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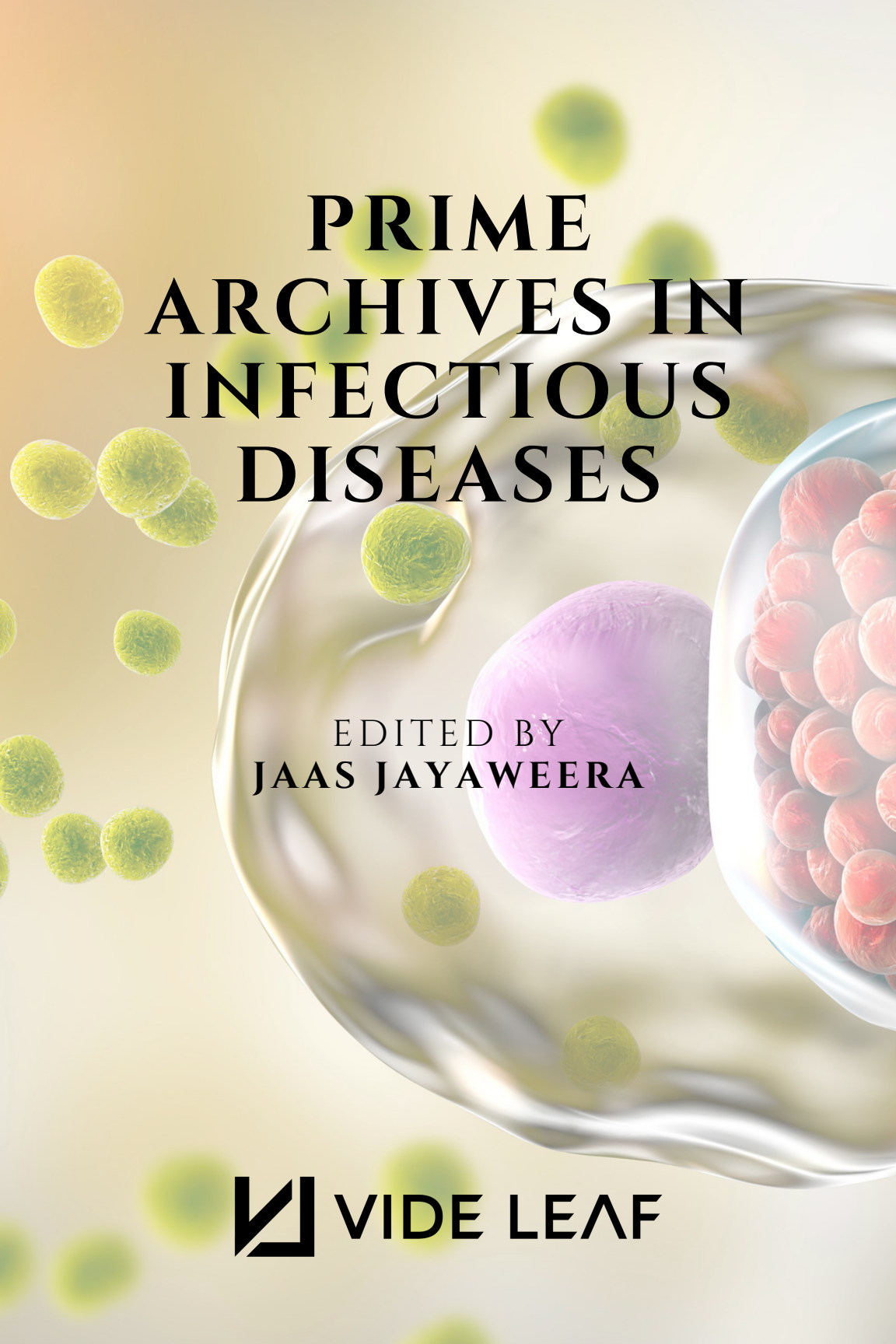

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Book Chapter

Prevalence of High-Risk Genotypes of Human Papillomavirus: Women Diagnosed with Premalignant and Malignant Pap Smear Tests in Southern Ecuador

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Abstract

Human papillomavirus (HPV) is the primary infectious agent for the development of cervical cancer, although the presence of the virus alone is insufficient for viral development and proliferation; this can be attributed to the increase in potential oncogenic risk, along with other risk factors. In the present investigation, the prevalence of high-risk HPV was determined from samples of premalignant or malignant cervical cytology in women from the southern region of Ecuador. The kit we used was able to detect genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59. In addition, 64.5% of the analyzed samples were positive for HPV, with genotypes 16 and 18 being the most prevalent (16 was detected in 148 samples and 18 in 108).

Genotypes 58 and 51 were the third most frequent simple and multiple infections, respectively. The data are very similar to those obtained worldwide, suggesting that the strategy of sex education, and the use of vaccines as primary prevention agents, could significantly decrease the incidence and mortality rate of cervical cancer in the southern region of Ecuador.

Introduction

According to data estimated for 2012, cervical cancer was the fifth most common type worldwide, behind breast, prostate, lung, and colorectal cancer. In the female population, it ranks fourth in incidence and mortality, with about 528,000 new cases and 266,000 deaths per year [1].

Data from the National Institute of Statistics and Censuses of Ecuador (INEC) that year found that cervical cancer was the second cause of death in women, due to oncological disease, with about 697 deaths, after stomach cancer [2].

In the southern region of the country, in the province of Loja, cervical cancer was reported as the most common cancer in women, with a total of 844 new cases diagnosed between 1997 and 2006. Loja province corresponds to 422 new cases, representing 23.1% of all malignant tumors in women. Of these 422 cases, 182 (43.1%) were carcinomas in situ. The standardized rate of the global incidence of invasive cervical cancer in this region was 31.5 × 100,000 inhabitants; in the case of in situ cancer, it was 23.8 × 100,000 inhabitants. Cervical cancer is also the number one cause of female death in the city of Loja, due to oncological disease, with a standardized rate of 9.6 × 100,000 inhabitants, identifying this disease as a major health problem in the southern region of Ecuador [3].

HPV is considered the main risk factor for the development of cervical cancer [4–7]. It is estimated that more than 50–75% of sexually active women are infected with one or more HPV genotypes throughout their lives [8, 9]. Once the infection occurs, the immune system achieves spontaneous regression [10,11] in 80–90% of cases after 18–24 months [8,12–14]. In

this way, it is demonstrated that HPV infection is a necessary but insufficient reason for development of cancer [15,16].

More than 150 HPV genotypes have been described. Approximately 40 of them have a special tropism via the anogenital and mucosal region [17–20]. They are classified into high oncogenic risk groups (16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, and 68), possible oncogenic risk (26, 30, 34, 53, 66, 67, 69, 70, 73, 82, 85, and 97), and low-risk group that cause benign lesions like cervical warts or condylomas (6, 11, 28, 32, 44, 43, 44, 54, 55, 57, 61, 62, 71, 72, 74, 81, 83, 84, 86, 87, and 89) [6, 20–24]. Among high-risk genotypes, the most common worldwide are 16 and 18, accounting for approximately 70% of cases of cervical cancer, including high-grade squamous intraepithelial lesions [16, 25–28]. Several factors (related to a high probability of developing HPV infection) are age at first intercourse and having several sexual partners [28–31].

The main objective of the present study is to determine the prevalence of high-risk HPV in samples of premalignant or malignant cervical cytology in women from the southern region of Ecuador. The kit was able to detect the presence of genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59. Papanicolaou test samples were collected according to the Bethesda Classification Diagnosis of any cellular epithelial abnormality. In addition, the possible association of different risk factors with HPV infection was evaluated. Given the high incidence and mortality rate of cervical cancer, and the absence of respective epidemiological studies in this area, the study may contribute to a reduction in its number of deaths.

Population and Methods

Population

This observational, cross-sectional, and prospective study was carried out from 2012 to 2013, having been approved by the research committees of the Universidad Técnica Particular de Loja (UTPL) and the Cancer Society of Ecuador (SOLCA) Store core. This study was supported by Universidad Técnica Particular de Loja: Projects PY1019 and PY1620.

A total of 431 women between 17 and 84 years of age used the gynecological services of the SOLCA Hospital, the UTPL Hospital, or the Ecuadorian Family Well-Being Association (APROFE) in the city of Loja, Ecuador, with the intention of learning their Pap test results.

Inclusion Criteria(i)Women domiciled in the Province of Loja, Zamora, or El Oro(ii)Cytological diagnosis of the premalignant or malignant pap smear (ASC-US, ASC-H, LSIL, HSIL, squamous cell carcinoma, AGC, or adenocarcinomas), which had been previously untreated(iii)Performing the diagnostic colposcopy(iv)Acceptance of participation in the study through signing an informed consent

Exclusion Criteria(i)Women who have given birth or had a Pap smear in the last 3 weeks(ii)Women who underwent colposcopy in the last 6 weeks(iii)Women who have undergone an invasive procedure in the uterine cervix (conization, biopsy, etc.) in the last 3 months

Methods

The sociodemographic data (age, place of residence, and marital status) and clinical data (age at first intercourse, number of partners, and history of sexually transmitted infections) of the volunteers were collected by survey.

Patients in the study underwent a cervical scraping, with a sterile cytobrush at the start of the diagnostic-therapeutic colposcopy. Samples were stored in 5 ml of PBS buffer (pH 7.5) between 6 and 8°C for a maximum period of 3 days until processing.

DNA extraction was performed with a commercial kit (Pure Link DNA, Invitrogen Co., Grand Isle, NY, USA). The quantity and quality of the DNA were measured by spectrophotometry, using the Nanodrop 200c, Thermo Scientific equipment. It was carried out by examining its optical density of 260/280-260/230 nm. Between 10 and 100 ng/μL of total DNA extracted for amplification was used.

HPV detection was done by real-time PCR, using the AmpliSens HPV genotype FTR kit (Bretonneux, FR) following the manufacturer's instructions. The kit recognizes 12 high-risk HPV genotypes (16, 18, 31, 33, 35, 56, 39, 45, 51, 52, 58, and 59). Positive and negative controls were used for each reaction. Amplification of the β-globin gene was a quality control measure of the genetic material.

The data obtained via amplification were analyzed by the Applied Biosystems Fast 7500 Software Detection System (Thermo Fisher Scientific, Waltham, MA, USA). We used a system of presence-absence detection of the genetic material for the virus with 4 fluorophores Cy5 (for β-globin), FAM (for genotypes 16, 39, 33, and 58), JOE (for genotypes 31, 45, 35, and 52), and ROX (for genotypes 18, 59, 56, and 51). The cycle threshold (CT) was 35, according to the manufacturer's instructions. Samples that exceeded this value were considered positive, by independently observing the behavior of the amplification curve of each genotype.

Statistical Analysis

The results were analyzed with descriptive statistics, identifying mean values, standard deviation, and/or percentages as appropriate. To assess the association of different risk factors and the presence of HPV infection, the OR (95% CI) was calculated, considering significant values as

Calculations were made with these programs: IBM SPSS Statistics (IBM, Armonk, NY, USA) and MedCalc® statistical software (Ostend, BE).

Results

Characteristics of the 431 participants in the study are shown in Table 1. Furthermore, 64.5% (278) of the samples tested positive for HPV. Genotypes 16 and 18 were the most prevalent, with 16 being detected in 148 samples (53.2% of positive samples) and 18 detected in 108 (38.8%). Another 110 samples (39.6%) involved a single viral type, while 168 samples (60.4%) had

multiple infections in samples with up to 7 different genotypes. Table 2 shows the frequency of each type of HPV infection, considering single and multiple displays.

Table 1: Characteristics of participants.

Characteristics	Values
Age	
Range	17–84
Average (SD)	40.7 (13.5)
Age of onset of sexual life	
Range	10–45
Average (SD)	18.9 (4.3)
Does not answer (%)	29 (6.7)
Number of sexual partners	
Range	1–10
Average (SD)	1.6 (1.0)
Does not answer (%)	2 (0.5)
Age at first pregnancy	
Range	11–39
Average (SD)	20.8 (4.6)
Does not answer (%)	218 (50.6)
Number of pregnancies	
0 (%)	46 (10.7)
<3 (%)	159 (36.9)
≥3 (%)	224 (52.0)
Does not answer (%)	2 (0.5)
Condom use	
No (%)	335 (77.7)
Yes (%)	49 (11.4)
Does not answer (%)	47 (10.9)
Civil status	
Married (%)	285 (66.1)
Divorced (%)	21 (4.9)
Single (%)	71 (16.5)
Does not answer (%)	54 (12.5)
Level of education	
Primary (%)	68 (15.8)

High school (%)	73 (16.9)
College (%)	87 (20.2)
Does not answer (%)	203 (47.1)
Learn about HPV	
No (%)	331 (76.8)
Yes (%)	71 (16.5)
Does not answer (%)	29 (6.7)
Abnormal cytology	
AGUS (%)	60 (13.9)
ASCUS (%)	94 (21.8)
ASC-H (%)	21 (4.9)
LSIL (%)	158 (36.7)
HSIL (%)	83 (19.3)
Cancer (%)	15 (3.5)

The percentage is calculated relative to the total positive samples of simple infections (110) and multiple infections (168), respectively.

Table 2: Frequency of HPV genotypes in positive cases.

Genotype	☒Simple infection		☒Multiple infection	
	n	%[a]	*N*	%[a]
HPV 16	52	47.3	96	57.1
HPV 18	17	15.5	91	54.2
HPV 31	3	2.7	41	24.4
HPV 33	1	0.9	11	6.6
HPV 35	0	0.0	11	6.6
HPV 39	2	1.8	23	13.7
HPV 45	0	0.0	7	4.2
HPV 51	9	8.2	72	42.9
HPV 52	1	0.9	27	16.1
HPV 56	5	4.6	27	16.1
HPV 58	11	10.0	63	37.5
HPV 59	9	8.2	42	25.0

Considering the cytological diagnosis and presence of HPV, 66.7%, 61.7%, 52.4%, 63.3%, 67.5%, and 86.7% of the samples with AGUS, ASCUS, ASC-H, LIE-AG, and cancer, respectively, had high-risk HPV. The first three HPV genotypes most frequent in each cytologic diagnosis are shown in Table 3.

Table 3: Frequency of HPV genotypes in all cytological diagnoses.

Cytology (HPV cases)	Genotype	*n*[a]	%[b]
AGUS (40)	HPV 16	23	57.5
	HPV 18	20	50.0
	HPV 51	11	27.5
	Other high-risk HPV[c]	39	97.5
ASCUS (58) 61.70%	HPV 16	29	50.0
	HPV 18	23	39.7
	HPV 58	15	25.9
	Other high-risk HPV[d]	56	96.6
ASC-H (11) 52.38%	HPV 16	4	36.4
	HPV 18	4	36.4
	HPV 51	4	36.4
	Other high-risk HPV[c]	11	100.0
LSIL (100) 63.29%	HPV 16	49	49.0
	HPV 18	35	35.0
	HPV 51	34	34.0
	Other high-risk HPV[c]	122	122.0
HSIL (56) 67.47%	HPV 16	35	62.5
	HPV 18	19	33.9
	HPV 51	15	26.8
	Other high-risk HPV[c]	43	76.8
Cancer (13) 86.67%	HPV 16	8	61.5
	HPV 18	7	53.9
	HPV 51	3	23.1
	HPV 56	3	23.1
	Other high-risk HPV[e]	10	76.9

[a]Simple and multiple infections together. [b]Percentage in total positive samples of each cytological alteration. [c]HPV 31, 33, 35, 56, 39, 59, 52, 58. [d]HPV 31, 33, 35, 56, 39, 45, 59, 51, 52. [e]HPV 33, 35, 39, 45, 59, 52, 58.

The association analysis between HPV infection and the risk factors studied, as well as the OR values, is shown in Table 4.

Table 4: Risk factors and HPV infection.

Variables	Positives	Total	OR (IC 95%)
Age of onset of sexual life			
≥21	65	97	
<21	195	305	0.87 (0.5–1.4)
Number of pregnancies			
<3	136	205	
≥3	141	224	0.86 (0.6–1.3)
Number of sexual partners			
1	171	262	
>1	105	167	0.90 (0.6–1.4)
Condom use			
Yes	35	49	
No	218	335	0.75 (0.4–1.4)
Civil status			
Married	185	285	
Single-divorced	56	92	0.84 (0.5–1.4)
Level of education			
High School-college	110	160	
Primary	55	68	1.92 (0.9–3.8)
Learn about HPV			
Yes	51	71	
No	211	331	0.69 (0.4–1.2)

[a]Primary education level included 7 years of study, and secondary level of education included over 7 years of study.

Discussion

In this first study of its kind for HPV genotypes, we determined the frequency of premalignant and malignant samples of cervical cytology, along with their association with cytological diagnosis and risk factors of infection. The most frequent cytopathology was LSIL at 36.7%, while cancer cases accounted for 3.5% of total samples. The prevalence of high-risk HPV in cytological

samples was 64.5%. In Quito, Ecuador, they reported other studies in cytological and/or histologically altered, in which the reported prevalence of HPV was found to be low risk at 67.7% and 86% [32,33]. In the city of Cuenca, in a study of 500 women from the general population, the prevalence of 25.6% of HPV for both high and low risk [34] was similar to that reported in Santa Elena. Brown et al. (2009) found [35] a prevalence of 24.2%. A higher prevalence of HPV with abnormal cytology studies indicates a relationship between the virus and malignant cells.

In the present study, genotypes 16 and 18 were the most frequent, for both simple infections (47.3% and 15.5%, resp.) and multiple infections (57.1% and 54.2%, resp.). Other oncogenic types showed significant incidence. Genotype 58 was presented as the third most common in simple infections (10%), while 51 ranked third in multiple infections (42.9%). These results agree with the meta-analysis of Bruni et al. (2010) [36], which reports genotypes 16, 18, 52, 31, and 58 as the most frequent. In the systematic review and meta-analysis of Ciapponi et al. (2011) [37], the prevalence of HPV in high-grade lesions and cancer of the uterine cervix in Latin America and the Caribbean (analyzing nearly 8,000 samples) was most frequently found in genotypes 16 and 18, followed in descending order by HPV 31, 58, 33, 45, and 52, similar to those obtained in our own study data. Mexico reported the 58 genotype as the second most common after HPV 16 in cervical cancer and abnormal cytology samples [38, 39] and, in Quito, in altered cervical biopsy samples [32].

Studies in Ecuador found genotype 16 as the most common [32,33,35,40], along with results of this investigation. The 18 genotype was not reported as the second most frequent, suggesting that genotypic variability by geographic region and sociodemographic factors had an effect, such that more research is necessary.

Considering the cytological diagnosis, the three most frequent HPV genotypes were 16, 18, and 51 in AGUS, ASC-H, LSIL, HSIL, while in cancer and ASCUS the type 58 was the third most frequent, behind genotypes 16 and 18. Some authors

suggest that the presence of these genotypes (high-grade lesions) would be more likely to progress to cervical cancer than other HPV types; this is due to the oncogenic potential of these genotypes [41].

There are several risk factors that can influence HPV infection. Some researchers have reported that early onset of sexual life and a higher number of sexual partners significantly increased risk of infection [42,43]. Some studies indicated that single or divorced women were more likely to have cervical cancer; however, according to reports in Latin America, men tend to have more than one sexual partner, limiting the use of marital status as a risk factor for contracting this virus [44]. To reduce risk of infection, condom use is recommended for sex, and, in some studies, it could reduce the risk of HPV infection by up to 70% [45,46]. However, there was no significant association in our study for increased risk of infection, given the factors analyzed; this could be due to a possible bias in the information provided by the participants, due to sociocultural factors; in addition, these questions are based on memory and a large number of people did not answer the questions, affecting significantly the results obtained.

In conclusion, in this study, genotypes 16 and 18 were identified as the main types of HPV in the samples analyzed for premalignant and malignant cytology. Genotypes 58 and 51 were presented as the third most frequent type, considering simple and multiple infections, respectively. The data are very similar to those obtained worldwide, again suggesting that the common strategies of sex education and vaccine use as primary preventions could significantly decrease the incidence and mortality rate of cervical cancer in the southern region of Ecuador. Particularly it is considered as potentially beneficial to introduce the integral sexual education in the primary school for 10-11-year-old children because of early beginning of sexual intercourse in the Ecuadorian population.

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Book Chapter

Association of Iron Deficiency Anemia with Recurrent Respiratory Tract Infections and Gastroenteritis in Children

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Abstract

Anemia affects approximately 30% of children all over the world. Acute respiratory tract infections (ARTI), urinary tract infections (UTI) and gastroenteritis (GE) are common infectious entities in children. Here, we assessed the association between anemia and development of recurrent ARTI, UTI, and GE in children. This was a case-control study in hospitalized 2–5 years old children in Professorial Pediatric Unit at Teaching Hospital Anuradhapura, Sri Lanka. An 18-month follow up was done to assess the risk factors for the development of recurrent ARTI, GE, UTI, and control presented without infections. Further, 6-month follow up done after 3-month iron supplementation to assess the occurrence of recurrences. Blood Hb concentration was measured using Drabking's reagent. Logistic regression was used to find the risk factors for the development of recurrences. In ARTI, 121/165 (73.3%), GE, 88/124 (71%), UTI 46/96 (47.9%) and control 40/100 (40%) were having anemia. Initial ARTI group, recurrent ARTI was 24 (14.5%, $p = 0.03$); initial GE group: recurrent GE was 14 (11.3%, $p = 0.03$), recurrent ARTI was 11 (8.9%, $p = 0.04$); initial UTI group, development of; recurrent UTI was 8 (8.3%, $p = 0.04$); control, recurrent ARTI was 11 (11%, $p = 0.03$). Following 3-month iron supplementation reduction of recurrences was significant: initial ARTI recurrent ARTI in 90%, recurrent GE in 77.7%; initial GE

recurrent GE in 83.3%, recurrent ARTI in 80%; initial UTI recurrent ARTI in 71.4% and control recurrent ARTI in 88.8%. Iron deficiency is a major type of anemia and anemic children are more prone to develop recurrent ARTI and GE. Once iron deficiency being corrected the rate of recurrent ARTI and GE was reduced. This would be a boost for policy developers to implement strategies at the community level to prevent iron deficiency in children to reduce ARTI and GE recurrences.

Introduction [MH]

Acute infective episodes in children are quite common and are associated with high morbidity and mortality [1]. Acute respiratory tract infections (ARTI), urinary tract infections (UTI) and gastroenteritis (GE) are such common infectious entities [2-5]. In the globe, infections following bacteria and viruses play a significant role while parasites and fungi are emerging and threatening [6,7].

The disease burden following childhood ARTI is greater than that of any other cause of disease[1]. In 2014, 18% of mortality for children younger than 5 years of age was caused by ARTI while the diarrheal disease is the next greatest [8,9]. UTI in children <5 years of age the associated burden is 4%. Further the overall conclusion one out of 20 girls and one out of 50 boys will have a UTI by the age of 5 years, with a predominance of boys during the neonatal period and early infancy [10]. Overall burden following these 3 major acute childhood infections is substantial thus impact on the globe and the country economy is enormous [11].

Following entry of microorganism to the organ vicinity several factors concurrently contribute for the development of the infection. When considering host-parasite interface nutritional status of the host is one of the key contributory factors for invasion and development of infections [12-14]. Hemoglobin (Hb) concentration is a parameter that reflects the chronic nutritional status and also blood oxygen carrying capacity [15,16]. Children are at a rapid growth state thus demand the nutrition is enormous [17]. Simultaneously, the tendency to

develop under-nutrition is also high. In such instances, the risk for development of infection is high and the vicious cycle continues leads to poor-nutrition [18].

Anemia is affecting approximately 30% of children all over the world [19,20]. Several factors are contributed to anemia. In childhood, nutritional anemia including iron, vitamin B-12, and folate deficiency is the commonest [21]. In addition to the nutritional, hereditary type of anemia including thalassemia, sickle cell anemia and aplastic variety following bone marrow suppression is observed [22]. Irrespective of the etiology following anemia, child suffers fatigability and its negative effect on growth is great. Iron deficiency anemia in children occurs most frequently between the age of 6 months and 3 years, the period of age when repeated infections occur [23]. Anemia associated lower ARTI occurs more commonly in children than in adults.

Recurrent ARTI and UTI are common in children and following development of recurrences the associated burden would worsen [24,25]. Recurrent infective episodes invariably lead to undernutrition [26]. Perhaps, anemia is a well-known risk factor for recurrent infective episodes [27]. In addition, studies on hemoglobin level and development of multiple episodes of otitis media in children been discussed [28]. In a cross-sectional case-control study anemia and occurrence of acute gastroenteritis also been discussed among children in Gaza strip [29]. In contrast, anemia and development of recurrent UTI are not been well described in the literature. Another hand if a child develops recurrent infections possibility of immunodeficiency need to be excluded [30].

Irrespective to the etiology of anemia, the relation between low hemoglobin (Hb) level and occurrence of infections has not been fully evaluated, and only a few reports are available [31]. This study would assess the Hb status and development of acute as well as recurrent ARTI, UTI, and GE in children.

Method

This was a case-control study in hospitalized 2–5 years old children with ARTI, UTI, and GE over March 2014 to August 2014. To participate in the study informed written consent obtained from the legal guardians/parents. As a control, children presented to the outpatient department to seek treatment for traumatic surgical cases who were having past 6-month period free of any acute or chronic infections were included. Children having pulmonary, cardiac, gastrointestinal and urogenital structural and functional anomalies were excluded from the study. Patients who undergo repeated blood transfusions were excluded from the study. Further, children with known immunodeficiencies were also excluded from the study. The study was performed at the pediatric ward, Professorial Unit, Teaching Hospital Anuradhapura, Sri Lanka. Participants including controls were followed up 18 months to assess the occurrence of recurrent ARTI, GE and UTI and the risk factors. This was done following weekly telephone conversations with the guardians. In each episode they were asked to admit to the Professorial Unit, Teaching Hospital Anuradhapura, Sri Lanka. Further, a period of 6 months was followed up following iron supplementation (3-month period) to assess the development of recurrent infections. They were followed up in similar manner. All methods and protocols were performed in accordance with the relevant approved guidelines and regulations.

ARTI cases with Severe Acute Respiratory Illness (SARI) defined by WHO were included. A child has a fever with dysuria (crying while micturition) and or hematuria was included as UTI. Sudden onset of diarrhea and/or vomiting, usually three or more bouts of diarrhea or vomiting were taken as GE. A hundred patients in age 2–5 years who visited the outpatient department for surgical problems were taken as a control. Definition of recurrent ARTI is arbitrary, too generic, restrictive and for our study: recurrent infectious rhinitis as more than five episodes per year and recurrent pharyngitis or tonsillitis more than three episodes within 12 months. For the lower respiratory tract, we have taken ≥3 episodes per 12 months. Similarly, for recurrent GE we have taken ≥3 episodes per 12 months while for the

clarity cases with chronic diarrhea were excluded. Recurrent urinary tract infection (UTI) refers to ≥2 infections in six months or ≥3 infections in one year. For anemia when hemoglobin level was considered age-specific 13-≤24 months [mean 12.0 g/dL (−2SD: 11.0 g/dL)] and 25-≤60 months [mean 12.5 g/dL (−2SD: 11.5 g/dL)] *Brian Yang Merritt's Haemoglobin concentration (http://emedicine.medscape.com/article/2085614-overview) below 2 standard deviation (SD) [32].

Children with ARTI nasopharyngeal aspirates (NPAs) collected with the help of recommended mucus extractor by the pediatrician/prior trained medical doctor (research candidate). Indirect immunofluorescence assay was performed by DAKO IMAGEN™ (United Kingdom) [33], respiratory screening reagents for 8 respiratory viruses and viral typing was done for each of RSV, adeno, parainfluenza 1, 2 & 3, influenza A & B and Human metapneumovirus (hMPV) viruses using monoclonal antibodies DAKO IMAGEN™ (United Kingdom). Children with UTI were having significant (single isolate or mixed > 10^5) and culture growth from clean caught urine was taken as having bacterial UTI. In GE stool full report was having a significant number of pus cells considered. The collected human fecal samples were tested in duplicate for Group A rotavirus and Adenovirus using a commercially available qualitative enzyme immunoassay (ProSpect™ Rotavirus Microplate Assay manufactured by Oxoid Ltd, UK and ProSpect™ Adenovirus Microplate Assay manufactured by Oxoid Ltd, UK, respectively), following the manufacturer's instructions.

Each stool was inoculated into selenite brilliant green sulfa enrichment broth (Oxoid Ltd, Basingstoke, UK) at 37 °C for 18 h, and was then plated onto Salmonella-Shigella agar (Oxoid Ltd, Basingstoke, UK) and xylose lysine deoxycholate agar (Oxoid Ltd, Basingstoke, UK) to detect non typhoidal salmonellosis and *Shigella* spp. strains, after an 18 h incubation at 37 °C. The suspicious colony was plated onto CHROMagar™ Salmonella medium (CHROMagar, Paris, France) and cultivated at 37 °C for 18 h. Each stool sample was directly inoculated onto alkaline peptone water (Oxoid Ltd, Basingstoke, UK) at 37 °C for 18 h to examine for *Vibrio cholera*, *Vibrio parahaemolyticus*,

Aeromonas spp., and *Plesiomonas* spp., and was then plated onto thiosulfate-citrate-bile salts-sucrose agar (Oxoid Ltd, Basingstoke, UK) at 37 °C for 18 h. Suspicious colonies were selected to conduct the oxidase experiment. If the oxidase test resulted in a positive reading, the systematic biochemical identification for these suspicious colonies was confirmed.

Stool iodine staining, wet smear, and microscopy were performed to assess amoebic cysts, oocytes, and other helminth oocytes and larvae. In addition to that fecal reducing substances, the level was taken to exclude lactose intolerance and malabsorption syndromes. Blood Hb concentration from all participants was measured using Drabking's reagent using a spectrophotometer. Blood picture analysis and serum ferritin levels were measured to define the etiology for anemia.

An investigator administered questionnaire was used to collect patients' demography, nutritional status, clinical presentation, and past medical history. For iron deficiency anemia following a period of 3-month of oral iron supplementation (weight/based) the subjects were further followed up over 6-month to observe the development of recurrent ARTI, GE and UTI. Children with hemoglobin 9–10 g/dL were supplemented with 60–120 mg of iron. Hemoglobin concentration, blood picture (normochromic and normocytic) and assessment of serum ferritin level was done to confirm the cure of iron deficiency anemia.

Data obtained were double entered into a spreadsheet database prepared with Microsoft® Excel and compared and cleaned for wrong entries. Statistical analysis was done using SAS version 9.1 (SAS, 2005, New Jersey) [34]. Association of each of the categorical variable with response variable was assessed by Chi-square test. Variables showing statistically significant association in univariate analysis with the outcome variable were considered as a risk factor. Only those variables were subjected to multivariate analysis. Logistic regression method was used to find the risk factor for the development of recurrent ARTI, UTI, and GE. In multivariate analysis, variables showing $P < 0.05$ were considered to be statistically significant. Continuous variables were expressed as a measure of central tendency.

Ethics Approval and Consent to Participate

Ethical approval for all experimental protocol/s were approved by ethical review and publication committee, University of Peradeniya, Sri Lanka and to participate in the study informed written consent obtained from the legal guardians/parents.

Results

Over the period of the initial 18 months, children with clinically suspected 165 cases of ARTI, 124 cases of GE, 96 cases of UTI and 100 control were enrolled in the study. Children with suspected ARTI, 65 (39.3%) cases of viral ARTI was detected based on IFA, mean age of presentation was 2.4 ± 0.5 years and mean hospital stay was 4 ± 2 days while 58% were males and 42% females. Children with GE, 62 (50%) viral, 12 (10%) bacterial cases detected based on viral ELISA and stool bacterial cultures respectively. The mean age of presentation of GE was 2.5 ± 0.8 years and mean hospital stay was 5 ± 2.5 days while 57% were males and 43% females. Children with UTI, 52 (54.1%) had culture-positive UTI mean age of presentation was 2.45 ± 0.7 years and mean hospital stay was 3 ± 2.4 days while 55% were males and 45% females. In control, 52% were males and 48% were females. In all including the control, male predominance ($p = 0.03$) with no significant difference between mean age and mean hospital stay was detected.

Out of 165 children with ARTI, 121 were anemics (73.3%). Eighty-eight children out of 124 with GE and (71%) and 46 out of 96 children with UTI were having anemia (47.9%). In control subjects, 40 of them were having anemia (40%). When compared the diseased groups and the control, children with ARTI and GE were having anemia significantly ($p = 0.03$ and 0.04 respectively) whiles children with UTI, anemia was not significant ($P > 0.05$) (Table 1).

Table 1: Details of recurrent infections before, after 3 month of iron supplementation and 6 months follow up.

Initial group (n)	**ARTI (165)**		**GE (124)**		**UTI (96)**	**Control (100)**	**P value and comments**
Percentage of Iron deficiency anemia (%)	**121 (73.3%)**		**86 (71.0%)**		**44 (47.9%)**	**40 (40%)**	**0.03**
Recurrent infections among anemics (%)	**ARTI 20 (16.5%)**	**GE 9 (7.4%)**	**GE 12 (14%)**	**ARTI 10 (11.6%)**	**ARTI 7 (16%)**	**ARTI 9 (22.5%)**	—
Recurrent infections among anemics (%)	**ARTI 4 (2.4%)**	**GE 2 (1.6%)**	**GE 2 (1.6%)**	**ARTI 1 (0.8%)**	**ARTI 1 (1%)**	**ARTI 2 (2%)**	—
Initial Hb (g/dl)	9.6 ± 0.8		9.5 ± 0.7		9.7 ± 0.8	9.6 ± 0.9	—
Initial serum ferritin (ng/ml)	4.5 ± 1.2		4.5 ± 1.1		4.5 ± 1.2	5.5 ± 0.3	—
3-month Iron supplementation							
Initial group	**ARTI**		**GE**		**UTI**		**Control**
At 3-month Hb (g/dl)	11.3 ± 0.2*		11.6 ± 0.4*		11.4 ± 0.3*	11.9 ± 0.3*	0.03*
serum ferritin (ng/ml)	12.6 ± 0.8**		12.6 ± 0.4**		12.6 ± 0.8**	12.2 ± 0.7**	0.02**
Follow up- 6 months							
Recurrent infection	**ARTI 2 (1.6%)**	**GE 2 (1.6%)**	**GE 2 (2.3%)**	**ARTI 2 (2.3%)**	**ARTI 2 (4.5%)**	**ARTI 1 (2.5%)**	—
At 6-month Hb (g/dl)	11.6 ± 0.4		12.1 ± 0.6		12.4 ± 0.3	11.9 ± 0.2	—
Reduction of recurrences over 6 month (%)	**ARTI** 90	**GE** 77.7	**GE** 83.3	**ARTI** 80	**ARTI** 71.4	**ARTI** 88.8	0.03

ARTI- acute respiratory tract infections, GE- gastro-enteritis, UTI- Urinary tract infections. $P < 0.05$ taken as significant. -: not significant.

When considering anemia, in initial ARTI group 121 (73.3%) of them were found to have iron deficiency anemia, 2 of them with megaloblastic anemia and 1 with asymptomatic sickle SD disease. Whiles in initial GE group 86 (71.0%) were found to have iron deficiency anemia, 2 of them with megaloblastic anemia. In UTI group 44 (47.9%) were found to have iron deficiency anemia and 2 of them were thalassemia trait. Further in control 38 (95%) were found to have iron deficiency anemia and 2 of them were thalassemia traits. In the control group, 35 (35%) found to have iron deficiency anemia. We have excluded patients having repeated blood transfusions. Therefore, patients having thalassemia major and other hemoglobinopathies demanding blood transfusions were not considered for the analysis. Based on blood picture analysis, iron deficiency anemia was further confirmed by serum ferritin assay. In initial ARTI patients, serum ferritin level in patients with anemia was 4.5 ± 0.3 ng/ml. In initial GE patients, it was 4.5 ± 0.4 ng/ml, in initial UTI patients it was 4.5 ± 0.35 ng/ml and in control with anemia it was 4.5 ± 0.3 ng/ml. It was significantly ($p = 0.03$) below the age-specific lower limit of the reference range (<6 ng/ml in both male and female <5 ng/ml years of age). In between anemic children in ARTI, GE, UTI groups and control the serum ferritin values were not significantly differed ($p > 0.05$). Further, no peripheral stigmata of iron deficiency anemia were observed. All of the subjects were on anti-helminth treatment once in six months. Further patients with GE, non-of them were having amoebic oocytes, cysts and any oocytes related to soil and non-soil inhabiting helminths in wet stool mounts. Also, on iodine staining, all tested diarrheal stool was negative for parasite cyst or ova.

Anemia was a risk factor for the development of ARTI with an odds ratio of 3.08 with 95% interval confidence of 2.03–4.80 ($P = 0.004$) Further anemia was a risk factor for the development of GE with an odds Ratio of 2.98 with 95% interval confidence of 1.93–4.40 ($P = 0.01$). In UTI group anemia was not either risk or a protective factor as the odds ratio of 1.03 ($p = 0.09$) with 95% interval confidence of 0.78–1.40.

Over the 18 months follow up period development of recurrent ARTI, GE and UTI in study groups and the control as follows. Among initial ARTI group, development of recurrent ARTI was 24 (14.5%, $p = 0.03$); recurrent GE was 11 (6.5%, $p = 0.06$) and recurrent UTI was 0. Among initial GE group, development of; recurrent GE was 14 (11.3%, $p = 0.03$), recurrent ARTI was 11 (8.9%, $p = 0.04$) and recurrent UTI was 4 (3.2%, $p = 0.07$). Among initial UTI group, development of; recurrent UTI was 8 (8.3%, $p = 0.04$), recurrent ARTI was 4 (4.1%, $p = 0.06$) and recurrent GE was 0. Among control, development of; recurrent ARTI was 11 (11%, $p = 0.03$), recurrent GE was 6 (6%, $p = 0.06$) and recurrent UTI was 2 (2%, $p = 0.08$). For the risk factor analysis, only significant ($p < 0.05$) recurrent infections among study groups was included (Table 2).

Table 2: Factors associated with development of recurrent acute respiratory tract infection (ARTI), gastroenteritis (GE) and urinary tract infection (UTI) among followed up ARTI, GE, UTI and control groups.

Initial disease	**ARTI**	**GE**		**UTI**	**Control**	**P value and comments**
Significant recurrent infection in above disease groups	**ARTI OR (95% CI)**	**GE OR (95% CI)**	**ARTI OR (95% CI)**	**UTI OR (95% CI)**	**ARTI OR (95% CI)**	
Risk factors						
Sex						
Male	1.6 (1. 5–1.8)	—	—		—	0.03
Female		—	—	1.6 (1.4–1.8)	—	0.04
Height for age (<−2 SD)	2.6 (2.1–3.1®)	1.6 (1.4–1.8)*	1.6 (1.4–1.8)$^{\mu}$	—	1.6 (1.4–1.8)	0.03®, 0.04*, 0.04$^{\mu}$, 0.04
Hb (<11 g/dL)	5.2 (4.5–5.9)®	3.6 (3.1–4.1)*	4.5 (4.0–4.9)$^{\mu}$	—	4.2 (3.4–4.9)	0.03®, 0.04*, 0.04$^{\mu}$, 0.04
Constipation	—	—	—	2.6 (2.2–3.0)	—	0.04
Water intake <1l	—	—	—	1.7 (1.4–2.0)	—	0.04
Hand washing prior to handling of child	—	1.5 (1.3–1.7)$^{\mu}$	1.8 (1.3–2.2)	—	—	0.03$^{\mu}$, 0.04
National program of immunization coverage	100%	100%	100%	100%	100%	—
Anti-helminth use-every 6 months	86%	88%	84%	90%	88%	—

ARTI- acute respiratory tract infections, GE- gastro-enteritis, UTI- Urinary tract infections, LSCS- lower segmental caesarian section, OR- odds ratio, SD- standard deviation. Only significant factors were included. $P < 0.05$ taken as significant. -: not significant.

Among initial ARTI group for the development of recurrent ARTI; male sex, height for age <−2 SD and Hb% <11 g/dL were significant risk factors whiles in initial GE group for the development of recurrent GE; height for age <−2 SD, Hb% <11 g/dL and not washing hands prior to handling of child were significant risk factors. Also, in initial GE group for the occurrence of recurrent ARTI; height for age <−2 SD, Hb% <11 g/dL and not washing hands prior to handling of the child were significant risk factors. Further, among initial UTI group for the occurrence of recurrent UTI; female sex, constipation, low water intake, and structural malformations were significant risk factors. In addition, among control group for the occurrence of recurrent ARTI; height for age <−2 SD and Hb% <11 g/dL were significant risk factors.

None of these factors were not associated significantly ($p > 0.05$) with recurrences in test subjects and the control. Birth weight (<2500 g), maturity (<36 weeks period of amenorrhea), mode of delivery (normal vaginal or caesarian section), weight for age (<−2 SD), type of drinking water source (tap, tank or spring), use of boiled cooled water, having daily bath and frequent (>2/day) body wash, exclusive breast feeding in first 4 months, family monthly income <30,000 rupees, mothers'/caregivers' education (only up to primary level), proper waste disposal, birth order (3^{rd} or more in order) and day care attendee, having congenital anomalies including cyanotic heart diseases, cystic fibrosis, structural anomalies in gastro-intestinal tract familial syndromes, having gastro-esophageal reflux and bronchial asthma.

Following a period of 3-month of iron supplementation (60–120 mg daily), the subjects were further followed up period of 6-month to observe the development of recurrent ARTI and GE. Meanwhile, advice on hand hygiene was also given. Recurrent infection among anemics as follow: initial ARTI subjects recurrent ARTI was detected in 20 (16.5%), recurrent GE was detected in 9 (7.4%); initial GE subjects recurrent GE was detected in 12 (10%), recurrent ARTI was detected in 10 (12%); initial UTI subjects development of recurrent ARTI was detected in 7 (16%) and control subjects development of recurrent ARTI was detected in 9 (25%). Following 3-month oral iron

supplementation hemoglobin concentration was increased significantly and (mean ± SD) mg/Dl as follows: initial ARTI- 11.3 ± 0.2 (p = 0.03), GE- 11.6 ± 0.4 (P = 0.03), UTI- 11.4 ± 0.3 (P = 0.03) and controls- 11.9 ± 0.3 (p = 0.03). serum ferritin was increased significantly and (mean ± SD) ng/ml as follows: initial ARTI- 12.6 ± 0.8 (p = 0.02), GE- 12.6 ± 0.4 (P = 0.02), UTI- 12.6 ± 0.8 (P = 0.02) and controls- 12.2 ± 0.7 (p = 0.02). Recurrent infection rates were reduced significantly.

Initial ARTI subjects recurrent ARTI was detected in 2 (1.6%), recurrent GE was detected in 2 (1.6%); initial GE subjects recurrent GE was detected in 2 (10%), recurrent ARTI was detected in 2 (12%); initial UTI subjects recurrent ARTI was detected in 2 (16%) and control subjects recurrent ARTI was detected in 1 (25%). Reduction of recurrences over 6-month follow up as follows: Initial ARTI subjects recurrent ARTI was reduced 90%, recurrent GE was reduced in 77.7%; initial GE subjects recurrent GE was reduced in 83.3%, recurrent ARTI was reduced in 80%; initial UTI subjects recurrent ARTI was reduced in 71.4% and control subjects recurrent ARTI was reduced in 88.8% (Table 1).

In subjects with normal hemoglobin the recurrent infections as follows. In initial ARTI group, development of; recurrent ARTI was 4 (2.4%, p = 0.09), recurrent GE was 2 (1.6%, p = 0.07) and recurrent UTI was 0. Among initial GE group, development of; recurrent GE was 2 (1.6%, p = 0.08), recurrent ARTI was 1 (0.8%, p = 0.1) and recurrent UTI was 0 (0%, p = 0.07). Among initial UTI group, development of; recurrent UTI was 1 (1%, p = 0.14), recurrent ARTI was 0 (0%, p = 0.06) and recurrent GE was 0. Among control, development of; recurrent ARTI was 2 (2%, p = 0.06), recurrent GE was 1 (1%, p = 0.07) and recurrent UTI was 1 (1%, p = 0.07). All were not significant (p > 0.05).

In initial ARTI patients out of 121 children with anemia viral etiology was detected in 52 (43%) cases. Respiratory syncytial virus (RSV) was commonly detected in the virus (60%) in children with ARTI. In initial GE patients out of 86, children with anemia etiology (viral-44, bacterial-2) were detected in 56 (65%) cases. Rotavirus (57%) was commonly detected in

children with GE. In initial UTI patients out of 44 children with anemia, etiology was detected in 36 (82%) cases. *E. coli* (64%) was commonly detected in children with UTI. Etiology of initial ARTI, GE and UTI groups and subsequent recurrences over the follow-up period following oral iron supplementation was displayed on Table 3. Recurrences following RSV was common in initial ARTI as well as other groups. Also, recurrences following RV was common in initial GE as well as other groups. The overall rate of RSV and RV recurrence in all groups have significantly reduced following oral iron supplementation (Table 3).

Further, primary or secondary immunodeficiency was not detected in any of the subjects including the control.

Table 3: Etiology of recurrent infections before, after 3 month of iron supplementation and 6 months follow up.

Initial group anemics (n)	**ARTI (121)**		**GE (86)**		**UTI (44)**	**Control (35)**
Etiology	Viral etiology -52 (43%) RSV- 31 (26%), PIV1-3 (2.5%), PIV 2–3 (2.5%), AV-2 (1.6%), hMPV-4 (3.3%), Influenza A- 6 (5%), Influenza B- 3 (2.5%), No etiology 69 (57%)		Etiology detected -56 (65%) RV-32 (37%), AV(g)- 12 (14%), *Shigella sonnei*-2 (2.3%) No etiology -30 (35%)		Bacterial etiology -36 (82%) *Escherichia coli*- 23 (53%), *Klebsiella pneumoniae*-6 (14%), *Staphylococcus aureus*-2(4.5%), proteus sp.-3(6.7%) No etiology -8 (18%)	—
Significant recurrent infections among anemics	ARTI 20 (16.5%)	GE 9 (7.4%)	GE 12 (10%)	ARTI 10 (12%)	ARTI 7 (16%)	ARTI 9 (25%)
Etiology	RSV- 18 (90%), Influenza A- 2 (10%)	RV-8 (89%), AV- 1 (11%)	RV-10 (83%), AV- 2 (17%)	RSV-10 (100%)	RSV-7 (100%)	RSV-8 (89%) AV-1 (11%)
3-month Iron supplementation						
	ARTI		GE		UTI	Control
Recurrent infection at 6 months follow up	ARTI 2	GE 2	GE 2	ARTI 2	ARTI 2	ARTI 1
Etiology	RSV-2 (100%)	RV-2 (100%)	RV-2 (100%)	RSV-2 (100%)	RSV-2 (100%)	RSV-1 (100%)

ARTI- acute respiratory tract infections, UTI- urinary tract infection, GE-gastro-enteritis, RSV-Respiratory syncytial virus, AV- Adenovirus, %), PIV1-parainfluenza virus-1, PIV 2- parainfluenza virus-2, hMPV-human Metapneumovirus, AV(g)- adenovirus causing gastro-enteritis

Discussion

Childhood nutritional anemia would reflect the status of chronic malnutrition [15,16]. The world health organization estimates that globally around 293 million young children suffer from anemia, among which 50% are estimated to be attributable to iron deficiency. Iron deficiency anemia can be present at early age and also in well-nourished children [21]. Iron deficiency is one of the most common micronutrient deficiencies in the world [22].

Children are vulnerable for various infections specially ARTI, UTI and GE [10,11]. Such infections could be associated with a low level of immunity. Frequent exposure and low level of hygienic practices are associated with recurrences [11,35]. Once recurrent infections are associated with childhood under-nutrition the outcome would worsen often ended up with frequent infections in early life. This is a modifiable risk factor for the development of infections [16,18]. While adherence to hygienic practices, consumption of nutritionally adequate diet would lead to alleviating the burden [16,35].

In our study, the risk of childhood ARTI was significantly associated with iron deficiency anemia. Blood iron deficiency is a risk factor for the development of recurrent ARTIs [25,26]. Mourad *et al*. and Ramakrishnan *et al*. shows that iron deficiency anemic children were two times and five times more susceptible to lower respiratory tract infection compared to the control group, respectively [31,36]. Adequate iron is important for proliferation and maturation of immune cells, particularly lymphocytes, for generation of specific response to infection [37]. Further the observed risk could be due to low oxygen carrying capacity in pulmonary vasculature and parenchyma leading to the low level of protectively towards invading pathogens. Among viral ARTI, recurrence was common following RSV. Is the most prevalent virus among childhood ARTI and frequent exposure would lead to recurrences. RSV is considered one of the earliest stimuli for recurrent wheezing in children [38]. The supplementation of iron in healthy childhood

community has reduced upper respiratory tract infections significantly [38-40].
Iron deficiency is a risk factor for the development of GE. Also, GE would be associated with malnutrition. Since gastric epithelium having a high turnover rate, it requires well nourish status for maintenance of mucosal integrity and absorptive capability [41,42]. Further cumulatively low level of nutrition leads to low immunity. Rotavirus was detected as commonest etiology for childhood GE as well as recurrent GE. Frequent exposure would lead to recurrences [43]. In here, the stool was tested only in single sample helminth infestation cannot be excluded. Continuous use of anti-helminth thus leads to the low incidence of helminth infestation.

Interestingly, anemia is neither a risk factor nor a protective factor for the development of childhood UTI. Level of immunity perhaps with low in mal-nutrition but low level of hydration is key to the development of UTI. In addition to that structural malformations and anatomical anomalies act simultaneously for the acquisition of childhood UTI [44].

Following iron supplementation hemoglobin concentration rose, became normal for age and sex. The increase in serum ferritin reflects the correction of iron storages. Over the follow-up period, recurrent ARTI and GE among tested groups were significantly reduced thus indicating replenishing iron in blood plays a significant protective role in childhood recurrent ARTI and GE. World Health Organization advice to supplement iron to prevent iron deficiency in the population to minimize infections [37,45,46]. Meanwhile, advice on hand hygiene and sanitary practices were also given as health education.

Sri Lanka comprises well spread public health facilities with great awareness but the majority of children suffer anemia. Although we did not collect data on dietary intake of infants (except breast milk intake and introduction of solid/semisolid foods), evidence from other studies from rural Sri Lanka suggests that dietary diversity is low and might also be responsible for anemia [47,48]. Poverty will be a key factor [23,46]. Also feeding mal-practices and lack of knowledge on

nutritive food materials would aggravate it. It is important that implementation of ground-level education on nutrition and supplementation of macro and micro-nutrients on a regular basis. This would reduce the level of childhood infections and associated burden.

Here, we haven't measured the level of adherence to hand hygiene and sanitary practices. This could be a confounding factor on the reduction of infections and the recurrences.

Conclusion

Children are vulnerable for developing various infections specially ARTI, UTI and GE. Iron deficient children are more prone to develop recurrent ARTI, GE and iron deficiency anemia would worsen the associated burden. Once iron deficiency being corrected the rate of recurrent ARTI and GE was reduced. This would be a boost for policy developers to implement strategies at the community level to prevent iron deficiency in children to reduce ARTI and GE recurrences.

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Book Chapter

High Frequency of *M. leprae* DNA Detection in Asymptomatic Household Contacts

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Availability of data and materials: All data generated or analysed during this study are included in this published article and its supplementary information files (Additional file 1).

Ethics approval and consent to participate: We hereby declare that this study was approved by the Ethics Committee of the Universidade Vale do Rio Doce—Univale, filed under N° PQ 022/09–009. All participants signed a free and informed consent at the first evaluation.

Competing interests: The authors declare that they have no competing interests.

Abstract

Background: Characterization of the *Mycobacterium leprae* genome has made possible the development of Polymerase Chain Reaction (PCR) systems that can amplify different genomic regions. Increased reliability and technical efficiency of quantitative PCR (qPCR) makes it a promising tool for early diagnosis of leprosy. Index cases that are multibacillary spread the bacillus silently, even before they are clinically diagnosed. Early detection and treatment could prevent transmission in endemic areas.

Methods: In this study, the qPCR technique is used to detect DNA of *M. leprae* in samples of slit skin smears (SSS) of the ear lobe and blood of leprosy patients and their asymptomatic household contacts residing in Governador Valadares, MG, Brazil, a hyperendemic area for leprosy. A total of 164 subjects participated in the study: 43 index cases, 113 household contacts, and, as negative controls, 8 individuals who reported no contact with patients nor history of leprosy in the family. The qPCR was performed to amplify 16S rRNA fragments and was specifically designed for *M. leprae*.

Results: Of asymptomatic household contacts, 23.89% showed bacillary DNA by qPCR in samples of SSS and blood. Also, 48.84% of patients diagnosed with leprosy were positive for qPCR while the bacillary load was positive in only 30.23% of patients. It is important to note that most patients were already receiving treatment when the collection of biological material for qPCR was performed. The level of bacillary DNA from household contacts was similar to the DNA levels detected in the group of paucibacillary patients.

Conclusion: Considering that household contacts comprise a recognizable group of individuals with a high risk of disease, as they live in close proximity to a source of infection, qPCR can be used to estimate the risk of progress towards leprosy among household contacts and as a routine screening method for a chemoprophylactic protocol.

Abbreviations

Any-Sample- Blood and/or Dermal Scraping; *BI*-Bacilloscopy Index; *CMB*-Contacts of Multibacillary; *CPB*-Contacts of Paucibacillary; *CREDEN-PES*-Center for Endemic Diseases and Special Programs; *Ct*-Cycle Threshold; *DNA*-Deoxyribonucleic Acid; *HHC*-Household Contact; *IC*-Informed Consent; *M. bovis*-Mycobacterium Bovis; *M. leprae*-Mycobacterium Leprae; *M. tuberculosis*-Mycobacterium Tuberculosis; *MB*-Multibacillary; *M-PCR*-Multiplex Polymerase Chain Reaction; *N*-Number of Patients; *PB*-Paucibacillary; *PCR*-Polymerase Chain Reaction; *PGL-1*-Phenolic Glycolipid I; *PNL*-Pure Neural Leprosy; *qPCR BL*-qPCR Blood; *qPCR DS*-qPCR Dermal Scraped; *Qpcr*-Quantitative Polymerase Chain Reaction; *RLEP*-Repeat Sequences; *rRNA*-Ribosomal Ribonucleic Acid; *µL*-Microliter;

Background

Leprosy is a chronic disease caused by *Mycobacterium leprae* that results in neurological and skin damage [1]. Despite advances toward the elimination of leprosy over the last two decades, new case detection rates have stabilized over the last decade, and leprosy remains an important health problem in many regions [2]. Leprosy is considered endemic in several countries with low rates of social and economic development, especially in India and Brazil, which contain the largest absolute number of cases. Given delays in the diagnosis of multibacillary (MB) leprosy, transmission of *M. leprae* from infected individuals to their contacts continues, and in many cases, irreversible nerve damage occurs before those infected are registered as patients [3,4]. It is remarkable that DNA amplification methods have been used for genomic studies and resistance-associated gene mutations [5].

The early diagnosis and prompt initiation of treatment is essential to the rapid interruption of the disease transmission chain. In this sense, the development of a sensitive test for the diagnosis of leprosy has been one of the main objectives of research related to the disease [6]. The *M. leprae* genome sequence has made it possible to target specific sequences of the bacillus. PCR is also a sensitive technique capable of detecting 25 fg (10^{-15} g) of *M. leprae* DNA [7,8]. The assays have been developed for regions such as the 36-kDa [9], 18-kDa [10] or 65-kDa [11] antigens as well as repeat sequences (RLEP) and the gene encoding the 16S rRNA of *M. leprae* [12,13]. The analysis of sensitivity and specificity of real-time quantitative PCR (qPCR) amplification of the sodA gene, 16S rRNA, RLEP, and 85BAg for the differential diagnosis of leprosy showed that the RLEP gene confers greater sensitivity (although a lower specificity) to the technique. The assay 16S rRNA, albeit less sensitive, was highly specifically suited for *M. leprae* [12]. Previous data [14] confirmed the suitability of the 16S rRNA primer for *M. leprae*, comparing it with nine other *Mycobacterium* species, including *M. tuberculosis* and *M. bovis* as well as bacteria of other genera, such as *Staphylococcus*, *Streptococcus*, and *Escherichia*. We screened and followed household contacts (HHCs) along with patients, testing the 16S rRNA qPCR assay to evaluate the presence of *M. leprae* DNA in patients and their asymptomatic HHCs.

Methods

Study Group

The study was conducted in the city of Governador Valadares in eastern Minas Gerais State, Brazil. This city is considered hyperendemic for leprosy: the new case detection rate () was 1.9/10,000 people in 2015. In Minas Gerais, the rate was 0.5/10,000 in 2016, and in Brazil as a whole, it is currently around 1.2/10,000. The study's participants included leprosy patients and HHCs who came to the Reference Center for Endemic Diseases and Special Programs (CREDEN-PES) at the Department of Public Health of Governador Valadares municipality. The index cases were diagnosed and biological

samples were collected for up to three months after treatment began. All contacts were subjected to careful clinical evaluation before being considered asymptomatic. One hundred sixty-four individuals participated in the study: 43 index cases and 113 HHCs. Eight individuals who reported no contact with patients or family history of leprosy were included as a negative control (NC) group. According to the classification used in CREDEN-PES, the index cases were identified as paucibacillary (PB) or MB based on the Guidelines of the Brazilian Health Ministry [15]. PB individuals showed Tuberculoid-Tuberculoid (2), Borderline Tuberculoid (18) in its clinical form (PB, $n = 20$), and were negative in bacillary load. MB individuals showed Borderline–Borderline (3), Borderline Lepromatous (7) and Lepromatous Lepromatous (13). HHCs were grouped and assigned as follows: contacts of PB patients (HHC-PB, $n = 52$) and contacts of MB patients (HHC-MB, $n = 61$).

Ethical Approval

The study was approved by the Ethics Committee of the Universidade Vale do Rio Doce—Univale, filed under N° PQ 022/09–009. All participants signed an informed consent (IC) at the first evaluation.

Sample Collection and DNA Extraction

The bacillary load technique was performed only in index cases, according to the guidelines to technical procedures: smear microscopy in leprosy [16]. The bacteriological index (BI) was calculated according to the work of Ridley and Jopling (1966) [17]. Slit skin smears (SSS) from the right earlobe and blood samples were collected for DNA extraction using the DNeasy kit (QIAGEN®). Samples of SSS stored in ethanol at 70% were thawed and centrifuged at 2000 rpm for 10 min. The extraction was performed according to protocols described by the manufacturer. The concentration of DNA in the eluate was determined by spectrophotometer (NanoDrop 1000 Spectrophotometer—Thermo Scientific). DNA extraction from blood samples followed the same procedure as mentioned above, using 50 μL of blood.

Real time PCR Assay—Qpcr

The qPCR was performed using the TaqMan qPCR amplification system. The amplification target was the gene 16S rRNA specific for *M. leprae* as previously described [14]. The threshold values to define positive samples were used as described (Martinez et al., 2011; Barbieri et al., 2014), and the number of genomes was calculated by interpolating the Ct values in a dilution curve with the known number of *M. leprae* genomes using an Excel spreadsheet (Martinez et al., 2011). All qPCR reactions were run in triplicates and in the same thermocycler under calibrated conditions where positive controls with known numbers of genomes were tested (Step One, Applied Biosystems).

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism version 5.0 software. The bacterial DNA levels among groups were evaluated by Mann-Whitney test and Kruskal-Wallis test (Dunn's Multiple Comparison Test).

Results

Efficiency of Bacillary Loads and qPCR for *M. leprae* Detection

Among the index cases, 25% of patients in the PB group showed *M. leprae* DNA in any sample (blood or SSS). It is important to note that all patients from the PB group had a negative BI. With respect to the MB group, it was found that 69.56% of patients were positive in both SSS or blood in qPCR (Any-Sample). On the other hand, in the MB group, the BI was positive in 56.52% of patients. It was observed that 30% of MB individuals that showed negative BI were qPCR positive. In addition, 100% of MB patients with positive BI showed a qPCR positive result. In summary, it was possible to identify *M. leprae* DNA in 48.84% of all index cases investigated while the BI was positive in only 30.23% (Table 1).

Table 1: Efficiency of bacilloscopy and qPCR for *M. leprae* detection.

Study Group	N	Bacilloscopy	qPCR blood	qPCR SSS	blood or SSS
		N (%)	N (%)	N (%)	N (%)
PB	20	0 (0.00)	2 (10.00)	4 (20.00)	5 (25.00)
MB	23	13 (56.52)	4 (17.39)	14(60.87)	16 (69.56)
Total	43	13 (30.23)	6 (13.95)	18 (41.86)	21 (48.84)

N number of patients, *qPCR* Slit Skin Smear = qPCR SSS

The Spearman test was used to correlate the genome numbers of *M. leprae* against the bacillary load. No association was observed ($p = 0.3894$, $r = 0.3571$) (Figure 1). In this analysis were included only patients from the MB group who were positive for qPCR and bacillary load.

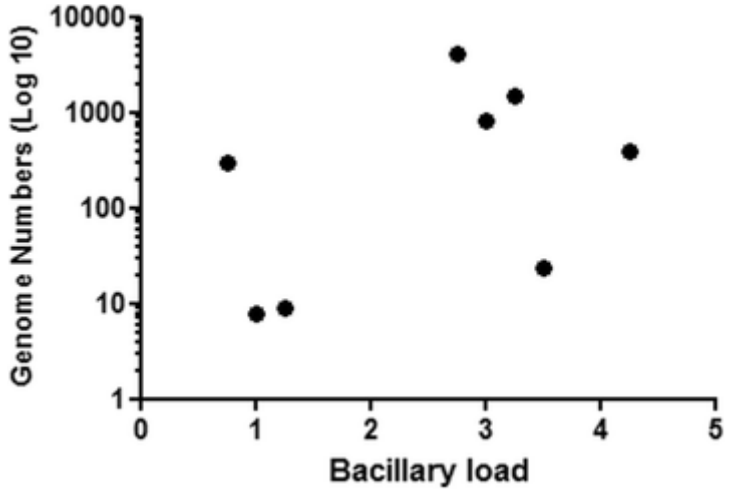

Figure 1: Spearman correlation between genome numbers and bacillary load for MB patients with positive bacilloscopy. ($N = 12$*; $p = 0.3894$; $r = 0.3571$) * Only individuals with bacillary DNA in SSS and positive bacilloscopy were selected

A comparison of bacterial genome numbers between the groups of index cases was performed using interpolation of the Ct

values as described above, obtained from the qPCR blood or SSS. It was observed that the genome number of *M. leprae* was significantly higher in the MB group than in the PB group (Figure 2).

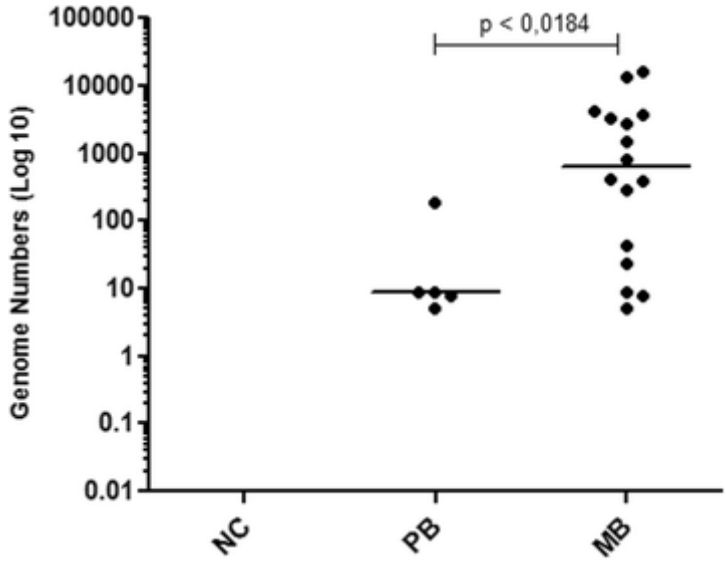

Figure 2: Comparison of *M. leprae* genome numbers between the groups of index cases. NC = Negative control ($n = 8$); PB = Paucibacillary ($n = 5$); MB = Multibacillary ($n = 16$). Each point represents the individual value of a genome number. The median is represented by the horizontal line. Mann-Whitney test was applied

M. leprae DNA Detection in Household Contacts

The 113 HHCs were evaluated by qPCR using blood samples and SSS of the right earlobe, and it was found that 19.23% of HHC-PB were positive for *M. leprae* DNA in blood or SSS. Likewise, in relation to the HHC of MB cases (HHC-MB), 27.87% were positive. Among 113 asymptomatic contacts assessed by qPCR, 23.89% had positive *M. leprae* DNA results (Table 2).

Table 2: Detection of *M. leprae* DNA in household contacts of index cases.

Study Group	N	qPCR blood	qPCR SSS	blood or SSS
		N (%)	N (%)	N (%)
HHC-PB	52	4 (7.69)	7 (13.46)	10 (19.23)
HHC-MB	61	7 (11.48)	11 (18.03)	17 (27.87)
Total	113	11 (9.73)	18(15.93)	27(23.89)

N Number of household contacts, *HHC-PB* House hold contacts Pauciballary, *HHC-MB* House hold contacts Multibacillary, *qPCR Slit Skin Smear* qPCR SSS

Comparison of DNA Levels of *M. leprae* between Household Contacts and Groups of Index Cases

Analysis of genome numbers obtained in qPCR blood or SSS of HHCs showed no statistical difference between the HHC-PB and HHC-MB groups (Figure 3). Interestingly, it was found that the median of the values of genome counts of all HHCs (HHC-PB and HHC-MB) was similar to the median of the index cases of PB. However, as expected, the median value of genome counts in MB cases was significantly higher than the median of the contact group ($p < 0.0001$). Note that two contacts showed similar genome numbers as the MB group (Figure 4). After a one-year follow-up, three individuals were diagnosed with leprosy. But only one of those three infected individuals was contacted; the others moved and were not found.

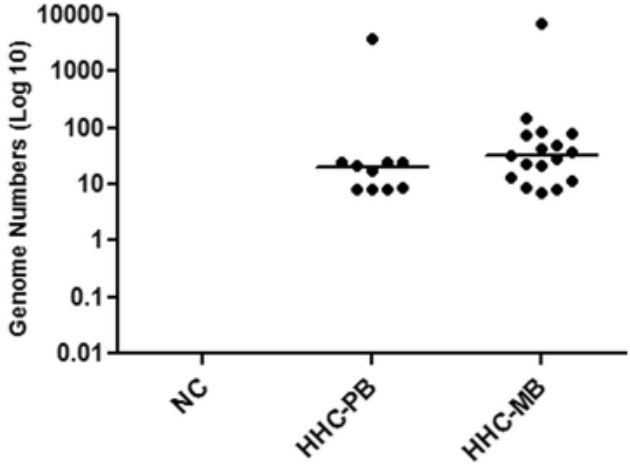

Figure 3: Comparison of *M. leprae* genome number among household contacts groups. NC = Negative control (n = 8); HHC-PB = Contact of paucibacillary (*n* = 10); HHC-MB = Contact of multibacillary (*n* = 17). Each point represents the individual value of a genome number. The median is represented by the horizontal line. Mann-Whitney test

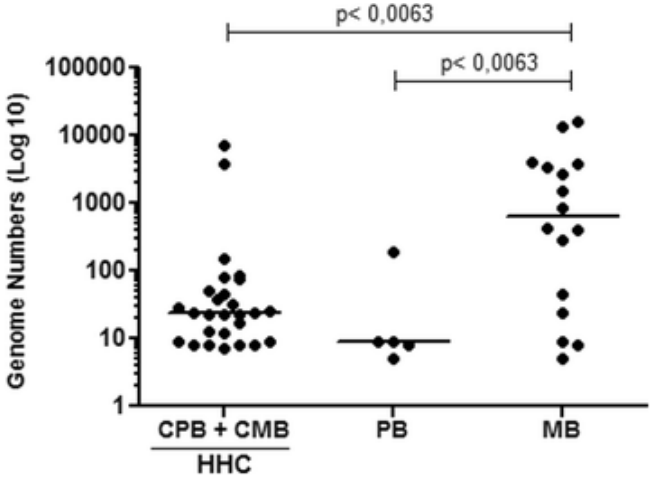

Figure 4: Comparison of the *M. leprae* genome number of all household contacts and index cases PB and MB. Household contacts (HHC) (*n* = 27); PB = paucibacillary (n = 5); and MB = multibacillary (n = 16). Each point represents the individual value of 1/Ct. The median is represented by the horizontal line. Kruskal-Wallis statistic and Dunn's Multiple Comparison Test were applied

Discussion

It is clear that effective control of leprosy requires, together with multidrug therapy, new diagnostic tools that can detect *M. leprae* infection at an early stage. The PCR technique has been evaluated for DNA detection of *M. leprae* in different systems [18-20].

The high sensitivity of qPCR in relation to the BI makes this an extremely important technique in supporting the clinical diagnosis, as reported by other authors [12,13,20]. In our study, we confirmed the significant potential of qPCR for *M. leprae* DNA detection in leprosy cases with negative BI as well as in asymptomatic HHCs. It was observed that 25% of PB and 30% of MB individuals who showed negative BI (dimorphous 3/10) were qPCR positive. Considering that, 100% of MB patients with positive BI (4 dimorphous and 9 virchowian) were also qPCR positive (Tables 3 and 1).

Table 3: Characterization of the study groups for clinical form and bacilloscopy.

Operational classification	Clinical form	Bacillary load	N
PB	TT	Negative	02
	BT	Negative	18
MB	BB	Negative	03
	BL	Positive	07
	LL	Positive	13
Total			43

Although our study did not present data about the sensitivity and specificity of the qPCR test, our assay was based in a series of previous works wherein the extensive evaluation of qPCR used here is 100% specific. The sensitivity is around 50% in patients with PB forms to near 100% in patients with MB forms of the disease [21].

Because collecting SSS is an invasive procedure, it was limited to one specific site (the right earlobe). Therefore, we believe that a higher frequency of positivity for qPCR could be achieved if

other collection sites could be used, as is standard for smear microscopy [16].

Importantly, some patients were already receiving treatment at the time of the collection of biological material, which may have reduced the positivity rate for qPCR, in accordance with Banerjee et al. (2010) [18].

Interestingly, we noted a moderate positive correlation ($p = 0.047$; $r = 0.5823$) between the values of 1/Ct (DNA levels) and the bacterial index (BI) in MB patients, reinforcing the association between the *M. leprae* DNA level detected by qPCR and infection. Therefore, it was found that, the higher the BI, the higher the bacterial DNA level (Figure 1) as demonstrated [22]. Evidence of a positive correlation between qPCR and BI led us to compare the *M. leprae* DNA levels among patients in an attempt to distinguish the groups. We found a significant difference in the level of bacterial DNA between the MB and PB groups (Figure 2). Studies on leprosy transmission have demonstrated that people living with index cases are exposed to a greater risk of progressing towards the disease [23,24]. An effective strategy for reducing the incidence of leprosy is contact tracing and diagnosis in the early stages of the disease. As shown by Banerjee et al. (2010) [18], MB contacts have increased the frequency of positivity in the multiplex PCR (M-PCR) compared to PB contacts; we also found a higher frequency of qPCR positive for HHC-MB (27.87%) in comparison with HHC-PB (19.23%). We consider that contacts of MB are more exposed to a higher bacterial load, possibly showing increased frequency of qPCR, positive in relation to HHC-PB contacts [25]. However, we did not detect significant differences in the number of genomes obtained from qPCR among HHC (HHC-PB + HHC-MB) groups.

It is important to note that all HHCs were considered asymptomatic for clinical evaluation. However, 23.89% of them had bacillary DNA in SSS of the earlobe. This high detection rate indicates a high dynamic transmission of leprosy in that study group. Knowing that leprosy has a long incubation period and that symptoms are difficult to detect in the early stages of the

disease, we emphasize that the contacts' surveillance with positive results for qPCR is extremely relevant. Six to 8% of HHCs develop clinical symptoms of leprosy within two years of diagnosis of the index case [26]. The fact that all contacts showed levels of bacterial DNA similar to the PB group can be explained by the slow progress of the disease; some of these contacts may develop leprosy in the future. In our study, with only one year of follow-up, we detected three new cases of leprosy among the examined contacts. Of these, two were CMB and one was positive for qPCR before the onset of clinical symptoms. The employment of strategy for early detection and/or identification of subclinical infection linked to chemoprophylaxis will certainly contribute to the effective control of leprosy [27]. More recently, the importance of serological and DNA-based techniques for the assessment and confirmation of diagnoses in suspected and early cases of leprosy was emphasized [28]. According Reis et al. 2014 [29], all HHC positive for qPCR should be addressed to control strategies to provide both chemoprophylaxis and immunoprophylaxis by vaccination to generate immediate protection that can be sustained in the long term.

Finally, we consider that the high sensitivity of qPCR allows the identification of a large number of asymptomatic contacts having *M. leprae* DNA. More qPCR tests need to be evaluated further as they could serve as a better diagnostic tool for early case detection and treatment to achieve faster control of leprosy.

Conclusion

The presence of bacterial DNA in the dermal scraping earlobe suggests subclinical infection, and therefore, contacts with positive qPCR should be monitored for disease development in the future. Early detection of leprosy cases and effective chemotherapy are the best strategies to reduce the incidence of new cases of leprosy and prevent transmission. Considering that HHCs comprise a recognizable group of individuals with a high risk of disease, as they live in close proximity to a source of infection, we suggest that, as a prevention strategy, qPCR should

be used to follow-up with leprosy HHCs to confirm or rule out subclinical infection.

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Additional files

Additional file 1: Bank of data. Description of data: this file contains all raw data of the study.
Additional file 1 can be accessed online at:
https://videleaf.com/wp-content/uploads/2020/11/PAINFD-20-04_Additional-File.pdf

Book Chapter

Risk Factors for Mycobacterium ulcerans Infection (Buruli Ulcer) in Togo - A Case-Control Study in Zio and Yoto Districts of the Maritime Region

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Ethics Approval and Consent to Participate: The study protocol was approved by the National Program for Buruli Ulcer Control, (Authorization No.006/2014/MS/DGS/DSSP/PNLUB-LP) and the Ministry of Health as an integral part of the surveillance of the disease. However, this study did not require a review of the ethics committee. The objectives of the study were explained to the participants and their inclusion was voluntary. For each participant, we obtained a signed consent. As for children, parents or legal representatives gave consent on their behalf.

Availability of Data and Materials: All data generated or analyzed during this study are included in this published article [and its supplementary information files]. However, the datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors have declared that no competing interests exist.

Authors' Contributions: IM, TT and DSK contributed to the study design, statistical analyses of data and wrote the paper. EP, KK were involved in the field investigation, data collection and reviewing the manuscript. MB provides advice for study methodology and performed critical review of the manuscript for important scientific content. KA, GB and FXW contributed to the critical review of the document. YK, KB and ABK contributed to the facilitation of the project, participated in its design, coordination and review the paper. All authors read and approved the final manuscript.

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Abstract

Background: Buruli ulcer (BU) is a neglected mycobacterial skin infection caused by *Mycobacterium ulcerans*. This disease mostly affects poor rural populations, especially in areas with low hygiene standards and sanitation coverage. The objective of this study was to identify these risk factors in the districts of Zio and Yoto of the Maritime Region in Togo.

Methods: We conducted a case-control study in Zio and Yoto, two districts proved BU endemic from November 2014 to May 2015. BU cases were diagnosed according to the WHO clinical case definition at the Centre Hospitalier Régional de Tsévié (CHR Tsévié) and confirmed by Ziehl-Neelsen (ZN) microscopy and IS2404 polymerase chain reaction (PCR). For each case, up to two controls matched by sex and place of residence were recruited. Socio-demographic, environmental or behavioral data were collected and conditional logistic regression analysis was used to identify and compare risk factors between BU cases and controls.

Results: A total of 83 cases and 128 controls were enrolled. The median age was 15 years (range 3-65 years). Multivariate conditional logistic regression analysis after adjustment for potential confounders identified age (<10 years (OR =11.48, 95% CI=3.72-35.43) and 10-14 years (OR = 3.63, 95% CI=1.22-10.83)), receiving insect bites near a river (OR = 7.8, 95% CI=1.48-41.21) and bathing with water from open borehole (OR = 5.77, (1.11-29.27)) as independent predictors of acquiring BU infection.

Conclusions: This study identified age, bathing with water from open borehole and receiving insect bites near a river as potential risk of acquiring BU infection in Zio and Yoto districts of the Maritime Region in south Togo.

Keywords

Buruli Ulcer; *Mycobacterium ulcerans*; District of Zio; District of Yoto; Togo; Risk Factor; Case-Control Study

List of Abreviations

INH - Institut National d'Hygiène ; ESTBA - Ecole Supérieure des Techniques Biologiques et Alimentaires ; CNRT-UB - Centre National de Référence pour le Traitement de l'ulcère de Buruli ; PNLUB-LP- Programme National de Lutte contre l'ulcère de Buruli, la Lèpre et le Pian ; DITM - Department of Infectious and Tropical Medicine ; DAHW-Togo - Association Allemande de lutte contre la Lèpre ; WHO - World Health Organisation; CHR - Centre Hospitalier Régional ; VIH - Human Immunodeficiency Virus; PCR - Polymerase Chain Reaction ; OR - Odds Ratio ; ASC - Health Community volunter; BCG - Bacille Calmette et Guérin ; EDST - Enquête de Développement Sanitaire du Togo ; SPSS - Statistical Package for Social Science

Background

Buruli ulcer (BU) is an emerging skin disease caused by an infection with Mycobacterium ulcerans [1–4]. BU represents the third most common mycobacterial disease after tuberculosis and leprosy in immunocompetent hosts. Infection with M. ulcerans often leads to extensive destruction of skin and soft tissue with the formation of large ulcers, commonly on limbs. About 60% of lesions occur on the lower limbs, 30% on the upper limbs and 10% on the rest of the body. Although the rate of mortality of Buruli ulcer is low, the serious morbidity caused by the disease includes functional disabilities that may result in permanent social, economic and developmental problems. At least 50% of those affected by BU are children aged < 15 years. Rate of infections among males and females are equal [1–5]. To date, BU cases have been reported in over 30 countries, particularly in tropical and subtropical climate regions but also in temperate climate zones such as Japan and southern Australia [1–5]. BU is a neglected tropical disease (NTD) with a poorly known global prevalence and mainly affects remote rural African communities [6]. According to the WHO, from an estimated 7,000 BU cases reported annually (2016) worldwide and more than 4,000 cases occurred in Sub-Saharan Africa. The largest numbers of reported BU cases were from West African countries, particularly from Ivory Coast (about 2,000 cases annually), Benin and Ghana as well, each of which reported about 1,000 cases a year (2016)[1–6].

In Togo, the first cases of BU have been described in 1996 by Portaels et al [7]. From 1996 to 2004, more than 100 cases were clinically diagnosed [8-9]. Between 2007 through 2010 [9], a joint research project between the German Leprosy and Tuberculosis Relief Organization in Togo (DAHWT) and the Department for Infectious Diseases and Tropical Medicine, University Hospital, Ludwig Maximilians-University, Munich, (Germany) allowed the first systematic study of laboratory confirmed BU cases from Togo and established prevalence of BU in the Maritime Region of south Togo. Since 2011, within the frame of the European Community funded research project “BuruliVac”, a National Reference Laboratory for BU (NRL-

UB) was established at the Institut National d'Hygiène (INH) and all BU cases notified were confirmed by IS2404 PCR [10].

Previous case-control studies [11-15] have reported a high risk of contracting Buruli ulcer by swimming in or wading through a river. Residence near marshy areas with stagnant or slow-flowing water bodies and farming activities near rivers were additionally described as risk factors [11–15]. Several epidemiologic studies in Africa [16-19] and Australia [20-21] have identified aquatic sources as possible reservoirs of M. ulcerans by detecting DNA of the pathogen in water filtrant and in a range of environmental samples. All these findings used PCR methodology which does not provide definitive proof for the presence of intact bacteria in a matrix. More recently, results from laboratory experiments [22-25] have suggested a new hypothesis that aquatic insects, fish, plants and terrestrial mammals may be reservoirs for M. ulcerans and that insect may be even involved in transmission to humans. In addition, the successful culture of M. ulcerans from an aquatic water bug collected in Benin [26] provides definitive evidence for the presence of M. ulcerans in an aquatic invertebrate as possible reservoirs or vectors of M. ulcerans.This considerable achievement showed that the M. ulcerans is present in the environment and that transmission to humans might occur through contact with water or environmental samples contaminated with or harboring the mycobacteria [27]. Inoculation of this pathogen into the subcutaneous tissue could occur when the exposed skin is traumatized. However, the exact mechanism of transmission of the bacterium remains unclear [27].

Human-linked changes in the aquatic environment such as dam constructions on rivers, deforestation, agriculture and mining have led to environmental disturbance and may contribute to the spread of M. ulcerans [28-29]. This could increase the incidence of Buruli ulcer cases in endemic areas and lead to the emergence of M. ulcerans in areas where the pathogen was previously absent [28]. Some studies, mainly clinical [7-10,30-32], were carried out in Togo on BU but little were focused on socio-demographic, environmental or behavioral factors. We

conducted this study to determine such risk factors for M. ulcerans infection in the Zio and Yoto Districts in the Maritime Region.

Material and Methods

Study Design

We conducted a case-control study in the Zio and Yoto districts of the maritime region (**Figure 1**) between November 2014 and May 2015. Buruli ulcer cases were selected at the National Reference Center for BU Treatment (CNRT-UB) located at CHR Tsévié. Patients enrolled were recruited from March 2013 to May 2015. Controls were recruited by active search during the survey. Patients infected with the human immunodeficiency virus (HIV) or with active tuberculosis were excluded from the study.

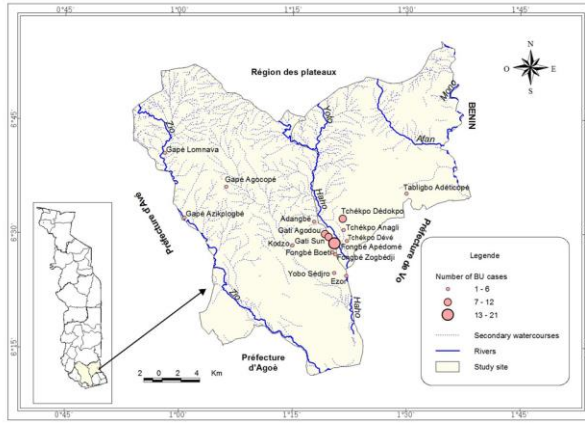

Figure 1: Maritime Region Map presenting villages surveyed, distribution of BU cases and hydrographic network:
The circles in red correspond to the number of Buruli ulcer cases and placed at the 17 villages location in Districts of Zio and Yoto of the Maritime Region. Most of BU cases are located arround the watercourse of Haho with few cases observed near the Zio watercourses. These watercourses are main sources of activities with water contact that are associated with increasing risk of *M. ulcerans* infection.

Case Definition

A probable case of Buruli ulcer was defined as any person aged ≥2 years who lived in Zio or Yoto district showing clinical symptoms according to the WHO clinical definition of BU [3]. A confirmed case was defined as a probable case with detection of M. ulcerans using Ziehl-Neelsen (ZN) microscopy and IS2404 PCR [9-10].

Recruitment of Controls

An eligible control was defined as any person aged ≥2 years without any history or clinical symptoms of Buruli ulcer. Up to two controls were randomly selected and matched to cases by sex and place of residence (home where lived the case or neighbor home in the same village).

Study Site

This study was conducted in 17 villages in districts of Zio and Yoto where more than 85% of confirmed BU patients originate. These districts are in the Maritime Region (South of Togo) which covers an area of 6.359 km². With an estimated population of 1,762,518 inhabitants in 2012, the climate is tropical and humid with two rainy seasons and two dry seasons. The maritime region has a flat topography, with a low contrast characterized by a sedimentary basin that covers 4/5 of the region, a low altitude (50-80m on average) and crossed by the depression of the Lama. The clay soil remains soggy and muddy in the rainy season. Water stagnates for several months in this region. The hydrographic network comprises 3 large rivers which are the Mono in the east, the Zio and the Haho in the center with several small tributaries that flow into the “lac Togo” (Figure 1). All these streams have a low flow, closely linked to seasonal variations of precipitations [33].

Laboratory Confirmation

Sample collection: Samples were collected according to standardized procedures as previously described [9-10]. Briefly,

fine needle aspirates (FNA) were collected from the center of non-ulcerative lesions or from undermined edges of ulcerative lesions including necrotic tissue. Swabs were collected by circling the entire undermined edges of ulcerative lesions. Samples taken were put in tubes containing cell lysis solution (CLS, Qiagen, Hilden, Germany) and sent for PCR analysis at INH.

Laboratory testing: Direct smears for microscopy were prepared from swab and FNA samples at peripheral care units or CNRT-UB and subjected to Ziehl-Neelsen staining for detection of acid fast bacilli at the laboratory of the CHR. Slides were analyzed by microscopy according to the WHO [34] recommended grading system. All slides were double checked at the INH by a second technician for external quality control.

All molecular analyses were conducted at the NRL for BU at INH as previously described [10]. For PCR analysis, DNA was extracted from FNA and swab samples with the Gentra Puregene DNA extraction kit (Qiagen) with minor modifications of the manufacturer's protocol. The conventional IS*2404*-PCR with gel-based amplicon detection was applied using dry-reagent-based consumables (DRB-IS*2404* PCR). Briefly, for the DRB-PCR, the primers MU5 (AGCGACCCCAGTGGATTGGT) and MU6 (CGGTGATCAAGCGTTCACGA) were lyophilized in reaction tubes. The Illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare) containing Taq polymerase, dNTPs and Mg^{2+} were added and dissolved in water before adding DNA template. All PCR assays included negative extraction, positive, negative (no template) and inhibition controls. All inhibited samples were repeated after 10-fold dilution.

Sample Size

We used the power calculation tool of Epi-Info (version 7; 2012) to determine the sample size by setting α to 5% and power β to 80%. The health and population development survey (EDST; 2014) in Togo showed that 36,2% of households use water from unprotected sources [35-36]. The odds ratio (OR) of the

association between cases and controls was set at 2, yielding a sample size of 100 BU cases and 200 controls.

Data Collection

The survey was conducted by a team of four people including a clinician from CHR Tsévié, a focal point of the national program of BU surveillance, a community volunteer and a laboratory technician. Case residence was identified by the community volunteer. Once at home, we selected one or two matched control subjects. A well-structured questionnaire was administered to all selected participants (Additional file 1: Questionnaire form S1). For participants who could not respond in French, the interview was conducted in the local language. For children under 10 years, we interviewed their parents mainly for their activities and behavior. All the participants gave their consent prior to data collection on socio-demographic characteristics, behavior, occupational and environmental factors as well as administration of BCG vaccination.

Statistical Analysis

Data collected were entered in a database designed through Epi-Info software (Version 7; 2012). Statistical analysis was carried out by SPSS software (Statistical Package for Social Science, Version 16.0, SPSS Inc. and Chicago, IL). Qualitative data were presented as number n (%) and quantitative data as mean ± standard deviation. Buruli ulcer was considered as the dependent variable and socio-demographic characteristics, occupational and environmental factors as independent variables. Student t-test was used for comparison of mean or median age and number of people in the household between patients and controls with significant level set at $p \leq 0.05$. Univariate logistic regression was used to determine the risk factors of *M. ulcerans* infection by determining the odds ratio (OR) and 95% confidence interval (CI). All variables obtained from the univariate analysis with p-value ≤ 0.1 were retained for the multivariate model. The final model was obtained after a step-by-step backward elimination step using multiple logistic regressions.

Results

Clinical Diagnosis, Laboratory Confirmation and Characteristics of BU Cases

During the study period, 129 probable cases were observed (Table 1). ZN microscopy confirmed the presence of acid-fast bacilli (AFB) in 67 (52%) among probable cases while PCR detected *M. ulcerans* DNA in 91 cases (71%). The two techniques were both positive in 58 cases (44.5%) and no AFB were detected from any of the PCR negative lesions (Table 1). Of all confirmed cases, lesions were mainly ulcers (41.7%), nodules (27.5%) and plaques (19.8%) (Table 2). Most of these lesions were found on the lower (40%) and the upper limbs (45%). The rest of lesions were localized on the buttocks, abdomen, back and head. Of 91confirmed cases, 83 (91%) responded to the questionnaire. The remaining 8 cases were absent at the time of the survey. Therefore, the case-control study was carried out with 83 cases and 128 control subjects. The socio-demographic characteristics of the participants are presented in Table 3.

Table 1: Yearly distribution of clinically suspected BU cases, laboratory tests used for confirmation and positive BU cases detected in Zio and Yoto Districts of Maritime Region, Togo, March 2013 to April 2015.

Parameters	Number of BU suspected cases, n
Yearly distribution of BU suspected cases	
2013 (March to December)	31
2014	63
2015 (January to April)	35
Total	129
Laboratory confirmation tests	Positive BU Cases, n (%)
Ziehl-Neelsen microscopy (129 cases analyzed)	67 (51.9)
PCR technique (129 cases analyzed)	91 (70.5)
ZN microscopy and IS2404 PCR	58 (44.4)

Table 2: Type and localization of observed lesions in 91 BU cases in Zio and Yoto Districts of Maritime Region, Togo, March 2013-May 2015

Clinical Characteristics	Number of BU cases, n (%)
Type of lesions	
Edema	10 (10.9)
Nodule	25 (27.5)
Plaque	18 (19.8)
Ulcer	38 (41.7)
Total	**91(100.0)**
Localization of lesions	
Abdomen	3 (3.0)
Back	2 (2.5)
Buttocks	3 (3.0)
Head	2 (2.5)
Lower limbs	37 (40.6)
Upper limbs	44 (48.3)
Total	**91 (100.0)**

Univariate Analysis

Socio-Demographic Characteristics of the Participants

Most of BU patients (66%) were under 15 years of age and were significantly younger (median age = 11 years) compared to controls (median age = 19 years with 73% of who aged more than 15 years) ($p = 0.001$) (Table 3). The primary school educational level was more frequent (59%) ($p = 0.03$) in children aged ≤10 years (28.2%) while the secondary school educational level was associated with the 15-24 age groups (56.2%) ($p = 0.007$) (Table 3). Among cases, women (60%) were more frequently affected than men (40%) ($p = 0.01$). There was no significant difference in the number of people living per household between cases and controls ($p = 0.58$) (Table 3).

Table 3: Socio-demographic characteristics of the participants of the case-control study in Zio and Yoto Districts of the Maritime Region, Togo, May 19-30, 2015

Characteristics	Cases n (%)	Controls n (%)	Total n (%)	p
Number of participants	83 (39.3)	128 (60.7)	211	
Sex				0.32
Female	50 (60.2)	68 (53.1)	118 (55.9)	
Male	33 (39.8)	60 (46.9)	93 (44.1)	
Age				
Median (range in years)	11 (3-65)	19 (8-60)	15 (3-65)	**0.001**
< 10	39 (47.0)	15 (11.7)	54 (25.6)	0.01
11-14	16 (19.3)	19 (14.8)	35 (16.6)	0.03
15-24	13 (15.7)	47 (36.7)	60 (28.4)	0.76
>= 25	15 (18.1)	47 (36.7)	62 (29.4)	
District of residence				0.75
Zio	62 (74.7)	98 (76.6)	160 (75.8)	
Yoto	21 (25.3)	30 (23.4)	51 (24.2)	
Education level				**0.03**
None	19 (22.9)	36 (28.1)	55 (26.1)	
Primary school	57 (68.7)	67 (52.3)	124 (58.8)	
Secondary school	7 (8.4)	25 (19.5)	32 (15.2)	
Ethnicity				0.48
Ewe	76 (96.2)	110 (97.3)	186 (96.9)	
Other (Lamba. Moba et Peulh)	3 (3.8)	3 (2.7)	6 (3.1)	
Number of people in household				0.58
Median (range)	8.5 (2-40)	8.0 (3-40)	8.0 (2-40)	

Environmental Factors

Exposure to Water Contact

We found that drinking or washing clothes with water taken from rivers (p = 0.95), open boreholes (p = 0.98) and boreholes with pump (p = 0.49) were not associated with an increased risk of contracting Buruli ulcer (Table 4). However, bathing with

water from an open borehole was associated with higher risk of contracting BU (OR = 5.07, 95% CI =1.33-19.31) (Table 4).

The frequent use of soap while bathing was not associated with reduced risk of BU (p= 0.69). In contrary, a significant decrease of risk of *M. ulcerans* infection was observed when using detergents for washing clothes or dishes (OR = 0.38, 95% CI = 0.32-0.45) (Table 4 continued). Walking in stagnant water or wading in mud did not significantly increase risk of *M. ulcerans* infection (p = 0.72). However, frequently crossing a river (OR = 1.93, 95% CI = 1.09-3.39) or swimming (OR = 1.98, 95% CI= 1.11-3.52) in a river were associated with an increased risk of BU. Receiving cuts or scratches (OR = 1.88, 95%=1.06-3.36) near rivers represented an additional increasing risk for contracting BU (Table 4).

Exposure to Insects

Our study showed that receiving insect bites near a river was significantly increase risk of *M. ulcerans* infection (OR=2.13, 95% CI=1.19-3.83) (Table 4). This risk was higher when it occurred on the forearm (OR = 1.88, 95% CI=1.08-3.31), the arm (OR = 1.77, 95% CI=1.01-3.10) and the hands (OR = 1.79, 95% CI=1.03-3.14) compared to the other parts of the body. We found that mosquito bites at home were not associated with an increased risk of *M. ulcerans* infection (p = 0.90) (Table 4). The use of mosquito coils (p = 0.27) or bednets (p = 0.26) did not provide any significant reduction in the risk of contracting BU (Table 4 continued).

Table 4: Univariate analysis of risk factors for Buruli ulcer disease in Zio and Yoto districts of Maritime Region, Togo, May 19-30, 2015

Characteristics	Cases n (%)	Controls n (%)	Univariable OR (95% CI)	p
Farming	77 (92.8)	120 (93.8)	0.86 (0.29-2.56)	0.78
Farming activities				
Plowing	79 (83.1)	117 (91.4)	0.46 (0.19-1.01)	0.07
Sowing	73 (88.0)	123 (96.1)	0.29 (0.09-0.90)	**0.03**
Harvesting	74 (89.2)	125 (97.7)	0.19 (0.05-0.75)	**0.01**
Exposure to water				
Primary source of drinking water				
River or stream	38 (45.8)	58 (45.3)	1.02 (0.58-1.77)	0.95
Open borehole	15 (18.1)	23 (18.0)	1.0 (0.49-2.01)	0.98
Borehole with pump	75 (90.4)	119 (93.0)	0.71 (0.26-1.92)	0.49
Primary source of washing water				
River or stream	42 (50.6)	60 (46.9)	1.16 (0.68-2.02)	0.59
Open borehole	21 (25.3)	28 (21.9)	1.21 (0.63-2.31)	0.56
Borehole with pump	73 (88.0)	116 (90.6)	0.75 (0.31-1.83)	0.54
Bathing with a water from an open borehole	9 (10.8)	3 (2.3)	5.07 (1.33-19.31)	**0.01**
Standing water in house	10 (12.0)	18 (14.1)	0.86 (0.38-1.97)	0.72
Swam, waded or bathed in a river or stream	37 (44.6)	37 (28.9)	1.98 (1.11-3.52)	**0.02**
Crossed a body of water	48 (57.8)	55 (43.0)	1.93 (1.09-3.39)	**0.02**
Received cuts, scratches and thorn pricks near a river	49 (62.8)	60 (47.2)	1.88 (1.06-3.36)	**0.03**
Exposure to insect bite				
Received insect bite near a river	50 (64.9)	59 (46.5)	2.13 (1.19-3.83)	**0.01**
location of insect bite on the body				
Head	48 (57.8)	56 (43.8)	1.76 (1.01-3.08)	0.05
Forearms	50 (60.2)	57 (44.5)	1.88 (1.08-3.31)	**0.03**
Arms	50 (60.2)	59 (46.1)	1.77 (1.01-3.10)	**0.04**
Hands	49 (59.0)	57 (44.5)	1.79 (1.03-3.14)	**0.04**
trunk	48 (57.8)	57 (44.5)	1.70 (0.98-2.98)	0.06
thigh	48 (57.8)	56 (43.8)	1.76 (1.01-3.08)	0.05
Legs	48 (57.8)	57 (44.5)	1.70 (0.98-2.98)	0.06
Feet	48 (57.8)	57 (44.5)	1.70 (0.98-2.98)	0.06
Mosquito bite in house	75 (90.4)	124 (96.9)	0.95 (0.48-1.89)	0.90
Exposure to animals				
Owned livestock or pets	60 (77.9)	100 (78.7)	0.95 (0.48-1.89)	0.89
Handled livestock or pets	12 (15.6)	26 (20.6)	0.71 (0.33-1.50)	0.37
Share indoor living space with livestock or pets	24 (32.4)	37 (29.4)	1.15 (0.62-2.14)	0.65
Bitten or scratched by animals	5 (6.7)	10 (7.8)	0.84 (0.27-2.56)	0.76
Exposure to infectious agents				
BCG vaccination	41 (51.2)	64 (50.4)	1.03 (0.59-1.181)	0.90

Table 4: Univariate analysis of risk factors for Buruli ulcer disease in Zio and Yoto districts of the Maritime Region, Togo, May 19-30, 2015 (**continued)**

Characteristics	Cases n (%)	Controls n (%)	Univariable OR (95% CI)	p
Soap use while bathing				
Sometimes	5 (6.4)	10 (7.9)	1	
Always	73 (93.6)	117 (92.1)	0.80 (0.26-2.43)	0.69
Soap use while washing				
Sometimes	5 (6.0)	0 (0.0)	1	
Always	78 (94.0)	128 (100.0)	0.38 (0.32-0.45)	**0.01**
Clothing worn while farming				
Trousers	40 (48.2)	92 (71.9)	0.36 (0.20-0.65)	**0.001**
Top shirt	76 (91.6)	120 (93.8)	0.72 (0.25-2.01)	0.55
Closed shoes	9 (10.8)	26 (20.3)	0.48 (0.21-1.08)	0.07
Dress	34 (41.0)	48 (37.5)	1.16 (0.66-2.03)	0.61
Open shoes	73 (88.0)	110 (85.9)	1.19 (0.522-2.73)	0.67
Hat	6 (7.2)	35 (27.3)	0.21 (0.08-0.52)	**0.001**
Clothing worn in non-farming activity				
Trousers	33 (39.8)	61 (47.7)	0.72 (0.41-1.27)	0.26
Top shirt	73 (88.0)	118 (92.2)	0.62 (0.25-1.56)	0.31
Closed shoes	4 (4.8)	5 (3.9)	1.25 (0.32-4.78)	0.74
Dress	33 (39.8)	54 (42.2)	0.90 (0.51-1.59)	0.73
Open shoes	72 (86.7)	114 (89.1)	0.80 (0.35-1.87)	0.61
Hat	1 (1.2)	7 (5.5)	0.21 (0.25-1.75)	0.15
Insect protection products use				
Sometimes	74 (96.1)	118 (92.2)	1	0.27
Always	3 (3.9)	10 (7.8)	0.59 (0.22-1.64)	
Bednets use				
Sometimes	40 (51.3)	76 (59.4)	1	
Always	38 (48.7)	52 (40.6)	0.72 (0.41-1.27)	0.26
Perception and etiology of the BUD	68 (88.3)	113 (88.3)	1.00 (0.42-2.42)	0.99
Behavior and beliefs				
Poor hygiene cause Buruli ulcer	57 (81.4)	116 (91.3)	0.42 (0.17-0.99)	**0.04**
Seeking treatment with plants	4 (5.3)	10 (7.9)	0.64 (0.19-2.12)	0.47

Farming Activities

Farming (93.8%) was the main activity of the participants of the study. However, there was no significant difference in practicing this activity between patients and controls (p = 0.78) (Table 4). In addition, some tasks such as sowing (OR = 0.29, 95% CI = 0.09-0.90) or harvesting (OR = 0.19, 95% CI = 0.05-0.75) during farming showed significant decrease in the risk of contracting BU (Table 4 continued). Frequently wearing trousers (OR = 0.36, 95% CI=0.20-0.65) or a hat (OR = 0.21, 95% CI= 0.08-0.52) while performing farming activities provided significant reduction in the risk. However, wearing clothes at home or in non-farming activities did not provided any significant reduction in the risk of BU disease (Table 4 continued).

Exposure to Animals

In our study, we found that living with (p = 0.89) or sharing indoor living space with livestock (p = 0.37) did not represent a significant increase in the risk of *M. ulcerans* infection neither did incurring bites or scratches from (p = 0.76) (Table 4). Also, hunting or handling of wild animals (p = 0.65) was not significantly associated with an increasing risk of BU infection.

BCG Vaccination

Most of participants showed BCG vaccine scars and there was no significant difference between cases and controls (p = 0.90) (Table 4).

Attitude, behavior and beliefs of BUD

Of the participants interviewed, 88.3% were familiar with BU symptoms and this attitude was similar between BU cases and controls (p = 0.99). Regarding treatment behaviors, most of cases (83.5%) indicated seeking help from hospital while 5.3% believed in herbal treatment as the first preferred treatment option (Table 4 continued). Considering the hygiene practice, BU cases as well as controls thought that personal poor hygiene

and dirty surroundings could increase the risk of contracting BU (Table 4 continued).

Multivariate analysis

After adjustment for potential confounders, we found that factors such as age (<10 years (aOR = 11.48, 95% CI=3.72-35. 43) and 10 to 14 years (aOR = 3.63, 95% CI=1.22-10.83)), receiving insect bites near a river in children aged 10 to14 years (aOR = 7.8, 95% CI= (1.48-41.24)) and bathing with water from open borehole (aOR = 5.77, 95% CI=1.11-29.27) (Table 5) remain as potential factors of increasing risk of *M. ulcerans* infection.

Table 5: Multivariate model for risk factors of Buruli ulcer disease in Zio and Yoto Districts of the Maritime Region, Togo, May 19-30, 2015

Characteristics	**aOR (95% CI)**	**p**
Age (Years)		
< 10	11.48 (3.72-35.43)	**0.001**
11-14	3.63 (1.22-10.83)	**0.02**
15-24	1.07 (0.39-2.97)	0.88
> 25	1	
Receiving insect bites near a river (Yes/No)		
<10 (years)	3.29 (0.77-14.04)	0.11
11-14 (Years)	**7.80 (1.48-41.21)**	**0.016**
15-24 (Years)	3.05 (0.71-12.99)	0.13
>25 (Years)	1.76 (0.48-6.45)	0.39
Bathing with water from open borehole	5.77 (1.11-29.27)	**0.03**

Discussion

The objective of this study was to identify risk factors for Buruli ulcer in the two endemic districts of Zio and Yoto of the Maritime region. This is the first study that has investigated these factors in Togo. In general, socio-demographic, behavioral or environmental factors have been considered as important risk factors for *M. ulcerans* infection.

Socio-Demographic Factors

The present study showed that children under 15 years of age were at higher risk of contracting Buruli ulcer than adults. This result is in accordance with other studies conducted in Benin [11] and Ivory Coast [13] as well as WHO reports [37]. Indeed, in this age group children appeared to be often less protected especially at the head and feet [11]. Also, children's behavior is usually driven by their parents' activities as they accompanied them to the river for washing and for farming where they were highly exposed to aquatic areas that are associated with an increasing risk of BU infection.

Environmental Factors

We found that bathing with water from an open borehole was associated with higher risk of contracting BU. Similar results were found in Ghana [38], Ivory Coast [39] and Cameroon [14]. Indeed, other studies [6,28,40] have also shown that using unprotected water sources for bathing was associated with M. ulcerans infection. It has also been observed that even when used with soap, unprotected water sources constitute an increased risk of M. ulcerans infection [37]. However, Raghunathan et al. [38] in Ghana found that using a detergent while bathing provides significant reduction in Buruli ulcer risk. This difference could be explained by the antibacterial power of the soap used.

Besides, in our study people from villages commonly used the local soap. On the other hand, we found that using soap to wash clothes or dishes was reducing the risk. This time, the type of the soap used for the laundry is provided from commercial brands

which are strongly enriched in detergents and acids. Our study also identified other water sources of M. ulcerans infection such as swimming in a river, frequently crossing a river, receiving insect bites or injuries of cuts near rivers. However, after adjustment for potential confounders, only receiving insect bites near a river remained as an independent predictor of acquiring BU infection. Similar results were found in Ghana [38] but in Ivory Coast [13] and in other study [12], it was found that swimming or wading in water did significantly increase the risk of BU infection. To explore the difference of our finding with other studies, we looked to determine any potential age confounding or effect modification. Therefore, we found that insect bites increase the risk of BU only in 10-14 years age group (aOR=7.80, 95%CI=1.48-41.21). Though, other studies did not determine in which age group swimming or wading in water significantly increased the risk of BU, we could explain the difference between these studies by the age of BU cases. Further, in our study, 63% of BU cases were aged < 15 years while in Ivory Coast, 75% of cases were aged more than 15 years who are able to swim or wad in a river.

Most of the people surveyed were perform agricultural activities. However, we did not find any significant association with the risk of contracting BU. Among agricultural activities, planting and harvesting activities were associated with decrease risk of M. ulcerans infection. Similar results were found in Cameroon [14]. We observed that wearing a long-sleeved shirt or a long dress while performing agricultural activities did not provide significant reduction of the risk of contracting of Buruli ulcer. This observation is in accordance with the study conducted in Cameroon [14]. On the other hand, we found that wearing pants or hats is associated with reduction in the risk of mycobacterial infection. This would explain the low frequency of wounds on head and legs observed in our investigation. These results are consistent with those found in Ghana [12,38] and Ivory Coast [13].

In Australia, Lavender et al. [20] showed that mosquito bites were significantly associated with Buruli ulcer. However, we did not find any risk of M. ulcerans infection associated to mosquito

bites in Togo. In general, results of studies on mosquito bites associated with the use of mosquito coils or bednets during M. ulcerans infection are often contradictory [11,14,38,39,41].

Some studies [12,13,42] have shown that animals such as chickens, goats, cats and pigs could harbour M. ulcerans and exposure to these animals may increase the risk of contracting BU disease. During this study, we did not observe significant increase in risk of contracting BU associated with contact with domestic animals.BCG vaccine is delivered against a mycobacterium. This vaccination could therefore provide a cross-protection against M. ulcerans infection [43]. In our study, we did not observe any significant difference in the percentage of BCG vaccination scar between patients and controls. The lack of a significant association with BCG vaccination with M. ulcerans infection has been also described in the literature [12,16,43]. However, data from Benin [11], Ivory Coast [13] and Cameroon [14] showed negative correlation between BCG vaccination and BU. Studies conducted to explore this possible cross-protection have often led to contradictory results. Indeed, a multicenter study [44] conducted in the DR Congo, Ghana and Togo did not reveal any significant association between BCG vaccination and BU disease.

Attitude, behavior and belief on BU

The attitude of the participants interviewed has considerably improved with their capacity to recognize some BU symptoms and their ability to refer suspected cases to medical treatment compared to the situation five years before [33]. This finding could be attributable to several awareness campaigns in the community that had influenced their behavior toward this disease [33]. However, there remains some effort to help recognizing early symptoms by the community as well as the herbalists because 5.3% of BU patients continue to believe in herbal treatment as the first preferred treatment option. Poor individual hygiene and dirty surrounding were recognized as a potential risk factor for participants in the present study. The impact of poor hygiene and its possible role as a risk factor has been underlined in studies in Benin [11,45] and Ghana [12].

This study had some limitation. We did not reach all participants especially some BU cases due to their unavailability during the survey time. The sample size was calculated based on the proportion of households using water from unprotected sources which was higher than the prevalence of BU. The number of newly confirmed BU cases in Togo every year is low and varies from 30 to 65 patients. During the study period, we found 91 BU cases but 8 patients were not available at the survey time. The main concern with the limit number of controls was due to the fact that in many households, there were often two to three patients and exceptionally in one house up to six. In those households, it was difficult to enroll two folds of controls. Moreover, as 47% of BU patients were under 10 years, it was difficult to interview children who were not capable to describe their activities which are driven by their parent's duties. The reason we had decided to use their parents as controls sometimes.

Conclusions

Our study identified some significant risk factors for BU infection including age, bathing with water from open boreholes and receiving insect bites near a river in Zio and Yoto Districts of the Maritime Region in south Togo.

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Book Chapter

Intravenously Administered Cloxacillin-Induced Neutropenia with Eosinophilia in a Patient with Infective Endocarditis: A Case Report

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Abstract

Background: Bacteremia following *Staphylococcus aureus* is a serious clinical condition which is often associated with distant metastatic infections. One of the most dreaded complications of *Staphylococcus aureus bacteremia* is infective endocarditis. Cloxacillin is a common antibiotic prescribed for suspected staphylococcal infections and confirmed methicillin-sensitive *Staphylococcus aureus* infections. Prolonged use of cloxacillin may lead to neutropenia.

Case Presentation: A 38-year-old Sinhalese man presented to Teaching Hospital Kurunegala, Sri Lanka, complaining of a 3-week history of fever; he was found to have a pansystolic murmur over the apex of his heart. He had leukocytosis with predominant neutrocytosis. His C-reactive protein was 231 mg/l and erythrocyte sedimentation rate was 100 mm/first hour. Transthoracic two-dimensional echocardiography revealed prolapsed mitral valve with 7×13 mm vegetation over the posterior mitral valve. On the following day, three blood cultures became positive and were subsequently identified as *Staphylococcus aureus*. Intravenously administered cloxacillin 3 g 6 hourly was started. Following day 24 of intravenously administered cloxacillin, our patient developed high spike fever. His total white blood cells were: 990/mm^3 with 34% neutrophils and 22% eosinophils. His hemoglobin concentration was 9.5 g/dL and platelet count remained normal (202×10^6/mm^3). His C-reactive protein was 78 mg/l, erythrocyte sedimentation rate was 95 mm/first hour, and he was otherwise comfortable, showing no signs of sepsis beside the high grade fever. His serum was negative for filarial and *Toxoplasma* antibodies while stool was negative for oocytes and amoebic cysts. Further, his serum was negative for dengue virus, Epstein–Barr virus, cytomegalovirus, and hepatitis B antibodies. He was clinically well on day 6 after stopping cloxacillin with 44% neutrophils and 18% eosinophils. His C-reactive protein and erythrocyte sedimentation rate became normal, and there was no further plan for cardiothoracic intervention or administration of antimicrobials. He was discharged from hospital and remained well 6 months later.

Conclusion: This case report signifies the potential fatal adverse effect of cloxacillin in methicillin-sensitive *Staphylococcus aureus* infections. Leukopenia is associated with prolonged use of high doses of cloxacillin. In addition to transthoracic two-dimensional echocardiography and inflammatory markers, sequential white blood cells and differential counts would help clinicians to assess the prognosis of patients with infective endocarditis.

Abbreviations

ALP-Alkaline Phosphatase; *ALT*-Alanine Transaminase; *CRP*-C-Reactive Protein; *Echo*-Echocardiography; *ESR*-Erythrocyte Sedimentation Rate; *GCSF*-Granulocyte Colony-Stimulation Factor; *IE*-Infective Endocarditis; *MRSA*-Methicillin-Resistant *Staphylococcus Aureus; MSSA*-Methicillin-Sensitive *Staphylococcus aureus; PT*-Prothrombin Time; *PTT*-Partial Thromboplastin Time; *WBC*-White Blood Cells; *WBC/DC*-White Blood Cell/Differential Counts

Background

Bacteremia following *Staphylococcus aureus* is a serious clinical condition which is often associated with distant metastatic infections [1,2]. One of the most dreaded complications of *Staphylococcus aureus* bacteremia is infective endocarditis (IE), which has been reported to occur in 6–32% of these patients [3,4].

Cloxacillin is one of several common antibiotics prescribed for suspected staphylococcal infections and confirmed methicillin-sensitive *Staphylococcus aureus* (MSSA) infections [5]. In IE, prolonged use of antimicrobials is advocated according to the guidelines of the Infectious Diseases Society of America; thus, it warrants complete cure of IE with minimal complications. However, prolonged (> 20 days) use of cloxacillin may lead to neutropenia [6]. In this case, prolonged use of cloxacillin in MSSA-associated IE led to neutropenia which is described here.

Case Presentation

A 38-year-old Sinhalese man presented to Teaching Hospital Kurunegala in Sri Lanka complaining of a 3-week history of fever, lethargy, and fatigability. He had not had any significant clinical conditions prior to this. He did not have any food or drug allergies while he was on regular anti-worm and anti-filarial prophylaxis. On clinical examination, he was febrile (39.4 °C), pale, and found to have a pansystolic murmur over the apex of his heart. He had leukocytosis (12.4 mm^3) with predominant neutrocytosis (81%). His hemoglobin was 11.2 g/dL, C-reactive protein (CRP) was 231 mg/l, and erythrocyte sedimentation rate (ESR) was 100 mm/first hour. Transthoracic two-dimensional echocardiography (echo) revealed grade II mitral regurgitation, myxomatous, prolapsed mitral valve with 7 × 13 mm vegetation over the posterior mitral valve. Three sets of blood cultures were obtained within 1 hour from three different venipuncture sites; intravenously administered ceftriaxone was started empirically.

The following day, the three blood cultures became positive and were subsequently identified as *Staphylococcus aureus*. Intravenously administered cloxacillin 3 g 6 hourly was initiated while ceftriaxone was omitted. The (72 hours following initial culture) clearance blood cultures revealed *Staphylococcus aureus* and repeated clearance cultures following 72 hours remained negative. From that day onwards, for duration of 42 days, intravenously administered cloxacillin therapy was determined while clinical response was monitored with quarter hourly temperature, transthoracic two-dimensional echo, white blood cell/differential counts (WBC/DC), CRP, and ESR (Table 1). Further, he was on acetaminophen and chlorpheniramine malate as required.

Table 1: Timeline: fluctuation of hematological parameters, inflammatory markers, and transthoracic two-dimensional echocardiography following intravenously administered cloxacillin treatment.

	Timeline								
Parameter	**On IV cloxacillin therapy**				**Omission of IV cloxacillin**				
Hematological	**On admission**	**Day 12**	**Day 17**	**Day 22**	**Day 24**	**Day 26**	**Day 30**	**Day 32**	**Day 34**
WBC/mm^3	12.4	8.2	8.1	3.5	2.5	3.1	4.2	4.4	4.4
N (%)	81	70	65	59	34	41	44	40	50
E (%)	1	1	1	6	22	22	18	18	9
Hemoglobin (g/dL)	11.2	10.9	10.8	10.0	9.5	10.1	10.9	10.9	10.0
Platelets ($10^6/mm^3$)	219	430	400	293	202	208	281	302	290
Inflammatory									
CRP (mg/l)	231	56	29	16	78	102	68	32	8
ESR mm/first hour	100	95	88	68	95	90	78	70	56
Transthoracic two-dimensional echo									
Vegetation	Yes	Yes	Yes	Yes	No	No	No	No	No
Size (mm)	7 × 13	7 × 10	6 × 10	4 × 8	–	–	–	–	–

CRP C-reactive protein, E eosinophils, echo echocardiography, ESR erythrocyte sedimentation rate, IV intravenously administered, N neutrophils, WBC white blood cells

Following day 24 of intravenously administered cloxacillin, our patient developed high spike fever (39.6 °C) and his full blood count showed: WBC 990/mm^3 with 34% of neutrophils and 22% eosinophils. His hemoglobin concentration was 9.5 g/dL, platelet count (202×10^6/mm^3), D-dimer (320 ng/mL fibrinogen equivalent units), and both prothrombin time (PT) and partial thromboplastin time (PTT) remained normal. His CRP was 78 mg/l, ESR was 95 mm/first hour, and he was otherwise comfortable, showing no signs of sepsis beside the high grade fever. His vital signs (blood pressure and pulse) were normal. Repeat transthoracic two-dimensional echo was normal thus no vegetations were detected. Mild elevation of liver enzymes was observed and an ultrasound of his abdomen revealed no hepatomegaly: gamma-glutamyl transferase 192 IU/ml, alanine transaminase (ALT) 15 IU/ml, and alkaline phosphatase (ALP) 136 IU/ml. We omitted intravenously administered cloxacillin and kept him without antimicrobials while arranging a septic screening with a close observation of clinical parameters, WBC/DC, and inflammatory markers. His blood picture showed leukopenia with profound neutropenia and he had eosinophilia. Red blood cells and platelets were normal. He was clinically well and on day 6 after stopping cloxacillin, white blood cells (WBC) became normal with 44% neutrophils and 18% eosinophils. Liver function tests also returned to normal after cloxacillin discontinuation. He was treated with anti-pyretic as required. Subsequently, his septic screening became negative and transthoracic two-dimensional echo showed complete healing with no vegetations.

His serum was negative for filarial and *Toxoplasma* antibodies while stool was negative for oocytes and amoebic cysts. Further, his serum was negative for Epstein–Barr virus, cytomegalovirus, and hepatitis B antibodies. Subsequently his CRP and ESR became normal, and there was no further plan for cardiothoracic intervention or administration of antimicrobials. He was discharged from hospital and remained well 6 months later.

Discussion

IE if left untreated is almost inevitably fatal. Prognosis mainly depends on whether or not complications develop. Early detection and appropriate treatment of this uncommon disease can be lifesaving. The overall mortality rate has remained stable at 14.5%. To eradicate the bacteria that remain inside the vegetation in IE requires prolonged and high-dose antimicrobial treatment. For community-acquired MSSA infection in individuals who do not abuse intravenously administered drugs, the cure rate is 60–70% [7].

Neutrophils are a type of immune cell, termed granulocytes, and they are the first cell types to travel to the site of an infection. Neutrophils help fight infection by ingesting microorganisms and releasing enzymes that kill the microorganisms. Agranulocytosis is a marked and profound decrease in the number of granulocytes, or an absolute lack of granulocytes in circulating blood, typically resulting in a neutrophil count below 0.5×10^9/L [8]. Most cases (70–90%) of agranulocytosis are caused by viral infections, drugs (drug or its metabolites), chemotherapy, and radiotherapy [9]. Beta lactams and especially penicillins have been reported to cause agranulocytosis since 1946 [10], and have long been associated with the inhibition of granulopoiesis. A few antibiotics other than beta lactam, such as linezolid, trimethoprim-sulfamethoxazole, clindamycin, gentamycin, and chloramphenicol, are known to cause agranulocytosis [11]. Simultaneously, development of eosinophilia signifies parasitic infestation or development of hypersensitivity or both.

In this case scenario, we had a fair amount of doubt about the definitive cause of the emergence of pyrexia and the hematological discrepancy. Top of the list was MSSA relapse, new infection with methicillin-resistant *Staphylococcus aureus* (MRSA) or different Gram-negative bacteria or viral agent, or parasitic infestation leading to pyrexia with leukopenia and eosinophilia. Often, isolated bacterial, viral, or parasitic infections do not produce a combined picture of neutropenia with eosinophilia. In parasitic infestation, eosinophilia can

develop without neutropenia, whereas in viral infections, neutropenia can develop without eosinophilia [12]. Co-infection of viral and parasitic agent can lead to the above picture [13]. However, we excluded common viral infections and parasitic infestations which are endemic in Sri Lanka. A repeat transthoracic two-dimensional echo was normal and excluded the possibility of valvular or myocardial abscesses. Also, his D-dimer, PTT, and PT were normal; the possibility of disseminated intravascular coagulation was excluded.

Following omission of cloxacillin and our patient's significant recovery with subsiding fever and normalizing of his hematological parameters, our attention was directed toward adverse drug reactions. The impact of this case report is that it signifies the potential fatal adverse effect of a commonly used antimicrobial in MSSA infections: cloxacillin. Often this phenomenon is associated with prolonged use of high doses. A case report in 2014 described the use of cloxacillin in a patient with MSSA-IE and agranulocytosis developed in 20 days of therapy [6]. In our case it was after 24 days and the total dose was 288 g of cloxacillin. It was postulated that total dose of > 150 g was associated with the development of neutropenia [6]. Our patient was on intravenously administered ceftriaxone as well but it was given over 2 days thus the effect could be negligible.

The exact pathogenesis behind the leukopenia was less understood and two mechanisms were proposed: direct toxic effect of the drug, and immunological allergic reaction and creation of neutrophil antibodies. This patient developed leukopenia associated with neutropenia while hemoglobin and platelets remained normal. This reflects suppression only in granulocyte axis of cell genesis. Also, during the leukopenia phase our patient had eosinophilia which suggests immune-mediated hypersensitivity [6,14].

Although this is a rare phenomenon, awareness among clinicians of such adverse reactions would alert them to assess the patient periodically with serial WBC/DC. In the documented cases, including this case, the patients only developed high grade fever

with high CRP and did not develop neutropenic sepsis [6]. Also, once cloxacillin was omitted WBC/DC became normal. The prolonged use of cloxacillin with high dose in clinical practice is quite common, but the occurrence of agranulocytosis is quite rare. For development of agranulocytosis there could be an existing genetic predisposition for the development of cloxacillin-induced hypersensitivity or drug-induced myelotoxicity.

In IE, prolonged (28–42 days) intravenously administered antibiotics are required to eradicate the microbes within the cardiac vegetation. Here, following early and appropriate therapy, our patient became afebrile and clinically stable. When febrile neutropenia develops, clinicians often tend to prescribe granulocyte colony-stimulation factor (GCSF) to stimulate granulocyte formation. This was practiced commonly among patients with cancer with IE. Here, however, the probable cause of neutropenia was drug induced and therefore we omitted the offending agent and did not give GCSF [9,15].

Conclusion

Prolonged administration of cloxacillin with high doses could lead to the development of leukopenia which puts patients at risk of developing neutropenic sepsis. Vigilant attention on blood WBC/DC would help clinicians to identify the leukopenia early and withdraw the cloxacillin. In addition to transthoracic two-dimensional echo, CRP, ESR, and sequential WBC/DC would help clinicians to assess the prognosis of patients with IE.

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