Annual Report

2015

IOM-NCGM
Research Collaboration Office

March 2016
Kathmandu, Nepal
Tokyo, Japan

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Preface

The Medical Education Project, which was implemented from 1980 to 1996, contributed to the establishment and enhancement of basic and the clinical medicine of the Institute of Medicine (IOM) at Tribhuvan University along with its attached teaching hospital (TUTH). During the project period experts dispatched from NCGM worked together with IOM staff and good relationship was built between the two institutions.

Nineteen years have passed since completion of the project. During this period IOM achieved further development and currently it is greatly contributing to medical education, medical care, researches and human resource development in Nepal. Besides, reliable relationship between NCGM and IOM has been maintained until today. This is one of the valuable outcomes and legacies of the project.

In January 2013, Memorandum of Understanding (MOU) was concluded between NCGM and IOM to start a unique cooperation focusing on research and related human resource development. Three years have passed since the start of the new cooperation between NCGM and IOM. Collaborations initiated based on the MOU are on track, and a number of fruitful outcomes have been obtained.

It is a great pleasure for us to summarize the outline report of our new collaboration as Annual Report 2015 following the publications of Annual Report 2013 and 2014. We also would like to thank all those who worked hard for the success of the project and toward the realization of new collaborations between NCGM and IOM. I sincerely hope that the relationship of mutual trust between the two institutions will be further strengthened.

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Preface

It is our immense strategy that Dr. Hiroshi Ohara has contributed to develop the Annual Report of National Centre for Global Health and Medicine (NCGM) 2015. In this regard, we would like to express our congratulations to Dr. Ohara and his team for their efforts and dedication. NCGM has been playing great role to strengthen the research capacity and additional components for the research of Institute of Medicine (IOM) and Tribhuvan University Teaching Hospital (TUTH). As a result, several scientific articles of Medical researchers of NCGM and IOM have been published in different journals of national and international.

The scientists of both institutions have now established close relationship to build up research collaboration and are conducting biomedical research to strengthen understanding of medical sciences through their research.

We would like to express our heartfelt gratitude to NCGM specially Dr. Hiroshi Ohara,

Dr. Teruo Kirikae and Dr. Tatsuya Tada for their continuous support and help.

With best wishes,

Prof. Dr. Bharat Mani Pokhrel
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Director
Research Department

Abbreviations

COPD	Chronic Obstructive Pulmonary Disease
CT	Computed Tomography
DM	Diabetes Mellitus
ESBL	Extended Stratum beta Lactamase
GFATM	Global Fund against AIDS, Tuberculosis and Malaria
IOM	Institute of Medicine
IT	Information Technology
JICA	Japan International Cooperation Agency
JOCVs	Japan Overseas Cooperation Volunteers
MBBS	Medicine Bachelor Bachelor Surgery
MOHP	Ministry of Health and Population
MOU	Memorandum of Understanding
NCD	Non Communicable Diseases
NCGM	National Center for Global Health and Medicine
NDM	New Delhi Methalo-β-Lactamase
ODA	Official Development Assistance
TUTH	Tribhuvan University Teaching Hospital
WHO	World Health Organization
WPRO	Western Pacific Regional Office

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I. Outline of Collaboration between NCGM and IOM

1. Introduction

The Institute of Medicine (IOM) at Tribhuvan University was established as the first medical school in Nepal with the support of Japan's Grant Aid in 1980 and then a technical cooperation project (Medical Education Project) was implemented from 1980 to 1996 supported by Japan International Cooperation Agency (JICA). During this period, the National Center for Global Health and Medicine (NCGM) dispatched a project team leader, doctors and other medical professionals. Currently, IOM functions as a core in medical education, medical care and medical human resource development in Nepal, and its attached Tribhuvan University Teaching Hospital (TUTH) has gained the trust and popularity of the people in Nepal.

Recently, NCGM has begun some unique activities focused mainly on research and human resource development based on the relationship of mutual trust built between NCGM and IOM during the JICA project. At the beginning of these activities, a memorandum of understanding (MOU) was concluded between NCGM and IOM, and the IOM-NCGM Research Collaboration Office was established.

2. Before the conclusion of MOU

(1) Grant-aid project and Technical cooperation project (1980-1996)

Since most of the medical education in Nepal was dependent on foreign countries and there were limited number of medical workers in the past, increasing the number of doctors was crucial in order to widespread modern medicine and improve the medical and hygienic standards. Hence, in 1978, the Nepalese government requested assistance from the Japanese government in order to establish a medical university.

After several surveys, a grant-aid project by JICA was initiated for the purpose of establishing a medical faculty as the 10th faculty of the Tribhuvan University located in the capital, Kathmandu. The project aimed to widely provide medical services to the general public by strengthening and developing the medical faculty as a core institution for medical education and healthcare in Nepal. The project was conducted by a combination of grant aid and technical cooperation.

Tribuvan University Institute of Medicine (TUTH) in 1995

For the grant aid project, main facilities such as hospital buildings, academic building for basic science and nursing school were constructed, and essential equipment was provided.

<Grant-aid project>

- 1981 Construction of outpatient building, etc.
- 1982 Construction of patients' ward, etc.
- 1990 Construction of academic building, etc.
- 1991 Extension of patient's ward, etc.
- 1992 Repair of operation theater, etc.

The Medical Education Project was conducted as a technical cooperation project in two phases. In the first phase (1980-1989), strengthening education, clinical practice, and the research infrastructure for basic medicine and clinical medicine were mainly implemented. In the second phase (1989-1996), a technical cooperation project was implemented, focusing mainly on improving medical education to have international accreditation, enhancing the function of basic medicine, clinical medicine, and research, as well as improving hospital management function.

Technical guidance in nephrology (1996)

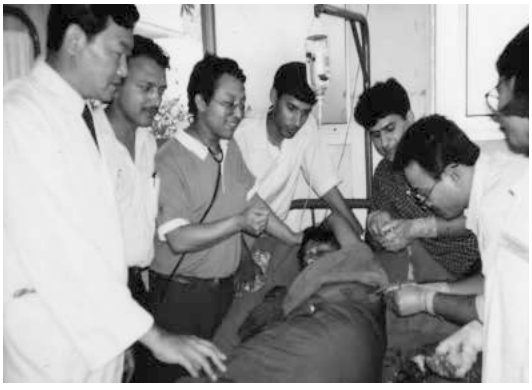
<Technical cooperation project: Medical Education Project>

Medical Education Project Phase 1 (1980-1989)

- Dispatch of Japanese Experts 12 (14JOCVs are included)
- Invitation for Nepalese Trainees 29
- Equipment Provision

Medical Education project Phase 2 (1989-1996)

- Dispatch of Japanese Experts 86
- Invitation for Nepalese Trainees 28
- Equipment Provision



International Symposium on Infectious and Tropical diseases (1996)

Local and International Seminar/ Symposium held by the Project

- Nepal-JICA Joint Symposium on Cholelithiasis 6-8 January 1991
- Congress on Altitude Medicine and Physiology 8 April 1992
- Nursing Research Conference 2-4 November 1993
- International Symposium on Obstructive Jaundice 17-18 February 1994
- International Symposium on Diabetes Mellitus 23-24 March 1995
- International Symposium on Infectious and Tropical Diseases 20-21 march, 1996

During the 16 year technical cooperation period almost all the basic and advanced techniques regarding diagnosis, examination, treatment and clinical record, which were indispensable to the daily medical care and medical education in IOM/TUTH, were guided (However, nosocomial infection control was not included as awareness on it was still low in those days even in advanced countries). IOM/TUTH had grown to be a medical institution that carried out a kernel function of Nepalese medical care, and had gained extremely high reliance from Nepalese people.

IOM/TUTH developed to be an internationally acknowledged medical institute; the MBBS (medicine bachelor bachelor surgery) degree awarded by IOM, Tribhuvan University was recognized by dental and medical councils of Bangladesh and Pakistan, and medical councils of India and Sri Lanka. Graduates of IOM had already pursued post-graduates education in Japan, Thailand, United Kingdom, U.S.A., and other countries.

As a result of the technical cooperation project over 16 years, the foundation for a medical education system, hospital management, clinical medicine, research, and nursing education was constructed at IOM.

(2) After Completion of the Technical Cooperation Project (1996-)

The Medical Education Project was completed in July 1996. Since then, IOM has greatly contributed to healthcare in Nepal, gaining the trust of the people as the most important medical institution in Nepal. Human resources trained by the technical cooperation project play leading roles not only in the capital Kathmandu but throughout the nation, furthermore globally, greatly contributing to the medical and healthcare field in Nepal.

After completion of the project, based on the good relationship developed during the project, NCGM and IOM have conducted small scale collaborative research activities on hepatitis, helicobacter infection, diarrhea, multi-drug resistant bacteria in respiratory tract infections, etc.

Nepal suffered political instability from 1996 to 2006 followed by a transition from the Kingdom of Nepal to the Federal Democratic Republic of Nepal in 2008. The political instability affected economic growth and compromised the delivery of social and public health interventions in the country. During this period, cooperative relations between IOM and NCGM were temporarily suspended, but with the stabilizing of political conditions in recent years, the good relationship has been recovering.

In September 2009, a joint symposium on nosocomial infection control was held at IOM jointly organized by NCGM and IOM, resulting in greater awareness of the importance of infection control and research collaboration. At the conference, successful technical cooperation in nosocomial infection control in Vietnam was introduced inviting director of Bach Mai Hospital in Hanoi. In 2013, "the 1st Joint Conference on Infectious Diseases with Growing Concern in Recent Years in Nepal" was held at IOM.

3. Conclusion of MOU and start of new cooperation (2013-2015)

On January 18, 2013, NCGM concluded an agreement of cooperation with IOM in Nepal for research and related human resource development, and IOM became the fifth overseas platform for NCGM. The Memorandum of Understanding (MOU) was signed by the President of NCGM in Tokyo firstly, and then, was signed by the Dean of IOM at IOM in the capital Kathmandu on January 18, 2013. Based on this MOU, unique activities of NCGM, such as collaborative research on infectious diseases, dual burden of infectious diseases and non-communicable diseases, and development of human resources related to these researches came to be actively promoted, seeking to improve healthcare in Nepal. Collaboration between NCGM and IOM including related institutions (Kathmandu University School of Medicine, Clinics in Kathmandu, etc.) mainly on research field will be continued hereafter. Conclusion of MOU is considered to be highly effective for the smooth and efficient implementation of collaborations including related institutions.

Joint Symposium on nosocomicao infection Control (September 2009)

Signing of MOU at NCGM and IOM (January 2013)

In September 2013, the IOM-NCGM Research Collaboration Office was established in the academic building of IOM, and the necessary equipment (desk, chair, computer, projector and scanner) was installed. One assistant was employed and given an orientation. Currently, activities utilizing grants from the International Health Cooperation Research (24-5, 24-5, 25-7), a grant from the Ministry of Health, Labor and Welfare of Japan, are progressing utilizing the office as a platform. IOM's Department of Microbiology and NCGM's Bureau of International Medical Cooperation manage the office operation.

The persons in charge of divisions of basic and clinical medicines are the professors for the department of Microbiology and Internal Medicine, respectively. In addition, there is a Research Department to manage research, which serves as a counterpart.

IOM-NCGM Research Collaboration Office

4. Purpose of the Collaboration between NCGM and IOM and expected outcomes

The purpose of this collaboration is to contribute to the healthcare in both countries by collaborative research and related human resource development, and to strengthen a reliable relationship.

Expected Outcomes

1. To strengthen research activities by the publication and presentation at academic conferences, etc. of the collaborative research by IOM and NCGM.
2. To obtain research results effectively through collaboration with IOM that is the main medical care and medical education institution in Nepal.
3. To provide benefits to younger staff members of NCGM by learning about the actual conditions of medical care in developing countries and how international collaborations are conducted.
4. To promote a relationship by utilizing a good relationship built on the results of the technical cooperation project.
5. To contribute to the quality improvement of medical care in Nepal by conducting researches focused on high-priority infectious diseases in Nepal and providing related technical assistance. These results are also beneficial for medical care in Japan.

6. To enable wider range of cooperation in the future, because the inclusion of other medical institutions other than IOM allows providing other possible collaboration fields.
7. To contribute to expansion of the benefit of ODA projects.

Main gate of Trubhuvan University Teaching Hospital (TUTH)

Nursing School

Academic building of Institute of Medicine (IOM)

II. Current IOM

1. General information

IOM was established as the first medical school in Nepal in 1980. Thereafter the number of medical schools (faculty of medicine, medical university) increased and at the time of project termination in 1986 there were 4 medical schools in Nepal. As of January 2015, the number of medical schools in Nepal is 22, in which 7 are affiliated with IOM. Post-graduate curriculums including diploma and degree programs have been improved so as to provide better medical personnel who can contribute efficiently to medical service.

Since its inception, IOM has produced more than 1,800 medical doctors (as of 1996 it was 307). These doctors are working, not only in Kathmandu Valley, but also in rural and remote areas of Nepal, and rendering valuable services. Beside, increasing number of doctors are studying or working in foreign countries. It is noteworthy that not a few graduates of IOM are working in medical schools across the country as professors. Similarly, nursing and co-medical staff trained in IOM/TUTH are rendering medical care to the Nepalese people. IOM is carrying out quite important role in medical care as well as medical education in Nepal.

Table 1 shows comparison of hospital general information between 1985 and 2015

2. Clinical Medicine

TUTH is a general hospital which has 740 beds (as of December 2015) under 28 Clinical Departments. The equipment which was provided during the project period has been gradually replaced with new ones, but not a few old equipment are still used. Advanced equipment such as CT scan, MRI, hemodialyzers are also equipped purchased by Nepalese government. TUTH can provide advanced medical care as well as essential medical care and examinations. TUTH has been conferred duties and roles as the most important top referral medical institution in Nepal. Since 1990, Cardiovascular Center, Obstetrics & Gynecology Center and Emergency Center have been constructed by Nepalese Government.

The number of out-patients, in-patients, and surgical operation has been ever increasing since its establishment, which means that the importance of TUTH in Nepalese medical care has been increasing year by year.

Patient waiting for doctor's consultation (TUTH)

3. Basic Medicine

Basic Medicine consists of 8 departments, which are responsible for giving lectures and practical studies to MBBS students and a variety of post-graduate courses to MBBS graduates. These departments are integrated in the academic building which was completed in 1992.

Research activities are another important role of the Basic Medicine departments in the university. The efforts to establish the basis for conducting research were made during the project period, and currently active researches are being done (currently more than 200 researches are conducted in IOM/TUTH). The results of research activities which were conducted in IOM/TUTH have been presented in various domestic and international medical congresses and journals. The Journal of Institute of Medicine, which was founded by IOM in 1984 as the first medical journal in Nepal developed to be a high level journal carrying a lot of valuable medical papers and it has become internationally acknowledged journal. Besides the 8 basic medicine departments, Research department, Department of Information Technology and National Center for Health Professional Education were set up after the project termination.

Bacteriological laboratory

4. Prospects

As a result of the 16-year technical cooperation, IOM/TUTH has developed to be one of the best medical institutions which receives the deep reliance of the nation and makes many contributions to medical care in Nepal. In addition, the friendly relationship between Nepalese staff and Japanese staff, and what is more, the Nepalese nation and the Japanese nation, has been firmly established. It is quite important to make the utmost effort to maintain these good results. It is true that the nationwide expectation for IOM/TUTH as well as duties and roles has been increasing. However, IOM/TUTH has problems such as small out-patient clinic space, obsolete equipment along with increasing number of non-communicable diseases cases. Besides, due to the catastrophic earthquake in 2015, a large number of equipment was damaged.

Hereafter, more and more importance in the quality of medical care will be attached. We expect IOM/TUTH will make every possible effort in order to live up to these expectations, addressing the existing challenges.

Patient's ward (TUTH)

Residents of Pulmonary Dept. (TUTH)

Clinical conference(TUTH)

Table 1 Situation and activities of IOM/TUTH : Comparison between 1985 and 2015

		1985	2015
1	Total No. of beds	401	740
2	No. of charity beds	40	63
3	No. of outpatients served (per year)	229,516	381,848
4	No. of in-patients served (per year)	11,973	23,107
5	Average length of hospitalization (days)	NA	6.73
6	No. of operations (per year)	5,237	12,531
7	Average bed occupancy rate (%)	NA	83%
8	Number of Clinical Departments	13	28
9	Number of Basic Science Departments (including forensic medicine and public health)	8	8
10	No. of students (per school year grade)	40	76
11	Research department	-	1
12	Department of Information Technology	-	1
13	National Center for Health Professional Education	-	1

III. Nepal earthquake in April 2015 and contribution of IOM/ TUTH to medical care for casualties

The recent Nepal earthquake of April 25, 2015 of magnitude 7.8 on the Richter scale had its epicenter in the area near Barpak, a mountain village between the capital, Kathmandu, and the tourist town of Pokhara. The earthquake was followed by many powerful after shocks on the same day and a very powerful one (6.7 on the Richter scale) hit Nepal on the very next day, Sunday April 26. The earthquakes, which caused extensive damage to buildings and thousands of deaths and injuries were even felt in Pakistan, India and Bangladesh.

Since immediately after the earthquake, the TUTH could start to offer medical services to a number of victims. This was because, even though some of the buildings in TUTH had cracks during the earthquake, all buildings in TUTH were not collapsed following the earthquake.

Furthermore, TUTH contributed to people in Kathmandu not only for medical services. TUTH was designated one of the two mortuaries in Kathmandu city, where many victims' dead bodies were placed. The dead bodies were kept cold during two months at the morgue. During that time, they tried their best to find their families, e.g. showing dead people’s belongings.

As of December 2015, there were still many of tents (shelters), tentative houses in skirt of Kathmandu city. Rubbles are still remained in these areas. Even though the difficult situation, medical staff actively offered medical services with compassion. They called for a long lasting supports.

(Cf. P. Earthquake disaster-associated health effects and the need for improved preventive measures)

IV. Research Activities

1. General information

To implement collaborative activities and strengthen the IOM-NCGM Research Collaboration Office, the following grants were utilized in 2015.

◆ Grants from the International Health Cooperation Research, grants from the Ministry of Health, Labor and Welfare of Japan.

1) 24-5: Studies on factors and trends of infectious diseases with growing concern in recent years in Nepal and Vietnam

2) 25-5: Studies on enhancement of human resource development utilizing the IOM-NCGM Research Collaboration Office - taking into consideration of the dissemination of the benefits of ODA project

3) 25-7: Fact-finding survey of nosocomial infection control in major hospitals in Nepal and planning of effective improvement

4) 27-4: Study on effective use of the surveillance results for drug resistant pathogens in nosocomial infection control

◆ Grant of Japan Society for the Promotion of Science: Scientific Research (B) (Overseas Academic Research) to NM: The survey of the present status of viral hepatitis treatment in Nepal

✧ The main theme of the ongoing researches is “Studies on Factors and Trends of Infectious Diseases with Growing Concern in Recent Years in Nepal.” Infectious diseases change over time, as can be clearly seen by the appearance of emerging or re-emerging infectious diseases and multi-drug resistant bacteria, and the control history of various infectious diseases by the appropriate measures. These changes in infectious diseases are considered to be associated with influential factors such as development, population movement, change in climate, nutrition, changes in the health system and disease structure, implementation of disease specific control programs, and support conditions of foreign counties. In this research, we summarized the overview of trends of infectious diseases chronologically and analyzed the factors that caused change in infectious diseases. Such overview and analysis are crucial to implement effective control measures.

As the results of preliminary study, we recognized some infectious diseases that fit this main theme. Among them we selected the following diseases and studies focusing on them were started. (Research grant 24-5)

① Malaria control and health system
② Diarrhea caused by emerging pathogens
③ Multi-drug resistant bacteria
④ Healthcare associated opportunistic infections
⑤ Dual burden of infectious disease and non-communicable diseases

✧ Recently in Nepal, although there has been increased awareness regarding nosocomial infection control, implementation of control measures is rather slow. In the Medical Education Project, technical guidance was provided in almost all fields of medical care, but only control measures against nosocomial infection were not included in the technical cooperation subjects. This was partly because of the poor awareness for nosocomial infection control even in developed countries including Japan at that time. Nosocomial infection control is also an important research theme for NCGM-IOM collaborative research.

Effective nosocomial infection control is crucial in the healthcare facilities of developing countries, but in actual fact, attention to it is still limited and control measures are not functioning well in many countries. This study has been conducted with the purpose to investigate the actual conditions of nosocomial infection control in Kathmandu City, Nepal as a basis for the possible contribution to its improvement. (Research grants 25-7, 27-4)

✧ Collaborative researches are being conducted in Nepal utilizing the IOM-NCGM Research Collaboration Office as a base (platform). Improvement of the base functions is essential to conduct research smoothly. We are trying to strengthen the function of the IOM-NCGM Research Collaboration Office aiming at smooth implementation of researches and human resource development. 1n 2014 the following activities were conducted. (Research grant 25-5)

① Strengthening of management capacity of IOM-NCGM Collaboration Office
② Comparative study on outcomes of Medical Education Project and current IOM/TUTH
③ Preparation for enhancement of human resource development
④ Making the Annual Report 2014, NCGM-IOM Research Collaboration Office

✧ In 2015 a new research “The survey of the present status of viral hepatitis treatment in Nepal” started with the Grant of Japan Society for the Promotion of Science.

Discussion on collaborative research Microbiology Dept. (IOM)

2. Progress of research activities up to 2015

2-1 Summary of the activities of Research Grant 24-5

Collaborative research based on the Research Grant 24-5 "Studies on factors and trends of infectious diseases with growing concern in recent years in Nepal and Vietnam" had been conducted for three years from April 2012 to March 2015, and fruitful results were obtained. The summary of the research is described below.

(1) Purpose:

The purpose of this study is to analyze the latest situation of the infectious diseases which have growing issues in recent years but appropriate studies along with control measures have not been done (namely, "Emerging health priorities in infectious diseases" as a new issue in the field of infectious diseases), and to discuss the cause of the spread of such diseases. This study has been carried out mainly in Nepal and partly in Vietnam with the aim of contribution to effective control of these diseases.

(2) Subjects and their significance

After the discussions with health authorities in each country, following subjects were selected and collaborative studies are going on: antibiotic resistance, health care-associated and opportunistic infections, malaria, diarrheal diseases caused by emerging pathogens (Rotavirus, Cryptosporisium, etc.), and dual burden of infectious diseases and non-communicable diseases. Necessities of conducting studies from the viewpoint of emerging health priorities for each subject are as follows:

- Antibiotic resistance: Bacterial resistance to antibiotics is increasing however studies about the spread of multi-drug resistant bacteria in developing countries along with measures to address such situation is still limited. To provide effective guidance for control, identification of the fact and analysis of the causative factors are needed.

- Antibiotic resistance: Bacterial resistance to antibiotics is increasing however studies about the spread of multi-drug resistant bacteria in developing countries along with measures to address such situation is still limited. To provide effective guidance for control, identification of the fact and analysis of the causative factors are needed.
- Health care-associated and opportunistic infections: These types of infections have been increasing even in developing countries alongside poor control measures, increase of compromised hosts, antibiotics abuse, application of modern technology (respirator, hemodializer, endoscope, etc.). However in general, these countries have a low awareness and control measures are not effectively done. To provide effective guidance for improvement, identification of the fact and analysis of the causative factors are needed.
- Malaria: Progress has been made in developing countries regarding this disease under the support of the Global Fund against AIDS, Tuberculosis and Malaria (GFATM). However, in Nepal and Vietnam (also in many other endemic countries) newly developed malaria-endemic areas have been reported, and the environment and social factors have been indicated as the leading cause. It is necessary to analyze these factors in order to take effective control measures against malaria.
- Diarrheal diseases caused by emerging pathogens: Diarrheal diseases are frequent in developing countries. In Nepal, diarrheal diseases caused by emerging pathogens such as Rotavirus, Cryptosporisium, Cyclospora are also frequently experienced at medical care settings. In order to conduct effective control, analysis of these pathogens is needed. (Basic research on the fact of these diseases and pathogens was carried out by this study group.)
- Dual burden of communicable diseases and non-communicable diseases: These have been raised as new problems in developing countries, and their detailed analysis is needed. To address this problem it is necessary to investigate the actual conditions of the issues and identify causative factors through basic studies.

(3) Major achievements

Major achievements of collaborative studies on these subjects up to now are shown below. These have been published in conference presentations, scientific papers and reports. Furthermore, through joint conferences (held in Kathmandu in January 2013, Nagasaki in October 2013, Kathmandu in December 2014) and a focal group discussions (held in Kathmandu in April 2014), study results have been shared with representatives of each country concerned (hospital personnel, WHO, Ministries of Health and Population, Embassy of Japan and JICA). Discussions have been held over the issues, and recommendations have been given based on study results.

- Factors related to successful measures against malaria and current issues were analyzed. The results are expected to serve as useful information that contributes to measures against malaria. Besides, our in-depth study revealed that in malaria endemic-areas, the distribution rate of bed-nets was low among poverty groups which did not receive aid from the government or other countries. Also, medical facilities were not properly utilized. These are considered to be important information in order to achieve universal health coverage.
- A high frequency of diarrheal diseases caused by emerging pathogens such as Rotavirus, Cryptosporisium, and Cyclospora in Nepal was indicated.
- In medical settings in Nepal, a significant growth in drug resistance of gram negative bacilli, which cause nosocomial infections, was clearly observed. (Multiple drug resistant bacteria with strong resistance to Carbapenem and Amynoglycoside were isolated.)
- The following new findings were obtained by analyzing nosocomial pathogens:
  - The new variant of New Delhi methalo-β-lactamase producers were identified from Escherichia coli and named NDM-8 and NDM-12 respectively (the first case in the world).
  - "AAC (6')-Ial" gene was identified for the first time in Serratia marcescens,
  - "AAC (6')-Iak" gene in Strenotrophomonas martophilia was identified (the first case in Nepal).
  - Providencia rettgeri producing NDM-1 Metallo-β-Lactamase and ArmA 16S rRNA
  - Methylase was detected (the second case in the world).
  - The new variant of New Delhi methalo-β-lactamase producers were identified from Escherichia coli in 2015 and named NDM-13.

✓ The following new findings regarding the epidemiology of extended-spectrum β-lactamase (ESBL)-producing E. coli were obtained: Patients infected with ESBL-producing E. coli were significantly younger than patients in Japan. Among them, infection rate was high in pregnant women. Moreover, E. coli 025b-ST131 clone, which is of growing global concern, was shown to have a high frequency in Nepal.

✓ A high rate of double burden of diabetes and tuberculosis was discovered. Furthermore, a trend toward an especially high dual burden rate of both diabetes and tuberculosis was seen among patients over the age of 40. From the above, the possibility of clinics for non-communicable diseases (NCD) and those for tuberculosis may be able to collaborate in early diagnosis of their counterpart illness.

Discovery of NDM variants

NDM variants	Organism and country	References
NDM-1	*E. coli and K. pneumoniae* (Sweden)	Yong D. et. al., 2009
NDM-2	*Acinetobactor baumanii* (Egypt)	Kaase M. et all., 2011
NDM-3	*E. coli* (Australia)	Rogers B.A. et. al., 2013
NDM-4	*E. coli* (India)	Nordmann P. et. al., 2012
NDM-5	*E. coli* (United Kingdom)	Hornsey M. et. al., 2011
NDM-6	*E. coli* (New Zealand)	Williamson D.A. et. al. 2012
NDM-7	*E. coli* (Canada)	Accession no. JX262694
NDM-8	***E. coli* (Nepal)**	**Tada T. et. al., 2013**
NDM-9	*K. pneumoniae* (China)	Accession no. KC999080
NDM-10	*K. pneumoniae* (India)	Accession no. KF361506
NDM-11	*Assigned* (not known)	www.lahey.org/studies
NDM-12	***E. coli* (Nepal)**	**Tada T. et. al., 2014**
NDM-13	***E. coli* (Nepal)**	**Shrestha B.,Tada T. et. al., 2015**

Joint conferences and information sharing carried out in this study have contributed to increased awareness of medical personnel in each country concerned (especially in Nepal) regarding emerging health priorities of infectious diseases (multi-drug resistant bacteria, equity in the distribution of bed-nets for malaria prevention, and double burden of infectious diseases and non-communicable diseases).

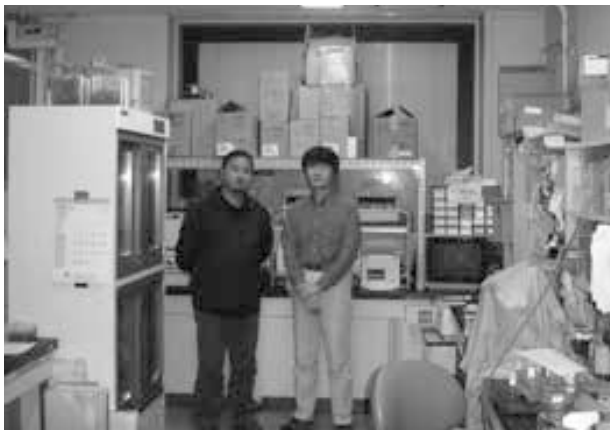
Opening ceremony of the 2nd Joint Conference (IOM, December 2014)

Especially in Nepal, the use of antibiotics, poor countermeasures for nosocomial infections, health knowledge of local residents, environmental change caused by development processes, population movement, and lifestyle habits including diet are considered to contribute to the increase and transition of infectious diseases (studied by this research group). It is important to seek measures that address these emerging priority issues taking account of such potential factors.

Our studies revealed the spread of multi-drug resistant bacteria in medical settings in Nepal. Measures must be urgently taken to address this situation, in which not only medical care but also countermeasures for nosocomial infections have great influence. To implement measures effectively, active intervention not only by medical facilities but also by governmental agencies, furthermore inter sectoral collaboration, is needed.

The significance of these diseases studied have not been fully recognized and active control measures have not been actively carried out. Our contribution to a heightened awareness by medical personnel of each country concerned can be considered as great progress. We aim to contribute to proper measures by further analyzing factors of growing issues regarding these infectious diseases (those studied by this study group).

Collaborative research on multi-drug resistant bacteria (IOM)



Collaborative research on multi-drug resistant bacteria (NCGM)

2-2 Outline of other researches

Research Grant: 25-5

The purpose of this study is to strengthen human resource development capacity at IOM, Tribhuvan University, by taking advantage of the overseas platform of the NCGM located at IOM (IOM-NCGM Research Collaboration Office). In the past, IOM received grant aid and technical cooperation projects from Japan's Official Development Assistance (ODA), and currently plays a leading and core role in Nepal's medical care. At IOM, studies have been carried out with the aim of sharing the benefits of medical care through human resource development, and to contribute to the improvement of medical standards in Nepal and the extension of ODA's achievements. In particular IOM focuses on improving management capacity at the IOM-NCGM Research Collaboration Office and human resource development in main areas of specialization (internal medicine and public health). In FY 2014, the following studies and activities were carried out:

1) Improvement of Management Capacity at the IOM-NCGM Research Collaboration Office:

The IOM-NCGM Research Collaboration Office was established in January, 2013. This well-maintained office contributes to the smooth implementation of study group activities. Management of the office and liaison with other study groups have been carried out in collaboration with Department of Microbiology, Institute of Medicine, Tribhuvan University. Of particular note is the well-established framework for cooperation and the continuing good relationship with departments at the Institute of Medicine, Tribhuvan University.

2) Improvements in Human Resource Development:

Planning, implementation and analysis (all or in part) of the following studies were carried out with the counterpart departments, contributing to human resource development by improving study and training capacity.

- Regarding “Knowledge, Attitude and Hand washing Practices among Health Care Professionals Working in Teaching Hospital, Kathmandu, Nepal”, the main study carried out by the Department of Study and Training, instructions were provided on data analysis and report preparation.
- Data analysis and report preparation of the “Study on malaria control and the equity in bed-net distribution” by the Department of Public Health were carried out with the counterpart department.
- In the “Study on pulmonary fibrosis using diagnostic imaging” carried out by Pulmonary Medicine, support was given regarding radiologic interpretation and data input, contributing to human resource development.
- Planning of “Training for Newly Employed Nurses on Measures for Nosocomial Infections” scheduled to be conducted at Nursing Department was carried out.
- It is noteworthy that IOM/TUTH contributed greatly to conduct medical care for the wounded people after the catastrophic earthquake which hit Nepal in April 2015

Research Grant: 25-7

Although important for the enhancement of quality in medical care, effective measures for nosocomial infection control have been limited in developing countries. The main purpose of this study group is to follow up JICA's “Training Course for Specialists of Nosocomial Infection Control and Prevention in Developing Countries” held at NCGM. This study includes Nepal, where such training has not been given up to now. In the study in Nepal, examination of actual conditions of measures for nosocomial infections, and analysis of problems was carried out, followed by important advice regarding improvement based on the obtained results and monitoring of the progress of improvement. In other words, the study in Nepal has

a meaning of a negative control in contrast to the countries where training has already been provided by the above JICA's training course.

In 2015, the following two surveys were carried out, followed by some advice for improvement. KAP Study on Medical Personnel regarding Nosocomial Infection Control.

This study was carried out with an aim to assess the level of awareness and actual practice of "hand-washing", which is a basic prevention method of nosocomial infection control among medical personnel, and then use the obtained results for improvement.

The study indicated that doctors at TUTH have a relatively good knowledge regarding hand-washing, but do not follow it in actual practice.

A large number of nurses and laboratory technicians lack knowledge, and have a low degree of hand-washing in actual practice prior to contact with patients. It is important to provide improved training for medical staff regarding nosocomial infection control and prevention, which are mainly based on standard precautions.

3. Outline of each research

Table 2 shows overview of the collaborative researches between NCGM and IOM. Progress status of each research (No. 1-7) is described on pages 28-42. We have already obtained some results, and these results were published at medical conferences and on scientific papers as shown on pages 43-46 (papers published in 2015 were put in full paper).

Among these researches it is particularly outstanding that new strains of New Delhi metalo-β-lactactamase producer were identified among the nosocomial infection cases and named NDM-8, 12 and 13.

Table 2 Collaborative researches in NCGM-IOM Collaboration Center, Nepal

No.	Chief Researchers in Japan and Nepal	Affiliation in Nepal	Subject	Source of fund
1	• Hiroshi Ohara (Bureau of International Medical Cooperation, NCGM) • Jeevan B Sherchand (Dept. of Public Health, Insitutie of Meidicne, Tribhuvan University)	• Institute of Medicine, Tribhuvan University • Faculty of Medicine, Kathandu University	Assessment of the interface between malaria control program and health system strengthening	24-5*
2	• Teruo Kirikae (Research Institute,NCGM) • Bharat M. Pokhrel (Dept. of Microbiology, Institute of Medicine, Tribhuvan University)	• Institute of Medicine, Tribhuvan University	Molecular epidemiology of nosocomial pathogens in developing countries	24-5*
3	• Norio Ohmagari, Kayoko Hayakawa (Disease Control and Prevention Center, NCGM) • Jatan Sherchan (School of Medicine, Kathmandu University)	• School of Medicine, Kathmandu University	Evaluation of changing epidemiology of infectious diseases in a developing country: The role of healthcare associated opportunistic infections	24-5*
4	• Naohiko Masaki (The Research Center for Hepatitis and Immunology, NCGM) • Pradeep K. Shrestha(Dept. of Internal Medicine, Teaching Hospital, Tribhuvan University)	• Institute of Medicine, Tribhuvan University	The survey of the present status of viral hepatitis treatment in Nepal	§
5	• Hiroshi Ohara (Bureau of International Medical Cooperation, NCGM) • Bharat M. Pokhrel (Dept. of Microbiology, Institute of Medicine, Tribhuvan University, Nepal)	• Institute of Medicine, Tribhuvan University	Fact-finding survey of nosocomial infection control in major hospitals in Nepal and discussion on effective improvement plans	25-7*
6	• Hiroshi Ohara (Bureau of International, Medical Cooperation, NCGM) • Bharat M. Pokhrel (Dept. of Microbiology, Institute of Medicine, Tribhuvan University)	• Institute of Medicine, Tribhuvan University, • School of Meicine, Kathmandu University	Studies on enhancement of human resource development utilizing the IOM-NCGM Collaboration Center - taking into consideration of the dissemination of the benefits of ODA project	25-5*
7	• Chieko Matsubara(Bureau of International Medical Cooperration, NCGM) • Bharat M. Pokhrel (Dept. of Microbiology, Institute of Medicine, Tribhuvan University)	• Institute of Medicine, Tribhuvan University, • School of Meicine, Kathmandu University	Fact-finding study of antibiotic stewardship programs in Nepali Hospitals	27-4*

* grants from the International Health Cooperation Research, a grant from the Ministry of Health, Labor and Welfare of Japan

§ Grant of Japan Society for the Promotion of Science : Scientific Research (B) (Overseas Academic Research) to NM

Research No.1

1.	Title(in English)	Assessment of the interface between malaria control program and health system strengthening
2.	Title(in Japanese)	マラリア対策とヘルスシステム強化に関する研究
3.	Main researcher	Hiroshi Ohara (Bureau of International Medical Cooperation, National Center for Global Health and Medicine)
4.	Co-Researcher(s)	Jeevan B. Sherchand (Dept. of Public Health, Institute of Medicine, Tribhuvan University, Nepal) Jatan B. Sherchan (Dept. of Microbiology, Faculty of Medicine, Kathmandu University)
5.	Resource of fund	Grants of National Center for Global Health and Medicine (24-5)
6.	Affiliation(s) in Nepal	Department of Public Health, Institute of Medicine, Tribhuvan University,
7.	Period of the research	January 2012- March 2016
8.	Publications in FY 2015	Ohara H, Sherchand JB, Pokhrel BM, et al. Assessment of health systems in relation to interface between malaria control programs and health system strengthening: comparative study between Nepal and Viet Nam. J Inst Med, 37(1): 11-20, 2015. Ohara H, Sherchand JB, Vu HN et al. Assessment of malaria control programs in relation to general health systems with special reference to equity in bed net use. NCGM report, available at: http://ncgmimcj.ecnet.jp/HP/library/research-_doc/ncgm_report_oct 2013.pdf
9.	Summary:	

Malaria has been a high priority issue in many tropical and sub-tropical countries. In order to implement malaria control program effectively, it is crucial to utilize health system effectively. In this study, interactions between malaria control program and health system strengthening was assessed.

The studies were conducted in Nepal and Vietnam with the methods of key informant interviews, investigation in malaria endemic areas and document review. As retrospective study, encountered challenges in malaria control and interventions for them were analyzed from the viewpoint of interactions between disease specific program and general health system using the 6 Building Blocks of Health System Strengthening of WHO (Leadership and Governance, Service delivery, Workforce, Information system, Medical products and technologies, and Financing). In addition, current challenges in malaria control were identified and possible interventions were discussed.

In Nepal, malaria was showing high morbidity and mortality rate until the middle of 1990s, however thereafter it decreased remarkably due to the effective control program. Leading factors contributed to the successful control were identified as the best practices.

The followings were recognized as leading current challenges in malaria control in Nepal: 1) Increase of malaria in some areas associated with population movement, 2) Shortage of health manpower in remote areas, 3) Poorly developed reporting system from the private health sector, 4) Difficulty in treatment due to increasing resistance of P. falciparum to anti- malaria drugs, 5) Low incentive for health workers, 6) existence of inequity of bednets distribution.

	In Vietnam, leading good practices included: 1)Strong government commitment for malaria control, 2)National strategy for rural development and intensified education for residents, 3)Effective vertical system from national to village level for malaria surveillance and service delivery, 4)Domestic antimalarial production and high coverage of control measures, 5)Strengthening the capacity of health workers along with mobilization of mass organizations, and 6)Support from international organizations. Effective implementation under the strong leadership of the governments utilizing the existing health system was outstanding in both countries. Besides, strengthening of the vertical health program appeared to have a good impact on the general health system, particularly at the primary level. We made an in-depth study in 4 districts in Terai areas in Nepal with the aim to examine variation in utilization of bednets by socioecomomic groups and inequities in access to malaria control services. This study revealed the wider disparity and pro-rich inequities in ownership of bednets. In area without bednet intervention, ownership was significantly higher in the rich households. There was significant variation in bednet ownership across caste/ ethnic groups. Disparity in ownership between the poorest and richest group appeared to be smaller in area with bednet intervention and people equally use bednets irrespective of caste and ethnic background. Free mass distribution of bednets allowed equitable ownership and reduce the inequality in usage of bednets across socioecomomic groups. The results suggested that if provided freely, bednet distribution program will be an important opportunity to reduce socioecomomic inequity in usage by allowing equitable ownership among the households of malaria risk area.

Research No.2

1.	Title (in English)	Molecular epidemiology of nosocomial pathogens in developing countries
2.	Title (in Japanese)	開発途上国の医療機関で分離される多剤耐性菌の推移に関する研究
3.	Main researcher	Teruo Kirikae (Department of Infectious Diseases, Research Institute, National Center for Global Health and Medicine)
4.	Co-Researcher(s)	Bharat M. Pokhrel (Dept. of Microbiology, Institute of Medicine, Tribhuvan University, Nepal)
5.	Resource of fund	Grants of National Center for Global Health and Medicine (24-5)
6.	Affiliation(s) in Nepal	Department of Microbiology, Institute of Medicine, Tribhuban University,
7.	Period of the research	April 2012- March 2015
8.	Publications In FY 2015	1. Int J Antimicrob Agents. 2015, 46(5):526-531 2. Microb Drug Resist. 2015, in press 3. Antimicrob Agents Chemother. 2015, 59(9):5847-5850

9. Summary:

Emergenece of multidrug-resistant pathogens has become one of the most serious problems in medical settings worldwide. There are serious concerns about dissemination of multidrug-resistant nosocomial pathogens in Nepal.

We started a study project of drug resistant pathogens isolated from inpatients hospitalized in Tribuvan University Teaching Hospital, Kathomandu, Nepal, in collaboration between Department of Microbiology, Institute of Medicine, Tribhuvan University and National Center for Global Health and Medicine from April 2012. Professor Dr. Bharat M. Pokhrel, his colleagues and his students obtained a total of 308 Gram-negative isolates by November 2014, including *Acinetobactor baumannii, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Providencia rettgeri, Serratia marcesces and Stenotrophomonas maltophilia* isolates.

Of *E. coli* clinical isolates, we detected a novel NDM-type metallo-β-lactamase variant, NDM-13. A novel New Delhi metallo-β-lactamase, NDM-13, was identified in a carbapenem-resistant Escherichia coli clinical isolate obtained from the urine of a patient in Nepal. The enzymatic activity of NDM-13 against β-lactams was similar to that of NDM-1. However, NDM-13 displayed significantly higher k cat/Km ratios for cefotaxime. The genetic environment of bla NDM-13 was determined to be *tnpA-IS30-bla*$_{NDM-13}$*-ble*$_{MBL}$*-trpF-dsbC-cutA-groES-groL,* with *bla*$_{NDM-13}$ located within the chromosome.

Of *S. marcescens* clinical isolates, we detected a novel 6′-*N*-aminoglycoside acetyltransferase-encoding gene, *aac(6′)-Ial*. The encoded protein AAC(6′)-Ial has 146 amino acids, with 91.8% identity to the amino acid sequence of AAC(6′)-Ic in *S. marcescens* SM16 and 97.3% identity to the amino acid sequence of AAC(6′)-Ian in *S. marcescens* WW4. The minimum inhibitory concentrations of aminoglycosides for *E. coli* expressing AAC(6′)-Ial were similar to those for *E. coli* expressing AAC(6′)-Ic or AAC(6′)-Ian. Thin-layer chromatography showed that AAC(6′)-Ial, AAC(6′)-Ic, or AAC(6′)-Ian acetylated all the aminoglycosides tested, except for apramycin, gentamicin, and lividomycin. Kinetic assays revealed that AAC(6′)-Ial is a functional acetyltransferase against aminoglycosides. The *aac(6′)-Ial* gene was located on a chromosomal DNA.

To clarify the genetic and epidemiological properties of MDR *A. baumannii* strains isolated from a medical setting in Nepal, 246 Acinetobacter spp. isolates obtained from different patients were screened for MDR *A. baumannii* by antimicrobial disk susceptibility testing. Whole genomes of the MDR *A. baumannii* isolates were sequenced by

	MiSeq™ (Illumina), and the complete genome of one isolate (IOMTU433) was sequenced by PacBio RS II. Phylogenetic trees were constructed from single nucleotide polymorphism concatemers. Multilocus sequence types were deduced and drug resistance genes were identified. Of the 246 *Acinetobacter* spp. isolates, 122 (49.6%) were MDR *A. baumannii,* with the majority being resistant to aminoglycosides, carbapenems and fluoroquinolones but not to colistin and tigecycline. These isolates harboured the 16S rRNA methylase gene armA as well as bla_{NDM-1}, bla_{OXA-23} or bla_{OXA-58}. MDR *A. baumannii* isolates belonging to clonal complex 1 (CC1) and CC2 as well as a novel clonal complex (CC149) have spread throughout a medical setting in Nepal. The MDR isolates harboured genes encoding carbapenemases (OXA and NDM-1) and a 16S rRNA methylase (ArmA).

Research No.3

1.	Title(in English)	Evaluation of changing epidemiology of infectious diseases in a developing country: The role of healthcare associated opportunistic infections
2.	Title(in Japanese)	途上国における感染症の変貌と要因に関する研究 - 特に医療に関連した日和見感染に関する検討 -
3.	Main researcher	Norio Ohmagari (Disease Control and Prevention Center, National Center for Global Health and Medicine)
4.	Co-Researcher(s)	Jatan Sherchan (Department of Medical Microbiology, Kathmandu University, School of Medical Sciences, Nepal) Kayoko Hayakawa (Disease Control and Prevention Center, National Center for Global Health and Medicine) Maki Nagamatsu (Disease Control and Prevention Center, National Center for Global Health and Medicine) Tohru Miyoshi-Akiyama (Pathogenic Microbe Laboratory, Research Institute, National Center for Global Health and Medicine)
5.	Resource of fund	
6.	Affiliation(s) in Nepal	Department of Medical Microbiology, Kathmandu University, School of Medical Sciences
7.	Period of the research	September 2012- March 2015
8.	Publications in FY 2015	Clinical epidemiology and molecular analysis of extended spectrum-β-lactamase-producing Escherichia coli in Nepal: characteristics of sequence types 131 and 648. Sherchan JB, Hayakawa K, Miyoshi-Akiyama T, Ohmagari N, Kirikae T, Nagamatsu M, Tojo M, Ohara H, Sherchand JB, Tandukar S. Antimicrob Agents Chemother. 2015;59(6):3424-32.

9. Summary:

Recently, CTX-M-type extended-spectrum-β-lactamase (ESBL)-producing *Escherichia coli* strains have emerged worldwide. In particular, *E. coli* with O antigen type 25 (O25) and sequence type 131 (ST131), which is often associated with the CTX-M-15 ESBL, has been increasingly reported globally; however, epidemiology reports on ESBL-producing *E. coli* in Asia are limited.

Patients with clinical isolates of ESBL-producing *E. coli* in the Tribhuvan University teaching hospital in Kathmandu, Nepal, were included in this study. Whole-genome sequencing of the isolates was conducted to analyze multilocus sequence types, phylotypes, virulence genotypes, O25b-ST131 clones, and distribution of acquired drug resistance genes. During the study period, 105 patients with ESBL-producing *E. coli* isolation were identified, and the majority (90%) of these isolates were CTX-M-15 positive. The most dominant ST was ST131 (n = 54; 51.4%), followed by ST648 (n = 15; 14.3%). All ST131 isolates were identified as O25b-ST131 clones, subclone H30-Rx. Three ST groups (ST131, ST648, and non-ST131/648) were compared in further analyses. ST64isolates had a proportionally higher resistance to non-β-lactam antibiotics and featured drug-resistant genes more frequently than ST131 or non-ST131/648 isolates. ST131 possessed the most virulence genes, followed by ST648. The clinical characteristics were similar among groups. More than 38% of ESBL-producing *E. coli* isolates were

	from the outpatient clinic, and pregnant patients comprised 24% of ESBL-producing *E. coli* cases. We revealed that the high resistance of ESBL-producing *E. coli* to multiple classes of antibiotics in Nepal is driven mainly by CTX-M-producing ST131 and ST648. Their immense prevalence in the communities is a matter of great concern.

Research No.4

1.	Title(in English)	The survey of the present status of viral hepatitis treatment in Nepal
2.	Title(in Japanese)	ネパールにおけるウイルス肝炎治療に関する実態調査
3.	Main researcher	Naohiko Masaki, MD, PhD (The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine)
4.	Co-Researcher(s)	Pradeep K. Shrestha (Dept. of Internal Medicine, Teaching Hospital, Tribhuvan University)
5.	Resource of fund	Grant of Japan Society for the Promotion of Science : Scientific Research (B) (Overseas Academic Research) to NM
6.	Affiliation(s) in Nepal	Department of Internal Medicine, Teaching Hospital, Tribhuvan University
7.	Period of the research	April 2015-March 2018
8.	Publications in FY 2015	Masaki N, Shrestha PK, Nishimura S, Ito K, Sugiyama M, Mizokami M. Use of nucleoside analogs in patients with chronic hepatitis B in Nepal: A prospective cohort study in a single hospital. **Hepatol Res** 45(12):1163-1169, 2015.

9. Summary:

According to WHO estimate, there are globally 240 millions and 150 millions of hepatitis B virus (HBV) and hepatitis C virus (HCV) carriers, respectively. Especially, most of the carriers are residents of Asian and African developing countries suffering from poor socio-economical problems and unstable political affairs, where the increase in numbers of death due to liver cirrhosis and hepatocellular carcinoma has been a serious burden on public health. Of note is that the people in those areas have been obliged to be far from the benefit of recent progress in diagnostic and therapeutic modalities, which, on the other hand, are regarded as universal in the developed countries.

We had performed epidemiological survey of HBV and HCV from 2007 to 2012 in Asia (Vietnum, Thailand, Nepal, Philippins, Taiwan, China, Parkistan, Uzbekistan, Indonesia, Bangladesh) and in Africa (Egypt, Kenya), and reported that 1) relatively cheaper generic drugs of nucleoside analogs for HBV were produced and distributed in those Asian countries, 2) use of expensive interferons for HCV was confined to only wealthy people, except for Egypt where interferon therapy was introduced as national policy, 3) there were many countries where domestic determinations of genotypes as well as viral load of HBV or HCV, which are essential to diagnosis and treatment of chronic liver diseases, were not available, and finally 4) doctors' prescriptions were not always mandatory for the patients to buy the anti-viral agents at pharmacies in those developing countries, where health insurance systems are not established, resulting in low drug adherence.

Our new research project will attempt to clarify the present status of public health administration in charge of viral hepatitis as well as of use of recently introduced anti-viral agents in clinical settings, in Nepal.

The objectives are as follows:

1. Epidemiological studies of HBV or HCV carriers, including HIV co-infection,among general populations
2. Prevalence of viral genotypes and their chronological changes in patients with chronic hepatitis B and C
3. Use of recently introduced anti-viral agents (nucleoside analogs such as lamivudine, adefovir, entecavir, tenofovir for HBV; directly-acting anti-virals [inhibitors of NS3/4A, NS5A and NS5B] for HCV) and their virological effects, including domestic coverage of virological monitoring systems, quality and commercial cost of anti-viral agents, medical expenses for standard regimens

	4. Emerging rates of drug-resistant HBV or HCV in real clinical settings 5. Coverage of universal hepatitis B vaccination, and evaluation of its effect 6. Present status of HBV reactivation and the preventive strategies against it: prevalence of anti-HBs and anti-HBc in blood donors 7. Serial determinations of whole genome sequence of HBV and HCV by next generation sequencer (deep sequencing), if applicable 8. A questionnaire survey of public health administration in charge of viral hepatitis and of the Nepalese Society of Hepatology and Gastroenterology, would be scheduled in the fiscal year 2016. Our research project was already approved by the Ethical Committees, TUTH, Tribhuvan University, Nepal as well as National Center for Global Health and Medicine, Japan. Collection of patients' serum samples and clinical information have just started since December, 2015.

Research No.5

1.	Title(in English)	Fact-finding survey of nosocomial infection control in major hospitals in Nepal and planning of effective improvement
2.	Title(in Japanese)	ネパールの主要病院における院内感染対策の実情分析と効果的な改善策に関する検討
3.	Main researcher	Hiroshi Ohara, Chieko Matsubara (Bureau of International Medical Cooperation, National Center for Global Health and Medicine)
4.	Co-Researcher(s)	Pokhrel BM, Shrestha RK, Dahal RK, Mishra SK, Kattel HP, Rijal BP (Dept. of Microbiology, Institute of Medicine, Tribhuvan University, Nepal) Jeevan B. Sherchand (Dept. of Public Health, Institute of Medicine, Tribhuvan University, Nepal) Shreshta DL (Dept. of Nursing Management, Tribhuvan University, Nepal) Teruo Kirikae, Yumiko Haneishi (Bureau of International Medical Cooperation, National Center for Global Health and Medicine)
5.	Resource of fund	Grants of National Center for Global Health and Medicine (25-7, 27-4)
6.	Affiliation(s) in Nepal	Department of Microbiology, Institute of Medicine, Tribhuban University,
7.	Period of the research	September 2012- March 2016
8.	Publications in FY 2015	Oral presentation 88th General Assembly of the Japanese Society of Infectious Diseases, June 2014, Fukuoka, Japan Scientific Paper *J Inst Med* 2014, 36(3): 38-48.

9. Summary:

In developing countries, where the incidence of infectious diseases is high and environmental conditions of healthcare facilities are poor, nosocomial infections may frequently occur. Effective nosocomial infection control is crucial in the healthcare facilities of developing countries, but in actual fact, attention to it is still limited and control measures are not functioning well in many countries. This study has been conducted with the purpose to investigate the actual conditions of nosocomial infection control in Kathmandu City, Nepal as a basis for the possible contribution to its improvement.

1. Fact-finding survey of nosocomial infection control in hospitals in Kathmandu, Nepal- a basis for improvement

The actual condition of nosocomial infection control were examined at the TUTH and five other referral hospitals in Kathmandu City with the method of questionnaire survey and interview. The obtained results were compared with the results of the last survey (2012-2013).

Both the frequency of the meetings of nosocomial infection control committee and the frequency of ICT rounds by infection control teams increased. Also, some improvement in the monitoring of causative agents and in the information provision system for clinical settings were recognized. Training for newly employed nurses on nosocomial infections control was enhanced and provided to all newly hired nurses in TUTH. Issues to be

addressed included improving the quality of the control system and training program, providing training to a wider range of staff members, improving waste disposal system, updating manuals, etc.

These findings clearly reflect that there is a need of further improvement of nosocomial infection control program in the hospitals of capital. Moreover, nosocomial infection control program should also have aim to estimate mortality, morbidity, additional financial burden and length of stay in the hospital due to nosocomial infection for public awareness.

1) KAP Study on Medical Personnel regarding Nosocomial Infection Control

This study was carried out with the aim to assess the level of awareness and actual practice of “hand-washing", which is a basic prevention method of nosocomial infection control among medical personnel, and then use the obtained results for improvement. 163 medical personnel (doctors, nurses and laboratory technicians) at Tribhuvan University Teaching Hospital (TUTH) were subjects of this study. A questionnaire, direct observation at important departments, and discussions were used. The following results were obtained

Knowledge: 74.1% of doctors had an accurate knowledge of hand-washing (significance, timing, methods and effects), while only 19.7% of nurses and 50.0% of laboratory technicians had such knowledge.

Attitude: 83.3% of laboratory technicians, 59.3% of nurses and 29.6% of doctors considered that they had an accurate knowledge. Regarding motive of hand-washing, fear for infections during medical practices accounted for a relatively high proportion (55.5% in doctors). Reasons given for not practicing hand-washing as instructed were: “I was busy” (46.0%), “I thought it wasn’t necessary because I wore gloves” (33.1%), “Something urgent came up” (11.7%). 38.7% answered that hand-washing is important. Poor levels of hand-washing practice among newly hired personnel and the importance of education for newly hired staff members were indicated.

Practice: The following results were obtained regarding the degree of hand-washing in actual practice: “Prior to contact with patients” (55.8%), “After contact with patients” (97.5%), and “At the end of work" (96.1%). Practice levels were higher in nurses than in doctors. Drying and wiping methods were: hand dryer (52.7%), shared-use towels (22.7%), personal towel or handkerchief (12.3%), and natural drying (3.7%).

The study indicated that doctors at TUTH have a relatively good knowledge regarding hand-washing, but do not follow it in actual practice. A large number of nurses and laboratory technicians lack knowledge, and have a low degree of hand-washing in actual practice prior to contact with patients. It is important to provide improved training for medical staff regarding nosocomial infection control and prevention, which are mainly based on standard precautions.

Research No.6

1.	Title(in English)	Studies on enhancement of human resource development utilizing the IOM-NCGM Collaboration Center — taking into consideration of the dissemination of the benefits of ODA project
2.	Title(in Japanese)	ネパール拠点を活用した人材育成能力強化に関する研究 - ODA プロジェクトの成果拡大を視野に入れて
3.	Main researcher	Hiroshi Ohara (Bureau of International Medical Cooperation, National Center for Global Health and Medicine)
4.	Co-Researcher(s)	Bharat M. Pokhrel Jeevan B. Sherchand (Dept. of Public Health, Institute of Medicine, Tribhuvan University, Nepal) Karbir N. Yogi (Dept. of Pulmonology, TUTH) Mitsuhiro Kamimura (Dept. of Pulmonology, National Disaster Medical Center) Pradeep Shrestha (Dept. of Internal Medicine, TUTH)
5.	Resource of fund	Grants of National Center for Global Health and Medicine (25-5)
6.	Affiliation(s) in Nepal	Department of Public Health, Institute of Medicine, Tribhuvan University,
7.	Period of the research	April 2013- March 2015
8.	Publications in FY 2015	Annual Report of the IOM-NCGM Collaboration Office in 2015 was made.

9. Summary:

Collaborative researches are being conducted in Nepal utilizing the IOM-NCGM Collaboration Office as a base. Strengthening of the base functions is essential to conduct researches smoothly. This study was started to strengthen the function of the IOM-NCGM Collaboration Office aiming at smooth implementation of researches and human resource development.

In FY 2014 the following activities were conducted.

1. Strengthening of management capacity of IOM-NCGM Collaboration Office:
   Management system to conduct researches/activities was clarified and some instructions were conducted to local staff. Management of the office and liaison with other study groups have been carried out.
2. Comparative study on outcomes of Medical Education Project and current IOM/TUTH:
   The overview of the Medical Education Project, which was implemented as Official Development Assistance by JICA from 1980 to 1996, was summarized and the situations of IOM/TUTH at the end of the project and the present time were compared.
3. Improvements in Human Resource Development:
   Planning, implementation and analysis (all or in part) of the following studies were carried out with the counterpart departments, contributing to human resource development by improving study and training capacity.

	➢ Regarding “Knowledge, Attitude and Hand washing Practices among Health Care Professionals Working in Teaching Hospital, Kathmandu, Nepal”, the main study carried out by the Department of Study and Training, instructions were provided on data analysis and report preparation. ➢ Data analysis and report preparation of the “Study on Malaria Control and the Equity in Bed-Net Distribution” by the Department of Public Health were carried out with the counterpart departments. ➢ In the “Study on Pulmonary Fibrosis using Diagnostic Imaging” carried out by Pulmonary Medicine, support was given regarding radiologic interpretation and data input, contributing to human resource development. ➢ Planning of “Training for Newly Employed Nurses on Measures for Nosocomial Infections” scheduled to be conducted at Nursing Department was carried out.

Research No.7

1.	Title(in English)	Fact-finding study of antibiotic stewardship programs in Nepali Hospitals
2.	Title(in Japanese)	ネパールの病院における抗生剤管理の現状調査
3.	Main researcher	Chieko Matsubara, Hiroshi Ohara (Bureau of International Medical Cooperation, National Center for Global Health and Medicine)
4.	Co-Researcher(s)	Jeevan Bahadur Sherchand (Dept. of Public Health, Institute of Medicine, Tribhuvan University, Nepal) Bharat Mani Pokhrel (Dept. of Public Health, Institute of Medicine, Tribhuvan University, Nepal) Jatan Sherchan (Dept. of Microbiology, Faculty of Medicine, Kathmandu University)
5.	Resource of fund	Grants of National Center for Global Health and Medicine (25-5, 27-4)
6.	Affiliation(s) in Nepal	Department of Public Health, Institute of Medicine, Tribhuvan University,
7.	Period of the research	December 2015
8.	Publications in FY 2015	In preparation for FY 2016
9.	Summary: Antibiotic resistance is a global emerging threat in public health issue. New multiple drug-resistant pathogens were reported in Nepal, however, few studies is publicly available to evaluate antibiotic stewardship programs in Nepali hospitals. The objective of this study is to investigate the actual conditions of antibiotic stewardship programs in Kathmandu City, Nepal. We are going to conduct a questionnaire survey and site visits at hospitals in Kathmandu City. Preferably, data collection will cover various levels of hospitals (for example, academic hospitals and non-academic hospitals). Potential interviewees are a director of antibiotic team and a responsible person of antibiotics in the hospital, e.g. a medical doctor, a pharmacist, and a microbiologist. We hope to conduct this study for 1-2 weeks in the middle of December, 2015. Major expected outcomes are to clarify the type of interventions implemented in hospitals for antibiotic stewardship programs. The outcomes would reveal the actual conditions of antibiotic stewardship programs and would contribute as a basis to decide/consider future plans for improvement in antibiotic stewardship at Nepali hospitals. In total 27 health facilities participated in this survey (Primary level: 6 facilities, Secondary level: 2 facilities, and Tertiary level: 19 facilities) and two of them skipped some questions. Among 21 secondary and tertiary hospitals, 6 facilities provided Infectious Control Team (ICT) and 2 facilities provided antibiotics committee. The 19 among 25 health facilities agreed that antibiotic resistance became an important issue. Among 17 secondary and tertiary hospitals, 15 facilities offered 24-hours laboratory services and conducted at least one blood-culture test every day. Further among 17 secondary and tertiary hospitals, 14 facilities offered 24-hours pharmacy services.	

	The results suggested that intervention for antibiotic stewardship is necessary to be introduced as soon as possible. Most of hospitals started 24-hours service at microbiology department and pharmacy. Therefore, if the hospitals developed their hospital system to connect ICT, clinical departments, microbiology department, and pharmacy, they will ensure a successful use of antibiotic resistance information as a shared information to proper use of antibiotic resistance, which will contribute to reduce antibiotic resistance. Further intervention is called for antibiotic stewardship in Primary Health Centers and Health Posts, where there are no microbiologists and pharmacitis.

Publication of scientific papers

Scientific papers which were published on international journals as results of collaborative studies during the period from 2012 to 2015 are shown below.

Full papers published in 2015 are put in this attachment.

Table 3 List of papers published on international journals (papers in press are included) 2012-2015: Achievement in NCGM-IOM Collaboration Office

◎ Full papers are put in this annual report ○ Only abstract is put in this annual report

	Papers	
1	Sherchan JB, Ohara H, Sakurada S, Basnet A, Tandukar S, Sherchand JB, Bam DS. Enteric opportunistic parasitic infections among HIV-seropositive patients in Kathmandu, Nepal. Kathmandu Univ Med J 2012; 38(2):14-17.	○
2	Sherchan JB, Ohara H, Sherchand JB, Tandukar S, Sakurada S, Gurung B, Ansari S, Rijal BP, Pokhrel BM. Molecular evidence based hospital acquired rotavirus gastroenteritis in Nepal. Prime J Mirobobiol Res 2011; 1(2): 16-21.	○
3	Shrestha S, Chaudhari R, Karmacharya S, Kattel HP, Mishra SK, Dahal RK, Bam N, Banjade N, Rijal BP, Sherchand JB, Ohara H, Koirala J, Pokhrel BM. Prevalence of nosocomial lower respiratory tract infections caused by multi drug resistant pathogens. J Inst Med 2012; 33(2): 7-14.	○
4	Tada T, Miyoshi-Akiyama T, Dahal RK, Sah MK, Ohara H, Shimada K, Kirikae T, Pokhrel BM. NDM-8 metallo-β-lactamase in a multidrug-resistant Escherichia coli strain isolated in Nepal. Antimicrob Agents Chemother 2013; 57(5): 2394-2396.	○
5	Shrestha RK, Dahal RK, Mishra SK, Parajuli K, Rijal BP, Sherchand JB, Kirikae T, Ohara H, Pokhrel BM. Ventilator associaed pneumonia in tertiary care hospital, Maharajgunj, Kathmandu, Nepal. J Inst Med 2013; 35(3): 21-28.	○
6	Ohara H, PokhrelBM, DahalRK, Mishra SK, Kattel HP, Shrestha DL, Haneishi Y, Sherchand JB. Fact-finding survey of nosocomial infection control in hospitals in Kathmandu, Nepal and trial to improvement. Tropical Med Health 2013; 41:113-119.	○
7	Tada T, Miyoshi-Akiyama T, Dahal RK, Sah MK, Ohara H, Shimada K, Kirikae T, Pokhrel BM. NDM-1 metalloβ-lactamase and ArmA 16S rRNA methylase producing Providencia rettgeri clinical isolates in Nepal. BMC Infect Dis 2014; 14:56-60	○
8	Tada T, Miyoshi-Akiyama T, Dahal RK, Mishra SK, Ohara H, Shimada K, Kirikae T, Pokhrel BM. Dissemination of multidrug-resistant Klebsiella pneumoniae clinical isolates with various combinations of carbapenemases (NDM-1 and OXA-72) and 16S rRNA methylases (ArmA, RmtC and RmtF) in Nepal. Int J Antimicrob Agents 2014; 42(4):372-374.	○
9	Tada T, Shrestha B, Miyoshi-Akiyama T, Shimada K, Ohara H, Kirikae T, Pokhrel BM. NDN-12, a Novel New Delhi Metallo-β-Lactamase Variant from a Carbapenem-Resistant Escherichia coli Clinical Isolate in Nepal. Antimicrob Agents Chemother 2014; 58(10):6302-6305.	○

	Papers	
10	Tada T, Miyoshi-Akiyama T, Dahal RK, Shyam MK, Shimada K, Ohara H, Kirikae T, Pokhrel BM. Identification of a Novel 6'-N-Aminoglycoside Aetyltransferase, AAC(6')-Iak from a Multidrug-resistant Clinical Isolates of Stenotrophomonas maltophilia. Antimicrob Agents Chemother 2014; 58(10):6324-6327.	○
11	Sah MK, Mishra SK, Ohara H, Kirikae T, Sherchand JB, Rijal BP, Pokhrel BM. Nosocomial bacterial infection and Antimicrobial resistant Pattern in a tertiary Care hospital in Nepal. J Inst Med 2014; 36(3): 38-48.	○
12	Sherchan JB, Hayakawa K, Miyoshi-Akiyama T, Ohmagari N, Kirikae T, Nagamatsu M, Tojo M, Ohara H, Sherchand JB, Tandukar S. Clinical epidemiology and molecular analysis of extended-spectrum β-lactamase (ESBL)-producing Escherichia coli in Nepal: Characteristics of sequence types 131 and 648. Antimicrob Agents Chemother 2015; 59(6): 3424-3432.	◎
13	Ohara H, Sherchand JB, Pokhrel BM, Hirayama T, Nam VH, Sherchan JB. Assessment of health systems in relation to interface between malaria control programs and health system strengthening: Comparative study between Nepal and Viet Nam. J Inst Med 2015; 37(1): 11-20.	◎
14	Shrestha S, Tada T, Miyoshi-Akiyama T, Ohara H, Shimada K, Satou K, Teruya K, Nakano K, Shiroma A, Sherchand JB, Rijal BP, Hirano T, Kirikae T, Pokhrel BM. Molecular epidemiology of multidrug-resistant Acinetobacter baumannii isolate in a university hospital in Nepal reveals the emergence of a novel epidemic clonal lineage. Int J Antimicrob Agents 2015; 46: 526-531.	◎
15	Shrestha B, Tada T, Miyoshi-Akiyama T, Shimada K, Ohara H, Kirikae T, Pokhrel BM. Identification of a novel NDM variant, NDM-13, from a multidrug-resistant Escherichia coli clinical isolate in Nepal. Antimicrob Agents Chemother 2015; 59(9): 5847-5850.	◎
16	Tada T, Miyoshi-Akiyama T, Shimada K, Dahal RK, Mishra SK, Ohara H, Kirikae T, Pokhrel BM. A novel 6'-N aminoglycoside acetyltransferase, AA(6')-Iai, from a clinical isolate of Serratia marcescens. Microbial Drug Resist 2015. (in press)	◎
17	Masaki N, Shrestha PK, Nishimura S, Ito K, Sugiyama M, Mizokami M. Use of nucleoside analogs in patients with chronic hepatitis B in Nepal: a prospective cohort study in a single hospital. Hepatology Research 2015; 45: 1163-1169.	◎
18	Sherchand JB. Earthquake disaster-associated health effects and the need for improved preventive measures. J Inst Med 2015; 37(1): 1-3.	◎

KATHMANDU UNIVERSITY MEDICAL JOURNAL

Enteric Opportunistic Parasitic Infections Among HIV-Seropositive Patients in Kathmandu, Nepal

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Citation

Sherchan JB, Ohara H, Sakurada S, Basnet A3, Tandukar S, Sherchand JB, et al. Enteric Opportunistic Parasitic Infections Among HIV-seropositive Patients in Kathmandu, Nepal. *Kathmandu Univ Med J* 2012;38(2):14-17.

ABSTRACT

Background

Enteric opportunistic parasitic infections are the major source of diarrheal disease in developing countries mainly in Human Immunodeficiency virus (HIV) infected patients.

Objective

The study was to detect enteric parasites causing diarrhea and their association with immune status in HIV-seropositive patients.

Methods

The present study was conducted in Dirgh-Jeevan Health Care Research Center and Tribhuvan University Teaching Hospital, Public Health Research Laboratory, Kathmandu, Nepal between June 2010 and May 2011 involving 146 Human Immunodeficiency virus (HIV) positive patients. Serostatus from these patients were detected by Enzyme Linked Immunosorbent assay. CD4+ T cell counts were done by flow cytometry. Stool was examined for enteric parasites by microscopy with special staining methods.

Results

A total of 146 HIV sero-positive patients with and without diarrhea age between 20 to 45 years were included in the study. Of the 146 patients, the protozoan parasitic infection was found in 30.13% (44/146). Out of 146 patients, 78 had diarrhea in which parasitic infection was 39 (50%) and 7.35% (5/68) protozoal parasites positive cases did not have diarrhea. A significant difference ($p<0.05$) was observed in the level of infection of intestinal protozoan between the HIV seropositive with diarrhea and HIV-seropositive without diarrhea. Out of 43 patients whose CD4+ T cells were <200/µl, 29 (67.4%) had opportunistic parasitic infection whereas out of 103 patients whose CD4+ T cells were ≥200/µl, only 15 (14.56%) had opportunistic parasitic infection ($P < 0.05$).

Conclusion

Enteric opportunistic parasitic infections were detected in 30.1% among HIV-seropositive patients and low CD4+ T count indicated high enteric opportunistic infection. Early detection of enteric parasitic infections will help in the management and to improve the quality of life for HIV-infected individuals.

KEYWORDS

Diarrhea, HIV, Opportunistic parasites

INTRODUCTION

Enteric opportunistic parasitic infections are major source of diarrheal disease in developing countries mainly in HIV infected patients. The progressive decline and ultimate destruction of immune system functions, which are characteristic for AIDS, usually result in morbidity and ultimately death due to opportunistic bacterial, viral, fungi and parasitic infections.[1] Gastrointestinal infections are very common in patients with HIV infection or AIDS.[2] Diarrhea is a common clinical presentation of these infections. Reports indicate that diarrhea occurs in 30-60 % of AIDS patients in developed countries and in about 90 per cent of AIDS patients in developing countries.[3] The presence of

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Full Length Research

Molecular evidence based hospital acquired rotavirus gastroenteritis in Nepal

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Rotavirus is the major pathogens of community acquired acute gastroenteritis in children, but their role in hospital acquired gastroenteritis is not fully understood. The aim of the study was to assess the incidence of hospital acquired gastroenteritis and molecular evidence among hospitalized children less than 5 years of age. A total of 154 children with hospital acquired acute gastroenteritis in children's hospital of Kathmandu were enrolled between January and December 2010. Acute gastroenteritis was classified as hospital acquired infection if diarrhea developed in 48 hours or more after admission. The incidence of hospital acquired infection due to rotavirus was 31.2% (48/ 154) by ELISA. The distribution of rotavirus genotypes G and P, serotype G12 represented 48% of rotavirus strains characterized by reverse transcription-polymerase chain reaction genotyping during the study, and was associated with P-types P[6], P[8] and P[4]. Further, a total of nine G/P type combination were identified, with G12 P [6] 30% being the most commonly detected rotavirus strain type. Most of the children who had hospital acquired rotavirus gastroenteritis found symptoms of diarrhea, vomiting, fever, poor sucking and dehydration. Additional findings showed that 2% cases of rotavirus co-infection with bacterial pathogens of *Esch coli* and *Shigella* species. The study revealed that G 12 and G12P [6] were found major genotypes causing hospital acquired rotavirus gastroenteritis in Nepal. Introduction of rotavirus vaccine along with strengthening hygienic measures could substantially reduce the incidence of hospital acquired acute gastroenteritis in children of Nepal.

Key word: Rotavirus, molecular, gastroenteritis, hospital acquired, Nepal

INTRODUCTION

Rotavirus is the major pathogen of community acquired acute gastroenteritis in children, but their role in hospital acquired gastroenteritis and molecular evidence is not fully understood. Although rotavirus is the most frequent cause of gastroenteritis in children under 5 years of age, but, the virus can cause severe diarrhea and dehydration, especially in children aged 6 to 24 months. In developing countries, acute gastroenteritis due to rotavirus infection causes the death of approximately 440,000 children every year (Parashar *et al.* 2009; Festini *et al.* 2010 and Parashar *et al.* 2006).The rotavirus genus of the *Reoviridae* family is very diverse, as it consists of different groups (A-G) and of different types based on the characteristics of the surface proteins VP7 (G = glycoprotein) and VP4 (P = protease-sensitive protein). To date, at least 23 G types and 31 P types of group A rotavirus, the group which most commonly infects humans, have been differentiated (Festini *et al.* 2010; Kang *et al.* 2006 and Ursu *et al.* 2009).

The virus is mainly transmitted by feco-oral route or by direct contact, but it can occasionally be transmitted through droplets. Since the virus is stable in the environment, transmission can occur through the ingestion of contaminated water and food, and through

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Prevalence of nosocomial lower respiratory tract infections caused by Multi- drug resistance pathologens

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Abstract

Introduction: Nosocomial infections caused by multi-drug resistant pathogens are major threat to the hospitalized patients. Extended spectrum beta-lactamase (ESBL) and metallo-beta-lactamase (MBL) producing bacterial strains causing hospital acquired lower respiratory tract infection are increasing in numbers. Only a limited number of studies related to MBL producers have been done in Nepal.

Objective: The goal of this study was to determine the etiology of nosocomial lower respiratory tract infections and to assess the current levels of antimicrobial resistance with special reference to ESBL and MBL producing bacterial strains.

Methods: A total of 100 specimens including sputum and endotracheal secretion from patients diagnosed of nosocomial lower respiratory tract infection were collected and processed according to the standard methodology. Combination disk method was done for the detection of ESBL and MBL producing isolates.

Results: Out of total 100 specimens, 87% was monomicrobial while the rest were polymicrobial. 96.5 % were gram negative while 3.5% were gram positive. All *E.coli, Klebsiella* spp and *S. aureus* were found to be MDR followed by *Acinetobacter* spp (97.2%) and *P. aeruginosa* (76.2%)

About 28.6 % of *E. coli*, 8.33% of *Klebsiella* spp and 2.4 % of *Pseudomonas aeruginosa* were ESBL producers. *Acinetobacter* spp. was not found to produce ESBL during the study. MBL was present in 17.4% of the gram negative isolates.

Conclusion: We found a high prevalence of MDR strains as a cause of nosocomial LRTI including significant proportions of ESBL and MBL producers. The rate of *Acinetobacter spp.*, including MBL producers, in our hospital setting was alarmingly high which prompts a special attention for the management of such patients as well as urgent need for implementation of infection control strategies.

Key words: MDR, LRTI, ESBL, MBL, nosocomial infection

Introduction

Nosocomial respiratory tract infections are major cause of excessive morbidity and mortality. Patients with serious underlying diseases have an especially high risk of acquiring these infections and that risk is magnified by exposure to respiratory therapy. Until recently, contaminated respiratory care devices were a major cause of infection, but procedures for the management of these devices have decreased their role substantially. Now aspiration of oropharyngeal flora appears to be responsible for most cases of bacterial respiratory infections. Therefore the techniques to alter the flora of the oropharynx and to diminish the risk of aspiration are important priorities for infection control. Exposure to intensive care units (ICUs) is also a major risk factor for nosocomial pulmonary infection and person to person spread of microorganisms within ICUs seems to be responsible for some of these infections[1].

Nosocomial pneumonia is the second most common infection after urinary tract infection and has the highest mortality rate amongst nosocomial infections. Nosocomial pneumonia accounts for 15% of all nosocomial infections and affects 0.5- 2.0% of hospitalized patient. The highest incidence rate was seen in ICU (15-20%) particularly in intubated patients on mechanical ventilation [2].

Almost three quarters of all antibiotic consumptions are for respiratory tract infections [3]. Beta-lactams remain a cornerstone for antimicrobial chemotherapy of a large number of bacterial infections, but their efficacy has been increasingly thwarted by dissemination of acquired resistance determinants among pathogenic bacteria [4]. The exposure of bacterial strains to a multitude of β-lactams has induced a dynamic and continuous production and mutation of β-lactamase in many bacteria, expanding their activity even against later

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NDM-8 Metallo-β-Lactamase in a Multidrug-Resistant *Escherichia coli* Strain Isolated in Nepal

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A novel metallo-β-lactamase, NDM-8, was identified in a multidrug-resistant *Escherichia coli* isolate, IOMTU11 (NCGM37), obtained from the respiratory tract of a patient in Nepal. The amino acid sequence of NDM-8 has substitutions at positions 130 (Asp to Gly) and 154 (Met to Leu) compared with NDM-1. NDM-8 showed enzymatic activities against β-lactams similar to those of NDM-1.

Metallo-β-lactamases (MBLs) produced by Gram-negative bacteria confer resistance to all β-lactams except monobactams (1). New Delhi metallo-β-lactamase-1 (NDM-1), a recently discovered MBL, was initially isolated from *Klebsiella pneumoniae* and *Escherichia coli* in 2008 in Sweden (2). Since then, NDM-1-producing members of the *Enterobacteriaceae* have been isolated in various parts of the world, including Australia, Bangladesh, Belgium, Canada, France, India, Japan, Kenya, the Netherlands, New Zealand, Pakistan, Singapore, Taiwan, and the United States (3, 4). In addition, isolates producing six NDM variants have been reported, including NDM-2-producing *Acinetobacter baumannii* strains from Egypt (5, 6), Israel (5), Germany (7), and the United Arab Emirates (8); an NDM-3-producing *E. coli* strain from Australia (accession no. JQ734687); an NDM-4-producing *E. coli* strain from India (9); an NDM-5-producing *E. coli* strain from the United Kingdom (10); an NDM-6-producing *E. coli* strain from New Zealand (11); and an NDM-7-producing *E. coli* strain from Canada (accession no. JX262694).

E. coli IOMTU11 (NCGM37) and *Pseudomonas aeruginosa* IOMTU9 (NCGM1841) were isolated from pus from a surgical site and from sputum of patients, respectively, in 2012 at Tribhuvan University Teaching Hospital in Kathmandu, Nepal. The isolates were phenotypically identified, and species identification was confirmed by 16S rRNA sequencing (12). MICs were determined using the microdilution method recommended by the Clinical and Laboratory Standards Institute (13). *E. coli* IOMTU11 was resistant to all antibiotics tested excepted fosfomycin (MIC, 4 μg/ml). The MICs of β-lactams are shown in Table 1, and those of other antibiotics were as follows: arbekacin, >1,024 μg/ml; amikacin, >1,024 μg/ml; colistin, 0.25 μg/ml; gentamicin, >1,024 μg/ml; and tigecycline, 0.5 μg/ml. MBL production was examined with an MBL Etest (Sysmex; bioMérieux Co., Marcy l'Etoile, France), with MICs of 256 μg/ml of imipenem and 2 μg/ml of imipenem-EDTA. PCR analysis for MBL genes (14, 15, 16) and 16S rRNA methylase genes (17) was performed. The isolates were positive for bla_{NDM} and *rmtB*. Sequence analysis showed that the *bla*NDM was a novel variant, and it was designated bla_{NDM-8}. Multilocus sequence typing (MLST) of IOMTU11 showed that it was ST101 (*Escherichia coli* MLST database [http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi.html]). *P. aeruginosa* IOMTU9 had bla_{NDM-1}, which was used as a reference gene.

The sequence of the bla_{NDM-8} gene showed mutations corresponding to two amino acid substitutions compared with bla_{NDM-1} (accession number JF798502). Analysis of the predicted amino acid sequence revealed two substitutions (D130G and M154L) compared with NDM-1, one substitution (D130G) compared with NDM-4, and one substitution (L88V) compared with NDM-5.

The bla_{NDM-8} and bla_{NDM-1} genes were cloned into the corresponding sites of pHSG398 (TaKaRa Bio, Shiga, Japan) with the primer set EcoRI-NDM-F (5′-GGGAATTCATGGAATTGCCCA ATATTATG-3′) and PstI-NDM-R (5′-AACTGCAGTCAGCGC AGCTTGTCGGCCAT-3′). *E. coli* DH5α was transformed with pHSG398-NDM-8 or pHSG398-NDM-1 to determine the MICs of β-lactams.

The open reading frames of NDM-1 and NDM-8 without signal peptide regions were cloned into the expression vector pQE2 (Qiagen, Tokyo, Japan) with the primer set SacI-NDM-F (5′-CCCC TCGAGCAGCAAATGGAAACTGGCGACCAACGGT-3′) and SalI-NDM-R (5′-CCCGAGCTCTCAGCGCAGCTTGTCGGCC ATGCGGGCC-3′). The plasmids were transformed into *E. coli* BL21-CodonPlus (DE3)-RIP (Agilent Technologies, Santa Clara, CA). The recombinant NDM proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose according to the manufacturer's instruction (Qiagen). His tags were removed by digestion with DAPase (Qiagen), and untagged proteins were purified by an additional passage over Ni-NTA agarose. The purities of NDM-1 and NDM-8 were over 90%, as estimated by SDS-PAGE. During the purification procedure, the presence of β-lactamase activity was monitored with nitrocefin (Oxoid Ltd., Basingstoke, United Kingdom). Initial hydrolysis rates were determined in 50 mM phosphate buffer (pH 7.0) at 25°C with a UV-visible spectrophotometer (V-530; Jasco, Tokyo, Japan). The K_m and k_{cat} values and the k_{cat}/K_m ratio were determined by analyzing β-lactam hydrolysis by use of the Lineweaver-Burk plot. Wavelengths and extinc-

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Original Article

Ventilator Associated Pneumonia in Tertiary Care Hospital, Maharajgunj, Kathmandu, Nepal

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Abstract

Introduction: Ventilator Associated Pneumonia (VAP) is the most common nosocomial infection among intensive care unit (ICU) patients and lack of much information in Nepal. So, the aim of this study was to determine prevalence and bacteriological profile of VAP with special reference to multi-drug resistant (MDR), Methicillin-resistant Staphylococcus aureus(MRSA),Metallo-β-Lactamase(MBL), Extended-Spectrum β-Lactamase(ESBL)-producing bacterial strains.

Methods: A total 150 tracheal specimens were studied during June 2011 to May 2012 at Department of Microbiology, TUTH as described by American Society for Microbiology (ASM). Combination disk method was done for the detection of ESBL and MBL producing isolates.

Results: Prevalence of VAP was found to be 34%. Acinetobacter calcoaceticus baumannii complex (44%) was the commonest isolate, followed by Klebsiella pneumoniae (22%), Pseudomonas aeruginosa (16%) and Staphylococcus aureus (12%). Among MDR Gram negative bacteria (GNB), 39% were MBL and 33% were ESBL-producers. All GNB (61) were sensitive to Polymyxin B and Colistinsulphate, whereas, 48% were found resistant to Carbapenems. Prevalence of MRSA was 75%, which were all sensitive to Vancomycin.

Conclusion: High prevalence of VAP, MDR along with MRSA or ESBL or MBL producing strains was found in the study. Thus, suitable control measures must be adopted to cope up this alarming situation with genetic characterization.

Key words: VAP, ICU, MDR, MRSA, ESBL, MBL.

Introduction

People with life-threatening injuries and illnesses need critical care and mechanical ventilation is must. It is often a life-saving intervention, but carries many potential complications, including pneumothorax, airway injury, alveolar damage, collapsed lung and ventilator-associated pneumonia.[1]

Ventilator-associated pneumonia (VAP) is defined as an episode of pneumonia in a patient who requires a device to assist or control respiration through a tracheostomy or endotracheal tube at the time of or within 48 hours before the onset of the infection.[2] Eighty-six percent of nosocomial pneumoni as are associated with mechanical ventilation.[3] This is associated with increases in morbidity and mortality, hospital length of stay, and costs.

In modern medical practice, extensive use of antibiotics have resulted in emergence and rapid dissemination of Multi drug resistant (MDR), Methicillin Resistant Staphylococcus aureus (MRSA), Extended-Spectrum β-Lactamase (ESBL) and Metallo-β-Lactamase (MBL) producing bacteria. Thus, their detection is crucial for the optimal treatment of patients and to control the spread of resistance.So this study is intended to address the issues regarding the prevalence of VAP, MDR, ESBL-, MBL-producing bacterial isolates, and MRSA.

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Original article

Fact-finding Survey of Nosocomial Infection Control in Hospitals in Kathmandu, Nepal—A Basis for Improvement

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Abstract: The purpose of this study was to investigate the actual conditions of nosocomial infection control in Kathmandu City, Nepal as a basis for the possible contribution to its improvement. The survey was conducted at 17 hospitals and the methods included a questionnaire, site visits and interviews. Nine hospitals had manuals on nosocomial infection control, and seven had an infection control committee (ICC). The number of hospitals that met the required amount of personal protective equipment preparation was as follows: gowns (13), gloves (13), surgical masks (12). Six hospitals had carried out in-service training over the past one year, but seven hospitals responded that no staff had been trained. Eight hospitals were conducting surveillance based on the results of bacteriological testing. The major problems included inadequate management of ICC, insufficient training opportunities for hospital staff, and lack of essential equipment. Moreover, increasing bacterial resistance to antibiotics was recognized as a growing issue. In comparison with the results conducted in 2003 targeting five governmental hospitals, a steady improvement was observed, but further improvements are needed in terms of the provision of high quality medical care. Particularly, dissemination of appropriate manuals, enhancement of basic techniques, and strengthening of the infection control system should be given priority.
Key words: Fact finding survey, nosocomial infection control, Kathmandu, Nepal

INTRODUCTION

Recently, nosocomial infections have become a global concern recognized as a major patient safety issue. They not only cause a significant burden on patients but also lower the quality of medical care. In addition, prolonged hospitalization due to nosocomial infections increases costs and unnecessary expenses for the hospital [1, 2]. In the healthcare setting, particularly in developed countries, various measures including the organization of infection control teams (ICTs), preparation of manuals, strengthening of surveillance systems, and training of staff have been taken to assure effective control. However, it is only some decades ago that importance was attached to nosocomial infection control and effective measures were employed, even in developed countries [3].

In developing countries, where the incidence of infectious diseases is high and environmental conditions of healthcare facilities are poor, nosocomial infections may frequently occur, and some studies have reported a high incidence at healthcare facilities in these countries [4–6]. Effective nosocomial infection control is crucial in the healthcare facilities of developing countries, but in actual fact, attention to it is still limited and control measures are not functioning well in many facilities. Furthermore, as implementation of control measures seems to be costly and to consume resources, nosocomial infection control is often given a low priority.

Severe acute respiratory syndrome (SARS), which originated in Guangdong Province, China in November 2002, spread to more than 30 countries. In many hospitals where SARS cases were encountered, nosocomial infections also broke out, causing many casualties along with economic havoc [7, 8]. It is not overstatement to say that such outbreaks have heightened awareness regarding nosocomial infection control even in developing countries. In

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RESEARCH ARTICLE Open Access

NDM-1 Metallo-β-Lactamase and ArmA 16S rRNA methylase producing *Providencia rettgeri* clinical isolates in Nepal

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Abstract

Background: Drug-resistant *Providencia rettgeri* producing metallo-β-lactamase and 16S rRNA methylase has been reported in several countries. We analyzed *P. rettgeri* clinical isolates with resistance to carbapenems and aminoglycosides in a hospital in Nepal.

Methods: Five clinical isolates of multidrug-resistant *P. rettgeri* were obtained in a hospital in Nepal. Antimicrobial susceptibilities were determined using the microdilution method and entire genomes were sequenced to determine drug-resistant genes. Epidemiological analysis was performed by pulsed-field gel electrophoresis.

Results: Four of the 5 isolates were resistant to carbapenems (imipenem and meropenem), with MICs ≥16 mg/L, with the remaining isolate showing intermediate resistance to imipenem, with an MIC of 2 mg/L and susceptibility to meropenem with an MIC ≤1 mg/L. All 5 isolates had bla_{VEB-1}. Of the 4 carbapenem-resistant strains, 3 had bla_{NDM-1} and 1 had bla_{OXA-72}. All isolates were highly resistant to aminoglycosides (MICs ≥1,024 mg/L) and harbored *armA*. As the result of pulsed-field gel electrophoresis pattern analysis in the 5 *P. rettgeri* isolates, 4 had identical PFGE patterns and the fifth showed 95.7% similarity.

Conclusions: This is the first report describing multidrug-resistant *P. rettgeri* strains harboring bla_{NDM-1} or bla_{OXA-72} and *armA* isolated from patients in Nepal.

Keywords: NDM-1, OXA-72, 16S rRNA methylase, *Providencia rettgeri*, Molecular epidemiology

Background

Providencia rettgeri has been associated with hospital acquired infections, including catheter-related urinary tract infections, bacteremia, skin infections, diarrhea, and gastroenteritis [1,2]. To date, there have been 5 reports of *P. rettgeri* isolates harboring metallo-β-lactamase (MBL) encoding genes, including IMP-type MBL producers in Japan [3,4]; VIM-type MBL, PER-1 extended-spectrum β-lactamase (ESBL) and 16S rRNA methylase ArmA in Korea [5]; and NDM-type MBL in Israel [6] and Brazil [7].

NDM-type MBL was initially identified in *Klebsiella pneumoniae* and *Escherichia coli* in 2009 in Sweden [8]. Since then, NDM-1-producing *Enterobacteriaceae* have been isolated in various parts of the world [9,10].

Exogenously acquired 16S rRNA methylase genes responsible for very high levels of resistance to various aminoglycosides are widely distributed among *Enterobacteriaceae* and glucose-nonfermentative microbes [11]. Gram-negative pathogens producing 16S rRNA methylase ArmA have been isolated in various countries [11].

Although co-production of several resistance determinants is not rare in *Enterobacteriaceae* [12-16], it is less common in *P. rettgeri* [5]. We describe here *P. rettgeri* clinical isolates from Nepal that produce carbapenemase (NDM-1 or OXA-72) and 16S rRNA methylase (ArmA).

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Table 1
Description of the 23 cefotaxime-resistant *Salmonella enterica* isolates.

Year of isolation	Isolate ID	Serovar	Phage type	Genes encoding cephalosporin resistance	Sex	Age (years)	Travel abroad	Resistance profile
2008	0812M7303	Typhimurium	193	$bla_{CTX-M-55}$	M	50	Thailand	CHL, CIP, FFN, GEN, SUL, STR, TET
	0811R10895	Typhimurium	RDNC	$bla_{CTX-M-1}$	M	1	Unknown	SUL,TET
	0809W37247	Stanley		$bla_{CMY-2-like}$	F	37	No	AMC, CHL, FFN, SUL, STR, TET
	0809F35063	Stanley		$bla_{CMY-2-like}$	F	6	Unknown	AMC, CHL, FFN, GEN, SUL, STR, TET
	0808S63221	Typhimurium	NT	$bla_{CMY-2-like}$	M	20	Thailand	AMC, CHL, FFN, SUL, STR, TET
	0807F21428	Stanley		$bla_{CMY-2-like}$	F	22	Thailand	AMC, CHL, FFN, GEN, SUL, STR, TET
	0806H16365	Stanley		$bla_{CMY-2-like}$	M	2	Unknown	AMC, CHL, FFN, GEN, SUL, STR, TET
	0806R9615	Typhimurium	U292	$bla_{CTX-M-3}$	M	12	No	None
	0805R9530	Typhimurium	NT	$bla_{CTX-M-14}$	M	47	Greece	AMC, CHL, GEN, SUL, STR, TMP
2009	0911W58164	Heidelberg		$bla_{CTX-M-14}$	M	40	Egypt	GEN, SUL, STR
	0910W56953	subsp. enterica (I)		$bla_{CMY-2-like}$	M	55	Thailand	AMC, CHL, CIP, FFN, GEN, NAL, SUL, STR, TET
	0910F48822	Isangi		$bla_{CMY-2-like}$, bla_{OXA-10}	M	<1	South Africa	AMC, CHL, CIP, FFN, GEN, NAL, SUL, STR, TET, TMP
	0909F36769	O:6,8; H:e,h:-		$bla_{CMY-2-like}$	M	49	No	AMC, CHL, FFN, SUL, STR, TET, TMP
	0905W18230	O:4,5,12; H:i:-	U302	$bla_{CTX-M-15}$	M	48	Unknown	CHL, CIP, GEN, SUL, STR, TET, TMP
	0904R11448	Enteritidis	1	$bla_{CTX-M-15}$	F	44	Egypt	GEN
	0904W9384	Typhimurium	193	$bla_{CTX-M-15}$	F	54	No	CHL, CIP, FFN, GEN, SUL, STR, TET
	0903T66197	O:4,5,12; H:i:-	193	$bla_{CTX-M-55}$	F	46	Unknown	GEN, SUL, STR, TET, TMP
2010	1003F13978	O:4,12; H:i: -	193	$bla_{CTX-M-55}$	M	16	Thailand	CHL, CIP, FFN, SUL, STR, TET
	1001M23541	Infantis		$bla_{CTX-M-55}$	F	56	Thailand	CHL, CIP, FFN, GEN, NAL, SUL, STR, TET, TMP
	1010H59657	Senftenberg		$bla_{CTX-M-15}$	M	36	Egypt	SUL, TMP
	1008R13307	Typhimurium	193	$bla_{CTX-M-55}$	F	21	Thailand	CHL, CIP, FFN, GEN, SUL, STR, TET
	1002H3270	Stanley		$bla_{CMY-2-like}$	F	58	Thailand	AMC, CHL, FFN, SUL, STR, TET
	1002W11208	O:4,5,12; H:i:-	193	$bla_{CTX-M-55}$	F	58	Unknown	CHL, CIP, FFN, GEN, SUL, STR, TET

CHL, chloramphenicol; CIP, ciprofloxacin, FFN, florfenicol; GEN, gentamicin; SUL, sulfamethoxazole; STR, streptomycin; TET, tetracycline; AMC, amoxicillin/clavulanic acid; TMP, trimethoprim; NAL, nalidixic acid.

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Dissemination of multidrug-resistant *Klebsiella pneumoniae* clinical isolates with various combinations of carbapenemases (NDM-1 and OXA-72) and 16S rRNA methylases (ArmA, RmtC and RmtF) in Nepal

Sir,

The carbapenemases NDM-1 and OXA-72 hydrolyse almost all β-lactams. NDM-1-producing Enterobacteriaceae and OXA-72-producing *Acinetobacter* spp. have been reported in various countries [1,2]. To date, OXA-72-producing isolates of bacterial species other than *Acinetobacter* spp. have not been reported.

Acquired 16S rRNA methylase genes responsible for high-level resistance to various aminoglycosides have been widely distributed among Enterobacteriaceae, including *Klebsiella pneumoniae* and glucose-non-fermentative bacteria [3]. 16S rRNA methylase-producing Gram-negative pathogens have been isolated in various countries [3], including Nepal [4]. The 16S rRNA methylases ArmA and RmtC are widely spread among various bacterial species, including Enterobacteriaceae and *Acinetobacter* spp.

In this study, 25 *K. pneumoniae* isolates were obtained from 25 inpatients during the period May–October 2012 at Tribhuvan University Teaching Hospital (Kathmandu, Nepal), of which 13 isolates were obtained from sputa and 12 were from pus samples. Isolates were identified phenotypically and species identification

Table 1
Summary of the characteristics of the 25 *Klebsiella pneumoniae* strains, including antimicrobial resistance profiles and resistance genes.

Strain	MIC (μg/mL)												Carbapenemases	ESBL	16S rRNA methylases	Mutations in DNA gyrase	
	PIP	TZP	CAZ	CTX	FEP	IPM	MEM	ATM	ABK	AMK	GEN	CIP				*gyrA*	*parC*
IOMTU 23	>1024	512	>1024	1024	256	16	32	512	>1024	>1024	>1024	128	NDM-1, OXA-72	CTX-M-15, SHV-158, TEM-1	RmtC, RmtF	S83I	S80I
IOMTU 25	>1024	1024	>1024	512	128	32	64	512	1024	1024	>1024	128	NDM-1	CTX-M-15, SHV-28, TEM-1	RmtF	S83I	S80I
IOMTU 40	>1024	256	>1024	1024	256	16	32	128	1024	>1024	1024	128	NDM-1	CTXM-15, SHV-28	ArmA	S83F, D87A	S80I
IOMTU 46	>1024	512	>1024	>1024	128	16	32	512	>1024	>1024	>1024	128	NDM-1	CTX-M-15, SHV-11, TEM-1	RmtC, RmtF	S83I	S80I
IOMTU 53	>1024	512	>1024	>1024	512	32	32	512	>1024	>1024	>1024	64	NDM-1, OXA-72	CTX-M-15, SHV-11, TEM-1	RmtC, RmtF	S83I	S80I
IOMTU 67	512	8	64	512	128	<0.5	<0.5	32	1	4	64	32	–	CTX-M-15, SHV-28, TEM-1	–	S83F, D87A	S80I
IOMTU 74	>1024	512	32	256	128	4	8	128	>1024	>1024	>1024	2	NDM-1, OXA-72	CTX-M-15, SHV-1	ArmA	S83F, D87A	S80I
IOMTU 76	>1024	1024	>1024	512	128	32	64	512	>1024	>1024	>1024	64	NDM-1	CTX-M-15, SHV-28, TEM-1	RmtF	S83I	S80I
IOMTU 83	1024	512	128	1024	128	32	32	128	>1024	>1024	>1024	128	NDM-1, OXA-72	CTX-M-15, SHV-28	ArmA	S83F, D87A	S80I
IOMTU 89	>1024	512	>1024	>1024	256	32	32	1024	>1024	>1024	>1024	128	NDM-1	CTX-M-15, SHV-28, TEM-1	RmtF	S83Y, D87F	S80I
IOMTU 100	>1024	1024	>1024	>1024	128	32	64	512	>1024	>1024	>1024	64	NDM-1	CTX-M-15, SHV-28, TEM-1	RmtF	S83F, D87A	S80I
IOMTU 102	>1024	512	>1024	1024	256	32	32	256	512	>1024	>1024	128	NDM-1	CTX-M-15, SHV-28	ArmA	S83F, D87A	S80I
IOMTU 103	>1024	8	64	>1024	64	<0.5	<0.5	128	1	8	32	128	–	CTX-M-15, SHV-28, TEM-1	–	S83F, D87A	S80I
IOMTU 111	>1024	1024	>1024	>1024	512	16	32	512	>1024	>1024	1024	64	NDM-1, OXA-72	CTX-M-15, TEM-1	RmtC	S83I	S80I
IOMTU 116.1	>1024	512	>1024	1024	512	8	8	256	>1024	>1024	512	64	NDM-1	CTX-M-15, SHV-28	ArmA	S83F, D87A	S80I
IOMTU 116.2	>1024	512	>1024	>1024	>1024	8	16	256	>1024	>1024	1024	64	NDM-1	CTX-M-15, SHV-28	ArmA	S83F, D87A	S80I
IOMTU 117	>1024	512	>1024	1024	128	4	4	128	1024	>1024	1024	64	NDM-1	CTX-M-15, SHV-28, TEM-1	ArmA	S83F, D87A	S80I
IOMTU 120	>1024	16	512	>1024	>1024	<0.5	<0.5	1024	>1024	>1024	>1024	128	–	CTX-M-15, SHV-28,TEM-1	RmtF	S83Y, D87N	S80I
IOMTU 122	>1024	512	>1024	>1024	512	16	16	256	>1024	>1024	>1024	128	NDM-1, OXA-72	CTX-M-15, SHV-28	ArmA	S83F, D87A	S80I
IOMTU 125	>1024	4	32	512	256	<0.5	<0.5	64	>1024	>1024	>1024	8	OXA-72	CTX-M-15, SHV-28, TEM-1	ArmA	S83F, D87A	S80I
IOMTU 138	>1024	128	256	>1024	>1024	<0.5	2	512	>1024	>1024	>1024	>1024	OXA-72	CTX-M-15, SHV-11, TEM-1	RmtF	S83F, D87N	No mutation
IOMTU 139	>1024	128	128	512	256	2	2	512	32	32	64	256	–	CTX-M-15, SHV-28, TEM-1	–	S83Y, D87G	S80I
IOMTU 145	>1024	8	32	1024	256	<0.5	<0.5	64	4	4	<0.5	2	–	CTX-M-15, TEM-1	–	No mutation	No mutation
IOMTU 154	>1024	1024	>1024	1024	256	16	32	64	>1024	>1024	1024	16	NDM-1, OXA-72	CTX-M-15, SHV-28, TEM-1	RmtC	S83F, D87A	S80I
IOMTU 164	>1024	8	64	512	256	<0.5	<0.5	64	4	4	<0.5	<0.5	–	CTX-M-15, SHV-83, TEM-1	–	No mutation	No mutation

MIC, minimum inhibitory concentration; PIP, piperacillin; TZP, piperacillin/tazobactam; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; MEM, meropenem; ATM, aztreonam; ABK, arbekacin; AMK, amikacin; GEN, gentamicin; CIP, ciprofloxacin; ESBL, extended-spectrum β-lactamase.

was confirmed by 16S rRNA sequencing. Minimum inhibitory concentrations of antibiotics were determined by the microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) M07-A9.

Entire genomes of the isolates were sequenced by MiSeq™ (Illumina, San Diego, CA). CLC Genomics Workbench v.5.5 (CLC bio, Tokyo, Japan) was used to search 923 drug resistance genes, including genes encoding β-lactamases, 16S rRNA methylases and aminoglycoside-acetyl/adenyltransferases, as well as point mutations in *gyrA* and *parC* associated with quinolone resistance. The genetic environments surrounding bla_{NDM-1}, bla_{OXA-72} and 16S rRNA methylase-encoding genes were determined. Multilocus sequence typing (MLST) and clonal complexes (CCs) were determined according to the *K. pneumoniae* MLST database website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html) and eBURST v.3 (http://eburst.mlst.net), respectively.

Pulsed-field gel electrophoresis (PFGE) analysis was performed and fingerprinting patterns were analysed by the unweighted pair-group method.

All isolates were resistant to piperacillin, of which 19 isolates were resistant to piperacillin/tazobactam. All isolates were resistant to ceftazidime, cefotaxime and cefepime. Seventeen isolates were resistant to carbapenems (imipenem and meropenem). All isolates are resistant to aztreonam. Twenty isolates were resistant to all aminoglycosides tested (arbekacin, amikacin and gentamicin). Twenty-two isolates were resistant to ciprofloxacin (Table 1).

The majority of isolates had various combinations of genes encoding carbapenemases (bla_{NDM-1} and bla_{OXA-72}) and 16S rRNA methylases (*armA*, *rmtC* and *rmtF*) (Table 1). These isolates also had extended-spectrum β-lactamase-encoding genes, including $bla_{CTX-M-15}$, bla_{TEM-1} and/or bla_{SHV}-type, as well as aminoglycoside-modifying enzymes, including *aac(6′)-Ib* and/or *aadA2*. Twenty-three isolates had two or three point mutations in the quinolone resistance-determining regions of *gyrA* and *parC*.

The genetic environment surrounding bla_{NDM-1} (GenBank accession no. AB824738) including *rmtC* was a unique structure, which was *orf1–tniB–orf2–orf3–rmtC–*bla_{NDM-1}*–*ble_{MBL}*–trpF–dsbC–cutA1–qroL*. The genetic environment surrounding *armA* (GenBank accession no. AB825954) from nucleotides 19 to 14 138 had >99.9% sequence identity to a nucleotide sequence from nucleotides 65 492 to 79 611 of the plasmid pCTX-M3 (GenBank accession no. AF550415). The genetic environment surrounding *rmtF* (GenBank accession no. AB824739) from nucleotides 268 to 9812 had >99.9% sequence identity to a nucleotide sequence from nucleotides 49 291 to 58 835 of the plasmid pKPX-1 (GenBank accession no. AP012055). The genetic environment surrounding bla_{OXA-72} (GenBank accession no. AB825955) from nucleotides 1 to 8970 was identical to that of pAB-NCGM253 (GenBank accession no. AB823544).

The clinical isolates of *K. pneumoniae* tested belonged to one of the following sequence types (STs): ST11; ST14; ST15; ST29; ST43; ST340; ST378; ST395; ST437; ST1231; and ST1232. Of these isolates, 14 belonged to CC14 and 5 belonged to CC11. These results mostly corresponded with the results of PFGE pattern analysis, which revealed two clusters showing >60% similarity (clusters I and II). Cluster I comprised 12 isolates belonging to CC14 and cluster II comprised 4 isolates belonging to CC11.

NDM-1-producers have epidemiological links to the Indian subcontinent as of 2011 [5]. There was, nevertheless, no report of NDM-1-producers in Nepal. We recently found NDM-1-producing *Pseudomonas aeruginosa* and a novel variant NDM-8-producing *Escherichia coli* isolates in Nepal [4].

This is the first report describing OXA-72-producers in South Asia, suggesting that OXA-72-producers have disseminated in this region. This is also the first report of OXA-72-producing *K. pneumoniae* clinical isolates. Up to now, OXA-72-producers were reported to be only *Acinetobacter* spp.

The present study suggests that aminoglycoside-resistant Gram-negative pathogens producing ArmA, RmtC and RmtF disseminated in medical settings in Nepal. These pathogens producing 16S rRNA methylases may also disseminate in neighbouring countries. Hidalgo et al. [6] recently reported that 14% of Enterobacteriaceae isolates from an Indian hospital had 16S rRNA methylases, of which 24% produced RmtF.

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Competing interests: None declared.

Ethical approval: This study protocol was ethically reviewed and approved by the Institutional Review Board of the Institute of Medicine, Tribhuvan University (Kathmandu, Nepal) [ref. 6-11-E].

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NDM-12, a Novel New Delhi Metallo-β-Lactamase Variant from a Carbapenem-Resistant *Escherichia coli* Clinical Isolate in Nepal

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A novel New Delhi metallo-β-lactamase variant, NDM-12, was identified in a carbapenem-resistant *Escherichia coli* clinical isolate obtained from a urine sample from a patient in Nepal. NDM-12 differed from NDM-1 by two amino acid substitutions (M154L and G222D). The enzymatic activities of NDM-12 against β-lactams were similar to those of NDM-1, although NDM-12 showed lower k_{cat}/K_m ratios for all β-lactams tested except doripenem. The bla_{NDM-12} gene was located in a plasmid of 160 kb.

Metallo-β-lactamases (MBLs) usually confer reduced susceptibility to carbapenems, cephalosporins, and penicillins but not monobactams (1). Acquired MBLs are produced by Gram-negative bacteria, including *Acinetobacer* spp., *Pseudomonas aeruginosa*, and several *Enterobacteriaceae* (1). MBLs are categorized by their amino acid sequences into various types (2–4), including AIM (5), DIM (6), FIM (7), GIM (8), IMPs (9), KHM (10), NDMs (11), SMB (12), SIM (13), SPM (14), TMBs (15), and VIMs (16). The most prevalent types of MBLs are IMP-, VIM-, and NDM-type enzymes (1, 2, 17). NDM-1 was initially isolated from *Klebsiella pneumoniae* and *Escherichia coli* in 2008 in Sweden (11). Subsequently, at least 11 NDM variants (www.lahey.org/studies) have been reported in several countries (4, 18–29).

This study was ethically reviewed and approved by the Institutional Review Board of the Institute of Medicine at Tribhuvan University (reference 6-11-E) and the Biosafety Committee at the National Center for Global Health and Medicine (approval no. 26-D-088 and 26-D-089).

E. coli IOMTU388.1 was isolated from a urine sample obtained from a patient in 2013 in a university hospital in Nepal. The isolate was phenotypically identified, and the species identification was confirmed by 16S rRNA sequencing (30). *E. coli* DH5α (TaKaRa Bio, Shiga, Japan) and *E. coli* BL21-CodonPlus(DE3)-RIP (Agilent Technologies, Santa Clara, CA) were used as hosts for recombinant plasmids and for expression of bla_{NDM-1} and bla_{NDM-12}, respectively.

MICs were determined using the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (31). The MICs of β-lactams for *E. coli* IOMTU388.1 are shown in Table 1, and the MICs of other antibiotics were as follows: amikacin, >1,024 μg/ml; arbekacin, >1,024 μg/ml; ciprofloxacin, 128 μg/ml; colistin, ≤0.125 μg/ml; fosfomycin, 8 μg/ml; gentamicin, >1,024 μg/ml; kanamycin, >1,024 μg/ml; levofloxacin, 32 μg/ml; minocycline, 8 μg/ml; tigecycline, ≤0.125 μg/ml; and tobramycin, >1,024 μg/ml. PCR analysis was performed to detect the MBL genes bla_{DIM}, bla_{GIM}, bla_{IMP}, bla_{NDM}, bla_{SIM}, bla_{SPM}, and bla_{VIM} (32, 33). The isolate was PCR positive for bla_{NDM} but negative for the other MBL genes tested. The DNA sequence of the PCR product revealed that the isolate had bla_{NDM-12}. Multilocus sequence typing (MLST) of IOMTU388.1 typed it as ST635 (*E. coli* MLST Database; http://www.pasteur.fr/recherche/genopole/PF8/mlst/EColi.html). bla_{NDM-1} obtained from *P. aeruginosa* IOMTU9 (29) was used as a reference gene.

The bla_{NDM-12} sequence had 2 amino acid substitutions (M154L and G222D) compared with bla_{NDM-1} (accession no. JF798502) and one substitution (G222D) compared with NDM-4 (accession no. JQ348841).

The bla_{NDM-1} and bla_{NDM-12} genes were cloned into the corresponding sites of pHSG398 (TaKaRa, Shiga, Japan) using the primer set EcoRI-NDM-F (5′-GGGAATTCATGGAATTGCCCAATATTATG-3′) and PstI-NDM-R (5′-AACTGCAGTCAGCGCAGCTTGTCGGCCAT-3′). *E. coli* DH5α was transformed with pHSG398-NDM-1 or pHSG398-NDM-12.

The open reading frames of NDM-1 and NDM-12 without signal peptide regions were cloned into the pET28a expression vector (Novagen, Inc., Madison, WI) using the primer set BamHI-TEV-NDM-F (5′-ATGGATCCGAAAACCTGTATTTCCAAGGCCAGCAAATGGAAACTGGCGAC-3′) and XhoI-NDM-R (5′-ATCTCGAGTCAGCGCAGCTTGTCGGCCATG-3′). The resulting plasmids were transformed into *E. coli* BL21-CodonPlus(DE3)-RIP (Agilent Technologies, Santa Clara, CA). Both recombinant NDM-1 and NDM-12 were purified simultaneously using Ni-nitrilotriacetic acid (NTA) agarose according to the manufacturer's instruction (Qiagen, Hilden, Germany). His tags were removed by digestion with TurboTEV protease (Accelagen, San Diego, CA) and untagged proteins were purified by an additional passage over the Ni-NTA agarose. The purities of NDM-1 and NDM-12, which were estimated by SDS-PAGE, were greater than 90%. During the purification procedure, the presence of β-lactamase activity was monitored using nitrocefin (Oxoid, Ltd., Basingstoke, United Kingdom). Initial hydrolysis rates were determined in 50 mM Tris-HCl buffer (pH 7.4) containing 0.3 M NaCl and 5 μM $Zn(NO_3)_2$ at 37°C, using a UV-visible spectrophotometer (V-530; Jasco, Tokyo, Japan). The K_m and k_{cat} values and the k_{cat}/K_m ratio were determined by analyzing β-lactam hydrolysis with a Lineweaver-Burk plot. Wavelengths and extinction coefficients for

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Identification of a Novel 6′-*N*-Aminoglycoside Acetyltransferase, AAC(6′)-Iak, from a Multidrug-Resistant Clinical Isolate of *Stenotrophomonas maltophilia*

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***Stenotrophomonas maltophilia* IOMTU250 has a novel 6′-*N*-aminoglycoside acetyltransferase-encoding gene, *aac(6′)-Iak*. The encoded protein, AAC(6′)-Iak, consists of 153 amino acids and has 86.3% identity to AAC(6′)-Iz. *Escherichia coli* transformed with a plasmid containing *aac(6′)-Iak* exhibited decreased susceptibility to arbekacin, dibekacin, neomycin, netilmicin, sisomicin, and tobramycin. Thin-layer chromatography showed that AAC(6′)-Iak acetylated amikacin, arbekacin, dibekacin, isepamicin, kanamycin, neomycin, netilmicin, sisomicin, and tobramycin but not apramycin, gentamicin, or lividomycin.**

Stenotrophomonas maltophilia is a globally emerging multidrug-resistant Gram-negative pathogen that is most commonly associated with respiratory infections in humans (1) and causes an increasing number of nosocomial respiratory tract and bloodstream infections in immunocompromised patients. *S. maltophilia* exhibits resistance to a broad spectrum of antibiotics, namely, β-lactam antibiotics, macrolides, cephalosporins, fluoroquinolones, aminoglycosides, carbapenems, chloramphenicol, tetracyclines, and polymyxins (1). Several intrinsic antibiotic resistance traits in *S. maltophilia* are known; an increase in membrane permeability and the presence of chromosomally encoded multidrug resistance efflux pumps have also been observed (2).

Aminoglycoside-resistant mechanisms involve primarily aminoglycoside-modifying enzymes (3) and 16S rRNA methylases (4). The 6′-*N*-aminoglycoside acetyltransferases [AAC(6′)s] are of particular interest because they can modify a number of clinically important aminoglycosides. There are two main AAC(6′) subclasses, which differ in their activities against amikacin and gentamicin. The AAC(6′)-I-type enzymes effectively acetylate amikacin but not gentamicin, whereas the AAC(6′)-II-type enzymes effectively acetylate gentamicin but not amikacin (5). To date, 45 genes encoding AAC(6′)-I types, designated *aac(6′)-Ia* to *-Iaj*, have been cloned, and their bacteriological or biochemical properties have been characterized (5–8).

S. maltophilia IOMTU250 was isolated from the endotracheal tube of a patient in a medical ward of a hospital in Nepal in 2012. *Escherichia coli* DH5α (TaKaRa Bio, Shiga, Japan) and *Escherichia coli* BL21-CodonPlus (DE3)-RIP (Agilent Technologies, Santa Clara, CA) were used as hosts for recombinant plasmids and protein expression, respectively. MICs were determined using the microdilution method (9). The MICs of tested aminoglycosides for *S. maltophilia* IOMTU250 are shown in Table 1. The MICs of other antibiotics were as follows: ampicillin, >1,024 μg/ml; ampicillin-sulbactam, 128 μg/ml; aztreonam, 128 μg/ml; ceftazidime, 8 μg/ml; cephradine, 1,024 μg/ml; cefepime, 64 μg/ml; cefotaxime, 64 μg/ml; cefoxitin, 512 μg/ml; chloramphenicol, 8 μg/ml; colistin, 32 μg/ml; fosfomycin, 128 μg/ml; imipenem, 256 μg/ml; levofloxacin, 1 μg/ml; meropenem, 64 μg/ml; minocycline, ≤0.25 μg/ml; penicillin, 512 μg/ml; ticarcillin-clavulanate, 8 μg/ml; tigecycline, ≤0.25 μg/ml; and trimethoprim-sulfamethoxazole, 4 μg/ml.

Genomic DNA was extracted from *S. maltophilia* IOMTU250 using DNeasy blood and tissue kits (Qiagen, Tokyo, Japan) and sequenced with a MiSeq system (Illumina, San Diego, CA). More than 20-fold coverage was achieved. A new 6′-*N*-aminoglycoside acetyltransferase variant was designated *aac(6′)-Iak*.

A synthetic *aac(6′)-Iz* gene (462 bp) was produced by Funako-

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TABLE 1 MICs of various aminoglycosides for *S. maltophilia* IOMTU250 and *E. coli* strains transformed with *aac(6′)-Iak* and *aac(6′)-Iz*

Strain[a]	MIC[b] (μg/ml)											
	ABK	AMK	APR	DIB	GEN	ISP	KAN	LIV	NEO	NET	SIS	TOB
S. maltophilia IOMTU250	512	64	>512	512	32	64	128	>512	512	512	64	64
E. coli DH5α/pSTV28	0.5	0.25	1	0.25	0.25	0.25	1	2	0.5	0.25	0.25	0.5
E. coli DH5α/pSTV28-*aac(6′)-Iak*	2	1	1	16	0.25	0.5	2	2	4	1	2	4
E. coli DH5α/pSTV28-*aac(6′)-Iz*	4	2	1	16	0.25	0.5	2	2	4	8	2	16

[a] The MICs for *S. maltophilia* and *E. coli* strains were determined with Mueller-Hinton broth preparations and individual aminoglycosides.
[b] ABK, arbekacin; AMK, amikacin; APR, apramycin; DIB, dibekacin; GEN, gentamicin; ISP, isepamicin; KAN, kanamycin; LIV, lividomycin; NEO, neomycin; NET, netilmicin; SIS, sisomicin; TOB, tobramycin.

Original article

Nosocomial Bacterial Infection and Antimicrobial Resistant Pattern in a Tertiary Care Hospital in Nepal

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Abstract

Introduction: Nosocomial infection is a global problem with multi facet outcomes. At present, the emergence of resistance to antimicrobial agents is a global public health problem which is well pronounced in developing countries.

Methods: The aim of this study was to determine the prevalence of bacteria causing nosocomial infections and their antibiotics resistant pattern among the patients admitted at Tribhuvan University Teaching Hospital (TUTH), Kathmandu, Nepal. The study was conducted during a period of March 2011 to February 2012. Nine hundred clinical specimens which included urine, sputum, endotracheal aspirates, pus & blood were subjected for bacterial culture and their antibiotics sensitivity test at the Department of Microbiology with the use of standard method as described by American Society for Microbiology (ASM).

Results: Prevalence of bacteria causing nosocomial infection was 34.4% (n=310). Out of 310 specimens, urine 122 (39.30%), sputum 78(25.2%), pus 78(25.2%), endotracheal secreation 24 (7.7%) and blood 8(2.6%). Three hundred thirty three bacteria were isolated from three hundred ten specimens. The most common isolates were Escherichia coli followed by Acinetobacter species, Klebsiella pneumonia and Staphylococcus aureus. In-vitro antibiotic susceptibility tests revealed that the Gram-negatives bacilli were only sensitive to fluroquinolones, ceftrixone, cefepime carbapenem, polymyxin B and colistin sulphate while the Gram-positive cocci were sensitive to fluroquinolones, Ceftroxone, cefepime and vancomycin.

Conclusion: The findings suggested the need for constant monitoring of susceptibility of specific pathogens in different populations to commonly used anti-microbial agents to cope up this alarming situation in the hospital for the management of such patients and prevent the dissemination of such strains.

Key words: Nosocomial infections, Bacteria and Antibiotics

Introduction

Nosocomial infections, also called healthcare acquired infections or health care-associated infections, is defined by the Center for Disease Control (CDC) as a localized or systemic condition that results from adverse reaction to the presence of an infectious agent(s) or its toxin(s) and that was not present or incubating at the time of admission to the hospital. For most bacterial nosocomial infections usually become evident after 48 hours (i.e., the typical incubation

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Clinical Epidemiology and Molecular Analysis of Extended-Spectrum-β-Lactamase-Producing *Escherichia coli* in Nepal: Characteristics of Sequence Types 131 and 648

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Recently, CTX-M-type extended-spectrum-β-lactamase (ESBL)-producing *Escherichia coli* strains have emerged worldwide. In particular, *E. coli* with O antigen type 25 (O25) and sequence type 131 (ST131), which is often associated with the CTX-M-15 ESBL, has been increasingly reported globally; however, epidemiology reports on ESBL-producing *E. coli* in Asia are limited. Patients with clinical isolates of ESBL-producing *E. coli* in the Tribhuvan University teaching hospital in Kathmandu, Nepal, were included in this study. Whole-genome sequencing of the isolates was conducted to analyze multilocus sequence types, phylotypes, virulence genotypes, O25b-ST131 clones, and distribution of acquired drug resistance genes. During the study period, 105 patients with ESBL-producing *E. coli* isolation were identified, and the majority (90%) of these isolates were CTX-M-15 positive. The most dominant ST was ST131 ($n = 54$; 51.4%), followed by ST648 ($n = 15$; 14.3%). All ST131 isolates were identified as O25b-ST131 clones, subclone H30-Rx. Three ST groups (ST131, ST648, and non-ST131/648) were compared in further analyses. ST648 isolates had a proportionally higher resistance to non-β-lactam antibiotics and featured drug-resistant genes more frequently than ST131 or non-ST131/648 isolates. ST131 possessed the most virulence genes, followed by ST648. The clinical characteristics were similar among groups. More than 38% of ESBL-producing *E. coli* isolates were from the outpatient clinic, and pregnant patients comprised 24% of ESBL-producing *E. coli* cases. We revealed that the high resistance of ESBL-producing *E. coli* to multiple classes of antibiotics in Nepal is driven mainly by CTX-M-producing ST131 and ST648. Their immense prevalence in the communities is a matter of great concern.

Escherichia coli is a part of the normal human and animal gastrointestinal flora; it is the most common cause of urinary tract infections and also causes various other infectious conditions, such as intra-abdominal infections, neonatal meningitis, and septicemia (1–3).

Recently, extended-spectrum-beta-lactamase (ESBL) producing *E. coli* strains, particularly strains producing CTX-M-type ESBLs, have emerged worldwide (4). In particular, *E. coli* with O antigen type 25 (O25) and sequence type 131 (ST131) is often associated with the CTX-M-15 ESBL and has been increasingly reported globally. These bacteria are resistant to classes of antibiotics distinct from β-lactams, such as fluoroquinolones and trimethoprim-sulfamethoxazole (5, 6). Epidemiology reports on ESBL-producing *E. coli* in Asia are limited to date. To the best of our knowledge, there has been no report on the prevalence of pandemic ESBL-producing *E. coli* ST131, or other potentially dominant ESBL-producing *E. coli* STs, or clinical and microbiological information pertaining to their isolation in Nepal. Nepal is located in south Asia and adjacent to India, where a high proportion of resistant Gram-negative bacteria has been reported (7); understanding the epidemiology of ESBL-producing *E. coli* in this region is therefore particularly important. In addition, the patients population and their clinical background in developing countries are different from those in developed countries, where the majority of studies on ESBL-producing *E. coli* have been conducted. It is thus imperative to reveal the clinical and microbiological characteristics of ESBL-producing *E. coli* in developing countries in order to better understand the global epidemiology of this pathogen. In this study, we aimed to elucidate the clinical and microbiological characteristics of ESBL-producing *E. coli* and specifically to reveal the unique aspects of the dominant ESBL-producing *E. coli* ST in Nepal.

MATERIALS AND METHODS

Study settings and design. Microbiological investigations and clinical epidemiological analyses of ESBL-producing *E. coli* were conducted among patients from whom ESBL-producing *E. coli* was isolated in the Tribhuvan University teaching hospital, which serves as a tertiary referral hospital in Kathmandu, Nepal. Institutional review boards at Tribhuvan University

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approved the study before its initiation. The study period, including chart reviewing, was from 1 February 2013 to 31 January 2014.

Patients and variables. Patients with clinical isolation of ESBL-producing *E. coli* between 1 February 2013 and 31 July 2013 were divided into three groups, i.e., ESBL-producing *E. coli* ST131, ESBL-producing *E. coli* ST648, and ESBL-producing *E. coli* non-ST131/648 (ESBL-producing *E. coli* isolates of ST other than ST131 and ST648), based on multilocus sequence type (MLST). For patients from whom more than one ESBL-producing *E. coli* strain was isolated during the study period, only the first episode was analyzed; this study therefore incorporated only unique patient episodes. Parameters retrieved from the patient records included (i) demographics, (ii) background conditions and clinical diagnosis. (iii) duration of hospital stay, and (iv) antimicrobial treatment during the current hospital stay (or at the outpatient clinic for outpatients).

Isolates. Standard identification and susceptibility testing of *E. coli* were performed and interpreted in accordance with the Clinical and Laboratory Standard Institute (CLSI) criteria (8), using an automated broth microdilution system (MicroScan; Siemens AG, Germany) unless otherwise stated. To determine the MIC of fosfomycin, an NC6.11J panel (Siemens AG, Germany) was used.

In addition, we tested MICs of flomoxef, cefoperazone-sulbactam, and fosfomycin, as they are potentially active against ESBL-producing *E. coli* (9–11). The breakpoints for susceptibility were ≤8 μg/ml for flomoxef (12), and ≤16/8 μg/ml for cefoperazone-sulbactam (13). ESBL production was confirmed with disc diffusion tests in accordance with the 2009 CLSI criteria (14).

Molecular analysis. Molecular analysis was conducted in the Pathogenic Microbe Laboratory, Research Institute, National Center for Global Health and Medicine, Tokyo, Japan. Whole-genome sequences of all 105 isolates were obtained using a MiSeq system with Nextera XT library kits (Illumina, Tokyo, Japan) for the analysis of MLST, phylotypes (15), H30 and H30-Rx subclones of ST131 (16), virulence genotypes (17), O25b-ST131 clones (18), and distribution of acquired drug resistance genes. Approximately one million 301-bp by 2 pair-end reads were obtained. After trimming based on base quality (quality score limit = 0.05; removing reads with more than 2 ambiguous nucleotides or of less than 15 bp in length), the reads were assembled *de novo* to construct contigs without annotation using the commercial software CLC genomics workbench (CLC Bio, Tokyo, Japan). The contigs were subjected to further analyses with the BLAST algorithm (19) and Resfinder (20). An identification rate of more than 98% was considered positive for each targeted gene. The virulence score was determined as the number of virulence genes detected, with *pap* elements counting collectively as a single trait (17).

We selected sequences of contigs that could contain mobile acquired elements carrying resistance genes in the isolates, based on BLAST (19) and ResFinder (20) analyses. We then conducted BLAST analyses of these sequences using the National Center for Biotechnology Information and identified sequences with close to 100% query cover and identity at the nucleotide level. We then identified isolates that contains the aforementioned sequences with >95% identity at the nucleotide level and >80% coverage level.

Statistical analysis. All analyses were performed using IBM-SPSS Statistics 20 (2012). Bivariate analyses were performed using Fisher's exact test or the chi-square test for categorical variables and the *t* test or the Mann-Whitney U test for continuous variables. All *P* values were two-sided. Throughout, the percentages displayed are the "valid percentage," which indicates the percentage excluding the missing data from the denominator.

RESULTS

A total of 105 patients with ESBL-producing *E. coli* isolation were identified during the study period. The STs and phylotypes of the isolated ESBL-producing *E. coli* are shown in Table 1. The most dominant ST was ST131, which accounted for 54 (51.4%) of all ESBL-producing *E. coli* isolates, followed by ESBL-producing *E. coli* ST648 ($n = 15$; 14.3%). One isolate of ST131 contained a new *fumC* allele (400), but the other 6 MLST locus sequences and phylotypes were consistent with ST131 and were therefore included in the ST131 group for further analysis. All ESBL-producing *E. coli* ST131 isolates were identified as the O25b-ST131 clone and the H30-Rx subclone. Among the 15 ST648 isolates, 12 had an identification rate of 97.4%, and 3 had a 96.0% identification rate of the *pabB* gene, which is reported to be specific for isolates of the O25b-ST131 clone (18). In order to further understand the characteristics of dominant STs, we conducted analyses comparing the three groups of ESBL-producing *E. coli* isolates (ST131, ST648, and non-ST131/648).

TABLE 1 Sequence typing and phylotypes of extended-spectrum-β-lactamase-producing *Escherichia coli* isolates in Nepal

Sequence type	No. (%) of isolates (total $n = 105$) in phylotype:			
	A	B1	D	B2
ST131				54 (51.4)
ST648			15 (14.3)	
ST405			5 (4.8)	
ST38			3 (2.9)	
ST167	2 (1.9)			
ST361	2 (1.9)			
ST410	2 (1.9)			
ST10	1 (1.0)			
ST14				1 (1.0)
ST44	1 (1.0)			
ST315			1 (1.0)	
ST393			1 (1.0)	
ST394			1 (1.0)	
ST421				1 (1.0)
ST443		1 (1.0)		
ST517				1 (1.0)
ST617	1 (1.0)			
ST624			1 (1.0)	
ST746	1 (1.0)			
ST1312	1 (1.0)			
ST2562		1 (1.0)		
New ST			7 (6.7)	1 (1.0)
Total	11 (10.5)	2 (1.9)	34 (32.4)	58 (55.2)

The comparison between the three groups with regard to their antimicrobial susceptibility is shown in Table 2. ST648 isolates were the most resistant to multiple antibiotics, including non-β-lactams such as levofloxacin, gentamicin, trimethoprim-sulfamethoxazole, and minocycline. More than 70% of ST131 ($n = 38$; 70%) and ST648 ($n = 12$; 80%) isolates were resistant to both levofloxacin and trimethoprim-sulfamethoxazole, compared to 14 (39%) of non-ST131/648 isolates (ST131 versus non-ST131/648, $P = 0.004$; ST648 versus non-ST131/648, $P = 0.013$).

We next analyzed and compared the profiles of resistance genes among the three isolate groups (Table 3). Overall, ST131 and ST 648 isolates had resistance genes more frequently than non-ST131/648. Importantly, *aac(3)-IIa* was most frequently identified in ST648 isolates, which would explain the higher rates of resistance to gentamicin in ST648 isolates. $bla_{CTX\text{-}M\text{-}15}$ was commonly isolated from all 3 groups.

To further elucidate the microbiological characteristics of ESBL-producing *E. coli* ST131 and ST648, we investigated the virulence-associated traits (Table 4). Overall, ST131 isolates had a

TABLE 2 Susceptibility profiles among extended-spectrum-β-lactamase-producing *Escherichia coli* isolates in Nepal

Antibiotic and parameter	Value for: Whole cohort (n = 105)	*E. coli* ST131 (n = 54)	*E. coli* ST648 (n = 15)	*E. coli* non-ST 131/648[b] (n = 36)	*P* value[a]: ST131 vs ST648	ST131 vs non-ST 131/648	ST648 vs non-ST 131/648
Levofloxacin							
No. (%) of resistant isolates[c]	92 (87.6)	52 (96.3)	15 (100)	25 (69.4)	>0.999	**0.001**	**0.022**
MIC_{50}, MIC_{90} (μg/ml)	>4, >4	>4, >4	>4, >4	>4, >4	NA		
Gentamicin							
No. (%) of resistant isolates	41 (39)	17 (31.5)	12 (80)	12 (33.3)	**0.001**	>0.99	**0.005**
MIC_{50}, MIC_{90}	2, >8	2, >8	>8, >8	2, >8	NA		
Amikacin							
No. (%) of resistant isolates	11 (10.5)	5 (9.3)	3 (20)	3 (8.3)	0.358	>0.99	0.343
MIC_{50}, MIC_{90}	8, 32	8, 16	8, 32	≤4, 16	NA		
Trimethoprim-sulfamethoxazole							
No. (%) of resistant isolates	69 (65.7)	39 (72.2)	12 (80)	18 (50)	0.743	**0.045**	0.064
MIC_{50}, MIC_{90}	>2/38, >2/38	>2/38, >2/38	>2/38, >2/38	≤2/38, >2/38	NA		
Minocycline							
No. (%) of resistant isolates	34 (32.4)	2 (3.7)	15 (100)	17 (47.2)	**<0.001**	**<0.001**	**<0.001**
MIC_{50}, MIC_{90}	4, >8	2, 4	>8, >8	4, >8	NA		
Amoxicillin-clavulanic acid							
No. (%) of resistant isolates	76 (72.4)	40 (74.1)	13 (86.7)	23 (63.9)	0.492	0.352	0.177
MIC_{50}, MIC_{90}	16/8, >16/8	16/8, >16/8	>16/8, >16/8	16/8, >16/8	NA		
Cefoperazone-sulbactam							
No. (%) of resistant isolates	32 (30.5)	19 (35.2)	2 (13.3)	11 (30.6)	0.125	0.82	0.297
MIC_{50}, MIC_{90}	≤16/8, 32/16	≤16/8, 32/16	≤16/8, ≤16/8	≤16/8, 32/16	NA		
Cefmetazole							
No. (%) of resistant isolates	5 (4.8)	0	0	5 (13.9)	NA	**0.009**	0.305
MIC_{50}, MIC_{90}	≤4, 8	≤4, ≤4	≤4, ≤4	≤4, 32	NA		
Flomoxef							
No. (%) of resistant isolates	2 (1.9)	0	0	2 (5.6)	NA	0.157	>0.99
MIC_{50}, MIC_{90}	≤8, ≤8	≤8, ≤8	≤8, ≤8	≤8, ≤8	NA		
Fosfomycin							
No. (%) of resistant isolates	0	0	0	0	>0.999	0.532	>0.999
MIC_{50}, MIC_{90}	≤4, 16	≤4, 16	≤4, 16	≤4, 16	NA		
Both levofloxacin and trimethoprim-sulfamethoxazole							
No. (%) of resistant isolates	64 (61)	38 (70.4)	12 (80)	14 (38.9)	0.534	**0.004**	**0.013**

[a] Bold *P* values represent statistically significant results. NA, data not available.
[b] ESBL-producing *E. coli* isolates with ST other than ST131 and ST648.
[c] Including intermediate and resistant isolates, based on 2013 CLSI criteria (M100-S23) unless otherwise noted.

higher prevalence of multiple virulence genes than ST648 and non-ST131/648 isolates, except for *hlyD*, which was more prevalent in ST648 isolates (n = 8; 53%) than in ST131 (n = 9; 17%) and non-ST131/648 (n = 11; 31%) isolates (ST131 versus ST648, P = 0.007; ST131 versus non-ST131/648, P = 0.131; ST648 versus non-ST131/648, P = 0.203). The median virulence score was highest in the ST131 group (score = 9; interquartile range [IQR], 6 to 10), followed by ST648 (score = 7; IQR, 5 to 8) and non-ST131/648 (score = 5; IQR, 3 to 7).

The clinical characteristics of patients with ESBL-producing *E. coli* isolates were also evaluated as a function of ST (Table 5). The mean age of the study cohort was 40.7 years (±23.2), and 39 patients (37%) were male. With regard to the demographics of the patients, the underlying conditions, the severity of illness, and the duration of hospitalization, there were no statistically significant differences among the ST groups. In female patients, the prevalence of pregnancy was high (n = 16; 24%), and in male patients, benign prostatic hyperplasia (n = 6; 15%) was common. There was a tendency that the ST131 group (n = 4; 8%) received appropriate empirical antimicrobial therapy less frequently than the non-ST131/648 group (n = 8; 23%); however, this trend did not reach statistical significance (P = 0.057).

TABLE 3 Resistance genes among extended-spectrum-β-lactamase-producing *Escherichia coli* isolates in Nepal

Drug and resistance gene	No. (%) of isolates				*P* value[a]		
	Whole cohort ($n = 105$)	*E. coli* ST131 ($n = 54$)	*E. coli* ST648 ($n = 15$)	*E. coli* non-ST 131/648[b] ($n = 36$)	ST131 vs ST648	ST131 vs non-ST 131/648	ST648 vs non-ST 131/648
Aminoglycoside							
aac(3)-IIa	37 (35.2)	15 (27.8)	12 (80)	10 (27.8)	**0.001**	>0.999	**0.001**
aac(3)-IId	3 (2.9)	1 (1.9)	0	2 (5.6)	>0.999	0.561	>0.999
aph(3′)-Ia	2 (1.9)	2 (3.7)	0	0	>0.999	0.515	NA
aph(3′)-Ic	1 (1)	0	0	1 (2.8)	NA	0.4	>0.999
aadA1	3 (2.9)	0	0	3 (8.3)	NA	0.061	0.54
aadA2	10 (9.5)	8 (14.8)	0	2 (5.6)	0.186	0.305	>0.999
aadA5	59 (56.2)	31 (57.4)	12 (80)	16 (44.4)	0.14	0.283	**0.03**
strA/B	32 (30.5)	20 (37)	1 (6.7)	11 (30.6)	**0.027**	0.652	0.083
Fluoroquinolone							
qnrB4	1 (1)	0	0	1 (2.8)	NA	0.4	>0.999
qnrS1	3 (2.9)	0	0	3 (8.3)	NA	0.061	0.546
qepA	2 (1.9)	0	0	2 (5.6)	NA	0.157	>0.999
Aminoglycoside and fluoroquinolone							
aac(6′)Ib-cr	69 (65.7)	39 (72.2)	14 (93.3)	16 (44.4)	0.163	**0.015**	**0.001**
Beta-lactams							
$bla_{\text{CTX-M-15}}$	99 (94.3)	51 (94.4)	15 (100)	33 (91.7)	>0.999	0.68	0.546
$bla_{\text{CTX-M-27}}$	2 (1.9)	2 (3.7)	0	0	>0.999	0.515	NA
$bla_{\text{OXA-1}}$	69 (65.7)	38 (70.4)	14 (93.3)	17 (47.2)	0.093	**0.046**	**0.004**
$bla_{\text{TEM-1B}}$	41 (39)	20 (37)	12 (80)	9 (25)	**0.004**	0.258	**<0.001**
$bla_{\text{SHV-12}}$	1 (1)	0	1 (6.7)	0	0.217	NA	0.294
$bla_{\text{CMY-42}}$	1 (1)	0	0	1 (2.8)	NA	0.4	>0.999
$bla_{\text{DHA-1}}$	1 (1)	0	0	1 (2.8)	NA	0.4	>0.999
Macrolide							
mphA	76 (72.4)	46 (85.2)	12 (80)	18 (50)	0.694	**0.001**	0.064
ermB	5 (4.8)	2 (3.7)	1 (6.7)	2 (5.6)	0.527	>0.999	>0.999
Chloramphenicol							
catA1	13 (12.4)	0	9 (60)	4 (11.1)	**<0.001**	**0.023**	**0.001**
catB3	66 (62.9)	37 (68.5)	14 (93.3)	15 (41.7)	0.093	**0.016**	**0.001**
Sulfonamide							
sul1	70 (66.7)	39 (72.2)	12 (80)	19 (52.8)	0.743	0.074	0.115
sul2	32 (30.5)	20 (37)	1 (6.7)	11 (30.6)	**0.027**	0.652	0.083
Trimethoprim							
dfrA12	10 (9.5)	8 (14.8)	0	2 (5.6)	0.186	0.305	>0.999
dfrA17	58 (55.2)	31 (57.4)	12 (80)	15 (41.7)	0.14	0.197	**0.016**
dfrA1	2 (1.9)	0	0	2 (5.6)	NA	0.157	>0.999
dfrA5	1 (1)	0	0	1 (2.8)	NA	0.4	>0.999
Tetracycline							
tetA	50 (47.6)	40 (74.1)	2 (13.3)	8 (22.2)	**<0.001**	**<0.001**	0.703
tetB	31 (29.5)	1 (1.9)	14 (93.3)	16 (44.4)	**<0.001**	**<0.001**	**0.001**
tetD	1 (1)	0	0	1 (2.8)	NA	0.4	>0.999

[a] Bold *P* values represent statistically significant results. NA, data not available.
[b] ESBL-producing *E. coli* isolates with ST other than ST131 and ST648.

We also searched for mobile acquired elements carrying resistance genes in the isolates (Tables 6 and 7). We identified five mobile acquired elements carrying multiple resistance genes: pEC958 (*E. coli* O25b:H4-ST131 strain EC958, plasmid pEC958), pKF3-140 (*Klebsiella pneumoniae* strain KF3, plasmid pKF3-140), IS*26* (*E. coli* DNA, insertion sequence IS*26*, insertion sequence ISEcp1, blaCTX-M-27), p6234 (*K. pneumoniae* strain 6234, plasmid p6234), and pEK499 (*E. coli* strain A, plasmid pEK499). The pEC958 element was the most common, and was identified in 70 isolates, followed by p6234, which was identified in 64 isolates, and pKF3-140, which was identified in 20 isolates. Eleven isolates carried three mobile acquired elements (pEC958, pKF3-140, and

TABLE 4 Virulence-associated traits among extended-spectrum-β-lactamase-producing *Escherichia coli* isolates in Nepal

Virulence-associated trait and gene[a]	No. (%) of isolates				*P* value[b]		
	Whole cohort ($n = 105$)	*E. coli* ST131 ($n = 54$)	*E. coli* ST648 ($n = 15$)	*E. coli* non-ST 131/648[c] ($n = 36$)	ST131 vs ST648	ST131 vs non-ST 131/648	ST648 vs non-ST 131/648
Adhesin							
papA	68 (64.8)	54 (100)	4 (26.7)	10 (27.8)	**<0.001**	**<0.001**	>0.999
papG II	60 (57.1)	35 (64.8)	10 (66.7)	15 (41.7)	>0.999	0.051	0.132
sfa/foc	1 (1)	0	0	1 (2.8)	NA	0.4	>0.999
focG	1 (1)	0	0	1 (2.8)	NA	0.4	>0.999
iha	70 (66.7)	53 (98.1)	4 (26.7)	13 (36.1)	**<0.001**	**<0.001**	0.746
hra	24 (22.9)	11 (20.4)	1 (6.7)	12 (33.3)	0.44	0.219	0.076
Toxin							
hlyD	28 (26.7)	9 (16.7)	8 (53.3)	11 (30.6)	**0.007**	0.131	0.203
cnf1	12 (11.4)	9 (16.7)	0	3 (8.3)	0.189	0.349	0.546
sat	71 (67.6)	54 (100)	5 (33.3)	12 (33.3)	**<0.001**	**<0.001**	>0.999
vat	3 (2.9)	0	0	3 (8.3)	NA	0.061	0.546
Siderophore							
iroN	6 (5.7)	0	0	6 (16.7)	NA	0.003	0.162
fyuA	92 (87.6)	53 (98.1)	14 (93.3)	25 (69.4)	0.39	**<0.001**	0.083
ireA	6 (5.7)	2 (3.7)	0	4 (11.1)	>0.999	0.213	0.307
iutA	83 (79)	54 (100)	14 (93.3)	15 (41.7)	0.217	**<0.001**	**0.001**
Capsule							
kpsM II	3 (2.9)	0	0	3 (8.3)	NA	0.061	0.546
K1 *kpsM*	2 (1.9)	0	0	2 (5.6)	NA	0.157	>0.999
K5 *kfiC*	14 (13.3)	11 (20.4)	1 (6.7)	2 (5.6)	0.44	0.067	>0.999
Miscellaneous							
usp	58 (55.2)	54 (100)	0	4 (11.1)	**<0.001**	**<0.001**	0.307
traT	87 (82.9)	47 (87)	15 (100)	25 (69.4)	0.333	0.059	**0.022**
ompT	71 (67.6)	54 (100)	11 (73.3)	6 (16.7)	**0.002**	**<0.001**	**<0.001**
H7 *fliC*	2 (1.9)	0	0	2 (5.6)	NA	0.157	>0.999
malX	104 (99)	54 (100)	15 (100)	35 (97.2)	NA	0.4	1.0
Median virulence score (IQR)[d]	9 (6–10)	9 (9–10)	7 (5–8)	5 (3–7)	**<0.001**	**<0.001**	0.074

[a] *papG* III, *pic*, and *ibeA* were tested, with no positive isolates detected.
[b] Bold *P* values represent statistically significant results. NA, data not available.
[c] ESBL-producing *E. coli* isolates with ST other than ST131 and ST648.
[d] The virulence score represents the number of virulence genes detected, with *pap* elements counting collectively as a single trait (17).

p6234), 10 of which belonged to ST131. All 64 isolates that carried p6234 also carried pEC958. All isolates which had these mobile elements had >95% identity at the nucleotide level and >80% coverage level.

DISCUSSION

To our knowledge, this is the first study to reveal the microbiological and clinical epidemiology of ESBL-producing *E. coli* in Nepal. We discovered that more than 90% of the ESBL-producing *E. coli* isolates in Nepal were CTX-M-15 positive, and more than half were ST131 isolates. All 54 ESBL-producing *E. coli* ST131 isolates were identified as the O25b-ST131 clone, subclone H30-Rx, which have been reported to be more antibiotic resistant and to have virulence profiles (16). These findings correlate with the multiple reports on a global spread of CTX-M-15-positive O25b-ST131 clones (21).

Another key finding of this study is the discovery that ST648 isolates made up 14% of ESBL-producing *E. coli* in Nepal and that their microbiological characteristics are distinct from those of ST131 isolates. Human isolates of CTX-M-producing *E. coli* ST648 have been reported from various geographical regions, such as Europe, North and South America, Africa, and Asia; the majority of these isolates belong to phylotype D (22–25). CTX-M-producing *E. coli* ST648 strains have also been isolated from wild birds in Mongolia and Germany (24, 26–28) and from companion and livestock animals in European countries (24, 29, 30). A recent study on ESBL-producing *E. coli* isolates ($n = 1,152$) sampled in Europe, predominantly from dogs, cats, and horses, identified 40 (3.5%) ESBL-producing *E. coli* strains as ST648 (phylotype D, CTX-M-15 positive; 72.5%), whereas ST131 isolates (phylotype B2, CTX-M-15-positive; 46.9%) occurred less frequently (2.8%) (30). The authors also found that a higher proportion of ST648 strains showed resistance to most non-β-lactam antibiotics, and virulence genes were less abundant in ST648 strains than in ST131strains (30). These findings are concordant with the current study. To our knowledge, this is the first study to systematically evaluate the microbiological and clinical characteristics of human isolates of CTX-M-producing ST648 strains, all of which were

TABLE 5 Bivariate analysis of clinical characteristics of patients with isolation of extended-spectrum-β-lactamase producing *Escherichia coli* as a function of sequence type

Parameter	Value[a] for:				*P* value		
	Whole cohort ($n = 105$)	*E. coli* ST131 ($n = 54$)	*E. coli* ST648 ($n = 15$)	*E. coli* non-ST 131/648[b] ($n = 36$)	ST131 vs ST648	ST131 vs non-ST 131/648	ST648 vs non-ST 131/648
Demographics							
Mean age, yr (SD)	40.7 (23.2)	41.7 (22.8)	45.7 (27.3)	37 (22.1)	0.541	0.266	0.251
Male patients	39 (37.1)	19 (35.2)	6 (40)	14 (38.9)	0.767	0.824	>0.999
Inpatients	65 (61.9)	35 (64.8)	10 (66.7)	20 (55.6)	>0.999	0.388	0.543
Departments							
Medicine	40 (38.1)	20 (37)	7 (46.7)	13 (36.1)	0.558	>0.999	0.539
Surgery	21 (20)	12 (22.2)	1 (6.7)	8 (22.2)	0.27	>0.999	0.251
Obstetrics and gynecology	22 (21)	13 (24.1)	2 (13.3)	7 (19.4)	0.494	0.796	0.709
Pediatrics	11 (10.5)	6 (11.1)	2 (13.3)	3 (8.3)	>0.999	0.736	0.624
Underlying conditions							
Benign prostatic hyperplasia	6 (15.4)	4 (21.1)	1 (6.7)	1 (7.1)	>0.999	0.366	0.521
Urolithiasis	6 (5.7)	2 (3.7)	0	4 (11.1)	>0.999	0.213	0.307
Uterine prolapse	6 (5.7)	4 (7.4)	0	2 (5.6)	0.57	>0.999	>0.999
Pregnancy	16 (24.2)	9 (25.7)	2 (22.2)	5 (22.7)	>0.999	>0.999	>0.999
Malignancy	3 (2.9)	2 (3.7)	0	1 (2.8)	>0.999	>0.999	>0.999
Diabetes mellitus	3 (2.9)	2 (3.7)	1 (6.7)	0	0.527	0.515	0.294
Chronic obstructive pulmonary disease	8 (7.6)	2 (3.7)	2 (13.3)	4 (11.1)	0.204	0.213	>0.999
Severity of illness							
Sepsis	10 (9.5)	6 (11.1)	2 (13.3)	2 (5.6)	>0.999	0.468	0.571
Antimicrobial therapy							
Appropriate empirical antimicrobial therapy	14 (13.6)	4 (7.5)	2 (13.3)	8 (22.9)	0.607	0.057	0.702
Median duration of hospitalization, days (IQR)	10 (8–14)	10 (8–13)	10 (6–14)	11 (9–16)	0.38	0.412	0.202

[a] Values are number (%) unless otherwise indicated.
[b] ESBL-producing *E. coli* isolates with ST other than ST131 and ST648.

TABLE 6 Mobile acquired elements carrying resistance genes among ESBL-producing *E. coli* isolates in Nepal

Sequence type	Isolate no.	Mobile acquired elements carrying resistance genes[a]				
		pEC958	pKF3-140	IS*26*	p6234	pEK499
ST131	1	+			+	
	2	+			+	
	3	+			+	
	5	+	+		+	
	8	+				
	14	+			+	
	16	+			+	
	19		+	+		
	23					+
	24	+			+	+
	25	+	+		+	
	26	+	+			
	28	+			+	+
	29	+	+		+	
	30	+			+	
	37	+			+	
	38	+	+		+	
	43	+	+		+	
	45	+	+		+	
	47	+			+	
	50	+			+	
	52	+	+			
	59	+			+	
	60	+				
	61	+	+		+	
	63	+			+	
	64	+			+	
	67	+				
	68	+			+	
	69	+	+		+	
	70		+			
	72	+			+	
	75	+	+		+	
	78	+			+	
	79	+			+	
	80	+			+	
	85	+			+	
	86	+			+	
	87	+			+	
	90	+	+			
	92	+	+	+		
	96	+			+	
	99	+			+	+
	101	+			+	
	102	+	+		+	
	104	+	+		+	
ST648	7	+			+	
	15	+			+	
	22	+			+	
	36	+			+	
	40	+			+	
	42	+			+	
	51	+			+	
	57	+			+	
	62	+			+	
	65	+			+	
	76	+			+	
	82	+			+	
	95	+			+	
	98	+			+	
ST405	12	+		+	+	
	31	+	+			+
	58	+			+	
	71	+			+	

TABLE 6 (Continued)

Sequence type	Isolate no.	Mobile acquired elements carrying resistance genes[a]				
		pEC958	pKF3-140	IS*26*	p6234	pEK499
ST38	77	+			+	
	81	+	+			
ST167	74	+				
ST361	105	+				
ST410	6	+			+	
	55	+			+	
ST44	27	+			+	
ST517	97	+			+	
ST617	46	+				
ST1312	10	+			+	
New ST	34	+			+	
	35	+			+	
	39	+			+	
	56	+			+	
	100	+	+		+	

[a] pEC958, *Escherichia coli* O25b:H4-ST131 strain EC958 plasmid pEC958; National Center for Biotechnology Information (NCBI) accession no. HG941719.1. pKF3-140, *Klebsiella pneumoniae* strain KF3 plasmid pKF3-140; NCBI accession no. FJ876827.1. IS*26*, *E. coli* DNA, insertion sequence IS*26*, insertion sequence ISEcp1, *blaCTX-M-27*; NCBI accession no. AB976590.1. p6234, *K. pneumoniae* strain 6234 plasmid p6234; NCBI accession no. CP010390.1. pEK499, *E. coli* strain A plasmid pEK499; NCBI accession no. EU935739.1.

positive for CTX-M-15. The fact that ST648 isolates, which show a higher drug resistance than ST131 isolates and contain profuse virulence genes, comprised 14% of all ESBL-producing *E. coli* isolates in Nepal is a concerning observation. In addition, previous reports have suggested the spread of CTX-M-producing ST648 strains among humans and animals worldwide (24). These find-

TABLE 7 Numbers of mobile acquired elements carrying resistance genes in each ST

Sequence type	No. (%) carrying resistance genes[a]				
	pEC958	pKF3-140	IS26	p6234	pEK499
ST131	43 (80)	17 (31)	2 (4)	36 (67)	4 (7)
ST648	14 (93)			14 (93)	
ST405	4 (80)	1 (20)	1 (20)	3 (60)	1 (20)
ST38	2 (67)	1 (33)		1 (33)	
ST167	1 (50)				
ST361	1 (50)				
ST410	2 (100)			2 (100)	
ST44	1 (100)			1 (100)	
ST517	1 (100)			1 (100)	
ST617	1 (100)				
ST1312	1 (100)			1 (100)	
New ST	5 (63)	1 (13)		5 (63)	
Total	76	20	3	64	5

[a] pEC958, *Escherichia coli* O25b:H4-ST131 strain EC958 plasmid pEC958; National Center for Biotechnology Information (NCBI) accession no. HG941719.1. pKF3-140, *Klebsiella pneumoniae* strain KF3 plasmid pKF3-140; NCBI accession no. FJ876827.1. IS*26*, *E. coli* DNA, insertion sequence IS*26*, insertion sequence ISEcp1, *blaCTX-M-27*; NCBI accession no. AB976590.1. p6234, *K. pneumoniae* strain 6234 plasmid p6234; NCBI accession no. CP010390.1. pEK499, *E. coli* strain A plasmid pEK499; NCBI accession no. EU935739.1.

ings raise the concern that ST648 strains could present another pandemic clone of ESBL-producing *E. coli*.

Other STs found in isolates from Nepal in this study have also been reported to be associated with ESBL production in *E. coli* and have been found in wildlife, livestock, and humans (24). The spread of ESBL-producing *E. coli* ST405, ST38, ST315, and ST410 has been reported in animals in Europe, as well as in humans worldwide (24). ESBL-producing *E. coli* ST393 was isolated from livestock animals in Germany and from humans globally (24). Global spread of ESBL-producing *E. coli* ST10, ST617, and ST167 has also been reported (24).

Due to the limited information available from medical charts, we could not evaluate detailed clinical variables in the current study. Nevertheless, it is noteworthy that more than 38% of ESBL-producing *E. coli* strains were isolated from the outpatient clinic, and about a quarter of patients with ESBL-producing *E. coli* were pregnant women. This would suggest a wide spread of CTX-M-producing *E. coli* across the communities in Nepal. Combined with the high resistance to orally available antibiotics, these community-isolated ESBL-producing *E. coli* strains present an emerging challenge for community practitioners and hospitals worldwide.

Data on the effectiveness of treatment using cephamycins and oxacephems for the ESBL-producing organisms are scarce (31, 32), and further studies regarding the optimal clinical uses of these drugs for the treatment of ESBL-producing *E. coli* are warranted.

In conclusion, we revealed that the high resistance of ESBL-producing *E. coli* to multiple classes of antibiotics in Nepal is driven mainly by CTX-M-producing ST131 and ST648 strains. Their prevalence in communities is a matter of great concern, and further studies are necessary to identify the epidemiology of CTX-M-ST648 to control the spread of ESBL-producing *E. coli*.

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We have no conflict of interest to declare.

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Original article

Assessment of Health Systems in Relation to Interface Between Malaria Control Programs and Health System Strengthening: Comparative Study Between Nepal and Viet Nam

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Abstract

Introduction: Malaria control has been a major health issue with high priority in endemic countries and various efforts have been made with the support of foreign assistant partners. In order to implement efficient and sustainable control, integration of the control program into general health system or effective interactions between them is one of the important strategies.

Methods: Studies were conducted in Nepal and Viet Nam. Information obtained from document reviews, interviews, and field surveys were analyzed from the viewpoint of interface between malaria control program and the health system in accordance with six building blocks of a health system, with special emphasis on good practices and challenges in the implementation of the malaria control program.

Results: Among good practices, strong government commitment towards the control programs to strengthen facilities and capacity of health workers at the primary level, utilization of health volunteers, setting up mobile team and intensified education for residents were noteworthy. Key challenges mainly involved remote areas. Introduction of malaria due to population movement and the emergence of new endemic areas have become growing issues. While strengthening of the vertical health program appeared to have some impact on the general health system, particularly at the primary level, dissociation between the vertical control program and horizontal general health system still remains.

Conclusion: It is crucial to implement an effective and equitable malaria control program that responds to these existing challenges and can create a sustainable health system. Addressing these issues will lead to further strengthening of the health system there and eventually lead to the effective implementation of various health programs.

Key words: Malaria control, Health system, Nepal, Viet Nam

Introduction

Over the past decades malaria control has been implemented intensively with the support of Global Fund against AIDS, Tuberculosis and Malaria (GFATM)and other assistant partners as one of the most important disease control programs, and good results have been obtained in some endemic countries.[1-3] Malaria control programs have been implemented under global malaria control strategies although key strategies have varied over times: spraying

of pesticide, community-based control, school health-based control, Roll Back Malaria etc. In recent years, the importance of disease control measures in relation to health system strengthening (HSS), particularly the integration of a control program (vertical health system) into the general health system (horizontal health system) with the aim of achieving a synergic effect among health programs, has been stressed as an important strategy.[4-6]

In the process of disease control, good practices have been recognized, but interaction between disease specific control programs and HSS has been debated, namely whether disease specific programs have actually contributed to the strengthening of the general health system and whether disease control programs are well integrated into the general health system.[7, 8]

In the program implementation process, smooth implementation of the disease specific control program has often been hindered by challenges or bottle necks that exist in the health system. Through the effective intervention to these challenges/bottlenecks,expansion of the health programs and strengthening of the general health system are expected, bringing a synergistic effect on other disease specific programs and furthermore general health system.

This study was undertaken in order to assess the interface between disease specific programs supported by GFATM and HSS, with special reference to interaction between malaria control programs and general health systems.

Methods

The primary surveys were conducted for Viet Nam (2009) and Nepal (2012), by setting the National Malaria Control Programs supported by GFATM as entry points with special emphasis on good practices and challenges/ bottlenecks in implementing malaria control program in relation to the components of health system. Data from the two countries was updated as long as possible, based on information obtained by the supplemental survey during 2012-14 and by documents. In each country, the survey was conducted at various levels (from central to primary level) by document reviews, key informant interviews, and observation of facilities. The results of the primary surveys were analyzed, summarized in reports and submitted to WHO Western Pacific Regional Office.[9]

Document reviews

Documents related to health systems, National Malaria Control Programs (NMCP), health statistics, GFATM supports, etc. were collected and reviewed.[10-15]

Key informant interviews

Key informant interviews were conducted with the health staff at each level (central, regional, district, and community level) of the health facility, health managers at the district and provincial levels, and program managers of Ministry of Health (MOH in Viet Nam) or Ministry of Health and Population (MOHP in Nepal),WHO, NGOs, and other partner agencies. Leading contents of the interview included an outline of the health system, general information on malaria control programs in relation to the health system. Good practices and strengths to overcome challenges/bottlenecks, and on-going interventions for existing bottlenecks, integration between NMCP and general health system, etc., were also considered

Field surveys

In addition to surveying the capital cities of each country, malaria endemic areas, where Malaria Control Programs have been implemented with the support of GFATM, were selected (Thanh Hoa and Dien Bien Provinces in Viet Nam, and Dhanusha District and Hetauda City in Nepal). General information on health and medical care, information on health system and health program implementation, etc. were collected at Provincial Health Offices and District Health Offices, followed by surveys at health facilities at the primary level (or community level).

Analysis

Information obtained from the interviews, field surveys, and documents were summarized from the viewpoint of interface between malaria control programs and the general health system in accordance with the six building blocks (components) of a health system (Leadership and governance, Service delivery, Workforce, Information system, Medical products and technology, and Financing) proposed by WHO.[16] Good practices and bottlenecks/ challenges in the implementation of the control programs were identified, and possible solutions for the challenges/ bottlenecks were discussed.

Results

1. Overview of malaria conditions in Nepal and Viet Nam

In the two countries in this survey, Viet Nam and Nepal, malaria morbidity and mortality rates were considerably high in the past and malaria was given the highest priority in the health policy by the governments. Since the mid-1990s, malaria controls were actively implemented in these countries based on the National Malaria Control Programs (NMCPs) and the principles of Roll Back Malaria, which

consisted of strategic priorities including vector control and personal protection through the distribution of bednets (insecticide-treated bed nets; ITNs and long lasting insecticidal nets; LLINs), early diagnosis and prompt treatment (EDPT)including rapid diagnosis tests (RDTs) and artemis in combination therapies (ACTs), malaria surveillance and epidemic preparedness, behavioral change communication (BCC), and improvement in program management along with setting up targets for control. In particular, since the early 2000s, GFATM has contributed a large budget to malaria control programs (60-65% in Viet Namand70-78% in Nepal of the total malaria control budget). As a result, malaria in these countries has decreased remarkably in recent years, reaching pre-elimination levels.

2. Outline of general health systems in Nepal and Viet Nam

Health networks in the two target countries have been created at the central to commune level in accordance with the administrative strata (e.g. in Nepal; central－regional－district－commune levels,in Viet Nam; central－provincial－district－commune levels). Treatment care systems as well as preventive care systems have also been basically constructed in accordance with the administrative strata. (i.e. central, provincial (or regional) and district hospitals and commune health stations, etc.).A referral system, as well as health information and supply systems, function based on this network. In addition, some health control programs have their own systems (e.g. national institutes, provincial or regional control centers, district control centers).

Commune Health Stations (CHSs) provide health care at the primary level and under the CHSs, there are health posts (HPs) and sub-health posts (SHPs). These CHSs along with HPs and SHPs have the tasks of providing primary health care services, first-aid and treatment, implementation of national health programs, assisting in normal deliveries, family planning practices, health promotion, etc. There are commune (village) health workers in each commune, under the direct management and direction of the CHSs and commune leaders.

3. Characteristics of malaria control in relation to the health system

A: Characteristics of general health system (horizontal health system)

B: Characteristics of malaria control (vertical health system)

3-1 Nepal

Leadership and governance

A: The current long-term health plan (1997-2017) aims to provide health services throughout the country, particularly extending the primary health care system to the rural population and improving the health status of vulnerable populations, such as women and children, the rural population, the poor, the underprivileged and the marginalized. In recent years, the government has attached high importance to the promotion of education among residents and many primary schools have been constructed.

B: Since the launch of the Insect Borne Disease Control Program in 1954, the government has given malaria control high priority, and since 1993, the Epidemiology and Disease Control Division (EDCD) under MOHP has taken the lead in the malaria control program. Currently, the control program is basically implemented using the existing health system.

Service delivery

A: Efforts have been made to design a health system hierarchy from MOPH to the primary level, in order to ensure that the majority of the population has access to public health care facilities and can receive minor treatment at affordable prices. The government has strengthened the HPs and SHPs, which serve as first contact to basic health services and the venue for community-based activities with the support of GFATM. The pull system for essential drug supply was expanded to all 75 districts in 2010.

B: Delivery of LLINs is managed by the Population Service International (PSI).At local level, distribution is managed by NGOs/other partners and implementation is carried out by a broad range of community based organizations. Monitoring teams for ITNs and LLINs were organized at the district level. Mobile teams were organized, and are responsible for prevention, diagnosis and treatment, in endemic areas. RDTs and microscopy have become available as diagnostic tools for malaria by HPs due to the support of GFATM, anda cold chain for vaccination was used for storage of RDTs.

Workforce

A: Since the establishment of the first school of medicine in 1980, the number has markedly increased (22 schools of medicine in 2014). The increased number of medical doctors, nurses, and other co-medical staff has contributed to increased health and medical care of the Nepalese people.

B: Female Community Health Volunteers (FCHVs) were organized at the commune level for integration activities including malaria control. Training on malaria control for the health staff has been integrated with other disease control and training has been conducted with the support of GFATM at the peripheral level.

Information system

A: The routine monitoring system has been improved over the years. The Health Management Information System (HMIS), Logistics Management Information System (LMIS) and Fiscal Management Information System (FMIS) have also been well developed over the last 10 years. Health related activities are recorded and reported from the lowest health unit right on up to the district hospitals. In addition to HMIS, other individual programs are also providers of information.

B: Sentinel sites were set up for malaria outbreak surveillance. Reporting and monitoring systems in the public sector, from the peripheral level up to MOHP level, have been strengthened.

Medical products and technologies

A: In 2007, the National Drug Strategy was revised and the National Essential Drug List was established. In order to manage the above processes effectively,HMIS is used.

B: A considerable amount of ITNs, LLINs, RDTs, ACTs, and slide glasses for microscopic testing were provided at the peripheral level with the support of GFATM.

Financing

A: The amount of the budget for health programs funded by the government, and the percentage of the health budget within the total budget, were increased (from 5.1% in 1998 to 6.3% in 2013).

B: Since 2004, GFATM has greatly contributed to the prevention and treatment of malaria, particularly through the distribution of LLINs, ACTs and RDTs in high risk areas, along with the training of health workers and BCC activities for the residents. Treatment drugs are administered to patients free of charge at public medical facilities nationwide and diagnostic services for malaria are provided free of charge at all public sector health facilities in high endemic areas.

3-2Viet Nam

Leadership and governance

A: Since the 1990s the government has worked hard to strengthen the general health system. The international community has also cooperated with policy implementation at various levels. For National Health Programs, National Steering Committees are organized and programs are managed more intensively and efficiently with strong leadership and inter sectoral collaboration. Related major national institutes are responsible for the executive centers of the respective health programs, as well as the provision of technical advice, operational research and staff training.

B: The government attached highest priority to malaria control in all health programs. NMCP, which began in 1991, has been reinforced by the strong leadership of the Government and National Steering Committee, which consists of multi-sector members,utilizing the vertical malaria control system along with general health system. The general health system has also been strengthened and used as a malaria control program. Recently, high priority has been attached to control in frontier areas. A high literacy rate, effective use of school health education, education for residents in endemic areas, and preparation of guidelines have also facilitated the smooth implementation of the program.

Service delivery

A: The local administration is organized into provincial, district and communal political units which are responsible for the implementation of the health programs. All medicines and medical equipment are supplied by the government through the administrative strata. Participation by health facilities under the military, police and other sectors providing medical services to the population has helped increase health care coverage. The referral system among medical institutions was strengthened in collaboration with foreign assistant partners.

B: Service delivery in malaria control was basically carried out utilizing the general health system. The Army Medicine, People's Committee, Women's Union and other local organizations helped deliver bed nets and other services in the control program.

Workforce

A: Since the 1990s, human resources in health care have been trained both quantitatively and qualitatively. Improvement in training capacity to increase human resources has been observed. Commune health workers have participated in various programs.

B: The government has implemented a policy to train health workers for malaria control with the cooperation of foreign donors. The role of public organizations, such as women's unions and youth unions, in the

implementation of malaria control programs and collaboration with the military in hard-to-reach areas, are also noteworthy.

Information system

A: The reporting and information system functions efficiently, and reports from the primary level are transmitted to upper levels. The role of the mobile team is outstanding in information transmission and guidance in program implementation.

B: Reports on malaria from the primary level are transmitted to upper levels and then feedback is provided. Currently, considerable parts of these systems are integrated into the general health system. There are many examples of prompt and appropriate responses in cases of disease outbreak. The role of the mobile team is outstanding in the transmission of information and in the provision of guidance for program implementation.

Medical products and technologies

A: Development of the pharmaceutical industry has contributed considerably to the implementation of essential drug policies targeting primary health care. Drug quality is managed following the good practice criteria based on standards and guidelines for drug production, quality control, storage and distribution. Currently, all facilities are to follow the standards of Good Manufacturing Practice (GMP)-WHO.

B: Production of drugs used for malaria treatment, insecticides, and bed nets within the control program has gradually shifted to local sources and the drugs are supplied to the peripheral level under the proper guidance of the government. Widespread distribution of artemis in suppositories at the primary level has greatly contributed to a lower mortality rate.

Financing

A: A broad orientation of health financing was set in the 1990s through the development of health insurance, the partial user fee policy, and the Government's resolution on "social mobilization" in areas of education, health and culture. The government also focused on subsidies to users of health services, such as health care for the poor and children under 6 years of age. The health budget has continued to increase in line with the economic development of the country.

B: Initially, the governments ought to increase the malaria program budget, and the People's Committee of Viet Nam and international community financially supported the program. GFATM has greatly contributed to the expansion of the malaria program by strengthening activities for high risk groups.

4. Good practices in malaria control

Table 1 summarizes the good practices in the general health system which are regarded as having a good effect on malaria control as shown in table. Table 2 summarizes the good practices in the malaria control programs.

5. Bottlenecks/challenges

Table 1 Good practices in the general health system (Strengthening of General Health System), which contributed to malaria control

	Leadership and governance
1	Effort of the government to strengthen the general health system
2	Efficient management of the National Health Programs with strong leadership (V)
3	High priority given to the promotion of education
	Service delivery
4	Strengthening of health posts and sub-health posts
5	Effective service delivery in accordance with the administrative strata (V)
6	Support of military and police to service delivery (V)
7	Contribution of the mobile teams
8	Expansion of the pull system for essential drug supply
	Workforce
9	Increased training opportunities for health workers.
10	Marked increase of the number of doctors and nurses (N)
11	Improved skills by commune health workers conducting various programs
	Information system
12	Improvement in the routine monitoring system
13	Contribution by the mobile teams (V)
	Medical products and technologies
14	Management of drug quality based on the standards and guidelines (V)
15	Development of the pharmaceutical industry and its contribution to the essential drug policy (V)
16	Revision of the National Drug Strategy (N)
	Health financing
17	Substantial support from the GFATM
18	Increase funding of the health budget by the government
19	Development of the health insurance, partial user fee policy, subsidies to users of health services (V)

(V): Outstanding in Viet Nam, (N): Outstanding in Nepal

Table 2 Good practices in malaria control

	Leadership and governance
1	High priority of malaria control by the government
2	Strong leadership of the government and the National Steering Committee (V)
3	Utilization of the general health system in the malaria control program
	Service delivery
4	Participation by the Army Medicine, People's Committee, Women's Union and other local organizations(V)
5	Monitoring teams for ITNs and LLINs at the district level (N)
6	Contribution by the mobile teams
	Workforce
7	Increased training opportunities for health workers
8	Contribution bywomen's unions, youth unions and military in hard-to-reach areas(V)
9	Contribution by Female Community Health Workers (N)
	Information system
10	Monitoring visits and periodical submission of reports
11	Setting up the sentinel sites for malaria outbreak surveillance(N)
12	Appropriate transmission of the information and feedback to primary level (V)
	Medical products and technologies
13	Provision of considerable amount of ITNs, LLINs, RDTs, ACTs
14	Domestic production of treatment drugs, insecticides and bed nets(V)
	Health financing
15	Considerable financial supportfor malaria control by GFATM
16	Treatment drugs provided free of charge at public facilities

(V): Outstanding in Viet Nam, (N): Outstanding in Nepal

ITNs: insecticide-treated bed nets, LLINs: long lasting insecticidal nets,

RDTs: rapid diagnosis tests, ACTs: artemisinin combination therapies

Table 3 Existing challenges and bottlenecks in malaria control

	A. Leadership and governance	Viet Nam	Nepal
1	Weak program management capacity		++
2	Introduction of malaria associated with population movement	++	++
3	Weak health system in remote (frontier & border) areas	+	++
4	Weak coordinationbetween medical institutions, public-private sectors and laboratories	+	++
	B. Service delivery	**Viet Nam**	**Nepal**
5	Inequality in the distribution of bed nets (to vulnerable people)		++
6	Many hard-to-reach areas		++
7	Weak coordination between local government and GFATM in the distribution of bed nets	+	++
	C. Workforce	**Viet Nam**	**Nepal**
8	Shortage of health workers and manpower in remote areas		++
9	Low skill level of health workers in remote areas	++	+
10	Frequent changes in health workers and manpower in remote areas	+	++
11	Limited number of entomologists	+	++
	D. Information system	**Viet Nam**	**Nepal**
12	Weak private health sector		+
13	Poorly developed reporting system from the private health sector	+	++
14	Inadequate disease surveillance system	++	+
	E. Medical products and technologies	**Viet Nam**	**Nepal**
15	Inadequate quality assurance system for malaria testing	+	++
16	Weak function of the National Reference Laboratory		++
17	Difficulty in treatment due to increasing anti-malaria drug resistance ofP. Falciparum	++	++
	F. Health financing	**Viet Nam**	**Nepal**
18	Sustainable supply of health products (ACT, RDT, LLIN)	+	++
19	Low incentive for health workers	++	++
20	Heavy dependence on GFATM (sustainability is a challenge)	+	++
	Other	**Viet Nam**	**Nepal**
21	New endemic areas have been reported (environmental and social factors are suspect)	++	++
22	Increased number of cases of imported malaria	+	++
++: major challenges/bottlenecks, +: intermediate, No mark: minor			

Discussion

The general health system in Nepal used to be fragile in the past, but has gradually been strengthened and has been utilized in greater part in the malaria control program.[17]During the period of political instability (1996-2006), health systems were affected, but malaria control was minimally affected compared to other disease control programs due to its high governmental priority and the continuous support of the international community.

The Vietnamese government has worked hard to strengthen the existing health systems since the 1990s (both malaria specific and general health systems). The international community has also cooperated with policy implementation of Viet Nam at various health system levels.[18]Malaria control measures were effectively implemented under the strong leadership of the National Steering Committee, which has further strengthened and utilized the existing health system and mobilized public organizations.

Generally, collaboration among disease specific programs at upper levels is limited, and a health staff, as well as an infrastructure, is dedicated to each program. However, health care at lower levels has greater integration(both in Nepal and Viet Nam). By coordinating with community and social organizations, health workers carry out various tasks, such as primary health care, implementation of national health programs, preventive medicine, IEC activities, etc. GFATM and other assistant partners provide support by promoting training and supplying essential medicine and equipment. Malaria control has gradually become integrated with the primary health care system.

Best practices were identified from survey results. Among these, intensified education for residents focusing on disease prevention, strengthening of facilities at the primary level such as health posts and training health workers, utilization of health volunteers at the primary level, giving frontier areas high priority, and setting up mobile teams, were noteworthy and were held in in common by both countries. In addition, effective implementation under the strong leadership of the National Steering Committee could be seen in Viet Nam, utilization of the existing heath system was outstanding.

A synergetic effect of disease specific programs(vertical health programs) such as malaria control on the general health system could be seen to some degree, particularly at the primary level. The management system of vertical health programs appeared to have a good impact on the general health system at various levels. However, dissociation between vertical malaria control program and horizontal general health system also seems to exist. In addition, similar to other reports,coordination between malaria control programs and other disease specific programs is limited in many cases.[5,6] It is true that carrying out a vertical health system is important in implementing a malaria control program, but intensification of the malaria control program does not automatically lead to strengthening of the general health system.[19]More effort is needed to realize maximum synergy between disease specific programs and the general health system, as well as among different health programs.

As seen in the results of a similar study in Laos, if the general health system appears weak, a strong vertical health system supported by GFATM can function separately from the general health system.[20,21] These findings were also observed in Nepal and Viet Nam, particularly at the early stage of support by GFATM, where disease specific programs utilized procurement, information, monitoring systems, etc., outside of the MOH (MOHP), with varying levels of support and input provided by the disease specific divisions.

One of the current leading challenges/bottle necks is the limited coverage and quality of malaria control measures among populations living in remote areas. Most health workers are concentrated in large cities and towns, while the health personnel and/or medical supplies of many health facilities at the primary level and at some district hospitals are still insufficient. A poorly developed reporting system from the private health sector, inadequate quality assurance system for malaria testing, and weak coordination between the local government and GFATM in the distribution of bed nets were also pointed out (particularly in Nepal). In Nepal it was suggested that bed nets are not always distributed to the vulnerable populations, as similarly reported in some African countries.[22, 23]In addition, the current heavy dependence on GFATM undermines the assurance of sustainable malaria control.

Introduction of malaria due to population movement, increased drug resistant malaria, and the emergence of new endemic areas have become growing issues in malaria control in recent years in many endemic countries. Although not always health system related, these issues do affect health systems and active health system strengthening seems to be crucial for their control. Such growing challenges are often related to political issues, poverty, and a changing environment due to indiscriminate development, global warming, etc.[24,25] In order to address these growing challenges, strong government leadership, a sector-wide approach, and inter sectoral collaboration are required.

It is crucial to implement effective malaria control programs which address these challenges and bottlenecks, seeking their elimination. Particularly, emphasis on strengthening the health systems in remote areas, training of the health staff at the peripheral level, diagnosis based on accurate quality assurance, promotion of public-private relationship and addressing the issue of imported malaria, are desired. To create sustainable health systems, serious consideration of issues regarding the availability of domestic resources, including workers, supplies and local participation, as well as budgetary resources, are needed. Moreover, good practices which have been identified in this survey are expected to provide useful lessons in the effective implementation of malaria control in endemic countries. Addressing these issues will directly lead to further strengthening of the health systems and eventually to the effective implementation of various health programs.

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Molecular epidemiology of multidrug-resistant *Acinetobacter baumannii* isolates in a university hospital in Nepal reveals the emergence of a novel epidemic clonal lineage

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ABSTRACT

The emergence of multidrug-resistant (MDR) *Acinetobacter baumannii* has become a serious medical problem worldwide. To clarify the genetic and epidemiological properties of MDR *A. baumannii* strains isolated from a medical setting in Nepal, 246 *Acinetobacter* spp. isolates obtained from different patients were screened for MDR *A. baumannii* by antimicrobial disk susceptibility testing. Whole genomes of the MDR *A. baumannii* isolates were sequenced by MiSeq™ (Illumina), and the complete genome of one isolate (IOMTU433) was sequenced by PacBio RS II. Phylogenetic trees were constructed from single nucleotide polymorphism concatemers. Multilocus sequence types were deduced and drug resistance genes were identified. Of the 246 *Acinetobacter* spp. isolates, 122 (49.6%) were MDR *A. baumannii*, with the majority being resistant to aminoglycosides, carbapenems and fluoroquinolones but not to colistin and tigecycline. These isolates harboured the 16S rRNA methylase gene *armA* as well as bla_{NDM-1}, bla_{OXA-23} or bla_{OXA-58}. MDR *A. baumannii* isolates belonging to clonal complex 1 (CC1) and CC2 as well as a novel clonal complex (CC149) have spread throughout a medical setting in Nepal. The MDR isolates harboured genes encoding carbapenemases (OXA and NDM-1) and a 16S rRNA methylase (ArmA).

1. Introduction

The emergence of multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR) *Acinetobacter baumannii* has become a serious medical problem worldwide [1]. MDR strains are defined as isolates not susceptible to at least one agent in three or more antimicrobial categories, XDR strains are defined as isolates not susceptible to at least one agent in all but two or fewer antimicrobial categories, and PDR strains are defined as isolates not susceptible to all classes of agent, including aminoglycosides, antipseudomonal carbapenems, antipseudomonal fluoroquinolones, antipseudomonal penicillins/β-lactamase inhibitors, extended-spectrum cephalosporins, folate pathway inhibitors, penicillins/β-lactamase inhibitors, polymyxins and tetracyclines [2]. MDR isolates are frequently involved in nosocomial outbreaks, mainly in intensive care units [3]. Nosocomial pneumonia caused by MDR isolates may affect critically ill patients, making them more difficult to treat [1].

Most MDR *A. baumannii* isolates harbour either genes or mutations in specific genes that are associated with resistance to antimicrobial agents [4]. For example, aminoglycoside-resistant strains may harbour genes encoding aminoglycoside-modifying enzymes (AMEs) and 16S rRNA methylases [5], whereas β-lactam-resistant isolates have been found to harbour genes encoding carbapenemases for all β-lactams except for monobactams [4]. Fluoroquinolone resistance has been associated with mutations in the quinolone resistance-determining region, which includes the *gyrA*

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and *parC* genes that encode DNA gyrase and topoisomerase IV, respectively [6].

Most MDR *A. baumannii* isolates resistant to aminoglycosides produce AMEs, including aminoglycoside *N*-acetyltransferases and aminoglycoside *O*-nucleotidyltransferases [7]. Increasing numbers of MDR *A. baumannii* isolates have been reported to produce 16S rRNA methylases [8]. To date, 10 types of plasmid-encoded 16S rRNA methylase genes have been identified in aminoglycoside-resistant clinical isolates (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *rmtF*, *rmtG*, *rmtH* and *npmA*), with isolates harbouring *armA* reported worldwide [8].

Carbapenemase-producing *A. baumannii* isolates have also shown increased global spread [1], with these carbapenemases identified as belonging to Ambler classes B and D [9]. Most of these isolates produce acquired carbapenem-hydrolysing class D β-lactamases (CHDLs), with others producing class B metallo-β-lactamases (MBLs) [4]. Carbapenem resistance in *A. baumannii* is mainly due to the production of CHDLs, including OXA-23, OXA-58, OXA-40 and OXA-143 [1,10]. Some carbapenem-resistant *A. baumannii* isolates produce an intrinsic OXA-51-like carbapenemase, whereas others produce class B MBLs, including IMP, NDM, SIM and VIM types [1].

This study analysed the molecular epidemiology of MDR *A. baumannii* isolates obtained from a university hospital in Nepal. The complete genome of one of these isolates, which belonged to a novel clonal complex 149 (CC149) lineage (ST622), was sequenced.

2. Materials and methods

2.1. Bacterial strains

A total of 246 *Acinetobacter* spp. isolates were obtained from 246 different patients treated at a university hospital in Kathmandu, Nepal, from September 2013 to June 2014. Clinical information on all patients and isolates is shown in Supplementary Table S1. One isolate (*A. baumannii* IOMTU433) was obtained in September 2013 from a sputum sample of a patient in a respiratory ward of the hospital. This patient had a history of acute exacerbation of chronic obstructive pulmonary disease with hypertension, type II diabetes mellitus and hypothyroidism. *Acinetobacter* spp. isolates were identified by biochemical methods and by the sequences of their 16S rRNA, *gyrB* and $bla_{\text{OXA-51-like}}$ genes.

Supplementary Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijantimicag.2015.07.012.

2.2. Antimicrobial susceptibility

Antimicrobial susceptibility testing was performed by the disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [11]. The disk method was used to screen MDR *Acinetobacter* spp. strains for susceptibility to amikacin, ampicillin/sulbactam, ciprofloxacin, cefepime, ceftazidime, ceftriaxone, doxycycline, gentamicin, imipenem, levofloxacin, meropenem, polymyxin, piperacillin/tazobactam and trimethoprim/sulfamethoxazole as described previously [2]. Carbapenem-resistant isolates were further screened for MBL production by a combination disk method using imipenem and ethylene diamine tetra-acetic acid (EDTA) [12]. Minimum inhibitory concentrations (MICs) were determined using the microdilution method as described in CLSI guidelines [11].

2.3. Whole-genome sequencing

Whole genomes of all of the MDR *A. baumannii* isolates were extracted using a DNeasy Blood & Tissue Kit (QIAGEN, Tokyo, Japan) and were sequenced by MiSeq™ (Illumina, San Diego, CA). More than 10-fold coverage was archived for each isolate. The whole-genome sequences of all 122 isolates have been deposited at GenBank as accession nos. DRX035998–DRX036119.

The whole genome of *A. baumannii* IOMTU433 was extracted using the cetyltrimethylammonium bromide DNA extraction procedure. Its entire genome was sequenced using the PacBio RS II platform (Pacific Biosciences, Menlo Park, CA) with a 20-kb insert library and P5-C3 chemistry. Prior to sequencing, libraries of >7 kb were selected using BluePippin targeted size selection system (Sage Science, Beverly, MA). De novo assembly was performed using the hierarchical genome assembly process (HGAP) workflow, including consensus polishing with Quiver. The resulting contigs were merged using Minimus2. The final, circularised assembly for the strain was polished again to finish the genome. Annotation of the genome sequences of the strain was accomplished by primary coding DNA sequence (CDS) extraction and initial functional assignment using the RAST automated annotation servers. The results were compared to verify the annotations and were corrected manually by in silico molecular cloning (In Silico Biology Inc., Kanagawa, Japan).

2.4. Phylogenetic analysis

Single nucleotide polymorphisms (SNPs) of the genome sequences of all MDR isolates tested were identified by comparison with the sequence of *A. baumannii* strain MDR-TJ (GenBank accession no. CP003500) [13], with all reads of each isolate aligned against the MDR-TJ sequence using CLC Genomics Workbench v.8.0.1 (CLC bio, Tokyo, Japan). SNP concatenated sequences were aligned using MAFFT (http://mafft.cbrc.jp/alignment/server/). Phylogenetic trees were constructed from the SNP concatemers. Two methods were used to evaluate robustness: a maximum-likelihood approach, PhyML 3.0; and the Bayesian MCMC framework, BEAST1. For PhyML, HKY and gamma were chosen for a nucleotide substitution model, and tree robustness was evaluated by an approximate likelihood-ratio test (aLRT). For BEAST, MCMC chains were run for 10 million generations, with sampling every 1000 generations. Convergences and effective sample sizes (ESSs) of the estimates were checked using Tracer v1.4 (http://beast.bio.ed.ac.uk/Tracer). Three of the phylogenetic trees constructed in this study had ESS values >200, suggesting sufficient mixing of the Markov chain.

2.5. Multilocus sequence typing (MLST) and drug resistance genes

MLST sequence types were deduced as described in the protocols of the Institut Pasteur MLST databases (http://pubmlst.org/abaumannii/). Clonal complexes were determined by eBURST v.3 (http://eburst.mlst.net). The sequences of drug resistance genes, including β-lactamase-encoding genes (http://www.lahey.org/studies), aminoglycoside resistance genes and quinolone resistance genes, were determined using CLC Genomics Workbench v.8.0.1.

3. Results

3.1. Screening for multidrug-resistant Acinetobacter baumannii

Of the 246 *Acinetobacter* spp. isolates tested, 129 unique MDR/XDR isolates were detected, including 122 *A. baumannii* isolates, 6 *Acinetobacter calcoaceticus* isolates and 1 *Acinetobacter bereziniae* isolate. No PDR isolate was detected. Of the 122 *A. baumannii* isolates, 109 and 13 were XDR and MDR, respectively (Table 1), including 60 (49.2%) from respiratory tract specimens, 30 (24.6%) from pus and wounds, 13 (10.7%) from

Table 1
Summary of the drug susceptibility profiles for 122 *Acinetobacter baumannii* isolates.

Drug resistance[a]	No. of isolates	No. of drug categories[b]	Resistance pattern[c]
XDR (109 isolates)	54	8	1
	55	7	5
MDR (13 isolates)	11	6	5
	2	5	2

XDR, extensively drug-resistant; MDR, multidrug-resistant.
[a] MDR strains are defined as isolates not susceptible to ≥1 agent in ≥3 drug categories, and XDR strains are defined as isolates not susceptible to ≥1 agent in all but ≤2 categories [2].
[b] Numbers of drug categories in which the isolate is not susceptible to at least one agent.
[c] Number of drug susceptibility patterns of the isolates.

Table 2
Minimum inhibitory concentrations (MICs) and percent antimicrobial resistance (%R) of *Acinetobacter baumannii* clinical isolates (n = 122).

Antimicrobial agent	Resistance breakpoint (μg/mL)[a]	%R	MIC (μg/mL)		
			Range	MIC_{50}	MIC_{90}
Amikacin	≥64	98	4 to >512	>512	>512
Arbekacin	–	–	1 to >512	>512	>512
Ceftazidime	≥32	100	64 to >512	>512	>512
Ciprofloxacin	≥4	99	1 to >512	64	512
Colistin	≥4	0	0.13–2	0.5	1
Meropenem	≥16	98	1–512	64	128
Tigecycline[b]	≥4	11	<0.031–8	1	4

$MIC_{50/90}$, MIC that inhibits 50% and 90% of the isolates, respectively.
[a] Breakpoints for antimicrobial resistance were determined according to the guidelines of the Clinical and Laboratory Standards Institute [11]. The breakpoint for tigecycline resistance was provided for Enterobacteriaceae but not for *Acinetobacter* spp. by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the US Food and Drug Administration (FDA), although it was proposed as ≥4 μg/mL [14].
[b] The MICs to tigecycline were 8 μg/mL for 4 isolates, 4 μg/mL for 9 isolates, 2 μg/mL for 19 isolates, 1 μg/mL for 61 isolates, 0.5 μg/mL for 21 isolates, 0.25 μg/mL for 7 isolates and <0.031 μg/mL for 1 isolate.

urinary tract specimens, 9 (7.4%) from blood, 7 (5.7%) from cerebrospinal fluid and 3 (2.5%) from other sources (Supplementary Table S1).

3.2. Antimicrobial susceptibility

Most of the 122 XDR/MDR *A. baumannii* isolates were resistant to amikacin, ceftazidime, ciprofloxacin and meropenem (Table 2), with 109 (89.3%) and 97 (79.5%) having MICs ≥ 512 mg/L to amikacin and arbekacin, respectively (data not shown). All isolates were resistant to ceftazidime and 119 (97.5%) were resistant to meropenem. All of the isolates were sensitive to colistin with MICs ≤ 2 mg/L. Moreover, 90 isolates (73.8%) had MICs ≤ 1 mg/L to tigecycline, whereas 4 isolates (3.3%) had MICs of 8 mg/L (data not shown). One XDR isolate (IOMTU433) was resistant to amikacin (MIC ≥ 512 mg/L), ceftazidime (MIC ≥ 512 mg/L), ciprofloxacin (MIC = 32 mg/L), meropenem (MIC = 128 mg/L) and arbekacin (MIC ≥ 512 mg/L) but was susceptible to colistin (MIC = 0.25 mg/L) and tigecycline (MIC = 0.25 mg/L).

3.3. Multilocus sequence typing and phylogenetic analysis

The 122 MDR *A. baumannii* isolates belonged to 15 different MLST sequence types (Fig. 1). eBURST analysis of these isolates, together with the Institut Pasteur MLST database, showed that 5 of the 15 sequence types could be grouped into three clonal complexes, CC1 (ST1 and ST623), CC2 (ST2) and CC149 (ST149 and ST622) (Fig. 1). CC1 contained 21 isolates (4 ST1 and 17 ST623), CC2 contained 36 isolates (all ST2) and CC149 contained 44 isolates (38 ST149 and 6 ST622). A maximum-likelihood phylogenetic tree constructed from the 122 MDR isolates (Fig. 1) was congruent with the tree constructed by the Bayesian method (Supplementary Fig. S1). These phylogenetic trees revealed three clades, corresponding to CC1, CC2 and CC149. All 122 isolates contained intrinsic bla_{ADC} as well as encoding $bla_{OXA\text{-}51\text{-}like}$ variants specific to STs. Of the 21 isolates belonging to CC1, the four ST1 isolates had $bla_{OXA\text{-}69}$ and the 17 ST623 isolates had $bla_{OXA\text{-}371}$. All 36 CC2 isolates belonging to ST2 had $bla_{OXA\text{-}66}$, and all 44 CC149 isolates belonging to ST149 and ST622 had $bla_{OXA\text{-}104}$ (Fig. 1; Table 3). Most CC1 isolates harboured $bla_{OXA\text{-}23}$ and $bla_{OXA\text{-}420}$ (a novel $bla_{OXA\text{-}58}$ variant), most CC2 isolates harboured $bla_{OXA\text{-}23}$, *aac(6′)-Ib* and *aadA1*, and most CC149 isolates harboured $bla_{NDM\text{-}1}$, $bla_{OXA\text{-}23}$, $bla_{PER\text{-}7}$ and the 16S rRNA methylase gene *armA* (Table 3).

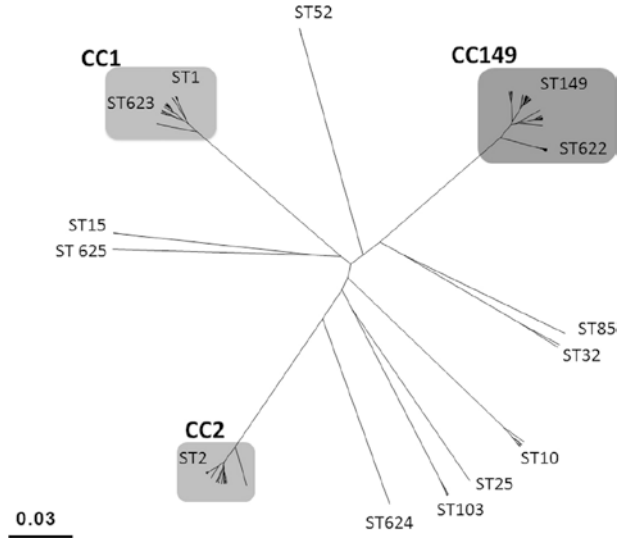

Fig. 1. Molecular phylogeny of the multidrug-resistant (MDR) *Acinetobacter baumannii* strains. A maximum-likelihood phylogenetic tree was constructed from the 122 MDR *A. baumannii* isolates, which were clustered into three clonal complexes (CC1, CC2 and CC149).

Supplementary Fig. S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijantimicag.2015.07.012.

3.4. Complete genome sequencing of IOMTU433

One isolate (IOMTU433) was selected for sequencing of the complete genome because it belonged to a novel sequence type (ST622) and a novel clonal complex (CC149) and harboured several drug resistance genes. The complete genome sequences of IOMTU433 and of the plasmid pIOMTU433 have been deposited in GenBank with the accession nos. AP014649 and AP014650, respectively. The complete genome sequence of IOMTU433 had 1030-fold coverage for a chromosome and 974-fold coverage for a plasmid. IOMTU433 consisted of a single circular chromosome of 4 000 970 bp, with an average GC content of 39.15%, whereas pIOMTU433 consisted of a single circular plasmid of 189 354 bp with an average GC content of 39.53% (Fig. 2). The chromosome was found to contain 3724 protein-encoding genes, including 74 tRNA genes and 1 tmRNA gene for all amino acids. The plasmid was found to contain 203 protein-encoding genes. MLST of IOMTU433 assigned it to ST622 belonging to CC149. IOMTU433 harboured four β-lactamase-encoding genes, including $bla_{NDM\text{-}1}$ encoding a MBL, $bla_{OXA\text{-}23}$ encoding a class D carbapenemase, $bla_{OXA\text{-}104}$ encoding

Table 3
Multilocus sequence typing (MLST) and drug resistance genes in *Acinetobacter baumannii* isolates.

Clonal complex	MLST	No. of isolates[a]	Carbapenemase- and ESBL-encoding genes	Aminoglycoside acetyl/adenylyl transferase-encoding genes
CC1	ST1	4	bla_{NDM-1}, bla_{OXA-23}, bla_{OXA-69}	*aacC1* (1/4), *aadA1*
	ST623	17	bla_{OXA-23}, $bla_{OXA-371}$, $bla_{OXA-420}$ (15/17), bla_{PER-7} (2/17), bla_{PSE-1} (3/17)	*armA* (4/17), *aadA2* (3/17), *aadB* (9/17)
CC2	ST2	36	bla_{OXA-23} (35/36), bla_{OXA-66}, bla_{PER-7} (4/36), bla_{TEM-1} (6/36)	*armA* (2/36), *aac(6′)-Ib* (26/36), *aac(6′)-Im* (1/36), *aacC1* (17/36), *aadA1* (30/36), *aadB* (1/36)
CC149	ST149	38	bla_{NDM-1} (19/38), bla_{OXA-23}, $bla_{OXA-104}$, bla_{PER-7} (35/38)	*armA* (29/38), *aacC1* (1/38), *aadB* (1/38)
	ST622	6	bla_{NDM-1} (4/6), bla_{OXA-23}, $bla_{OXA-104}$, bla_{PER-7} (4/6)	*armA* (3/6)
Other	ST10	7	bla_{OXA-23}, bla_{OXA-68}, bla_{PER-7}	*armA* (5/7)
	ST15	2	bla_{OXA-23}, bla_{OXA-51} (1/2), $bla_{OXA-132}$ (1/2), bla_{PER-7} (1/2), $bla_{TEM-166}$ (1/2)	*armA*
	ST25	4	bla_{OXA-23} (3/4), bla_{OXA-64}, bla_{PER-7}	*armA* (3/4), *aacC1* (2/4), *aacC2* (1/4)
	ST32	2	bla_{NDM-1}, bla_{OXA-32}, $bla_{OXA-420}$ (1/2), bla_{PSE-1}	*armA* (1/2), *aacA2* (1/2)
	ST52	2	bla_{PER-7}, bla_{OXA-98}, bla_{PSE-1}	*armA*, *aadB*
	ST85	1	bla_{NDM-1}, bla_{OXA-94}	*aadB*
	ST103	1	bla_{OXA-70}, bla_{PER-8}, bla_{PSE-1}	*armA*
	ST624	1	bla_{OXA-23}, $bla_{OXA-203}$	*aadB*
	ST625	1	bla_{OXA-23}, bla_{OXA-58}, bla_{OXA-90}	*aacC2*

ESBL, extended-spectrum β-lactamase.
[a] Total number of isolates belonging to the same sequence type.

a variant of an OXA-51-like intrinsic carbapenemase, and bla_{PER-7} encoding an extended-spectrum β-lactamase (ESBL), as well as one aminoglycoside resistance gene (*armA*) encoding a 16S rRNA methylase. Three of these genes (bla_{NDM-1}, bla_{OXA-23} and $bla_{OXA-104}$) were located in various regions of the chromosome (Fig. 2A), whilst two (*armA* and bla_{PER-7}) were located on the plasmid pIOMTU433 (Fig. 2B).

IOMTU433 was found to have two *gyrA* mutations resulting in the amino acid substitutions Ser83Leu and Asp87Glu as well as a mutation in *parC* (Ser80Leu) resulting in fluoroquinolone resistance. The bla_{NDM-1} was found to be located between two copies of IS*Aba125*, from nucleotide 132 336 to nucleotide 142 373 (GenBank accession no. PRJDB3154) (Fig. 3A). This sequence showed 100% homology with that of the *Citrobacter freundii* plasmid

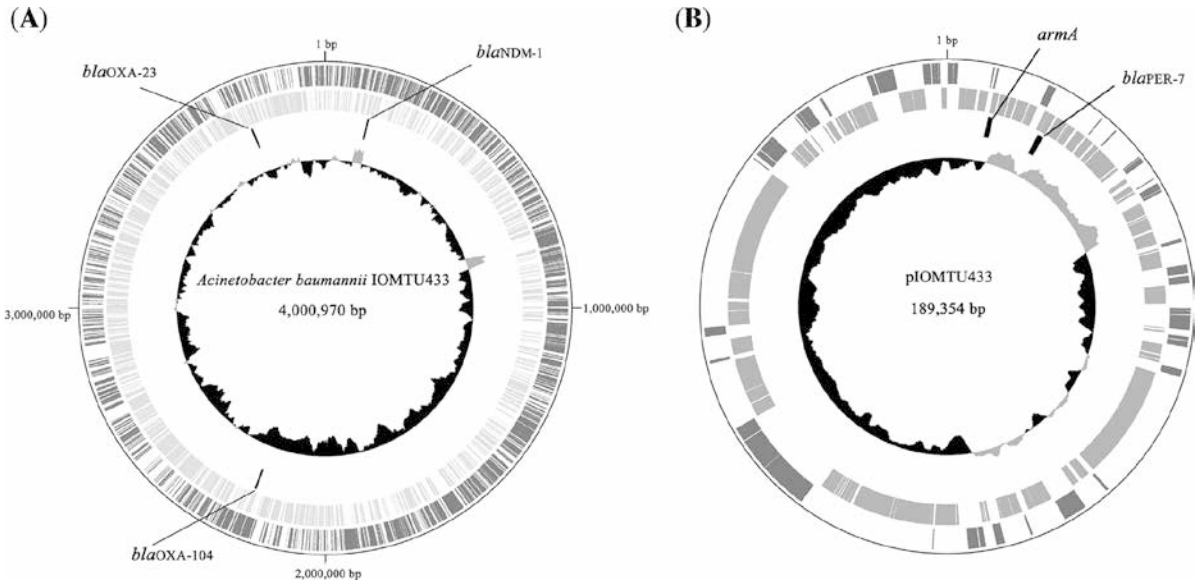

Fig. 2. Circular representation of (A) the genome of *Acinetobacter baumannii* IOMTU433 and (B) the plasmid pIOMTU433. The outermost circle indicates the coordinates of the complete genome. The second and third circles show annotated coding DNA sequences (CDS) encoded by the forward (dark grey) and reverse (light grey) strands, respectively. The fourth circle (black) shows antibiotic resistance genes, including bla_{NDM-1}, bla_{OXA-23}, $bla_{OXA-104}$, *armA* and bla_{PER-7}. The G+C plots are indicated in the innermost circle, ranging from below (black) to above (grey) average.

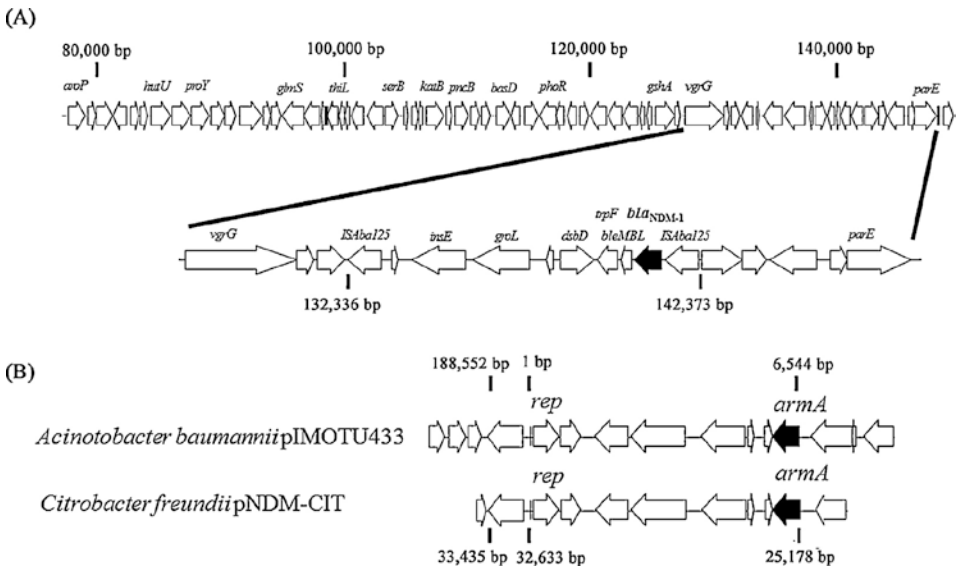

Fig. 3. Genetic environments of (A) bla_{NDM-1} in *Acinetobacter baumannii* IOMTU433 and (B) *armA* in pIOMTU433 and pNDM-CIT. The sequence of pNDM-CIT was obtained from GenBank (accession no. JX182975).

pNDM-CIT (GenBank accession no. JX182975.1) [15] and of the *Klebsiella oxytoca* E718 plasmid pKOX_R1 (GenBank accession no. CP003683) [16] and 99% homology with *A. baumannii* 161/07 (GenBank accession no. HQ857107) [17]. The NDM-1 in *A. baumannii* 161/07 was integrated into the *mfs* gene adjacent to a homoserine lactone synthase gene (*cepI*) on the chromosome [17]. In contrast, the NDM-1 in *A. baumannii* IOMTU433 was integrated into a different site, in IS*Aba125*, which was located between two housekeeping genes. Specifically, NDM-1 was downstream of the *vgrG* gene, which encodes part of a type IV secretion system, and upstream of *parE*, which encodes a topoisomerase IV gene. The sequence of the genetic environment surrounding *armA*, from nucleotide 188 552 to nucleotide 6544, showed 100% homology with the sequence of *C. freundii* pNDM-CIT from nucleotide 33 435 to nucleotide 25 178.

4. Discussion

To our knowledge, this is the first molecular epidemiological analysis of MDR *A. baumannii* clinical isolates in Nepal. CC149 is apparently a novel clonal complex of *A. baumannii*, to which one-third of MDR isolates in Nepal belong. An *A. baumannii* ST149 isolate was first identified in Japan (http://pubmlst.org/abaumannii/). Of the 122 isolates analysed in this study, 44 (36.1%) belonged to CC149, indicating that MDR *A. baumannii* isolates belonging to CC149 are spreading in other medical settings in Nepal. Further studies are needed to determine whether MDR isolates belonging to ST149 are emerging and spreading in other South Asian countries. In addition to CC149 isolates, most of the ST623 isolates belonging to CC1 harboured the novel bla_{OXA-51} variant $bla_{OXA-371}$ and the bla_{OXA-58} variant $bla_{OXA-420}$, indicating that MDR *A. baumannii* may evolve in a unique manner in South Asian countries.

In contrast to CC149, CC1 and CC2 are clonal complexes of MDR isolates more common in Europe (European clones I and II) as well as globally (International clonal complexes 1 and 2) [18]. The isolates belonging to ST10, ST15, ST25, ST32 and ST52 belonged to international clone III, members of which have disseminated throughout Europe, including in the Czech Republic, Denmark, Germany, Greece, Italy, The Netherlands, Norway, Portugal, Spain and Sweden [19].

High levels of drug resistance in these MDR *A. baumannii* isolates may be due to their harbouring various drug resistance genes. NDM-1, bla_{OXA-23} and bla_{PER-7} may be associated with high levels of ceftazidime resistance, and bla_{NDM-1}, bla_{OXA-23} and $bla_{OXA-104}$ may be associated with high levels of carbapenem resistance. Overexpression of the $bla_{OXA-51-like}$ gene has been reported to cause carbapenem resistance [18], whereas *armA* can result in high levels of amikacin and arbekacin resistance. Producers of ArmA have been shown to be resistant to most clinically used aminoglycosides, including amikacin and arbekacin [8].

This study strongly suggests that *A. baumannii* isolates producing NDM-1 and 16S rRNA methylase ArmA have been spreading throughout medical settings in Nepal. Bacteria producing NDM-1 and 16S rRNA methylases are resistant to clinically important carbapenems [9] and aminoglycosides [8], respectively. Gram-negative pathogens producing NDM enzymes and 16S rRNA methylases, including *Escherichia coli*, *Klebsiella pneumoniae* and *Providencia rettgeri*, have spread throughout medical settings in Nepal [20–23]. Although the subject of this study was MDR and XDR *A. baumannii* isolates, it is also necessary to survey aminoglycoside- and carbapenem-resistant *A. baumannii* isolates in Nepal.

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Competing interests: None declared.

Ethical approval: This study was reviewed and approved by the Institutional Review Board of the Institute of Medicine at Tribhuvan University (Kathmandu, Nepal) [ref. 6-11-E] and the Biosafety Committee at the National Center for Global Health and Medicine (Tokyo, Japan) [approval nos. 26-D-088 and 26-D-089].

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Identification of a Novel NDM Variant, NDM-13, from a Multidrug-Resistant *Escherichia coli* Clinical Isolate in Nepal

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A novel New Delhi metallo-β-lactamase, NDM-13, was identified in a carbapenem-resistant *Escherichia coli* clinical isolate obtained from the urine of a patient in Nepal. The enzymatic activity of NDM-13 against β-lactams was similar to that of NDM-1. However, NDM-13 displayed significantly higher k_{cat}/K_m ratios for cefotaxime. The genetic environment of bla_{NDM-13} was determined to be *tnpA*-IS*30*-bla_{NDM-13}-ble_{MBL}-*trpF*-*dsbC*-*cutA*-*groES*-*groL*, with bla_{NDM-13} located within the chromosome.

The emergence of metallo-β-lactamases (MBLs) and increased carbapenem resistance among Gram-negative pathogens has become a serious problem worldwide (1). MBLs, which are produced by many Gram-negative bacterial species (1) and by Gram-positive *Bacillus* spp. (2, 3), confer resistance or reduce bacterial susceptibility to carbapenems, cephalosporins, and penicillins, except for monobactams (1). NDM-1 was initially isolated in Sweden from *Klebsiella pneumoniae* and *Escherichia coli* in 2008 (4). Subsequently, 16 NDM variants (www.lahey.org/studies) have been reported in several countries (5–17).

E. coli IOMTU558 was isolated from a urine sample obtained from an inpatient at a university hospital in Nepal in 2013. The bacterial species was identified by biochemical analysis and confirmed by 16S rRNA sequencing (18). MICs were determined using the microdilution method (19). The MBL genes (bla_{DIM}, bla_{GIM}, bla_{IMP}, bla_{NDM}, bla_{SIM}, bla_{SPM}, and bla_{VIM}) were amplified by PCR (20, 21), and the sequence of the entire genome was determined by MiSeq (Illumina, San Diego, CA). Sequences of all drug resistance genes registered at the Lahey Clinic website (www.lahey.org/studies), including MBL genes, were determined using CLC genomics workbench version 5.5. Multilocus sequence typing (MLST) was performed according to protocols appropriate for *E. coli* sequences in the MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). *E. coli* transformants expressing bla_{NDM} genes were constructed as described previously (22), with bla_{NDM-1}, which originated from *Pseudomonas aeruginosa* IOMTU9 (17), used as the reference gene. Recombinant NDMs were purified as described previously (22), with β-lactamase activities during the purification process monitored using nitrocefin (Oxoid, Ltd., Basingstoke, United Kingdom). Initial rates of β-lactamase activities were determined as described previously (22), with some modifications. In the modified protocol, the substrate solutions were prewarmed to 37°C in a water bath to minimize fluctuations in the temperature of the reaction mixture. K_m values, k_{cat} values, and k_{cat}/K_m ratios were determined by analyzing β-lactam hydrolysis using Lineweaver-Burk plots. Previously reported wavelengths and extinction coefficients were used for analysis of β-lactam substrates (23–25). K_m and k_{cat} values were determined using triplicate analyses. A DNA plug, digested for 3 h with I-CeuI, was separated by pulsed-field gel electrophoresis and subjected to Southern hybridization as described previously (26). Probes for 16S rRNA and bla_{NDM} were prepared as described previously (27, 28).

The MICs of β-lactams for *E. coli* IOMTU558 are listed in Table 1. The MICs of the other antibiotics were as follows: amikacin, 16 μg/ml; arbekacin, 32 μg/ml; ciprofloxacin, >1,024 μg/ml; colistin, 1 μg/ml; fosfomycin, 8 μg/ml; gentamicin, 256 μg/ml; kanamycin, 256 μg/ml; levofloxacin, 32 μg/ml; minocycline, 4 μg/ml; tigecycline, 2 μg/ml; and tobramycin, 128 μg/ml. PCR showed that the isolate was positive for bla_{NDM} and negative for the other genes. Sequencing of the PCR product showed that it was a novel bla_{NDM} variant. This new variant was designated bla_{NDM-13}, and the sequence was deposited in GenBank. IOMTU558 belonged to sequence type 101 (ST101). Based on the predicted amino acid sequence, NDM-13 had two amino acid substitutions (D95N and M154L) compared to NDM-1 and one amino acid substitution each compared to NDM-3 (M154L) and NDM-4 (D95N). The isolate harbored $bla_{CTX-M-15}$, $bla_{TEM-166}$, and *ampC*. The promoter region of *ampC* (nucleotides [nt] −42 to −1) included a mutation at nt −18 (G to A), but there were no other mutations or nucleotide insertions compared with *E. coli* strain K-12. The isolate harbored *ompC* and *ompF* and their positive regulator, *ompR*. The *ompR* gene had no mutation compared with that of the K-12 strain. IOMTU588 harbored efflux pump genes (the *acrAB-tolC* operon), the *marR* repressor gene, and the gene (*yedS*) encoding the putative outer membrane protein. The *marR* gene had a mutation resulting in an amino acid substitution (Y137H).

E. coli DH5α expressing bla_{NDM-1} or bla_{NDM-13} showed a significant reduction in susceptibility to all tested β-lactams, except for aztreonam, compared with the DH5α strain expressing a vector control (Table 1). *E. coli* DH5α expressing bla_{NDM-13} showed a

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TABLE 1 MICs of various β-lactams for *E. coli* strain IOMTU558 and *E. coli* DH5α transformed with plasmids encoding NDM-1 or NDM-13

Antibiotic(s)	MIC (μg/ml) for:			
	IOMTU558	DH5α(pHSG398/NDM-1)	DH5α(pHSG398/NDM-13)	DH5α(pHSG398)
Ampicillin	>1,024	256	512	4
Ampicillin-sulbactam	>1,024	256	256	4
Aztreonam	>1,024	≤0.063	≤0.063	≤0.063
Cefepime	>1,024	1	1	≤0.063
Cefoselis	>1,024	16	16	≤0.063
Cefotaxime	>1,024	16	64	≤0.063
Cefoxitin	>1,024	64	64	≤0.063
Cefpirome	>1,024	4	4	≤0.063
Ceftazidime	>1,024	256	512	≤0.063
Ceftriaxone	>1,024	16	32	≤0.063
Cephradine	>1,024	512	256	16
Doripenem	128	0.25	0.25	≤0.063
Imipenem	32	1	2	0.125
Meropenem	128	0.25	0.25	≤0.063
Moxalactam	>1,024	32	32	0.25
Penicillin G	>1,024	256	256	32

4-fold-higher MIC of cefotaxime than *E. coli* DH5α expressing bla_{NDM-1} (Table 1).

The recombinant NDM-1 and NDM-13 hydrolyzed all tested β-lactams, except for aztreonam (Table 2). The enzymatic activity profiles of NDM-13 against the tested β-lactams were similar to those of NDM-1; however, NDM-13 showed a higher k_{cat}/K_m ratio for cefotaxime, which may have been due to NDM-13 having a lower K_m value than NDM-1 (Table 2). The kinetic parameters for NDM-1 (Table 2) differed from those of our previous report (22). The modified protocol utilized in this study may have affected the results of the kinetic assays.

The genomic environment of bla_{NDM-13} was *tnpA*-IS*30*-bla_{NDM-13}-ble_{MBL}-*trpF-dsbC-cutA-groES-groL*, and the sequence was deposited in GenBank. This genomic structure, except for bla_{NDM-13}, was identical to that of pPMK1 expressed by *K. pneumoniae* PMK1 (isolated in Nepal) (29), an *Enterobacter hormaechei* CCHB10892 plasmid (from Brazil) (30), pKPX-*1* from *K. pneumoniae* KPX (from Taiwan) (31), and pNDM-*MAR* isolated from *K. pneumoniae* (from Morocco) (32, 33). However, the bla_{NDM-13} gene was located on the chromosome (Fig. 1).

The two substitutions in NDM-13 compared to NDM-1 (i.e., D95N and M154L) increased the affinity of the enzyme for cefotaxime and affected the catalytic activity of the enzyme against this drug (Table 2). The D95N amino acid substitution was reported to reduce the k_{cat} values of NDM-3 compared with NDM-1 for all β-lactamases tested (28). An NDM-4 mutant containing M154L was found to have increased hydrolytic activity toward carbapenems and several cephalosporins compared with that of NDM-1 (12). NDM-13 with the D95N and M154L substitutions did not show increased hydrolytic activity against the tested carbapenems, cephalosporins, and penicillins, except for cefotaxime. Among the known NDM variants (NDM-1 to NDM-13), amino acid substitutions were observed at 13 amino acid positions: 28, 32, 36, 69, 74, 88, 95, 130, 152, 154, 200, 222, and 233. Positions 28, 32, and 36 are located in the signal peptide region. Positions 95, 130, and 154 have been reported to affect β-lactam hydrolyzing activity (12, 17, 28), although they are not located at the active site of NDM-1 or in amino acid residues that bind to zinc ions (34, 35). The effects of the remaining 10 substitutions on hydrolyzing activity have not yet been reported. Determination of the hydrolyzing activities of

TABLE 2 Kinetic parameters of NDM-1 and NDM-13 enzymes

β-Lactam	Result for[a]:					
	NDM-1			NDM-13		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
Ampicillin	76 ± 6	30 ± 0.6	0.40	140 ± 26	52 ± 3	0.38
Aztreonam	NH[b]	NH	NH	NH	NH	NH
Cefepime	129 ± 8	9.9 ± 0.4	0.077	83 ± 11	8.2 ± 0.6	0.099
Cefotaxime	49 ± 6	41 ± 2	0.85	22 ± 2	38 ± 1	1.7
Cefoxitin	13 ± 3	1.0 ± 0.03	0.076	12 ± 2	1.1 ± 0.1	0.093
Ceftazidime	54 ± 4	8.5 ± 0.2	0.16	30 ± 3	7.5 ± 0.3	0.25
Cephradine	14 ± 3	23 ± 1	1.8	28 ± 4	27 ± 1	0.98
Doripenem	39 ± 2	13 ± 0.3	0.34	49 ± 4	15 ± 1	0.31
Imipenem	56 ± 7	14 ± 1	0.26	71 ± 6	17 ± 1	0.24
Meropenem	21 ± 4	21 ± 1	1.1	37 ± 6	26 ± 1	0.73
Penicillin G	19 ± 6	19 ± 1	1.1	37 ± 5	49 ± 2	1.3

[a] The proteins were initially modified by a His tag, which was removed after purification. The K_m and k_{cat} values are means ± standard deviations from 3 independent experiments.
[b] NH, no hydrolysis was detected under conditions with substrate concentrations up to 1 mM and enzyme concentrations up to 700 nM.

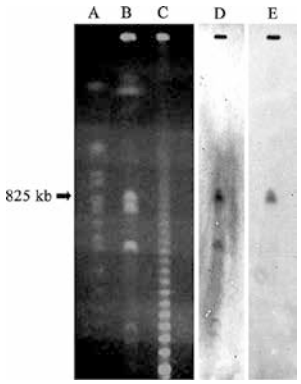

FIG 1 Localization of the bla_{NDM-13} gene on the chromosome of *E. coli* strain IOMTU558, separated by pulsed-field gel electrophoresis (PFGE). Lanes: A, contour-clamped homogeneous electric field (CHEF) DNA size marker (*Saccharomyces cerevisiae*; Bio-Rad, Hercules, CA); B, I-CeuI-digested total DNA of *E. coli* IOMTU558; C, lambda ladder pulsed-field gradient (PFG) marker (New England BioLabs, Ipswich, MA); D, hybridization of I-Ceu-I-digested total DNA of IOMTU558 with a 16S rRNA-specific probe; and E, hybridization of I-Ceu-I-digested total DNA of IOMTU558 using a bla_{NDM-13}-specific probe.

all NDM variants is critical to understanding the mechanisms underlying the molecular evolution of the NDMs.

The profile of β-lactam resistance in IOMTU558 (Table 1) could be explained by the presence of bla_{NDM-13}, as well as $bla_{CTX-M-15}$, $bla_{TEM-166}$, and *ampC*. Among these genes, $bla_{CTX-M-15}$ confers high resistance against most β-lactams (except carbapenems), including aztreonam, cefepime, cefotaxime, and ceftriaxone (36). *ampC* could not be overexpressed in the isolate. The β-lactam-susceptible *E. coli* wild-type strain has been found to express *ampC* at low levels due to degenerated promoter boxes upstream of *ampC* (37). Two mutations in the *ampC* promoter sequence, at positions −42 (C to T) and −18 (G to A), generate two alternative promoter boxes, resulting in high-level expression of *ampC* (37). IOMTU558 had a mutation at position −18 (G to A) but not at position −42. It is unclear whether the mutation at position −18 alone could affect the *ampC* expression level in IOMTU558. The other mechanisms of β-lactam resistance are decreased cell permeability due to loss or alteration of the *ompC* and *ompF* porins and activation of efflux systems (38). Loss or alteration of porins was not detected in IOMTU558. The *marR* repressor gene of *marAB* and the *yedS* gene encoding a putative outer membrane protein were reported to be involved in carbapenem resistance via downregulation of efflux pump genes (the *acrAB-tolC* operon). The *marR* mutation and *yedS* could contribute to carbapenem resistance in IOMTU558 (39).

NDM-producing *E. coli* belonging to ST101 seems to be an epidemic strain in several regions of the world, although ST101 *E. coli* strains are not recognized as a cause of pandemics, as is *E. coli* clone ST131 producing CTX-M types of β-lactamases (40). NDM-1-producing *E. coli* isolates belonging to ST101 have been reported in Bulgaria (41), Canada (42), England (43), India (44), Pakistan (43), South Korea (45), and the United States (46).

This is the first report describing an NDM-13-producing *E. coli* isolate. NDM-type MBLs have evolved under the pressure of antibiotic usage. Therefore, NDM-producing pathogens must be monitored.

Nucleotide sequence accession number. The sequence of bla_{NDM-13} has been deposited in GenBank under accession no. LC012596.

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MECHANISMS

A Novel 6′-*N*-Aminoglycoside Acetyltransferase, AAC(6′)-Ial, from a Clinical Isolate of *Serratia marcescens*

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Serratia marcescens IOMTU115 has a novel 6′-*N*-aminoglycoside acetyltransferase-encoding gene, *aac(6′)-Ial*. The encoded protein AAC(6′)-Ial has 146 amino acids, with 91.8% identity to the amino acid sequence of AAC(6′)-Ic in *S. marcescens* SM16 and 97.3% identity to the amino acid sequence of AAC(6′)-Iap in *S. marcescens* WW4. The minimum inhibitory concentrations of aminoglycosides for *Escherichia coli* expressing AAC(6′)-Ial were similar to those for *E. coli* expressing AAC(6′)-Ic or AAC(6′)-Iap. Thin-layer chromatography showed that AAC(6′)-Ial, AAC(6′)-Ic, or AAC(6′)-Iap acetylated all the aminoglycosides tested, except for apramycin, gentamicin, and lividomycin. Kinetics assays revealed that AAC(6′)-Ial is a functional acetyltransferase against aminoglycosides. The *aac(6′)-Ial* gene was located on chromosomal DNA.

Introduction

THE MAIN MECHANISMS underlying resistance to aminoglycosides are the production of aminoglycoside-modifying enzymes[12] and 16S rRNA methylases.[22] The aminoglycoside 6′-*N*-acetyltransferases [AAC(6′)s] modify a number of clinically important aminoglycosides. The AAC(6′)-I type confers resistance to amikacin, dibekacin, isepamicin, kanamycin, netilmicin, sisomicin, and tobramycin, whereas the AAC(6′)-II type acetylates dibekacin, gentamicin, kanamycin, netilmicin, sisomicin, and tobramycin. To date, 45 genes designated as *aac(6′)-Ia* to *-Iaj*, which encode AAC(6′)-I enzymes, have been cloned and characterized.[10,12,17,20]

Serratia marcescens is well recognized as a nosocomial pathogen and has been responsible for outbreaks in neonatal and pediatric intensive care units[21] and outbreaks of bloodstream infection associated with intravenous drug solutions.[15] Moreover, respiratory colonization with *S. marcescens* has been a risk factor for outbreaks of bloodstream infection.[18] Drug-resistant *S. marcescens* isolates have been emerging in medical settings; these isolates produce aminoglycoside modification enzymes [AAC(6′)-Ib, AAC(6′)-Ib-cr, AAC(6′)-Ic, AAC(6′)-Iid, and ANT(3")-Ii],[3,4,13,19] 16S rRNA methylases (ArmA and RmtB),[1,2] and metallo-β-lactamases,[9,11,23] although *S. marcescens* is naturally resistant to tetracycline, amoxicillin, amoxicillin-clavulanate, cephalothin, and colistin.[14]

Materials and Methods

Bacterial strains, plasmids, and antimicrobial susceptibility

S. marcescens IOMTU115 was isolated from an endotracheal aspirate of a patient in a medical ward of a hospital in Nepal in 2012. *S. marcescens* WW4 was originally isolated from environmental water samples in a paper factory in Taiwan.[6] *Escherichia coli* DH5α (Takara Bio, Shiga, Japan) and *E. coli* BL21-CodonPlus (DE3)-RIP (Agilent Technologies, Santa Clara, CA) were used as hosts for recombinant plasmids and proteins, respectively. Plasmids pSTV28 (Takara) and pQE2 (Qiagen, Tokyo, Japan) were used for cloning of *aac(6′)s* and purification of recombinant AAC(6′)s, respectively, as described previously.[16] Minimum inhibitory concentrations (MICs) for *S. marcescens* IOMTU115 and *E. coli* transformants with *aac(6′)* genes were determined using the microdilution method.[8]

Genome sequencing and cloning

Genomic DNA of *S. marcescens* IOMTU115 was extracted using the DNeasy Blood and Tissue kit (Qiagen) and sequenced with MiSeq (Illumina, San Diego, CA). More than 20-fold coverage was achieved for IOMTU115. A new 6′-*N*-aminoglycoside acetyltransferase variant was designated as *aac(6′)-Ial*. *aac(6′)-Ial* and its genetic environment

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FIG. 1. Alignment of AAC(6′)-Ic, AAC(6′)-Ial, and AAC(6′)-Iap amino acid sequences. Identical residues are marked with *black boxes*.

(315,403 bp) was deposited in GenBank with the Accession Nos. AB871481 and AB894481, respectively. A putative *aac(6′)* gene of *S. marcescens* WW4 was designated as *aac(6′)-Iap* and deposited in the GenBank with the Accession No. AB979699.

The *aac(6′)-Ic* gene was synthesized by Funakoshi, Tokyo, Japan. *aac(6′)-Ic*, *aac(6′)-Ial*, and *aac(6′)-Iap* genes were cloned into the multiple cloning site of pSTV28, as described previously.[17] *E. coli* DH5α was transformed with pSTV28--*aac(6′)-Ic*, *-aac(6′)-Ial*, and *-aac(6′)-Iap* to determine the MICs for aminoglycosides.

Amino acid sequence alignment was calculated using the CLUSTAL W2 program. Genetic environments surrounding drug-resistant genes were annotated and compared using the BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome).

Analysis of aminoglycoside acetyltransferase activity

The open reading frames (ORFs) of *aac(6′)-Ic*, *aac(6′)-Ial*, and *aac(6′)-Iap* were cloned into the pQE2 expression vector with the same primer sets as described above. Purification of the recombinant AAC(6′)-Ic, AAC(6′)-Ial, and AAC(6′)-Iap proteins and thin-layer chromatography analysis were performed as described previously.[17]

Results

Drug resistance profile of S. marcescens *IOMTU115*

The MICs of *S. marcescens* IOMTU115 were as follows: ampicillin, 32 μg/ml; ampicillin–sulbactam, 16 μg/ml; arbekacin, 8 μg/ml; amikacin, 8 μg/ml; aztreonam, <0.125 μg/ml; ceftazidime, 0.25 μg/ml; cephradine, 1,024 μg/ml; cefepime, 0.25 μg/ml; cefotaxime, 0.25 μg/ml; cefoxitin, 32 μg/ml; ciprofloxacin, <0.125 μg/ml; colistin, >1,024 μg/ml; fosfomycin, 32 μg/ml; gentamicin, 2 μg/ml; imipenem, 8 μg/ml; kanamycin, 16 μg/ml; meropenem, <0.125 μg/ml; penicillin, >1,024 μg/ml; tigecycline, 2 μg/ml; and tobramycin, 4 μg/ml.

Amino acid sequence of AAC(6′)-Ial enzyme

AAC(6′)-Ial consists of 146 amino acids. Multiple sequence alignments among AAC(6′) enzymes revealed that AAC(6′)-Ial had 97% identity to AAC(6′)-Iap from *S. marcescens*,[6] 92% identity to AAC(6′)-Ic from *S. marcescens*,[13] 38% identity to AAC(6′)-Iag from *Pseudomonas aeruginosa*,[5] and 37% identity to AAC(6′)-Iy from *Salmonella enterica*.[7] Both AAC(6′)-Ial and AAC(6′)-Iap are newly described in this study, although AAC(6′)-Iap was deposited as a putative protein in a complete genome sequence of *S. marcescens* WW4 (Accession No. NC020211). Biological properties of AAC(6′)-Iap had not been analyzed. As shown in Fig. 1, amino acid substitutions existed at positions 4, 18, 23, 24, 25, 26, 28, 56, 64, 84, 86, 95, and 122 among the three AAC(6′) variants.

Aminoglycoside susceptibility of E. coli *transformants*

As shown in Table 1, a vector control of *E. coli* DH5α/pSTV28 was susceptible to all the aminoglycosides tested, whereas *E. coli* DH5α/pSTV28-*aac(6′)-Ic*, *aac(6′)-Ial*, and *aac(6′)-Iap* were resistant to all aminoglycosides, except for apramycin, gentamicin, and lividomycin, with 2- to 64-fold higher MICs than those of the vector control. The MICs of apramycin, gentamicin, and lividomycin in *E. coli* expressing *aac(6′)-Ic*, *aac(6′)-Ial*, and *aac(6′)-Iap* were the same as those in the vector control. The MIC profiles were essentially similar among the *E. coli* expressing *aac(6′)-Ic*, *aac(6′)-Ial*, and *aac(6′)-Iap* (Table 1). *E. coli* expressing *aac(6′)-Ial* and

TABLE 1. MICS OF AMINOGLYCOSIDES FOR *ESCHERICHIA COLI* TRANSFORMANTS

Strain[a]	*MIC (μg/ml)*											
	ABK	*AMK*	*APR*	*DIB*	*GEN*	*ISP*	*KAN*	*LIV*	*NEO*	*NET*	*SIS*	*TOB*
E. coli DH5α/pSTV28	0.5	0.5	2	0.25	0.125	0.125	0.5	2	0.5	0.5	0.25	0.5
E. coli DH5α/pSTV28-*aac(6′)-Ic*	4	1	2	8	0.125	0.5	4	2	2	8	4	8
E. coli DH5α/pSTV28-*aac(6′)-Ial*	4	1	2	16	0.125	0.5	8	2	2	8	4	8
E. coli DH5α/pSTV28-aac(6′)-*Iap*	4	2	2	16	0.125	0.25	4	2	2	8	2	8

MICs of aminoglycosides for *E. coli* DH5α transformants with *aac(6′)-Ic*, *aac(6′)-Ic*, and *aac(6′)-Ic* were determined using the microdilution method.[8]

[a]MICs for *E. coli* strains were determined with Mueller–Hinton broth preparations and individual aminoglycosides.

ABK, arbekacin; AMK, amikacin; APR, apramycin; DIB, dibekacin; GEN, gentamicin; ISP, isopamicin; KAN, kanamycin; LIV, lividomycin; MIC, minimum inhibitory concentration; NEO, neomycin; NET, netilmicin; SIS, sisomicin; SPM, spectinomycin; STM, streptomycin; TOB, tobramycin.

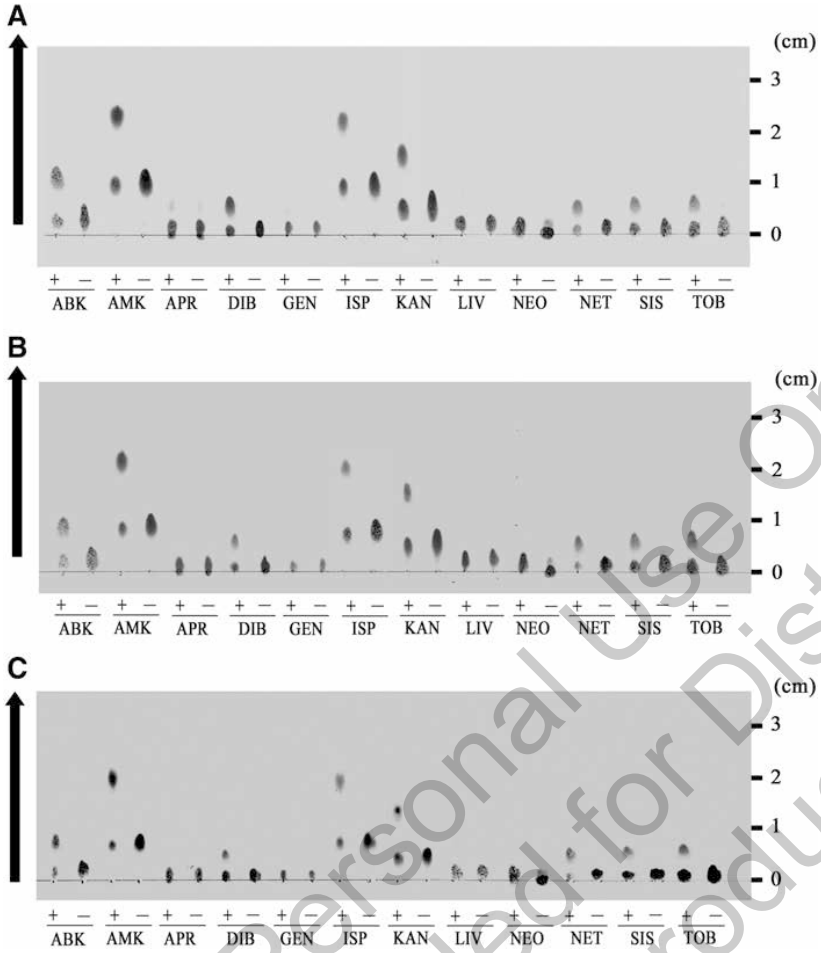

FIG. 2. TLC analysis of acetylated aminoglycosides by AAC(6′)-Ic **(A)**, AAC(6′)-Ial **(B)**, and AAC(6′)-Iap **(C)**. AAC(6′) and various aminoglycosides were incubated in the presence (+) or absence (−) of acetyl-coenzyme A. The *arrow* indicates the direction of development. ABK, arbekacin; AMK, amikacin; APR, apramycin; DIB, dibekacin; GEN, gentamicin; ISP, isopamicin; KAN, kanamycin; LIV, lividomycin; NEO, neomycin; NET, netilmicin; SIS, sisomicin; TOB, tobramycin.

aac(6′)-Iap were resistant to dibekacin, with MICs 64-fold higher than those of the other aminoglycosides.

TLC analysis and kinetics against aminoglycosides

The results of TLC analysis of aminoglycosides acetylated by AAC(6′) enzymes were shown in Fig. 2. Lividomycin was used as a negative control because it has a hydroxyl group instead of an amino group at the 6′ position and, therefore, cannot be acetylated by AAC(6′). In total, 9 out of the 12 aminoglycosides tested were acetylated by AAC(6′)-Ic, AAC(6′)-Ial, and AAC(6′)-Iap, with the exception of apramycin, gentamicin, and lividomycin (Fig. 2). Kinetics parameters of AACs(6′) against the nine aminoglycosides were determined, and the results are shown in Table 2. The profiles of the kinetics parameters were essentially similar among the three enzymes. The k_{cat}/K_m values against dibekacin were relatively high compared with those against other aminoglycosides, whereas the values against amikacin were relatively low (Table 2). The substrate specificity of AAC(6′)-Ial is similar to that of AAC(6′)-Ic and AAC(6′)-Iap. The kinetics parameters of the three enzymes against dibekacin were relatively high among the three enzymes, whereas those against amikacin were relatively low (Table 1). These differences in substrate specificity will be associated with an amino group at position 1 of ring I. Dibekacin has an amino group, whereas amikacin has a hydroxyl group at position 1 of ring I.

Genetic environments surrounding aac(6′)-Ial *and* aac(6′)-Iap

As shown by the genome rearrangement map in Fig. 3, a genetic region of *S. marcescens* IOMTU115 from nucleotide (nt) 1 to 315,403 (Accession No. AB894481) had 97% identity to a region of *S. marcescens* WW4 from nt 4,490,655 to 4,842,041 (Accession No. CP003959) (Fig. 3). Bacteriophage-derived genes with 33,097 bp were inserted from nt 4,583,432 to 4,616,528 in WW4. The downstream region of the genetic environment surrounding *aac(6′)-Ial* was similar to the one surrounding *aac(6′)-Iap* (Fig. 3). The downstream region of the genetic environment surrounding *aac(6′)-Ial* contained several housekeeping genes, including *dnaG*, *rpoD*, and *mug* (Fig. 3), indicating that *aac(6′)-Ial* was located in the chromosome.

Discussion

Amikacin and isepamicin share a characteristic structure of an L-hydroxyaminobuteroyl amide moiety at N-3 in the

Table 2. Kinetics Parameters of Aac(6′)-Ic, Aac(6′)-Ial, and Aac(6′)-Iap Enzymes

	AAC(6′)-Ic			*AAC(6′)-Ial*			*AAC(6′)-Iap*		
Aminoglycoside	K_m (μM)[a]	k_{cat} (s^{-1})[a]	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	K_m (μM)[a]	k_{cat} (s^{-1})[a]	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)	K_m (μM)[a]	k_{cat} (s^{-1})[a]	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
ABK	152 ± 59	10 ± 4	0.064	399 ± 36	17 ± 2	0.048	210 ± 134	9 ± 5	0.042
AMK	55 ± 18	0.24 ± 0.05	0.005	86 ± 48	0.2 ± 0.1	0.002	24 ± 4	0.27 ± 0.03	0.011
DIB	405 ± 136	44 ± 11	0.11	582 ± 182	85 ± 24	0.15	487 ± 84	48 ± 7	0.10
ISP	149 ± 27	10 ± 2	0.069	404 ± 125	25 ± 6	0.061	551 ± 205	26 ± 7	0.048
KAN	185 ± 53	2.8 ± 0.5	0.015	322 ± 73	10 ± 2	0.032	280 ± 104	6 ± 2	0.020
NEO	465 ± 58	6.3 ± 0.7	0.013	470 ± 27	7.8 ± 0.4	0.025	696 ± 155	12 ± 2	0.017
NET	20 ± 5	0.6 ± 0.1	0.029	23 ± 6	0.73 ± 0.06	0.032	7 ± 1	0.15 ± 0.01	0.022
SIS	16 ± 7	0.6 ± 0.1	0.041	12 ± 3	0.49 ± 0.03	0.041	15 ± 3	0.6 ± 0.1	0.043
TOB	20 ± 4	0.8 ± 0.1	0.039	28 ± 3	0.67 ± 0.05	0.024	12 ± 3	0.4 ± 0.1	0.037

The proteins were initially modified by a His-tag, which was removed after purification.
[a]K_m and k_{cat} values represent the means of three independent experiments ± standard deviations.

2-deoxystreptamine ring II, whereas the remaining aminoglycosides do not. This moiety will affect the substrate specificities between the three enzymes. As shown in Table 2, kinetics parameters against dibekacin were high compared with those against other aminoglycosides, whereas those against amikacin were low. The differences of the kinetics parameters between dibekacin and amikacin will be associated with an amino group at position 1 of ring I. Dibekacin has an amino group, whereas amikacin has a hydroxyl group at position 1 of ring I. A total of 13 amino acid substitutions, found among the 3 AAC(6′)-Ic, AAC(6′)-Iai, and AAC(6′)-Iap, may not be associated with the aminoglycoside modifications because of no difference between these enzymatic properties (Table 2).

These intrinsic *aac(6′)* variants in *S. marcescens* may be evolved while mutating in their unique ways in the conserved genetic environment, because these genes were not observed in other organisms. Our study suggests that *aac(6′)* variants in *S. marcescens* have potential to become more resistant to aminoglycosides by causing further genetic selection. Shaw *et al.* reported that expression of the AAC(6′)-I aminoglycoside resistance profiles can be due to (1) mutation of a gene encoding a negative regulator, (2) insertion upstream or within the 5′ coding region of the aac(6′)-I gene in *S. marcescens*, or (3) point mutations that create a new promoter.[13]

The *aac(6′)* genes may contribute to aminoglycoside resistance in *S. marcescens*. The *aac(6′)-Ic*, *aac(6′)-Ial*, and *aac(6′)-Iap* were detected in clinical and environmental

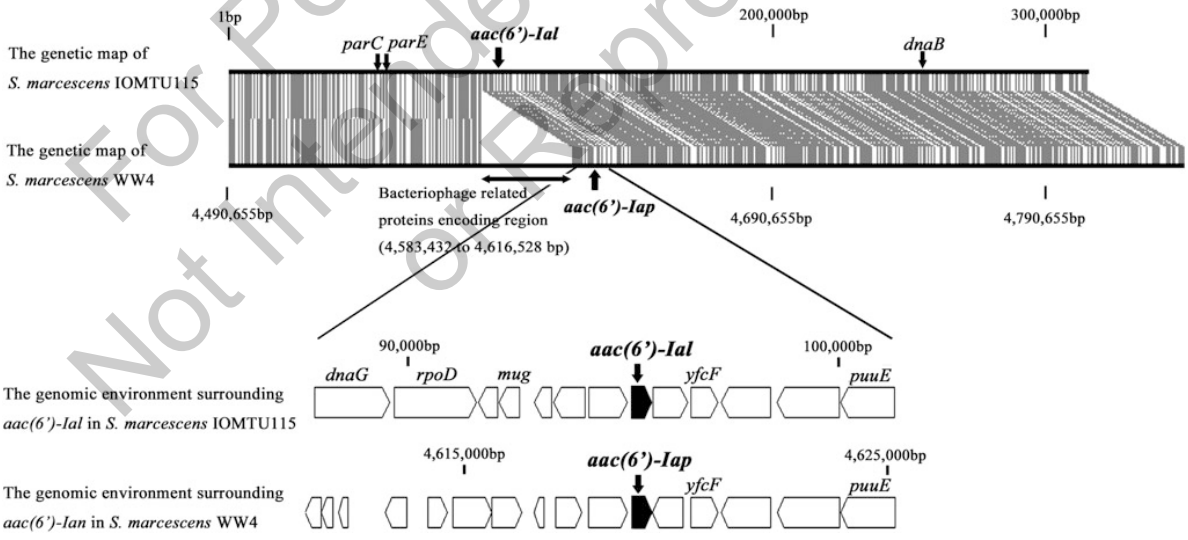

FIG. 3. A genome rearrangement map between *Serratia marcescens* IMOTU115 (Accession No. AB894481) and *S. marcescens* WW4 (Accession No. CP003959), and the comparison of the genomic environments surrounding *aac(6′)-Ial* in IOMTU115 and *aac(6′)-Iap* in WW4. The genetic map from nt 1 to 315,403 in *S. marcescens* IMOTU115 had 97% identity compared to that from nt 4,490,655 to 4,842,041 in *S. marcescens* WW4. The upstream region of genomic environment surrounding *aac(6′)-Ial* was similar to that surrounding *aac(6′)-Iap*. The genomic environment surrounding *aac(6′)-Ial* contained several housekeeping genes, including *dnaG*, *rpoD*, and *mug*.

isolates of *S. marcescens*.[6,13] Therefore, further monitoring and investigation of aminoglycoside-resistant *S. marcescens* are necessary to understand the diversity and evolution of *S. marcescens* intrinsic *aac(6′)* genes.

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Author Disclosure Statement

No competing financial interests exist.

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Original Article

Use of nucleoside analogs in patients with chronic hepatitis B in Nepal: A prospective cohort study in a single hospital

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Aim: There still remain many concerns about the present status of antiviral therapy for chronic hepatitis B in developing countries in Asia, where the monitoring systems of virological markers have not been well established, despite the high prevalence of hepatitis B virus (HBV) infection. To investigate it in Nepal, this prospective cohort study was conducted at the Teaching Hospital of Tribhuvan University in Kathmandu.

Methods: From 2007 to 2012, 65 patients were consecutively enrolled, 44 of whom received nucleoside analogs (NA), such as lamivudine (LMV), adefovir or tenofovir (TDF), on the decision of the local hepatologist. Virological determinations were performed in Japan, by using the serially collected serum samples at the Teaching Hospital. Statistical analysis was performed, using Mann–Whitney *U*-test or Fisher's exact test.

Results: The younger, especially female patients of reproductive age were more frequently prescribed with these NA, and an increased preference for the use of TDF was observed over time. However, there was insufficient follow up of the NA-treated patients in this cohort, and not a few patients developed emergence of NA-resistant HBV: known resistance to LMV in 3 patients and incidental resistance to non-administrated NA in four patients.

Conclusion: The results of the present study indicate that education for physicians as well as for infected patients regarding the proper use of NA, together with establishment of appropriate monitoring systems for virological markers, is warranted to prevent an increase in NA-resistant HBV infections in Nepal.

Key words: antiviral therapy, chronic hepatitis B, hepatitis B virus, nucleoside analogs, resistance

INTRODUCTION

ACCORDING TO ESTIMATES by the World Health Organization, almost 240 million people are infected by hepatitis B virus (HBV) worldwide, and up to 780 000 deaths related to liver cirrhosis or hepatocellular carcinoma caused by chronic HBV infection occur annually.[1] HBV infection is endemic, particularly in Asian and African countries, and the prevalence is reported to be as high as 20% of the general population in some countries.[2] In the rural areas of Nepal, one of the poorest countries in the world, the prevalence of HBV and hepatitis C virus (HCV) among blood donors was reported to be 0.86% and 0.52%, respectively, in 2001–2002.[3] Our epidemiological study conducted between 1997 and 2002 also demonstrated that HBV and HCV carrier rates among 540 blood donors in the urban area of Nepal were 2.4% and 1.1%, respectively (e.g. N. Masaki, unpubl. data, 2003).

The recent introduction of nucleoside analogs (NA) such as lamivudine (LMV), adefovir (ADF), entecavir (ETV) and tenofovir (TDF) into clinical settings has significantly attenuated the progression of liver fibrosis and hepatocarcinogenesis,[4] leading to improvements in the

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[*]Author contribution: N. M. designed the research protocol, obtained funding and wrote the first draft of the manuscript. P. K. S. was the counterpart of this research in Nepal and contributed to the collection of serum samples as well as clinical information. S. N. and K. I. contributed to the acquisition of data and the analysis. M. S. carried out the molecular analyses of hepatitis B virus. M. M. supervised the entire research project. All authors read and approved the final manuscript.

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prognosis of HBV-related liver diseases. However, because the pharmacological nature of these NA is chiefly confined to competitive inhibition of HBV DNA polymerase, semi-permanent use of NA is principally necessary to prevent the relapse of hepatitis accompanying HBV proliferation. However, the long-term use of NA may result in emergence of NA-resistant HBV,[5] therefore, it is necessary to regularly monitor the serum viral load during treatment.

Although the HBV and HCV carrier rates in Nepal appear to be relatively lower than in other developing countries in Asia, it is important to understand the current use of therapeutic strategies. However, little information about the present status of antiviral therapy for chronic HBV is available in this country, especially in terms of NA use. Furthermore, there are several serious socioeconomic problems to be addressed in Nepal. In relation to chronic HBV, domestic laboratories in Nepal are not able to perform virological determinations of HBV genotypes or HBV viral load, and sending serum samples to overseas commercial labs is costly. For example, the measurement of HBV viral load in India costs approximately 8000 Nepalese rupees (i.e. $US 127). In contrast, NA are relatively inexpensive in Nepal, because of an influx of generic drugs produced by foreign pharmaceutical companies. One tablet of LMV, ADF, ETV or TDF costs 18 ($US 0.28), 30 ($US 0.48), 80 ($US 1.26) and 43 Nepalese rupees ($US 0.68), respectively. As a result, NA may be disorderly administrated in this country. This study aimed to document NA use for HBV and discuss the socioeconomic problems that need to be addressed in most of the developing countries in Asia.

METHODS

CONSECUTIVE NEPALESE PATIENTS with chronic HBV infection in the outpatient clinic of the Tribhuvan University Teaching Hospital in Kathmandu were enrolled between August 2007 and August 2012, and the observation period extended from the first day of enrollment to February 2013. The Teaching Hospital, which was established in 1983 with the support of the Japanese Government as an integral part of the Institute of Medicine of Tribhuvan University, provides academic training programs (basic, graduate and postgraduate) for the Institute and, as a national hospital, renders medical care and services to the Nepalese people.

Nucleoside analog administration was based on the decision of the local hepatologist (P. K. S.), and the clinical course was prospectively analyzed. NA treatment was categorized into three groups: LMV-, ADF- and TDF-based. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees of the National Center for Global Health and Medicine, Japan, on human experimentation and with the Declaration of Helsinki of 1975, as revised in 2008. At that time, when we had started this project, the ethics committee of Tribhuvan University Teaching Hospital was not organized yet; hence, oral or written informed consent was obtained by the local hepatologist (P. K. S.), and this study was conducted as part of routine clinical practice.

Determination of HBV markers

Only a qualitative determination of hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (HBsAb), hepatitis B e-antigen (HBeAg) and hepatitis B e antibody (HBeAb) was available in Nepal, therefore, quantitative determination of the virological parameters was performed in Japan using serum samples collected at the Teaching Hospital. In addition to the samples collected at enrollment, the local hepatologist was advised to collect serial serum samples at least every 6 months in NA-treated patients.

Hepatitis B surface antigen, HBeAg and HBeAb were determined by chemiluminescent microparticle immunoassay (ARCHITECT; Abbot Japan, Tokyo, Japan). The HBV viral load was determined by transcription-mediated amplification (Chugai Diagnostics Science, Tokyo, Japan) or AccuGene m-HBV (Abbott RealTime HBV; Abbot Japan; lower limits of detection, 1.7 log copies/mL), according to the manufacturers' instructions. HBV genotype was determined by restriction fragment length polymorphism, as described previously.[6] NA-resistant HBV was determined by INNO-LiPA HBV DR version 2, according to the manufacturer's instructions (INNOGENETICS, Ghent, Belgium). This line-probe assay can simultaneously detect HBV mutations resistant to LMV, ADF, ETV and TDF.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test for comparison of the means of continuous variables or Fisher's exact test for categorical variables. A two-tailed *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Demographic features and laboratory data at enrollment

TABLE 1 PROVIDES the characteristics of the 65 patients (72% male) who were enrolled during the study period. The average ± standard deviation age was 33 ± 13 years, and 32% and 28% were aged in their

Table 1 Demographic features and laboratory data at the enrollment

	n (%)[†]
Sex (men/women)	47 (72%)/18 (28%)
Age (years)	33 ± 13[‡] (range, 16–71)
Serum ALT (IU/mL)	34 ± 32[‡] (range, 2–176)
HBeAg/HBeAb	
+/−	29 (46.0%)
+/+	1 (1.6%)
−/−	1 (1.6%)
−/+	32 (49.2%)
HBV viral load (log copies/mL)	
≤3.9	24 (36.9%)
4.0–6.9	16 (24.6%)
≥7.0	25 (38.5%)
HBV genotype	
Aa (A)	12 (18.5%)
C	14 (21.5%)
C/D recombinant	1 (1.5%)
D	33 (50.8%)
Not detected	5 (7.7%)

[†]Unless otherwise indicated.
[‡]Mean ± standard deviation.
ALT, alanine aminotransferase; HBeAb, hepatitis B e antibody; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus.

Table 2 Profiles of the NA-treated 44 patients enrolled in this prospective study

	No. of patients[†]
Sex (male/female)	28/16
Age (years)	31 ± 12[‡] (range: 16–71)
Treatment history of NA at enrollment	
Naive cases	22
for LMV/for ADF/for TDF	7/3/12
On NA treatment	19
of LMV/of ADF/of LMV + ADF/of TDF	8/3/2/6
Previous history of NAs	3
of LMV/of LMV + ADF	2/1
Treatment profiles of NA during observation period[§]	
LMV-based	12
on LMV alone/LMV alone→stop	9/3
ADF-based	6
on ADF alone/on LMV + ADF/LMV + ADF→stop	2/3/1
TDF-based	23
LMV→TDF/ LMV→ADF→TDF/on TDF alone	4/2/17

[†]Unless otherwise indicated,
[‡]Mean ± standard deviation.
[§]After excluding three patients with only previous history of NA, the treatment profiles were evaluated in 41 patients, as of February 2013.
ADF, adefovir; LMV, lamivudine; NA, nucleoside analogs; TDF, tenofovir.

20s and 30s, respectively. The extent of liver inflammation was relatively mild. The proportions of HBeAg positive and HBeAb positive patients were similar, and 38.5% of the patients had a viral load of 7.0 log copies/mL or more. The grades of viral load were quite consistent with the HBeAg/HBeAb status, namely, 21 (72%) out of 29 patients positive for HBeAg and negative for HBeAb had a higher viral load (≥7.0 log copies/mL), while 20 (63%) out of 32 patients negative for HBeAg and positive for HBeAb had a lower viral load (≤3.9 log copies/mL, $P < 0.001$). In this cohort, HBV genotype D was the most prevalent, affecting as many as 50% of the patients.

Profiles of the NAs-treated patients

Of the 65 enrolled patients, 44 (68%) received NA during the observation period or before enrollment. NA were prescribed in 16 of the 18 female patients (88.9%) and 28 of the 47 male patients (59.6%, $P < 0.05$). The average age of the NA-treated patients was significantly younger than that of the NA-untreated patients (31 ± 12 years vs 39 ± 15 years, $P < 0.05$).

In Table 2, the profiles of the NA-treated patients are detailed. Among 44 patients enrolled in this study, 22 patients (50%) were naive cases for NA, and LMV, ADF and TDF were administrated in seven, three and 12 patients, respectively. Nineteen patients had already been of NA treatment at enrollment: LMV, ADF, LMV + ADF and TDF were administrated in eight, three, two and six patients, respectively. Three patients had only previous history of NA, and received no further treatment. As for the treatment profiles of the 41 patients treated with NA during the observation period until February 2013, LMV-, ADF- and TDF-based therapies were carried out in 12, six and 23 patients, respectively. Four patients stopped NA: one receiving LMV alone and one receiving LMV + ADF because of HBsAg loss during the observation period, while the remaining two stopped LMV due to the decision of the patients. The preference for TDF as the first or second line of treatment was clearly demonstrated in this prospective study (56.1%).

Follow up of the NA-treated patients and emergence of NA-resistant HBV

The number of patients who returned to the hospital for follow-up visits decreased over time. As detailed in Figure 1, 44 NA-treated patients were classified according to both the time after enrollment (~every 6 months) and

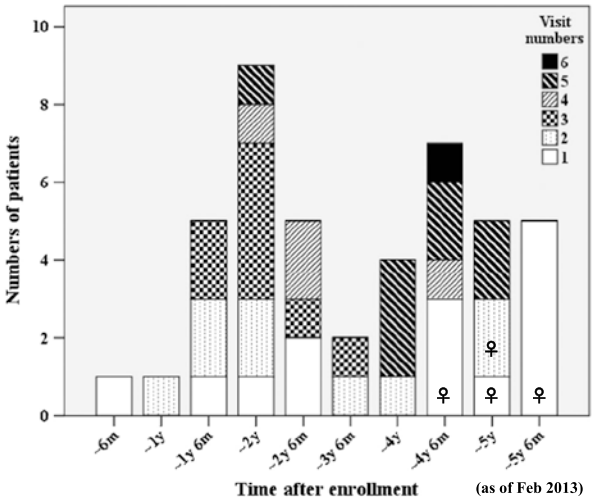

Figure 1 Subsequent hospital visits decreased in this prospective cohort of patients treated with nucleoside analogs. The local hepatologist was advised to collect serial serum samples approximately every 6 months in patients with chronic hepatitis B treated by nucleoside analogs. The numbers of hospital visits during the observation period were evaluated as of February 2013. For the patients who were enrolled more than 3 years from the end of observation period, the numbers of follow-up visits to the Teaching Hospital were significantly less than expected. ♀ denotes the four female patients who were lost to follow up before February 2013.

the numbers of follow-up visits to the Teaching Hospital (range, 1–6). Especially for the patients who were enrolled more than 3 years prior from the end of the observation period (i.e. February 2013), the numbers of follow-up visits were significantly less than expected. Under these circumstances, serial determinations of serum HBV DNA levels and the emerging rate of NA-resistant HBV were difficult to achieve in clinical settings.

Of those patients who were followed, seven developed NA-resistant HBV during the observation period (Fig. 2); as the first line treatment, five of these patients were treated with LMV and two patients were treated with TDF. In three patients (patients CHB-16, CHB-22, CHB-23) with LMV-resistant HBV, the clinical course varied. A change to TDF dramatically decreased the HBV viral load in patient CHB-16; however, serum levels of HBV DNA rose up again thereafter, probably due to poor adherence to TDF, considering absence of NA-resistant HBV; the M204I mutation spontaneously disappeared in patient CHB-22 despite the continuation of LMV; and in patient CHB-23, the M204I mutation disappeared immediately after replacement with TDF, without viral breakthrough. Interestingly, in the remaining four patients (i.e. CHB-25, CHB-28, CHB-38 and CHB-49), incidental and unrelated resistance to NA that had not been administrated (i.e. ETV or ADF) was detected; however, except in one patient in whom LMV was withdrawn (i.e. CHB-28), the HBV DNA levels were fully suppressed, despite the emergence of incidental resistance. In addition, in TDF-treated patients (i.e. CHB-38 and CHB-49), such incidental resistance (i.e. ADF resistance and ETV resistance, respectively) was detected just transiently and spontaneously disappeared.

To evaluate the effects of TDF, serial changes of serum HBV DNA were examined in 16 patients naive to TDF treatment, for whom multiple serum samples were available. As shown in Figure 3, 14 out of 16 patients (88%) achieved a fair response to TDF, except for two patients whose HBV DNA levels were unchanged, likely as a result of poor adherence. In 13 patients (81%), HBV DNA levels decreased to less than 3.0 log copies/mL within 12 months of the start of TDF. As detailed above, even though two patients (i.e. CHB-38 and CHB-49 in Fig. 2) developed incidental resistance to either ADF or ETV that was not administered, the virological response to TDF was satisfactorily maintained (Fig. 3).

DISCUSSION

IN THIS PROSPECTIVE study, we found that NA were prescribed preferably in younger female patients with chronic hepatitis B and that the monitoring systems for HBV markers were extremely insufficient in this country. As a consequence, not a few patients developed emergence of NA-resistant HBV: known resistance to LMV in three patients and incidental resistance to non-administrated NA in four patients.

In developing countries, where health insurance systems are not available, patients can obtain medications without a doctor's prescription after the first visit to the pharmacy. Moreover, socioeconomic problems such as persistent poverty and insufficient infrastructure may prevent regular medical checkups,[7] even with chronic liver disease, as was confirmed in the present study.

One concern in younger patients and, in particular, female patients of reproductive age, is the use of NA without appropriate checkups. All of the approved NA have prominent warnings regarding the potential risk of fetal anomaly with their use just before and during pregnancy. Telbivudine and TDF are considered Pregnancy Category B drugs (i.e. no known teratogenicity or embryotoxicity in animal studies, but inadequate human studies) by the US Federal Drug Administration, while LMV, ADF and ETV are Category C (i.e. embryotoxic or teratogenic in animal studies, but inadequate human studies).[8]

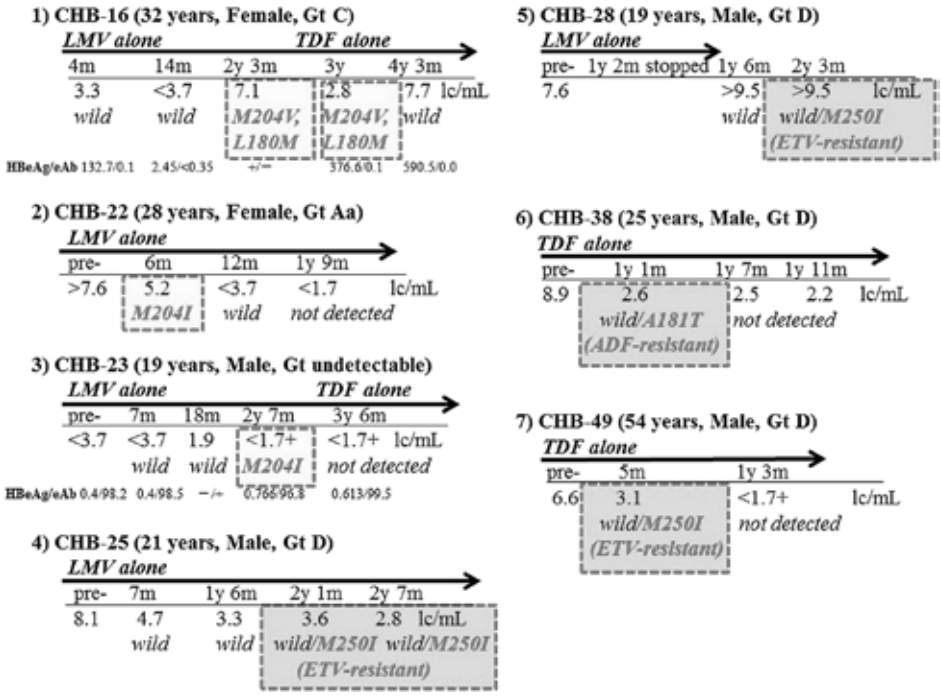

Figure 2 Emergence of NA-resistant hepatitis B virus. Seven patients developed NA-resistant hepatitis B virus in this prospective cohort. Patients 1–5 received LMV-based treatment, and patients 6 and 7 received TDF-based treatment. In two patients (i.e. CHB-25 and CHB-28) treated with LMV and two patients (i.e. CHB-38 and CHB-49) treated with TDF, incidental resistance to unused NA such as ETV or ADF was also detected. ADF, adefovir; ETV, entecavir; Gt, genotype; lc, log copies; LMV, lamivudine; NA, nucleoside analog; TDF, tenofovir.

Accordingly, their use in men and women of child-bearing age should be limited.

Also of concern is that long-term NA use may result in NA-resistant HBV. The first approved NA for chronic HBV was LMV, and the cumulative incidence of LMV resistance, including M204V/I or L180M, has been reported up to 70% at treatment year 5,[4] leading to hepatitis flare-up as well as viral breakthrough. ADF add-on therapy is effective for the prevention of these adverse events; however, additional use of medication may increase patients' mental and financial burden. In contrast, ETV and TDF are potent HBV inhibitors with a high barrier to resistance.[9–13] Of the limited number of patients with follow-up data in the current study, seven developed NA-resistant HBV; five were treated with LMV and two with TDF. The classic YMDD mutation was detected in three patients treated with LMV, and, in the remaining four patients, incidental resistance to other non-administrated NA (ETV and ADF) was found. This information is relevant to future consideration of drug choice in these patients. Because, in CHB-38 and CHB-49 treated with TDF, we did not check the NA-resistant HBV at pretreatment, the possibility of pre-existing ADF-resistant or ETV-resistant HBV could not be excluded. According to the recent systematic review and meta-analysis,[14] the incidence of naturally occurring resistance rates in naive chronic hepatitis B was reported to be 5.39% globally (including China, Japan, Turkey, Korea, Iran, India, Pakistan, Thailand, Australia, USA, Brazil, Spain, Italy, France and South Africa; 95% confidence interval, 4.54–6.24%); the highest incidence was 7.83% (6.48–9.18%) in China, while 1.39% (0.67–2.10%) in other countries. In subgroup analysis, it was shown that genotype C HBV infection, male and HBeAg negative patients had a slightly higher natural mutation rate, although the precise mechanisms were not elucidated. In contrast, in CHB-25 and CHB-28, ETV-resistant HBV first emerged during the observation period. In CHB-25, the virological response to LMV seemed to be relatively slow, and in CHB-28, LMV was stopped owing to the patient's will at 1 year and 1 month before. Although these two cases could not yet be classified as multidrug resistance evolution in patients failing NA,[15] further follow ups

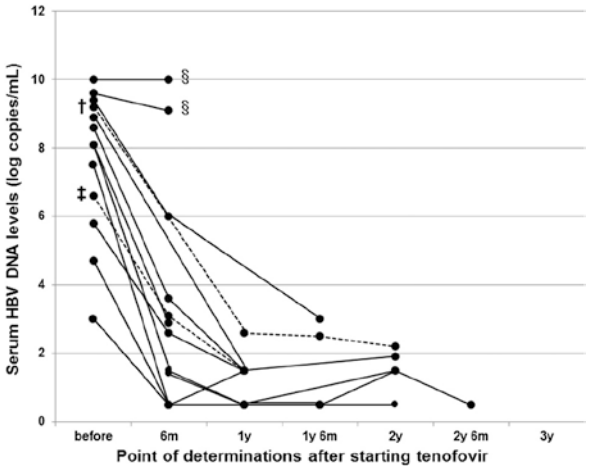

Figure 3 Serial changes in serum HBV DNA levels in patients naive to tenofovir treatment. In 16 patients naive to TDF treatment, multiple serum samples were available for determination of serum HBV DNA levels. Fourteen of 16 patients (88%) achieved a fair response to TDF, except for two patients (§) who likely had poor adherence, considering no emergence of NA-resistant HBV. In 13 patients (81%), HBV DNA levels decreased to less than 3.0 log copies/mL within 12 months after the start of TDF. Two patients (i.e. CHB-38 [†] and CHB-49 [‡] in Fig. 2) developed incidental resistance to either ADF or ETV, that was not administrated, the virological response to TDF was satisfactorily maintained (dashed line). ADF, adefovir; ETV, entecavir; HBV, hepatitis B virus; LMV, lamivudine; NA, nucleoside analog; TDF, tenofovir.

should be mandatory to prevent progression of chronic liver diseases.

A recent study reported that no resistance to TDF was detected after 6 years of treatment in patients with HBeAg positive and HBeAg negative chronic HBV, and regular use of TDF could satisfactorily suppress HBV proliferation,[16] which was also demonstrated in the present cohort. These lines of evidences may explain the recent preference for TDF in Nepal, even though the monitoring systems for virological markers have not been well established.

There are several limitations in this study. First, owing to the small sample size, we must be prudent in drawing conclusions. However, we believe that the use of antiviral medications documented in this study are representative of general practice at this hospital, the affiliated hospital of the only national university of Nepal. Second, it is difficult to determine the specific reasons other than poor drug adherence in patients with poor response to NA, because we could did not have enough data regarding the quality or storage conditions of generic drugs from neighboring countries.

In conclusion, this prospective study documented the present status of antiviral therapy in patients with chronic HBV in Nepal, one of the representative developing countries in Asia. Owing to a lack of monitoring systems, there is a risk of an increase in NA-resistant HBV infections. Given the prevalence of HBV in these countries, this could represent a global public health problem. In addition, education for physicians as well as for infected patients regarding the appropriate use of NA is urgently required.

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Editorial

Earthquake disaster-associated health effects and the need for improved preventive measures.

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During the past 20 years, earthquakes alone have caused more than a million deaths worldwide[1]. Nine countries (Armenia, Chile, China, Guatemala, Iran, Italy, Japan, Peru, and Turkey) account for more than 80% of all fatalities in this century, and almost half of the total numbers of earthquake casualties in the world during this period have occurred in China alone[2].

The recent Nepal earthquake of April 25, 2015 of magnitude 7.8 on the Richter scale had its epicenter in the area near Barpak, a mountain village between the capital, Kathmandu, and the tourist town of Pokhara. The earthquake was followed by many powerful after shocks on the same day and a very powerful one (6.7 on the Richter scale) hit Nepal on the very next day, Sunday April 26.

The earthquakes, which caused extensive damage to buildings and thousands of deaths and injuries were even felt in Pakistan, India and Bangladesh.

In Nepal many historic and recently-built buildings collapsed or were very badly damaged, temples have been ruined, roads destroyed. There were nearly 8,800 deaths and more than 23,000 injured in Nepal and tens of deaths in India & Tibet. The quake was followed by more than 1000 after shocks and another huge earthquake (7.3 on the Richter scale) on May 12.

Despite remarkable scientific progress in seismology and earthquake resistant engineering during the past few years, achieving high standards of safety against earthquakes is a goal that has yet to be achieved in many parts of the world. But in Nepal, due to lack of resources and an unstable political situation, no proper safety measures were established a head of time, resulting in great loss of innocent Nepalese (and foreigners) people's lives and earthquake related injuries and illness.

People's health impact: In most earthquakes disasters, people are killed by mechanical energy as a direct result of being crushed by falling building materials. Deaths resulting from major earthquakes can be instantaneous, rapid, or delayed[3]. Instantaneous death can be due to severe crushing injuries to the head or chest, external or internal hemorrhage or drowning from earthquake-induced tidal waves (tsunamis). Rapid death occurs within minutes or hours and can be due to asphyxia from dust inhalation or chest compression, hypovolemic shock, or environmental exposure (e.g., hypothermia). Delayed death occurs within days and can be due to dehydration, hypothermia, hyperthermia, crush syndrome, wound infections, or postoperative sepsis[4,5]. As with most natural disasters, the majority of people requiring medical assistance following earthquakes have minor lacerations and contusions caused by falling elements, like pieces of masonry, roof tiles and timber beams[6]. The next most frequent reasons for seeking medical attention are simple fractures not requiring operative intervention[7].

Such light injuries usually require only outpatient treatment and tend to be much more common than severe injuries requiring hospitalization. Major injuries requiring hospitalization include skull fractures with intracranial hemorrhage (e.g., subdural hematoma); cervical spine injuries with neurologic impairment; and damage to intrathoracic, intraabdominal, and intrapelvic organs, including pneumothorax, liver lacerations, and ruptured spleen. Most seriously injured people will sustain combination injuries, such as pneumothorax in addition to an extremity fracture. Am-putations and chronic sequelae of orthopedic and neurologic injuries, especially spinal cord injuries, can be expected[8]. Huge amounts of dust are generated when a building is damaged or collapses, and dust clogging the air passages and filling the lungs is a major cause of death for many building-collapse victims[2,9,10]. Fulminant pulmonary edema from dust inhalation may also be a delayed cause of death[11]. Dust has hampered rescue and clean-up operations by causing eye and respiratory-tract irritation. Burn sand smoke inhalation from fires used to be major hazards after an earthquake.

During the urbanization process, cities are laden with chemical and petroleum products that could contribute substantially to the generation of toxic substances following an earthquake[11,12]. Industrial storage facilities for hazardous

materials might explode or leak and damage to a nuclear power plant could lead to widespread contamination by radioactive materials. In a major earthquake, pipelines carrying natural gas, water, and sewage can be expected to be disrupted.

In earthquakes, people over 60 years of age are at increased risk for death and injury and can have a death rate five times higher than that of the rest of the population. Children between 5 and 9years of age, women, and the chronically ill also seem to be at an elevated risk for injury and death[12]. Lack of mobility to flee collapsing structures, inability to withstand trauma, and exacerbation of underlying disease are factors that may contribute to the vulnerability of these groups. Mortality distribution by age will also be affected to a certain degree by the social attitudes and habits of different communities. In general, the morbidity and mortality rates are significantly greater among people who are indoors than among those who are outdoors when the tremor begins [9]. Although the probability of finding live victims diminishes very rapidly with time, entrapped people have survived for many days. People have been rescued alive 5, 10 and even 14 days after an earthquake[13], and these "miracle rescues" are often the result of exceptional circumstances. For example, someone with very light injuries could be trapped in a void deep in the rubble with air and possibly water available.

What efforts need to be taken to improve preventive measures?: Prevention and control efforts need to be multidisciplinary and should include public education programs, as well as better building design and improved quality of construction in those areas most likely to suffer an earthquake[14]. Avoiding unnecessary residential and commercial construction on or near active faults and in areas subject to landslide slope failures, soil liquefaction, and rock falls is technically a secondary prevention measure for earthquakes, but it is a primary prevention measure for earthquake related injuries[15]. Prompt rescue should improve the outcome of victims, and early medical treatment should lessen the sequelae of the primary injuries (e.g., wound complications, chronic neurological disabilities). Provision of adequate food, water, and shelter should especially help people in vulnerable age groups and those with pre-existing diseases. Effective environmental control measures should prevent secondary environmental health problems, such as gas leaks, fallen or loose wires, damaged appliances and pipelines, sewage backup, and water contamination. Public health officials need to establish in advance how the affected areas will be surveyed. Just as speed is required for effective search and extrication, it is also essential for effective emergency medical services, since the greatest demand occurs within the first 24 hours[9].The medical and public health impacts of a severe earthquake may well be compounded by significant damage to medical facilities, hospitals, clinics, and supply stores within the affected area [16].

In the worst-case scenario, a hospital building may itself be damaged by the earthquake, and the hospital staff may have to continue emergency treatment without using the buildings[17]. Hospital emergency plans in earthquake areas should provide for the contingency of evacuating patients from the wards; safely removing critical equipment from operating theaters, radiology departments, and other parts of the hospital; and re-establishing routine patient-care services[18].

In addition, a comprehensive training course conducted in advance is essential for provision of sophisticated medical care to victims in the first hours after a catastrophic earthquake. In such a scenario physicians, nurses, and other health care providers who will encounter situations for which they have not been prepared. Therefore, a thorough training course should be required for all medical facility staff. As outlined in the medical disaster-response model, the subjects covered in the training course would be mass-casualty triage, airway management, use of intravenous fluids, anesthesia and analgesia in the field, crush-injury treatment, and command and control.

Problems and challenges: The first need in earthquake assistance is medical care and medicines for the injured in a situation where all government hospitals in the area have been destroyed. Teams of independent doctors from other parts of the country, along with army medical staff, are providing some basic access in such areas in Nepal. However, some recently created medical camps in different areas, mainly in rural Nepal, seem to have very few or no bandages, gauze or painkillers. There is also a need for basic provisions, including food, water and tents.

Earthquakes will continue to affect human populations into the distant future. With technologic advances, increasingly complex infrastructures, and new building designs, the built environment will evolve over time. Shifts in population locations and characteristics will accompany these environmental changes. Every earthquake is different, as is every population affected. Thus, despite the significant challenges involved, there is a critical need for evidence-based prevention and preparedness efforts to ensure the best possible chances of limiting earthquake related death, injury and destruction in the future.

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