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AIMS OF THE JOURNAL

- To serve as an important medium for the publication of original research works in the field of medical science and health research, thus filling gaps in health knowledge for effective utilization of research findings
- To disseminate recent basic, applied and social research findings among health personnel of different strata for enhancing nation-wide health development in Myanmar
- To offer current medical knowledge and updated scientific information obtained from research to health professionals for better and appropriate health care management

EDITORIAL

The year 2014 is the time of the Silver Jubilee publication of the Myanmar Health Sciences Research Journal since it was established in 1989. This Silver Jubilee year is coincident with one of the accomplishments of Department of Medical Research (Lower Myanmar), i.e. the opening ceremony of Laboratory for Communicable Diseases Research in Myanmar (Advanced Molecular Research Centre) supported by Korea International Cooperation Agency (KOICA) which was successfully held in August 2014. With its advanced facilities, there will be significant improvements in molecular-based diagnostic methods in our department, thereby contributing towards reduction in morbidity and mortality resulted from communicable diseases such as HIV/AIDS, Viral Hepatitis, Tuberculosis, etc., in Myanmar.

Accordingly, the leading article we chose in this issue is about HIV infection, an important communicable disease of global and national concern. To prevent transmission of HIV from person to person, early and accurate screening of HIV is critical. Previously, first to third generation of HIV tests cannot detect antibody to HIV within the window period ranged from 3 weeks to 6 months when the risk of HIV transmission is quite high. Although further studies are still needed, this study highlighted that early case of HIV infection during the window period can be detected by the fourth generation ELISA when nucleic acid amplification tests are not available. The findings of this antigen/antibody combination assay, commercially available in Myanmar, are very useful for detection of early HIV patients in our country.

As only one course meal is not the favorite taste for everyone, there are a variety of course meals included at the special dinner party. With this basic concept, we present different categories of important health issues to scientists from various disciplines and those who are interested in health knowledge. Other articles mentioned in this issue are: youth

behaviour on smoking, alcohol drinking and betel chewing practices, purification and characterization of metalloproteinase from Myanmar russell's viper (*Vipera Russelii*) venom, relationship of plasma proteins and red cell in malaria patients, evaluation of bed nets treated with insecticide for reduction of malaria transmission, detecting rotavirus genotypes in under five years of children, detection of respiratory syncytial viruses in Infants with acute respiratory infection, antioxidant activity total phenol content and ascorbic acid content of Noni fruit juices, simple extraction and evaluation of effectiveness in wound healing of collagen from fish, heavy metal contamination in selected medicinal plants and soils in Mandalay Region, antibiotic susceptibility pattern among *Helicobacter pylori* isolates from chronic dyspepsia patients, and bacteriological analysis of household drinking water. Regarding with well-informative articles included, we fully appreciated the concerted efforts of authors for conducting their respective works.

Finally, it is certain that a variety of articles providing up-to-date health research information in this issue will be invariably attractive to scientists and researchers including medical students who are searching for local references to their theses.

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Detection of Early Cases of HIV Infection by Using Fourth Generation Enzyme-Linked Immunosorbent Assay (ELISA)

Htin Lin[1], Hlaing Myat Thu[1], Khin Mar Aye[1], Thida Kyaw[1], Mo Mo Win[1], Khin Thet Wai[1], Amy Khine[2], Myat Min Zaw[2] & Min Zaw[2]

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Fourth generation HIV enzyme-linked immunosorbent assay **(**ELISA) detects both HIV p24 antigen and antibody to HIV-1 or 2 simultaneously. The aim of this study was to determine the role of fourth generation ELISA in the detection of early cases of HIV infection. It was a cross-sectional, descriptive study conducted at QC centre of PSI/Myanmar and Virology Research Division. The investigators collected 100 HIV-seronegative samples of anonymous individuals from QC centre. These individuals had possible exposure to HIV within 2 to 8 weeks before coming to the centre. When these samples were tested with fourth generation ELISA (RecombiLISA HIV antigen-antibody Test, CTK Biotech), 6 samples (6%) showed HIV positive. Two out of six samples revealed HIV-RNA, when confirmed by real time polymerase chain reaction using HIV RT-PCR kit (careHIV-1 RT-PCR Kit V2, Qiagen, Shenzhen). These two cases had the 4 weeks onset and 8 weeks onset of possible exposure to HIV previously. In conclusion, fourth generation ELISA can provide detection of some early cases of HIV infection during the window period. The results suggest the use of antigen/antibody combination assays rather than the antibody assay in the screening of HIV cases when nucleic acid amplification tests are not available.

INTRODUCTION

HIV infection is an important health problem of global concern. About 33 million people are living with HIV with 2 million deaths annually worldwide.[1] In many countries, screening of HIV is usually recommended in antenatal cases, preoperative cases, blood transfusion cases and organ transplantation cases to prevent transmission of HIV from person to person.[2, 3, 4]

First generation to third generation HIV tests detect the various isotypes of antibody to HIV and are commonly used in the screening of HIV cases. Since these tests can detect antibody to HIV only, they can diagnose HIV infection only after the window period ranging from 3 weeks to 6 months.[5] During that period, risk of HIV transmission is usually high relative to chronic HIV infection.[6] Nucleic acid amplification test such as viral RNA test can diagnose HIV infection in very early phase and can reduce the window period up to one to two weeks.[7, 8] Due to the cost and technology of the test, the use of such test is limited in the screening of HIV cases in resource-limited settings. HIV Combo enzyme linked immunosorbent assay (ELISA) is the fourth generation HIV test that detects both HIV p24 antigen and antibody to HIV 1 or 2 simultaneously. This test can detect HIV infection beyond 16 days after exposure to HIV and can also reduce the window period up to 3 to 4 weeks.[5, 9]

The UK national guidelines for HIV testing recommends the fourth generation HIV assay as the first-line of HIV screening.[10] Now, these fourth generation assays are commercially available in Myanmar and are applied in some health care settings. So, the role of these assays in the detection of early

HIV infection cases is needed to be determined. The objective of this study was to determine the role of fourth generation ELISA in detection of early HIV infection cases.

MATERIALS AND METHODS

Study design

It was a cross-sectional, descriptive study conducted at QC centre of Population Services International (PSI)/Myanmar and Virology Research Division of Department of Medical Research (Lower Myanmar). The study period was from March to October, 2012.

Study population

The investigators collected 100 left-over blood samples of anonymous individuals from QC centre. These individuals had possible exposure to HIV within 2 to 8 weeks before coming to the centre. They had negative results for HIV-antibody when tested with both Determine and Unigold HIV-antibody immunochromatographic test (ICT) at the centre. Any person taking post-exposure prophylysis (PEP) was excluded from this study.

Fourth generation ELISA

The investigators tested all blood samples with fourth generation ELISA (Recombi LISA HIV Antigen-Antibody Test, CTK Biotech). The samples were centrifuged at 1000 rpm for 10 minutes and sera were collected. Each 75 µl of test sera and control specimens were added to the wells of ELISA plate and then, 25 µl of biotynated p24 antibody were added to each well. After the incubation of the plate at 37˚C for 60 minutes, the wells were washed with wash buffer for 5 times.

Each 100 µl of horserish peroxidase-conjugate was added to the wells. The plate was incubated at 37˚C for 30 minutes. The wells were washed with wash buffer for 5 times. After this step, each 50 µl of TMB substrate A and B was added to the wells. Then, the plate was incubated at dark at 37˚C for 10 minutes and 50 µl of stop buffer were added to each well. The plate was read immediately by a microplate reader at 450 nm wavelength.

Adding 0.1 to optical density (OD) value of negative control specimen revealed cut-off value. Cut-off ratio of the test specimen was calculated by dividing cut-off value with OD value of specimen. Specimen with a cut-off ratio less than 1 was taken as negative for either p24 antigen or HIV-antibody. Specimen with a cut-off ratio more than or equal 1 gave a positive result for p24 antigen or HIV-antibody. Positive samples were confirmed for HIV-RNA by real time polymerase chain reaction (RT-PCR) using HIV RT-PCR kit (careHIV-1 RT-PCR Kit V2, Qiagen, Shenzhen).

Extraction of RNA from samples

RNA was extracted from sera by using QIAamp^{R} Viral RNA Mini Assay (Qiagen, Valencia, CA). Lysis buffer (AVL) (560 µl) was put into 1.5 ml eppendorf tube. Serum (140 µl) was added to AVL buffer and incubated at 25˚C for 10 minutes. Absolute ethanol (560 µl) was then added. The solution (630 µl) was passed through silica columns by centrifugation at 8000 rpm for 1 minute. After the supernatant was removed, the silica pellets were resuspended in 500 µl of wash buffer (AW1). Then, the pallets were transferred to 2 ml tubes, centrifuged at 8,000 rpm for 1 minute. The supernatant was removed and the pellets were resuspended in 500 µl of wash buffer (AW2).

Then, the pallets were transferred to 2 ml tubes and centrifuged at 14,000 rpm for 3 minutes. After the supernatant was removed, the pellets were resuspended in 60 µl of elute buffer (AVE), and incubated at room temperature for 1 minute. After centrifugation at 8,000 rpm for 1 minute, template RNA solution was transferred to fresh tubes. The template RNA solution was stored at -70˚C until it was used.

Reverse transcription and real time-PCR amplification

Reverse transcription and real time-PCR amplification were done by using Rotor-Gene Q 36 rotor and HIV RT-PCR kit (careHIV-1 RT-PCR Kit V2, Qiagen, Shenzhen). Reaction mixture contained 13 µl of HIV RT-PCR solution, 2 µl of enzyme mix and 35 µl of template RNA solution. Rotor Gene Q Series Software (version 2.0.2) analyzed PCR amplification throughout the process. Hold temperature was 50˚C for 30 minutes and 95˚C for 15 minutes. Cycling temperature was 95˚C for 15 seconds, 50˚C for 15 seconds and 72˚C for 45 seconds for a total of 45 cycles. Channel acquiring was at the step of 50˚C. Both positive and negative control samples were tested along with the test samples.

Data analysis

Statistical Package for Social Sciences software, version 16 was applied for the entry and cross-tabulation of the data.

Ethical consideration

Approval for the conduct of this study was obtained from the Institutional Ethical Review Committee of Department of Medical Research (Lower Myanmar). Anonymous sera samples were taken in this study. There was no identifier to be traced back to the individuals who came to the centre for HIV testing.

RESULTS

In this study, a total of 100 HIV-seronegative samples were tested for HIV by fourth generation ELISA. When all seronegative cases were tested with fourth generation ELISA, 6 cases (6%) showed HIV positive (Table 1 & Fig. 1).

Table 1. Number of HIV cases detected by HIV-antibody tests and HIV-Combo ELISA

No. of cases	HIV antibody test		HIV Combo ELISA (RecombiLISA HIV)
	Determine HIV ICT(%)	Unigold HIV ICT(%)	
HIV positive	0(0)	0(0)	6(6)
HIV negative	100(100)	100(100)	94(94)
Total	100(100)	100(100)	100(100)

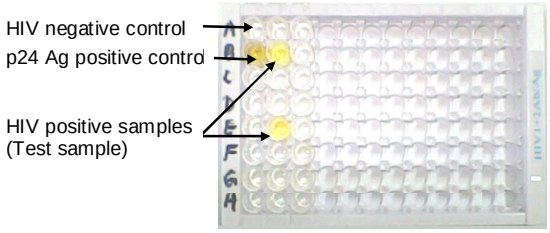
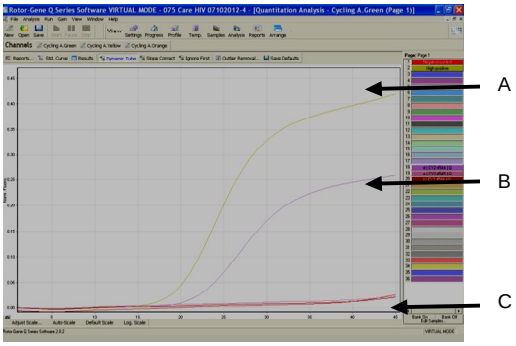


Fig. 1. HIV fourth generation ELISA plate showing results of HIV p24 antigen/ antibody

Two out of six positive samples revealed HIV-RNA, when confirmed by real time PCR using HIV RT-PCR kit (Table 2). These two cases had a possible previous exposure to HIV 4 weeks and 8 weeks, respectively.

Table 2. List of HIV (antigen/antibody) positive cases with HIV-RNA result

No.	Case	Onset of possible exposure to HIV (week)	HIV p24 Ag/Ab	HIV-RNA
1	M03WAMMY	8	Positive	Not detected
2	M08TATHL	4	Positive	Detected
3	M15NNPBD	4	Positive	Not detected
4	M19AAMT	2	Positive	Not detected
5	M08SMLMD	8	Positive	Detected
6	F12TTAKMD	1	Positive	Not detected



A= Channel of HIV highly positive control samples
B= Channel of HIV positive test samples
C= Channel of HIV negative control samples

Fig. 2. The target channel of control and test samples analyzed by Rotor Gene Q Series Software 2.0.2

DISCUSSION

This study involved testing of 100 sero-negative samples for HIV infection. Six out of 100 samples (6%) showed HIV positive when they were tested with fourth generation ELISA. So, these samples were

most probably positive for HIV p24 antigen. When these samples were confirmed by real time PCR, 2 samples revealed HIV-RNA. According to the results, HIV Combo ELISA can detect additional 2 cases of HIV infection among 100 seronegative cases. In agreement to this study, one study had also found that 2.3% of additional HIV positive cases were detected in the window period by estimating HIV p24 antigen.[11]

In this study, only two out of six positive samples revealed HIV-RNA when tested with real time PCR. The remaining 4 samples showed HIV-RNA negative in PCR. This may be due to the false-positive reactivity of the Combo ELISA. The conditions like autoimmune diseases, recent vaccination, recent influenza infection and hepatitis C virus infection sometimes give false-positive result in ELISA.[12, 13, 14, 15]

Besides, these PCR negative results may be due to the use of left-over sera samples since serum samples have less and in-consistent level of HIV RNA when compared with plasma samples.[16]

The two confirmed HIV cases had the history of possible exposure to HIV within 4 weeks, and 8 weeks, respectively, before coming to the centre. The diagnostic window for HIV Combo ELISA could not be calculated in this study because the number of antigen positive cases was very few. National AIDS Trusts (NAT) estimates that the window period for HIV Combo assays is from 11 days to 4 week.[5] However, British Association for Sexual Health and HIV (BASHH) recommends the use of fourth generation HIV assays at 4 weeks after exposure.[17]

HIV p24 antigen usually appears in the blood after 16 days of infection and become undetectable after the antibody to p24 antigen appears in the blood.[18] The window period exists before the level of p24 antigen peaks in the blood. During that time, fourth generation ELISA cannot detect p24 antigen or HIV-antibody. So, there may be some HIV cases among the remaining 94 seronegative cases especially the cases with a few weeks onset of exposure to HIV. However, it was obvious that fourth generation ELISA can detect more HIV cases than HIV-antibody tests in the window period because the combination test can detect the cases earlier than the antibody tests. A study had observed that fourth generation assays can diagnose HIV infection earlier than third-generation HIV-antibody assays.[15] In agreement to that finding, another study had reported a case of early HIV infection in 2005 in which fourth generation ELISA and PCR showed HIV-positive result whereas HIV antibody test showed negative result.[19]

Conclusion

Fourth generation ELISA can provide detection of some early cases of HIV infection during the window period. The results suggest the use of antigen/antibody combination assays rather than the antibody assay in the screening of HIV cases when nucleic acid amplification tests are not available. Further studies are needed to validate the various kits of such combi-nation assays since this was a preliminary study of the fourth generation HIV assay in Myanmar.

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Factors Influencing Smoking, Alcohol Drinking and Betel Chewing Practices among Third-year Male Students at University of Agriculture (Yezin)

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A cross-sectional, descriptive study was carried out with all the 166 third-year male students at University of Agriculture (Yezin). The study objectives were to determine the practices of smoking, betel chewing and alcohol drinking among third-year male students at University of Agriculture, and to identify factors influencing these practices. Face-to-face interviews were performed using a pre-tested questionnaire. It was found that the age of initiation of smoking, betel chewing and alcohol drinking was between 16 to 20 years. There were statistically significant associations between parental use and smoking practice ($p<0.05$); parental use and alcohol drinking practice ($p<0.05$); between influence of peer and smoking practice ($p<0.01$); between influence of peer and betel chewing practice ($p<0.01$); and between influence of peer and alcohol drinking practice ($p<0.01$). This situation revealed that there is a necessity for launching a program towards changing youth's behavior on smoking, betel chewing and alcohol drinking and to further strengthen the smoke-free campus strategy.

INTRODUCTION

According to Myanmar Global Health Professions Students' Survey (GHPSS) 2006, current cigarette smoking among medical students was 12.6% and that among dental students was 21.7%.[1] It was reported for Myanmar that smoking prevalence in adult males was 44.7% and that among females was 7.8%.[2] Heavy episodic drinkers among Myanmar males (aged 15-85+ years) were reported to be 18.7%.[3] According to the report made in GHPSS 2006, smokeless tobacco use among third-year medical and dental students was 11.3% and 13.3%, respectively.[1]

GHPSS being carried out among medical and dental students, this study explored the practices related to smoking, betel chewing and drinking alcohol, and factors influencing these practices among third-year male students of the University of Agriculture (Yezin). The reasons for selecting third-year students were that in Myanmar those above 18 years of age only are allowed to buy tobacco and alcohol-related substances and majority of the students will enter 18 years of age only on reaching third-year. The reason for excluding female students was that majority of female students neither chew, nor drink and smoke. If female population was included, a larger sample size would have to be considered to ensure those females who smoke, drink or chew betel.

The study objectives were to determine the practices of smoking, alcohol drinking and betel chewing among third-year male students at University of Agriculture (Yezin), and to identify factors influencing these practices.

MATERIALS AND METHODS

Study design

The study was school-based, cross-sectional study.

Study area

The study was carried out in University of Agriculture (Yezin), Nay Pyi Taw, which is designated as a smoking, betel chewing and alcohol drinking free campus.

Study period

The study was done from April to November, 2012.

Study respondents

There were altogether 432 third-year students at the University of Agriculture (Yezin). Among them, there were 166 male and 264 female students. So, this study included all of third-year male students.

Data collection method and tools

Quantitative data were collected, after receiving informed consent, by face-to-face interview method using a pre-tested structured questionnaire. The questionnaire consisted of three sections: general back-ground characteristics; perception of smoking, alcohol drinking and betel chewing; and practice of smoking, alcohol drinking and betel chewing. Prior to interviewing, informed consent was obtained from each interviewee and the purpose of study and nature of interviewing were explained.

Data management and analysis

Data were thoroughly checked in the field by trained research team members for consistency and accuracy of each and every questionnaire. Checking for wrong entries and missing answers was also made to allow for necessary correction and validation. The data were analyzed using SPSS version 16 software.

Ethical consideration

Ethical clearance was obtained from Post Graduate Board of Studies, Defense Services Medical Academy.

RESULTS

Background characteristics of the third-year male students

The age of the third-year male students who participated in the study ranged from 18 to 40 years, with the majority of students (62%) being in the age group of 18 to 20 years. About 98% of the students were single, and 89.8% of the students lived at private hostels.

Practices of smoking, betel chewing and alcohol drinking

Table 1. Practices of smoking, betel chewing and alcohol drinking among third-year male students

Type	Current users		Ex-users		Non-users		Total	
	No.	%	No.	%	No.	%	No.	%
Smoking	73	44.0	31	18.7	62	37.3	166	100
Betel chewing	57	34.3	36	21.7	73	44.0		
Alcohol drinking	61	36.7	34	20.5	71	42.8		

Table 1 indicates that among the students studied, current smoking prevalence was 44%, betel chewing prevalence was 34.3% and alcohol drinking prevalence was 36.7%.

Table 2. Categories of smoking, betel chewing and alcohol drinking practices

Category of practice	Frequency	%
Current users		
Current smokers (smoking only)	17	10.2
Current betel chewers (betel chewing only)	15	9.0
Current drinkers (drinking only)	9	5.4
Current smokers and betel chewers	9	5.4
Current smokers and drinkers	19	11.4
Current betel chewers and drinkers	5	3.0
Current smokers, betel chewers and Drinkers	28	16.9
Ex-users and non-users		
Smoking, betel chewing and alcohol drinking	64	38.6
Total	166	100

In Table 2, it shows that those who are currently practicing either smoking, betel chewing or drinking only are 10.2%, 9.0% and 5.4%, respectively. Ex-users and non-users of smoking, betel chewing and alcohol drinking constitute 38.6%.

Table 3. Age of initiation of smoking, betel chewing and drinking among total current users

Age of Initiation (years)	Smokers		Betel chewers		Alcohol drinkers	
	No.	%	No.	%	No.	%
6-10	0	0.0	1	1.8	0	0.0
11-15	2	2.7	3	5.3	1	1.6
16-20	69	94.5	48	84.2	55	90.2
21-25	1	1.4	4	7.0	3	4.9
26-32	1	1.4	1	1.8	2	3.2
Total	73	100	57	100	61	100

Table 4. Associations between influencing factors and the practices of smoking, betel chewing and alcohol drinking among third-year male students

Influencing factor	Category of practice				Total	
	Current user		Ex- and non-user			
	No.	%	No.	%	No.	%
Parental						
*Smoking #, **						
Yes	24	57.1	18	42.9	42	100
No	43	38.1	70	61.9	113	100
Betel chewing #						
Yes	20	40.8	29	59.2	49	100
No	30	28.3	76	71.7	106	100
*Alcohol drinking #, **						
Yes	9	60	6	40	15	100
No	47	33.6	93	66.4	140	100
Peer pressure						
*Smoking***						
Can withstand	30	24.6	92	75.4	122	100
Cannot withstand	43	97.7	1	2.3	44	100
*Betel chewing***						
Can withstand	28	20.6	108	79.4	136	100
Cannot withstand	29	96.7	1	3.3	30	100
*Alcohol drinking***						
Can withstand	35	25.4	103	74.6	138	100
Cannot withstand	26	92.9	2	7.1	28	100
Having problems while attending the University						
Smoking						
Yes	32	46.4	37	53.6	69	100
No	41	42.3	56	57.7	97	100
Betel chewing						
Yes	29	42.03	40	57.9	69	100
No	28	28.9	69	71.1	97	100
Alcohol drinking						
Yes	27	39.1	42	60.9	69	100
No	34	35.1	63	64.9	97	100

= Did not include 11 students who lived with guardians
* =Association was statistically significant at 0.05
**= Association was statistically significant at 0.01

Age of initiation of smoking, betel chewing and drinking among current users

Table 3 shows age of initiation for the total users of each practice category of smoking, betel chewing and alcohol drinking. Almost all of them started the respective practices between 16 and 20 years of age.

Associations between influencing factors and the practices of smoking, betel chewing and alcohol drinking among third-year male students

From Table 4, it was observed that there were statistically significant associations between parental use and smoking practice ($p<0.05$); parental use and alcohol drinking practice ($p<0.05$); between influence of peer use and smoking practice ($p<0.01$); between influence of peer use and betel chewing practice ($p<0.01$); and between influence of peer use and alcohol drinking practice ($p<0.01$).

DISCUSSION

It was found in this study that current smokers constituted 44% among the male students. It was higher than the findings of a study among male university students of Bangladesh (36.1%),[4] more than the finding among male university students in Malaysia (29%)[5] and higher than the finding of smoking prevalence among Myanmar males (33.3%).[6]

As regards the betel chewing practice, current betel chewers were 34.3% and slightly higher than prevalence of smokeless tobacco use among Myanmar males (31.8%),[6] more than the finding in Malaysian adults (8.2%)[7] and more than the finding among youth of Western Nepal (30.2%).[8] In alcohol drinking practice, currently drinkers were 36.7%. This was lower than the findings in a study in Korea (50.4%)[9] and in a study in South-east Nigeria (78.4%).[10]

Parental risk taking behaviours influenced on risk taking behaviours among the third-year male agricultural students. This finding was similar to the findings of a study from Bangladesh male university students.[4]

Regarding age of initiation of these three behaviours, 16 to 20 years old age group was the most common starting point (18.08± 2.15 years in smoking, 18.44±2.96 years in

betel chewing and 18.72±2.63 years in alcohol drinking). The mean age of smoking initiation was a little bit higher than mean age of starting point (16.8±2.7 years) of male students of a university of Bangladesh[4] and lower than mean age of onset (19.4±3.3 years) found in the study in Ayeyawady Naval Regional Command of Myanmar Navy.[11]

In this study, there were statistically significant associations between parental smoking and student's smoking, and between parental alcohol drinking and student's alcohol drinking. Such a significant association was not found for betel chewing behavior. In another study in Myanmar, conducted among Final Part II MB.,BS students,[12] significant associations were found between parental smoking student's smoking behaviour and between parental betel chewing and student's betel chewing behavior; however, there was no such association for alcohol drinking behaviour.

Peer's use was statistically significantly associated with student's risk behaviours among those who were current smokers, current chewers and current drinkers. Similar findings were observed in the study among Myanmar Final Part II MB.,BS students.[12]

Conclusion and recommendation

University students are one of the important segments of the young adult population. In the context of this study, current tobacco smoking was 44%, betel chewing prevalence was 34.3% and alcohol drinking prevalence was 36.7%. This situation revealed that there is a necessity for launching a program towards changing youth's behavior on smoking, betel chewing and alcohol drinking so as to prevent tobacco and alcohol-related risks and illnesses. Smoke-free campus strategy should be further strengthened thereby reducing the prevalence of smoking among the current smokers and protecting all the students from the risk of experiencing to environmental tobacco smoke. Further in-depth studies of peer pressure, including examination of overt and covert pressures to use smoking, betel chewing and alcohol drinking should be done.

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Purification and Characterization of Metalloproteinase from Myanmar Russell's Viper (*Vipera russelii*) Venom

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A basic metalloproteinase was purified from the venom of Myanmar Russell's viper (*Vipera russelii*) by gel filtration on Sephadex G-75 followed by ion-exchange chromatography on Q-Sepharose and SP-Sepharose. The enzyme was purified by 8.5 fold and its recovery was 82%. The purified metalloproteinase constituted approximately 9.6% of total venom protein. The approximate molecular weight as seen on SDS-PAGE was 75 kDa. From 2-D electrophoresis, its isoelectric point is approximately 8.0. The purified metalloproteinase exhibits haemorrhagic, edema inducing and myonecrotic activities. It also possesses coagulant, fibrinolytic and fibrinogenolytic activities.

INTRODUCTION

Snake bite is a national health problem in Myanmar and 60% of the morbidity (fatality rate 8.2%) resulted from Russell's viper bite envenomation.[1] WHO estimated that there are 8832 envenoming and 336.7 deaths from snake bite each year in Myanmar.[2] Snake venom is a complex mixture of proteins that synergize and complement each other in function. Symptoms following Viperid snakebites are local and systemic haemorrahge and retard blood coagulation. Snake venom metalloproteinases (SVMPs) have been considered as the key toxins involved in snake venom-induced pathogenesis, such as hemorrhage, edema, hypotension, hypovolemia, inflammation and necrosis.[3]

They belong to the metazincin family characterized by the presence of a conservative zinc-binding sequence, HEXXHXXGXXH, an essential motif for the proteolytic activity. SVMPs are classified according to their domain structure into three basic groups, P-I (protein class I) to P-III.[4] All three groups share a metalloproteinase domain (about 200 residues) containing the Zn^{2+}-binding motif. The P-I class (20-30 kDa) has only a metalloproteinase-domain structure. The P-II class (30-60 kDa) (disintegrin precursor) has an additional domain carboxy to the proteinase domain, a disintegrin or disintegrin-like domain. The P-III class (60-100 kDa) has both a disintegrin-like domain and a high-cysteine domain in addition to the proteinase domain. In Myanmar, a major protease enzyme from Russell's viper venom which has caseinolytic activity was partially purified and characterized. The protease showed autolysis and was more susceptible to inhibition by EDTA than Ca^{++} and Mg^{++}.[5]

Khin Pa Pa Kyaw, *et al* (unpubished data) purified a neutral protease which appeared to be a dimeric protein with a native molecular weight of 120 kDa composed of 2 subunits with molecular weight of 60 kDa. The enzyme had a K_m value for casein of 0.7 mg/ml and showed maximum activity at pH 8.0. Although few proteinase enzymes from Myanmar Russell's viper venom were partially purified and their biochemical

properties were characterized, the biological properties of a fully purified metalloproteinase contributing to hemorrhage have not been studied. The present study was to describe the purification and characterization of a metalloproteinase from the Myanmar Russell's viper venom.

MATERIALS AND METHODS

Venom and reagents

The desiccated form of Russell's viper venom (RVV) was provided by Myanmar Pharmaceutical Factory, Yangon. Sephadex G-75, Q-Sepharose FF and SP-Sepharose HP (Pharmacia Biotech, Sweden), vivaspin 20 MWCO 10,000 and Immobiline^{TM} DryStrip Gels (GE Healthcare, Sweden), molecular weight markers for SDS-polyacrylamide gel electrophoresis (PAGE) (Bio-Rad, USA), caesine (Sigma, USA), bovine fibrinogen (Sigma, USA) and thrombin (Sigma, USA) were used in this study.

Animals

The male ICR mice (25-30 gm) were provided from Laboratory Animal Services Division, Department of Medical Research (Lower Myanmar), Yangon, Myanmar.

Purification of metalloproteinase

Sephadex G-75 gel filtration chromatography

Fifty milligrams of RVV were dissolved in 1.0 ml of 0.02 M Tris-HCl buffer (pH 7.0) and centrifuged at 10,000 x g for 5 minutes. The clear supernatant was applied on Sephadex G-75 column (1.5 x 30 cm) pre-equilibrated with two column volumes of 0.02 M Tris-HCl buffer (pH 7.0). Protein elution was carried out with the same buffer. The flow rate was adjusted to 18 ml per hour and 2 ml fractions were collected. The absorbance of all fractions was monitored at 280 nm. Four fractions (P_1, P_2, P_3 and P_4) were collected from the pool of venom fractionated on the Sephadex G-75 column and aliquots were taken for testing enzymatic activities. The pooled Sephadex G-75 fraction with the highest proteinase activity was further fractionated by Q-Sepharose anion-exchange chromatography.

Q-Sepharose ion-exchange chromatography

The sample obtained from previous step was applied on Q-Sepharose ion-exchange column (2.5 x 10 cm) pre-equilibrated with starting buffer (0.02 M Tris-HCl buffer, pH 7.4). Eighty milliliters each of starting buffer and limiting buffer (0.5 M NaCl in 0.02 M Tris-HCl buffer, pH 7.4) were mixed in a gradient mixer GM-1 (Pharmacia) to produce a combined ionic strength. The flow rate was adjusted to 15.7 ml per hour and 1.5 ml fractions were collected.

The absorbance of all the fractions was monitored at 280 nm. A total of 2 new pooled fractions (P_{11} and P_{12}) were obtained and screened for enzyme activity. The fraction which showed the highest proteinase activity was dialyzed against 0.05 M phosphate buffer (pH 7.0) with vivaspin 20 (MWCO 10,000 Da).

SP-Sepharose ion-exchange chromatography

The P_{11} fraction containing concentrated proteins obtained from previous step was applied on SP-Sepharose ion-exchange column (2.5 x 4 cm) pre-equilibrated with two column volumes of 0.05 M phosphate buffer, pH 7.0 (starting buffer). Proteins were eluted with a linear gradient of NaCl from 0 to 0.5 M obtained by mixing an equal volume of starting buffer (40 ml) and limiting buffer (0.5 M NaCl in starting buffer; 40 ml). The flow rate was 15 ml per hour and 1 ml fractions were collected. The absorbance of all the fractions was monitored at 280 nm.

Protein determination

The protein content in the fraction was calculated from the absorbance at 280 nm in a 1 cm cell, based upon the assumption that the absorbance of 1 mg/ml of crude venom was 1.12.[6]

Caseinolytic activity assay and inhibitory study

The proteolytic activity was estimated by hydrolysis of heated casein by Anson method.[7] The reaction mixture, consisting of 500 µl casein (20 mg/ml) in 0.1 M Tris-HCl (pH 8.0), 50 µl venom in Tris buffer, was incubated for 15 minutes at 37˚C. The reaction was quenched by the addition of 500 µl of 5% trichloroacetic acid (TCA) at room temperature. After centrifugation at 10,000 rpm for 5 minutes, the hydrolyzed substrate un-precipitated with TCA was determined by optical absorption by Folin Ciocalteau method.[8] Then, 400 µl of the supernatant was mixed with 1 ml of 0.5 M Na_2CO_3 and 200 µl of diluted (1:5) Folin & Ciocalteau's phenol reagent. The mixture was then incubated at 37˚C for 30 minutes and absorbance was measured at 660 nm. One enzyme unit is defined as the amount of enzyme which hydrolyzes casein to produce color equivalent to 1.0 µmole of tyrosine per minute at pH 7.5 at 37˚C. The effect of protease inhibitor on the proteolytic activity of the sample was observed by pre-incubated the sample with EDTA (final concentration was 10 mM) at 37˚C for 10 minutes. The mixture was then assayed the activities in the corresponding assay systems. Percent inhibition for the caseinolytic activity of metalloproteinase was calculated for the assayed samples as compared with that of control.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The apparent molecular mass and homogeneity of metalloproteinase was analyzed on SDS containing polyacrylamide slab gels according to a modified method of Laemmli.[9] The venom samples (10 µg of proteins) were mixed 3:1 (v/v) with sample buffer and loaded onto SDS-PAGE (12.5% resolving gel and 3.75% stacking gel containing 0.1% SDS) under non-reducing conditions. Electrophoresis was performed at room temperature using 30 mA for 90 minutes in running buffer (25 mM Tris-glycine, pH 8.8). The precision plus protein standards (5 µl) were used as molecular weights markers.

Two-dimensional gel electrophoresis (2-DE)

The fraction from tube 15 of SP Sepharose column was purified by gel electrophoresis. The first dimension (Isoelectric focusing, IEF) of 2-D gel electrophoresis was performed on Multiphor II (Pharmacia) using 24 µg of venom protein and 7 cm immobiline dry strip gel with a linear range of pH 3-10 (GE Healthcare). Electrofocusing was carried out at 30 kVh using an IPGphor at 20˚C according to the manufacturer's instruction. The second dimension was run on 12.5% SDS-PAGE gel with initially 20 mA for 15 minutes, followed by 40 mA. The separated gel proteins were visualized through Coomassie brilliant blue staining.

Hemorrhagic property

Hemorrhagic property was tested by the skin method of Kondo.[10] Different concentration of metalloproteinase enzyme (5 µg, 10 µg, 15 µg) and 10 µg of RVV in 100 µl were injected subcutaneously into the skin of on left side of ICR mice (n=3). The right side of skin was injected only 100 µl of normal saline which served as control. After 2 hours, the mice were scarified under ether. Then, the skin were removed and examined from inside of skin for hemorrhagic spot.

Edema-forming property

Edema-forming property was tested according to the method as described by Vishwanath, *et al.*[11] Metalloproteinase fraction (5 µg in 20 µl normal saline) and crude RVV (5 µg in 20 µl normal saline) were injected into the left foot pad of mice (n=3). The right foot pad was injected 20 µl of normal saline which served as control. After 45 minutes, all mice were scarified under ether. Then, the foot pads were cut at the ankle joint and weighed separately. The increase in weight can be expressed in edema ratio. Edema ratio is the percentage increase in weight of the edematous leg compared to the control leg.

Myonecrotic property

Myonecrotic property was tested according to the method as described by Kasturi, *et al.*[12] Five micrograms of purified metalloproteinase in 0.1 ml normal saline were injected intramuscularly into left thigh muscle of mice (n=3). The right thigh was injected 0.1 ml of normal saline which served as control. After 4 hours, the mice were scarified under ether. The thigh muscle was taken out, fixed in 10% formalin solution, embedded in paraffin waxed, sectioned by microtome into 5-10 µm, and stained by haematoxylin and eosin stain for microscopic examination.

Coagulation activity

The coagulation activity was measured by the method of Dimitrov and Kankonkar.[13] The citrated human plasma (0.2 ml) was incubated with 0.1 ml each of sample and 0.01 M Tris-HCl buffer, pH 7.3 at 37˚C for 5 minutes. After incubation, 0.1 ml of 0.05 M calcium chloride was added to preincubating mixture. The clotting of plasma was noted and clotting time was recorded in seconds. A measure of coagulant activity was the decrease in the time of coagulation compared with that of a sample which did not contain enzyme solution.

Fibrinolytic activity

The fibrinolytic activity was assayed by the fibrin-plate method, as prescribed by Jespersen and Astrup.[14] A fibrin-agarose gel was prepared by mixing equal volumes of a 1 mg/ml bovine finbrinogen solution with a preheated solution of 2% agarose in 50 mM Tris-HCl (pH 7.5) buffer containing 200 mM NaCl, 50 mM $CaCl_2$ and 10 U of thrombin. Samples were applied to wells of the solidified gel and incubated at 37˚C for 1 hour. At the end of this period, the hydrolyzed areas were measured.

Fibrinogenolytic activity

The fibrinogenolytic activity was assayed on SDS-polyacrylamide slab gel (5% staking/ 12% resolving gel) as described by Ouyang and Teng.[15] Five hundred microliters of 2 mg/ml bovine fibrinogen dissolved in 5 mM Tris-HCl buffer (pH 7.5) containing 10 mM NaCl were incubated at 37˚C with equal volume of sample solution (10 µg/ml). At various time intervals, 0, 5, 15, 30, 60 and 120 minutes, 100 µl of aliquots were withdrawn from the digestion mixture, and then denatured and reduced by boiling for 10 min with 100 µl of 1M Tris-HCl, pH 6.8 containing glycerol, β-mercaptoethanol, SDS and bromophenol blue.

RESULTS

Purification of metalloproteinase

The crude venom of *Vipera russelii* was initially separated by gel-filtration chromatography using a Sephadex G-75 column. Protein elution showed 4 peaks, P_1 to P_4 (Fig. 1a).

All fractions were tested for caseinolytic activity. The first peak (P_1), displayed much higher protease activity than others, accounted for 24.2% of the total soluble protein (Table 1). This fraction was concentrated and further purified by Q-Sepharose FF column. Two protein peaks were obtained, only the first peak (P_{11}) exhibiting proteinase activity (Fig. 1b). The active fraction was subjected to SP-Sepharose HP column (Fig. 1c). Only one peak (P_{111}) was obtained, the proteinase activity was found throughout the protein peak. The amount of proteinase in the crude venom was estimated to be 9.6% (w/w) and its specific activity towards casein was 1709.5 µmoles/min/mg protein (Table 1).

Proteolytic assay and inhibitory study

The proteolytic activity of metalloproteinase upon casein was measured in the presence of metal chelating agent EDTA to verify that it possesses a metal ion (Zn^{2+}) in the functional group. The caseinolytic activity of the purified metalloproteinase (P_1 fraction) was inhibited by 63-84% and P_{111} fraction by 98-99% in 10 mM EDTA.

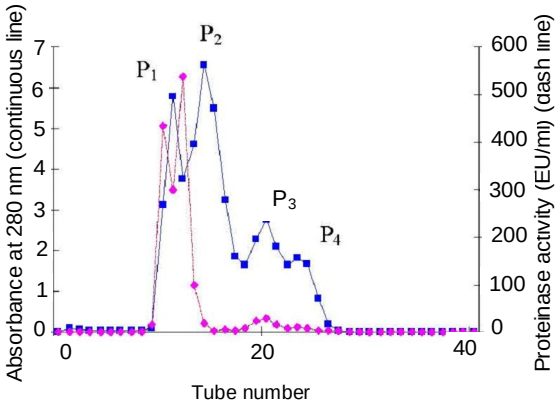


Fig. 1. a. Crude venom (50 mg/ml) in 0.02 M Tris-HCl buffer, pH 7.0 was chromatographed on a Sephadex G-75 column

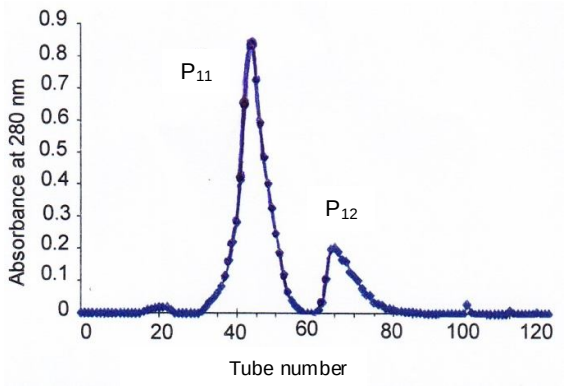


Fig. 1. b. The proteinase fraction P_1 was further separated on a Q-Sepharose FF column equilibrated with 0.02 M Tris-HCl buffer, pH 7.4 and eluted with a 0-0.5 M NaCl gradient in the same buffer

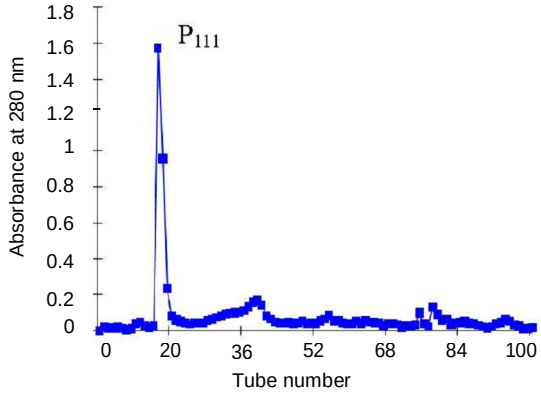


Fig. 1. c. The peak P_{11} showing proteinase activity was finally fractionated by cationic exchange chromatography on SP-Sepharose HP column equilibrated with 0.05 M phosphate buffer, pH 7.0 and eluted with a 0-0.5 M NaCl gradient in the same buffer

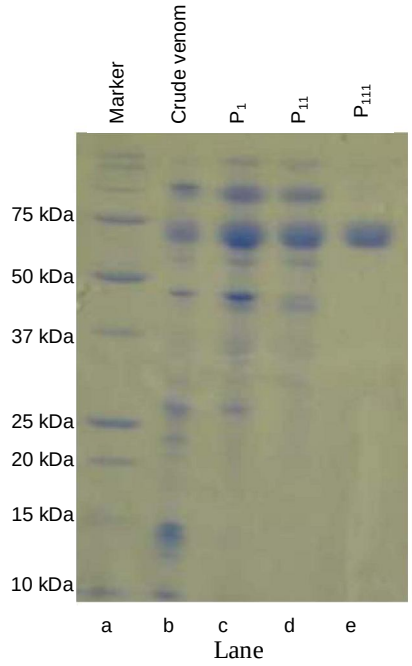
Fig. 1. The enzyme purification scheme

Table 1. Summary of purification of metalloroteinase from Russell's viper crude venom

Purification steps	Total protein (mg)	Percent of protein yield	Specific proteinase activity (μmoles/min/mg)
Crude venom	50.0	100.0	200.4
Sephadex-G75	12.1	24.2	718.2
Q-Sepharose	7.3	14.6	1140.4
SP-Sepharose	4.8	9.6	1709.5

Gel electrophoresis of metalloproteinase

The purity of the purified metalloproteinase was determined by SDS-PAGE. The molecular weight of the purified metalloproteinase was estimated to be 75 kDa (Fig. 2). The 2-DE of the purified protein showed its isoelectric point is approximately 8.0.



Lane a=marker proteins of molecular weight
Lane b=crude venom
Lane c=proteinase peak (P_1) of Sephadex G-75 elutes
Lane d=proteinase peak (P_{11}) of Q-Sepharose chromatography
Lane e=proteinase peak (P_{111}) of SP-Sepharose chromatography

Fig. 2. SDS-PAGE of the purified metalloproteinase (The sample of 10 μg was used in each lane.)

Hemorrhagic activity

Crude Russell's viper venom produced subcutaneous hemorrhage when 10 μg was injected subcutaneously into the mice skin.

The purified metalloproteinase also produced subcutaneous hemorrhage after injection of 5-15 µg range of metalloproteinase was used. The purified enzyme (10 µg) induced hemorrhage area of 1.78 cm in diameter.

Edema forming activity

Edema formation was noted by increase in weight after injection of purified metalloproteinase into the foot pad of mice. The increase in weight was expressed in terms of edema ratio. The edema ratios were 151.79 ±13.11% (mean±SE) and 170.24±6.75% when 5.0 µg each of RVV and the purified metalloproteinase were injected, respectively.

Myonecrotic activity

The myonecrotic activity of the purified metalloproteinase was observed when 5 µg was injected into thigh muscle of mice. Five microgram of RVV also caused muscle necrosis. Macroscopically, the thigh muscle became swollen and reddish brown in color.

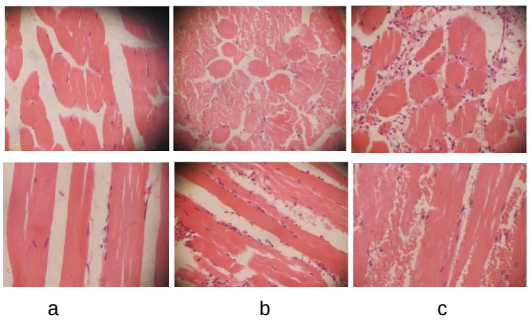


Fig. 3. Light micrograph of sections of mouse thigh muscle 4 hours after injection of (a) 100 µl normal saline (b) 5 µg RVV in 100 µl normal saline (c) 5 µg enzyme in 100 µl normal saline. Top row representing cross section and bottom row representing longitudinal sections of muscle fibres (b, c) Notice in inflammatory infiltration of polymorphornuclear leucocytes. 200x., and (c) Prominent hemorrhage is observed in endomysium. 200x.

Microscopically, disintegration of intact nuclei, myofibril disarrangement and infiltration of inflammatory cells were seen in injected muscle of both RVV and the enzyme, while normal intact muscle fibers were seen in saline-injected muscle. There was a marked infiltration of red blood cells in the muscle fibers injected with the purified enzyme (Fig. 3).

Coagulant activity

The clotting time of recalcified plasma was 143±1 sec, whereas, the recalcification time of plasma containing 1 µg RVV was 36.33 ±1.53 second and the recalcification time of plasma containing 1 µg enzyme was 46±1 second. The shortened clotting time caused by 1 µg of enzyme was 97±1 second. Thus, the purified metalloproteinase showed coagulant activity.

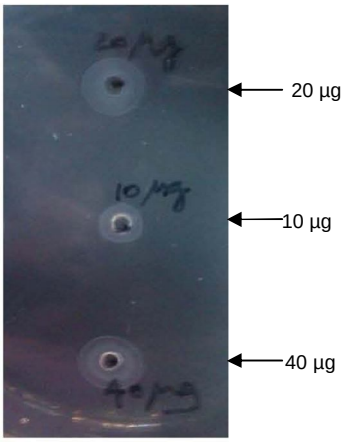


Fig. 4. The hydrolyzed areas produced by different doses of purified enzyme, 10 µg, 20 µg and 40 µg, on a fibrin plate

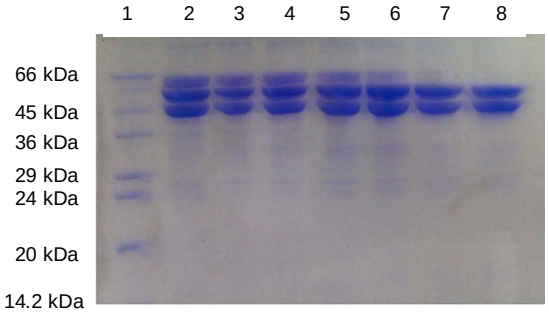


Fig. 5. Fibrinogenolytic activity of metalloproteinase (1) Protein standards (2) Reduced fibrinogen, (3-8) Fibrinogen incubated with metalloproteinase for 0, 5, 15, 30, 60 and 120 minutes, respectively, at room temperature. Fibrinogen (1 mg/ml) was incubated for the indicated periods with 10 µg/ml of metalloproteinase in deionized water

Fibrinolytic activity

The purified enzyme at 10 µg, 20 µg, 40 µg and 100 µg showed a fibrinolytic activity of 35.00±4.58 mm^2, 53.83±5.62 mm^2, 66.83 ±10.30 mm^2 and 98.33±10.40 mm^2 (n=3), respectively. The enzyme has dose dependent finbrinolytic activity (Fig. 4).

Fibrinogenolytic activity

The purified enzyme is able to degrade Aα-chain of bovine fibrinogen. The time course digestion of fibrinogen by the enzyme revealed that within 60 minutes, the Aα-chain has been completely destroyed while Bβ- and γ-chains remained intact throughout the course of time (Fig. 5).

DISCUSSION

The yield of the purified metalloproteinase was similar to that reported for major proteinase enzyme purified by a variety of procedure; 9.3% in a study[5] and 5.5% daborhagin-M in another study[16] Inhibition of the proteinase activity by a metal chelation agent, EDTA suggested that the purified enzyme is a metalloproteinase.

The SDS-PAGE indicated that the apparent molecular weight of the enzyme is 75 kDa which is approximate to 65 kDa for the Russell's viper hemorrhagic metalloproteinases, daborhagin-K and daborhagin-M;[16] to 70 kDa for *Vipera ammodytes ammodytes* haemorrhagic metalloproteinase, VaH1 and VaH2;[17] to 68 and 75 kDa for *Bitis arietans* hemorrhagins, BHRa and BHRb, respectively;[18] to 69 kDa for *Bothrops asper* hemorrhagic metalloproteinase, BaH4[19] and to 60 kDa for *Vipera palaestinae* hemorrhagic factor, HR1.[20] According to their molecular masses and SVMP-classification,[3, 21] the purified metalloproteinase belongs to P-III class of SVMPs.

Both crude venom and purified enzyme induced haemorrhagic spots at 10 µg dose. Comparing the texture of the hemorrhagic spots, it was obvious the hemorrhagic spot induced by crude venom showing the intact vascular network while that induced by the purified enzyme showing red, edematous, amorphous bleeding spot without vascular network appearance. This suggested that the purified enzyme cause bleeding by affecting the microvasculature and interstitial matrix. Lesions in the walls of small blood vessels are caused by proteolysis of components of the basal lamina of the microvasculature and substrates of all the major proteins of the extracellular matrix showed degradation by venom metalloproteinase.[22]

The larger edema ratio resulting from the metalloproteinase injected foot pad indicated that the enzyme contributes the edema inducing property of the crude venom in large proportion. In external appearance, more dark red hemorrhage was seen in the edematous food pad in which the enzyme injected than those in which crude venom injected. The venom metalloproteinase plays a relevant role in the complex and multifactorial inflammatory response characteristic of viperine envenomation.[23] The proteolytic domain of SVMPs per se is able to trigger inflammatory events both *in vivo*[24] and *in vitro* experimental models[25] as in BaP1 from *Bothrops asper* venom.

The massive extravasation of erythrocytes in histological view (Fig. 3c) may be due to per diapedesis, i.e., erythrocytes escape through widened intracellular junctions of endothelial cells[26] or per rhexis mechanism via which erythrocytes escape to the interstitial through gaps or lesion within the endothelial cells.[27] The hemorrhagic toxins induce myonecrosis as a consequence of ischaemia. Histological evidence of myonecrosis was observed at relatively late time intervals after BaH1 injection, i.e., after 6 hours, whereas hemorrhage develops within minutes.[28]

The results on fibrin plates evidenced that the purified enzyme has fibrinolytic activity suggesting that it acted directly on fibrin in dose dependent manner. Similarly, Porthidin-1, a metalloproteinase from the venom of Lansberg's hog-nosed pitvipers (*Porthidium lansbergii hutmanni*),[29]

ahpfibrase, P-II class SVMP from *Gloydius halys*[30] and fibrolase, a non-hemorrhagic fibri(ogen)lytic metalloproteinase isolated from the southern copperhead snake (*Agkistrodon contortrix contortrix*)[31] showed fibrinolytic activity on the fibrin plates. The fibrinolysis by enzyme could have repercussions on the final balance of patient hemostasis and could induce a more intense hemorrhagic syndrome.

At the concentration of 5 µg, the purified enzyme cleaved the Aα-chain of bovine fibrinogen within 60 minutes, but no apparent effect on Bβ- and γ-chain. Thus, it is an α-fibrinogenase, similar to daborhagin-M of *Daboia siamensis* from Myanmar,[16] EoVMP2, haemorrhagic group P-III metalloproteinase of the venom of the West African saw-scaled viper *Echis ocellatus*,[32] VaH1 haemorrhagic P-III metalloproteinase from *Vipera ammodytes ammodytes*,[17] albofibrase from green pit viper (*Trimeresurus albolabris*) venom[33] and patagonfibrase from *Philodryas patagoniensis* venom.[34] The purified enzyme is so able to interfere with the haemostatic system by degrading plasma fibrinogen.

Conclusion

A high molecular mass metallo-proteinase has been purified from the venom of Myanmar Russell's viper. Its molecular weight (75 kD), and its inhibition by EDTA suggest that it is a P-III class snake venom metalloproteinase. This toxin induced hemorrhage, edema and myonecrosis following local injection demonstrated its involvement in local tissue damage in viper envenomation.

It also possesses coagulant, fibrinolytic and fibrinogenolytic activities. As a member of the SVMPs family, the purified enzyme would be likely to show multiple effects on hemostasis. Further studies are required to determine the full structure of the metalloproteinase enzyme from Myanmar Russell's viper venom to elucidate its mechanism of action.

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Relationship of Plasma Proteins (Albumin & Fibrinogen) and Red Cell Aggregations (Rouleaux & Rosette Formations) in Falciparum Malaria Patients

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This cross-sectional, comparative study was done to compare and correlate some plasma proteins (albumin and fibrinogen) and red cell aggregations (rouleaux and rosette formations) among healthy adult males, uncomplicated falciparum malaria and moderately severe falciparum malaria in Myanmar male patients. Rouleaux formation (determined by ESR) and fibrinogen concentration were significantly greater ($p<0.05$) in uncomplicated malaria (56.17±27.91 mm/1st hour and 1317.89±676.74 mg/dl) as well as in moderately severe falciparum malaria (72.42±38.04 mm/1st hour and 1342.17±789.74 mg/dl) than healthy subjects (23.83±18.39 mm/1st hour and 461.94±310.62 mg/dl). Fibrinogen concentration showed no significant difference between uncomplicated and moderately severe falciparum malaria patients although ESR was significantly higher in moderately severe malaria. Rosette formation rate (%) was different between uncomplicated (8.39±19.03) and moderately severe falciparum malaria (20.67±26.11), but not statistically significant. Albumin concentration was decreased in uncomplicated falciparum malaria, but not significant (4.28±1.31 g/dl) and significantly decreased in moderately severe falciparum malaria (3.72±0.98 g/dl) than normal subjects (5.29±1.18 g/dl). Parasite count was significantly higher in uncomplicated falciparum malaria blood (56500.00 ±68510.25 asexual form/microliter of blood) than that of moderately severe falciparum malaria (42283.33±67452.47). Rosette formation rate was inversely correlated with fibrinogen concentration in moderately severe falciparum malaria (r=-0.586), but not in uncomplicated falciparum malaria patients (r=-0.140). According to the findings, it was evident that higher rosette formation in moderately severe malaria patients may be due to fibrinogen level which was not obviously higher than that of uncomplicated malaria patients. It was concluded that the lower the fibrinogen concentration, the higher the rosette formation which may lead to lesser parasite count in moderately severe falciparum malaria when compared with uncomplicated falciparum malaria.

INTRODUCTION

The parasitized red blood cells (pRBCs) in severe falciparum malaria increase adhesiveness to a number of other cells including the vascular endothelium (cytoadherence)[1] and non-parasitized RBCs (rosette formation)[2] to form sequestration that can lead to cessation of local blood supply and decreased availability of oxygen to cells including neurons, thus contributing to manifestations of severe malaria. Plasma protein fibrinogen known to participate in rouleaux formation of normal erythrocytes also produces stable rosettes in conjunction with the parasite-derived rosetting ligand *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1).[3] Albumin can be used to estimate status of liver function in malaria[4, 5] but its influence on red cell aggregation was not found yet. This study aimed to find out relationship between plasma proteins (albumin, fibrinogen) and red cell aggregations (rosette and rouleaux)

in confirmed uncomplicated falciparum malaria (CUM) and confirmed moderately severe falciparum malaria (CSM) patients.

MATERIALS AND METHODS

Cross-sectional, comparative study was done from January to November, 2006. Eighteen confirmed uncomplicated falciparum malaria (CUM) patients and 12 confirmed moderately severe falciparum malaria (CSM) patients from Defence Services General Hospital (DSGH), No. 11 Defence Industry (DI 11) and Vector Born Disease Control Department (VBDC) and 18 apparently healthy volunteers: 10 from Defence Services Medical Academy (DSMA) and 8 from University of Medicine 2 (UM 2) included in the study.

Experimental procedure

Falciparum malaria was confirmed by examining *Plasmodium falciparum* parasites on thick and thin blood films. After thorough explanation about the study, informed consent taking and venous blood sampling were done after confirming and counting the parasite. Subjects were selected and grouped according to WHO criteria, 2000.[6] ESR was determined by the modified Westergren method of the International Committee for Standardization in Haematology (ICSH).[7] Fibrinogen determination was done by using precipitation method.[8]

Albumin determination was done by colorimetric method with bromocresol green[9] using Hospitex Diagnostics srl kit from Italy. *In vitro* culture of *plasmodium falciparum* was done by using Candle-Jar technique[10] on blood group O normal red blood cells with the help of a technician from Parasitology Research Division, Department of Medical Research (lower Myanmar). Rosette formation rate was estimated in cultivated parasitized red blood cells containing mature trophozoite stage (at least 5%-judged by thin film examination) by staining with Acridine Orange on a drop of diluted blood (culture media: red cell was 99:1) under light microscope and pRBCs were confirmed by fluorescent microscope.[2] The pattern of rosette was photographed through tri-ocular microscope.

RESULTS AND DISCUSSION

Comparison of data between normal and malaria cases was done by ANOVA. Correlations between the variables were done by Pearson correlation test. Significant level was set at $p<0.05$.

Table 1 shows ESR, rosette formation rate, fibrinogen concentration, albumin concentration and parasite count in the study groups. ESR was significantly higher in malaria patients than healthy control subjects. Rosette formation was found only in malaria patients and higher in CSM than CUM, but not significant statistically.

Table 1. Comparison of ESR, rosette, albumin, and fibrinogen concentration between healthy control subjects and falciparum malaria patients

Variables	Healthy control subjects (n=18) Mean±SD	Falciparum malaria patients	
		CUM (n=18) Mean±SD	CSM (n=12) Mean±SD
ESR (mm/1st hour)	23.83 ±18.39	56.17 ±27.91*	72.42 ±38.04*
Rosette (%)	0	8.39 ±19.03	20.67 ±26.11
Fibrinogen (mg/dl)	461.94 ±310.62	1317.89 ±676.74*	1342.17 ±789.74*
Albumin (g/dl)	5.29 ±1.18	4.28 ±1.31	3.72 ±0.9*
Parasite count (asexual form/ µL of blood)	0	56500.00 ±68510.25	42283.33 ±67452.47*

*=$p<0.05$, CUM=confirmed uncomplicated falciparum malaria, CSM=confirmed moderately severe falciparum malaria

Plasma fibrinogen concentration was significantly higher in malaria patients than in normal subjects, but not significantly different between CUM and CSM patients. No significant difference in fibrinogen level of CUM and CSM has generally been explained by activated coagulation and fibrinolytic system in blood of malaria patients.[11] It may also be due to hepatitis[12]

and impaired liver function from some hepatic enzymes derangement[13] in malaria.

In the present study, plasma albumin concentration was decreased in malaria patients, but not significant in CUM and significantly decreased in CSM ($p<0.05$). The level of plasma albumin is important in consideration of liver involvement in malaria patients.[4] Hepatic protein biosynthesis shifts from albumin synthesis to the synthesis of proteins involved in the acute inflammatory response such as C-reactive protein, coagulation factors, fibrinogen and complement components.[5] Significantly reduced albumin concentration of CSM patients in the present study may be related to impaired liver function of plasma fibrinogen synthesis.

In the present study, liver function impairment in some CSM patients as significantly lower albumin than CUM patients was observed. Parasite count in circulation of CSM patients was significantly lower than that of CUM patients. It may be consequence of cytoadherence and sequestration of most parasitized RBCs in microcirculations of falciparum malaria infection.[1]

Table 2. Correlation between erythrocyte sedimentation rate and plasma proteins

ESR	Plasma proteins (r value)	
	Fibrinogen	Albumin
Normal	0.367	-0.492*
CUM	0.327	-0.237
CSM	0.376	-0.522

*=$p<0.05$, CUM=confirmed uncomplicated falciparum malaria, CSM=confirmed moderately severe falciparum malaria

Table 2 shows direct relationship of ESR with fibrinogen concentration and inverse relationship of ESR with albumin concentration both in normal and malaria patients. It indicated effect of fibrinogen and albumin on ESR in falciparum malaria blood.

Table 3 shows that inverse correlation of rosette formation both with fibrinogen and albumin concentration in malaria patients. Significant inverse correlation was found in CSM ($p<0.05$). It indicated that in falciparum malaria patients, rosette formation will be higher if fibrinogen is decreased. In other words, rosette formation will be decreased if fibrinogen is increased. This effect can be seen more obviously in CSM patients. It has been postulated that fibrinogen may hinder the formation of rosettes by interfering with expression of adhesive molecules such as PfEMP1 on the membrane of pRBCs.[14] It also explained the reason why rosette formation rate was higher in moderately severe falciparum malaria patients when they showed no significantly higher fibrinogen concentration than that of uncomplicated falciparum malaria patients in the present study.

Table 3. Correlation between rosette formation and plasma proteins

Rosette formation	Plasma proteins (r value)	
	Fibrinogen	Albumin
CUM	-0.14	-0.32
CSM	-0.586*	-0.313

*=$p<0.05$, CUM=confirmed uncomplicated falciparum malaria, CSM=confirmed moderately severe falciparum malaria

Microscopic findings

Figure 1, 2 and 3 show cultivated red cell in culture media. Normal red cell did not form rouleaux formation in culture media. Malaria red cells form aggregations not in rouleaux, but in rosette pattern.

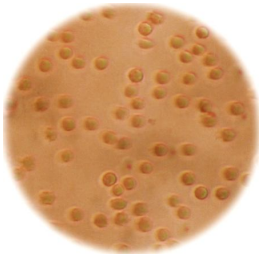

Fig. 1. Normal RBCs in culture media (x16)

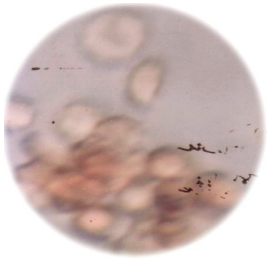

Fig. 2. Rosettes of cultivated RBCs in culture media (x40)

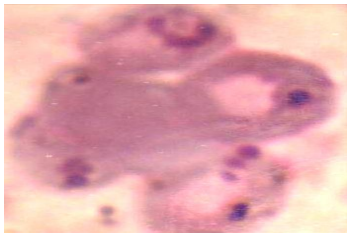

Fig. 3. Reversed rosette formation in fresh blood film of CSM patient (x100)

Conclusion

It was concluded that the lower the fibrinogen concentration, the higher the rosette formation which may lead to lesser parasite count in moderately severe falciparum malaria when compared with uncomplicated falciparum malaria.

Recommendation

In the present study, a plenty of reversed rosettes and large white blood cells aggregations were found in moderately severe malaria patients. Further research should be done on relationships of reversed rosette (a non-parasitized RBC surrounded by two or more parasitized RBCs) formation, WBC aggregations and severity of malaria. Generally, globulin affects on ESR similar to fibrinogen. So, it may affect on rosette as well. Further research should be performed to find out the effect of globulin on ESR and rosette formation to use it as the index of rosette formation. Relationship of albumin, fibrinogen and liver functions in falciparum malaria patients should be studied as the further research.

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Evaluation of Deltamethrin Impregnated Bed Nets for Reduction of Malaria Transmission in Forested Foothill Area of Bago Region

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The field trial was conducted in Gyobingauk Township, Bago Region from January 2010 to April 2012 to identify the efficacy of deltamethrin (55 mg/m2) treated mosquito nets against malaria vectors in Thayetchaung Village as test area, and Bawbin dam Village as control. Before deltamethrin impregnation, entomological and parasitological baseline data were collected from March 2010 to February 2011. Mosquito nets were impregnated 6 monthly in Thayetchaung Village in March, August 2011 and February 2012. Adult mosquitoes and finger-pricked blood samples (parasite positivity) were collected and identified seasonally in both study villages. Three-minute bioassay test was done according to WHO cone test methods. Mortality was measure after 24 hours exposure time in paper cup with glucose in damp condition in dry (March and April), rainy (July and August), cold (January and Febuary) seasons and after six months of used insecticide treated nets. Overall, *An. vagus* predominated (34-37%), followed by *An. Annularis* (20-26%), *An. culicifacies* (12-14%), *An. maculatus* (10-12%), *An. philippinensis* (2-3%), *An. stephensi* (1-3%), *An. aconitus* (0.7-1.0%), and the main vector *An. minimus* (4-7%). After intervention, seasonal parasite positivity rates reduced from 1.6%, 3.2%, and 3.2% to 0%, 0.7% and 0.7% in intervention village. In the control village, man-biting rates and parasite positivity rates were gradually increased across three seasons. Indoor man-biting rate of *Anopheles* mosquitoes significantly reduced from 3, 4.4 and 5.4 to 0.8, 0.8 and 1 in intervention village (p=0.4986). The main vector *An. minimus* also reduced from 0.8, 1.2 and 1 to 0, 0, and 0.2 in three seasons, respectively. In contrast, there was no significant reduction of outdoor man-biting rates in both test and control villages. The insecticide susceptibility test of dry, rainy, cold season and 6 months after impregnated nets for 3-minute bioassay tests showed 100% mortality of *An. maculatus, An. vagus, An. annularis, An. culicifacies* and *An. minimus.* Therefore, deltamethrin treated nets could reduce indoor man-biting rate and malaria transmission effectively.

INTRODUCTION

WHO report (2005) estimated that 350-500 million clinical malaria cases occur annually, most of these areas caused by *Plasmodium falciparum* and *Plasmodium vivax*.[1] Malaria is still the most important parasitic and vector-borne diseases in many tropical and subtropical countries. It is one of the main health problems in the Southeast Asia. Malaria has been a priority health problem in Myanmar and there are 600,000 cases annually.[2] The Roll Back Malaria partnership set on ambitious target for 2010 of 80% protection of high risk groups by a locally appropriate vector control measure.[3]

As a consequence of this decision, ITNs are being implemented as part of national malaria control programmes around the world.[4] Insecticide-treated mosquito nets have emerged as a potent tool globally in preventing morbidity and mortality due to

mosquito-borne diseases.[5] Main mosquito-borne diseases are malaria transmitted by *Anopheles mosquitoes*, dengue fever and dengue haemorragic fever transmitted by *Aedes* mosquitoes, Japanese encephalitis and filariasis transmitted by *Culex* mosquitoes.

Malaria is considered to be the most prevalent disease of tropical and sub-tropical regions worldwide. It is necessary to have an appropriate and timely care program for patients and successfully integrate and selective control of the vector species.[6] Bet nets have been used against mosquitoes or other biting insect for a long time. Untreated mosquito nets were reported to give some protection against malaria and mosquitoes.[7] They did not reduce incidence[8] or prevalence of malaria,[9] splenomegaly, or parasitaemia rate.[10] Bed nets cannot provide complete protection against blood questing mosquitoes.[11]

A study of untreated nets were similar to nonusers in terms of fever, reported axillary temperature, parasite density and hemoglobin level.[12] Treatment of mosquito nets with synthetic pyrethroid like permethrin or deltamethrin has been studied in various community level settings and recommended as a malaria control measure.[13] Insecticide-treated nets (ITNs) and long lasting insecticide nets (LLINs) lead to a reduction of human-vector contact and reducing mosquito population and also provide a physical barrier with high coverage levels that benefit the whole community. A large-scale trial of deltamethrin-treated bed nets in China and small-scale trial of India revealed that treated nets gave high protection against mosquitoes.[14, 15] Same trials were carried out in various countries of Asia[16, 17, 18] and Africa.[1]

It is well known that one of the elements for successful malaria prevention is reduction in man-vector contact.[19] Pyrethroid treated bed nets have been shown in recent trials to have an important impact on cases of malaria, incidence of infection and prevalence of anemia and all caused child deaths in several parts of Africa.[20] In Myanmar, different brands of long lasting insecticidal nets (LLINs) were distributed in malaria prone areas by Ministry of Health, local NGOs and INGOs but in some areas, where malaria is endemic any LLINs have not been distributed and villagers encounter difficulties, and distance making malaria prevention uneasy. Villagers could not afford to buy LLINs because they are more expensive than untreated nets. They used 1 to 2 untreated bed nets to protect from mosquito bite. These nets can be impregnated with WHO recommended insecticide as deltamethrin. Study areas of Gyobingauk Township, Bago Region are forest foothill areas and malaria is endemic and insecticide treated mosquito nets are never distributed. There is a need to introduce locally available, convenient and inexpensive WHO recommended insecticide as innovative measures for villagers.

Therefore, it was decided to impregnate the untreated nets with deltamethrin and evaluate their effectiveness on malaria transmission and occurrence of *Anopheles* mosquitoes in intervention and non-intervention forest foothill areas of Gyobingauk Township, Bago Region.

MATERIALS AND METHODS

Study design and areas

A quasi-experimental study was done in Thayetchaung Village as intervention area and Bawbin Village as non-intervention area in Gyobingauk Township, Bago Region.

Study period

It was started from January, 2010 to April, 2012. Baseline data collection was done from March 2010 to February 2011 and intervention was done from March 2011 to February 2012, seasonally. The seasons are divided as hot season (March to May), rainy season (June to October), and cold season (November to February).

Thayetchaung and Bawbin villages

These two villages are situated beside the Bawbin dam of the foothill area of Bago

Yoma. Bawbin dam Village is situated at eastern part of the dam and Thayetchaung Village is situated at western part of the dam. Thayetchaung is about 6 km away from Bawbin and both villages are about 40 km far away from Yangon Pyi Road. One RHC, one government primary school and one monastery primary school are available in Thayetchaung.

About 58 houses consisted in Bawbin and 61 houses consisted in Thayetchaung. Total populations are about 286 populations live in Bawbin and 315 populations live in Thayetchaung. In both villages, households have 1 to 2 untreated mosquito nets. In Bawbin, a total of 76 untreated mosquitoes nets were owned by 58 houses and 61 houses of Thayetchaung have 96 untreated nets. In both areas, over 85% of the populations were farmers and remaining were wood cutters, charcoal makers, fisher-men, school teachers, government staff, and dam staff.

Study population

Mosquitoes and blood samples were collected seasonally as baseline year from March 2010, August 2010 and February 2011 and intervention year in March, August 2011 and February 2012. A total of 286 populations from Bawbin and 315 populations from Thayetchaung, at least 50% of finger-prick blood samples were collected for malaria prevalence study in each village seasonally.

Intervention description

Impregnation of mosquito nets by deltamethrin 2.5 EC from selected households used a total of 96 mosquito nets of 61 households from Thayetchaung intervention area) were impregnated with deltamethrin insecticide for six-monthly for one year in March, August 2011 and February 2012. The rate of dipping was deltamethrin 55 mg/m^2. All impregnated mosquito nets were dried under the shade of the house and trees. At the same time, finger-prick blood slides were taken from all selected household populations of both areas seasonally.

Thick and thin blood films

In both intervention and control areas, at least 50% of finger-prick blood of thick and thin blood films were collected seasonally from selected household's populations by trained technician through house-to-ouse fortnightly visit. Collected blood films were stained with 10% Giemsa's stain and malaria parasites were identified under oil emersion lens of compound microscope.

Mosquitoes collection

Indoor and outdoor human bait catching were done using WHO sucking tube and cattle bait catching using a big mosquito net (330x 3300x 180 cm), *An.* mosquitoes were collected according to WHO collection method[21] by well-trained insect collectors for 5 days in both areas. Larvae were collected in and around 3 kilometers away from villages. Collected *Anopheles* mosquitoes and adult mosquitoes emerged from larval survey were identified according to standard morphological methods.[22-26]

Mosquito susceptibility test

Three-minute bioassay test of unwashed deltamethrin impregnated nets was done seasonally according to WHO cone test methods[27] with wild caught *Anopheles* mosquitoes. After three-minute bioassay test, Knockdown effect was measured 60 minutes of exposure period and mortality was counted after 24 hours exposure periods in paper cups with glucose under moisture condition.

Data analysis

Computing parasite positive rate, spleen positive rate, infant parasite rate, indoor and outdoor man-biting rate, insecticide susceptibility as % knockdown, % mortality, were calculated using Microsoft EXCEL software.

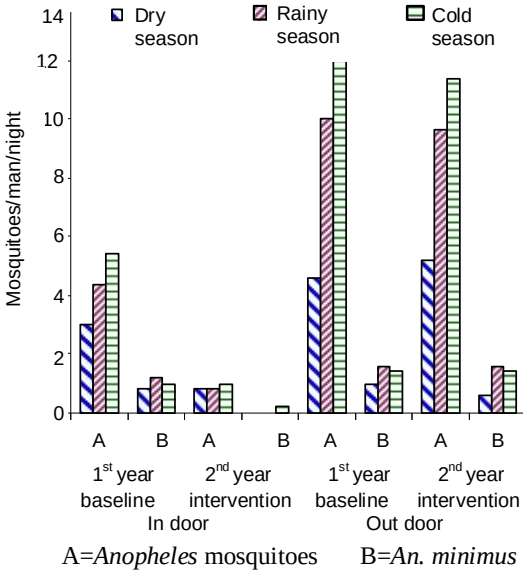
RESULTS

Anopheles mosquitoes collection

Detail of seasonally *Anopheles* mosquitoes catching by different catching methods of baseline and interventions were shown in Table 1.

Table 1. Year-round total collected *Anopheles* mosquitoes species by different methods in intervention and non-intervention villages

Mosquito species	Thayetchaung				Bawbin			
	Baseline		Intervention		Baseline		Non-intervention	
	Total	%	Total	%	Total	%	Total	%
An. vagus	556	36.75	512	37.40	569	34.38	576	34.18
An. annularis	392	25.91	344	25.13	411	24.83	353	20.95
An. culicifacies	212	14.01	172	12.56	209	12.63	237	14.07
An. maculatus	160	10.58	161	11.76	186	11.24	196	11.63
An. minimus	64	4.23	60	4.38	99	5.98	106	6.29
An. kichi	22	1.45	29	2.12	27	1.63	30	1.78
An. babirostris	27	1.78	25	1.83	53	3.20	67	3.98
An. philippnansis	43	2.84	34	2.48	49	2.96	59	3.50
An.aconitus	11	0.73	11	0.80	12	0.73	16	0.95
An.tessellatus	1	0.07	1	0.07	1	0.06	1	0.06
An.stephensi	25	1.65	20	1.46	39	2.36	44	2.61
Total	1513	100	1369	100	1655	100	1685	100
	x^2=4.704, p=0.9100				x^2=10.026, p=0.4382			



A=*Anopheles* mosquitoes B=*An. minimus*

Fig. 1. Indoor and outdoor man-biting rate of *Anopheles* mosquitoes and main vector *An. minimus* in baseline year and intervention year of Thayetchaung Village

Overall, *An. vagus* predominated (34-37%) in both areas, followed by *An. annularis* (22-26%), *An. culicifacies* (13-14%), and *An. maculatus* (11-12%), moderate amount of *An. minimus, An. kochi, An. babirostric, An. philippnonsis* and *An. stephensi* (6-2%) and very small amount of *An. aconitus* and *An. tessellatus.* The main vector *An. minimus* was caught (4-6%) by indoor, outdoor and animal bait catching methods. The peak biting time of *An. minimus* was observed 22:00 to 01:00 hours. *An. minimus* is a main vector of malaria in these areas. Mostly, *An. minimus* larvae were collected in rice field. Highest density of *Anopheles* mosquitoes and larvae were collected in the rainy season followed by the cold season.

Man-biting rate

Indoor man-biting rate of main vector *An. minimus* was significantly reduced from 0.8, 1.2 and 1 to 0, 0, and 0.2 in hot, rainy and cold seasons, respectively. Indoor man-biting rates of *Anopheles* mosquitoes were also significantly reduced from 3, 4.4 and 5.4 to 0.8, 0.8 and 1 in intervention village (p=0.4986) (Fig. 1).

Malaria parasite positive rates

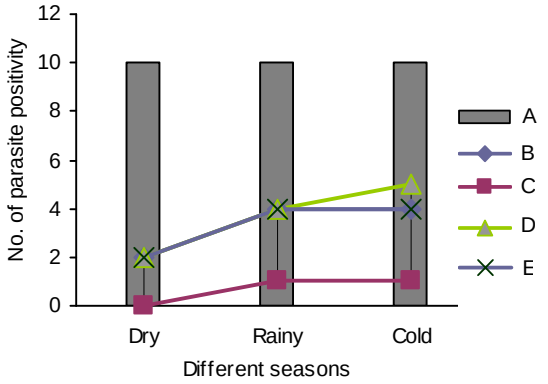
In the study period, slide positive rate (SPR) and slide *falciparum* rate (SFR) were not significantly different between baseline years of both villages (Table 2).

Table 2. Seasonal malaria parasite rates of Thayetchaung and Bawbin during baseline and intervention years

	Baseline year 1			Intervention year 2		
Intervention (Thayetchaung Village)						
Seasonally	Dry M-M	Rainy J-O	Cold N-F	Dry M-M	Rainy J-O	Cold N-F
Total examination	125	124	124	138	139	141
Total positive	2	4	4	0	1	1
Pf	1	2	2	0	1	1
Pv	1	2	1	0	0	0
Mixed	0	0	1	0	0	0
SPR%	1.6	3.2	3.2	0	0.72	0.71
SFR%	0.8	1.6	1.6	0	0.72	0.71
	x^2= 3.715, p=0.4162					
Non-intervention (Bawbin Village)						
Seasonally	Dry M-M	Rainy J-O	Cold N-F	Dry M-M	Rainy J-O	Cold N-F
Total examination	123	128	138	126	130	138
Total positive	2	4	5	2	4	4
Pf	1	2	2	1	2	2
Pv	1	1	2	1	2	1
Mixed	0	1	1	0	0	1
SPR%	1.62	3.1	3.62	1.58	3.07	2.89
SFR%	0.81	1.56	1.45	0.79	1.53	1.45
	x^2= 3.3463, p=0.5016					

M-M=March to May, J-O=June to October
N-F=November to February

During intervention, seasonal parasite positivity rates were reduced from 1.6%, 3.2%, and 3.2% to 0%, 0.7% and 0.7% in intervention village of Thayetchaung (Fig. 2).



A =ITNs impregnation C=Intervention E=Non-intervention
B=Baseline D=Baseline

Fig. 2. Malaria parasite positivity against impregnation of mosquito nets in different seasons of intervention (Thayetchaung) and non-intervention (Bowbin) areas

Year-round total malaria parasite positive rates of baseline and intervention years of both areas found that *P. falciparum* 50% (5/10), *P. vivax* 40% (4/10) and mixed 10% (1/10) in baseline year and *P. falciparum* 100% (2/2) in intervention year in Thayetchaung; in Bawbin, *P. falciparum* 45.46% (5/11), *P. vivax* 36.36% (4/11) and mixed 18.18% (2/11) in baseline year and *P. falciparum* 50% (5/10), *P. vivax* 40% (4/10) and mixed 10% (1/10) in intervention year (Table 2). *Plasmodium falciparum* was slightly dominant in these areas.

Spleen positive rates

Spleen positive rate of 2-9 years old children in both areas were found more or less similar between intervention and non-intervention villages (i.e., 24% in Thayetchaung and 25% in Bawbin). Infant parasite rates were observed zero in both villages during survey periods.

Age specific parasite rate

Age specific parasite rate was found the highest in 15-30 years age group followed by 9-15 years age group in both areas. Highest-risk age group was observed in 15-30 years age group.

Three-minute bioassay test

Before and after treating of bed nets with deltamethrin in Thayetchaung Village, bioassay test was done seasonally and after six months impregnation according to WHO cone test methods with wild caught *Anopheles* mosquitoes. There were 90-100% knockdown and 100% mortality of *Anopheles* mosquitoes and 100% knockdown and 100% mortality of *An. minimus* against deltamethrin treated nets in seasonally and after six months of treated nets.

DISCUSSION

Insecticide treated nets (ITNs) are the best alternative control method where malaria is endemic and could not affordable to buy LLINs or are lacking or not distributed areas. Bago Yoma mountain range and it's foothill areas are highly prevalent for malaria.[28, 29] *An. dirus* is a main vector of malaria in endemic areas and they are abundantly found in deep forest of Bago Yoma and *An. minimus* are found in forest fringe and foothill areas of Bago mountain range.[26, 29, 30]

A total of 11 *Anopheles* mosquitoes species were collected from both areas and *An. vagus* was predominantly distributed in both areas (34-37%), followed by *An. annularis* (20-26%), *An. culicifacies* (13-14%), *and An. maculatus* (11-12%), moderate amount of *An. minimus, An. kochi, An. babirostric, An. philippnonsis* and *An. stephensi* (6-2%) and very small amount of *An. aconitus* and *An. tessellatus*. The main vector *An. minimus* was caught (4-6%) by indoor, outdoor and animal bait catching methods (x^2=4.704, p=0.9100 for Thayetchaung, x^2=10.026, p=0.4382 for Bawbin). *An. minimus* are early mid-night vector and its peak biting time was observed at 22:00 to 23:00 hour. The results were agreed with previous researchers worked in Thabwewa Village, Oktwin Township, Bago Region and Yeasitkan Village, Taikkyi Township, Yangon Region.[29, 31, 32] *An. minimus* is a main vector of malaria in these areas because the main

vector *An. dirus* was not caught in all mosquitos' collection methods in the study periods. Some researchers revealed that *An. dirus* are hilly or forested breeder and *An. minimus* are found in forest fringe foothill areas and plain areas.[28, 29, 30]

Highest numbers of *An. minimus* larvae were collected in slowly running water of creeks followed by sand pools and rice fields in Thyetchaung than Bawbin. *An. dirus* larvae are abundantly present in wells in coastal areas of Mon State and Tanintheryi Region of Myanmar.[33, 34] Mostly *An. minimus* larvae were collected in rice field.[35] Highest density of *Anopheles* mosquitoes and larvae were found in rainy season followed by cold season. These results agreed with the study in forest foothill area of Bago Yoma.[28] After deltamethrin impregnation of mosquito nets, indoor man-biting rate was significantly reduced when compared with preintervention periods. Outdoor man-biting rate was higher than indoor man-biting rate and also outdoor man-biting rates were not significantly different in both areas in baseline year and intervention periods. Insecticide treated nets cannot effect the outdoor man-biting rates and outdoor biting *Anopheles* mosquitoes and zoophilic *Anopheles* mosquitoes.

This is the first report of the evaluation of deltamethrin treated bed net to malaria control in Bawbin Dam area in Gyobingauk Township, Bago Region. The number of *An. minimus* in indoor biting in house with treated bed nets were significantly reduced compared with baseline and untreated nets used in control areas (x^2=3.365 p=0.4986, x^2=3.6463 p=0.4559). In longitudinal trials in India, reductions in the indoor resting densities of *An. culicifacies* by use of nets treated with lambadacyhalothrin,[36, 37] of *An. minimus* by using deltamethrin[15] and of *An. fluviatilis* by cyfluthrin treated nets,[38] have been reported. Similar reductions against different malaria vectors were found in Kenya.[18, 39] The reduction in numbers can be due to excite repellency, deterrence or mass killing of mosquito vector or any of these combinations.[40] The treated nets significantly reduced the survival of *An. culicifacies* as shown by the decreased parous rate.[14, 40] The present study found that indoor biting rate of malaria vector mosquito was significantly reduced in deltamethrin treated net village compared to untreated nets village during intervention but outdoor biting rates were not significantly reduced, it was as same as baseline outdoor biting rate. Deltamethrin treated nets not only protected the volunteers but also killed the mosquitoes attracted to the sleepers.[15]

Although untreated nets are reported to give some protection compared with no nets,[11] they do not provide complete protection against blood questing mosquitoes. The biting rhythm of *An. minimus* showed that it feed throughout the night but the peak biting occurred between 22:00 and 01:00 hours. This is important epidemiologically because most of the people would be under the nets during this period (20:00 to 04:00) which covers the peak biting time, and can thus escape being bitten and transmission of malaria. At the same time, the treated nets would kill the vector mosquitoes attracted by the sleepers. Deltamethrin treated nets in regular use caused 100% mortality of malaria vector *An. minimus* and secondary vector *An. vagus, An. maculatus, An. culicifacies,* and *An. annularis* up to 6 months in 3 minutes bioassay.[27] Two studies revealed that the insecticide activity declined after 6 months with treated various pyrethroids. In this study, indoor biting rate and malaria cases declined more markedly when mosquito nets treated with deltamethrin every 6 months because 100% mortality in vectors was recorded up to 6-month treated nets.[41, 36]

Baseline year study showed that malaria positivity rate was higher than intervention year in Thayetchaung and also positive rate was slightly higher in non-intervention village than in intervention village in baseline year. In the study period, slide

positive rate (SPR) and slide *falciparum* rate (SFR) were not significantly different between baseline years of both villages. During intervention, seasonal parasite positivity rates were sharply reduced from 1.6%, 3.2%, and 3.2% to 0%, 0.7% and 0.7% in intervention village of Thayetchaung (x^2=3.715, p=0.4462) (Table 2).

Total malaria parasite positive rates of baseline and intervention year of both areas found that *P. falciparum* 50% (5/10), *P. vivax* 40% (4/10) and mixed 10% (1/10) in baseline year and 100% (2/2) *P. falciparum* in intervention year in Thayetchaung Village but in control village, parasite positive rate was not significantly reduced, *P. falciparum* 45.46% (5/11), *P. vivax* 36.36% (4/11) and mixed 18.18% (2) in baseline year and *P. falciparum* 50% (5/10), *P. vivax* 40% (4/10) and 10% (1/10) mixed in nonintervention year in Bawbin were detected (x^2=3.3463, p=0.5016). *P. falciparum* was slightly dominant in these areas. In Myanmar, *P. falciparum* is a dominant species[42] but in some areas *P. vivax* infection rate is gradually rising.[43]

Highest parasite positive rate was observed among 15-30 years followed by 31-45 years and 9-15 years age groups. Two *P. falciparum* cases were observed, one in rainy and one in cold season during intervention period. It may be due to the fact that these age groups went to deep forest to collect wood, bamboo, bamboo shoot, making charcoal, fishing and hunting. Thabyewa Village of Bago Yoma had high malaria positivity.[31, 33]

Present study found that spleen positive rate of 2-9 years children in both areas were found more or less similar between intervention and non-intervention villages (i.e., 24% in Thayetchaung and 25% in Bawbin). It was 1 to 2 folds lesser than the spleen studies[29, 31] of Thabyewa Village of Bago Yoma in which the spleen positive rate was high (50 to 70%). The spleen positive rates of nearly 25% in both areas revealed that the areas are holo endemic areas of malaria.[19] Infant parasite rates were observed zero in both villages during survey periods compared to high rate (70 to 100%) in Thabyewa Village.[29, 31] Similar results have been found in Letpankhon Village, Bago Yoma foothill area, Oungpinquin Village, Kanbock Township, Thanintharyi Region, Kyaungkan and Weyat villages of Thanbuezayat Township, Mon State.[29, 34, 44] Infant parasite positive rate was zero%, it means that there was not indoor malaria transmission or very low transmission and it may be due to the reduction of indoor man biting rate in both areas using mosquito nets.[19]

Present study concluded that deltamethrin treated mosquito nets prevent malaria transmission certifiably in test area. Although ITNs are recommended for malaria control purposes, their performance should be monitored in the field under various ecological settings to assess their durability and long-term effectiveness for malaria prevention and control. Strategic plan of each area should be carefully designed to preserve the effectiveness of ITNs. The different methods of bioassay which are easier to perform than the other methods should be considered.

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Detecting Rotavirus Genotypes in Children under Five Years of Age Admitted for Diarrhea in Yangon Children's Hospital, 2010-2011

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Rotavirus is one of the leading causes of diarrheal disease and diarrheal deaths in infants and under five year-old children worldwide. The development of vaccines against rotavirus diarrhea is based upon the identification of the most common circulating genotypes. This study involved detection and characterization of rotavirus genotypes in stool specimens from children under 5 years admitted with diarrhea to the three medical wards of the Yangon Children's Hospital. The stool samples were tested for the presence of rotavirus antigen by a commercial enzyme immunoassay (ProSpect T^{TM} Rotavirus, OXOID, UK). Among 1208 stool samples collected and tested from January 2010 to September 2011, rotavirus was detected in 670 cases (55%). Rotavirus diarrhea was most prevalent in the 6-11 months age group (49.7%) and occurred more in males than females (1.7:1). A subsample of rotavirus positive stool samples were selected and subjected to G and P genotyping by employing multiplex reverse transcription-polymerase chain reaction (RT-PCR). Of the 204 samples subjected to genotyping, 167 (81.9%) were genotype G12, followed by 13(6.4%) of G2, 9(4.4%) of G1, 3(1.5%) of mixed type (G2+G12), 1 (0.5%) of G3 and the remaining 11 samples (5.3%) were untypable. The most common G and P combination was G12 P[8]. This study is the first to report a rotavirus genotype G12 in Myanmar. The emergence of G12 genotype and distribution of G and P genotypes provides valuable information in the consideration of the effectiveness of rotavirus vaccination.

INTRODUCTION

Rotavirus infection is the most important cause of severe gastroenteritis among children under five years worldwide.[1] In developed nations like the US, around 70 child deaths are attributed each year to this diarrhea.[2] In developing countries, however, nearly 1,400 children die from rotavirus infections each day.[1] A hospital-based surveillance for rotavirus diarrhea in children in Yangon revealed that 10% of all hospitalization in children was due to rotavirus diarrhea.[3]

Rotavirus is a 100-nm virus with a characteristic wheel-shaped structure (rota) and belongs to the family *Reoviridae*. The virus has three shells, an outer capsid, an inner capsid and a core. They surround 11 segments of double-stranded RNA, which encode for six structural proteins (VP1-VP4, VP6, VP7) and five non-structural proteins (NSP1-NSP5). Two structural proteins, VP7 (the glycoprotein or G protein) and VP4 (the protease-cleaved protein or P protein), make up the outer shell and are considered important for vaccine development since they define the serotype of the virus and are the major antigens involved in virus neutralization.[4]

Because the genes encoding these proteins segregate independently of each other during reassortment, a dual-serotyping system to account for the specificities of both VP7 and

VP4 has been adopted.[5] Thus, the classification of rotaviruses is based on differences in the VP7 (G) and VP4 (P) capsid proteins. At present, 15 G types and 26 P types have been identified.[6] G genotypes 1-4, and P genotypes P[8] and P[4] predominate worldwide.[7]

Initial observation of the natural history of rotavirus infection noted a child's first infection with rotavirus, which occurs early in life, is usually most severe and results in immunity against subsequent illnesses. This protective immunity against severe disease is boosted by subsequent infection.

This disease pattern makes researchers believe that a vaccine could protect against the severe disease and death caused by rotavirus. A universal childhood vaccine may be the best mean to prevent rotavirus disease.[7]

The first rotavirus vaccine, Rotashield, was licensed worldwide in August, 1998 [8, 9] but voluntarily withdrawn by the manufacturing company within 3 months due to epidemiological data supporting a causal link between the vaccine and intussusception.[10]

Recently, 2 new candidate vaccines, RotaTeq and Rotarix, were licensed for use in over 40 countries. RotaTeq was developed with the aim of providing immunity against the 4 common G types (G1-G4) and 1 common P type (P[8]). In contrast, Rotarix vaccine, a monovalent vaccine contained a single G1P[8] strain.[11]

However, the increasing number of reports of the emergence of novel G and P types in various countries raises concerns about the adequacy of current vaccination strategies.

In this study, a reverse transcription-polymerase chain reaction (RT-PCR) for G and P genotyping of rotavirus isolates was used to determine the rotavirus genotypes prevailing in Yangon as strain surveillance is essential for monitoring the pattern of distribution of genotypes and the emergence of novel strains.

MATERIALS AND METHODS

A total of 1208 stool samples were collected from children under five years of age, admitted to the three medical wards of Yangon Children's Hospital for diarrhea from January 2010 to September 2011. Approximately 5 mls of stool sample was collected using wide-mouth screw-capped bottles and stored at -20˚C until testing was done. The presence of rotavirus antigen was determined by commercial enzyme immunoassay (ProSpect T^{TM} Rotavirus, OXOID, UK). Briefly, diluted stool samples, positive and negative controls were added to the wells of 96-well EIA plates precoated with anti-rotavirus antibody.

The plates were incubated and then washed. Then, anti-rotavirus antibody conjugated to horseradish-peroxidase was added and incubated. After washing, the enzyme substrate was added to develop colour in the wells. The absorbance (optical density) of the wells was read in an EIA reader at 490 μm wavelenghth. The cut-off value was calculated form the absorbance readings of the positive and negative controls.

A subsample of the monthly rotavirus EIA-positive stool samples were randomly chosen and G and P genotyped by reverse transcription-polymerase chain reaction (RT-PCR).[12, 13] RNA was extracted by using QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Germany) according to manufacturer's instructions.

The extracted dsRNA was amplified by reverse transcription-polymerase chain reaction (RT-PVR) using specific oliginucleotide primers. The extracted RNA was used as a template to produce and amplify full-length complementary DNA (cDNA) of the VP7 and VP4 region by RT-PCR. The cDNA was used a template for a second and subsequent rounds of PCR using genotype-specific primers to amplify cDNA. G types were identified by multiplex RT-PCR assay using consensus primers Beg9 and End9, and typing primers specific for the VP7 genes of G types 1- 4, 8, 9 and 12 (Table 1).

P types were identified by multiplex RT-PCR assay using consensus primers VP4F and VP4R, and primers specific for the VP4 genes of P types 4, 6, 8, 9 and 10 (Table 2).[13] The amplified products together with a molecular weight marker were subjected to electrophoresis in a 2% agarose gel containing 0.5 µg of ethidium bromide per ml. The cDNA bands and the molecular weight marker were observed with molecular imager (Gel Doc^{TM} XR^{+} Imaging System (BIO-RAD). Data entry was done by using Microsoft Excel program and analyzed by SPSS software.

Table 1. Consensus and type-specific primers for G typing

Primer name	Sequence (5' to 3')	Strain/ [G] type	Nt position	Polarity	PCR product (bp)
1st amp consensus primers					
Beg 9	ggc ttt aaa aga gag aat ttc cgt ctg g		1-28	pos	1062
End 9	ggt cac atc ata caa ttc taa tct aag		1062-1036	neg	1062
G12S	ccg atg gac gta acg ttg ta		548-567	pos	501
2nd amp typing primers					
RVG 9	ggt cac atc ata caa ttc t		1062-1044	neg	
aBT1	Caa gta ctc aaa tca atg atg g	G1	314-335	neg	749
aCT2	caa tga tat taa cac att ttc tgt g	G2	411-435	neg	652
aET3	cgt ttg aag aag ttg caa cag	G3	689-709	neg	374
aDT4	cgt ttc tgg tga gga gtt g	G4	480-498	neg	583
aAT8	gtc aca cca ttt gta aat tcg	G8	178-198	neg	885
aFT9	cta gat gta act aca act ac	G9	757-776	neg	306
G12B	ccg atg gac gta acg ttg ta	G12	548-567	neg	501

Table 2. Consensus and type-specific primers for P typing[13]

Primer name	Sequence (5' to 3')	Strain/ [P] type	Nt position	Polarity	PCR product (bp)
1st amp consensus primers					
VP4F	tat gct cca gtn aat tgg		132-149	pos	876
VP4R	att gca ttt ctt tcc ata atg		775-795	neg	876
2nd amp primers (VP4F was also included)					
1T-1	tct act tgg ata acg tgc	P[8]	339-356	neg	345
2T-1	cta ttg tta gag gtt aga gtc	P[4]	474-494	neg	483
3T-1	tgt tga tta gtt gga ttc aa	P[6]	259-278	neg	267
4T-1	tga gac atg caa ttg gac	P[9]	385-402	neg	391
5T-1	atc ata gtt agt agt cgg	P[10]	575-594	neg	583

RESULTS

When 1208 stool samples were tested with enzyme linked-immunosorbant assay, 670 samples (55%) showed positive for rotavirus antigen. Thus, rotavirus was found to be responsible for more than half of diarrhoea cases in under 5 children. Of 670 rotavirus-positive cases, 419 cases (62.5%) were boys and 251 cases (37.5%) were girls. The ratio of male to female was 1.7:1. The rotavirus infection was most prevalent between 6 to 11 months of age that accounted for nearly 50% of all rotavirus-positive cases. The lowest number of cases (5.2%) was found in the 24 to 59 months age group.

Rotavirus-positive cases were found throughout the whole study period with peak occurrences in January 2010 and February 2011 (Fig. 1).

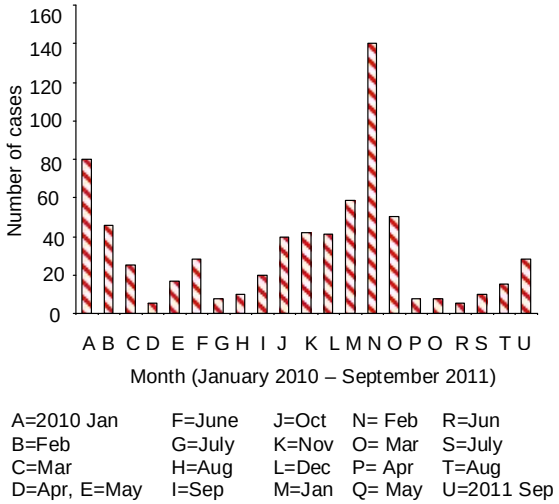

Fig. 1. Monthly distribution of rotavirus-positive cases from January 2010 to September 2011

Of 204 samples identified for genotyping, 193 samples were typable for G-genotypes among which 81.9% were of genotype G12 (n=167), 6.4% were G2 (n=13), 4.4% were G1 (n=9), 3 samples, i.e., 1.5% were of mixed-type (G2+G12) and 0.5% (n=1) were G3. The remaining 11 samples (5.3%) were untypable (Fig. 2).

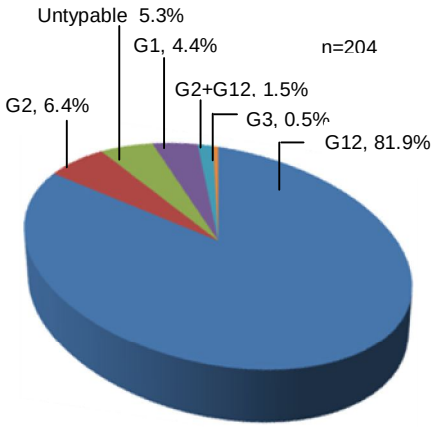

Fig. 2. Distribution of G-genotypes among selected cases of rotavirus infection

Of 193 samples identified for genotyping, 129 samples were typable for P-genotypes among which 50.8% were of genotype P[8], 7.3% were P[6], 5.7% were P[4], 2% were of mixed-type P[4]+P[8], 0.5% of P[6]+P[8] and 0.5% of P[10]. The remaining 33.2% were untypable (Fig. 3).

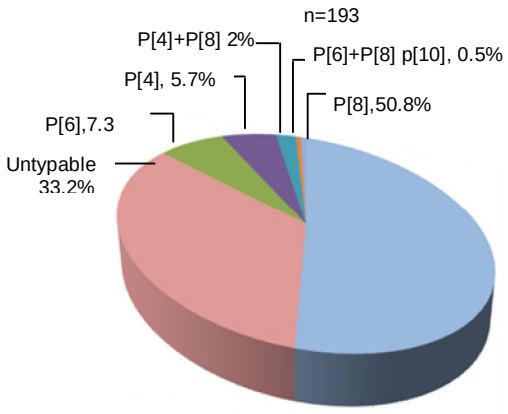

Fig. 3. Distribution of P-genotypes among the cases of typable G-genotypes

Regarding the clinical presentations, all rotavirus-positive cases presented with diarrhea (100%), 85.4% with fever and 85.1% with vomiting. Regarding the degree of dehydration, majority of rotavirus-positive cases (76.6%) showed some dehydration, and only 7 cases (1%) were found to be severely dehydrated.

Table 3. Distribution of combination of G and P-genotypes among the selected cases of rotavirus infection

G geno-type	P[4]	P[6]	P[8]	P[10]	P[4] +P[8]	P[6] +P[8]	Untyp able P	Total samples selected for P typing
G1			5				3	8
G2	11		1					12
G3							1	1
G12		14	92	1	4	1	58	170
G2+12							2	2
Total	11	14	98	1	4	1	64	193

DISCUSSION

Acute gastroenteritis accounts for millions of deaths each year in young children, mostly in the developing countries. World-wide, 3-5 billion cases of acute gastro-enteritis with nearly 2 million deaths occur each year in children under 5 years. Most cases are due to viral aetiology, which contributes up to 70% with rotaviruses being top of the list. The rate of detection of rotavirus diarrhea among hospitalized children in Yangon has been more than doubled, from 22% in the 1980s to 55% according to this study (Fig. 1).

Rotavirus persists as the etiology of child-hood diarrhea throughout the year with the peaks in cold dry season in the tropical regions including Myanmar. For both 2010 and 2011, a distinct seasonality of rotavirus diarrhoea was identified. The peak detection of rotavirus occurred during the months of the cool dry season, in January of 2010 and February of 2011. In this study, rotavirus was more commonly identified in boys than girls (the ratio being 1.7:1). This was also seen in a study conducted from early 2002 through late 2003 in which 62% of children with rotavirus diarrhea were boys.[3] The most common age group tested to be rotavirus positive by ELISA was the 6 to

12 months group which represents 49.7% of the total positive cases. This data is consistent with the data of a previous two-year study in Yangon from 2004-05, when the most vulnerable age group to rotavirus was between 7-12 months.[14] The current study reveals that the under one age group contributed to over 63% of ELISA-positive cases and over 94% of children had rotavirus diarrhea by the age of 2 years (Fig. 3).

Regarding the genotype distribution, the most common G genotype in this study is G12. This genotype accounted for 88% of the total typable samples in 2011. The second most common G genotype in this study is G2, which accounts for 6.4% and G3 being the least identified genotype accounting for 0.5%. This genotypic distribution of strains varied remarkably from previous studies as in 2005, G3 was the most predominant strain identified, representing over 80% of total samples.[14]

This current study is the first to report isolation of G12 strains in Myanmar. Genotype G12 has emerged in countries around the world since its first appearance in the Philippines in 1990[15] more than a decade later in the United States[16] and Thailand,[17] circulating for the first time in Saudi Arabia in 2004[18] and Vietnam in 2011.[19] A very limited number of strains were also isolated in Japan, Brazil, and Argentina.[20]

The high detection rate of G12 has direct implication for rotavirus vaccination, because the protective immunity of the two available vaccines against G12 genotype is currently undetermined. As the current vaccines do not include the G12 genotype, it remains to be determined whether these vaccines will cross protect against G12 infections.

In a study of rotavirus G12 in South India, severe rotavirus infection by a G12 strain was seen among children previously having rotavirus infections. Though the numbers described in that study were small, these infections raise the possibility that there may be no cross-protection against G12 strains from previous rotavirus infections. These data reinforce the necessity that a strain surveillance component be included in all countries for monitoring of circulating strains and detection of emerging rotaviruses before and after the introduction of a rotavirus vaccine.[21]

Regarding the P genotypes, in 2010 and 2011, over half (50.8%) were P[8], which meant the predominant P genotype had not changed from previous studies as in 2004, 74% of P genotypes were identified as P[8].[14]

However, interestingly, of the 7 cases in this study presenting clinically with severe dehydration, 5 cases were G12 and in all 5 cases the P genotype was untypeable. Although the numbers are small, this led us to speculate that there may be an emergence of new P genotypes unusual for Myanmar and thus further characterization and sequence analysis of untypeable strains should be considered in the future.

The major G-P combinations usually identified in Myanmar are the globally common combinations G1P[8] and G3P[8].[14] But in the current study, G1 P[8] comprised only the minority i.e., 3.8% and the predominant combination was G12 P[8] which accounted for over 71% of typeable strains.

Conclusion

The extreme diversity of rotavirus strains due to the reassortable nature of the segmented genome and the incomplete global geographical data together poses a problem that the strains used in a candidate vaccine may differ from those currently in circulation and thus the vaccine used would have little or no effect on the target population.

This highlights the need of updated data and consecutive virological surveillance which gives information of viruses prevailing in the area and timely detection of new emerging strains being essential especially in countries where rotavirus-related mortality and morbidity are major health problems.

ACKNOWLEDGEMENT

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Detection of Respiratory Syncytial Viruses in Infants with Acute Respiratory Infection

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Acute respiratory infection (ARI) is a clinical condition which causes high morbidity and mortality, especially in infants and young children. There are many viruses responsible for ARI. Among them, respiratory syncytial virus (RSV) is one of the major viral pathogens for annual epidemic ARI outbreaks in infants and preschool children, worldwide. This study involved the detection of respiratory syncytial viruses in infants with acute respiratory infection (ARI) attending medical wards of Yangon Children's Hospital, Yankin Children's Hospital and child wards of North-Okkalapa General Hospital from January to August, 2012. It aimed to determine the positivity rate of respiratory syncytial viruses in ARI cases attending these hospitals. Nasopharyngeal aspirates were collected from infants and they were inoculated into Human Epithelial (HEp-2) cells line for virus isolation. After fourth passage, respiratory viruses were screened by indirect immuno-fluorescence (IF) using screening antibody. Respiratory syncytial viruses were detected among screening positive samples by IF using specific monoclonal antibody. On the screening of 100 nasopharyngeal aspirates, 56 samples (56%) were positive for respiratory viruses. Among them, 39/56 samples (69%) were positive for respiratory syncytial virus. Most RSV positive cases were between 5 months to 1 year of age and sex distribution was nearly equal. Positive cases presented mainly with cough (100%), wheezing (84%), fever (77%) and tightness of chest (71%). Most cases had severe pneumonia (54%). This study highlights the role of respiratory syncytial viruses in ARI cases. The obtained evidence-based information may serve as an input for preventive messages.

INTRODUCTION

Acute respiratory infections (ARI) are a major cause of death among under 5 years old children. About two million children die worldwide yearly due to ARI. The incidence of ARI in children under 5 years of age is about 151 million with 5 million new episodes each year in both developing and developed countries.[1] In Myanmar, ARI is one of the major health problems of the country. It has been ranked as the nineteenth priority disease of Myanmar National Health Plan for the years 2006 to 2011.[2] There are many clinical presentations of ARI ranging from the common cold or cough to severe respiratory diseases. Among them, pneumonia, bronchiolitis and acute laryngitis are known to be a threat to the children's lives[3] and bronchiolitis is the most common cause in first year of life. Although it may occur in any age, severe symptoms are usually only evident in young infants. There are many viruses responsible for ARI, among them respiratory syncytial virus (RSV) is responsible for annual epidemic ARI outbreaks in infants and preschool children, worldwide. It frequently causes bronchiolitis and pneumonia, mostly in infants less than six months old. RSV infection is the cause of 50 to 90 percent of hospitalizations for bronchiolitis, 5 to 40 percent of those for pneumonia among children.[4]

Current methods for the detection of RSV include viral isolation in tissue culture,

immunofluorescence assay (IFA), and enzyme immunoassay (EIA). Tissue culture is still considered to be the standard against which all other methods are compared. Due to slow detection, the tissue culture system has been largely replaced by rapid techniques such as IFA and EIA. IFA has a sensitivity of 95 percent to 98 percent and specificity of the test is 90 percent to 100 percent. EIA and immunofluorescence tests have similar sensitivity and specificity, but some authors found that the sensitivity of EIA to be 85 percent to 95 percent which is same as immunofluorescence but the specificity is lower than immunofluorescence.[5]

So, in this study isolation and indirect immunofluorescence assay were performed on nasopharyngeal aspirate specimen from infants at North-Okkalapa General Hospital, Yangon Children's Hospital and Yankin Children's Hospital for RSV isolation. It can provide a definitive diagnosis of infection. Because there have been no previous studies done for RSV in Myanmar, this study may provide evidence-based information regarding respiratory syncytial virus infection and also aid in the management of the disease in that study hospitals.

The general objective of this study was to detect respiratory syncytial virus in infants with acute respiratory tract infection attending child wards of North-Okklapa General Hospital, child medical wards of Yangon Children's Hospital and Yankin Children's Hospital.

MATERIALS AND METHODS

Study design

It was a laboratory-based, cross-sectional descriptive study conducted at pediatric wards of North-Okklapa General Hospital, medical wards of Yankin Children's Hospital and Yangon Children's Hospital and Virology Research Division, Department of Medical Research (Lower Myanmar). The study period was from January to August, 2012.

Study population

A total 100 infants from pediatrics wards of North-Okklapa General Hospital, medical wards of Yankin Children's Hospital and Yangon Children's Hospital presenting with signs and symptoms of acute respiratory infection were studied. Under one month old children and children who cannot withstand the procedures were excluded from this study.

Collection and transport of specimen

Written informed consent was obtained followed by relevant history taking from the gurdian. Nasopharyngeal aspirates (NPAs) were collected by inserting a feeding tube through a nostril into the nasopharynx and applying gentle suction by syringe. The aspirated contents were put into a sterile test tube containing 1-2 ml volume of viral transport medium. Each sample was labeled and sent to the laboratory of Virology Research Division of the Department of Medical Research (Lower Myanmar) in a cool box within 4 hours.

Sample storage

At laboratory, nasopharyngeal aspirates were centrifuged at 2000 rpm for 10 minutes. Then the fluid was filtered through acetate membrane into the vials (2 ml). The vials were labeled and stored at -80°C until they were processed.

Laboratory methods for identification of RSV

Respiratory syncytial virus was isolated from nasopharyngeal aspirates by culture in Human Epithelial (HEp-2) cells in T-75 tissue culture flask. After inoculation of clinical samples into HEp-2 cell monolayers, the culture tube were incubated at 37°C for 7 days. Cytopathic effect (CPE) in viral culture tube was examined daily under the inverted microscope at 10X magnification. The virus isolation was done up to the fourth passage. After the fourth passage, the cell pellets of the tissue culture fluid were tested for a group of respiratory viruses as well as respiratory syncytial virus.

Identification of respiratory syncytial virus by indirect immunofluorescent assay

The cell pellets were fixed on the wells of the slide with chilled acetone for 10 minutes. The slide was air-dried completely. For screening of respiratory viruses, pooled monoclonal antibody was used as the first antibody and FITC conjugated anti-mouse IgG was used as the second antibody. For identification RSV, monoclonal antibody for respiratory syncytial virus was used as the first antibody and FITC conjugated anti-mouse IgG was used as the second antibody. Then, the slide was examined under the fluorescent microscope at 100X magnification. Detailed examination was carried out at 400X magnification.

Interpretations of IFA results for identification of respiratory syncytial virus

The presence of at least two or more fluorescent cells per field at 400X magnification was interpreted as positive result for that type of virus in the well. Absence of those cells was interpreted as a negative result. The pattern of fluorescent staining of respiratory syncytial virus exhibit an apple green fluorescent of cytoplasmic, dustlike, granular characteristic.

Data analysis

After collection of data, data entry and data analysis were done using appropriate Microsoft Excel 2003.

Ethical consideration

Approval for the conduct of this study was obtained from the Institutional Ethical Review Committee of Department of Medical Research (Lower Myanmar).

RESULTS

A total of 100 ARI cases were recruited from medical wards of Yangon Children's Hospital and Yankin Children's Hospital and child wards of North-Okkalapa General Hospital from January to August, 2012. One hundred ARI children were screened by viral polyclonal antibodies, 56(56%) children were respiratory virus positive cases by indirect immunofluorescence (indirect-IF) method and the rest 44(44%) children were negative.

Screening of RSV with specific monoclonal antibody by using indirect-IF was done in 56 virus positive cases, 39 cases (39/56, 69.6%) were positive with RSV antibody. The rest 17/56 (30.4%) were not detected by RSV antibody (Fig. 1).

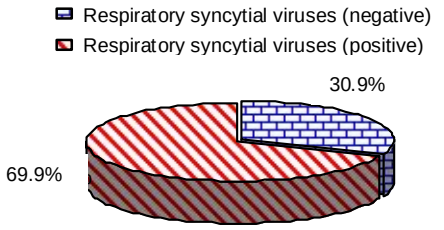
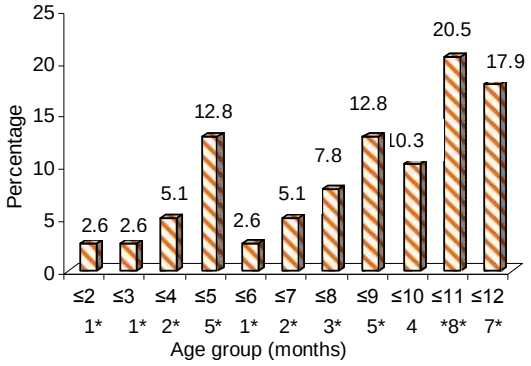


Fig. 1. Dection of respiratory syncytial viruses

Out of 39 RSV-positive cases, the virus was mostly detected in the age group between 10-11 months (8/39, 20.5%) followed by 11-12 months (7/39, 17.9%), 4-5 months and 8-9 months (5/39, 12.8% each), 9-10 months (4/39, 10.3%), 7-8 months (3/39, 7.8%), 3-4 months and 6-7 months (2/39, 5.1%, respectively). RSV was least detected in the age groups 1-2 months, 2-3 months and 5-6 months age group, each accounting for 1 case only (i.e., 2.6% for each group) (Fig. 2).



*=Number

Fig. 2. Distribution of respiratory syncytial virus infection cases by age group

In a total of 39 RSV-positive cases, all cases (100%) had cough. The second commonest presentation was wheezing (84%), followed

by fever (77%), tightness of chest (71%) and respiratory distress (59%), rhinorrhoea (38%), fast breathing (26%) and vomiting (18%) (Fig. 3).

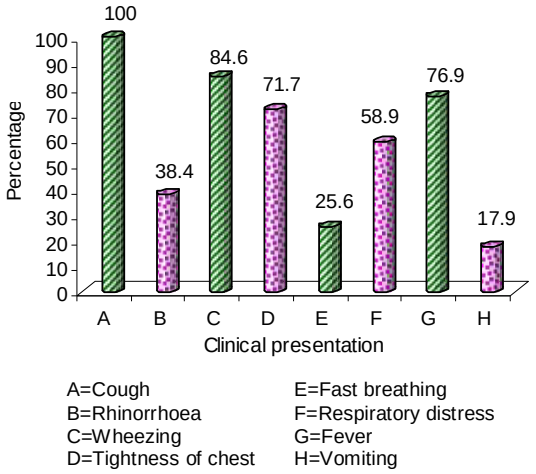


Fig. 3. Distribution of different clinical presentations in respiratory syncytial virus positive cases

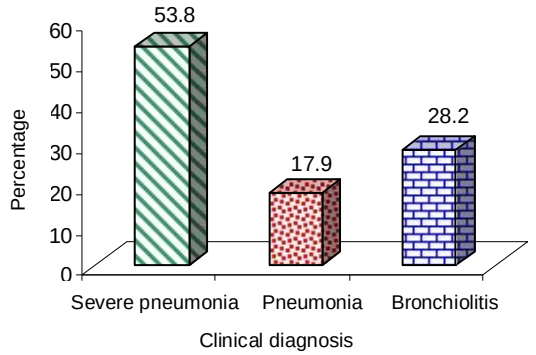


Fig. 4. Clinical diagnosis of respiratory syncytial virus infection cases

Figure 4 showed clinical diagnosis in RSV positive cases. Out of 39 children, 21/39 (53.8%) children were diagnosed as severe pneumonia, 11/39(28.2%) children were diagnosed as pneumonia and 7/39(17.9%) children were diagnosed as bronchiolitis.

DISCUSSION

In this study, one hundred samples were tested for respiratory viruses with pool monoclonal antibodies by using indirect immunofluroscence method after the fourth passage. From those, 56 samples (56%) showed screening positive and remaining 44 samples (44%) showed screening negative. The finding of the present study showed that respiratory viruses can be considered as main pathogens of ARIs in infants. A similar study reported that 49.4% was caused by respiratory viruses among ARI cases.[6] Similarly in Iran study, respiratory viruses were detected in 109 (54%) ARI cases.[7]

Subsequently, when 56 viral ARI samples were screened for RSV with specific monoclonal antibody by indirect IF method, 39 cases (39/56, 69.6%) were positive with RSV antibody. The rest 17/56 (30.4%) were not detected by RSV antibody and these samples may be positive for other respiratory viruses such as influenza virus, parainfluenza, adenovirus and rhinovirus. A study in Brazil showed that among 316 viral ARI-positive samples, 26.2% was positive for RSV, 6% for adenovirus, 1.7% for influenzaviruses, 1.5% for parainfluenza viruses, and 1.2% for mixed infection.[8]

Out of 39 RSV-positive cases, the virus was mostly isolated (8/39, 20.5%) in the age group between 10-11 months and followed by 11-12 months (7/39, 17.9%), 4-5 months and 8-9 months (5/39, 12.8% each, respectively), 9-10 months (4/39, 10.3%), 7-8 months (3/39, 7.8%), 3-4 months and 6-7 months (2/39, 5.1% each, respectively). RSV was leastly isolated in 1-2 months, 2-3 months and 5-6 months age group, accounting for 1 case of each (2.6%, each group). In an Indian study, 39% of positive RSV cases were detected in less than 6 months old and children aged 6-11 months comprised 24% of cases and overall 63% of infected children were under 1 year of age.[9] A study conducted in Japan identified that nearly 50% of children hospitalized for RSV infection were younger than 6 months old, and 66% were younger than 1 year of age.[10]

In this study, out of 39 RSV positive cases, male children were more infected in the age groups 1-2 months, 5-6 months, 6-7 months, 9-10 months and 11-12 months. But, in the age groups 2-3 months, 3-4 months, 4-5 months, 7-8 months and 8-9 months,

female children were more affected. But in the age group 10-11 months, RSV occurrence was nearly equal in both sexes. Several studies reported that the sex distribution of the patients had a male predominance and overall 60% of infected children were males.[11]

In this study, the samples were collected within 7 months (from January to August, 2012). So, seasonality of RSV cases could not be determined in this study. Within the study period, RSV was mostly isolated in February 2012. In most published studies, RSV has a high seasonal variation. RSV outbreaks in temperate or Mediterranean climates occur mainly during the cold months as in Western Europe and North America.[12, 13] In desert climates, such as Kuwait and Saudi Arabia,[14] cases were also seen in the cold months. Two countries, Islamabad in Pakistan and Chandigarh in India, have similar climates but RSV outbreaks in Pakistan were reported mainly in the cold season,[15] whereas an outbreak in Chandigarh was associated with the rainy season.[16]

When the presenting symptoms of RSV infection cases were analyzed, all these cases present with cough (100%) followed by wheezing (84%), fever (77%), tightness of chest (71%) and respiratory distress (59%). Thus, these symptoms can be regarded as the major symptoms of RSV infection cases but in this study, ARI without RSV virus also presented with cough, wheezing, fever and tightness of chest. Addition to the clinical presentations described above, some of these infection cases presented with rhinorrhoea (38%), fast breathing (26%) and vomiting (18%). In a similar study conducted in Brazil, fever and rhinorrhoea were found in all RSV cases.[17]

A study showed that wheezing, rhinorrhea, vomiting, and diarrhea were significantly frequent in the infants infected with RSV.[18] In one study, the most frequent clinical findings among 355 RSV-positive out-patients were cough (98%), fever (75%), and wheezing (65%).[19]

In this study, out of 39 children, 21/39, (53.8%) children were diagnosed as severe pneumonia, 11/39 (28.2%) children as pneumonia and 7/39 (17.9%) children as bronchiolitis. A study found that in 26.2% of patients with bronchiolitis were RSV positive and, 21% with pneumonia were RSV positive.[20] A study reported that the most common admitting diagnosis for RSV infection were bronchiolitis (37.4%) and pneumonia (32.5%).[21]

Conclusion

This study highlights the role of respiratory syncytial virus in ARI cases in children. Moreover, there have been no previous studies done for RSV in Myanmar, and this study may provide the evidence-based information regarding respiratory syncytial virus infection. The results will assist in the management of the disease and preventive messages for the future. The findings expand knowledge about the epidemiological features of respiratory viruses in hospitalized children with clinical evidences of ARI.

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Antioxidant Activity, Total Phenol Content and Ascorbic Acid Content of Three Different Preparations of Noni Fruit Juice (&J)(

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Noni (*Morinda citrifolia* L.) is among the traditional medicinal plants used as remedies in Myanmar. Nowadays, noni manufacturers produce various forms of noni fruit juice from ripe noni fruits as dietary supplements and herbal drug in market. This study was done to evaluate the *in vitro* antioxidant activity, total phenol content, ascorbic acid content and acute toxicity of three different preparations of noni fruit juices: fresh expressed juice (EJ), fermented juice (FJ) and boiled juice (BJ). Three different preparations of noni fruit juices (4 µg/ml) possess antioxidant activity and percent inhibitions show 83.8% (EJ), 72.3% (FJ) and 66.04% (BJ), respectively, compared with standard ascorbic acid by DPPH method using UV spectrophotometer. Expressed juice possessed highest amount of total phenol and ascorbic acid contents (4.1±0.1 mg/ml quercetin equavalent and 1.12±0.1 mg/ml ascorbic acid equivalent, respectively). Boiled juice have lowest amount of total phenol and ascorbic acid contents (2.23±0.2 mg/ml and 0.48±0.1 mg/ml, respectively). Fresh expressed juice of noni fruit contains high amounts of total phenol and ascorbic acid with strong antioxidant activity. In fermented juice, there were 32.5% decrease in total phenol content, 31.3% decrease in ascorbic acid content and 13.7% decrease in antioxidant activity when compared with expressed juice. In boiled juice, there were 45.6%, 57.1% and 21.3% decrease in total phenol concentration, ascorbic acid concentration and antioxidant activity, respectively, compared with expressed juice. Three different preparations of noni fruit juice showed no lethal effect when tested for acute toxicity with the maximum dose of 67 ml/kg body weight on mice model.

INTRODUCTION

Morinda citrifolia is the scientific name of noni plant. Noni (Yeyo) is a native plant of Panama and grows in hot tropical environments.[1] In Myanmar traditional medicine, noni has been used as a remedy for various ailments. Nowadays, noni manufacturers produce various forms of juice from ripe noni fruits as dietary supplements and herbal drug in market. Noni fruits are used to treat diseases in many countries and regions.[2, 3] Noni fruits are used as emmenagogues, blood purifier, antiemetic agents, tonic supplements and for digestive disorders, tuberculosis, urinary-tract ailments, stimulation of appetite and central nervous system, hypertension, diabetes and depression. Noni helps the immune system work better.[3] Noni juice has been used by people with a variety of conditions. It has been used by people with immune compromising diseases like chronic fatigue syndrome to boost the immune system function. The results of a study in smokers showed that the levels of total cholesterol and triglycerides improved after one month of drinking noni juice.[4]

Noni fruit is an excellent source of nutrients including vitamin C, vitamin A, niacin,

potassium, calcium and sodium. Ten essential vitamins, 7 dietary minerals and 18 amino acids have been identified from noni fruit and it also contains a mixture of anthraquinones, organic acids and xeronine.[5] Dietary foods contain a wide variety of free radical-scavenging antioxidants, for example, flavonoids and antioxidant vitamins such as ascorbic acid and α-tocopherol. These compounds are particularly rich in vegetables, fruits, tea and wine. On the other hand, colorimetry with 1, 1-diphenyl-2-picrylhydrazyl (DPPH), a stable free radical, has been reported as a simple method for evaluation of the free radical-scavenging activity.[6]

In this study, the free radical-scavenging activity in three different preparations of noni fruit juice was determined by using *in vitro* DPPH method which is simple and rapid. The antioxidant activity of noni fruit was found to be quite all dependent on polyphenols including vitamin C, vitamin A, flavonoid and polyphenol. In fermentation method, noni fruit was fermented under sunlight for 6 weeks. Fresh expressed juice and boiled juice are commonly used in traditional medicine. High temperature applied in boiling may lead to phenolic depletion while polyphenols are mainly degraded by exposure to large amount of oxygen.[7]

Therefore, this study was conducted to evaluate the antioxidant activity of three types of noni fruit juice.

MATERIALS AND METHODS

Collection of samples

In this study, mature white hard noni fruits were collected from Yangon area from September 2012 to March 2013. The ripe soft fleshes of collected noni fruits were tested.

Preparation of three types of noni fruit juice

Noni fruit juice was prepared in three ways.[8, 9]

Expressed juice (EJ)

Expressed juice is the liquid product obtained by crushing and blending fresh noni fruits. These juices were squeezed, filtered, and then centrifuged at 3000 rpm for 30 minutes. Expressed juice is the freshly squeezed unfermented sweeter version.

Fermented juice (FJ)

Firstly, ripe noni fruits were washed with water, and put inside a sterile airtight glass container. The container was put under sunshine for 6 weeks. When the fruits were fermented, they were filtered and the filtrate was used as test sample.

Boiled juice (BJ)

Freshly ripe noni fruits with distilled water 1:2 (w/v) were boiled at high temperature of 150˚C- 200˚C for 30 minutes to evaporate all the water. Boiled fruits were squeezed, filtered, and then centrifuged at 3000 rpm for 30 minutes. The juice was collected in the sterilized container to be tested.

Determination of physico-chemical characterization

Determination of pH value in three different preparations of noni fruit juice was done by pH meter (pH-700, Eutach) and sugar content was tested by refractometer (Pal-1, Atago).

Qualitative identification by thin layer chromatography

Ascorbic acid and polyphenol compounds were identified in three different preparations of noni fruit juices by thin layer chromatography[10] using solvent system of n-butanol, acetic acid, water (4:1:5) on silica gel $60F_{254}$ precoated TLC plate. 10% ferric chloride spray was used as visualizing reagent for polyphenol compounds and ascorbic acid was seen under short wave ultraviolet light.

Quantitative determination of total phenol

The total phenolic contents of three types of noni fruits juice were measured with the Folin-Ciocalteu regent.[11, 12] Firstly, 1.6 ml

of diluted noni fruit juices and 100 µl of Folin-Ciocalteu regent were mixed, then 300 µl of saturated Na_2CO_3 (20%) were added. After the solution was incubated at 40˚C for 30 minutes, the absorbance of the solution was measured at 765 nm with the UV spectrophotometer (UV-1601). Total phenol content of noni fruit juices was calculated from quercetin standard curve.

Quantitative determination of ascorbic acid

Ascorbic acid content of three different preparations of noni fruit juices were determined by Unani method with UV Spectrophotometer.[10, 13] The fruit juice was diluted with oxalic acid and mixed with 1: 9 of 2, 6 dichlorophenolindophenol solution. Exactly 15 seconds after adding the dye, the solution was measured at 520 nm by UV spectrophotometer (UV-1601). The ascorbic acid contents of noni fruit juices were calculated based on the ascorbic acid standard curve.

Determination of antioxidant activity by DPPH method

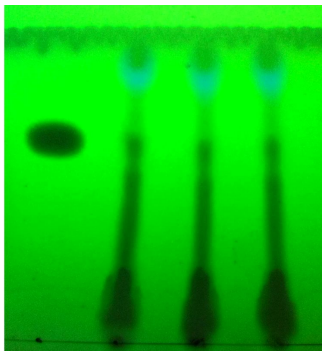
Serial dilutions of three different preparations of noni fruit juices were diluted with 50% ethanolic solution.[12, 14, 15] Test samples and 60 µM DPPH (1, 1-diphenyl-2-picrylhydrazyl) solution were mixed (1:1) vigorously by a vortex mixer. All solutions were allowed to stand at room temperature in dark place for 30 minutes, after which measurement of absorbance was done at 517 nm using UV Spectrophotometer (UV-1601). Absorbance measurements were done in triplicate and calculated by the formula to show percent inhibition compared with standard ascorbic acid.

Acute toxicity study on animal model

Acute toxicity study of three different preparations of noni fruit juice was done according to Litchfield & Wilcoxon (1949).[16] Both sexes of hundred albino mice (DDY strain) in 10 groups (10 mice/group) were given orally three types of fruit juices at the dose of 17.0, 33.5 and 67.0 ml/kg body weight per day. The control group received 10 ml/kg body weight of distilled water. All the animals were kept under observation for toxic effect for two weeks.

RESULTS AND DISCUSSION

Ascorbic acid was identified in EJ, FJ and BJ on thin layer chromatogram observed R_f value (0.67), dark color under short wave ultraviolet light (254 nm) comparing with standard compound (Fig. 1).



AS=Standard ascorbic acid FJ=Fermented juice
EJ=Expressed juice BJ=Boiled juice

Fig. 1. Thin layer chromatogram of ascorbic acid and three types of noni fruit juices

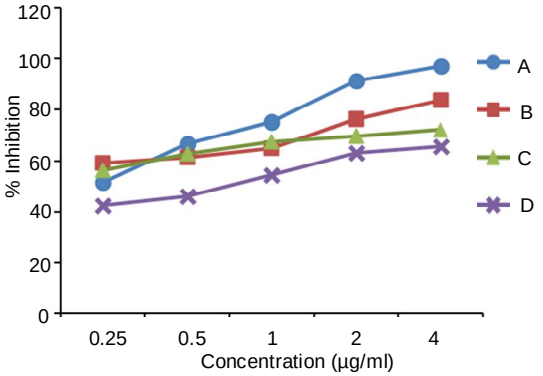
Thin layer chromatogram of phenolic compounds in EJ, FJ and BJ showed bluish black color after spraying with 10% ferric chloride reagent. Physico-chemical characterization of three different preparations of noni fruit juices is shown in Table 1. Fresh expressed juice of noni fruit exhibited total phenols content and ascorbic acid content significantly higher than those of fermented juice and boiled juice.

Table 1. Physico-chemical characterization of noni fruit juices

Test parameter	Expressed juice (EJ)	Fermented juice (FJ)	Boiled juice (BJ)
Yield percent (v/w)	40.0%	48.0%	43.0%
Soluble matter (w/v)	9.6%	7.3%	9.7%
pH value	3.8	3.6	3.9
Sugar content (w/v)	9.5%	8.9%	9.1%
Total phenol (mg/ml)	4.1±0.1	2.77±0.7	2.23±0.2
Ascorbic acid (mg/ml)	1.12±0.1	0.77±0.1	0.48±0.1

The loss of total phenol and ascorbic acid contents in fermented juice by fermentation under sunlight for 6 weeks were 32.5% and 31.3%, respectively, compared with fresh expressed juice. In fermentation process, fermented juice was subjected to strong UVA and UVB light and temperature ranged from 28˚C to 32˚C, which is a typical tropical environment. In boiled juice, high temperature of 150˚C-200˚C for 30 minutes, the loss of total phenol concentration and ascorbic acid concentration were 45.6% and 57.1%.

Three different preparations of noni fruit juices (EJ, FJ, BJ) 4 µg/ml possessed antioxidant activity and percent inhibitions are shown in Figure 2. In this study, free radical scavenging activities of fermented juice and boiled juice were 13.7% and 21.3% lower than expressed juice.



A=Standard ascorbic acid C=Fermented juice
B=Expressed juice D=Boiled juice

Fig. 2. Antioxidant activities of std. ascorbic acid and three types of noni fruit juices

In acute toxicity test, toxic effect was not found in all three different preparations of noni juice in maximal permissible dose. Therefore, the median lethal dose (LD_{50}) was more than 67 ml/kg body weight that is equal to 0.84 ml/ kg body weight human dose.

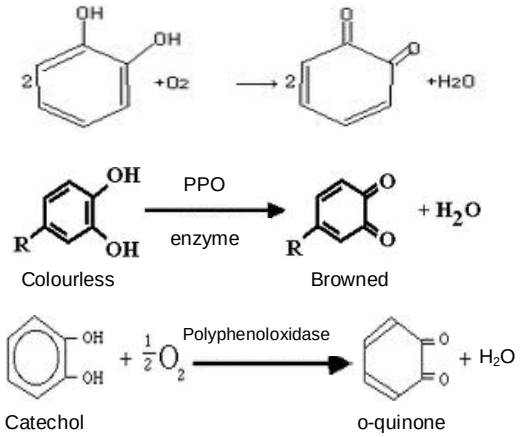
From the results, antioxidant activities of different preparations of noni fruit juices depend on total phenol content and ascorbic acid content. Expressed juice has the highest antioxidant activity, ascorbic acid content and total phenol content. The boiled juice of noni fruit showed lowest antioxidant activity due to high temperature of the juice processing method. Fresh noni fruit is a good source of antioxidants activity and phenolic content. But traditional fermentation practice and storage at room temperature dramatically decreased its free radical scavenging activity. Noni fruit juice lost a significant percentage of radical scavenging activity within 1 week when stored at 24˚C but not within 1 week at 4˚C or within 2 weeks at -18˚C freezing in refrigerator.[17]

Ascorbic acid is unstable, especially in alkali solution, readily undergoing oxidation even by atmospheric oxygen, the change being accelerated by light and heat.[18] The loss of vitamin C of *Phyllanthus emblica* L. fruit by different drying processes were 86.58% when dried in shade for four days, 80.90% when dried in oven at 75˚C for four hours, 71.33% when kept directly in sunshine for four hours, 66.2% when dried in oven at 50˚C for four and half hours, and 47.3% when dried in microwave oven for six minutes, respectively.[10]

In this study, the highest ascorbic acid content was 1.12 mg/ml in fresh expressed juice of noni fruit. High temperature was not used during the fruit juice process. During fermentation process of fermented juice, there was 31.3% decrease in ascorbic acid content due to low temperature for 6 weeks. In boiled juice, there was 57.1% decrease is ascorbic acid concentration due to high temperature for 30 minutes. This finding indicates that the loss of ascorbic acid content in fruit juice also depends on temperature.

A study reported that during 9 months of blueberry nectar storage, there was 3-9.9% decrease in polyphenol concentrations and antioxidant activity decreased by 20.9-24.2%.[19] The higher antioxidant activity was observed in the fresh expressed juice of noni fruit than fermented juice and boiled juice. This study showed that changes in total polyphenol concentrations

and ascorbic acid concentrations showing antioxidant activity changes. Since oxygen can destroy the antioxidant ability of polyphenols.



These equations show the degradation of polyphenol compound due to oxidation from environment effect.[20] The DPPH radical scavenging assay determined free antioxidants in noni juice, whereas the assay of total phenols with Folin-Ciocalteu reagents determined both free phenolic and bound phenolic in noni juice.[21] Nonphenolic antioxidants such as ascorbic acid exhibit rapid reactions to the DPPH radical.[22]

The present study indicated that the polyphenol present in the fruit juice are destroyed (oxidized) to some extent during the processing method, ie., in sunlight and/ or heating. As polyphenolic antioxidants are known to positively influence human health, stability determination is crucial. Degradation of polyphenol was higher at increasing temperatures. The antioxidant activity of fermented juice and boiled juice is affected by the temperature during the noni juice preparation method. This study showed that antioxidant activity depends on ascorbic acid and total phenol contents in three different preparations of noni fruit juice.

Conclusion

It can be concluded that freshly expressed juice contains high amount of ascorbic acid and total phenol than other fermented juice and boiled juice. These results suggest that expressed juice of noni fruit provides antioxidant properties and offers effective protection from free radicals and support that noni fruit is a promising source of natural antioxidants. Therefore, it is needed to prevent noni fruit juice from thermal degradation of polyphenol compound for antioxidant activity. Fresh expressed juice rather than fermented juice and boiled juice is recommended for its substantial antioxidant properties.

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Establishment of the Simple Extraction of Collagen from Some Fishes in Myanmar and Evaluation of Effectiveness in Wound Healing

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Collagen is major component of connective tissue found in the body. Collagen has a wide range of application in cosmetic, biomedical, pharmaceutical industrial fields. Fishery production in Myanmar is growing with a huge amount of by-product released. It can be used for collagen extraction. The aim of this research was to extract collagen from fish skins by using simple extraction method and investigate the some properties of the extracted fish collagen for the application. Collagen was extracted from the skin of Ngamyitchin (*Labeo rohita*) and Ngaphe (*Notopterus notopterus*) by using common salt (NaCl). The yields of collagen for Ngamyitchin and Ngaphe were 7.65 and 6.45%, respectively. The effect of pH and salting out on the sample were determined. The maximum solubility of collagen of Ngamyitchin and Ngaphe was observed at pH 2 and 3, respectively. No changes in solubility were observed in the presence of NaCl up to 3% (w/v). However, a sharp decrease in solubility was found above 3% NaCl. Fourier transform infrared (FTIR) spectra of both samples showed the presence of N-H, O-H, C=O, $-CH_2$, $-CH_3$ groups in the samples. FTIR spectra of the collagen sample from both fishes showed to coincide with typical protein spectra. In this study, *in vivo* study was carried out for the determination of wound healing in 24 Wistar rats. Wound was induced under anesthesia in every rat and treated with collagen 1 mg/kg dressing for 18 days. Histological results showed that extracted collagen from Ngamyitchin was more completed wound healing in skin lesion than that of Ngaphe.

INTRODUCTION

Collagens are generally extracellular structural proteins involved in formation of connective tissue structure and are known to occur in genetically distinct forms identified as type I through XIX. They vary considerably in their complexity and the diversity of their structure. Also, the different types show variations in amino acid composition and physical properties that also seems to be correlated with the temperature of the source animal's environment. The main sources of industrial collagen are limited to those from pig and bovine skin and bones. Due to collagen's unique chemical features, they have been used in various ways, such as leathers and film, beauty aids and cosmetics, biomedical and pharmaceutical applications, and food.[1, 2]

However, the occurrence of bovine spongiform encephalopathy (BSE), and foot and mouth disease (FMD) along with religious constraints has resulted in an anxiety among users of collagen and collagen-derived products from land-based animals in recent years, and thus, increasing attention has been paid to alternative collagen sources, especially fish skin and bones which comprise about 30% of the total fish weight available after fish fillet preparation.[3] So far, skin and bone collagen from several fish species have been isolated and characterized.[4] As there is an increased consumer

demand for its fillet, optimal utilization of their wastes after filleting, especially evaluation of its potential use as an important source of collagen could be profitable. Hence, the objective of the present study was to extract collagen from the skins of Ngamyitchin (*Labeo rohita*) and Ngaphe (*Notopterus notopterus*), and to evaluate of effectiveness in wound healing.

MATERIALS AND METHODS

Sample collection

Fish skins of Ngamyitchin (*Labeo rohita*) and Ngaphe (*Notopterus notopterus*) were collected from the market.

Fish skins preparation

The skins which have been peeled off from the body of Ngamyitchin and Ngaphe fishes were obtained from the Hlaing market. The skins were washed well with water (tap-water) to remove scales, fats and partial flesh from the skins. After cleaning the skins, they were cut in squares by the proper small dimensions.

Extraction of collagen

The properly cut small pieces of skin were weighed. Sodium chloride (local made) which was three times of fish skins were added into the small pieces of skin. The salted skin pieces were left cold at the temperature 4˚C about one week.

At this stage, the salted skin pieces were closely enclosed with a filtration cloth. Then, the filtration cloth containing the salted skin pieces was exposed to a stream of water and washed well therein for about 30 minutes to 1 hour, thereby causing most of non-collagen substances or portions to be decomposed into minute pieces and escaped out through the fine meshes of cloth, whereby collagen portions of the skin pieces were caught by the meshes against leakage.

At this step, the residual water on the skin pieces was wiped-off well. Thereafter, the pieces were immersed in distilled water heated at 80˚C and incubated therein for 2 hours under the same temperature, that the quantity of distilled water must be ten times as much as that of the skin pieces. After lapse of 2 hours, a gelatinous collagen was obtained.

At this filtration step, impure collagen solution was mixed with 1% (w/v) of activated carbon. Then, the mixture of activated carbon and impure collagen solution was stirred by a stirrer for 1 hour. Then, the fluid mixture (activated carbon and collagen solution) was sucked and filtered via that filtered material by a sucking means (any suitable suction filter) so as to filter out most of impurities from the collagen solution.

The drying mode is such that hot air of 70˚C was applied by a hot air blower to the refined collagen for 12 hours, whereby one or more bars of solidified collagen were obtained. Finally, the collagen bars were broken into fine powders by a pulverizer so as to obtain collagen powder readily available in the commercial and industrial fields. [5]

Effect of pH on collagen solubility

Eight milliliters of collagen solution (3 mg/ml) in a centrifuge tube were adjusted across the pH range of (1 to 12) with HCl or NaOH dilution series, and then the volume was made up to 10 ml with distilled water. The solutions were centrifuged at 20,000 g for 30 minutes at 4˚C. Protein concentration in the supernatant was determined by Biuret method[6] and weighed out bovine serum albumin (BSA, ZISTCHIMI, Tehran, Iran) was used as a standard.

Protein solubility was calculated using the following equation:

$$\text{Solubility} = \frac{\text{Protein content of the supernatant}}{\text{Total protein content in the sample}}$$

$$\text{Relative solubility} = \frac{\text{Solubility at given pH}}{\text{The highest solubility in the range of pH}}$$

Effect of NaCl on collagen solubility

Five milliliters of collagen (6 mg/ml) in 0.05 M acetic acid were mixed with 5 ml of NaCl in 0.05 M acetic acid at various

concentrations (0, 2, 4, 6, 8, 10) and 12% (w/v). The process thereafter was like that for pH solubility.

Fourier transform-infra red spectrum analysis

FTIR spectroscopy of solid samples was relied on a Bio-Rad FTIR-40 Model, USA. Sample (10 mg) was mixed with 100 mg of dried potassium bromide (KBr) and compressed further to prepare as a salt disc (10 mm diameter) for reading the spectrum.[7]

In vivo test

Albino Wistar rats of either sex weighing 200-225 gm were used. Animals were maintained under hygienic conditions and they were provided with DMR-LM food pellets and tap water *ad libitum*. Cleaning and sanitation work were done daily throughout the experiment. Wood shaving was provided as bedding material, which was changed twice a week. The cages were also changed twice a week.

The animals were anesthetized by using chloroform in the anesthesia chamber. An impression was made on the dorsal thoracic region 1 cm away from vertebral column and 5 cm away from ear on the anaesthetized rat. Para vertebral straight incision of 1.5 cm length was made through the entire thickness of the skin, on either side of the vertebral column with the help of a sharp scalpel (surgical blade). Haemostatic was achieved by blotting the wound with cotton swab soaked in normal saline.

The animals were then grouped and treated as follows:

Group I: No treatment
Group II: Cicatrin (Neomycin sulphate and Bacitracin zinc) [1g/kg]
Group III: Ngamyitchin collagen [1g/kg]
Group IV: Naphae collagen [1g/kg]

Wound area was measured by tracing the wound with a millimeter scale ruler on predetermined days i.e., 2, 4, 6, 8, 10, 12, 14, 16, 18 days post-wounding for determination of wound contraction - 50%. Falling of scar leaving no raw wound behind was taken as the end-point of complete epithelization.

Histological study

The tissues from the skin lesions of control and test rats obtained by necropsy were formalin-fixed paraffin embedding rim for routine histological processing. A 3-5 micronmeter section obtained from each paraffin block was stained with Haematoxylin and Eosin (H&E) and evaluated in a blinded manner by two observers using a light microscope. Histological appearance of wound healing was observed in epidermis, dermis and subcutaneous areas of control and test of the rats.

RESULTS AND DISCUSSION

In this study, collagen was extracted using salt-based method. The yield percent of collagen from Ngamyitchin and Ngaphe were 7.65% and 6.45%, respectively. Since the inexpensive salt was used for effective removal of non-collagen substances or portions and to obtain the collagen portion, the method itself was simplified and economical.

The effect of pH and NaCl on the solubility of collagen extracted from the skins of Ngaphe and Ngamyitchin were determined. The maximum solubility of Ngaphe and Ngamyitchin was observed at pH 3 and 2 whereas the minimum solubility was observed at pH 10 and 9, respectively. The solubility of the skin of collagen of fishes varied widely and the pH for the highest solubility differed with the fish species.

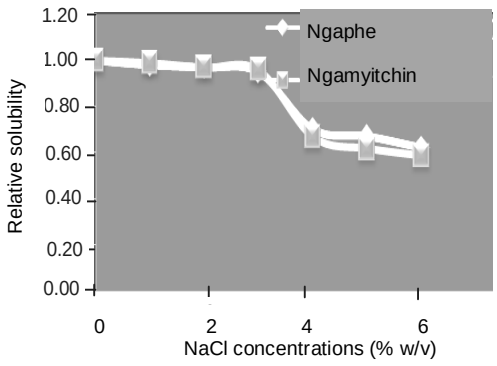

Fig. 1. Solubility of extracted collagen samples in 0.5 M acetic acid at different NaC1 concentrations

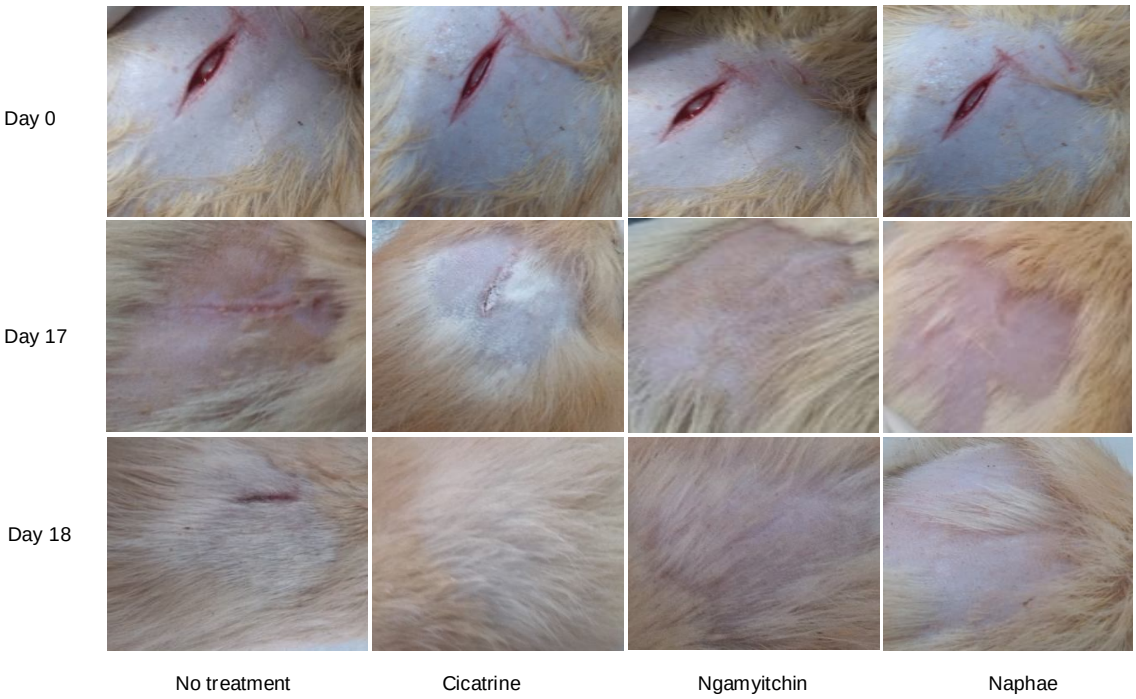

Fig. 2. Effects of the collagen treatment, cicatrin powder and no treatment on incision wound area-macroscopic appearance of healing

From the results, the solubility up to 3% NaCl concentration was remained nearly constant. The relative solubility was decreased significantly above 3% NaCl concentration due to the salting-out effect (Fig. 1).

FTIR data of the extracted collagen samples indicated the presence of N-H, O-H, C=O, $-CH_2$ and $-CH_3$ groups in the samples (Table 1). FTIR spectra of the collagen sample from both fish happened to coincide with typical protein spectra.

From the *in vivo test*, a significant decrease in period of epithelialization was observed in Naphae collagen treatment groups and no treatment control group. Comparative analysis revealed that Ngamyitchin collagen and Cicatrin (neomycin sulphate and bacitracin zinc) had almost equal wound healing activity. There was a significant reduction in wound contraction - 50% in all the treatment groups except no treatment control group (Fig. 2).

Histopathological finding of the skin lesions after 18 days were done by H&E method and recorded by using the light microscope with specific images. Delayed wound healing

Table 1. FTIR spectral data assignments of samples

No.	Wave number (cm^{-1})		Assignments
	Ngaphe	Ngamyitchin	
1	3495	3425	ν_{N-H}, amide group
2	3371	3279	ν_{O-H}, -COOH group
3	3084	3065	ν_{C-H}, $-CH_2$ group
4	1666	1651	$\nu_{C=O}$, -amide group
5	1450	1450	δ_{C-H}, $-CH_2$ group
6	1327	1327	δ_{C-H}, $-CH_3$ group
7	1080	1080	ν_{C-O}, -COO group

was observed in no treatment group. This section of rat skin showed incomplete epithelialization in epidermis, dermis and cutaneous layers. Standard group of rat skin showed re-epithelialization of three layers of skin in this histological section. It was the appearance of normal wound healing process. Well degree of wound healing was observed in Ngamyitchin collagen group. This section of rat's skin showed complete epithelialization in epidermis, dermis and cutaneous layers. Normal wound healing of rat's skin was observed in Ngaphe collagen group. This section of rat skin showed normal epidermis, dermis and subcutaneous layers.

Histological comparisons of healed skin wounds (No treatment, Cicatrine, Ngamyitchin and Ngaphe) are shown in Figure 3.

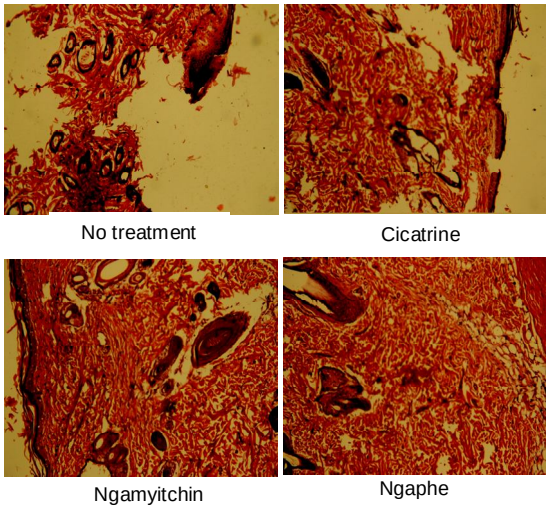

Fig. 3. Histological comparisons of healed skin wounds

Conclusion

Collagen was extracted from the skin of Ngamyitchin (*Labeo rohita*) and Ngaphe (*Notopterus notopterus*). The yield percent of collagen from Ngamyitchin was higher than that of Ngaphe. The maximum solubility of Ngaphe and Ngamyitchin was observed at pH 3 and 2 whereas the minimum solubility was observed at pH 10 and 9, respectively. These results showed high solubility in the acidic pH ranges.

However, the solubility decreased in the presence of NaCl at concentration above 3%. The extracted collagen from Ngaphe and Ngamyitchin were present triple helical structure. The extracted collagens from the skin of Ngamyitchin (*Labeo rohita*) and Ngaphe (*Notopterus notopterus*) were applied in the treatment of incision wound by using Wistar rats. The results which were compared with standard Cicatrine (neomycin sulphate and bacitracin zinc), showed that Ngaphe collagen had normal wound healing activity as standard and Ngamyitchin had well degree of wound healing. This study pointed out that extracted collagen from fish skin can be used in biomedical, pharmaceutical applications and skin care.

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Comparative Study of Heavy Metals in Selected Medicinal Plants and Soils from Different Sites of Mandalay Region

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Medicinal plants play a vital role in healthcare sector for developing nation and potent source of therapeutic molecules to heal various diseases in the world. World Health Organization estimated about 75-80% of the world's population use plant-based medicines. The purpose of the current study was to determine various indigenous medicinal plants for heavy metals contamination and to make awareness among the public regarding its safer use and collection areas, containing high level of heavy metals and their adverse health effects. The atomic absorption spectrophotometer was employed for estimation of heavy metals of four different plant species that were collected from different locations in Mandalay Region. The content of (Cd, Cr, Cu, Fe, Pb and Zn) in *Andrographis paniculata*, *Eclipta alba*, *Curcuma longa* and *Alternanthera pungens* and their soils in which they grown were selected for their analysis. Most of the selected medicinal plants and all soils from Mandalay Region contained the metals which are within permissible limit and they are generally safe for use. In few cases; *Eclipta alba* from Department of Medical Research (Upper Myanmar) contains Cd (0.46±0.04 ppm) and Cr (8.1±0.64 ppm) which is above permissible limit. And also, *Alternanthera pungens* from Chanayethazan Township and Amarapura contain more Zn than permissible limit (29.71±5.57 ppm and 33.29±2.29 ppm, respectively). All plants contain high amount of Fe than permissible limit set by FAO/WHO (1984) in edible plants (20 mg/kg). Consumption of these two plants as drugs may constitute possible health hazards to consumers at the time of the study.

INTRODUCTION

Traditional medicinal plants are used for the treatment of various ailments. The World Health Organization (WHO) estimated about 75-80% of the world's population use plant-based medicines. All plants may not be as useful as claimed, or may have more therapeutic properties than are known traditionally.[1] Herbs may be contaminated with heavy metals during growing in the field, processing and handling. It is important to protect consumers from contamination. Herbs which are contaminated with heavy metals can be toxic and produce undesirable side effects. Some of the heavy metals are essential in very low concentrations for the survival of all forms of life. Heavy metals such as iron, chromium, copper and zinc are essential metals since they play an important role in biological system, whereas lead and cadmium are non-essential metals which can be toxic even in trace amounts.[2]

Plants may absorb heavy metals from soil, water or air. The ability of plants to selectively accumulate essential element varies in different species and is subjected to certain geochemical characteristics depending on the type of soil.[2] Monitoring of the contamination of soil with heavy metals is of interest due to their influence on plants, animals and humans.[3]

After collection and transformation of herbs into dosage form, the heavy metals confined in plants finally enter the human body and may disturb the normal functions of central nervous system, liver, lungs, heart, kidney and brain, leading to hypertension, abdominal pain, skin eruptions, intestinal ulcer and different types of cancers. The concentration of essential and non-essential heavy metals in medicinal herbs beyond permissible limit is a matter of great concern to public safety all over the world.[2]

Myanmar being a developing country has insufficient medical set-up in its rural areas. Thus, people of these areas depend on pre-used medicines prepared from different medicinal plants.

Andrographis paniculata is used as anti-inflammatory, antiviral and liver protective. *Eclipta alba* is used as hepatoprotective, antiviral and antioxidant. *Curcuma longa* is used traditionally as anti-inflammatory, antioxidant and hepatoprotective in South-east Asian countries.[4] *Alternanthera pungens* is used traditionally against dysentery, venereal diseases, cholera, many parasitic diseases and kidney diseases.[5] The WHO recommends that medicinal plants which form the raw materials for the finished products may be checked for the presence of heavy metals.[2]

The purposes of the current study were:

- To standardize various indigenous medicinal plants (*Andrographis paniculata*, *Eclipta alba*, *Curcuma longa* and *Alternanthera pungens*, and soils [They were grown at the Department of Medical Research (Upper Myanmar) (site I), University of Traditional Medicine, Mandalay (site II), Chanayethazan Township, Mandalay (site III) and Amarapura (site IV)] for heavy metals contamination, and
- To make awareness among the public regarding its safer use and collection areas, containing high level of heavy metals and their adverse health effects.

MATERIALS AND METHODS

Chemicals and reagents

Analytical grade reagents (Merck) of cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), lead (Pb) and zinc (Zn) standard were used as standard reference materials, while 70% nitric acid (HNO_3), 69% hydro-chloric acid (HCl), and double de-ionized water (DDW) were used for digestion purpose.

Instruments and apparatus

Atomic Absorption Spectrophotometer (AAS), AA 6650, Shimadzu, Japan was used for measurement under the standard operating condition. Muffle furnace (LEF 1035), oven, analytical balance, vortex mixer, heating magnetic stirrer, volumetric flask, measuring cylinder, beaker, pipette, crucible and desiccator were used.

Calibration of equipment

For the elements under investigation, the following sensitivity and detection limits were established respectively for the used AAS apparatus: Cd 0.1 ppm, 0.2 ppm, 0.4 ppm and 0.8 ppm, Cr 0.5 ppm, 1 ppm, 2 ppm and 4 ppm, Cu 0.5 ppm, 1 ppm, 2 ppm and 5 ppm, Fe 0.5 ppm, 1 ppm and 2 ppm, Pb 1 ppm, 2 ppm, 5 ppm and 10 ppm, Zn 0.2 ppm, 0.4 ppm, 0.8 ppm and 1.6 ppm.

Plant authenticity

The plants were identified and confirmed for their specific botanical name by competent taxonomist from the Department of Botany, Mandalay University, Mandalay.

Sample collection

Soil samples were collected from each site of plants at about 8-10 cm depth of the soil and both plants and soil samples were placed in the polyethylene sampling bags. Plants samples of the medicinal plants *Androgiaphis paniculata, Eclipta alba, Curcuma longa* and *Alternanthera pungens* and their soil samples were collected from four different places, Department of

Medical Research (Upper Myanmar) (site I), University of Traditional Medicine, Mandalay (site II), Chanayethazan Township, Mandalay (site III and Amarapura site IV).

Sample preparation

The plant materials were washed thoroughly with tap water and finally with double de-ionized water. The plants samples were dried in shade at room temperature. The dried plant samples were crushed, powdered and homogenized using a mortar and pestle, and kept in polyethylene sampling bags for analysis.

Digestion of soil samples

Soil samples were dried in an oven at 110˚C for 2 hours until they were brittle and crisp. A portion (1g) of dried soil samples were placed separately in 50 cm^3 pyrex beakers and then digested with 12 cm^3 of a mixture of HNO_3-HCl (in the ratio of 1:3 v/v) to near dryness in an oven at 110˚C for 3 hours and cooled. Then, 20 ml of 2% HNO_3 were added into the beaker on a hot plate to boil for 10 minutes and cooled. The digests were filtered into a 100 cm^3 volumetric flask using Whatman no. 42 filter paper and the volumes made up to the marks with double de-ionized water.[6]

Digestion of plant samples

Specified weight (2.5 g) of crushed and powder portion from each plant of *Androgiaphis paniculata*, *Eclipta alba*, *Curcuma longa* and *Alternanthera pungens* was placed into crucible for heating in an oven at 110˚C for 2 hours to remove moisture. Then, the dried sample after charring was placed in furnace. The furnace temperature was gradually increased from room temperature to 550˚C in 30 minutes.

The sample was ashed for about 4 hours until a white or grey ash residue was obtained. The content of crucible was cooled in desiccators and weighed. Then, 5 ml of 6M HNO3 were added to the ash samples of each plant to dissolve and digest the contents. The solutions were filtered by Whatman (no. 42) filter papers, transferred to 50 ml volumetric flasks and were diluted with de-ionized water.[7]

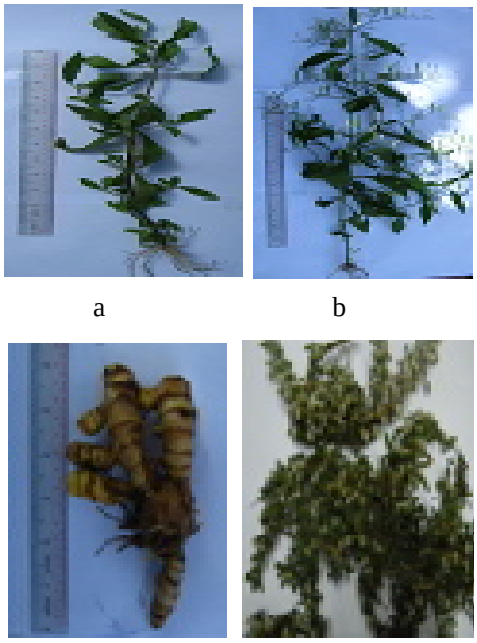
Statistical analysis

Data were analyzed by using Microsoft Excel, 2007. Results were presented as mean±SD.

RESULTS AND DISCUSSION

Plant authenticity

Morphology, taxonomy and anatomy of the plants were observed to agree with the following botanical names; *Andrographis paniculata* (Saykhagyi), *Eclipta alba* (Kyatemhan), *Curcuma longa* (Nanwin) and *Alternanthera pungens* (Myaekhatkyetmauk) (Fig. 1).



a=*Andrographis paniculata* (Saykhagyi)
b=*Eclipta alba* (Kyatemhan)
c=*Curcuma longa* (Nanwin)
d=*Alternanthera pungens* (Myaekhatkyetmauk)

Fig. 1. Plants studied in the study

Cadmium

In studied soil samples, 'Cd' concentrations in site I and site IV were 0.41±0.120 ppm and 1.11±0.25 ppm, respectively, but these

values were lower than the maximum allowable limit (MAL) of 3 mg/kg.[8] But, it was not detected in site II and site III.

In the studied plants, 'Cd' was detected in *Eclipta alba* from site I (0.46±0.04 ppm) and *Alternanthera pungens* from site IV (0.16±0.02 ppm). However, for medicinal plants, the permissible limit for 'Cd' set by WHO (2005),[2] China and Thailand was 0.3 ppm. *Eclipta alba* from site I was found to contain 'Cd' above this limit. But, 'Cd' was not detected in *Andrographis paniculata* and *Curcuma longa* L. collected from both site I and site II.

Table 1. Level of heavy/toxic metals (ppm) in soil samples from different sites

Metals	Site I	Site II	Site III	Site IV	Ref. value
Cd	0.41 ±0.12	ND	ND	1.11 ±0.25	3*
Cr	46.54 ±7.51	23.80 ±1.36	ND	46.69 ±4.55	100**
Cu	16.15 ±4.73	12.45 ±1.22	17.64 ±9.06	25.08 ±3.68	140*
Fe	3412.41 ±670.28	2906.47 ±164.98	2058.98 ±260.49	3094.002 ±621.97	50000**
Pb	24.94 ±5.20	132.36 ±40.99	16.73 ±3.07	177.34 ±35.65	600**
Zn	41.07 ±8.30	65.67 ±9.66	38.82 ±7.77	142.56 ±26.29	300*

*****=Ref 8, ******=Ref 9, Cd=Cadmium, Cr=Chromium, Cu=Copper, Fe=Iron, Pb=Lead, Zn=Zinc

Table 2. Level of heavy/toxic metals (ppm) in plant samples from different sites

Metals	*Andrographis paniculata*		*Curcuma longa* L.		*Eclipta alba*		*Alternanthera pungens*		Ref. value
	Site I	Site II	Site I	Site II	Site I	Site II	Site III	Site IV	
Cd	ND	ND	ND	ND	0.46±0.04	ND	ND	0.16±0.02	0.3*
Cr	ND	0.41±0.09	ND	ND	8.1±0.64	ND	ND	1.49±0.01	2*
Cu	5.88±1.43	8.58±5.50	2.34±1.07	1.86±1.12	14.12±5.15	8.70±2.51	6.09±1.29	14.38±0.41	20*
Fe	119.74±24.41	142.44±1.85	76.78±30.27	127.71±28.42	127.14±12.5	276.42±56.27	237.74±58.3	356.05±126.72	20*
Pb	3.75±1.92	ND	3.42±1.86	ND	4.33±0.63	ND	ND	8.61±0.11	10*
Zn	19.02±4.22	24.69±1.10	23.60±1.02	24.30±5.78	22.81±8.35	13.03±3.78	29.71±5.57	33.29±2.29	27.4*

*****=Ref 2, Cd=Cadmium,Cr=Chromium, Cu=Copper, Fe=Iron, Pb=Lead, Zn=Zinc

And also, it was not detectable in *Eclipta alba* from site II and *Alternanthera pungens* from site III. 'Cd' causes both acute and chronic poisoning, adverse effect on kidney, liver, vascular and immune system.[10]

Chromium

In studied soil samples, **'**Cr' was detected in soil from site I, site II and site IV. Their concentrations of 'Cr' ranged between 23.80 ±1.36 ppm and 46.69±4.55 ppm, which were lower than the maximum allowable limit (MAL) of 100 µg/g (FAO/ WHO, 2001).[9] **'**Cr' was not detected in soil from site III.

In the studied plants, 'Cr' was detected in *Eclipta alba* (8.1±0.64 ppm) from site I and *Alternanthera pungens* (1.49±0.01 ppm) from site IV. After comparison of the metal permissible limit in the studied medicinal plants with those proposed by Canada (2 ppm) in raw medicinal plant material,[2] it was found that *Eclipta alba* accumulate 'Cr' above this limit. 'Cr' was not detected in *Andrographis paniculata* collected from site I and *Curcuma longa* L. collected from site I and site II.

And also, it was not detectable in *Eclipta alba* from site II and *Alternanthera pungens* from site III. Chronic exposure to 'Cr' may result in liver, kidney and lung damage.[10]

Copper

The concentrations of 'Cu' in soil ranged between 12.45±1.22 ppm and 25.08± 3.68 ppm. But 'Cu' concentrations in all soil samples were below its MAL of 140 mg/kg of Australian and New Zealand Guideline.[8] The range of 'Cu' in studied plants varied between 1.86±1.12 ppm in *Curcuma longa* L. from site II and 14.38±0.41 ppm in *Alternanthera pungens* from site IV. For medicinal plants, the WHO (2005) limits had not yet been established for 'Cu'. But, these values were lower than the permissible limits for 'Cu' in medicinal plants set by

China and Singapore (20 and 150 ppm, respectively).[2]

In the present study, lower amount for 'Cu' in plant materials collected from site I was found showing no harmful effect of this metal on plant. 'Cu' is one of the essential elements for plants and other living organisms. Fumes of 'Cu' may cause metal fumes fever with flu-like symptoms, hair and skin discoloration while dermatitis has not been reported.[1]

Iron

The range of 'Fe' in the studied sites was high with a minimum of 2058.98±260.49 ppm in site III and a maximum of 3412.41 ±670.28 ppm in site I, which were lower than the maximum allowable limit (MAL) of 50000 µg/g (FAO/WHO, 2001).[9] 'Fe' concentrations in the studied plants ranged from 76.78±30.27 ppm in *Curcuma longa* L. from site I to 356.05±126.72 ppm in *Alternanthera pungens* from site IV. For medicinal plants, the WHO (2005) limit has not yet been established for 'Fe'. All of them were found to be higher than the permissible limit of 'Fe' set by FAO/WHO (1984) in edible plants (20 mg/kg).[2] But, 'Fe' concentrations in this study was comparable to that of a study in which the range of 'Fe' was between 261 and 1239 ppm in selective medicinal plants of Egypt.[10]

'Fe' is necessary for the formation of haemoglobin and also plays an important role in oxygen and electron transport in human body systems.[10] Excess of 'Fe' can cause haemosiderosis.

Lead

In the studied soil samples, 'Pb' concentrations ranged from 16.73±3.07 ppm to 177.34±35.65 ppm and found to be lower than MAL (600 mg/kg) from Nigeria.[8] Among the investigated medicinal plants, 'Pb' was detected and ranged from 3.42 ±1.86 ppm in *Curcuma longa* L. from site I to 8.61±0.11 ppm in *Alternanthera pungens* from site IV. These values were lower than the permissible limits of 'Pb' for medicinal plants set by China, Thailand, Malaysia and WHO (10 mg/kg).[2] But it was not detected in all plants from site II and site III. 'Pb' causes both acute and chronic poisoning, and also poses adverse effects on kidney, liver, vascular and immune system.[10]

Zinc

In the studied soil samples, 'Zn' concentrations ranged from 38.82±7.77 ppm to 142.56±26.29 ppm and found to be lower than the maximum allowable limit (MAL) of 300 mg/kg of Australian and New Zealand Guideline.[8] The content of 'Zn' in the plant samples ranged between 13.03 ±3.78 ppm in *Eclipta alba* from site II and 33.29±2.29 ppm in *Alternanthera pungens* from site IV. The permissible limit set by FAO/WHO (1984)[2] in edible plants was 27.4 ppm. After comparison with the metal limits proposed by FAO/WHO (1984),[2] it is found that all plants accumulate Zn below this limit except *Alternanthera pungens.*

The general sources of 'Zn' contamination are agro-chemicals, burning of fossil fuels and sewages. Due to its importance, 'Zn' is present in blood and about 85% of it combines with protein for transport after absorption and its turn-over is rapid in pancreas. Deficiency of 'Zn' causes diabetic hyposomia, hypogensia or coma.[1]

Conclusion

There is no own soil and plant quality standard regarding the limit of heavy metal contamination in Myanmar yet. Therefore, Myanmar still has to establish soil and plant quality standards and the data generated in this study may also be useful as a basis for formulation of standard guideline.

Most of the selected medicinal plants from Mandalay Region contained minerals which are within permissible limit and they are generally safe for use. However, few plants contain more heavy/toxic metals than the permissible limit. The possible reasons for accumulation of hazardous metals in plants may include the growing of these plants in contaminated soil which is near roadways or

mining and industrial areas or irrigated by toxic water. The other reasons may be that the studied plants were hyperaccumulators even their soil contain the lower elemental level.[11]

Therefore, special care must be taken during the administration of these plants as a remedy. Medicinal plants should be collected from areas not contaminated with heavy metals. It is also necessary to have a look on good quality control methods and research practices for these plant-based herbal medicines and screening in order to protect humans from heavy metal toxicity.

The present study will also provide useful reference informative data for the standardization of medicinal plant materials regarding the limit of heavy metal contamination.

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Antibiotic Susceptibility Pattern among *Helicobacter pylori* Isolates from Chronic Dyspepsia Patients

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The importance of *H. pylori* as an etiological agent in gastroduodenal disease had suggested antibiotic treatment as the main target for the elimination of infection. A cross-sectional, descriptive study was conducted from September 2012 to August 2013 to isolate the *H. pylori* from chronic dyspepsia patients and also to determine the drug sensitivity profile of isolated *H. pylori*. Two hundred gastric biopsy specimens were taken from dyspeptic patients comprising 100 gastritis cases and 40 duodenal ulcer cases and 60 gastric ulcer patients. *Helicobacter pylori* were isolated from 30 cases (15%), comprising 20 cases (20%) of gastritis, 6 cases (10%) of gastric ulcer and 4 cases (10%) of duodenal ulcer. Antibiotic susceptibility profile of isolated *H. pylori* was determined by using the Epsilometer test (E test). Nineteen (63.3%) of the isolates were resistant to clarithromycin (Minimum Inhibitory Concentration) MICs>256 µg/ml and all isolates were (100%) resistant to metronidazole (MICs>256 µg/ml). However, all isolates were sensitive to amoxicillin (MICs 0.50 µg-1.5µg) and levofloxacin (MIC 0.25 µg-0.125 µg), respectively. Data from this research were expected to provide the necessary information to select the drug of choice for eradication of *H. pylori* and also in the management of gastritis and peptic ulcer, and ultimately, to prevent cancer of the stomach.

INTRODUCTION

Helicobacter pylori is a gram-negative bacterium, firstly isolated in gastric mucosa by Marshall and Warren in 1983. Epidemiological studies strongly suggested that more than 50% of the world's populations are colonized by *H. pylori.* However, the prevalence of *H. pylori* infection varies from 10% to 90%, depending on age, geographic location, and socioeconomic status of the populations. In developing countries, the prevalence of *H. pylori* infection was found in more than 70% of the populations.[1] Among Southeast Asian countries, the reported seroprevalence rate was 35.9% in Malaysia, 31% in Singapore and 57% in Thailand.[2]

A study of *H. pylori* positivity on chronic gastritis and peptic ulcer patients was done in 2000 in Myanmar, which revealed that 20% of cases were *H. pylori* positive.[3] Similar study was done in 2005, with results of 30% of culture positivity rate.[4] In Myanmar, the overall seroprevalence of *H. pylori* is 69% and found to be significantly increased with age.[5]

Persistent *H. pylori* infection often induces gastritis and is associated with the development of peptic ulcer disease, atrophic gastritis, and gastric adenocarcinoma.[6] The importance of *H. pylori* as an etiological agent in gastroduodenal disease had suggested antibiotic treatment as a main target for the elimination of infection. The successful eradication of *H. pylori*

infection was shown to resolve the gastritis, dramatically accelerate ulcer healing and reduce ulcer recurrence and the prophylactic effect of the recurrence of ulcer bleeding.[7]

Antimicrobial susceptibility testing of *H. pylori* isolates can be carried out by disc diffusion and determination of minimum inhibitory concentration (MIC) by agar dilution tests or E tests. The Epsilometer test (E Test BIOMERIEUX, France) is a newly developed technique for the quantitative determination of susceptibility to antimicrobial agents in a variety of bacteria and fungi.[8] The test is based on the combination of concepts of both the agar dilution and disk diffusion methods but differs from the conventional disk diffusion method by the use of the predefined exponential gradient of antibiotics with the lowest concentration and the highest concentration. The E test is also much less labor intensive and is easier to perform than agar dilution and broth dilution method, which also allows the test to be quickly and economically adapted into the laboratory work flow.

Widespread use of antimicrobial drugs has resulted in worldwide increase in the prevalence of antibiotic resistance in *H. pylori.* There are only limited studies on *H. pylori* antibiotic sensitivity pattern in Myanmar. In 2005, one study reported that metronidazole resistance was 54.2% and clarithromycin resistance was 12.5% and there was low detection rate for amoxicillin resistance (8.3%).[4] Similar study was done in Thailand in 2008 with resistance rates of 30.2% for metronidazole, 5% for clarithromycin and 2.4% for amoxicillin.[9]

In India, studies reported a relatively high resistance to metronidazole and clarithromycin i.e., between 90%-100% and 30%-50%, respectively.[10] Resistance to amoxycillin was reported to be 40%. Thus, as the result of changing patterns of antibiotic sensitivity between different geographic regions and also within the same region, it is suggested valuable to test the sensitivity regularly before deciding the therapeutic regime.

The objective of the study was to determine the antibiotic susceptibility pattern among *H. pylori* isolates from chronic dyspepsia patients.

MATERIALS AND METHODS

Study design and study period

A cross-sectional, laboratory-based descriptive study was carried out from September 2012 to August 2013.

Place of study

Department of Gastroenterology, Yangon General Hospital (YGH) and Bacteriology Research Division, Department of Medical Research (LM) were places of the study.

Selection of patients

Gastric biopsy specimens were taken from the patients with gastroduodenal disorders who had undergone endoscopy at Department of Gastroenterology, Yangon General Hospital (YGH) according to the following inclusion and exclusion criteria. Written informed consents were obtained from eligible patients.

Inclusion criteria

- Patients with gastroduodenal diseases such as gastritis, gastric ulcers and duodenal ulcers; diagnosed by endoscopy
- Patients older than 18 years, both sexes

Exclusion criteria

- Patients with oesophageal varieces
- Patients with active gastric bleeding
- Complicated cases such as intestinal obstructions, or patients with haematemesis and melena

Study population

A total of 200 patients who fulfilled the inclusion criteria were studied.

Procedure

After obtaining the written informed consent from eligible patients, relevant history taking was done according to the proforma. Specimens were taken and sent to the Bacteriology Research Division,

Department of Medical Research (LM) for culture and antibiotic sensitivity.

Methods of sample collection

Two bites of biopsy sample were taken from patients with a sterile fibreoptic endoscope with biopsy forceps by gastroenterologists. The site of gastric biopsy specimens were from gastric antrum. One sample was tested for urease test and the other for culture.

Processing of samples

One endoscopic biopsy sample was transferred to 0.2 ml of thioglycolate transport broth and the other into microtitre plate for urease test, and then brought back to the laboratory in an ice box within two hours of collection.

Primary isolation and drug sensitivity testing of bacteria

Before inoculating on the culture media, the specimens were minced and gently homogenized in thioglycolate broth with a ground glass grinder. The amount of inoculum was 200 µl for each plate and this will be inoculated onto the Muller-Hinton agar (Oxoid) with Skirrow's supplement with 5-10% horse blood. The culture plates were incubated at 37˚C in an anaerobic jar (Gas pak jar) under microaerophilic condition. The optimal temperature for incubation was 35-37˚C.

The primary biopsy cultures were incubated for 3 to 5 days. Plates showing presence of contamination were discarded. The media which did not show any growth after 7 days incubation were regarded as negative culture. The organisms were identified as *H. pylori* by colony morphology, gram stain reaction, and positive reactions to oxidase, catalase, and urease activities. The confirmed *H. pylori* isolates were preceded for drug susceptibility testing by the E test using metronidazole, clarithromycin, levofloxacin and amoxicillin strips.

E test procedure

The bacterial inoculum was prepared from isolates grown on selective media. Colonies were taken from a plate and suspended in Brain Heart Infusion broth until a turbidity equivalent to 3 McFarland standard was obtained. The cell suspension was inoculated onto Mueller-Hinton agar containing 5% horse serum and 2% supplement by gently spreading with a sterile cotton-tipped swab.

The plates were dried for 3-4 minutes, then the E test strip was placed in the centre of plate, then incubated at 37˚C in a microaerophilic conditions. MICs were read after 48 hrs of incubation on the basis of the intersection of the elliptical zone of the growth inhibition with the MIC scale on the E test strip. Based on MIC values described by the manufacturer (BIOMERIEUX, France), the isolates were interpreted as susceptible, intermediate and resistant.[11]

RESULTS

Two hundred gastric biopsy specimens were taken from dyspeptic patients, consisted of 125 women and 75 men with the age range of 23-75 years (mean age of 45.5 years) presenting with different clinical conditions; comprising 100 gastritis cases and 40 duodenal ulcer cases and 60 gastric ulcer patients. *Helicobacter pylori* were isolated from 15% of total cases (30/200), comprising 20% of gastritis cases (20/100), 10% of gastric ulcer cases (6/60) and 10% of duodenal ulcer cases (4/40) (Table 1). All 30 cases of *H. pylori* culture positive cases were urease positive.

Table 1. Different clinical conditions of *H. pylori* infection

Total number of different types of diseases	*H. pylori* culture isolates			
	positive		negative	
	No	Percent	No	Percent
Gastritis (n=100)	20	20	80	80
Gastric ulcer (n=60)	6	10	54	90
Duodenal ulcer (n=40)	4	10	36	90
Total (n=200)	30	15	170	85

Results of our study showed 100% resistance to metronidazole (MIC>256 µg/ml) and 63.3% of the isolates were resistant to

clarithromycin (MICs>256 µg/ml). However, all isolates were sensitive to amoxicillin (MICs 0.50 µg-1.5 µg) and levofloxacin (MIC 0.25 µg-0.125 µg) and 36.7% of sensitivity to clarthromycin (MICs 2 µg-4 µg), respectively (Table 2).

Table 2. Antibiotic susceptibility pattern of *H. pylori* determined by E test (n=30)

Tested antibiotics	Susceptible (S) no. (%)	Intermediate (I) no. (%)	Resistant (R) no. (%)
Metronidazole	-	-	30(100)
Clarithromycin	11(36.7)	-	19(63.3)
Amoxicillin	30(100)	-	-
Levofloxacin	30(100)	-	-

E test BIOMERIEUX's Interpretative criteria according to MIC (µg/ml): metronidazole (R≥32, I=16, S≤8), clarithromycin (R≥32, I=16, S≤8), amoxicillin (R≥8, I=4, S≤2), levofloxacin (R≥4, I=2, S≤1)

Table 3. Distribution of drug resistant *H. pylori* infection among different clinical conditions

Name of drug	Diseases	Drug resistant cases	
		Number	Percent
Metronidazole	Gastritis (n=20)	20	100
	Gastric ulcer (n=6)	6	100
	Duodenal ulcer (n=4)	4	100
Clarithromycin	Gastritis (n=20)	12	60
	Gastric ulcer (n=6)	6	100
	Duodenal ulcer (n=4)	1	25

Although the distribution of metronidazole resistant infections were found in almost all *H. pylori* isolates (100%) of different clinical conditions, the clarithromycin resistant isolates were discovered among 100% of gastric ulcer cases, 60% of gastritis cases and 25% of duodenal cases, respectively (Table 3).

DISCUSSION

In vitro sensitivity testing of *H. pylori* is regarded as an important test as no regimen is proved to be universally successful. Sensitivity profile is recommended to be determined regionally and periodically before starting the treatment.[11] In the past, *H. pylori* isolates were susceptible to many different antibiotics, but now the successful treatment of it is challenging.[12] Antibiotic resistance in *H. pylori* isolates is widespread and increasing of resistant isolates can make considerable clinical problem for antibiotic therapy.

In this study, four commonly used antibiotics were tested by E test against 30 *H. pylori* isolates obtained from different diseased group. In this study, 30 isolates showed 100% sensitivity to amoxicillin (MICs 0.50 µg-1.5 µg) and levofloxacin (MIC 0.25 µg-0.125 µg) and 36.3% of sensitivity to clarthromycin (MICs 2 µg-4 µg), respectively. But all the clinical isolates were found to be resistant to metronidazole (MIC>256 µg) and 63.6% of the isolates were resistant to clarithromycin (MIC>256 µg/ml).

According to the review of the World Gastroenterology Organization Global Guidelines in 2010, antibiotic resistance rates for *H. pylori* are increasing throughout the world and they vary geographically and are higher in developing countries. According to the data from 2006 report on Southeast Asia region, the resistance rates for metronidazole and clarithromycin are 100% and 28%.[13]

A 2003 multicenter Indian study showed that the *H. pylori* resistance rate was 78% for metronidazole and 45% for clarithromycin.[14] In Japan, a working group of the Japanese Society for Helicobacter Research undertook a surveillance study to determine the current antimicrobial susceptibility profiles of *H. pylori* isolates during the period 2002-2005 revealed the resistance rate to clarithromycin increased from 19% to 28% over the 3-year period. Unlike other parts of Asia, it showed that resistance to metronidazole in Japan remained low at 3.3-4.9% during the study period, reflecting the restricted use of metronidazole in Japan.[15]

High resistance observed for metronidazole could be due to the frequent use of the antibiotic in the empiric treatment of diarrhea. The use of metronidazole for dental infections may also add to selection pressure.[16] In addition, antibiotics self-medication is encouraged by free access and

over the counter purchase and by ineffective drug control policy. This could be a contributing factor for the very high level of resistance of *H. pylori* to metronidazole (100%) and clarithromycin (63.3%).

Previous study in Myanmar in 2005 has shown that metronidazole resistance was 54.2% and clarithromycin resistance was 12.5%. Thus, decreased susceptibility of isolates to metronidazole and clarithromycin were noted in the present study. According to the *in vitro* susceptibility results, amoxicillin and levofloxacin were found to be effective against almost all of the tested *H. pylori* strains and there was also low detection rate for amoxicillin resistance (8.3%) in previous study done in 2005. A study reported that resistance rate to amoxicillin was 2.4% in Iran.[17] In Europe and in the USA, resistance rates are less than 1%,[18] however, there have been recent reports of resistance to this antibiotic. In contrast, high resistance rates have been reported in South Korea (18.5%) and in Indonesia (19.4%).

Levofloxacin, a newly introduced antibiotic for treatment of *H. pylori* infection and it was reassuring to note that resistance was zero to this antibiotic. A study from Malaysia in 2011 also described the zero resistance to levofloxacin.[19] However, in countries where prescription of this antibiotic is widespread, an exponential increase in resistance has been observed. For example, in Taiwan, levofloxacin resistance has increased 2.8% in 2003 to 11.8% in 2007, in France from 3.3% 1999 to 17.2% in 2005, and in Korea from 0% in 1999 to 21.5% in 2006.[20, 21]

The present study highlighted that majority of the *H. pylori* isolates were susceptible to amoxicillin and levofloxacin and the occurrence of high level metronidazole and clarithromycin resistant strains in the study population. These data suggested that metronidazole and clarithromycin should be used in first-line therapy with caution.

Considering the increasing resistance rate in many countries, monitoring of susceptibility of *H. pylori* to antibiotics appears to be necessary in the choice of effective therapy in order to eradicate *H. pylori* infections and to optimize the regimen in case of treatment failure.

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Bacteriological Analysis of Household Drinking Water in Selected Wards in North Dagon Township, Yangon

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Worldwide, an estimate of 2.5 billion cases of diarrheal diseases are annually reported and major contributors being unsafe drinking water, poor sanitation and unsatisfactory hygienic conditions. A cross-sectional, descriptive study was carried out in two selected wards which included households with reported fairly high attack rate of acute diarrheal disease in North Dagon Township, Yangon from December 2012 to August 2013 to determine the bacteriological quality of household drinking water. One sample of drinking water was collected from currently using drinking water containers of each study household. It was founded that most of the study households used water from common water tanks for drinking. Bacteriological parameters including total coliform and faecal coliform counts were detected by Multiple Tube Method at Bacteriology Research Division, Department of Medical Research (Lower Myanmar). Of 137 water samples tested, coliforms were detected in 94.9% (130/137) and faecal coliforms were detected in 93.4% (128/137), ranging from most probable number (MPN) of 2.2 cfu/100 ml to indeterminate. *Escherichia coli* was isolated from 45.3% (58/128) of faecal coliform positive water samples. The results indicated that the majority of the tested drinking water samples were contaminated with bacteria of faecal origin. Potential risk factors for drinking water contamination might include existing patterns of drinking water storage, methods used for treating drinking water, waste disposal and insanitary latrines that required further confirmation. The present study highlighted the poor bacteriological quality of drinking water and unhygienic sanitation practices in the study households. The results may assist in developing intervention programs for household drinking water treatment and safe storage (HWTS) strategies in the vulnerable communities.

INTRODUCTION

At least one third of the population in developing countries and almost one fifth of the global population have no access to safe drinking water. As a result, drinking water related diseases continue to be one of the major health problems globally.[1] The lack of adequate water supply and sanitation facilities causes a serious health hazard and exposes many to risk of water-borne diseases. Those at greatest risk of water-borne disease are infants, young children, people who are debilitated or living under unsanitary conditions and the elderly. Worldwide an estimated 2.5 billion cases of diarrheal diseases are annually reported and major contributors being unsafe drinking water, poor sanitation and unsatisfactory hygienic conditions that can ultimately lead to 1.5 million deaths mostly among children under five years of age.[2]

In Myanmar, according to 2008 data, total population of 14396,000 did not have an access to safe water supply.[3] A recent review estimates that 106,000 children under five years of age die in Myanmar each year and 22,260 (21%) of them die of

diarrhea.[4] It has frequently been observed that the microbiological quality of water in vessels at home is lower than at the source, suggesting that contamination is widespread during collection, transport, storage and drawing of water.[5] Household-based water treatment and safe storage was associated with a 35% reduction in diarrheal disease.[6]

The UNICEF-WHO 7 point strategy also highlighted the role of households in safe water storage and treatment to reduce diarrhoea in children under-five years.[7] Previous studies in Africa and Asia reported direct correlation between poor drinking water quality and incidence of diarrheal diseases in a community and an improvement in water quality will greatly reduce the occurrence of diarrhea in any given population.[8] In Myanmar, a previous study showed that 63% of drinking water from households and public-used pots in Yangon were contaminated with faecal coliforms.[9]

It was also reported that coliform contaminations were found in 66-100% of drinking water in schools in 10 townships in Yangon.[10] In a recent study on environmental risk factors related to an attack rate of acute diarrhea was found to be fairly high in two wards from North Dagon Township, Yangon.[11] Thus, this study was carried out to determine the bacteriological quality of household drinking water in above two selected wards in North Dagon Township, Yangon.

MATERIALS AND METHODS

Type of study

This study was cross-sectional, field-and laboratory-based descriptive study.

Study period

It was conducted from December 2012 to August 2013.

Study population

A total of 137 households (67 from Ward 41 and 70 from Ward 42) in North Dagon Township included in the study.

Sample collection

Before collection of water samples, written informed consents were taken from the head/responsible persons of the selected households. One water sample from each house was collected by currently using drinking cup and transferred to the sterile bottle and kept in an ice box during transportation. Bacteriological analysis was carried out within 4-6 hours of collection at the Bacteriology Research Division, Department of Medical Research (Lower Myanmar).

Field survey was carried out with one visit per week and about 10-15 water samples were collected from ten to fifteen households per visit. While taking water sample, date and time of sample collection, source of water sample, background information related to existing practices on drinking water such as drinking water source, pattern of storage, use of water treatments, types of utensils used for drinking water etc., were recorded in the proforma.

Determination of coliform count and faecal coliform count

Coliform and fecal coliform counts were done by Multiple Tube Method according to the WHO guidelines for drinking water quality.[12]

Procedure

In the Multiple Tube Method, a "presumptive coliform test" is performed first. Samples (10 ml, 1 ml, 0.1 ml) were inoculated into a series of tubes containing MacConkey Broth. The tubes were incubated at 37˚C for 24 hours and each tube was observed qualitatively for gas formation (presumptive test).

This test is used to detect and estimate coliforms population of a water sample. In the second stage, the positive tubes obtained in the presumptive test were again inoculated in two sets of brilliant green bile broth (confirmatory test). Each set of tubes were incubated at 37˚C (for coliform count) and 44˚C (for faecal coliform count), respec-

tively, for 24 hours and examined for gas formation. The most probable number (MPN) of bacteria present in the samples were statistically interpreted by using McCrady's Table.

Isolation and identification of pathogenic bacteria

For isolation and identification of bacteria, the undiluted samples were inoculated onto selective agar plates (MacConkey agar, Salmonella Shigella agar and Thiosulphate Citrate Bile Sucrose agar and Nutrient agar). Colonial morphology and microscopic examination of isolated bacteria were performed followed by routine biochemical tests. The isolates were identified according to the scheme of Cowan and Steel's Manual for the identification of medical bacteria.[13]

Data entry and analysis

EPI DATA software was used for data entry. Range and consistency checks were done. Data analysis was carried out by SPSS version 19.0.

Ethical considerations

This study was approved by the Institutional Ethical Review Committee, Medical Research Involving Human Subjects, Department of Medical Research (Lower Myanmar) by the letter number 1/Ethics 2012, Date: 1.3.2013.

RESULTS

Socio demographic characteristics of study households

In the present study, a total of 137 household drinking water samples (67 drinking water samples from Ward 41, 70 from Ward 42) were collected (Table 1).

Coliforms and faecal coliforms in drinking water samples

Coliforms were detected in 94.9% (130/137) and faecal coliforms were detected in 93.4% (128/137), ranging from most probable number (MPN) of 2.2 cfu/100 ml to indeterminate (Table 2).

Table 1. Socio-demographic characteristics of study households (n=137)

Characteristics	Frequency	%
Age in completed years of head or responsible persons of study households		
<25	13	9.5
>25-50	110	80. 3
>50-75	14	10.2
Number of under 5 years old children in the households		
Nil	25	18.2
1 to 2	109	79.6
3 to 4	3	2.2
Type of housing		
Pucca	3	2.2
Semi-pucca	4	2.9
Wooden	100	73.0
Bamboo	30	21.9
Type of latrine		
Pit latrine	63	46.0
Sanitary fly-proof latrine	71	51.8
No latrine	3	2.2
Way of waste disposal from household		
Composing	12	8.8
Specific dumping site	63	45.9
Stream/lake/river	50	36.5
YCDC trolley	12	8.8
Source of water supply in the household		
Artesian well	45	32.8
Shallow well	1	0.7
Natural pond	4	2.9
Public reservoir in the wards collecting pipe water	87	63.5
Type of water used for drinking		
Public reservoir in the wards collecting pipe water	62	45.3
Artesian well	54	39.4
Shallow well	1	0.7
Purified water	16	11.7
Others (Direct pipe water)	4	2.9
Type of utensils for drinking water storage at home		
Purified water bottle (20 lit)	34	24.8
Earthen drinking water pot	99	72.3
Purified water machine	1	0.7
Others (Plastic pot)	3	2.2
Type of water treatment method currently used for drinking water		
Boiling	4	2.9
Cloth filter (Handmade)	28	20.4
Cloth filter(Bought from market)	81	59.1
Purified water machine	1	0.7
Not used any method	23	16.8
Presence of covers in drinking water storage containers		
Fully	122	89.1
Half	1	0.7
No cover	14	10.2

Table 2. Coliform and faecal coliform in drinking water samples

Ward No.	No. of household	Percent of detected houses			
		Coliform		Fecal coliform	
		Positive	Negative	Positive	Negative
41	67	95.5 (64/67)	4.5 (3/67)	92.5 (62/67)	7.5 (5/67)
42	70	94.3 (66/70)	5.7 (4/70)	94.3 (66/70)	5.7 (4/70)
Total	137	94.9 (130/137)	5.1 (7/137)	93.4 (128/137)	6.6 (9/137)

Table 3. Isolated bacteria from contaminated drinking water samples

Name	Number	Percent
E. coli	58	45.3
Citrobacter freundii	61	47.7
Bacillus spp	9	7
Total	128	100

Isolated bacteria from contaminated drinking water samples

Escherichia coli were isolated from 45.3% (58/128), *Citrobacter freundii* from 47.7% (61/128) and environmental organisms (*Bacillus* spp) from 7.0% (9/128) of fecal coliform positive water samples. All the water samples investigated for presence of enteric pathogens like *Salmonella*, *Shigella* and *Vibrio cholera* were negative (Table 3).

DISCUSSION

The present study revealed the quantification and extent of microbial contamination of drinking water in the study households from peri-urban areas of Yangon showing only 6.6% (9/128) of all drinking water was bacteriologically satisactory. Thus, the study areas were highly polluted in bacteriological respect. The source of household drinking water in the study area in North Dagon Township is mainly public reservoir in the ward and artesian well water.

Water of present study areas was not suitable for drinking. Insects or other media may carry bacteria to enter the well, pond or supply water. The source of contamination may be septic system, too close to the well or the well casing isn't deep enough to assure that recharge water receives sufficient filtration to remove bacteria.[14]

Presumptive coliform or most probable number (MPN) test is used extensively for drinking water quality analysis. It can be used as indicator both for treatment efficiency and of the integrity of distribution system.[15] In this study, 93.4% (128/137) of water samples were contaminated with faecal coliforms and this finding was similar to a study[16] which showed 81% of the untreated water samples were positive for coliforms most probable coliform numbers, showing a high contamination rate and risk to public health. Another two studies [17, 18] also observed the poor water quality in 67% of the households in Brazil and 68.4% in Aligarh.

The detection of faecal coliform organisms provides definite evidence of fecal contamination and they were found in 45.3% (58/128) of the positive samples. This finding is similar to a study in Peshawar which showed 43.28% of *E. coli* was found in drinking water from urban and peri-urban areas of Peshwar and lower than that of a study conducted in urban areas of Nepal where 70-100% of water samples were found to be contaminated with fecal coliform organisms.[17, 19]

If any water sample is positive for *E. coli*, it is not suitable for drinking unless it is boiled for at least one minute, longer time may be needed at high altitudes. Contaminated drinking water can be treated using alum, aqua tab, chlorine, ultra-violet light, or ozone, all of which act to kill or inactivate contaminated organisms.

The obtained results revealed that *Citrobacter freundii* was found in 47.7% (61/128) of contaminated drinking water that might be due to lack of proper treatment and cleaning of containers or contamination of water sources. The percent of *Citrobacter freundii* in present study was found to be higher than the findings in Makkah city and Khamis Mushait, Sauid Arabia, which represent 6.3% and 8.8%, respectively.[20, 21] Present finding is nearest to a study which showed 30% of *Citrobacter freundii* contamination in rural area in Saudi Arabia.[22]

The environmental bacteria such as *Alcaligenes* spp, *Acinetobacter* spp, *Bacillus* spp were most saprophytic in origin and 14.92% and 55.56% were recovered in Peshawar and Makkha studies,[16, 20] respectively, indicating no treatment/improper treatment or post-treatment contamination. Their eradi-

cation is also essential for better sanitary value of drinking water supplies. In this study, only 7% (9/128) of environmental bacteria, *Bacillus* spp were isolated from contaminated water and this percent was lower than other's findings.

The major contributors of diarrheal diseases are unsafe drinking water, poor sanitation and unsatisfactory hygienic conditions and the most effected population is children under five years of age. In the present study, 93.4% of drinking water samples of study households had poor bacteriological quality.

Of these households, 81.8% had children under 5 years of age. Previous studies in Myanmar also showed that 63% of drinking water from households and public-used pots in Yangon were contaminated with faecal coliforms[9] and in 66-100% of drinking water in schools in 10 townships in Yangon were contaminated[10] This finding highlighted the need of intervention strategies to improve the drinking water quality in the vulnerable communities.

United Nations set the goals of halving the proportion of people without sustainable access to safe drinking water and of reducing under-five mortality by two thirds by 2015 under the Millennium Development Goals (MDG) (UNICEF-WHO, 2009).[7] The present study will link to ongoing epidemiological studies on the same area providing information on microbiological quality of drinking water in the study population and assist in developing intervention programs for household drinking water storage and treatment strategies.

Recommendation

Proper sanitary survey, design and implementation of water and/or sanitation projects; regular disinfections, maintenances and supervisions of water sources; and regular bacteriological assess-ment of all water sources for drinking should be planned and conducted. The results may assist in developing intervention programs for household drinking water treatment and safe storage (HWTS) strategies in the vulnerable communities.

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