4-2.1	0.84	0.69	0.4-2.0
st2904.1	13 (5.1)	7	6	1.15	0.81	0.4-3.5	1.15	0.80	0.4-3.5	1.13	0.83	0.4-3.5
66.0	12 (4.7)	9	2	4.65	0.05	1.0-22.0	4.64	0.05	1.0-21.9	8.70	0.04	1.1-70.5
87.0	9 (3.6)	5	4	1.23	0.76	0.3-4.7	1.22	0.77	0.4-7.1	1.29	0.71	0.3-5.0
49.3	8 (3.2)	5	3	1.65	0.50	0.4-7.1	1.66	0.50	0.4-7.1	1.13	0.88	0.2-5.8
27G.0	8 (3.2)	1	7	0.13	0.06	0.0-1.1	0.13	0.06	0.0-1.1	0.14	0.07	0.0-1.1
Unique	22 (8.7)	10	12	0.80	0.61	0.3-1.9	0.79	0.61	0.3-1.9	0.65	0.39	0.3-1.7

[a] Adjusted for age

[b] Adjusted for age, income, number < 15 yrs. in household

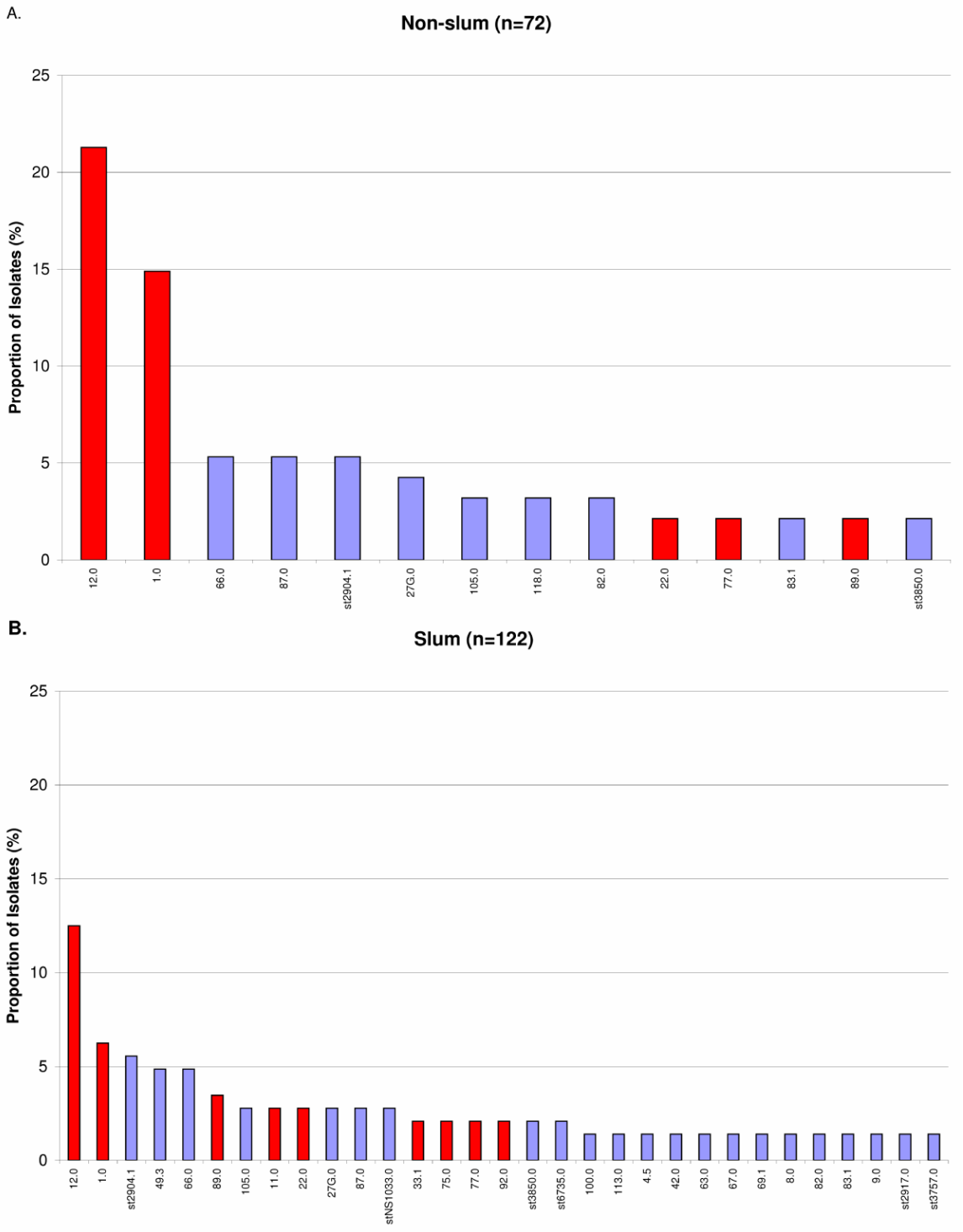

Figure 1. *Streptococcus pyogenes* (GAS) *emm* types in non-slum (A) and slum (B) populations. Only *emm* types represented by more than one isolate are included in the graphs. Bars in red indicate *emm* types included in the 26-valent vaccine. Blue bars indicate *emm* types not included in the vaccine.

Appendix 1. Reason for visit of patients for patients without sore throat

	Clinic A N= 566	**Clinic B N= 363**	**Clinic C N= 628**
Gastrointestinal	186 (32.9)*	138 (38.0)*	157 (25.0)*
Nausea	1	1	11
Stomach ache	40	44	58
Vomit	118	78	58
Diarrhea	27	15	30
Upper Respiratory	371 (65.5)	167 (46.0)	254 (40.4)
Nasal Congestion	6	4	9
Cough	168	78	105
Allergies	13	8	27
Phlegm	10	4	16
Difficulty breathing	2	4	10
Asthma	172	96	87
Other	353 (62.4)	230 (63.4)	282 (44.9)
Fever	190	88	106
Chest pain	11	3	3
Flu	14	4	13
Eye infection	6	1	5
Chicken pox	7	2	4
Skin infection	18	11	10
Body ache	10	9	9
Headache	42	37	50
Injury	18	13	7
With patient but not seeking care	16	51	45
Urinary tract infection	7	6	10
Earache	14	5	20
Other	22	26	38
Don't know	55 (9.7)	24 (6.6)	150 (23.9)

*Sum may be greater than 100% because patients may have given more than one complaint

Appendix 2. Demographic characteristics and streptococcal group distributions of children attending slum clinics A and B

	Clinic A N=759	Clinic B N=518	p-value
Cases	193 (25.4)	144 (27.8)	0.24
Controls	566	363	
Mean Age in years (95%CI)	7.3 (7.1-7.5)	7.1 (6.8-7.3)	0.14
Sex			
Female	372 (49.0)	245 (47.3)	0.55
Male	387	273	
Monthly salary (in reais)			
≤415	410 (57.6)	238 (48.3)	0.02
416-830	208 (29.2)	181 (36.7)	
831-1660	74 (10.4)	58 (11.8)	
1661-2490	14 (2.0)	8 (1.6)	
≥2491	6 (0.8)	8 (1.6)	
Mean # people /house (95%CI)	4.4 (4.3-4.5)	4.5 (4.4-4.7)	0.22
Mean # people ≤ 15 yrs. / house (95%CI)	2.1 (2.0-2.1)	2.0 (1.9-2.1)	0.33
Group A *Streptococcus*	85 (11.2)	70 (13.5)	0.22
Group B *Streptococcus*	2 (0.3)	8 (1.5)	0.02
Group C *Streptococcus*	13 (1.7)	14 (2.7)	0.22
Group F *Streptococcus*	17 (2.2)	10 (1.9)	0.71
Group G *Streptococcus*	40 (5.3)	47 (9.1)	0.01

Chapter 4: Epidemiologic characterization of patients with sore throat in slum and non slum settings: possible protective role of Group G Streptococcus colonization

A. INTRODUCTION

While *Streptococcus pyogenes* (Group A *Streptococcus*; GAS) is clearly a cause of pharyngitis, the role of *Streptococcus dysgalactiae equisimilis* (SDE) as a causative agent of endemic pharyngitis has been debated for more than 50 years [1-7, 8]. SDE has been implicated as a causative agent of pharyngitis in outbreak situations, but its role in endemic disease remains uncertain [9-16]. More than seven decades of nomenclature changes among non-Group A β-hemolytic streptococci has contributed to the difficulty of generating consensus regarding the role of these organisms in causing disease [17]. In 1933, Rebecca Lancefield developed a technique which classified β-hemolytic strains by specific carbohydrate "group" antigens [18]. Additional speciation techniques have revealed streptococci with carbohydrate groups C and G (GCS, GGS) to be comprised of several streptococcal species, in which some species can express either group C or G carbohydrate antigen.

The most common human ß-hemolytic GGS and GCS include SDE and *S. anginosus*. GGS/GCS are most commonly considered commensal organisms with the capacity to cause severe opportunistic infections in individuals with underlying medical conditions such as malignancy, vascular disease or diabetes [19, 20]. However, in recent decades, GGS/GCS have been increasingly implicated in diseases that occur in healthy individuals [21, 22]. GGS/GCS and GAS inhabit the same tissue sites, and the disease spectrum caused by GGS/GCS overlaps with that caused by GAS [10, 11, 13, 19, 23-28]. In some medical centers, GGS has been shown to exceed GAS as the leading cause of invasive streptococcal infection [26, 29, 30].

SDE produces an M protein that has structural and functional features similar to the M protein of GAS. *S. anginosus* does not produce an M protein. The M protein is an important virulence factor and the most common target of strain typing methods for GAS and SDE. One typing method based on sequence analysis of the 5' end of the gene that encodes M protein (*emm*) has been a valuable tool for characterizing *Streptococcus* strains for epidemiologic studies [31]. Correlations between *emm* type and disease potential have been shown for GAS and SDE [21, 32-34].

In environments endemic for SDE and GAS, intra- and interspecies lateral gene transfer between the organisms occurs [35-38]. Virulence factors and housekeeping genes may be transferred across species via mobile genetic elements such as transposons and phages [36, 39-44]. Such exchanges could affect the pathogenic potential of SDE [35]. This process may occur more frequently in highly dense population settings where contact between organisms is more likely to occur, such as in urban slums in developing

countries. Therefore, we chose to study this question in Salvador, Brazil, by comparing slum and non-slum populations. We investigated the epidemiologic features associated with the prevalence of ß-hemolytic GGS/GCS in the throat in children seen in outpatient emergency clinics serving slum and non-slum communities, and whether sore throat is associated with any of the GGS/GCS species or genotypes in these study populations.

B. METHODS

4.1 Study sites

Study participants were recruited from three pediatric outpatient emergency clinics (clinics A, B, and C). Clinical services at Clinics A and B are offered free to patients through the publicly funded Unified Health System (SUS). The socioeconomic status and demographic characteristics (household density, income, education level of parents) of patients seeking care at Clinic A and Clinic B are similar. Clinic C serves wealthier clientele and only those with private health insurance.

4.2 Patient recruitment

Patients aged 3-15 years were consecutively recruited from Clinics A, B and C from April 17, 2008 to October 31, 2008. Recruitment occurred while patients waited for their medical evaluation or immediately following their appointment. Parents/guardians of children were approached by a research team member for recruitment and consent to participate in the study. A brief description of the study, risks, benefits, and issues of confidentiality was provided. Following consent from parents/guardians and verbal assent from minors, a member of the research team administered a standardized questionnaire and collected a throat swab sample from each study participant.

Institutional Review Board (IRB) approval was obtained from all hospitals, the Comissão Nacional de Ética em Pesquisa (*Conep*) (National Bioethics Commission of Brazil), the Comitê de Ética em Pesquisa-Centro de Pesquisa Gonçalo Moniz – Fiocruz (Ethics Committee for Research – Fiocruz), and the University of California, Berkeley Committee for the Protection of Human Subjects.

Definitions: Cases were defined as children whose chief complaint was sore throat. GGS/GCS culture-positive sore throat was defined as a child with a sore throat in whom GGS/GCS was isolated from the throat swab. Controls were defined as children visiting the clinics for reasons other than sore throat. Exclusion criteria were use of antibiotics in the past two weeks or any illness requiring inpatient hospitalization on the day of recruitment.

4.3 Data collection

The following variables were recorded: reason for visit to the clinic, date of birth of patient, sex of patient, household income, home address, enrollment in school or daycare, location of school or daycare, total number of people living in household, number of children ≤ 15 years of age in household, occurrence of sore throat in the case in the past six months, and mother's and father's education level.

4.4 Isolation of streptococci and group carbohydrate identification

A sterile cotton swab tip was applied to the posterior pharynx and tonsils of each participant, as recommended by the Infectious Disease Society of America (IDSA) [45]. All study technicians were observed periodically at the clinic sites for proper and consistent swabbing technique. Swabs were immediately placed in Stuart transport medium, transported to the laboratory and plated the day of collection on 5% sheep blood agar. Plates were incubated at 37ºC with 5% CO_2 for 24 - 48h. Streptococci were phenotypically identified by beta-hemolysis, colony morphology, and the catalase test. Carbohydrate group identification (Groups A, B, C, F, G) was performed by positive latex agglutination (Remel PathoDx Strep Grouping Latex Test Kit, Remel, Lenexa, KS, USA). Cultures were stored in 5% glycerol at -80ºC.

DNA Isolation: DNA isolation was performed using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) as indicated.

16S rRNA Polymerase Chain Reaction (PCR) for Species Designation: Amplifications were carried out in a total volume of 25 µl with 2µl of template DNA, 0.5µl 50µM each of forward (16s-8F: 5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer (16s-806R: 5'-GGA CTA CCA GGG TAT CTA ATC C-3'), 2.5µl 10X Buffer 15mM $MgCl_2$, 0.5µl 10mM dNTP mix, and 0.1 µl 5,000 U/ml Taq (New England Biolabs, Ipswich, MA). Thermal cycling conditions were as follows: 5 minute denaturation at 94ºC, 33 cycles of 30 second denaturation at 94ºC, 30 second annealing at 62 ºC, 90 second extension at 72ºC, and a final 7 minutes at 72ºC.

emm typing: *emm*-typing of SDE isolates was performed according to the Centers for Disease Control and Prevention (CDC) protocol (http://www.cdc.gov/ncidod/biotech/strep/protocol_emm-type.htm).

4.5 Statistical analysis

Analyses were conducted using STATA 11.0 (Stata Inc., College Station, Texas). Patients who reported sore throat were initially compared with patients who did not report sore throat by bivariable methods. Bivariable analyses were also used to compare those with and without GGS/GCS and those from slum and non-slum communities. Categorical variables were compared using the chi-square test or the two-tailed Fisher's

exact test. Student's t-test was used to compare means. Statistical analysis was carried out by computing 95% confidence intervals and two tailed p-values. Two tailed p-values less than 0.05 were considered statistically significant. Selection of variables into the multivariable model was done by backward stepwise logistic regression with a cut-off of p=0.20. The multivariable model was used to evaluate the association between presence of SDE and *S. anginosus* and case status while controlling for the covariates: age, sex, slum versus non-slum residence, and culture-positive status of GAS. We evaluated effect modification between covariates using the Mantel-Haenszel test of homogeneity following bivariable stratification, and by using cross-product terms in the multivariable model.

C. RESULTS

4.6 Demographic and clinical characteristics of study population

Between April 17, 2008 and October 31, 2008, 2194 eligible children aged 3-15 years (759 in Clinic A, 518 in Clinic B, 917 in Clinic C) were enrolled in the study. Of 2181 children with data on case status, 624 (28.6%) reported sore throat (cases), and 1557 (71.4 %) presented with other illnesses. Reasons for hospital visit among the controls were comparable across all three clinics (data not shown).

Patients from the slum population were younger ($p<0.001$), less likely to be cases ($p=0.015$), had lower income ($p<0.001$), and lived in higher density households ($p<0.001$), compared to those in the non-slum population (Table 1). Patients colonized with SDE were older ($p=0.004$), lived in households with higher density of children (aged $\leq$ 15 years)($p=0.04$), and were less likely to be cases ($p=0.03$) than those without SDE (Table 1).

4.7 Microbiological studies

In total, 133 (6.1%) GGS isolates and 57 (2.6%) GCS isolates were obtained from 2194 children. Of 133 GGS isolates, we were able to speciate 122; 60 were SDE, 61 were *S. anginosus*, and one was *S. constellatus*. Using PCR, we were not able to amplify the target sequence for 11 strains; speciation of the remaining 11 isolates is currently underway using biochemical methods. Of 57 GCS isolates, 39 were speciated; 12 were SDE, 26 were *S. anginosus*, and one was *S. constellatus.* Speciation using biochemical methods is currently underway for the 18 GCS isolates which we were not able to speciate using PCR techniques.

4.8 Risk factors for sore throat by bivariable analyses

In bivariable analyses, those who presented with sore throat were more likely to be female ($p<0.001$), from the non-slum study population ($p=0.015$), 3-5 years old ($p=0.03$), living in a household with earnings at least six times the minimum wage ($p=0.002$), or in a household with a mother and/or father with a university or higher education level ($p\leq0.01$) (Table 2).

Those with sore throat were less likely to be colonized with GGS overall ($p=0.01$), and specifically with SDE ($p=0.03$) or *S. anginosus* ($p=0.06$) (Table 2). Those with sore throat were more likely to be colonized with GAS ($p<0.001$). Colonization with GCS was not associated with sore throat ($p=0.49$).

4.9 Risk factors for sore throat by multivariable analyses

Several variables were found to be independently associated with absence of sore throat by multivariable logistic regression (Table 3). Living in a slum was associated with reduced odds of sore throat (OR=0.79, CI: 0.65-0.96; $p=0.018$), age 3-5 years was associated with increased odds of sore throat compared with children aged 6-8 years ($p=0.011$).

We found two interaction terms which remained significant following adjustment of age and slum residence in the multivariable model. The first interaction term represented effect modification by sex in the association between SDE and sore throat ($p=0.031$). Effect modification by sex was also evident in the association between GAS and sore throat; the interaction term approached statistical significance ($p=0.057$) and was left in the model for adjustment purposes. To evaluate these interactions, we established females with no SDE and no GAS infection as the reference (Figure 1). Figure 1 demonstrates the interactions between GAS and SDE and sex. In this figure, it is evident that GAS increased the odds of sore throat in both males and females (males: OR: 2.64, CI: 1.82-3.83; females: 2.33, CI: 1.58-3.43; both $p<0.001$). Compared to baseline, male sex alone was protective against sore throat, and those without GAS or SDE had lower odds of being a case (OR=0.67, CI: 0.54-0.83, $p<0.001$). In males, the additional colonization with SDE increased the protective effect against sore throat by more than five and a half times (OR=0.12, CI: 0.03-0.51, $p=0.004$). Interestingly, the protective effect of SDE was not seen in females colonized with this organism (OR=1.09, CI: 0.50-2.35, $p=0.831$).

4.10 Streptococcus dysgalactiae equisimilis emm typing

Of 60 GGS SDE isolates, we were able to *emm* type 51 isolates. We were not able to amplify the *emm* target sequence using PCR for any of the 12 SDE GCS samples, and could not type these isolates. Four *emm* types--stG480.0 (n=11), stG4831.0 (n=10), stC6979.0 (n=9) and stG166b.0 (n=5)--collectively accounted for 69% of the typed SDE

strains. The remaining 12 *emm* types were represented by two (emm57.4, stG485.0, stG5063.0, stG6792.0) or one isolate each (emm31.1, stC58.0, stG10.0, stG245.0, stG4974.0, stG643.1, stG652.1, stG866.0). None of the four predominant *emm* types were significantly associated with clinical outcome.

D. DISCUSSION

We found an unexpected association of GGS colonization, specifically SDE, with lower odds of sore throat in pediatric populations in Salvador, Brazil. In an attempt to discern whether GGS is associated with a clinical syndrome, we detected the opposite effect. To our knowledge a statistically significant association of SDE colonization with endemic sore throat has not been previously reported in the literature.

This finding may reflect a spurious association. However, the protective association persisted after controlling for age, sex, and living in a slum in a multivariable analysis with evaluation of interaction terms. There may be other confounders which were not considered in this study, and the findings presented in this study may be due to bias or chance. Further investigations would help elucidate the generalizability of this reported association.

In our study, an interaction between sex and colonization with SDE was detected. The evaluation of these interaction effects were displayed in Figure 1. Comparing females and males with no GAS or SDE it was found that females had higher odds or reporting sore throat than males. Furthermore, colonization with SDE in females was not protective, although SDE was protective in males. Finally, a male with SDE demonstrated lower odds of sore throat compared to a female with or without SDE.

In 2009, Steer et al. published their findings from a study of prospective surveillance for sore throat in a tropical country in children aged 5-14 [46]. In this study, sore throat was defined as patient complaint of sore throat. Extracting data from their tables, we found what appears to be an interaction by sex in the association of sore throat and GGS/GCS (excluding *S. anginosus* isolates). From our calculations, a lower odds of sore throat was reported with GGS/GCS colonization in males (OR=0.76; p=0.01) but not in females (OR=1.2; p=0.06) (See Appendix for calculations). Similar to our findings, an interactive effect was not seen for sex and GAS. The consistency between this study and ours provides preliminary evidence for an association that may warrant investigation in future studies.

The biological plausibility of this finding may be analogous to what is seen in other human bacterial ecosystems, in which the presence of a non-pathogen inhibits colonization by more pathogenic species, thereby preventing disease in the host. Protection can occur through steric hindrance of pathogen adhesion sites, or stimulation of cross-protective immunity. Co-colonization of several streptococcal species is rare or transient in pharyngeal colonization with *Streptococcus*. The presence of non-pathogenic GGS in a mucosal site may prevent colonization by more pathogenic species such as

GAS or viral respiratory pathogens. This idea is supported by the increased protective effect of GGS (OR=0.32) or SDE (OR=0.32) when analysis was confined to children colonized with any *Streptococcus* spp.

An interesting discovery from these data was that only one of the GGS species, namely SDE, was found to be associated with lower odds of sore throat. Whereas SDE is morphologically similar to GAS and shares structural features such as the M protein, *S. anginosus* is morphologically distinct [47]. The hypervariable terminal region of the M protein is known to be immunogenic. It is possible that prior or long-term SDE colonization induces a cross-protective mucosal immune response against subsequent GAS colonization or infection.

In this study, living in a slum was protective against sore throat. This finding may be the consequence of the case definition being a proxy for health care seeking behavior. It is possible that people with more resources are more likely to seek care for self-limited, relatively benign symptoms such as sore throat. It is also possible that slum residents experience repeated exposures to causative agents and are therefore more likely to develop immunity relatively earlier in age against symptomatic infections. The small sample size of the study limited our power to detect associations with particular SDE *emm* types and clinical outcome. Two of the *emm* types found in this study were recently shown to be associated with invasive disease in Japan [32]. Larger studies are needed to further elucidate the role of these *emm* types in endemic pharyngitis.

The results which are represented in this study may be influenced by laboratory identification techniques. The common laboratory protocol of sub-culturing (for isolation of a single species) a primary plating of a swab sample with selection of only one colony from the initial plate may contribute to underestimation of co-colonization. We were careful to evaluate primary plates for presence of all beta-hemolytic colonies which appeared to have distinct phenotypes (we did find some children in our study colonized with more than one beta-hemolytic *Streptococcus* species), however, SDE strains can visually appear very similar to GAS colonies. If GAS was the predominant strain in a child (and therefore also on the plate) with complaint of sore throat, but this child was simultaneously colonized with SDE that was similar in phenotype to GAS on agar plate, it is likely that the single colony selected for sub-culture would be GAS. If this event occurred repeatedly in the study, we could be underestimating the number of children with sore throat and SDE, thus biasing our results to detect the protective effect that we found. Furthermore, we were not able to generate hypotheses of biologic plausibility to explain the interaction we detected between sex and SDE on sore throat. This limitation weakens our ability to propose that our findings represent a causal association.

The diversity of *emm* types detected in this study (16 types among 51 isolates) is greater than what has been previously published [21, 48, 49]. One quarter of our SDE isolates displayed *emm* types that are associated with GCS or GAS. This may reflect a particularly high degree of genetic exchange among streptococci in this population. These dynamics may have important implications for changes in the pathogenicity of SDE.

SDE has been increasingly implicated as a cause of invasive disease and other diseases . SDE may be developing increased capacity for virulence due to genetic exchange with pathogenic species, or this may be an artifact of improved laboratory methods for distinguishing β-hemolytic streptococci (such as latex agglutination for Lancefield grouping) [25]. A great deal of GGS/GCS literature does not identify the bacteria to the species level; this might have contributed to the historical uncertainty about the role of GGS, specifically SDE, in clinical disease. Clearly, further studies of the epidemiology of GGS stratified by species are needed, particularly in tropical regions where the prevalence of these bacteria appears to be high, and where environmental and socioeconomic conditions favor high rates of transmission.

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Table 1. Demographic characteristics of children colonized with *Streptococcus equisimilis dysgalactiae* and those attending slum and non-slum clinics in Salvador, Brazil

	Total Population (n=2194)	SDE‡ (n=71)		Non-slum* (n=917)	Slum** (n=1277)	
			p-value			p-value
Cases†	624	12	0.03	287	337	0.015
Controls	1557	59	--	628	929	--
Mean Age in years (95%CI)	7.4 (7.3-7.6)	8.6 (7.8-9.4)	0.004	7.8 (7.6-8.0)	7.2 (7.1-7.4)	<.001
Sex						
Female	1060	31	0.43	443	617	1.0
Male	1134	40		474	660	
Monthly salary	N=2075	N=68		N=870	N=1205	
≤415	685	23	0.26	37	648	<.001
416-830	550	25		161	389	
831-1660	336	9		204	132	
1661-2490	195	5		173	22	
≥2491	309	6		295	14	
Mean # people /house (95%CI)	4.3 (4.2-4.4)	4.4 (3.9-4.9)	0.64	4.0 (3.9-4.1)	4.5 (4.4-4.6)	<.001
Mean # people ≤ 15 yrs. / house (95%CI)	1.8 (1.8-1.9)	2.1 (1.7-2.5)	0.04	1.6 (1.5-1.6)	2.0 (2.0-2.1)	<.001
Group A *Streptococcus*	252	1	--	99	154	0.36
Group G *Streptococcus*	133	--	--	46	87	0.08

*Includes all children from Clinic C

**Includes all children from Clinics A and B

† Case/control status missing for 13 individuals (2 in non-slum population, 11 in slum population)

‡ SDE = *Streptococcus dysgalactiae equisimilis*

N: Total number with available response

Table 2. Risk factors for sore throat in a pediatric outpatient population in Salvador, Brazil

Covariates	Sore throat Positive (n=624)*	Sore throat Negative (n=1557)*	Odds Ratio	95% Confidence Interval	p-value
Male	285 (45.7)	843 (54.1)	0.71	0.59-0.86	<.001
Slum	337 (54.0)	929 (59.7)	0.79	0.66-0.96	0.015
Age					
3-5 years	226 (36.2)	511 (32.8)	1.0	--	--
6-8 years	165 (26.4)	481 (30.9)	0.78	0.61-0.98	0.03
9-11 years	148 (23.7)	365 (23.4)	0.92	0.72-1.17	0.49
12-15 years	85 (13.6)	200 (12.8)	0.96	0.71-1.29	0.79
Monthly salary					
≤415	189 (32.3)	494 (33.5)	1.0	--	--
416-830	129 (22.0)	414 (28.1)	0.81	0.63-1.06	0.12
831-1660	92 (15.7)	241 (16.3)	1.00	0.74-1.34	0.99
1661-2490	60 (10.2)	134 (9.1)	1.17	0.83-1.66	0.38
≥2491	116 (19.8)	193 (13.1)	1.57	1.18-2.09	0.002
Mean # people /house (SD)	4.31 (0.07)	4.26 (0.04)	--	4.18- 4.34	0.53
Mean # people ≤ 15 yrs. / house (SD)	1.86 (0.04)	1.83 (0.03)	--	4.18- 4.44	0.67
Education Mother					
Primary Incomplete	159 (25.8)	425 (27.7)	1.0	--	--
Primary Complete	37 (6.0)	125 (8.2)	0.79	0.53-1.19	0.26
Secondary Incomplete	44 (7.1)	151 (9.8)	0.78	0.53-1.14	0.20
Secondary Complete	241 (39.1)	573 (37.4)	1.12	0.89-1.42	0.33
≥ University	133 (21.6)	247 (16.1)	1.44	1.09-1.90	0.01
Illiterate	3 (0.49)	13 (0.85)	0.62	0.17-2.19	0.46
Education Father					
Primary Incomplete	124 (22.4)	345 (25.0)	1.0	--	--
Primary Complete	45 (8.1)	122 (8.8)	1.03	0.69-1.53	0.90
Secondary Incomplete	33 (6.0)	113 (8.2)	0.81	0.52-1.26	0.35
Secondary Complete	220 (39.7)	585 (42.4)	1.05	0.81-1.35	0.73
≥ University	126 (22.7)	207 (15.0)	1.69	1.25-2.29	0.001
Illiterate	6 (1.1)	9 (0.7)	1.85	0.65-5.32	0.25
School	552 (88.6)	1394 (89.5)	0.91	0.67-1.24	0.53
Daycare	40 (6.4)	75 (4.8)	1.36	0.89-2.04	0.13
Streptococcal Colonization					
Group G (all species)	25 (4.0)	108 (6.9)	0.56	0.34-0.88	0.01
Group C (all species)	14 (2.2)	43 (2.8)	0.81	0.41-1.52	0.49
S. Dysgalactiae equisimilis	12 (1.9)	59 (3.8)	0.50	0.24-0.94	0.03
S. Anginosus	17 (2.7)	69 (4.4)	0.60	0.33-1.05	0.06
Group A *Streptococcus*	128 (20.5)	124 (8.0)	2.98	2.26-3.93	<.001

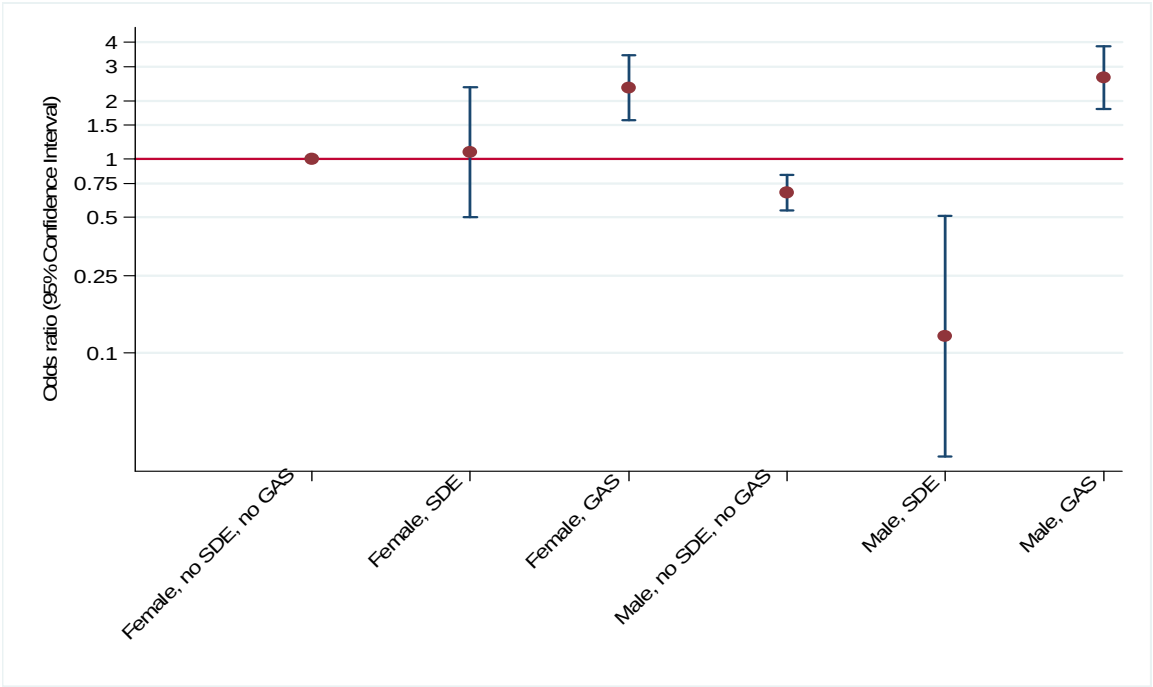
*Thirteen subjects missing case/control status

Table 3. Association of colonization with *S. dysgalactiae equisimilis* and sore throat in Salvador, Brazil.

Covariate	Adjusted Odds Ratio* (95% Confidence Interval)	P-value
Slum	0.79 (0.65-0.96)	0.018
Age		
3-5 years	1.0 (Reference group)	--
6-8 years	0.73 (0.57-0.93)	0.011
9-11 years	0.87 (0.68-1.25)	0.293
12-15 years	0.92 (0.67-1.25)	0.575

*Adjusted for *S. pyogenes*, *S. dysgalactiae equisimilis*, sex, the interaction term for sex and *S. pyogenes*, and the interaction term for sex and *S. dysgalactiae equisimilis*.

Figure 1. Odds ratios of sore throat as the outcome predicted by throat colonization with *S. dysgalactiae equisimilis* or *S. pyogenes* as the exposure, among subgroups defined by sex



SDE = *Streptococcus dysgalactiae equisimilis*
GAS = *Streptococcus pyogenes*

Appendix 1

Data collected by Steer et al. in a prospective surveillance study of streptococcal sore throat in a tropical country (1)

	Sore Throat	Carriage (no sore throat)	Risk Ratio (95% Confidence Interval)	p-value
Total	667	665	--	--
Male	340	348	--	--
Female	327	317	--	--
GAS Culture Positive	61	40	1.23	0.03
Male & GAS Culture Positive	33	23	1.21	0.14
Female & GAS Culture Positive	28	17	1.25	0.11
GGS/GCS Culture Positive	120	126	0.97	0.65
Male & GGS/GCS Culture Positive	48	75	0.76	0.01
Female & GGS/GCS Culture Positive	72	51	1.20	0.06

*****GAS = Group A *Streptococcus*; GGS/GCS = Group G *Streptococcus*/Group C *Streptococcus*

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Chapter 5. Discussion

A. SUMMARY OF OBJECTIVES AND SPECIFIC AIMS

The objectives of this thesis were to determine the burden of end-stage RHD in Salvador, Brazil, and to evaluate whether strain characteristics of *Streptococcus spp.* which may influence the outcome of RHD differ between slum and non-slum communities in this city. Often, RHD takes decades to manifest. Therefore, the chapters in this thesis investigated RHD epidemiology at the two stages of the disease process: the infectious events that initiate the RHD immune process, and end-stage disease at the point of surgical intervention.

Chapter 2 demonstrated that valve surgery in Salvador is associated with substantial morbidity and mortality in a young population, and that RHD was the main cause of valve surgery in this city from 2002-2005. In Chapter 3 we found that the bacterial ecology, as measured by genotype diversity, of *S. pyogenes* differed between the slum community and the non-slum community that we studied. Chapter 3 further provided preliminary data which identified genotypes associated with sore throat in analyses unadjusted for multiple comparisons. In Chapter 4, we evaluated *S. dysgalactiae equisimilis* and *S. anginosus*, two other streptococcal species which colonize the throat and are known to cause outbreaks of pharyngitis and are hypothesized to be associated with RHD. We found an unexpected finding of lower odds of sore throat with colonization with *S. dysgalactiae equisimilis*. We discuss the possible explanations for this finding, including biologic plausibility and alternate explanations.

This final chapter summarizes our findings according to our stated specific aims, and discusses the contributions of this thesis to general knowledge of current RHD epidemiology. Finally, this thesis concludes with a discussion of the implications of our findings for possible public health interventions, and the identification of possible future directions for RHD research.

5.1 Specific Aim 1: Determine the burden of end-stage rheumatic heart disease in Salvador, Brazil

This study was the first population-based study of cardiac valve surgeries in Brazil. We found that despite Brazil's emerging economy, valve surgery in Salvador more closely reflects that of developing countries where RHD is the predominant etiology.

We found that the mean age of the population undergoing RHD valve surgery was 38 years, and that RHD patients were more likely to be female, non-white, have multiple valve involvement and corrective procedures, tricuspid insufficiency, and be poor compared with those undergoing valve surgeries for other reasons. Those undergoing

surgery for endocarditis due to RHD were even younger, averaging less than 32 years of age.

Mortality due to RHD surgery was more than twice as high as what was found in large studies conducted in Canada and France. In our study, 9% of those undergoing RHD surgery died.

In this study, implant of a mitral prosthesis was more than 5 times as frequent as mitral valve repair. Prostheses are associated with higher mortality and post-operative complications than repair procedures. That implant of prostheses was much more common than repairs may reflect the severe disease state in which patients presented for surgery. An additional finding in this study was that 46% of those less than 30 years of age in our study received biologic prostheses, which are associated with poor long-term prognosis. These findings raise serious concerns about the enormous economic burden placed on the long-term management of these patients and provide, for the first time, the data that can inform resource allocation for this public health problem.

5.2 Specific Aim 2: Determine risk factors for death during hospitalization in rheumatic heart disease patients and for valve surgery patients due to all causes

In multivariable analyses restricted to RHD patients, a striking finding was that every additional surgery due to RHD in the medical record was associated with three times the odds of death during hospitalization. Of all RHD patients in the study, 28% had evidence of previous surgery due to RHD. Furthermore, many of the patients undergoing surgery during the study period were very young in age and receiving biologic prostheses that will need to be replaced at least once and likely several, times in their lifetime.

Young, poor patients with RHD have high odds of a negative health outcome over their lifetime. Surgeons in Salvador may choose biologic valves over metallic valves in young patients in part due to knowledge that the patient population with RHD has limited access to the health care that is required to maintain anti-coagulation treatment for these patients over the long term. Inconsistent anti-coagulation therapy is associated with high probability of potentially lethal complications. For these patients, these risks are being weighed against the high risks inherent in future re-operative procedures. Our data demonstrate that RHD is a disease associated with a great deal of morbidity and mortality in a young population.

Payment by SUS for valve surgery was associated with approximately five times the odds of death in RHD patients in multivariable analysis, and the majority of surgeries in RHD patients are paid for with SUS. Those paying for surgery with SUS had higher odds of being black or racially mixed and may represent poorer populations with limited access to health care. Those paying with SUS may present for surgery with more advanced disease.

We found a surprisingly high case fatality ratio among those undergoing cardiac valve surgeries in Salvador, Brazil. Overall, 12% of patients identified in the medical chart review died during hospitalization. This proportion is two to three times higher than what is seen in the United States for overall cardiac valve surgical procedures in much older populations. In multivariable analyses, we found that older age, additional days in the ICU, and several post-operative complications, were associated with higher odds of dying.

5.3 Specific Aim 3: Determine diversity of group A *Streptococcus* in slum and non-slum communities

We found significant differences in *emm* type diversity in GAS isolates obtained from slum versus non-slum populations. The diversity index was significantly higher among GAS isolates from children residing in slum communities (97%) compared to those living in wealthier neighborhoods (92%). The dissimilarity in these diversity indices is similar to what has been shown in comparisons of developing and developed countries.

By comparing two communities in the same urban center, we were able to control for factors that could affect the diversity when comparing countries on distant continents. Comparisons between countries on different continents are common in the literature. What has never been asked is whether one might see the same distinctions in genotypic diversity when comparing two communities of contrasting economic development in the same city. In this study, we found that distinct bacterial population distribution occurred between different neighborhoods in the same city. Our study was limited in size; these data alone do not definitively demonstrate that local factors contribute to the diversity of GAS *emm* types, however, the findings from this study provide important preliminary data which can inform hypotheses for future investigations.

We also found certain *emm* types appear to be more or less frequent among children with sore throat. These samples are available for future laboratory studies to determine why certain *emm* types predominate or are rarely encountered in clinical cases.

The implications of this study are that the 26-valent vaccine, or any other M-protein-based vaccine with a restricted number of *emm* types in the formulation, would result in different levels of coverage in slum versus non-slum populations. We found that only 36% of the total isolates in the slum communities, and 48% of isolates from non-slum communities would be covered by the 26-valent M-protein-based GAS vaccine.

5.4 Specific Aim 4: Determine species and clonal composition of group G and C *Streptococcus* in slum and non-slum communities

Previous studies conducted in tropical climates have found very high prevalences of GGS and GCS in the throat. We sought to determine if these GGS/GCS are also highly prevalent in pediatric populations in Salvador, and whether GGS/GCS differ in species or

clonal constitution between slum and non-slum communities. First we sought to characterize GGS and GCS at the species level. We analyzed the population distribution of *S. dysgalactiae equisimilis*, at the level of genotype. Of 2194 children, we found that 6% were colonized with GGS and 3% were colonized with GCS.

Among the *S. dysgalactiae equisimilis* isolates that we were able to genotype, we found that a diversity of *emm* types that was greater than what has been previously published. We found that one quarter of our *S. dysgalactiae equisimilis* isolates displayed *emm* types that are associated with GCS or GAS. This may reflect a particularly high degree of genetic exchange among streptococci in this population. In this context, *S. dysgalactiae equisimilis* may be more likely to encounter and incorporate virulence factors that could result in changes in its capacity to cause disease in humans.

5.5 Specific Aim 5: Determine if group G or C *Streptococcus* (*S. dysgalactiae equisimilis* and/or *S. anginosus*) cause endemic pharyngitis

The role of GGS and GCS in endemic pharyngitis remains unknown. What is known is that exchange of genetic material between GAS and GGS/GCS is ongoing. Urban settings such as slums may be more likely to have high rates of transmission of *Streptococcus* and high levels of exchange of virulence-coding genetic material between streptococcal species. We hypothesized that these communities may be more likely to harbor GGS species that can cause endemic pharyngitis.

However, we found an unexpected association of *S. dysgalactiae equisimilis* with lower odds of sore throat in pediatric populations in Salvador, Brazil, particularly in males and those living in slums. We have not seen similar findings reported in the literature. These findings may be due to bias introduced with laboratory techniques, and/or bias introduced by differences in health care seeking behavior between slum and non-slum populations. However, these findings may also reflect biologic processes; we hypothesize that the presence of non-pathogenic GGS/GCS in a mucosal site may prevent colonization by more pathogenic species such as GAS or other respiratory pathogens. We further suggest that colonization with *S. dysgalactiae equisimilis* may induce a cross-protective mucosal immune response against subsequent GAS colonization or infection. Slum residents may experience different levels of exposure to these organisms and therefore develop greater immunity. Our data are not sufficient to support or negate any of these hypotheses, or to generalize our findings beyond the scope of our study. Future studies are needed to ascertain the validity of these findings and to investigate if there is an association between SDE and sore throat, and the biologic or cultural role of sex and environment in the association.

B. CONTRIBUTIONS OF THE RHD WORK AND FUTURE DIRECTIONS

5.6 Prevention

That *Streptococcus pyogenes* remains susceptible to penicillin presents an auspicious opportunity to improve RHD prevention strategies. With antibiotic prophylaxis strategies, the burden of end-stage disease RHD can be significantly reduced [3-5]. Penicillin is inexpensive and widely available. Therefore, the treatment itself is not the primary barrier to prevention.

Two stages, known as “primary prophylaxis” and “secondary prophylaxis” have been identified as the most effective periods to administer preventative measures against RHD [6]. Primary prophylaxis of RHD is defined as antibiotic treatment for streptococcal pharyngitis. Secondary prophylaxis consists of administration of antibiotics to patients with a previous attack of ARF or documented RHD. Antibiotics are administered by intramuscular injection of penicillin every three weeks for a duration which ranges from five years to lifelong treatment. Secondary prophylaxis is a difficult treatment regimen to follow for many reasons, including access to health services, absenteeism from work or school for patients seeking treatment, and travel costs to health clinics.

Achieving effective primary and secondary prophylaxis programs is not only about antibiotic delivery. In order for secondary prophylaxis programs to be effective and for people to initiate treatment early in their disease course, it is important to identify people with mild RHD. The new data from Marijon et al. powerfully demonstrate the utility of population-based echocardiographic screening to identify undiagnosed RHD. However, expensive technologies are often neither feasible nor affordable in poor countries with the highest disease burden. Marijon et al. estimated that 2-3% of school-age children in Cambodia and Mozambique had undiagnosed RHD. This is an alarmingly high prevalence. As our data in Chapter 1 demonstrate, and as other data show as well, cases in children to represent a small proportion of all RHD cases in the population, and the burden in the total population is expected to be considerably higher [7].

However, reducing the incidence of RHD situation in Brazil is possible. Improvements in the economy in the past decade and the significant amount of funding that is being spent on surgical procedures provide excellent justification for increased investment and energy into prophylaxis campaigns. A great deal of effort and consideration has already been dedicated to the planning of prophylaxis programs in developing countries. The World Health Organization has been publishing recommendations for more than 20 years [4]. Furthermore, a 2005 meeting of cardiology experts in Africa produced The Drakensberg Declaration on the Control of Rheumatic Fever and Rheumatic Heart Disease in Africa. This document presents the A.S.A.P. Programme, consisting of: increased **A**wareness of RF/RHD among the general public and practitioners; the establishment of **S**urveillance programmes to measure the burden of disease in the population; **A**dvocacy to increase allocation of resources for the treatment of affected children and young adults; and the

implementation of primary and secondary **P**revention schemes in all countries of Africa [8]. Brazil has more resources than many African countries to invest in these strategies.

5.7 Slums revisited

Our studies of *Streptococcus* may speak to the larger issues of infectious disease dynamics in slums. Our preliminary finding that bacterial diversity differed between a slum and a non-slum population is limited to only two communities. However, if this finding is representative of genotype diversity distributions that are also found in other cities with slums, this pattern could also apply to other infectious diseases as well. The slum environment may constitute conditions that favor genetic exchange between infectious organisms. This may have implications for vaccine efficacy in the short and long-term. In the short term, serotypes circulating in slums may not be well represented in vaccines which are formulated to protect against a limited number of serotypes; particularly those vaccines which are created by and for developed countries. In the long term, the presence of high genotype diversity may enhance vaccine type replacement, as has been suggested for the 7-valent pneumococcal conjugate vaccine [9]. The circulation of infectious organisms under crowded conditions facilitates increased opportunities for genetic exchange. Frequent horizontal gene transfer events could contribute to increased dissemination of virulence factors, or the spread of drug resistance genes in bacterial populations. Finally, repeated exposure to infectious agents with different serotypes may induce host immune responses that lead, in some, to immune-mediated disease, such as RHD.

5.8 Future Directions

There is a great deal more to learn about RHD in Salvador, Brazil. The medical chart review raised several questions that merit further investigation. We found that even those undergoing valve surgery caused by degenerative disease are dying at much higher proportions than what we found in studies conducted with patients undergoing valve surgery due to degenerative disease in different populations. We were able to identify post-operative complications and more time in the ICU as increasing the odds of death for valve surgeries due to all etiologies. Perhaps those who die are presenting to surgery with more severe disease. Why are patients in Salvador arriving for surgery with more severe disease? The same questions can be posed for RHD patients; why is paying with SUS associated with higher odds of death? Is it because of severity of disease in the patient base due to delayed diagnosis or quality of hospital care, or both? Ultimately, we will present our data and enter into conversation with the participating surgery teams of our study to discuss opportunities for feasible intervention.

Currently, we are conducting additional analyses in collaboration with a geo-referencing specialist at the Oswaldo Cruz Foundation in Salvador to determine which of the patients in the medical chart review are from slums, and which are not from slums. This information is important to our study and may elucidate some of these questions.

This project generated a large collection of *Streptococcus* isolates which have corresponding epidemiological data. A duplicate of this bacterial collection exists in Brazil. Many bacterial collections contain only isolates from symptomatic patients. However, our collection contains isolates from asymptomatic children as well, which is valuable for pathogenicity studies to investigate why some bacteria cause disease while others do not.

5.9 Final Remarks

As the urban population expands in size and number every year, the issues surrounding the health of these populations will grow as well. Many of these residents will have little to no political or economic power with which to make their voices heard. It is important that we continue to increase acknowledgement and awareness of the health risks of these populations. Furthermore, in the five years of conducting these projects, many students from Brazil and the United States participated in this research and gained epidemiology and microbiology skills in the public health context. We hope that an additional outcome of this study is the training and inspiration of a new generation of public health professionals interested in neglected diseases in neglected populations.

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