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Mussel RL, De Sa Silva E, Costa AM, Mandarim-De-Lacerda CA (2003). Mast cells in tissue response to dentistry materials: an adhesive resin, a calcium hydroxide and a glass ionomer cement. J. Cell. Mol. Med. 7:171-178.

Booth M, Bundy DA, Albonico P, Chwaya M, Alawi K (1998). Associations among multiple geohelminth infections in school children from Pemba Island. Parasitol. 116: 85-93.0.

Fransiscus RG, Long JC (1991). Variation in human nasal height and breath, Am. J. Phys. Anthropol. 85(4):419-427.

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International Journal of Medicine and Medical Sciences

Table of Contents: Volume 5 Number 7 July 2013

ARTICLES

Research Articles

Hematological response in human toxocariasis patients 320
Zubair Ahmad Dar, Syed Tanveer, G. N. Yattoo, Bashir Ahmad Sofi, Showkat Ahmad Wani and Perviz Ahmad Dar

Examination methicillin-resistant *Staphylococcus aureus* (MRSA) prevalence in cockroaches from hospital in Chaharmahal-va-Bakhtiari province, Iran by polymerase chain reaction (PCR) 324
Ehsan Heidari Soureshjani and Abbas Doosti

No differences in total bilirubin levels in neonates from Tzeltal, Chol and Mestizo ethnic groups in the state of Chiapas, Mexico 330
López-Narváez María Lilia, Martínez-Hernández Fátima Cristell, Juárez-Rojop Isela Esther, López-Narváez Amelia and Tovilla-Zárate Carlos Alfonso

The risk factor indicators of malaria in Ethiopia 335
Dawit Getnet Ayele, Temesgen T. Zewotir and Henry G. Mwambi

Anti-osteoclastogenic activity of butanol fraction of rice bran extract via downregulation of MAP kinase activity and c-Fos/NFATc1 expression 348
Jungsun Moon, Seong-Hee Moon, Sik-Won Choi, Sookyeon Lee, Seong Hwan Kim and Dongsool Yim

Evaluation of National Health Insurance Scheme (NHIS) awareness by civil servants in Enugu and Abakaliki 356
Ndie Elkenah Chubike

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International Journal of Medicine and Medical Sciences

Full Length Research Paper

Hematological response in human toxocariasis patients

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The present study was carried out on the human population in Kashmir valley to study the status of hematological parameters of *Toxocara* infection. Blood samples were collected from 514 individuals; 298(57.97%) males and 216 (42.02%) females; 187 (36.38%) tested seropostive for human toxocariasis. In the present study, *Toxocara* infection and hemoglobin value were correlated. It was found that infected persons had less mean values of hemoglobin (10.23±1.5 g/dl) than uninfected individuals (10.51±1.5 g/dl). The mean erythrocyte value of infected and uninfected individuals was 4.82±0.39 × 10^6/ mm^3 (4.0-6.2, 95% CI 4.76-4.88) and 4.84±0.44 × 10^6/ mm^3 (3.4-6.7, 95% CI, 4.79-4.89) respectively. The mean total leukocyte count in *Toxocara* positive individuals was 8.73±1.45 × 10^4 and in uninfected individuals was 7.70±1.23 × 10^4, respectively. *Toxocara* infection considerably increased the total number of leukocytes. Individuals with *Toxocara* infection had higher values of eosinophil in their blood, 9.0±4.30% (range 1-23; 95% CI 8.36-9.63), where as the mean values of eosinophil in uninfected individuals was 3.22±2.47% (range 1-17.00; 95% CI 2.77-3.67).

Key words: Toxocariasis, humans, hematological study.

INTRODUCTION

Toxocariasis is a zoonotic disease caused by the ascarid of dogs and cats, the main representative of which is *Toxocara canis* (Glickman and Schantz, 1981). The eggs of *Toxocara canis* are unembryonated when passed out in the faeces of dogs into the environment. Under optimal temperature and humidity the eggs develop into embryonated eggs that are infective to both final and paratenic hosts. Infective eggs are reported to survive optimal circumstances for at least one year. Humans may acquire the infection by oral ingestion of infective *Toxocara* eggs from contaminated soil (sapro-zoonosis), unwashed hands or consumption of raw vegetables (Ahmad et al., 2002).

The disease manifests itself in three distinct forms; visceral larva migrans (VLM), ocular larva migrans (OLM) and covert toxocariasis. The signs and symptoms of VLM vary from an asymptomatic state with mild eosinophilia to a severe and potentially fatal disorder including hepatomegaly, hyperglobulinemia, pneumonitis and neurological disorders (Gillespie, 1993). The disease has a chronic state and the symptoms can even persist for more than a year. Patients with OLM also show variable clinical signs varying from asymptomatic state to acute lesions including endophthalamitis accompanying loss of vision and mass similar to retinoblastoma (Mirdha and Kokhar.,2002; Shimizu et al., 2005; Fomda et al., 2007).

Distribution of the disease is world wide. There is no definitive method in diagnosing *Toxocara* infection. As the larvae of *T. canis* are arrested in the paratenic host-larvae during migration and do not mature into adults, a stool examination of the patient will not give any clue about the infection. However, numerous studies have

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shown that immunoassay for detection of antibodies using a purified excretory-secretory antigen from the larval stage significantly improves sensitivity and specificity compared to assays using crude antigens (Fenoy et al., 1996; Kenny et al., 1995). The most widely used test, because of its high sensitivity and specificity is the enzyme linked immunosorbent assay (ELISA) in which antibodies to *T. canis* larval excretory-secretory antigens (De Savigny et al., 1979; Glickman and Schantz, 1981) or to larval extracts are measured (Fan and Suke, 2004; Glickman et al., 1985).

In India cases of human toxocariasis have been reported (Ahmad et al., 2002; Mirdha and Khokar, 2002) but there are limited studies from the Kashmir valley (Ahmad et al., 2002). The present study was conducted to determine the effect of *Toxocara* infection on hematological parameters in the human population of Kashmir valley.

MATERIALS AND METHODS

Strategically located, Jammu and Kashmir (J&K) State constitutes the Northern most extremity of India. J&K is situated between 32.17° and 36.58° North latitude and 37.26°and 80.30° East longitude. The projected population of the state is 76.77 lacs. The state with its summer and winter capital at Srinagar and Jammu respectively is divided into 14 districts. For this study, samples were randomly selected from six districts of Kashmir valley. Blood samples were collected from 514 individuals consisting of 298 males and 216 females.

Before taking a blood sample, each individual's consent was sought and obtained. Blood was collected by commercially available 5 ml disposable syringes. The blood samples collected were stored in two separate bottles; one with anticoagulant EDTA (ethylene diamine tetra acetic acid) and another without anticoagulant from which serum was later separated. The bottles with blood samples were simultaneously labeled to prevent intermixing after which the samples were transported to the laboratory for further investigation.

The samples were stored at –20°C until tested. ELISA was used for the qualitative screening of serum IgG antibodies to *Toxocara* infection. Commercially available ELISA kit which uses an inactivated purified specific excretory secretory antigen (Toxocara Micro well serum, ELISA, IVD, Research, Inc., Carlsabad, CA_92008) was used to carry out tests on microtitre plates for the detection of Anti-*Toxocara canis* antibodies IgG. The whole procedure was carried out according to the manufacturer's instructions. Optical density (OD) value was recorded in an automatic ELISA reader (Anthos) at 450 nm. The samples were considered positive if absorbance reading was equal to or greater than 0.3 OD units and negative if the absorbance value was less than 0.3 OD units.

Estimation of blood hemoglobin was done by Sahli's acid hematin method. Counting of the total leukocyte count is clinically significant when accompanied by a differential leukocyte count. Improved Neubauer chamber was used to obtain total leukocyte count. The differential leukocyte count calculates the relative proportion of 5 types of leukocytes. It is expressed as a percentage of each type of 100 leukocytes counted in a suitable area of the smear. Red blood cell count was done by improved Neubauer chamber. Simple Interactive Statistical Analysis (SISA) software was used for data analysis. The descriptive data were given as mean ± standard deviation (SD). The differences were considered to be significant when the p-value obtained was less than 0.05.

RESULTS

Blood samples were collected from 514 individuals, 187(36.38%) tested seropositive for human toxocariasis. *Toxocara* infection and hemoglobin, erythrocyte, leukocyte and eosinophil values were correlated. It was found that infected persons had less mean values of hemoglobin (10.23±1.5 g/dl) than uninfected individuals (10.51±1.5 g/dl) ($P<0.05$). The Hb value in infected persons ranged between 5-14 g/dl (95% CI 10.0-10.4) and 5.4-14.6 g/dl (95% CI 10.34-10.68) in uninfected persons. The mean erythrocyte values of infected and uninfected individuals were $4.82\pm0.39 \times 10^6/mm^3$ (4.0-6.2, 95%CI 4.76-4.88) and $4.84\pm0.44 \times 10^6/mm^3$ (3.4-6.7, 4.79-4.89), respectively.

The mean total leukocyte count in *Toxocara* positive individuals was $8.73\pm1.45 \times 10^4$ and in uninfected individuals was $7.72\pm1.23 \times 10^4$, respectively. *Toxocara* infection considerably increased the total number of leukocytes. Individuals with *Toxocara* infection had higher values of eosinophil (9.0±4.30%; range 1-23; 95% CI 8.36-9.63) in their blood, whereas the mean values of eosinophil in uninfected individuals was 3.22±2.47% (range 1-17.00; 95% CI 2.77-3.67) ($P<0.05$). This shows a threefold increase in eosinophil count on infection with *Toxocara* as shown in Table 1.

DISCUSSION

In the present study, *Toxocara* infection and hemoglobin (Hb) value were correlated and it was observed that infected individuals had lower mean Hb values as compared to uninfected ones. The reasons for this difference are numerous. Due to poverty, people are already at risk of having low Hb value and when infected by *Toxocara* conditions get aggravated. The present results are supported by many other studies such as Rayes et al. (2001) who reported Hb value of <12.5 g/dl in 88% of toxocariasis patients studied; Sharma et al. (1984) while conducting experimental work on chickens infected with *T. canis* found a significant decrease in hemoglobin; Thakur et al. (1998) and Baldisserotto et al. (1999) found hemoglobin levels of below normal values in toxocariasis patients; Arango and Fla (1998) in visceral larva migrans case also found low Hb value. Similarly, in other studies like Singh et al. (1992) and Alonso *et al.* (2000) found hemoglobin level falls below the normal value in toxocariasis patients. From the above discussion it is clear that toxocariasis is associated with a condition leading to anemia.

Leukocytes play an important role against various types of infectious diseases by lessening their effect on the human body. During any type of infection in the human body, leukocytes increase in number so as to quickly fight against the infecting pathogenic organism. In the present study, it was found that *Toxocara* infected individuals had high numbers of leukocytes compared to uninfected

Table 1. Hematological parameters in *Toxocara* seropositive and seronegative individuals.

Parameter	Type	Mean ± SD	Range	95% CI	*p*
Hemoglobin (g/dl)	+	10.23±1.5	5.0-14.0	10.0-10.4	0.040
	-	10.51±1.5	5.4-14.6	10.34-10.68	
Erythrocyte 10^6/mm^3	+	4.82±0.39	4.0 -6.2	4.76-4.88	0.62
	-	4.84±0.44	3.4 - 6.7	4.79-4.89	
Leukocyte 10^4/mm^3	+	8.73±1.45	4.50-1.35	8.73-8.83	0.00
	-	7.72±1.23	4.40-1.24	7.5-7.86	
Eosinophil %	+	9.0±4.30	1-23	8.36 -9.63	0.001
	-	3.22±2.47	1-17	2.77-3.67	

(+): Seropositive, (-): Seronegative.

individuals. Arango and Fla (1998) reported visceral larva migrans displaying a white blood cell count of 42,000 cells per mm^3. The other studies that are in agreement with the present study include Baldisserotto et al. (1999); Singh et al. (1992); Ashwath et al. (2004); Vidal et al. (2003); Xinou et al. (2003); Sommerfelt et al. (2006) and Sharma et al. (1984). Yarsan et al. (2003) while conducting an experimental work on mice infected with *Toxocara* found significant increases in leukocyte counts occurring only after 8 days of *Toxocara* infection. In the present study eosinophils levels were found to be raised in individuals who were *Toxocara* seropositive and the difference was significant. These results are supported by other authors like Sommerfelt et al. (2001) who in an experimental work found that eosinophils were significantly higher in pigs inoculated with *Toxocara* eggs compared to control groups. Figueiredo et al. (2005) observed extremely significant association between seropositivity and eosinophilia. Tonz et al. (1983) found eosinophilia as an excessive and sustained symptom in 6 clinical observations. Giacometti et al. (2001) found all *Toxocara* seropositive individuals, with the exception of the subject in the control group, showed an increase above normal in the number of eosinophils per unit volume of peripheral blood. Alonso et al. (2000) found very high values of total eosinophilia in *Toxocara* seropositive children. Marmor et al. (1987) found that all cases of *Toxocara* infection had higher mean percentages of eosinophils than controls (2.6±4.3% in cases vs. 1.3±2.8% cells/mm^3) in controls; mean difference = 1.3% and higher absolute number of eosinophils (211±36.2 cells/mm^3 in cases vs. 121±290 cells/mm^3 in controls; mean difference = 90 cells/mm^3). Havasiova et al. (1993) found that clinical manifestations of *Toxocara* in the studied group of patients were highly variable. The most frequent were leukocytosis and eosinophilia (46%). Berrocal (1980) reported that in many cases of toxocariasis eosinophilia was predominantly high and acted as a diagnostic feature in these cases. Hayashi et al. (2005) reported that 24 of the 34 subjects (70.6%) had hypereosinophilia with five of these showing extreme hypereosinophilia. Santos et al. (2004) found that individuals with higher eosinophil counts presented a greater frequency of anti- *Toxocara* antibodies and the relation between eosinophilia and *Toxocara* infection was found to be statistically significant. Sommerfelt et al. (2001) reported a significant relation between eosinophil count and groups inoculated by *T. canis* as compared to control. Various other studies that report a correlation between eosinophil count and *Toxocara* infection include Ashwath et al. (2004), Arango and Fla (1998), Yarson et al. (2003), Xinou et al. (2003), Vidal et al. (2003), Sharma et al. (1984), Sugane and Oshuma (1984), Inan et al. (2006), Alonso et al. (2000), Yokoi et al. (2003), Shimizu et al. (2005), Thakur et al. (1998), Singh et al. (1992), Baldisserotto et al. (1999), Azuma et al. (2002) and Taranto et al. (2003). Thus from the present study and the above discussion it is concluded that while going for the *Toxocara* serological examination in humans, patients should be advised for the eosinophil count so as to get the proper diagnosis of the disease easier.

In the present study, the effect of *Toxocara* infection on the total erythrocyte count showed no significant difference between infected and uninfected individuals. Similar results have been reported by other workers such as Sommerfelt et al. (2001) who found that in *T. canis* infected pigs there were no significant changes in RBC count. Similarly various other studies supporting the present observation include Xinou et al. (2003), Inan et al. (2006), Ashwath et al. (2004) and Alonso et al. (2000). Therefore it was found that in the case of *Toxocara* infection in humans there is no effect on RBC count.

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Examination methicillin-resistant *Staphylococcus aureus* (MRSA) prevalence in cockroaches from hospital in Chaharmahal-va-Bakhtiari province, Iran by polymerase chain reaction (PCR)

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This study gives the reported prevalence of cockroaches and the medical importance of the insects due to the transmission of nosocomial infections. Besides, one of the major reasons of hospital and community infections all over the world is methicillin-resistant *Staphylococcus aureus* (MRSA). The aim of this study was to determine inset the hospital cockroaches as the main factor for MRSA infections transmission and also determine antibiogram pattern MRSA. In this study, going to hospitals, over 100 cockroaches were collected using hand and Telesticky methods and enriching the intended strain on a specific medium. Then was a designed primer for mecA gene and amplification in polymerase chain reaction (PCR). The disk diffusion method was used for specifying resistance patterns in line with guidelines of Clinical and Laboratory Standards Institute (CLSI). Results showed that a total of 44 (62.86%) of 100 samples were contaminated with *S. aureus* isolated from cockroaches hospitals, also with molecular sieve of PCR, an addition of 8 (19.56%) of the strain contained the mecA gene. The overall resistance of isolated MRSA strains to antimicrobial agents was 8 (100%) for methicillin, 7 (87.5%) for cefixime and 6 (75%) for vancomycin, which had more resistance, respectively. This study implies that cockroaches, as a potential factor in transmission of MRSA function and medical resistance pattern of MRSA are different in different areas.

Key words: Antibiogram, resistance, methicillin-resistant *Staphylococcus aureus* (MRSA), Chaharmahal-va-Bakhtiari hospitals, polymerase chain reaction (PCR).

INTRODUCTION

In nineteenth century, the role in the transmission of disease to humans from insects was demonstrated (Service, 1979). Among these insects are cockroaches, which can influence human health; the reason of which medical importance of insects is of top priority among the food habits and stooling at various intervals, poor bites especially between the toes, that is potentially dangerous to human health (Zaeim et al., 2008). Cockroaches are normally carriers of about 40 different species of pathogenic bacteria that infect vertebrates (Vatandoost and Mousavi, 2009). They have ability to transfer at least 7 species of intestinal worms, among which include bilharziver, teniasis, askaryazys, necatoriasis (Doroodgar et al., 2005) and the transmission of poliomyelitis and

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Aspergillus fungi. More than what is thought, naturally, cockroaches are infected with pathogenic bacteria, thus causing leprosy, yeki, bloody diarrhea, pimples, Hungarian unitary tract abscesses, and food poisoning. Almost all cockroaches have co-existence with 150 species of bacteria and 60 species of yeast species, and 90 species protozoa and 45 species of the pathogenic ring worm (Salehzadeh et al., 2007). So far, numerous pathogenic bacteria, including *Salmonella* spp, *Shigella* spp, *Campylobacter* spp, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* have been isolated from cockroaches (Fotedar et al., 1991; Foster 2004).

Staphylococcus aureus is a Gram-positive bacterium, and about 20% of the human population carries this commensal bacterium without any clinical symptoms. However, *S. aureus* has the potential to cause a broad range of infections, including wound infections, skin abscesses, pneumonia, bacteremia, meningitis, and toxic flick syndrome (Iandolo 1989). It is well documented that strains of *S. aureus* produce a variety of extracellular protein toxins, including enterotoxins, toxic shock syndrome toxin 1 (TSST-1), exfoliative toxin (ET), hemolysins, and coagulase. The two ETs, exfoliative toxins A and B (ETA and ETB), in continued or independently, are implicated in the cause of staphylococcal scalded-skin syndrome (Iandolo 1989). In the early 1960s, only 2 years after the introduction of methicillin as a drug versus *S. aureus* infections, hospital-acquired methicillin-resistant *S. aureus* (HA-MRSA) strains were first isolated.

Since the 1990s, virulent association-acquired MRSA (CA-MRSA) strains, which are characterized by the presentment of the toxin Panton-valentine leukocidin (PVL), and have been encountered in the community and health care (Deurenberg and Stobberingh 2008). MRSA is an eminent cause of nosocomial infections worldwide and has also emerged as a community-associated pathogen (Chambers and Deleo, 2009). The risk agents for infection with these pathogens that are unique to the hospital population are well established (Lowry, 1998). Over the last few decades, there has been an enormous gain and emergence of MRSA as a cause of infection in the community in patients who have never been hospitalized and have no other known risk factors, for MRSA infection causes significant worry. Infections caused by these organisms have been described in earlier studies as having specified strain, virulence, and epidemiologic properties (Diep et al., 2006a; Diep et al., 2006b; Tenover et al., 2006). According to serological classification, to date, six staphylococcal enterotoxin (SE) groups have been recognized: staphylococcal enterotoxin A, B, C, D and E (SEA SEB, SEC, SED and SEE) (Martins et al., 2007) and the recently described SEH (Mehrotra et al., 2000). Community-associated MRSA (CA-MRSA) strains differ from health care-associated *S. aureus* strains in that they are more often improved from skin and soft tissue sources, with at least two clones, designated USA300 and USA400.

For both groups of patients with CA-MRSA (*S. aureus* resistant to methicillin patients) and patients with CA-MSSA (sensitive methicillin patients with *S. aureus*) skin infections were the most common location (80% of patients infected with MRSA and 93% of patients infected with MSSA), other infection sites included the respiratory tract (13% for MRSA-infected patients and 6% for MSSA infected patients), blood (4% for MRSA infected patients and 1% for MSSA infected patients), and urine (3% for MRSA-infected patients) (Davis et al., 2007). The *S. aureus* strains resistance to these antibiotics is attributed to the presence of mecA, whose product is a 78 kDa protein called penicillin binding protein 2a (Mehrotra et al., 2000), whose transcriptional control may be mediated by a repressor (mecI) and a sensor/inducer (mecR1). The mecI-mecR1-mediated induction of mecA takes various hours, rendering the strains phenotypically susceptible in spite of the presence of the resistance gene. Therefore, it has been proposed that the full resistance to b-lactams observed in many contemporary clinical *MRSA* strains requires a non-functional mecI-mecR1 regulatory system. The mecA gene is embedded in a large chromosomal cassette (the SCCmec element) for which several structural types have been characterize.

The MRSA characteristic phenotype is due to the presence of mecA which encodes a penicillin-binding protein (PBP, PBP2a), with degraded affinity for b-lactams (Oliveira and De Lencastre, 2011). Methicillin resistance is associated with the presence of a chromosomal mobile genetic element entitled the staphylococcal cassette chromosome mec (SCCmec) (IWG-SCC 2009). So far has been identified five different types of Sccm (V, IV, III, II and I). 20 KP to 68 KP have the variable type III which is the most dangerous (Martins et al., 2007; Zhang et al., 2005). Some epidemic MRSA clones, typically expressing full b-lactam resistance, carry SCCmec elements that contain an intact mecI-mecR1 locus (for example, SCCmec types II and III) (Oliveira and De Lencastre 2011). Of cause, one of the efficient ways of controlling the spread of MRSA is through determination of the genotypic characteristics as well as species genetic relatedness in geographic other regions (Gomes et al., 2006; Enright et al., 2002). Since the hospital environments provide them with suitable temperature, humidity and a ready source of food, presence of cockroaches here is uncommon.

Many researches in recent years have shown that drug resistant bacteria are of great importance in hospitals that are potential carriers of microorganisms, and their presence makes the problem more significant. The aim of this research was to use PCR to determine the prevalence MRSA in cockroaches from a hospital in Chaharmahal VA Bakhtiar, Iran. The study also tried to determine the pattern of antibiotic resistance in summer of 2011.

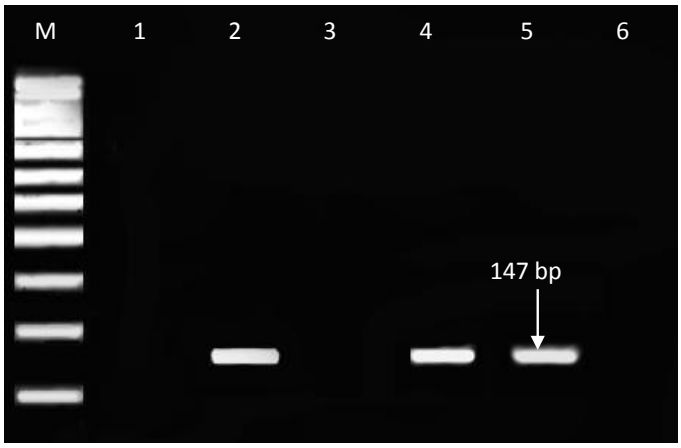

Figure 1. Agarose gel stained with ethidium bromide, with PCR products of MRSA isolates. Line M is 100 bp DNA ladder, line 1 is negative control, line 2 is positive control, line 3 and 6 are MRSA negative samples, line 4 and 5 are MRSA positive samples.

Table 1. Antimicrobial resistance of isolated *S. aureus* strains from different samples to studied antibiotics.

Antimicrobial agent	Resistance (%)
Nitrofurantoin	25 (56.8)
Methicillin	32 (72.72)
Vancomycin	26 (59.09)
Cefixime	39 (88.63)
Trimethoprim sulfamethoxazole	16 (36.37)
Amikacin	3 (6.81)
Ciprofloxacin	1 (2.27)
Gentamcin	5 (11.36)

MATERIALS AND METHODS

Sample collection

A total of 100 cockroaches were collected over a period of two month, from different wards of six hospitals located in Chaharmahal VA Bakhyiari province (Hajar Shahrekord, Ayatollah Kashani Shahrekord, Shohada Farsan, Imam Javad Naghan, Imam Reza Lordagan and Valiasr Boroujen). In these cockroaches' samples, 21, 24, 14, 12, 14, 15 specimens were obtained from hospitals, respectively. The collection of sample was done using manual and sticky trap methods from kitchens, wardrobe departments, and pediatric patients and they were transmitted to the Biotechnology Research Center of Shahrekord Islamic Azad University using separate sterile tube to prevent any contamination mixing of the samples.

Picking up the cultivation of susceptibility

The collected samples were incubated in sterile conditions on beard parker environment for 24 h at 37°C.

Antimicrobial susceptibility testing

Antimicrobial susceptibility profiles were determined by the dilution method on Mueller-Hinton agar, according to the guidelines of Clinical and Laboratory Standards Institute (CLSI 2012). The antimicrobial agents tested included nitrofurantoin, methicillin, vancomycin, cefixime, trimethoprim sulfamethoxazole, amikacin, ciprofloxacin and gentamcin. Clinical and Laboratory Standards Institute breakpoints were used for minimum inhibitory concentrations (MIC) interpretation (CLSI 2012). The results were interpreted after 24 h of incubation at 37°C, as sensitive, intermediately sensitive, and resistant according to the zone diameter around each antibiotic disk.

PCR

To confirm the presence of MRSA mecA gene, PCR test was performed. Therefore, the final volume of 25 microliter PCR reaction containing 2 microliter of deoxyribonucleic acid (DNA) template, $MgCl_2$ concentration of 1.5 mM, 2 micromoles dNTPS, 2 mMol of each primers mecA-F: 5´-AACAGGTGAATTATTAGCACTTGTAAG-3 and mecA-R: 5´-ATTGCTGTTAATATTTTTTGAGTTGAA-3, and a single DNA polymerase was performed; thermal PCR conditions consisted of 5 min at 95°C and then 31 cycles Vasrsht initial temperature of 94°C, temperature of 61 and 72°C connector at each end for 1 min, and final extension was for 5 min at 72°C. For analysis of PCR products, the amplification products were analyzed in 1% agarose gel electrophoresis. Electrode buffer was TBE (Tris-base 10.8 g, 89 mM, boric acid 5.5 g, 2 mM EDTA (pH 8.0) 4 ml of 0.5 MEDTA (pH 8.0), with all components combined in sufficient H_2O and stired to dissolve). Gels were stained with ethidium bromide, aliquots of 10 µl of PCR products were applied to the gel. Constant voltage of 80 V for 30 min was used for products separation. After electrophoresis, images were obtained in ultra violet imager (UVI) doc documentation systems (UK).

Statistical analysis

The numbers of cockroaches presenting airsacculitis and the prevalence of re-isolation of *S. aureus* from the swap were analyzed by the chi-square test using the statistical package for social sciences (SPSS) 17 (SPSS Inc. Chicago, IL, USA) software. The probability level for significance was $p \leq 0.05$.

RESULTS

The quality of extracted DNA from samples was examined by electrophoretic analysis through a 2% agarose gel. Of 100 cockroaches collected from hospital in this study, 44 samples (62.86%) were infected with *S. aureus*. The mecA gene of MRSA was successfully amplified with the MecA-F and MecA-R primers. Agarose gel electrophoresis of the PCR amplified products is show in Figure 1. From 44 *S. aureus* samples that assayed by PCR in this research, only 8 samples (19.56%) were positive to MRSA (147 bp fragment).

There were antimicrobial susceptibility pattern of isolated *S. aureus* strains to studied antibiotics as shown in Table 1. The overall susceptibility of isolated *S. aureus* strains to antimicrobial agents was 97.73% for ciprofloxacin, 93.19% for amikacin, 88.64% for gentamicin, 63.63% for trimethoprim sulfamethoxazole, 43.2% for nitrofurantoin, 40.91% for vancomycin, 27.28%

Table 2. Antimicrobial resistance of isolated MRSA strains to studied antibiotics.

Antimicrobial agent	Resistance (%)
Nitrofurantoin	5 (62.5)
Methicillin	8 (100)
Vancomycin	6 (75)
Cefixime	7 (87.5)
Trimethoprim sulfamethoxazole	3 (37.5)
Amikacin	0 (0.0)
Ciprofloxacin	0 (0.0)
Gentamcin	0 (0.0)

Table 3. Comparison of resistance pattern of *S. aureus* strains to antimicrobial agents in different studies.

Antimicrobial agent	Resistance (%) present study (Iran, 2011)	Resistance (%) Ekrami & Samarbafzadeh (Iran, 2007; Ekrami et al., 2010)	Resistance (%) R Baral & B Khanal (Nepal, 2008; Baral et al., 2011)	Resistance (%) Khalili (Iran,2008; Soltani et al., 2010)
Nitrofurantoin	56.8	-	-	-
Methicillin	72.72	-	26	-
Cefixime	88.63	-	-	-
Trimethoprim sulfamethoxazole	36.37	-	-	-
Ciprofloxacin	2.27	68.2	11.36	41.9
Gentamicin	11.36	30.6	22	44.9
Amikacin	6.81	25	-	39.3
Vancomycin	59.09	0	0	0

(Ekrami et al., 2010), (Baral et al., 2011); (Soltani et al., 2010).

for methicillin, and 11.37% for cefixime. According to these results, ciprofloxacin, amikacin, and gentamicin were the most effective agents against isolated *S. aureus*. It also showed that isolated *S. aureus* of cockroaches from hospital has more resistance to vancomycin rate of 59.09%, rather than other studies. Antimicrobial susceptibility of MRSA isolates from cockroaches is shown in Table 2. Gentamcin (100%), amikacin (100%), ciprofloxacin, and trimethoprim sulfamethoxazole (62.5%) were the most effective agents against these isolates. Comparison of resistance pattern of *S. aureus* strains to antimicrobial agents in different studies is shown in Table 3.

DISCUSSION

S. aureus is a gram-positive bacterium that can be part of the normal human microbiota as a colonizer of the mucosal membranes and skin. However, *S. aureus* has the potential to reason a wide range of infections, including wound infections, skin abscesses, pneumonia, bacteremia, meningitis, and toxic shock syndrome (Foster, 2004). MRSA is the origin cause of nosocomial infection worldwide (Lowry, 1998). MRSA is a main nosocomial isolate in hospitals that is responsible for higher morbidity. Sources of MRSA are infected patients, asymptomatic colonized hospital party and hands of health care workers serving in intensive care units (ICUs) on MRSA positive cases (Nickersin et al., 2009). Variegation types of the staphylococcal cassette chromosome mec (SCCmec) are known to confer methicillin resistance on the human pathogen *S.*

Aureus (Chlebowicz et al., 2010). *S. aureus* is responsible for skin infection in mice (Nippe et al., 2011) and finding genes in *S. aureus* strain isolated from raw and pasteurized milk (Rall et al., 2008).

The results of the present conciliate revealed a contamination of almost all cockroaches collected from hospitals with different microorganism that is significantly higher in collation to control group (Salehzadeh et al., 2007). Although in the present study, only one isolate showed infections to cockroaches, *S. aureus* and MRSA, but reports from other studies indicate that infections can be seen in more bacteria. For instance, (Fotedar et al., 1991) showed that bacterial pathogens like *Klebsiella* spp, *P. Aeruginosa* and *S. aureus* were infections to cockroaches. This is similar with the study of Salehzadeh et al. (2007) that reported *S. aureus* of (16.5%) isolated from cockroaches of hospital of Hamadan Iran (Salehzadeh et al., 2007).

In a study performed by Fotedar (1991) in India, fewer than 5% of all *S. aureus* isolates were reported as MRSA (Fotedar et al., 1991). In the present study, also, a high percentage of test cockroaches (62.86%) were showed to carry *S. aureus*, some of them are of medical importance. However, only few numbers of cockroaches collected from hospitals (19.56%) showed to have MRSA contamination. It appears that cockroaches infected with MRSA are important endemic pathogen in our hospitals. Virtually all *S. aureus* strains were susceptible to penicillin G until 1994 when the first reports of penicillin-resistant *S. aureus* were reported, and today virtually all strains of *S. aureus* are resistant to natural penicillins.

Methicillin and other resistant penicillins were developed to treat infections reasoned by penicillin-resistant *S. aureus* and met with initial success; however, over time, strains of MRSA began to spread (Rice, 2006). Table 3 shows reported resistance pattern of *S. aureus* strains to antibiotics in some other studies compared to the present document. In a related study in Taleghani Burn Hospital of Ahvaz, Iran Medical University, MRSA was resistant to gentamycin (64.3%), ciprofloxacin (81.2%) (Ekrami and Kalantar, 2007). But in our study, MRSA was resistance to ciprofloxacin (0.0%) and gentamycin (0.0%) were high levels. It appears that MRSA has emerged as an important endemic pathogen in our hospitals. According to the reports, burst of MRSA is increasing in Europe. In Austria, 21.6%; Belgium, 25.1%; Spain, 30.3%; and France, 33.6% of isolated *S. aureus* strains are methicillin resistant (Ekrami et al., 2010). In a survey performed in Pakistan, 61.29% of isolated *S. aureus* strains were resistant to oxacillin (Farzana and Hameed 2006). In a survey performed by H. Khalili in Iran, 100% of strains sensitive to vancomycin was recorded among isolated *S. aureus* strains (Soltani et al., 2010). In the study performed in Pakistan, 100% of isolated *S. aureus* strains were sensitive to vancomycin (Farzana and Hameed, 2006). The difference in the current study was that about 75% of MRSA and 59.09% *S. aureus* isolates were sensitive to vancomycin. In Iran, vancomycin resistance rates of 11, 21, and 42.5% have been reported in different studies that have evaluated pediatric population (Mamishi et al., 2005; Haghi-Ashteiani et al., 2007; Kalantari et al., 2007).

CONCLUSION AND RECOMMENDATION

Finally, as stated earlier, our study showed isolated *S. aureus* of cockroaches from hospital have more resistance to vancomycin rate of 59.09%, than other studies. Amikacin, ciprofloxacin, and gentamcin seems to be the only antimicrobial agent that showed 100% sensitivity and may be used as the drug of choice for treating multidrug resistant MRSA infections. However, regular monitoring of amikacin, ciprofloxacin, and gentamcin sensitivity, and routine testing of other newer glycopeptides like teicoplanin should be carried out. Furthermore, the regular surveillance of hospital associated infections including monitoring antibiotic sensitivity pattern of MRSA and formulation of definite antibiotic policy may be helpful for reducing the incidence of MRSA infection.

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Full Length Research Paper

No differences in total bilirubin levels in neonates from Tzeltal, Chol and Mestizo ethnic groups in the state of Chiapas, Mexico

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Ethnicity is an important factor for neonatal hyperbilirubinemia. This study explored total serum bilirubin levels in three ethnic groups of Chiapas, Mexico (Tzeltal, Chol and Mestizo groups). The aim of the study was to examine whether the level of total serum bilirubin is associated with any specific ethnic group. This study was conducted in 113 neonates of three different ethnic groups from Chiapas, México. Total serum bilirubin was not significantly higher in neonates of any of the three ethnic groups (p = 0.38). In addition, when we analyzed for differences between ethnic and mestizo groups, no significant differences were encountered (p = 0.20). Our results showed no significant differences in total serum bilirubin levels among three ethnic groups in a population sample from Chiapas, Mexico. However, more studies are necessary in which larger samples must be considered to determine conclusively this non association.

Key words: Bilirubin, neonates, jaundice.

INTRODUCTION

Neonatal hyperbilirubinemia affects 60% of full-term newborns and remains a significant cause of hospital readmission during the first week of life (Burgos et al., 2008; Grupp-Phelan et al., 1999; Hanchard et al., 2011). This is manifested by jaundice, the yellow-orange tint found in the sclera and skin of infants, with total serum bilirubin levels greater than 5 mg/dl (Schwartz et al., 2011). Despite the cause- and effect relationship, the terms neonatal hyperbilirubinemia and neonatal jaundice are used fairly interchangeably.

Various reports in the literature ascertain that severe neonatal jaundice is caused by isoimmune hemolytic disease (ABO and/or Rh incompatibility), infections, prematurity, low birth weight, polycythemia, closed-space bleedings such as cephal hematoma, metabolic disturbances, and intestinal obstruction. Sex, race, premature

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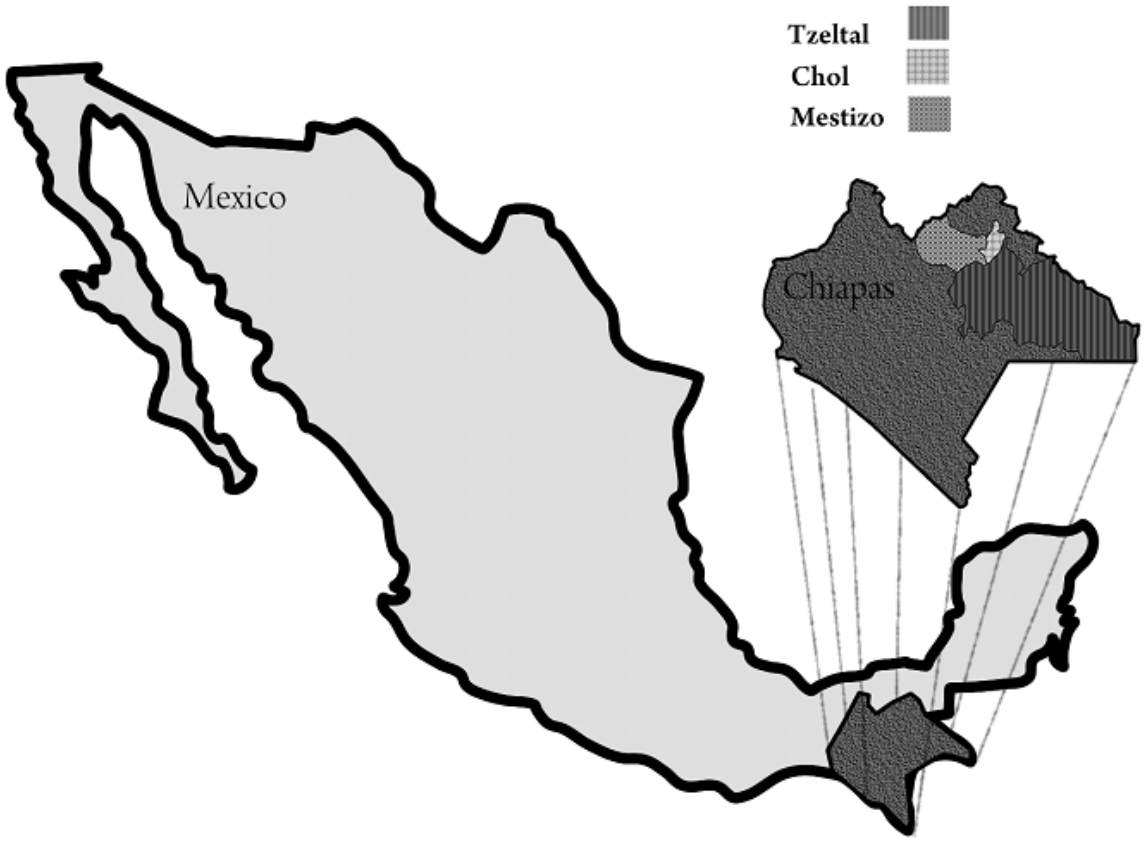

Figure 1. Map of Mexico showing the three different regions when was performed this study.

labor, insufficient fluid and caloric intake, type of delivery, premature rupture of membranes, maternal and neonatal medications and low birth weight are risk factors for jaundice. However, there are significant differences in the prevalence and severity of neonatal jaundice and hyperbilirubinemia among various populations (Setia et al., 2002; Desandre et al., 2006; Narter et al., 2011; Wasser and Hershkovitz, 2010). For example, one study reported differences in serum bilirubin levels between neonates of East Asian ancestry and European or African ancestry (Linn et al., 1985). Similarly, other study identified differences between Chinese ethnic ancestry and non-Chinese infants (Huang et al., 2009). In addition, several studies conducted in different populations have encountered that hyperbilirubinemia is associated with mutations in the gene UGT1A1 (Kadakol et al., 2000; Zhang et al., 2007; Chang et al., 2009; Carvalho et al., 2010; D'silva et al., 2012). To explore possible differences among populations, we conducted a study in three ethnic groups of the Mexican population to examine whether the bilirubin level is associated with any particular ethnic group.

METHODOLOGY

Sample

This was a prospective study conducted at the General Hospital of Yajalón Chiapas, México, during a 6-month period, starting from January, 2008. Approval for this study was granted by the bioethics committee of the General Hospital of Yajalón and the Health Ministry of Chiapas, Mexico. The initial sample consisted of 320 neonates from the General Hospital of Yajalón who were invited to participate in the study. However, only a total of 113 term neonates with ages older than 37 weeks and 2,500 g were eligible for enrollment. Hemolytic anemia, hypoxia/asphyxia, dehydration/ vomiting, cephalohematoma, sepsis, liver dysfunction, hypothyroidism were excluding criteria. Bilirubin analysis (direct, indirect and total) was determined within the first 24 h of life of the neonates. The study was performed in three different populations of the southeast of Mexico: two Indian and one urban population. Populations were identified according to Mexican National Institute of Statistic, Geography, and Informatics (INEGI) regulations (Figure 1). An urban area is defined as a settlement consisting of more than 15,000 inhabitants. An Indian population is classified on the bases of tribal language and self-declared ethnic identity. The life in rural areas has less than 2,500 inhabitants and low socioeconomic level.

Ethnia A: Tzeltal is an ethnic group that inhabits a broad

Table 1. Clinical characteristics of neonates from different ethnias.

Parameter	Tzeltal	Chol	Mestizo
Gender: male (%)/female (%)	15 (13.1) /9 (7.8)	33 (29.2)/36 (31.8)	12 (10.5)/8 (7.6)
Gestational age (weeks)	39.29±1.15	38.82±2.78	39.22±0.91
Weight at delivery (kg)	3.08±34.8	2.96±35.5	3.13±42.9
Mode of delivery: cesarean section/vaginal delivery	18 (15.9)/6 (5.3)	46 (40.7)/23 (20.4)	12 (10.6)/8 (7.1)
Apgar score at 1 min	9 (8/9)	9 (8/9)	9 (8/9)
Apgar score at 5 min	10 (10/10)	10 (10/10)	10 (10/10)

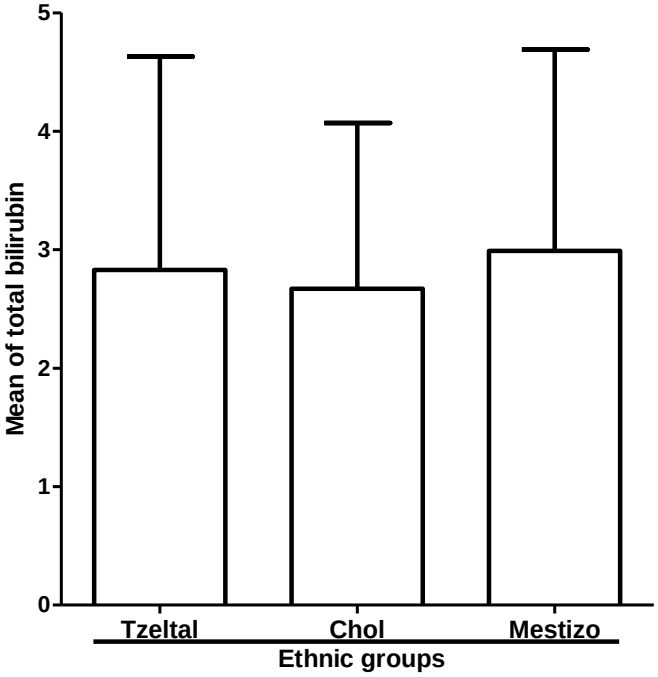

Figure 2. Mean and standard deviation of total bilirubin level Tzeltal, Chol and Mestizo groups living in Yajalón, Chiapas, México.

geographic area in southeast Mexico. The region extends across several municipalities of Chiapas. We selected mainly three municipalities: Yajalón, Chilón and Bachajón. This indigenous group lives in small villages, or pueblos, located in mountainous terrain with difficult access (900 meters above sea level on average). This group speaks its own particular dialect. Twenty four neonates from these three municipalities whose mother tongue was Tzeltal, were included.

Ethnia B: Chol; currently, this indigenous group lives in many municipalities of the states of Chiapas and Tabasco in Mexico. However, we studied neonates whose mothers live in semi-urban or rural locations of Tumbala and Tila municipalities in which Chol is their native tongue. Sixty nine neonates were included.

Group C: Mestizo; a third group constituted of 20 neonates whose mothers lived in the city of Yajalón. The inhabitants of Yajalón are mostly Spanish-speaking subjects who are considered mestizos. In this group, we wanted neonates whose mothers lived in urban settlements.

Bilirubin determination

Quantitative determination of total bilirubin concentration was performed in blood serum using a Beckman synchron analyzer according to previously published methods described elsewhere (Abdel Ghany et al., 2012)

Statistical analysis

The data are described as number and percentages for qualitative variables and as mean and standard-deviation for quantitative variables. A general linear model analysis of variance (ANOVA) was performed to identify differences in the quantitative variables of interest. Independent sample t-tests were used to compare ethnic and mestizo groups. The level of significance was set at $p < 0.05$.

RESULTS

Among the 113 neonates who participated in this study, 60 were male and 43 female. The mean gestational age was 38.98 weeks with a standard deviation (SD) of 2.27. Of these, the mean weight at delivery was 3.020 kg with an SD of 37.15 g. Demographic parameters and total bilirubin levels in the study groups are shown in Table 1, according to their socioeconomic characteristics. The mean of total bilirubin level for the Tzeltal, Chol and Mestizo groups was 2.83 ± 1.8, 2.67 ± 1.4, and 2.99 ± 1.7 mg/dl, respectively (Figure 2). Differences between groups were not significant ($F = 0.29$, $p = 0.38$). Therefore, we investigated the existence of a potential difference between ethnic and mestizo populations. However, significant differences were not observed (mean of Tzeltal and Chol groups: 2.72 ± 0.16 mg/dl, $t = 0.67$, $p = 0.20$). Finally, as well as a not significant correlation between the level of total bilirubin and gender, gestational age and weight at delivery was encountered ($p > 0.05$) (Table 2).

DISCUSSION

To examine the level of bilirubin is essential, since this

Table 2. Correlation between at level of total bilirubin and gender, gestational age and weight at delivery.

Parameter	Pearson´s correlation	Significance (2-tailed)
Gender	-0.59	0.539
Gestational age	-0.049	0.60
Weight at delivery	0.003	0.97

information can predict cerebral palsy (chroreoathetotic type), sensorineural hearing impairment, psychological impairment, and disturbances of visual perception (Chen et al., 2006). In addition, it has been recently described that neurological damage due to severe neonatal jaundice is increasing mainly to early discharge from the hospitals (Gazzin and Tiribelli, 2011). Chol and Tzeltal populations live in mountainous terrain with difficult access, so neonates are readmitted to hospitals only after severe jaundice. Therefore, we wanted to identify whether there was a group of major risk for severe neonatal jaundice in this region of Mexico.

In this study, we explored total bilirubin levels in three ethnic groups in the Mexican population. To our knowledge, this is the first report assessing differences in total bilirubin levels among ethnic groups in southeast Mexico. The three groups selected have differences in culture, including lifestyle, diet and possibly gene structure. However, we encountered no significant differences in the level of total serum bilirubin among the three ethnic groups. There are some possible explanations to account for this finding. First, the size of the sample was small. We observed that most of the parents in these populations refused to have their children enrolled in the study; hence future studies must include larger samples to determine more conclusively possible differences among these ethnic groups. Second, the sample could consist of a homogeneous population. One study, analyzing the frequency of the *UGT1A1* promoter gene in a sample from 14 states of Mexico did not find significant differences (Arambula and Vaca, 2002). However, this study included Mexican mestizos and did not analyze any ethnic group in particular.

It has been well documented that differences in development exist according to the various ethnic groups with respect to total serum bilirubin. For example, there are differences between black and white infants in the United States. Black infants as a group exhibit lower levels of total serum bilirubin than white infants (Newman et al., 1990). Similarly, differences have been reported between neonates of East Asian ancestry and European or African ancestry (Linn et al., 1985), or between white and mixed East Asian infants (Setia et al., 2002). Conversely, we could not find differences in total bilirubin levels among the three ethnic groups in the population of Chiapas, Mexico.

In the same form, we analyzed if the level of bilirubin could be associated with gender, mode of delivery or gestational age. However our analysis revealed no significant difference. Previous studies have reported these not to be related (Chang et al., 2009; Chen et al., 2006). One possibility is what the level of bilirubin is associated with; other factors could be genetics or clinic condition. Finally, this study has some limitations. First, we did not perform follow-up studies of the neonates studied. Second, we did not go to the communities to enroll the neonates included in this study, we only included neonates of mothers that came to the hospital for medical care. Finally, we did not consider sociodemographic factors such as the diet or lifestyle.

Conclusion

We did not find differences in total serum bilirubin concentration among the three ethnic groups of Chiapas, Mexico. Similarly, we did not encounter differences between the ethnic and mestizo populations. This outcome suggests that the level of total serum bilirubin may not be associated with a particular ethnic group. However, further studies must analyze this non association in larger samples in these and other ethnic groups of Mexico.

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Full Length Research Paper

The risk factor indicators of malaria in Ethiopia

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This study evaluates the effects of socio-economic, demographic and geographic indicators on the malaria rapid diagnosis test (RDT), using the baseline malaria indicator survey of 2007. This survey covered the Amhara, Oromiya and Southern Nations, Nationalities, and People's Region (SNNPR) of Ethiopia. A total of 224 clusters of, on average, 25 households each were selected. In total, 28,994 individuals participated in the survey. A generalized linear mixed model was used to analyze the data where the response variable was the presence or absence of malaria using the RDT. The results showed that for households with toilet facilities, clean drinking water and more living space, the chances of testing positive for malaria RDT decreased. Moreover, using malaria nets and spraying the house walls with anti-mosquito were found to be effective control measures.

Key words: Cluster sampling, interaction effect, mixed model, odds ratio, rapid diagnostic test.

INTRODUCTION

While malaria has long been a cause of human suffering and mortality in Sub-Saharan Africa (Eisele et al., 2010), in Ethiopia the problem is particularly severe. Here, it is the major cause of illness and death (Schabenberger and Gotway, 2005), with 75% of the total area being malarious (Cressie, 1991), and approximately 68% of the Ethiopian population living in these affected areas. Annually, about 4 to 5 million Ethiopians are affected by malaria (Federal Ministry of Health (FMH), 2004a; World Health Organization (WHO), 2006a). Malaria transmission in Ethiopia is seasonal, depending mostly on altitude and rainfall, with a lag time varying from a few weeks before the beginning of the rainy season to more than a month after the end of the rainy season (Deressa et al., 2003; Tulu, 1993).

Malaria epidemics in Ethiopia are relatively frequent (WHO, 2006b; Zhou et al., 2004), involving highland or highland fringe areas, mainly 1,000 to 2,000 meters above sea level (Adhanom, 2006; FMH, 2006; Tulu, 1993). Malaria transmission peaks bi-annually from September to December and April to May, coinciding with the major harvesting seasons (FMH, 2004a). This seasonality has serious consequences for the subsistence economy of Ethiopia's countryside and for the nation in general. Early diagnosis and prompt treatment is one of the key strategies in controlling malaria. For areas where laboratory facilities are not available, clinical diagnosis is widely used (FMH, 2004b; WHO, 1999). To diagnose malaria, microscopy remains the standard method. However, it is not accessible and affordable in most peripheral health facilities. The recent introduction of rapid diagnosis test (RDT) for malaria has become a significant step forward in case detection, management and reduction of unnecessary treatment in Ethiopia (Tekola et al., 2008).

In order to estimate the prevalence of malaria parasites in Ethiopia, a population based survey was conducted in 2006/2007. Rapid diagnostic tests as well as the conventionally accepted diagnostic tests using standard microscopy of peripheral blood slides were used for this survey. Both tests use *finger-stick* or *venous* blood. The level of disagreement in this survey between the results of microscopy and RDT was studied by Tekola et al. (2008) and found to be insignificant.

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The objective of this study is to identify the socio-economic, demographic and geographic risk factors associated with the prevalence of malaria obtained from the rapid diagnosis tests.

METHODS AND MATERIALS

Study design

The Carter Center (TCC) conducted a baseline household cluster malaria survey in Ethiopia in 2007. The questionnaire was developed as a modification of the malaria indicator survey (MIS) household questionnaire. The questionnaire had two parts; the household interview and malaria parasite form.

For the baseline household cluster malaria survey which was conducted by TCC, a multi-stage cluster random sampling was used. By assuming the lowest measurement of prevalence malaria indicator, the sample size was estimated. Therefore, for TCC baseline household cluster malaria survey in Amhara, Oromiya and the Southern Nations, Nationalities and People's (SNNP) regions of Ethiopia which was conducted in 2007, the design was a population-based household cluster survey. Based on these clusters, zone-level estimates of indicators were obtained for Amhara region, and sub-regional estimates were obtained for Oromiya and SNNPR. Furthermore, the sampling design was involved to select households within each first-stage cluster, or Kebele (smallest administrative unit in Ethiopia). From the 224 selected Kebeles, 25 households were chosen, from which even-numbered households were selected for the malaria (RDT). All individuals in these 12 households (even-numbered households) were eligible for individual interviews. Furthermore, each room in the house was listed separately. By using the mosquito nets as a guide, it was possible to determine the number of persons sleeping in each room. This information was useful in determining the number of sleeping rooms both within and outside the house. In addition to the number of rooms and number of nets, the persons sleeping under each net were listed. Further studies on the sampling procedure for the survey were studied by different researchers (Emerson et al., 2008; Shargie et al., 2008).

Malaria parasite testing was performed on consenting residents. The blood sample for malaria RDT was collected by taking finger-prick blood samples from participants. The RDT used was ParaScreen which is capable of detecting both *Plasmodium falciparum* and other *Plasmodium* species. Participants with positive rapid tests were immediately offered treatment according to national guidelines.

Variable of interest

Response variables

The outcome of interest is the RDT result. RDTs assist in the diagnosis of malaria by detecting evidence of malaria parasites in human blood and are an alternative to diagnosis based on clinical grounds or microscopy, particularly where good quality microscopy services cannot be readily provided. Thus, the response variable was binary, indicating that either a person was positive or not positive.

Independent variables

The independent predictor variables consisted of baseline socio-economic, demographic and geographic variables, which were collected from each household. The socio-economic variables were the following: main source of drinking water; time taken to collect water; toilet facilities, availability of electricity, access to radio and television, total number of rooms, main construction material of the rooms' walls, main construction material of the room's roof and main construction material of the room's floor, incidence in the past 12 months of anti-mosquito spraying, use of mosquito nets and total number of nets. Geographic variables were region and altitude, and demographic variables were gender, age and family size. Of these variables, age and sex were collected at the individual level, while altitude, main source of drinking water, time taken to collect water, toilet facilities, availability of electricity, radio, television, total number of rooms, main construction material of walls, roof and floor, incidence of anti-mosquito spraying and use of mosquito nets were all collected at the household level.

The statistical model

A generalized linear mixed model (GLMM) was used to analyze the data. Classical linear models can be generalized using the generalized linear models (GLMs) to the exponential family of sampling distributions. These models have an immense impact on both theoretical and practical aspects in statistics. The term 'mixed' in the GLMMs means that the random effects and the fixed effects are mixed together to get a modified model. This can overcome the over-dispersion in the data and at the same time, accommodate the population heterogeneity. Therefore, the addition of random effects allows accommodating correlation in the context of a broad class of models for non-normally distributed data. These models become more applicable in practical situations. The logistic regression model, which includes the mixed effects, is a common choice for analysis of multilevel dichotomous data. In the GLMM, this model utilises the logit link, namely:

$$g(\mu_{ijk}) = logit(\mu_{ijk}) = log\left[\frac{\mu_{ijk}}{1-\mu_{ijk}}\right] = \eta_{ijk},$$

The conditional expectation $\mu_{ij} = E(Y_{ijk}|v_i, x_i)$ equals $p(Y_{ij} = 1|v_i, x_{ij})$, i.e., the conditional probability of a response given the random effects. Here, Y_{ijk} corresponds to the i^{th} respondent in the j^{th} household within k^{th} probabilistic sampling unit (PSU). Therefore, this model can also be written as:

$$P(Y_{ijk} = 1|v_i, x_{ijk}, z_{ijk}) = g^{-1}(\eta_{ijk})$$

Where, the inverse link function $g^{-1}(\eta_{ij})$ is the logistic cumulative distribution function (cdf), namely:

$$g^{-1}(\eta_{ijk}) = [1 + exp(-\eta_{ijk})]^{-1}.$$

Table 1. Type 3 analysis of effects for the GLMM.

Effect	Num DF	F value	P > F
Age	1	10.16	0.0014
Gender	1	0.12	0.7257
Family size	1	75.32	<0.0001
Region	2	0.02	0.9761
Altitude	1	215.47	<0.0001
Main source of drinking water	2	6.59	0.0014
Time to collect water	3	7.46	<0.0001
Toilet facilities	2	5.2	0.0055
Availability of electricity	1	17.61	<0.0001
Availability radio	1	2.82	0.0732
Availability television	1	4.5	0.034
Number of rooms/person	1	38.49	<0.0001
Main material of the room's wall	2	12.94	<0.0001
Main material of the room's roof	2	2.07	0.1262
Main material of the room's floor	2	13.37	<0.0001
Spraying of anti- mosquito	1	986.9	<0.0001
Number of months room sprayed	1	944.72	<0.0001
Use of mosquito nets	1	11.62	0.0027
Number of nets/person	1	13.48	0.0002
Age and gender	1	0.027	0.9784
Main source of drinking water and main material of the room's roof	4	4.57	0.0004
Gender and use of mosquito nets	1	11.59	<0.0001
Time to collect water and main material of the room's floor	4	14.57	0.0024
Gender & main source of drinking water	1	33.46	<0.0001
Gender and main material of the room's floor	2	5.67	0.0035
Gender and spraying anti-mosquito spray	1	849.57	<0.0001
Use of mosquito nets and number of nets per person	1	849.57	<0.0001
Age, gender and source of drinking water	4	8.42	<0.0001
Age, gender and availability of electricity	2	7.8	0.0004

Num DF = Number difference.

The logistic distribution simplifies parameter estimation because the probability density function (pdf) is related to the cdf (Agresti, 2002).

The survey logistics model is an alternative statistical methodology (Natarajan et al., 2008) used to identify factors affecting the malaria risk. Studies conducted by Ayele et al. (2012), using survey logistic method, concluded that malaria epidemic in Amahara, Oromia and SNNP regions of Ethiopia is associated with the socio-economic, demographic and geographic factors (Ayele et al., 2012). But this model is survey based, whereas the Kebeles are chosen at random which could result in some variability between the sampling units. Such a study of the identification of the socio-economic, demographic and geographic risk factors is helpful to identify households who are in a critical need of intervention. Generalized linear mixed models (GLMM) explore the idea of statistical models that incorporate random factors into generalized linear models. GLMMs add random effects or correlations among observations to a model, where observations arise from a distribution in the exponential family. The generalized linear mixed model has many advantages. The use of GLMMs can allow random effects to be properly specified and computed, and errors can also be correlated. In addition to this, GLMMs can allow the error terms to exhibit non constant variability while also allowing investigation into more than one source of variation. This ultimately leads to greater flexibility in modelling the dependent variable.

RESULTS

Model selection was achieved by first including into the model all predictor variables and then evaluating whether or not any interaction terms needed to be incorporated. This was determined by fitting to the model, one at a time, each of the interaction terms formed from the predictor variables, and retaining in the model only those interaction terms which were significant. This process continued until the final maximal model was obtained. The final chosen model for the malaria rapid diagnosis test contained all main effects as well as six two-way interaction terms, and two three-way interaction terms. The final model is presented in Table 1.

Age, family size, altitude, main source of drinking water, time taken to collect water, availability of toilet facilities, availability of television, number of rooms per

person, main construction material of the rooms' walls, roof and floors, incidence in the past 12 months of anti-mosquito spraying, number of months the room sprayed and total number of nets per person were found to be significant main effects. From these main effects, the following were involved in the interaction effects: main source of drinking water; time to collect water; availability of electricity; main construction material of the rooms' walls, roof and floor; incidence of anti-mosquito spraying; and the use of mosquito nets. There are two three-way and eight two-way significant interaction terms. The three-way interaction term is between age, gender and main source of drinking water and between age, gender and availability of electricity. The two-way interaction terms are between source of water and roof material; between number of nets per person and use of mosquito nets; between gender and availability of electricity; between gender and floor material; between time to collect water and construction material of room's floor; between gender and application of anti-mosquito spray; and between gender and number of months the room was sprayed. The interpretation of the results is presented as follows.

Tables 2 and Table 3 presents odds ratio estimates associated with age, gender, family size, region, altitude, toilet facilities, main source of drinking water, time to collect water, availability of electricity, radio and television, number of rooms per person, main construction material of room's roof, wall and floor, application of anti-mosquito spray, number of months the room sprayed, use of mosquito nets and number of nets per person. Our result reveals that malaria risk is high for young household members (OR = 0.992, P-value < 0.0002). Based on the results, for a unit increase in family size, the odds of positive RDT for individuals increases by 3.76% (OR = 1.0376, P-value < 0.0001). Furthermore, for a unit increase in altitude, the odds of positive RDT decreases by 2.2% (OR = 0.978, P - value <0.0001). With reference to individuals with no toilet facility, malaria RDT was seen to be positive for more individuals with toilet with flush (OR = 0.894, P-value = 0.0141) followed by pit latrines (OR = 0.878, P-value = 0.005). Moreover, for a unit increase in the number of total rooms, the odds of malaria diagnosis test for individuals decreased by 5.5% (OR = 0.945, P-value = 0.004).

Interaction effects

Figures 1 and 2 shows the distribution of malaria RDT against the main source of drinking water for both males and females, respectively. As age increased, positive malaria diagnosis was less likely for males than for females who were using protected, unprotected and tap water for drinking. Furthermore, as age of respondents increased, malaria RDT was less likely to be positive for individuals who used tap water for drinking (OR = 0.98, P - Value < 0.0001) for males and (OR = 1.077, P - Value < 0.0001) for females. More specifically, positive malaria diagnosis rates increased with age for females whereas it decreased for males as age increased (Figures 1 and 2). The figures further show that the gap in the RDT between respondents using unprotected, protected and tap water for drinking widens with increasing age.

The relationship between age, gender and availability of electricity is presented in Figure 3. As the figure indicates, positive malaria RDT decreases as age increases for both male and female respondents, whether or not they had access to electricity. However, the rate of decrease was not the same for males and females after controlling for other covariates in the model. The rate of increase for females who responded positively to having electricity was 9.14% higher than the other categories (OR = 1.0914, p-value < 0.001). Probabilities for this interaction are presented in Figure 3.

Interaction effects between main source of water and main construction material of the room's roof is presented in Figure 4. From the figure, it is clearly seen that with respondents who reported using tap water as well as protected and unprotected water for drinking, positive rapid diagnosis of malaria was significantly higher when the roof of the house was thatched, followed by those who occupied a stick and mud roof and finally respondents living in a house with a corrugated iron roof. The difference in RDT between the respondents' use of tap, protected and unprotected sources of drinking water and having a thatch or stick/mud roof was particularly significant. It has also shown that for a corrugated iron roof, positive RDT was significantly lower for respondents who reported using tap water for drinking than for those who were using protected and unprotected water. The other two-way interaction effect which is significant is between the time taken to collect water and main construction material of the room's floor (Table 1). This result is presented graphically in Figure 5. Positive RDT was significantly higher in a room with an earth or dung and plaster floor than in one with cement or wooden floors for respondents who took < 30 min and > 90 min to collect water. But for respondents who took less than 90 min to collect water and had a cement floor, positive rapid diagnosis is low. Furthermore, with respondents who took between 30 to 40 min to collect water, there was lower positive RDT for respondents with an earth or dung and plaster floor and a wooden floor.

The relationship between the main construction material of the room's floor and gender for a household is presented in Figure 6. As the figure indicates, positive RDT was significantly higher for males than females with respondents who reported having an earth or dung and plaster floor (OR = 4.911, P-value = 0.001) as well as for

Table 2. Estimates of odds ratio for main effects.

Effect	Estimate	OR	95% CI		P-value
			Lower	Upper	
Intercept	0.622	1.863	1.369	2.536	<0.0001
Age	-0.009	0.992	0.987	0.996	0.0002
Gender (Ref. Male)					
Female	-0.027	0.973	0.637	1.487	0.8995
Family size	0.037	1.038	1.018	8.118	<0.0001
Region (Ref. SNNP)					
Amhara	0.004	1.044	0.972	1.036	0.8271
Oromiya	0.002	1.072	0.963	1.043	0.9053
Altitude	-0.007	0.978	0.945	0.998	<0.0001
Main source of drinking water (Ref. protected water)					
Tap water	1.591	4.909	1.892	7.751	<.0001
Unprotected water	0.725	2.065	1.066	3.902	0.031
Time to collect water (Ref. less than 30 min)					
30 - 40 min	0.721	2.056	1.066	3.900	0.031
40 - 90 min	1.470	4.349	2.284	8.373	<0.0001
> 90 min	0.069	1.071	0.959	1.065	0.6932
Availability of toilet facility (Ref. No facility)					
Pit latrine	-0.130	0.878	0.694	0.940	0.005
Toilet with flush	-0.112	0.894	0.610	0.956	0.0141
Availability of electricity (ref. no)					
Yes	0.166	1.181	0.987	1.133	0.1098
Availability of radio (ref. yes)					
No	-0.022	0.978	0.980	1.009	0.4328
Availability of television (ref. yes)					
No	-0.104	0.901	0.845	0.960	0.0013
Number of rooms/person	-0.057	0.945	0.908	0.982	0.004
Main material of room's wall (Ref. cement block)					
Corrugated metal	-0.329	0.719	0.700	0.740	<0.0001
Mud block/stick/wood	-0.322	0.725	0.570	0.922	0.0086
Main material of room's roof (Ref. Corrugate)					
Thatch	0.006	1.006	0.995	1.018	0.0269
Stick and mud	0.045	1.046	1.016	1.077	0.0024
Main material of room's floor (Ref. /Local dung plaster)					
Cement-floor	-0.174	0.840	0.624	1.132	0.2532
Wood-floor	-0.136	0.872	0.657	1.158	0.3456
Use of anti-mosquito spray (ref. No)					
Yes	-0.396	0.673	0.656	0.690	<0.0001
Number of months the room sprayed	-0.053	0.949	0.945	0.953	<0.0001
Use of mosquito nets (ref. No)					
Yes	-0.009	0.991	0.999	1.019	0.0778
Number of nets/person	-0.034	0.966	0.949	0.984	0.0002

Table 3. Estimates and odd ratios for interaction effects.

Effect	Estimate	OR	95% CI		P-value
			Lower	Upper	
Main source of drinking water and main material of the room's roof (ref. Protected water and cement block)					
Tap water and mud block/stick/wood	-0.034	0.967	0.944	0.991	0.006
Tap water and corrugated metal	-0.264	0.768	0.626	0.829	0.019
Unprotected water and Mud block/stick/wood	-0.008	0.992	0.966	1.000	0.020
Unprotected water and Cement block	-0.032	0.968	0.906	1.035	0.549
Time to collect water and material of room's floor (ref. less than 30 min and earth/local dung plaster)					
Greater than 90 min and Cement	-0.039	0.962	0.857	1.079	0.5048
Greater than 90 min and Wood	-0.294	0.745	1.201	1.500	<0.0001
Between 30 - 40 min and Cement	-0.016	0.985	0.980	1.053	0.3901
Between 30 - 40 min and Wood	0.145	1.156	1.147	1.165	0.0048
Between 40 - 90 min and Cement	-0.172	0.842	1.226	1.151	<0.0002
Between 40 - 90 min and Wood	0.200	1.221	1.312	1.137	0.3901
Gender and main source of drinking water (ref. male and protected water)					
Female and tap water	0.0169	1.017	0.941	1.099	0.0488
Female and unprotected water	-0.0795	0.924	0.854	0.999	0.0467
Gender and material of room's floor (ref. male and earth/local dung plaster)					
Female and cement	-0.0175	0.983	0.619	0.998	0.0408
Female and wood	0.2741	1.315	0.859	2.014	0.0075
Gender and use of mosquito nets (ref. male and yes)					
Female and no	-0.034	0.967	0.964	0.969	<0.0001
Gender and use of anti-mosquito spray (ref. male and no)					
Female and yes	0.0018	1.002	0.985	1.030	0.0055
Number of nets per person and use of mosquito nets (ref. No)					
Yes	0.00491	1.005	1.000	1.010	0.0467
Age and gender (ref. Male)					
Age and female	0.0336	1.034	0.992	1.002	0.4011
Age, gender, main source of drinking water (ref. male and protected water)					
Female and tap water	-0.00098	0.999	0.998	1.000	0.0119
Female and unprotected water	0.00199	1.002	1.001	1.003	<0.0001
Age, gender and electricity (ref. Male and yes)					
Female and no	0.00335	1.003	0.995	1.105	0.0003

those who reported having a wooden floor in their house (OR = 2.039, P-value = 0.031). There was however, no significant difference in positive RDT between females and males who reported having a room with a cement floor. The interaction effect between gender and main source of drinking water is presented in Figure 7. The figure shows that the risk of malaria for households using unprotected water is significantly higher than for those households who reported having protected and tap water for both males and females. Moreover, for female members of the household, the risk of malaria was higher for those households who reported having unprotected

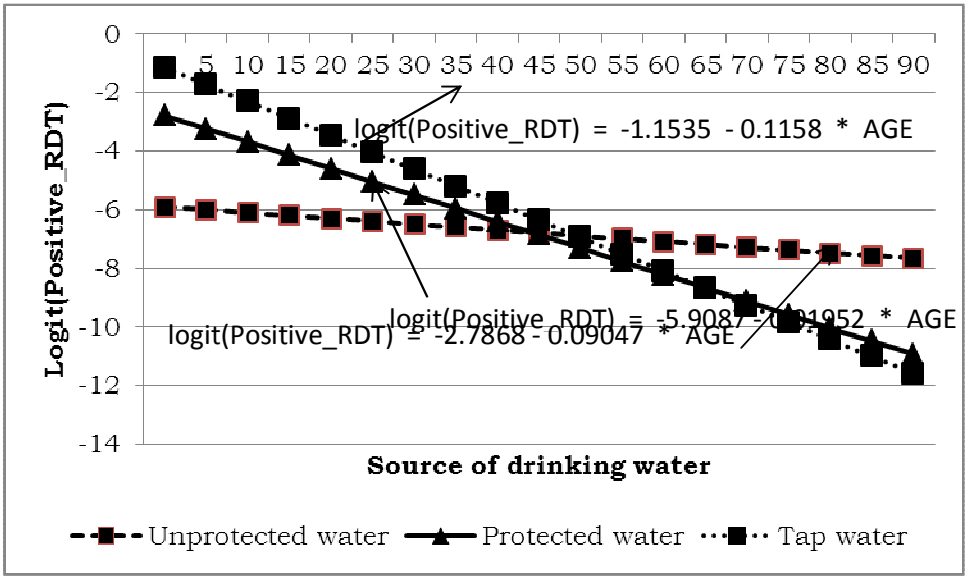

Figure 1. Log odds associated with rapid diagnosis test and age for male respondents with source of drinking water.

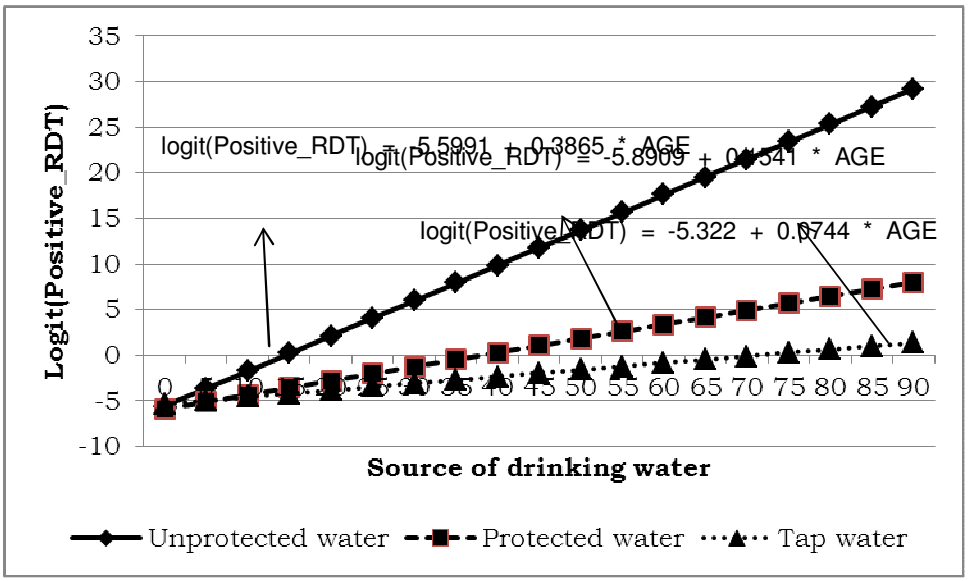

Figure 2. Log odds associated with rapid diagnosis test and age for female respondents with source of drinking water.

water.

Figure 8 presents the interaction effect between the use of anti-mosquito spray and gender for individuals. Prevalence of malaria was significantly higher for male than for female respondents who were living in a house treated with anti-mosquito spray. For males living in a house, which was not treated with anti-mosquito spray, the positive malaria result was significantly higher than it was for females. Similarly, the interaction effect between use of mosquito nets and gender is presented in Figure 9. As the figure indicates, the risk of malaria is higher for males than for females using mosquito nets when sleeping. As the number of mosquito nets increased, the risk of malaria was less likely for household members with and without nets. However, the risk of malaria was found to be much lower for individuals as the number of nets increased (Figure 10). This figure shows that for individuals with and without the use of mosquito nets, the

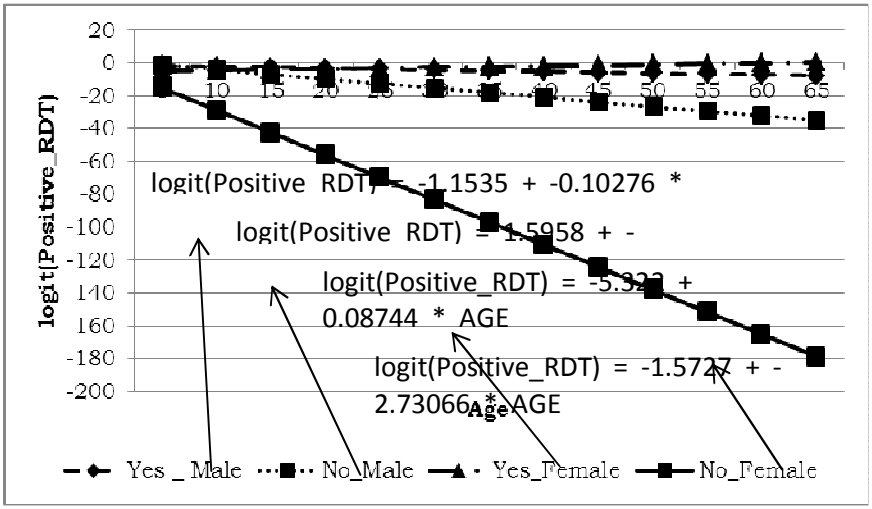

Figure 3. Log odds associated with rapid diagnosis test with age for male and female respondents with availability of electricity.

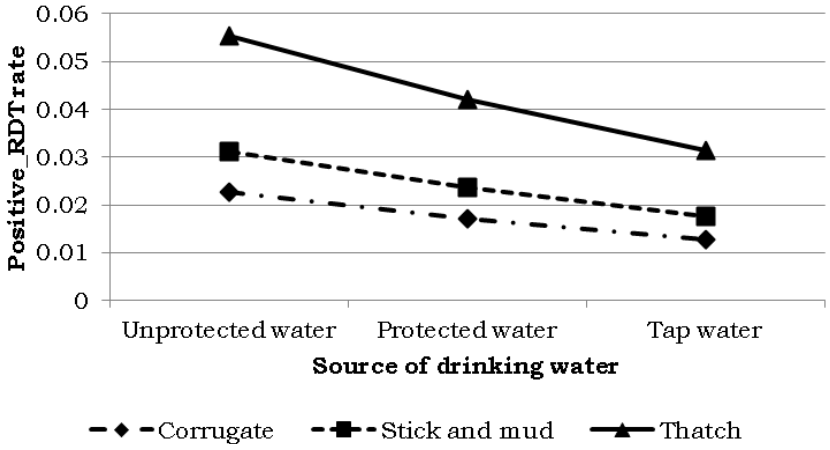

Figure 4. Log odds associated with rapid diagnosis test and source of drinking water with material of the room's roof

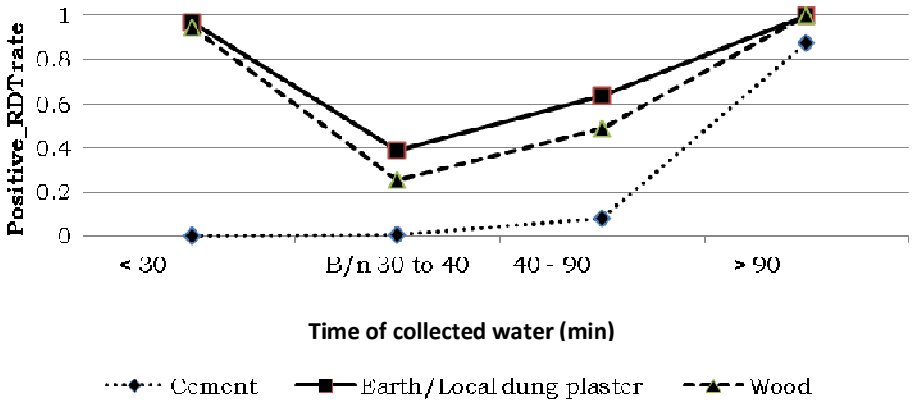

Figure 5. Log odds associated with rapid diagnosis test and time to collect water with material of the room's floor.

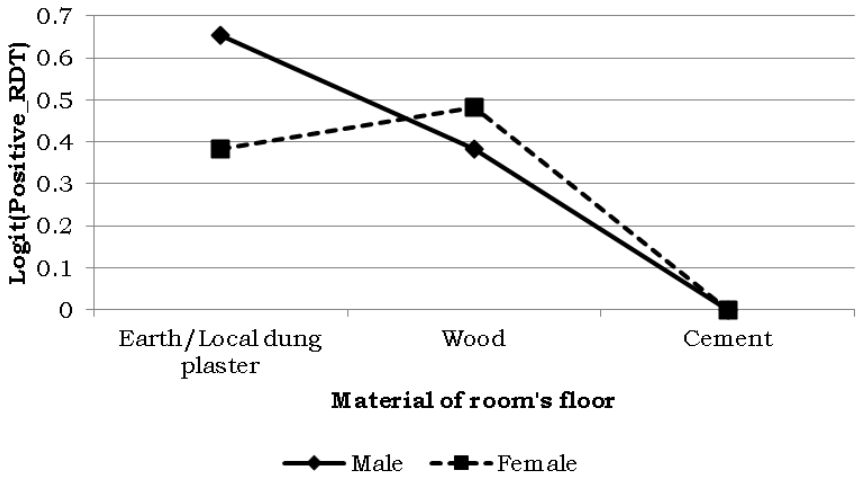

Figure 6. Log odds associated with rapid diagnosis test and material of room's floor with gender.

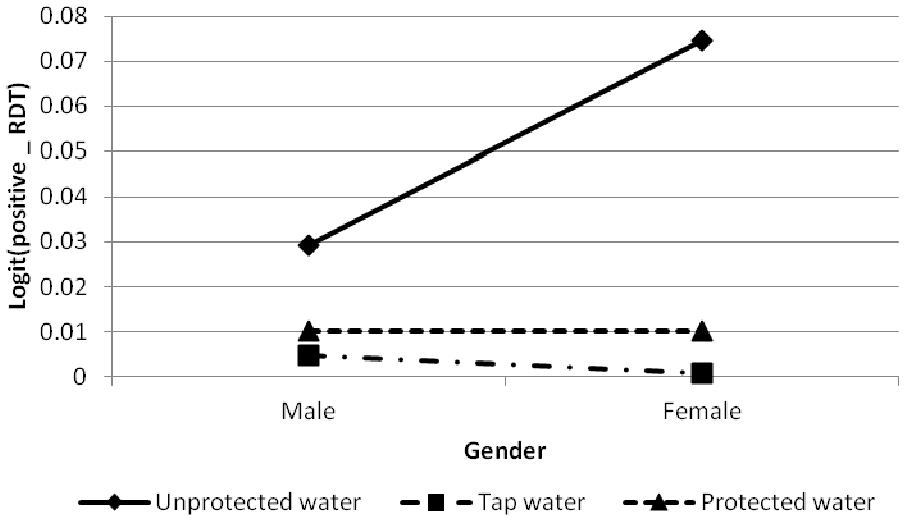

Figure 7. Log odds associated with rapid diagnosis test and main source of drinking water with gender.

risk of malaria decreased as the number of net ownerships in the household increased.

DISCUSSION

Malaria is normally referred to as a disease of poverty and related to poor socio-economic factors (Hay et al., 2004). Malaria disproportionately affects poor people who cannot afford treatment or have limited access to health care. Families and communities are then trapped in a downward spiral of poverty (Worrall et al., 2002). Since poverty is related to socio-economic factors, it is important to understand the linkages between malaria and poverty. Identifying the factors that increase the risk of malaria can be used to guide government policy-makers into creating and implementing more effective policies to tackle the disease.

SAS version 9.2 was used for the analysis of the data. Because of the nature of the methodology of the study and socio-economic, demographic and geographic variables are related. This might cause the confounding

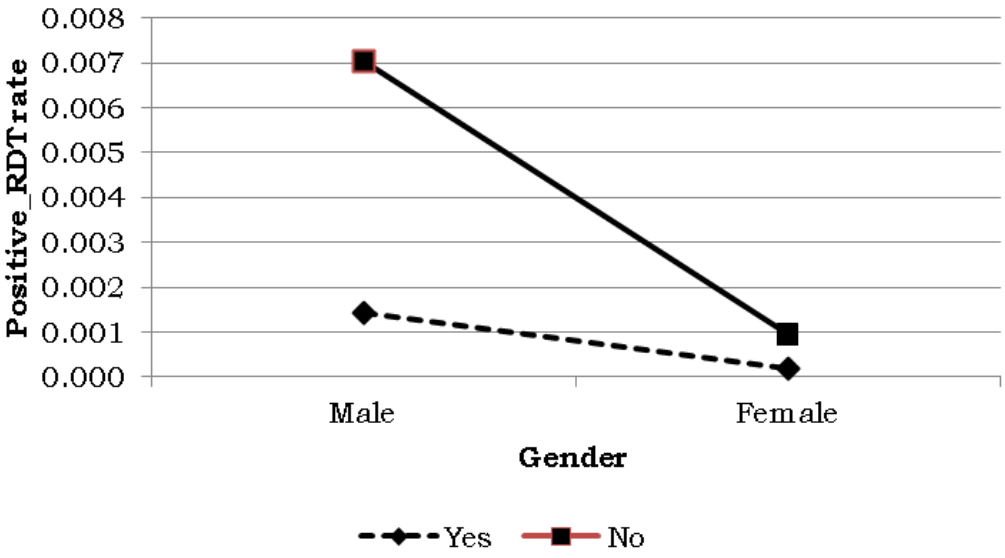

Figure 8. Log odds associated with rapid diagnosis test and anti-mosquito spraying of respondents with gender.

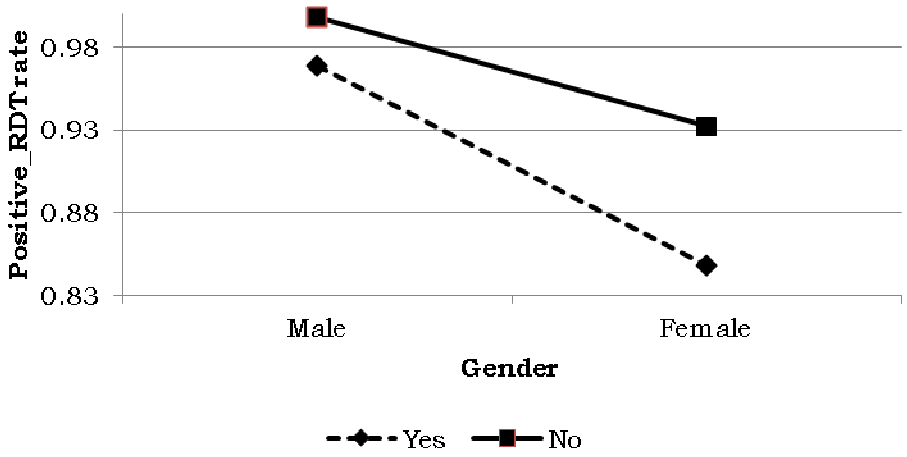

Figure 9. Log odds associated with rapid diagnosis test and use of mosquito nets with gender at individual level.

problem. Therefore, to avoid confounding effects, the model was fitted in two steps. The model was fitted to each predictor variables one at a time. In stage two, the significant predictors were retained in the model. In addition to the main effects, possible combinations of up to three-way interaction terms were added and assessed to further avoid and mitigate the problem of confounding.

Majority of studies conducted so far have suggested that malaria could be linked to poverty. The global distribution of malaria also supports this claim because malaria is concentrated to the poorest continents and countries. Therefore, our study supports the fact that malaria is related to poverty. The study indicates that socio-economic, demographic and geographic factors are responsible for the transmission of malaria. These factors are age, family size, region, altitude, main source of drinking water, time taken to collect water, toilet facilities, availability of electricity, availability of radio, total number of rooms, main construction material of the room's walls, main construction material of the room's floor, use of anti-mosquito spray, use of mosquito nets and total number of nets were the major factors associated with malaria RDT results. In addition to the main effects, three-way and two-way interaction effects were identified. The three-way interactions were between age, gender and main source of drinking water and age, gender and availability of electricity. The two-way interaction effects were between main source of drinking water and main construction

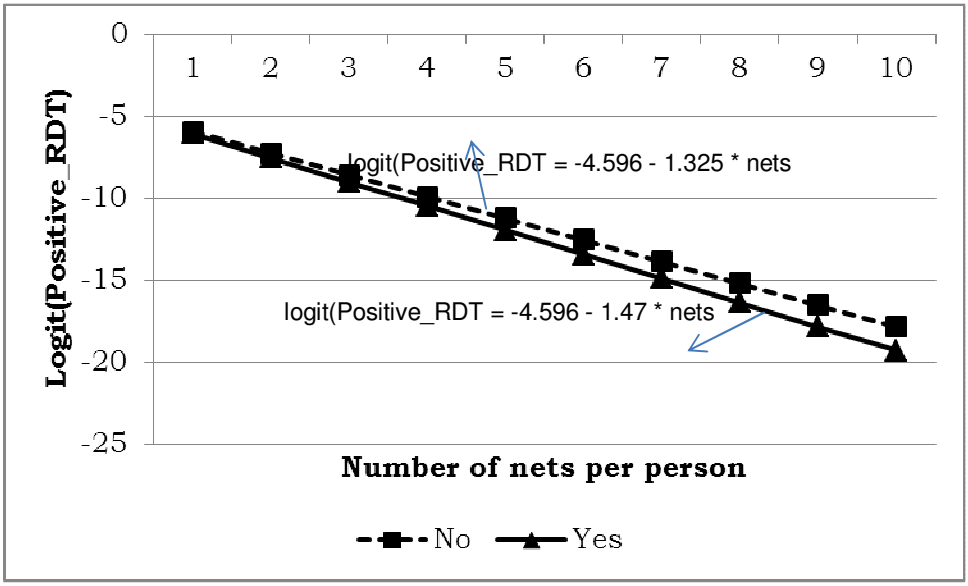

Figure 10. Log odds associated with rapid diagnosis test and use of mosquito nets with number of nets per person.

material of the room's roof, time taken to collect water and main construction material of the room's floor, age and gender, gender and main source of drinking water, gender and availability of electricity, and gender and main construction material of the room's floor.

In the present study, the effect of socio-economic factors shows that residents with no toilet facilities were found to be at more risk of malaria than those with toilet facilities. Additionally, malaria prevalence is low for households with a greater number of rooms in the house. On the other hand, having more mosquito nets over beds was found to be one way of reducing the risk of malaria. The prevalence of malaria for households with access to clean water was found to be less. Malaria rapid diagnosis was found to be higher for those respondents living in thatched houses, or ones with stick and mud roofs. Therefore, having a house with a corrugated iron roof was found to reduce the risk of malaria. Furthermore, the prevalence of malaria for households with earth and local dung and plaster floors was found to be higher. Moreover, the treatment of walls of houses with anti-mosquito spray was found to be one means of reducing the risk of malaria.

Based on demographic factors associated with malaria, our findings showed that females and children are at a greater risk. Furthermore, the malaria prevalence rate was found to be less for households with fewer people in the house. Malaria prevalence was similarly associated with geographic factors. The association between malaria and altitude showed that malaria prevalence is higher for households who are living at lower altitudes.

The result of this study supports the result from the majority of previous studies. These studies were conducted to understand the distribution of malaria. Moreover, these studies have suggested that malaria could be linked to poverty. Therefore, better understanding of the relationships between malaria and poverty is important to design effective policies (Hay et al., 2004; Mendis et al., 2009). Furthermore, the findings of this study have similar results to some of the results from previous studies (Banguero, 1984; Koram et al., 1995; Sintasath et al., 2005). In 1998 and 2000, study was conducted by (Ghebreyesus et al., 2000; Snow et al., 1998) in Ethiopia and Kenya, respectively. In this study, the assessment of different types of materials used in the construction of walls, roofs and floors of a house was done. Therefore, from the study, it was possible to observe association between any roof, wall and floor material and risk of malaria. Therefore, the finding of this study supports the result from the previous studies. Similarly, the use of mosquito nets was studied by different researchers. Therefore, the findings of these studies support the outcome of this study (Messina et al., 2011).

CONCLUSION

The government of Ethiopia has adopted various strategies to control malaria. These include early diagnosis, prompt treatment, selective vector control, epidemic prevention and control. In addition to this, the government has supporting strategies such as human resource development, monitoring and evaluation. One of the government's key goals in the control of malaria is to achieve the complete elimination of malaria within those geographical areas with historically low malaria trans-

mission and achieve near zero malaria transmission in the remaining malarious areas of the country. For this reason, evidence based strategies to prevent malaria is an attractive strategy for the country (Goovaerts, 1997). Therefore, the results from this study showed that malaria is associated with socio-economic, demographic and geographic factors, mainly influenced by poverty levels. Malaria is generally regarded as a disease of poverty. The more wealthy households who can afford to have toilet facilities, a greater number of rooms in the house, clean drinking water, and well built houses were found to be less affected by malaria. Furthermore, it was found that women and children are more vulnerable to malaria. Lack of bed nets contributes to this vulnerability. Moreover, as our results indicate having more bed nets is one means of reducing malaria and evidence suggests that households which are unable to afford sufficient mosquito nets, due to large families and low incomes, are more affected by malaria. Women and children are also exposed to mosquito bites while they are travelling long distances to fetch water. As the wealthier households were found to be less vulnerable to malaria than the poor households, improving the living conditions of the communities could be one way of achieving the malaria control goals set by the health professionals.

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Full Length Research Paper

Anti-osteoclastogenic activity of butanol fraction of rice bran extract via downregulation of MAP kinase activity and c-Fos/NFATc1 expression

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Osteoclasts are responsible for bone metabolic diseases including osteoporosis, rheumatoid arthritis, multiple myeloma and peritonitis. Recently, the anti-osteoporotic activities of natural product extracts have become the subject of research interest. Rice bran (RB) extracts exhibits anti-inflammatory activity and ameliorates anti-oxidative stress. However, the effects of RB extracts on osteoclastogenesis-related diseases such as osteoporosis have not been investigated. Here, we investigated the effects of RB extracts and of its fractions on RANKL-induced osteoclast differentiation. Interestingly, the butanol extracts of RB (RB-BuOH) dose-dependently inhibited osteoclast differentiation by down-regulating the RANKL-induced activations of mitogen-activated protein (MAP) kinases. Moreover, the mRNA expression of osteoclast-mediating molecules such as c-Fos, NFATc1, DC-STAMP and cathepsin K were dose-dependently attenuated by RB-BuOH during osteoclast differentiation. Furthermore, RB-BuOH depressed the protein levels of NFATc1 and its promoter activity. The findings of this study show that RB-BuOH and its components might prevent osteoclast-related bone loss.

Key words: Rice bran, osteoclast differentiation, NFATc1, mitogen-activated protein (MAP) kinase.

INTRODUCTION

A tight balance between osteoclast-mediated bone resorption (or destruction) and osteoblast-mediated bone formation is required to maintain bone homeostasis. However, an imbalance induced by increasing osteoclastic bone resorptive activity or decreasing osteoblastic bone-forming activity can lead to a variety of bone metabolic diseases, such as osteoporosis (Boyle et al., 2003). Therefore, two strategies are adopted to reduce the incidence of osteoporosis; that is, the reduction of bone resorption using anti-resorptive agents, like bisphosphonates, and the induction of bone formation using anabolic agents, like parathyroid hormone (PTH). Apparently, anti-resorptive agents still remain the therapeutic mainstay for osteoporosis, but the most common anti-resorptive agents, bisphosphonates, pose risks of side effects like bisphosphonate-related osteonecrosis of the jaw (Dannemann et al., 2007) and atypical femoral fracture (Meier et al., 2012). Anabolic PTH is currently available for stimulating bone formation, but its use is limited by its costs and concerns regarding its long-term safety. Thus, there is an urgent need for new anti-osteoporotic agents that are both effective and. safe for long-term

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management.

Rice is the most important food in Asia, including Korea, and generally white rice which has the bran removed from brown rice is usually consumed. Rice bran (RB) has long been considered an agricultural waste, but recently, it has been reported to contain various nutrients including vitamins, minerals and amino acids that could be used to prevent and treat chronic diseases. Therefore, RB extracts have been reported to exhibited anti-inflammatory, anti-oxidative, anti-diabetic, anti-mutagenic and anti-carcinogenic activities (Nam et al., 2005; Roschek et al., 2009; Norazalina et al., 2010; Rao et al., 2010; Jun et al., 2012; Kaup et al., 2012). These studies have attracted considerable research attention regarding the biological activity of RB, and its evident safety assessment has accelerated the commercial use of RB and its ingredients.

The biological activity of RB could result from components such as γ-oryzanols, tocopherols, tocotrienols and phenolic compounds which have been shown to have potential roles in disease prevention and treatment (Henderson et al., 2012; Verschoyle et al., 2007; Flight and Clifton, 2006). Among the bioactive components in RB, several substances such as ferulic acid, hydroxycinnamic acid, tocopherols and tocotrienols have been shown to inhibit osteoclastogenesis or prevent bone loss (Sassa et al., 2003; Lai and Yamaguchi, 2007; Ha et al., 2011). However, the effect of RB extracts on osteoclast differentiation has not been previously studied. Therefore, in this study, we investigated the effects of RB extracts on osteoclast differentiation and sought to determine the molecular mechanism responsible for its action.

MATERIALS AND METHODS

Preparation of rice bran extracts

Rice bran from grain rice (*Orysa sativa* L., Japanica) obtained by milling rice cultivated in Pocheon, Kyunggi Province, Korea, was used in this study. Totally, 8 kg of rice bran was extracted with 90% (v/v) hot ethanol (EtOH) three times for 4 h. The extract was filtered and concentrated in a vacuum to obtain the EtOH extract (yield 1,787 g). The concentrated extract was suspended in water and partitioned sequentially with *n*-hexane (Hx; yield 1,049 g), ethyl acetate (EA; yield 8 g) and *n*-butanol (BuOH; yield 20 g) to afford three fractions and an aqueous residue. These fractions were evaporated to dryness in a rotary vacuum evaporator (Büchi, Swiss). The dried extracts were dissolved in dimethyl sulfoxide (DMSO) to the final concentration of 200 mg/ml. Further dilution was done by phosphate buffered saline (PBS).

Osteoclast differentiation

Bone marrow cells were obtained from 5 to 8-week-old male imprinting control region (ICR) mice by flushing femurs and tibias with α-minimum essential medium (MEM) supplemented with antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin; Hyclone, UT). Cells were cultured for 1 day in 10 cm culture dishes in α-MEM containing 10% fetal bovine serum (FBS; Gibco, Paisley, UK), antibiotics, and macrophage colony stimulating factor (M-CSF; 10 ng/ml; Peprotech, NJ). Non-adherent bone marrow cells were plated on 9 cm petri dishes and cultured for 3 days in the presence of M-CSF (30 ng/ml). After removing non-adherent cells by washing, remaining adherent cells were bone marrow-derived macrophages (BMMs). BMMs were induced to differentiate into osteoclasts by culture (1×10^4 cells/well in a 96-well plate or 3×10^5 cells/well in a 6-well plate) for 4 days in the presence of M-CSF (30 ng/ml) and receptor activator of nuclear factor kappa-B ligand (RANKL; 5 ng/ml; R&D Systems, MN).

Tartrate-resistant acid phosphatase (TRAP) staining

To visualize mature osteoclasts, we stained cells with label tartrate-resistant acid phosphatase (TRAP), a biomarker of osteoclast differentiation. Cells were then fixed with 10% formalin for 10 min and 0.1% Triton X-100 for 10 min, and stained with the leukocyte acid phosphatase kit 387-A (Sigma, MO). Images were captured with a microscope equipped with the DP controller (Olympus Optical, Tokyo).

Cell viability assay

BMMs were suspended in α-MEM containing 10% FBS and plated in a 96-well plate at a density 1×10^4 cells/well. Cells were treated with various concentrations of RB-BuOH in the presence of M-CSF (30 ng/ml) and incubated for 1 or 3 days. Cell viability was then measured with the cell counting Kit-8 (Dojindo Moleculer Technologies, MD) according to the manufacturer's protocol. Measured absorbances were converted to cell numbers using a standard curve.

Western blot analysis

Briefly, cells were homogenized and centrifuged at 10,000 × *g* for 15 min. Supernatants were collected for the isolation of cytoplasmic proteins. Denatured proteins were separated on the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, CA). After incubation with antibody, the membranes were developed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce), with the LAS-3000 luminescent image analyzer (Fuji Photo Film Co., Ltd., Japan). All antibodies used in this study were purchased from Santa Cruz (CA).

Real-time PCR

Primers were chosen using the on-line primer 3 design program (Rozen and Skaletsky, 2000), and are listed in Table 1. Total RNA was isolated using TRIzol reagent, according to the manufacturer's instructions. First strand cDNA was synthesized using the Omniscript RT kit (Qiagen, CA) and 1 µg of total RNA, 1 µM of oligo-dT_{18} primer, 10 units of RNasin (RNase inhibitor; Promega, WI), according to the manufacturer's instructions. Quantitative polymerase chain reaction (PCR) (QPCR) was performed using the Stratagene Mx3000P Real-Time PCR system and Brilliant SYBR Green Master Mix (Stratagene, CA). Briefly, first-strand cDNA was diluted 1:10, and primers (10 pmol) were added according to the manufacturer's instructions. The thermocycling protocol consisted of three parts. The first part involved incubation at 95°C for 10 min to activate the polymerase; the second involved 40 amplification cycles of 94°C for 40 s (denaturation), 53°C for 40 s (annealing), and 72°C for 1 min (extension); and the third incubation at 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s to generate PCR product temperature-dissociation curves (also called 'melting curves'). All reactions were run in triplicate, and data were analyzed using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001). Glyceraldehyde-3-

Table 1. Primer sequences used in this study.

Target gene	Forward (5'-3')	Reverse (5'-3')
c-Fos	CCAGTCAAGAGCATCAGCAA	AAGTAGTGCAGCCCGGAGTA
NFATc1	GGGTCAGTGTGACCGAAGAT	GGAAGTCAGAAGTGGGTGGA
Cathepsin K	GGCCAACTCAAGAAGAAAAC	GTGCTTGCTTCCCTTCTGG
DC-STAMP	CCAAGGAGTCGTCCATGATT	GGCTGCTTTGATCGTTTCTC
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

phosphate dehydrogenase (GAPDH) was used as an internal standard. Statistical significances were determined using the Student's *t*-test using GAPDH-normalized $2^{-\Delta\Delta C}{}_{T}$ values.

Luciferase activity assay

Human embryonic kidney 293T cells were plated in a 24-well plate, and then transfected with different amounts of the following reporter plasmids: (1) the DNA-binding sequence of the nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) fused to the firefly-luciferase sequence and, (2) the sequence of receptor activator of nuclear factor κB (RANK) fused to the renilla-luciferase sequence in pGL4 vector. After 48 h, transfected cells were lysed with lysis buffer (Promega, Madison, WI, USA), and luciferase activity was measured using the dual-luciferase assay system (Promega). Luciferase activity was normalized versus renilla luciferase activity in each sample.

Statistical analysis

All experiments were performed in triplicates and quantitative values are presented as mean ± standard deviation (SD). The significances of differences were determined using the Student's *t*-test. Statistical significance was accepted for p values < 0.05.

RESULTS

RB-BuOH inhibits RANKL-induced osteoclast differentiation

Rice bran ethanol extract (RB-EtOH) was further fractionated to obtain hexane (RB-Hx), ethyl acetate (RB-EA) and butanol (RB-BuOH) fractions. The anti-osteoclastogenic effects of all four extracts were evaluated during the RANKL-induced osteoclast differentiation of primary mouse BMMs. At 50 μg/ml, RB-EtOH and RB-Hx did not exhibit anti-osteoclastogenic activity, but RB-BuOH did inhibit the formation of TRAP-positive multinucleated osteoclasts (Figure 1A). RB-EA was cytotoxic to BMMs. RB-BuOH significantly and dose-dependently inhibited the RANKL-induced formation of TRAP-positive multinucleated osteoclasts (Figure 1B), numbers of multinucleated osteoclasts (Figure 1C) and the mRNA expression of TRAP (Figure 1D). To ascertain that the inhibitory effect of RB-BuOH on RANKL-induced osteoclast differentiation was not due to its cytotoxicity, we evaluated its effect on cell viability. At the concentrations used in this study, RB-BuOH did not have any cytotoxic effect on BMMs (Figure 1E).

RB-BuOH inhibits RANKL-induced MAP kinase activation

To gain insight of the mechanism by which RB-BuOH blocks osteoclast differentiation, we investigated the effect of RB-BuOH on the activities of signaling molecules involved in osteoclast differentiation. First, we considered MAP kinases because they are known to be involved in the progress (Asagiri and Takayanagi, 2007). By evaluating the effect of RB-BuOH on the activations of MAP kinases, we found that RANKL strongly induced the activations of p38, JNK, and ERK, but these activations were strongly inhibited by RB-BuOH at 40 μg/ml (Figure 2).

RB-BuOH inhibits the RANKL-induced expressions of c-Fos and NFATc1 and the expressions of their regulatory genes

The inhibitory effect of RB-BuOH on osteoclast differentiation was further explored by evaluating the expressions of the transcription factors, c-Fos and nuclear factor of activated T cells (NFATc1) which are known to regulate genes required for osteoclast differentiation. In addition, we investigated the NFATc1-regulated mRNA levels of dendrite cell-specific transmembrane protein (DC-STAMP) and cathepsin K. As shown in Figure 3, mRNA expression levels of c-Fos and NFATc1 were strongly induced by RANKL, but these inductions were significantly inhibited by RB-BuOH. The expression levels of DC-STAMP and cathepsin K were also strongly elevated by RANKL, but these inductions were also significantly inhibited by RB-BuOH.

RB-BuOH inhibits RANKL-induced NFATc1 activity

We hypothesized that the anti-osteoclastogenic activity of RB-BuOH might result from its ability to inhibit the expression and activity of NFATc1 via down regulation of MAP kinase activity. To explore this hypothesis, the effect of RB-BuOH on the protein expression and activity of NFATc1 was further evaluated in BMMs. As shown in Figure 4A, western blot analysis revealed that the RANKL-mediated induction of NFATc1 protein was inhibited by RB-BuOH (Figure 4A). Furthermore, the NFATc1-

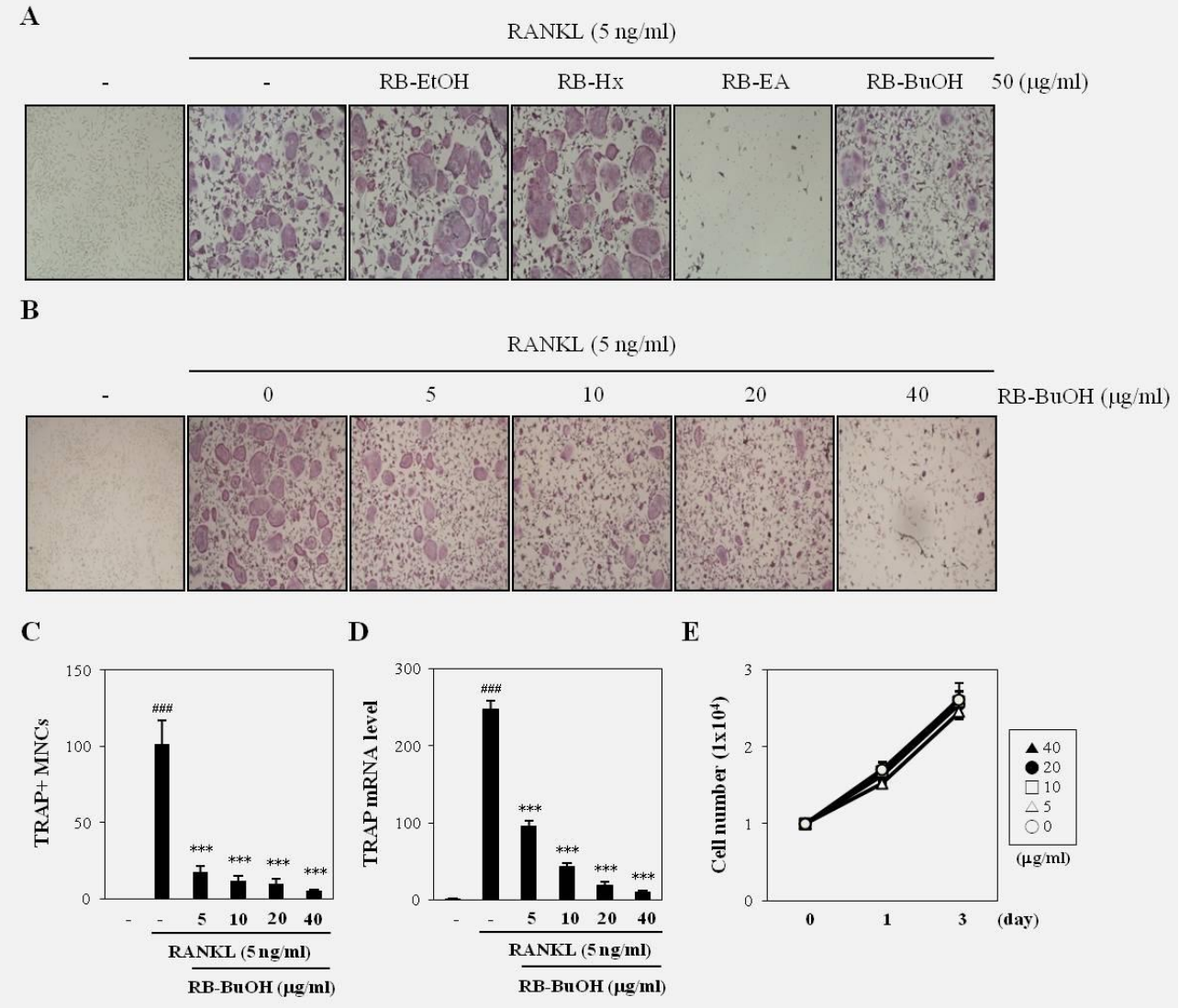

Figure 1. RB extracts inhibit RANKL-induced osteoclast differentiation. (A) BMM cells were cultured for 4 days in the presence of RANKL (5 ng/ml) and M-CSF (30 ng/ml) with RB extracts. Multinucleated osteoclasts were visualized by TRAP staining. (B) RB-BuOH dose-dependently inhibited RANKL-induced osteoclast differentiation. (C) RB-BuOH dose-dependently inhibited RANKL-induced formation of TRAP positive-multinucleated osteoclasts ($TRAP^+$MNCs). $TRAP^+$MNCs were counted. $^{###}p < 0.001$ (versus the negative control); ***$p < 0.001$ (versus the RANKL-treated group). (D) RB-BuOH suppressed RANKL-induced mRNA expression of TRAP. BMMs were treated with RANKL (5 ng/ml) and RB-BuOH for 4 days then mRNA expression level of TRAP was analyzed by the real-time PCR. $^{###}p < 0.001$ (versus the negative control); ***$p < 0.001$ (versus the RANKL-treated group). (E) The effect of RB-BuOH on the viability of BMMs was evaluated by CCK-8 assay.

luciferase reporter activity assay showed that the RANKL-mediated activation of NFATc1 was dose-dependently inhibited by RB-BuOH (Figure 4B). These results suggest that NFATc1 could be a major mediator of the anti-osteoclastogenic activity of RB-BuOH.

DISCUSSION

The incidence of osteoporotic fracture can be reduced by prescribing anti-resorptive agents or anabolic agents, but their uses are limited by their side effects, costs and by concerns regarding long-term safety (Dannemann et al., 2007; Meier et al., 2012). An alternative strategy for reducing those limitations is to find natural compounds with anti-osteoporotic potentials. Natural products have historically yielded a variety of therapeutic agents, and healthy nutrients or foods with medicinal properties are both effective and safe for the long-term management of disorders. In fact, recent studies have aimed to identify natural products or healthy foods that prevent and/or ameliorate osteoporosis with minimal adverse effects (Morabito et al., 2002).

In the present study, we found that RB-BuOH

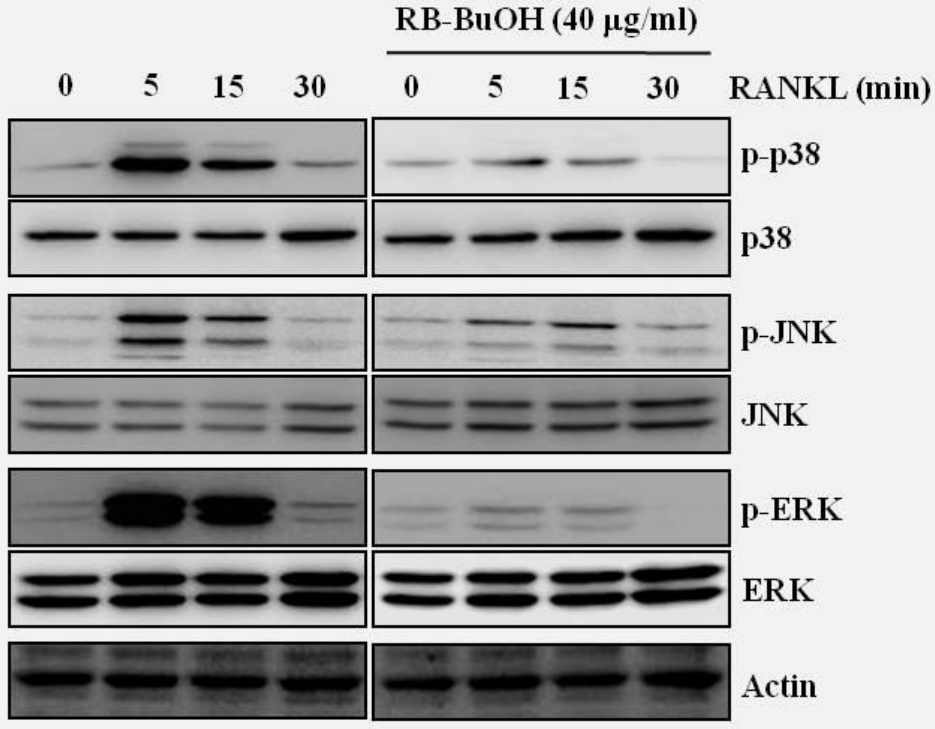

Figure 2. RB-BuOH inhibits RANKL-induced MAP kinase activity. BMMs were pretreated with or without RB-BuOH (40 μg/ml) for 1 h prior to RANKL stimulation (5 ng/ml) at the indicated times. Protein expression levels were evaluated by western blot analysis.

significantly inhibited the RANKL-induced differentiation of BMMs into osteoclasts without any cytotoxicity at the concentration used in this study. Since osteoclasts play an essential role in bone resorption, the modulations of osteoclast differentiation and functional maturation are considered a major strategy for treating bone metabolic diseases such as osteoporosis (Raisz, 2005). Osteoclast differentiation and subsequent maturation are mainly triggered by RANKL signaling, which is considered an important target for preventing pathological bone loss. Docking of RANKL to its receptor, RANK, rapidly activates MAP kinases such as p38, ERK and JNK, and these MAP kinases are essential for the differentiation, survival and activation of osteoclasts (Boyle et al., 2003; Lee and Kim, 2003). Interestingly, RB-BuOH strongly attenuated the RANKL-induced activation of MAP kinases in the present study, and others have reported that RB and its components such as γ-tocotrienol inhibit MAP kinase activations (Bi et al., 2010; Hoshino et al., 2010; Kannappan et al., 2010; Tanaka et al., 2012).

The activations of MAP kinases lead to the stimulation of transcription factors such as activator protein (AP)-1 and NFATc1 (Boyle et al., 2003; Lee and Kim, 2003). c-Fos (an AP-1 family member) is essential for osteoclast differentiation (Wang et al., 1992), and NFATc1 has been shown to rescue osteoclastogenesis in cells that lacked c-Fos (Matsuo et al., 2004). Interestingly, c-Fos is expressed during the early stages of osteoclast differentiation and regulates NFATc1 expression by binding to its promoter region (Matsuo et al., 2004; Asagiri et al., 2005). In the present study, RB-BuOH significantly attenuated the RANKL-induced mRNA expressions of c-Fos and NFATc1. In a previous study, the antioxidant α-tocotrienol in RB was shown to inhibit RANKL-induced osteoclast differentiation by suppressing c-Fos expression, and this anti-osteoclastogenic effect was reversed when c-Fos or an active form of NFATc1 was overexpressed (Xu et al., 2001; Ha et al., 2011).

After NFATc1 is expressed during the middle or late stages of osteoclast differentiation, it subsequently regulates a number of osteoclast-specific genes such as DC-STAMP and cathepsin K (Kim et al., 2008; Balkan et al., 2009). In the present study, RB-BuOH also significantly attenuated the RANKL-induced mRNA expressions of DC-STAMP and cathepsin K, probably due to the inhibition of NFATc1 mRNA expression. Thus, considering DC-STAMP is essential for osteoclast fusion (Kukita et al., 2004; Yagi et al., 2005; Asagiri and Takayanagi, 2007) and cathepsin K is a major player in osteoclastic bone resorption (Gelb et al., 1996; Ishikawa et al., 2001), RB-BuOH might have the potential to inhibit osteoclast fusion and resorptive activity as well as osteoclastogenesis.

Finally, our hypothesis that RB-BuOH inhibits osteoclastogenesis by down regulating MAP kinase-c-Fos-NFATc1 signaling axis was confirmed by our observations

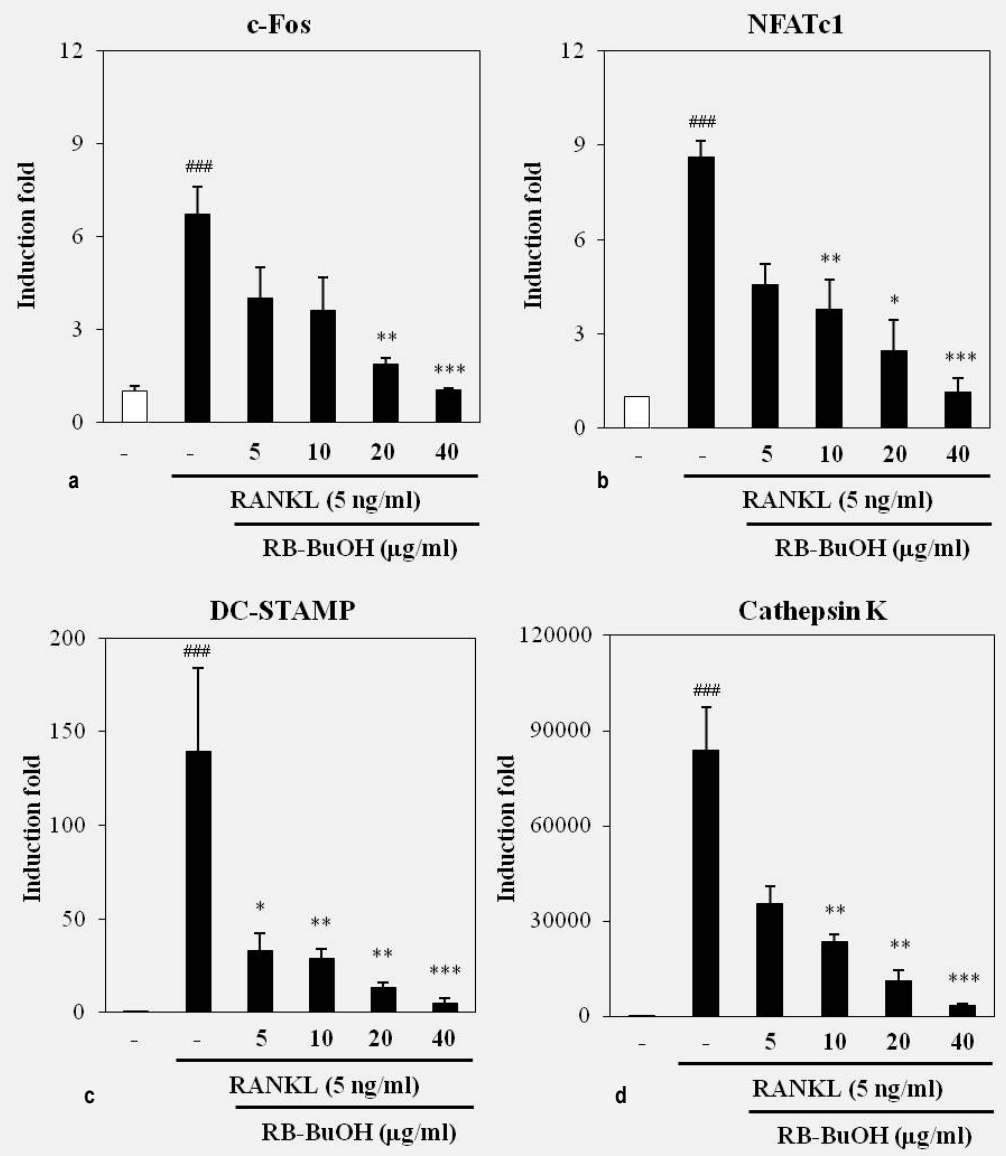

Figure 3. RB-BuOH suppresses RANKL-induced mRNA expression of c-Fos, NFATc1, DC-STAMP and Cathepsin K. BMMs were treated with RANKL (5 ng/ml) and RB-BuOH for 4 days and then mRNA expression levels were analyzed by real-time PCR. $^{###}p < 0.001$ (versus the negative control); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (versus the RANKL-treated group).

of its effect on the protein expression and activity of NFATc1. Interestingly, RB-BuOH was found to inhibit the protein expression and activity of NFATc1 dose-dependently.

To the best of our knowledge, this is the first study to report that RB-BuOH has the potential to inhibit RANKL-induced osteoclast differentiation. The ability of RB-BuOH to attenuate the RANKL-induced activations of MAP kinases could block the inductions of c-Fos and NFATc1 that down regulate the expressions of NFATc1-controlled osteoclast-specific genes such as DC-STAMP and cathepsin K, which are essential factors for cell fusion and bone resorption. Further study is required to identify the components in RB-BuOH that confer anti-osteoclastogenic activity and to evaluate their anti-resorptive activity in mature osteoclasts. Finally, the anti-osteoclastogenic property of RB-BuOH could provide benefits for bone health, and we believe it should be considered as a potential treatment for osteoclast-related disorders.

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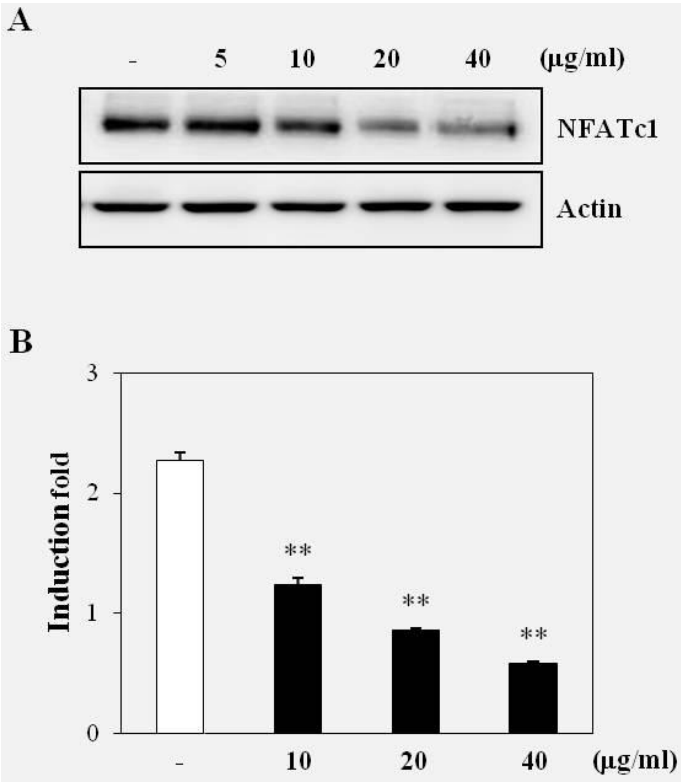

Figure 4. RB-BuOH suppresses NFATc1 expression and its activity. (A) BMMs were treated with RANKL (5 ng/ml) and RB-BuOH for 4 days. The protein expression level of NFATc1 was evaluated by western blot analysis. (B) 293T cells were co-transfected with NFAT firefly-luciferase (100 ng) and RANK (100 ng) together with pGL4 renila-luciferase (20 ng). After 6 h, transfected cells were co-treated with RANKL (50 ng/ml) and RB-BuOH. Treated cells were cultured for 48 h and lysed; luciferase activity is expressed as fold inductions versus the activity of NFAT luciferase only. pGL4 renila-luciferase activity was used to normalize transfection efficiency and activity. **$p < 0.01$ (versus the control).

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Short Communcation

Evaluation of National Health Insurance Scheme (NHIS) awareness by civil servants in Enugu and Abakaliki

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The awareness of the National Health Insurance Scheme (NHIS) by civil servants residing in Enugu and Abakaliki were evaluated using questionnaire. The results show that the level of awareness was very low with most of the respondents not knowing the mode of payment and benefits of the NHIS and were of the opinion that NHIS may not succeed in Nigeria. The recommendation is that the operators of the NHIS should embark on educating the citizenry on the mode of operation and benefits of the NHIS. A good knowledge of the scheme will improve its utilization.

Key words: NHIS, awareness, civil servants.

INTRODUCTION

Ever since Emperor Otto Von Bismarck of Germany enacted the mandatory legislation on the “sickness funds” for working Germans in 1883, different models of health insurance have continued to evolve worldwide albeit with the same general insurance principles. In the developed world, insurance in one form or the other is a veritable and sustainable tool for financing healthcare. The National Health Insurance (NHIS) was launched in Nigerian on October 15, 1997 and was passed into law in May 1999. The original scheme has been modified to include healthcare for less privileged persons in the country (FMH, 1998).

According to the World Health Organization (WHO) in 2005, Nigeria was ranked 197th out of 2000 nations; life expectancy was put at 48 years for male and 50 years for female while healthy life expectancy (HALE) for both sexes was put at 42 years. Nigeria accounts for 10% of global maternal mortality with 59,000 women dying annually from pregnancy and child birth; only 39% are delivered by skilled health professionals. In order to provide equitable distribution of health, the NHIS was introduced in Nigeria.

The need for the establishment of the scheme was informed by the general poor state of the nation’s healthcare services, excessive dependence and pressure on the government’s provision of health facilities, dwindling funding of health care in the face of rising cost, poor integration of private health facilities in the nation’s healthcare delivery system and overwhelming dependence on out-of-pocket expenses to purchase health. Like any other insurance scheme, the premium for the NHIS is the amount charged by the insurance compared with the promise to pay for any eventual “covered medical treatment” for the designated “coverage”. Consequently health insurance makes it possible to substitute a small but certain cost for a larger but uncertain loss (chain) under an arrangement in which the healthy majority compensate for the risks and costs of the unfortunate ill minority. The NHIS currently represents 15% of one’s basic salary. The employer is to pay 10% while the employee contributes 5% of his/her basic salary to enjoy healthcare benefits. The contribution made by the insured person entitles his/her spouse and four children under the age of 18 to full health benefits (FMH 2005).

NHIS was designed to provide minimum economic security for workers with regard to unfavorable losses resulting from accidental injury, sickness, old age,

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Table 1. Awareness Response to NHIS by Civil Servant

No.	Question	Nurses = 240				Artisans =120				Clerical officer =136				Teacher = 200			
		Yes		No		Yes		No		Yes		No		Yes		No	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
1	Do you know about National Health Insurance Scheme (NHIS)	154	64	86	36	24	20	96	80	27	28.1	69	71.9	20	10	180	90
2	Are you a registered member of NHIS	53	2	235	98	-	00	120	100	1	1	95	99	2	1	198	99
3	Do you know anybody who is a registered member of NHIS	113	47	127	53	-	00	120	100	3	3.1	93	96.9	5	2.5	195	97.5
4	Do you know a healthcare facility that have NHIS accreditation certificate	134	56	106	43.9	15	12.5	105	87.5	20	20.8	76	79.2	17	8.5	183	91.5
5	NHIS is earning related	149	62	91	63	11	9.2	109	90.8	25	26	71	74	10	5	190	95
6	NHIS is 15% basic salary	149	62	91	38	11	9.2	109	90.8	9	9.4	87	90.6	3	1.5	197	9835
7	Percentage to be paid by employer is 10%	149	62	91	66	11	9.2	109	90.8	9	9.4	87	90.6	3	1.5	197	98.5
8	Percentage to be paid by employee is 5%	149	62	91	66	13	10.8	107	89.2	17	17.7	79	82.3	3	1.5	197	98.5
9	NHIS covers employee, spouse and 4 children below 18 years of age	96	64	86	36	9	7.5	110	92.5	12	12.5	84	87.5	12	6	188	94
10	Do you think health insurance policy will work in Nigeria	48	20	192	80	5	4.2	115	95.8	8	8.3	88	91.7	10	5	190	95

unemployment and premature death of family wage earner. NHIS is made compulsory because the government based on past experiences predicted that some citizens cannot engage in the scheme and the government also has the duty to protect the general welfare of all citizens (Ibiwoye and Adedeke, 2007). It is also the government's belief that NHIS will help to break the vicious cycle of poverty in the country. It is also a form of social support for workers (Jutting, 2003).

There is lack of health care coverage and little equity. Access to healthcare is limited and most Nigerians are unable to pay for health services and health facilities are far from being equitably distributed. All these contributed to the limitation in health services (Samin and Awe, 2009). The available health services are very expensive and the common man cannot afford it; only the privileged few can get access to good health. This study aims at assessing the level of knowledge and attitude of civil servants resident in Enugu and Abakaliki to NHIS.

METHODOLOGY

A descriptive survey design was adopted for the study examining the awareness of Civil servants in Enugu and Abakaliki on NHIS. The population of the study comprised nurses, artisans, clerical officers and teachers resident in Enugu and Abakaliki. Convenient sampling procedure was used to select 696 civil servants resident in both study areas.

The instrument used for data collection was the questionnaire. The self constructed questionnaire was validated using test and retest method. The final corrected copy was used on a pilot study before being adopted for the study. The researcher administered the questionnaire to the respondents in their various offices and homes.

The respondents were given 24 h to fill the questionnaires before returning them to the researcher. Data were analyzed statistically using descriptive statistics and SPSS package was used for the analysis.

RESULTS

The result of response of civil servants tothe awareness of NHIS is shown on Table 1. The results show that 64% of the nurses know about NHIS, 20% of artisan, 28.1% of clerical officer while 20% of the teachers know about the NHIS. 2% of Nurses are registered members of NHIS while 47% of the Nurses know other registered members of NHIS. None of the artisans is a registered member of NHIS and none also know anybody that is a registered member. 1% of the clerical officers and teachers are registered members while 3.1% clerical officers know other registered members of NHIS and only 2.5% of teachers know other registered members.

56.1% of nurses know healthcare facilities accredited for NHIS while 12.5%, 20.8% and 8.5% of artisans, clerical officer and teachers respectively know healthcare facility accredited for NHIS. 62% of the nurses agreed that NHIS is earning related (15% of the basic salary and employee pay 5%). 9.2% of artisans are of the opinion that it is earning related and employers pay 10% while 15.8% of the artisan agreed that it is 15% of the basic salary of the members and 10.8% agreed that the employee pays 5%. 26% of clerical officers agreed that NHIS is earning related, 14.5% agreed that it is 15% of the basic salary while 9.6% agreed that the employer pays 10% while 17.7% agreed that the employee pays 5% of the premium.

64% of the nurses agreed that the NHIS covers the employee, spouse and 4 children below 18 years of age. Only 20% of the nurses agreed that the NHIS policy will work in Nigeria. 7.5% of the artisans agreed that the NHIS covers the

employee, the spouse and four children below the age of 18 years of age while only 4.2% agreed that NHIS policy will work in Nigeria. 12.5% of the clerical officers agreed that the policy covers the employee, the spouse and four children below the ages of 18 years of age and 8.3 agreed that NHIS policy will work in Nigeria 6% of the teachers agreed that the policy covers the employee, spouse and four children below the ages of 18 years of age while only 5% agreed that NHIS policy will not work in Nigeria.

DISCUSSION

This result indicates that civil servants working with Ebonyi and Enugu State governments do not know much about the NHIS. Nurses have the greatest knowledge about NHIS when compared to artisans, clerical officers and teachers. This is not surprising since the insurance policy has to do with health care provision which nurses play a very important role in. Considering the fact that nurses are involved in the implementation of the policy, 64% awareness among the nurses is therefore too low. The extent of the success of NHIS is shown from the fact that the policy launched in 1997 and passed into law in 1999 is not yet known by civil servants whom this policy should serve. This is a policy designed over 12 years ago to provide minimum economic security for civil servants resulting from accidental injuries sickness, old age and unemployment (Olanrinwaju 2013; FMH, 2005). 12 years after the introduction of the policy, civil servants in Enugu and Ebonyi States do not know what the policy is all about. This must be part of the reasons the National House of Representatives' Committee on Health in November 2011 whilst investigating the implementation of the scheme declared NHIS "a national embarrassment, disaster and colossal failure".

Most of the respondents do not know that NHIS is earnings related and currently represents 15% of the workers basic salary. The employer pays 10% while the remaining 5% is paid by the employee and that NHIS cares for the employer, the spouse and four children below 18 years of age. This level of awareness has indicated that it may not be possible to meet the objectives of the scheme whose establishing act states that the scheme is to ensure that all Nigerians are covered by the scheme by 2015 by eliminating problems associated with accessing health care delivery. Unlike primary health care that aims at brining medi-care to the doorstep of rural dwellers and is funded by government, NHIS is funded by the citizens through their own contribution. Most of the respondents do not agree that NHIS will work in Nigeria. This opinion may be based on the level of their awareness about how the scheme operates or due to previous experiences with insurance schemes in Nigeria whose operations are not clear and contributors never benefit from it.

RECOMMENDATION

It is recommended from the study that the operators of NHIS should provide massive education programme to enable citizens understand what it is all about and facilities available to its contributors. If there is knowledge and availability of healthcare facilities, Nigerians are likely to patronize the scheme.

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UPCOMING CONFERENCES

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