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To contact us:

Western Pacific Surveillance and Response
World Health Organization
Office for the Western Pacific Region
United Nations Avenue
1000 Manila, Philippines
wpsar@wpro.who.int
www.wpro.who.int/wpsar

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A decade of gains in public health emergency preparedness and response at points of entry

Shahrokh Roohi^a and Todd Wilson^a

Correspondence to Shahrokh Roohi (e-mail: sroohi@cdc.gov).

The role of air travel in rapid translocation of infectious disease is indisputable.¹ The global health community has long been concerned about the movement across borders of vaccine-preventable diseases, tuberculosis and other diseases of public health concern. These concerns escalated following the September 2001 terrorist attack and the anthrax bioterrorism incident in the United States of America; the worldwide spread of severe acute respiratory syndrome (SARS) in 2003; and the reemergence of H5N1 avian influenza soon thereafter, which stoked fears about the possibility of a severe influenza pandemic. To better prepare and coordinate countries to respond to all-hazards health emergencies at their borders, in the past 10 years the global public health community has formed numerous domestic and international alliances.

In the international arena, country public health officials contributed to the revision of the World Health Organization (WHO) International Health Regulations. Prior to the 2005 revision, Member States were required to report cholera, plague and yellow fever. The revised regulations² are risk-based rather than prescriptive and mandate the recognition and notification of any unusual public health event or emergency of international concern that meets certain criteria, including biological, chemical and radiological risks. As a result, much has been done to strengthen core public health capacities and preparedness for emergency response at points of entry and exit, contributing to community and global health security. Additionally, international partners supported the United Nations International Civil Aviation Organization's Cooperative Arrangement for the Prevention of Spread of Communicable Disease through Air Travel. Through this collaboration, Member States engaged in planning

for communicable disease response at airports, including drills, exercises and response coordination between the aviation and public health sectors. Furthermore, to facilitate the sharing of information and expertise, public health officials supported the WHO Ports, Airports and Ground Crossings Network (PAGNet). This network provides a forum to address international travel health and transport issues in real time. PAGNet members and WHO regional staff meet periodically for training and information sharing. Topics of discussion include preparedness at air, sea and land points of entry and exit, and coordination of response between governmental entities. Another noteworthy effort is the Global Health Security Initiative. The participants in this group, which include the health ministers of Canada, France, Germany, Italy, Japan, Mexico, the United Kingdom and the United States of America, as well as the European Commission and WHO officials, work to strengthen global preparedness for pandemics and bioterrorism.

Within the United States of America, governmental interagency planning efforts in the past decade have bolstered emergency preparedness planning, trainings, drills and exercises at the 20 points of entry that receive about 80% of inbound international airline passengers. These collaborations were energized in 2003 post-SARS, redoubled in 2006 through the development of response plans for communicable diseases in airline passengers and further expanded in 2009 by adding a passenger health screening component.

Because of these and related collaborations in the last decade, coordination between border health authorities has improved markedly in addressing travel and points-of-entry public health issues. In 2009, the H1N1 influenza pandemic tested the world's preparedness with its rapid spread across the globe from

^a US Centers for Disease Control and Prevention
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its origin in North America. The public health community ably responded at international borders by issuing travel advisories and health information for travellers and implementing health declarations, contact tracing or screening in some cases.³

Within the United States of America, border planning efforts also improved readiness and coordination for other unanticipated public health emergencies. Examples include the border public health response to the 2010 Haiti earthquake and the subsequent cholera outbreak, the Fukushima Daiichi nuclear power plant accident caused by the 2011 Great East Japan Earthquake, the repatriation of citizens during these disasters and the communication of health risk to travellers following the global resurgence of measles in 2011.

Clearly, much progress has been achieved in preparedness and response planning in the last decade, although more remains to be done, especially as it relates to improving linkages between domestic and global health security.⁴ However, in an era of budget cutting and global austerity, we may be at risk of slipping backward. Progress can be undermined if health authorities fail to connect the dots between border health and traditional public health activities. Border planning, training and exercise activities, along with timely cross-fertilization of ideas and information sharing can protect the health of the travelling public. This in turn can mitigate the burden of disease in the travellers' destination communities during global outbreaks.

Moving forward, it is essential that the international border health community facilitate dialogue on research, evaluation, publication of data and discourse on published reports with differing conclusions and recommendations on border measures. This will help unify our understanding of the role that border interventions can play under different circumstances and promote balanced and evidence-based decision-making. More work remains to be done in defining benchmarks for preparedness, metrics for impact, use of risk assessment to inform decision-making and the establishment of

scientific guiding principles for just-in-time decision-making on when to initiate and end border measures. Without agreed-upon scientific principles, definitions and performance standards, it will be difficult to measure our progress and prioritize future programmatic and scientific investments.

Throughout recorded history, travel has been a major factor in the spread of disease. This will continue to be the case in the foreseeable future given the volume and speed of travel, the just-in-time global shipping of goods and the limited availability of local commercial supplies. Early detection, rapid public health response, and all-hazards coordination for biological, zoonotic, chemical and radiological incidents at our borders are more important now than ever. We must therefore continue to learn from and expand upon the gains we have made in the past decade by advancing the evaluation of border public health activities, publishing border intervention data and sharing lessons learnt on all-hazards public health emergency preparedness and response.

Disclaimer:

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the United States Department of Health and Human Services or the Centers for Disease Control and Prevention.

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Cholera in Papua New Guinea and the importance of safe water sources and sanitation

Paul Horwood^a and Andrew Greenhill^a

Correspondence to Paul Horwood (e-mail: paul.horwood@pngimr.org.pg or paul.horwood@hotmail.com).

Until recently cholera had never been reported in Papua New Guinea despite the close proximity of cholera-endemic countries and the presence of environmental and social characteristics that are considered risk factors for cholera outbreaks. The current outbreak began in July 2009¹ and rapidly spread throughout the coastal regions of the country. Initial characterization studies using variable-number tandem repeat analysis indicate that the outbreak was a recent clonal incursion from South-East Asia. By mid-2011 the outbreak had resulted in the reporting of more than 15 500 cases of cholera and over 500 deaths: a case fatality ratio (CFR) of approximately 3.2%.² Following an outbreak of cholera, interventions such as the introduction of oral rehydration therapy aim to reduce the CFR to below 1%. This elevated CFR is likely a reflection of the inaccessibility of much of the country, the lack of health care services available in remote regions and the general unpreparedness for an outbreak of this kind. This premise is supported by the differences in CFRs between the relatively well-serviced National Capital District (0.1%) and more remote regions such as the Western Province (8.8%).

When cholera spreads to a non-endemic area, or a new epidemic emerges within an endemic country, it is often preceded by a natural or human-induced disaster.³ This was not the case here, with no notable disaster impeding health care delivery or access to safe drinking-water. It seems that in parts of the country, the combination of an increasing population, reduced access to health care and lack of safe drinking-water has reached a critical point, thereby facilitating the spread of cholera once it was introduced. The worst affected are people living in settlements where crowding and unsanitary conditions are the norm. However, rural

villages have also been affected where service delivery is poor. The presence of cholera in Papua New Guinea is a timely reminder of the declining standard of service delivery in much of the country, which is exemplified by the poor epidemiological data that were collected during the outbreak and the lack of ongoing active surveillance for cholera cases.

The concern now is that cholera will persist in the environment and Papua New Guinea will officially become a cholera-endemic country with periodic outbreaks of variable severity. Factors such as the large, slow moving, saline river systems and the lack of adequate sanitation and hygiene in many communities increase the potential for endemicity. *Vibrio cholerae* is highly adapted to the aquatic environment, and lives naturally in riverine and estuarine ecosystems.⁴ With the sustained and widespread transmission of cholera for over two years in Papua New Guinea, it is likely that an environmental reservoir will be, or already has been, established. Cholera endemicity has broad implications beyond the health risk to its citizens, including the possibility of temporary trade barriers, reduction in tourist numbers and an increased burden on the health care systems.

Access to safe drinking-water and adequate sanitation are widely recognized as the key factors to preventing cholera outbreaks. In Papua New Guinea, only approximately 40% of people have access to a safe water supply and adequate sanitation, one of the lowest rates in the Western Pacific Region.^{5,6} Moreover, there has been no significant improvement in recent years. The importance of improved drinking-water was highlighted by the outbreak in the Central Province where communities with access to reticulated water

^a Papua New Guinea Institute of Medical Research, Goroka, Papua New Guinea.
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supplies were largely untouched by the outbreak. Yet similar villages in the same region without access to safe water supplies were severely affected by cholera cases. Cholera vaccination is increasingly being used in endemic regions as an adjunct to improving water supplies and sanitation. However, vaccination for cholera in an outbreak setting has not been trialled on a large scale and questions remain as to the logistical and financial challenges of using multidose vaccines in an epidemic situation where people displacement and civil unrest may hinder access.⁷

Poor access to safe drinking-water and sanitation is no doubt a major driving factor behind the spread of cholera in Papua New Guinea – a significant event in itself. However, the implications of inadequate safe water sources and poor sanitation and hygiene are much broader. Enteric diseases remain an important cause of morbidity and mortality in Papua New Guinea and in other low-income countries. In Papua New Guinea, diarrhoea is the fifth most common reason for presenting to health clinics and contributes to over 15% of deaths in children under five years of age.^{5,8} Other enteric diseases such as shigellosis and typhoid fever are also important causes of morbidity and mortality in Papua New Guinea, but their exact burden is unknown due to the lack of in-country diagnostic capacity. Poor access to safe water and poor hygiene contribute to other disease burdens, such as enteric parasites, which may contribute to poor nutritional status,⁹ skin infections, which are the leading cause of outpatient visits,⁵ and increased spread of respiratory infections.¹⁰ Moreover, improving sanitation and hygiene and safe water can improve educational outcomes, particularly for girls.¹¹ Clearly, improving access to safe water and improved sanitation and hygiene would reduce the risk of future outbreaks of cholera, and, if widely implemented, these measures could greatly improve health and social outcomes in Papua New Guinea.

The current lull in cholera cases throughout most of the country should not be regarded with complacency. Health authorities need to be aware that cholera presents as a seasonal disease in areas where it is endemic. Factors such as rainfall, salinity, temperature and copepod (zooplankton) blooms have all been linked to periodic outbreaks in countries such as Bangladesh and India.⁴ Papua New Guinea is likely to face further challenges from cholera outbreaks and the extent of

preparations to assist affected communities and limit the spread of the disease will determine the impact that the next outbreak has on the people and economy of Papua New Guinea.

A strategy needs to be planned and implemented to limit and contain cases in the likely event of a further regional outbreak of cholera. Environmental and syndromic surveillance, backed up by rapid and appropriate response, need to be conducted to mitigate the impact of another nationwide outbreak. Safe, clean water supplies and associated educational campaigns need to be provided to at-risk communities to limit transmission. A clear strategy to deal with subsequent outbreaks will save lives and limit the extent of the outbreaks. Moreover, any measures taken to prevent further outbreaks of cholera are likely to have a positive impact on the burden of other infectious diseases.

Conflicts of interest

None declared.

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Epidemiological and clinical characteristics of patients who died from Influenza A(H1N1)pdm09 in Viet Nam

Phan Thanh Tinh,^a Phan Trong Lan,^{ab} Patel Mahomed^a and Tran Minh Nhu Nguyen^{ac}

Correspondence to Phan Thanh Tinh (e-mail: mdtinh@gmail.com).

We describe the epidemiological and clinical characteristics of patients who died from influenza A(H1N1)pdm09 in hospitals in Viet Nam between August 2009 and March 2010.

Of 58 fatal cases, 32 (55%) were below 30 years of age and 14 (24%) were pregnant females. Forty-five (78%) patients had at least one underlying medical condition including chronic heart, kidney or lung diseases or pregnancy. Twelve (21%) cases sought medical attention on the day of symptom onset. Only 13 (36%) of 36 cases for whom treatment data were available had been given antiviral drugs within the recommended two days of symptom onset.

The clinical and epidemiologic characteristics of the patients who died from influenza A(H1N1)pdm09 are similar to those reported from other countries. To improve preparedness and response to future pandemics, Viet Nam needs to strengthen the surveillance of influenza; increase laboratory capacity to test for influenza viruses; and develop strategies for promoting the timely attendance of at-risk individuals at health facilities and the early administration of antiviral drugs, particularly for persons with underlying medical conditions and pregnant females.

INTRODUCTION

The emergence and spread of A(H1N1)pdm09 was first reported in Mexico in the spring of 2009.¹ This novel virus then spread rapidly across the world. On 11 June 2009, the World Health Organization (WHO) declared this event the first influenza pandemic of the 21st century. By March 2010, 213 countries had reported cases with laboratory-confirmed A(H1N1)pdm09 and 17 483 associated deaths.^{2,3}

The pandemic was introduced into Viet Nam via Ho Chi Minh City in early June 2009 by passengers flying in from countries affected by the pandemic, particularly the United States of America and Australia. The number of cases increased in August as the virus spread to other regions, and by March 2010, 11 208 laboratory-confirmed cases were reported across Viet Nam. To strengthen Viet Nam's preparedness, surveillance and response capacities, we conducted a retrospective study of the epidemiological and clinical features of patients who died from A(H1N1)pdm09 to determine the frequency of underlying medical conditions and the use of antiviral drugs by health staff.

METHODS

The definition of a suspected case with A(H1N1)pdm09 was the sudden onset of fever (>38°C) plus symptoms of an acute respiratory infection that started within a week of travel to an affected area or history of close contact with a patient who had laboratory-confirmed A(H1N1)pdm09. Confirmed influenza A(H1N1)pdm09 was defined as a suspected case who tested positive on real-time RT-PCR assay in accordance with the protocol from the US Centers for Disease Control and Prevention.⁴ Between August 2009 and March 2010, a A(H1N1)pdm09 related death was defined as death in a person who had laboratory-confirmed A(H1N1)pdm09, regardless of the underlying cause of death. We selected this study period because the first known death from influenza was in August 2009, and we continued the enhanced hospital-based surveillance for deaths until March 2010.

In response to the pandemic, a system was established to enhance surveillance across Viet Nam. All hospitals in Viet Nam were requested to collect naso-pharyngeal swabs from all patients who had

^a Viet Nam Field Epidemiology Training Programme.

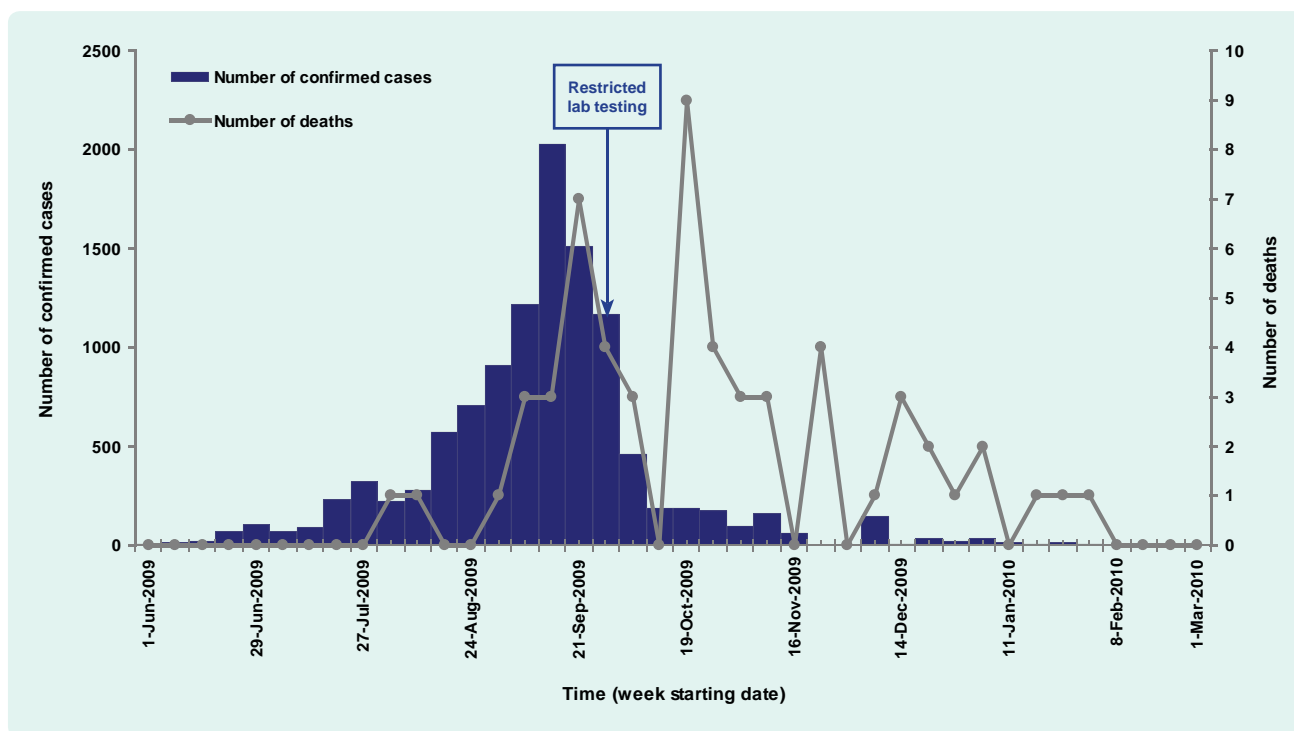
^b General Department of Preventive Medicine, Ministry of Health, Viet Nam.

^c World Health Organization Representative Office, Hanoi, Viet Nam.

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Figure 1. Cases of laboratory-confirmed influenza A(H1N1)pdm09 and associated deaths ($n=58$) by date of illness onset, Viet Nam, June 2009 to March 2010



an acute respiratory infection. The swabs were sent initially to the four reference laboratories accredited by the Ministry of Health, namely, the laboratories at the National Institute of Hygiene and Epidemiology in Hanoi, the Ho Chi Minh City Pasteur Institute, the Nha Trang Pasteur Institute and the Tay Nguyen Institute of Hygiene and Epidemiology in Buon Me Thuot. However, as the number of cases rose rapidly by October 2009, the Ministry of Health authorized laboratories at 16 tertiary level hospitals with adequate facilities, equipment, appropriately trained staff and standard operating procedures to conduct the confirmatory RT-PCR testing. Furthermore, from the first week of October 2009 onward, testing was limited to persons at risk of severe complications (pregnant females, persons with chronic diseases and young children), and to patients with severe respiratory illness (labelled as restricted laboratory testing in Figure 1).

Hospitals reported any deaths among patients with laboratory-confirmed A(H1N1)pdm09 on a standard form to the provincial preventive medicine centres; the latter forwarded the reports to the respective regional Institutes of Hygiene and Epidemiology or Pasteur Institutes that in turn submitted them to the

Department of Communicable Disease Control, Ministry of Health, in Hanoi. The data included identifying and demographic information, date of onset of symptoms and of the first attendance for health care, presence of underlying medical conditions and pregnancy, details of antiviral administration, date and place of death, medical examiner reports and the death certificate.

The denominator for calculating the mortality rate was based on Viet Nam's 2009 population census, i.e. a total population of 85.8 million.

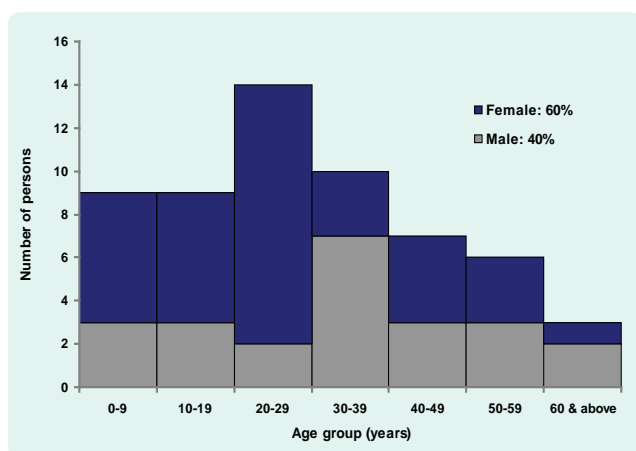
RESULTS

Deaths from influenza A(H1N1)pdm09

The first death in a person with laboratory-confirmed A(H1N1)pdm09 was reported in August 2009 in the central province of Khanh Hoa, and by March 2010 a total of 58 deaths had been reported. The overall mortality was 0.7 per million population.

The number of deaths increased gradually in August 2009 to peak in late September 2009, in parallel with the number of notified laboratory-confirmed

Figure 2. Age and sex distribution of persons who died from influenza A(H1N1)pdm09 ($n=58$), Viet Nam, 2009–2010



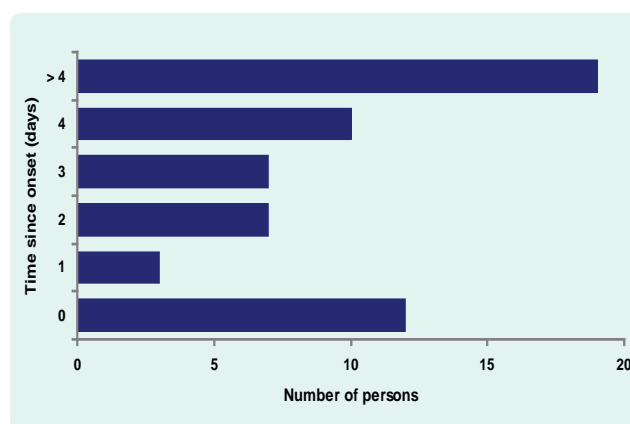
cases, but dropped sharply over the following three weeks (Figure 1). The drop in number of deaths coincided with the drop in the number of laboratory-confirmed cases, and this occurred one week before the Ministry of Health recommended it was no longer necessary to test everyone with symptoms of an acute respiratory illness. The number of deaths then increased sharply to reach the second peak in late October 2009 and early November 2009.

The age range of the 58 persons who died was between 10 months and 64 years, with a median of 29 years. There were 32 (55%) deaths in patients below 30 years of age, 49 (84%) deaths below 50 years of age, and seven deaths (12%) in children less than five years of age. Females accounted for 35 (60%) of the deaths (Figure 2).

Twelve (21%) of the 58 patients who died sought health care on the day the symptoms first appeared (Figure 3); eight of them had underlying conditions, and four females were pregnant, one of whom also had asthma. A further 10 (17%) patients attended within one to two days, seven (12%) attended on day three after symptom onset, 10 (17%) on day four and the remaining 19 (33%) attended more than four days after symptom onset.

The first place of attendance for health care was as follows: two (4%) at commune-level and private health care clinics, 18 (31%) at a district or municipal level hospital, 28 (48%) at a provincial level hospital and 10 (17%) at a tertiary level hospital.

Figure 3. Time interval between onset of symptoms and first health care attendance of persons who died from influenza A(H1N1)pdm09 ($n=58$), Viet Nam, 2009–2010



Underlying medical conditions

Of the 58 persons who died, 45 (78%) had at least one underlying medical condition (Figure 4). An underlying medical condition was reported in nine of 10 cases (90%) aged over 50 years, in 24 of 30 cases (80%) aged between 20 and 49 years and in 12 of 18 cases (67%) aged below 20 years. The mean interval between onset of symptoms and death in these patients with underlying medical conditions was 10 days while in the remaining 13 cases the interval was 12 days.

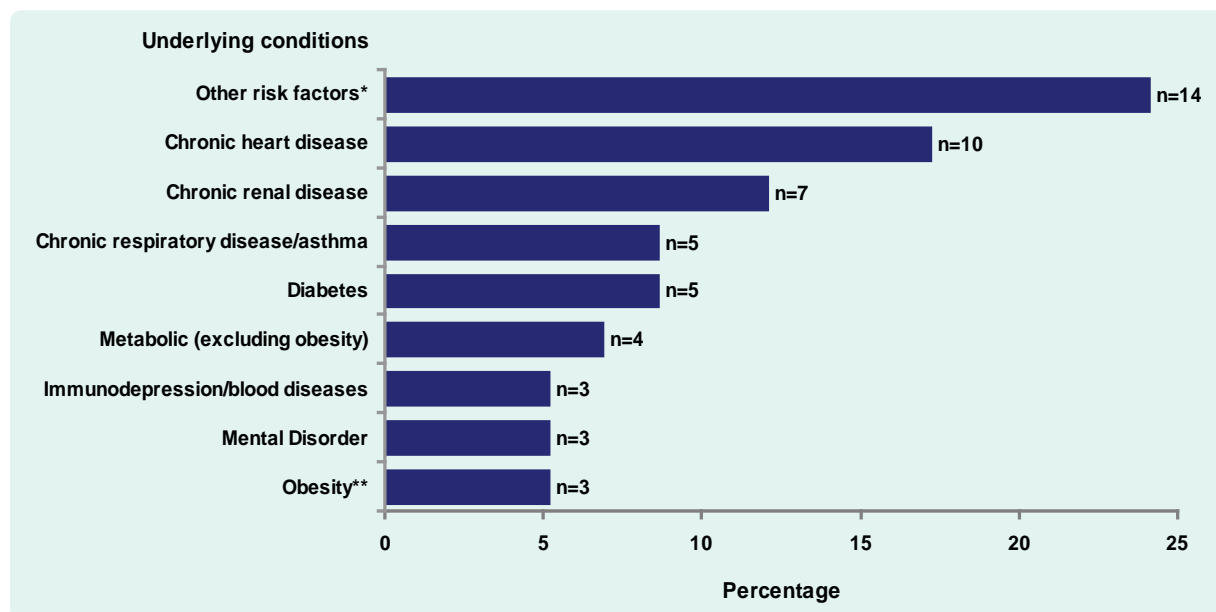
Data on the use of antiviral treatment (oseltamivir) was available on 36 (62%) of the 58 cases. Of these, only 13 (36%) had received medical care within two-days of symptom onset and received the drug within this period as recommended by WHO.⁵ Treatment data on the other nine cases who received medical care in this period was not available. The remaining 23 cases received treatment after this period, while treatment data on the other 13 cases who sought care more than two days after symptom onset was not available.

Thirty-three (55%) of 58 cases required mechanical ventilation; the median interval between symptom onset and start of ventilation was five days.

Pregnancy

Of the 35 females who died, 14 (40%) were pregnant, and their ages ranged from 15 to 39 years, with a median

Figure 4. Underlying medical conditions in persons who died from influenza A(H1N1)pdm09 (n=58), Viet Nam, 2009–2010



* Cases may have more than one underlying medical condition; the category labelled “Other risk factors” included arthritis, malaria, Down’s syndrome, hepatitis B, and sequelae of polio.

** Obesity was defined as a body mass index equal to or more than 30.

of 24 years; eight (57%) of the 14 pregnant females had an additional underlying medical condition, including heart disease, kidney disease, chronic lung disease and other metabolic disorders. Ten females (71%) were in the third trimester of pregnancy and the remaining four (29%) were in the second trimester.

DISCUSSION

Between August 2009 and March 2010, 58 persons were reported to have died from laboratory-confirmed A(H1N1)pdm09 in Viet Nam, yielding an overall mortality of 0.7 per million population. Most patients were young or middle-aged, and had an underlying medical condition or were pregnant. Only 13 of 22 patients who were treated within the first two days of symptom onset were given oseltamivir within the recommended two days after onset of symptoms; this included six of the seven pregnant females who sought care within two days of symptom onset.

The overall A(H1N1)pdm09 mortality in Viet Nam is low when compared with reports ranging between 2.2 and 3.3 deaths per million in northern hemisphere countries, except in Japan, where the mortality rate was 0.2 per million.⁶ The most likely explanation for the low mortality in our study, as elaborated under

‘limitations’ below, is an underascertainment of the number of deaths.

Of the 58 deaths, 49 (84%) were aged below 50 years, and just over half (55%) were below 30 years. Consistent with other reports,^{6–9} deaths in this younger age group contrasts with the experience from seasonal influenza in the United States of America where about 90% of deaths are in those aged over 65 years.¹⁰ This observation probably reflects an age-cohort effect as the elderly were probably protected from severe disease due to previous exposure to antigenically similar viral strains.¹¹

Of all deaths, 45 (78%) had an underlying medical condition. This finding is also similar to the experience in other countries. In The United Kingdom and Greece, for example 72% and 82% of the deaths attributed to A(H1N1)pdm09, respectively, had at least one underlying risk factor.^{8,9} In France, over half the patients had an underlying risk factor, including obesity, although obesity is usually not considered a risk factor for deaths from seasonal influenza.⁷ However, it is possible we may have overestimated the frequency of an underlying medical condition in our study because from October 2009 onwards, laboratory confirmation of influenza was selectively recommended only in people

with an underlying medical condition or with severe disease.

Fourteen (40%) of the 35 females who died were pregnant. The United Kingdom,⁸ but not Greece,⁹ reported pregnancy as a risk factor for dying. A study in the United States also revealed pregnancy as a risk factor for dying, but showed that antiviral treatment within two days of onset of symptoms reduced this risk compared with treatment after four days of onset of symptoms.¹²

Although the trend in the number of reported deaths mirrored the rising incidence of confirmed cases from late August to late September, we were unable to explain the sharp drop in deaths through mid-October. It is possible that health staff may not have been collecting specimens for laboratory testing from all cases with severe disease who died up to this period. Interestingly, the number of cases reported with confirmed influenza also started dropping at the same time and before laboratory testing of all cases was stopped as the disease started spreading rapidly across the country. Possible explanations for the drop in the number of confirmed cases include: fewer persons seeking health care once the relatively mild nature of the disease was apparent and inability of the laboratories to maintain a surge capacity to test all specimens. The sharp rise in deaths in late October to early November 2009 probably reflects the priority accorded to testing a larger proportion of patients at high risk of severe complications such as pregnant females, persons with chronic diseases, young children and patients with severe illness.

The major limitation of this study is the likely underascertainment of the number of deaths across Viet Nam, but this assumption was not evaluated formally. Possible explanations for this may include lack of reporting of deaths that may have been due to influenza but were not tested for influenza and failure to test all patients with severe disease who died subsequently (post-mortem examinations of unexplained deaths were not conducted). An additional limitation was that we did not have data on whether nine of 22 patients who attended within two days of symptom onset had received anti-viral treatment, thus precluding further analysis on whether health staff provided timely treatment with antiviral drugs.

In conclusion, our findings on the clinical and epidemiologic characteristics of people who died from

A(H1N1)pdm09 are similar to those in other countries. The key lesson for Viet Nam is the need to strengthen the surveillance of influenza in the context of addressing the multiple challenges for strengthening surveillance and control of all the communicable diseases, particularly those with epidemic potential. It is relatively simple to recommend from our study that Viet Nam must develop and fund strategies to strengthen surveillance of influenza-like illness, promote early health care attendance of at-risk individuals with influenza and offer timely antiviral treatments. In recent years, Viet Nam has been developing and implementing a range of strategies to address these challenges in collaboration with its national experts and international partners.

Conflicts of interest

None declared.

Funding

None.

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Epidemiologic characteristics of haemorrhagic fever with renal syndrome in Mainland China from 2006 to 2010

Xiaoxia Huang,^{*a} Hongmei Yin,^{*b} Lei Yan,^a Xiaofang Wang^a and Shiwen Wang^a

Correspondence to Shiwen Wang (e-mail: wangshiwencdc@163.com) and Xiaofang Wang (e-mail: wangxiaofang90@163.com).

Objective: To design effective prevention and control strategies for haemorrhagic fever with renal syndrome (HFRS) in Mainland China, we evaluated the epidemiologic characteristics and trends of HFRS cases reported between 2006 and 2010.

Methods: HFRS data from 1970 to 2010 were obtained from the China Notifiable Disease Reporting System (CNDRS). The cases analysed included clinical cases and laboratory-confirmed cases. Data was extracted for statistical analysis by time, region and profession; the incidence rate was obtained directly from CNDRS. In this study, we analysed the morbidity and mortality data of HFRS from 2006 to 2010.

Results: HFRS cases trended downward from 2006 (15 098) to 2009 (8745), but exhibited a slight increase in 2010 (9526). Twenty-nine of 31 provinces reported HFRS cases between 2006 and 2010. Five provinces, namely, Heilongjiang, Jilin and Liaoning in the North-east, Shandong in the east, and Shaanxi in the central part of China, were characterized as high-endemic areas. Seasonal case distribution was bimodal, with peaks of cases in spring and winter. Young male farmers were the most susceptible population to HFRS. Early- to middle-aged adults (20–50 years old) represented the largest groups of HFRS cases.

Conclusion: The overall number of cases of HFRS in China has trended downward possibly due to national vaccine and rodent vector control programmes implemented in the past 25 years. However, this trend slowed down in the last five years. High-endemic regions and at-risk population groups still exist and will benefit from further targeted prevention strategies.

Haemorrhagic Fever with Renal Syndrome (HFRS), a rodent-borne viral disease caused by different species of Hantaviruses, is characterized by fever, haemorrhagic manifestations and renal dysfunction.^{1,2} In Mainland China, there are two predominant species of Hantavirus, Hantaan and Seoul virus, and either or both species may circulate in a given area. However, studies have revealed that a heterogeneous, gradually evolving, co-circulation of Hantaan and Seoul viruses is most common in Mainland China.^{3,4} This dynamic type of epidemiology is characterized by co-circulation of both viruses with a predominance of Hantaan virus in north-eastern China and a predominance of Seoul virus south-western China.

HFRS is transmitted by contact with rodent urine, feces or saliva.^{5–7} Of the major endemic countries for HFRS, China accounts for 90% of total HFRS cases worldwide.⁸ The annual reported cases of HFRS surpassed 110 000 in 1986.⁹ HFRS cases have been reported in all 31 Chinese provinces in Mainland China.

Although environment management, host surveillance and HFRS vaccine implementation have played an important role in controlling HFRS, it is still a serious disease in Mainland China.^{2,9–11}

We analysed data reported from all the provinces of Mainland China between 2006 and 2010 to attain an in-depth understanding of HFRS in recent years. Hopefully, the findings from this study will contribute to the development of more effective HFRS prevention and control strategies.

METHODS

HFRS is one of the Class B Notifiable Diseases, and data have been reported since 1950 according to a standard protocol.¹ In this study, HFRS data from 1970 to 2010 was obtained from the China Notifiable Disease Reporting System (CNDRS).^{1–3} The cases analysed included clinical cases and laboratory-confirmed cases. Clinical diagnosis

^a National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention (China CDC), Beijing, China

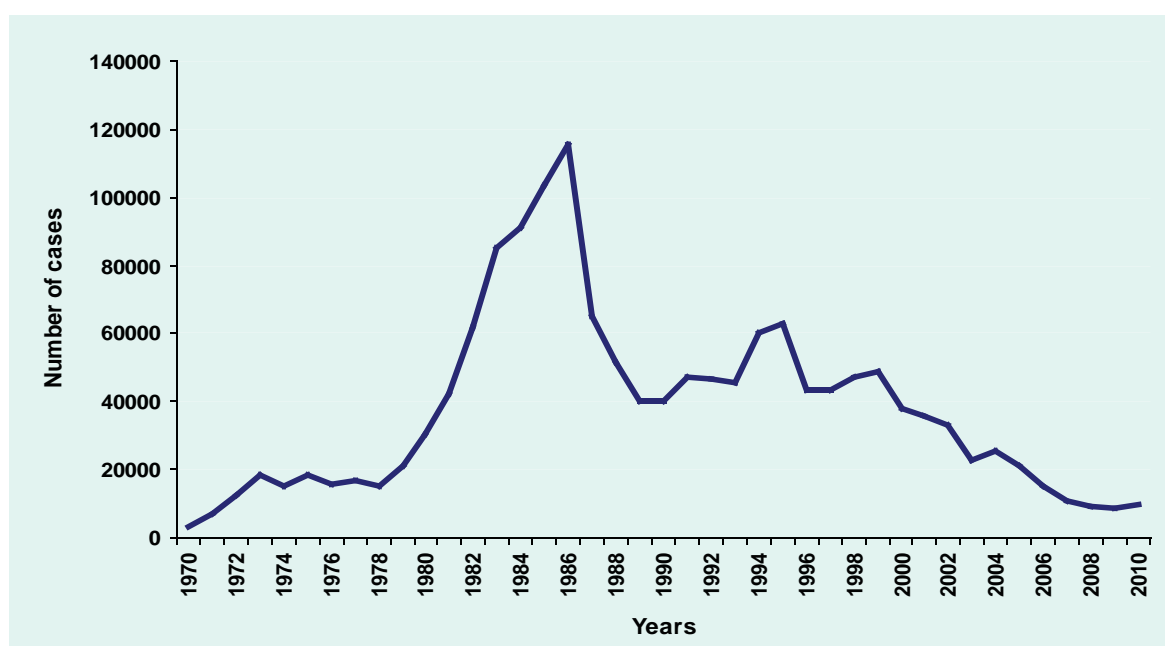
^b Chinese Center for Disease Control and Prevention (China CDC), Beijing, China

* Both authors contributed equally in this article

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Figure 1. Annual haemorrhagic fever with renal syndrome incidence, China, 1970 – 2010



criteria included: exposure history (i.e. direct or indirect exposure to rodents and their excreta and saliva within two months before the onset of illness); acute onset with at least two of the following clinical symptoms (i.e. fever $>38^{\circ}\text{C}$, chills, haemorrhagic manifestations, headache, back pain, abdominal pain, acute renal dysfunction and hypotension); experience or partial experience of the five phases of disease course (i.e. fever, hypopiasis, oliguresis, hyperdiuresis and recovery) and abnormal blood and urine routine parameters. Laboratory-confirmed case diagnosis criteria were clinical diagnosis with one of the positive laboratory tests (HV anti-IgM positive, four-fold increasing of anti-IgG and virus isolated from serum or detected HV RNA).^{1,12,13}

All data recorded for HFRS cases from 1970 to 2010 were extracted for statistical analysis by time, region and profession; the incidence rate was obtained directly from CNDRS. We analyzed the morbidity and mortality data of HFRS from 2006 to 2010. This period was China's Eleventh Five-Year Plan, during which time much work was done to prevent and control communicable diseases including HFRS. In 2008, China began the expanded immunization programme for HFRS vaccine, which targeted susceptible people in high-endemic districts to control the HFRS incidence.

Data were organized in Microsoft Excel spreadsheets and processed with SPSS 13.0 software.

RESULTS

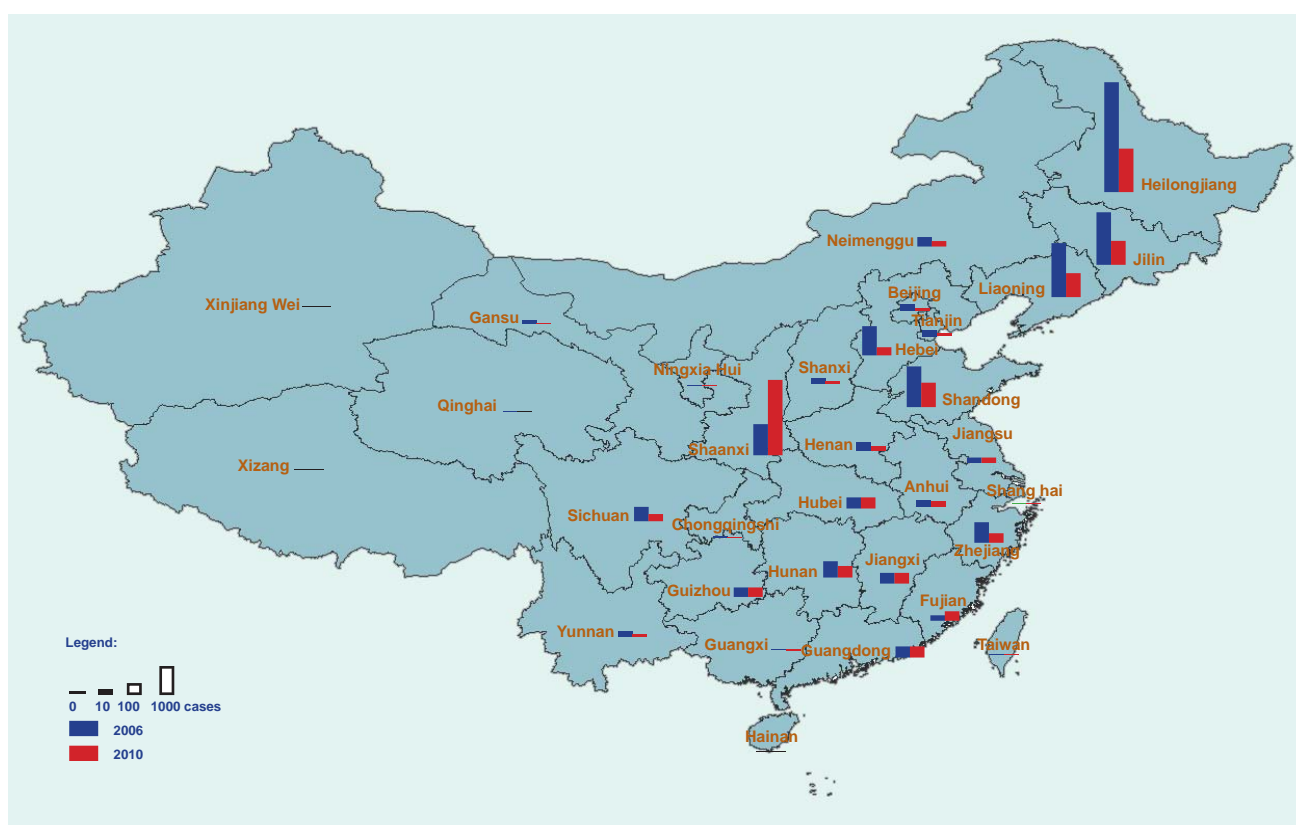
Overview of the HFRS in Mainland China

From 1970 to 2010, 1 546 063 HFRS cases were recorded. The annual HFRS incidence rose steadily in the early 1970s but experienced an alarming increase in the early 1980s. Case numbers peaked in 1986, when 115 804 cases were reported in Mainland China. From 1987 to 2010, however, HFRS case numbers decreased, with occasional small fluctuations. Eventually, in 2009, HFRS case numbers reached its lowest number (8745) since 1986, followed by a slight rise in 2010 (9526) (Figure 1).

Analysis of HFRS epidemiologic characteristics from 2006 to 2010

In recent years, the number of HFRS cases had experienced a steady decline. The per cent decline between 2006 and 2009 ranged from 26.73% (2006 to 2007) to 18.30% (2007 to 2008) and 3.25% (2008 to 2009). In 2010, however, the number of reported cases rose. While the number of cases in 2010 (9526 total HFRS cases) was slightly higher than that reported in 2008 (9039) and 2009 (8745), it was still lower than the annual mean number of cases (10 986) between 2006 and 2009.

Figure 2. Haemorrhagic fever with renal syndrome regional distribution, China, 2006 – 2010



The HFRS incidence rates (per 100 000 population) for each year from 2006 to 2010 were 1.15, 0.84, 0.68, 0.66 and 0.71, while corresponding death numbers and case fatality ratios were 173 (1.15%), 145 (1.31%), 103 (1.14%), 104 (1.19%) and 118 (1.24%), respectively. The incidence rate in 2010 was determined to be 7.58% higher than that in 2009.

From 2006 to 2010, the number of laboratory-confirmed cases were 5628 (37.28% of the total cases reported), 3940 (35.61%), 3202 (35.42%), 3411 (39.01%) and 4830 (50.70%), respectively.

HFRS regional distribution

From 2006 to 2010, a total of 53 471 HFRS cases were reported from 29 provinces. Eight provinces accounted for 80.44% of cases: Heilongjiang, Shaanxi, Jilin, Shandong, Liaoning, Zhejiang, Hunan and Hebei provinces (listed from highest to lowest number of HFRS cases). **Figure 2** shows the HFRS regional distribution in the years 2006 and 2010 and **Figure 3** shows the number of cases in the above eight high incidence provinces from 2006 to 2010.

HFRS seasonal distribution

Two peaks of HFRS cases were observed annually. One peak occurred in the winter and appeared as a relatively high and narrow spike in November. The spring peak was lower and broader, spiking in June. These seasonal distribution profiles were similar for all years between 2006 and 2010, with only a small difference in 2010. In the months before September 2010, number of cases was consistently lower than that of the same period in the years 2006 to 2009. From September onward, however, HFRS case numbers increased, resulting in the highest annual number of cases in all the five years examined (**Figure 4**).

HFRS population distribution

From 2006 to 2010, 85.60% of the HFRS cases were represented by the late adolescent-to-adult age group (16–59 years old), ranging from 87.36% in 2006 to 81.87% in 2010 (**Figure 5**). When cases were broken down by 10 year age groups, early- to middle-age (ages 20, 30, 40 and 50) represented the largest groups of HFRS cases. The overall male-to-female ratio of HFRS cases for 2006–2010 was 3.2:1. This distinctive

Figure 3. Haemorrhagic fever with renal syndrome case numbers in eight high incidence provinces, China, 2006 – 2010

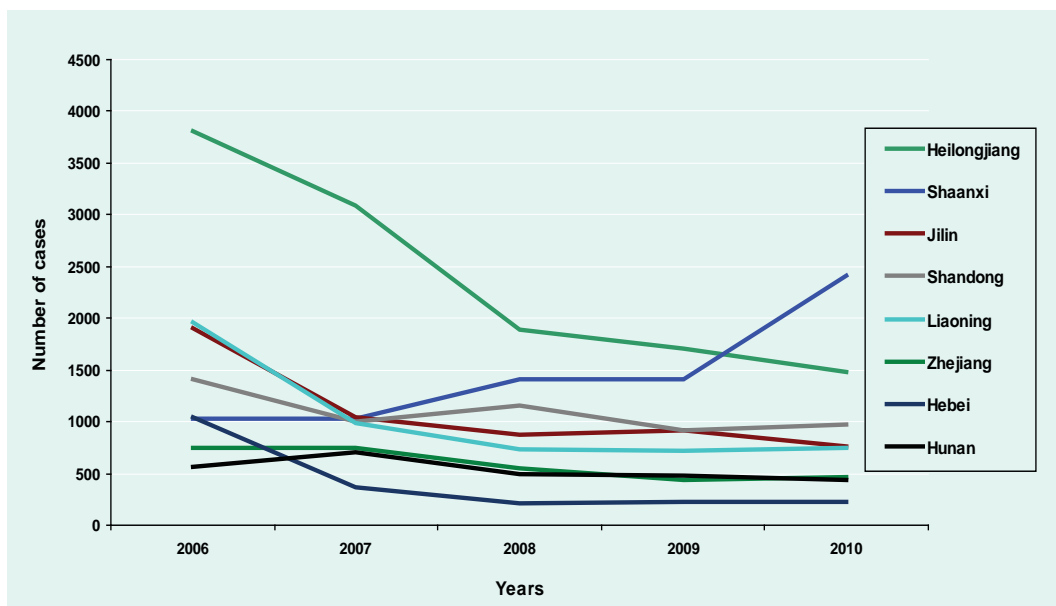
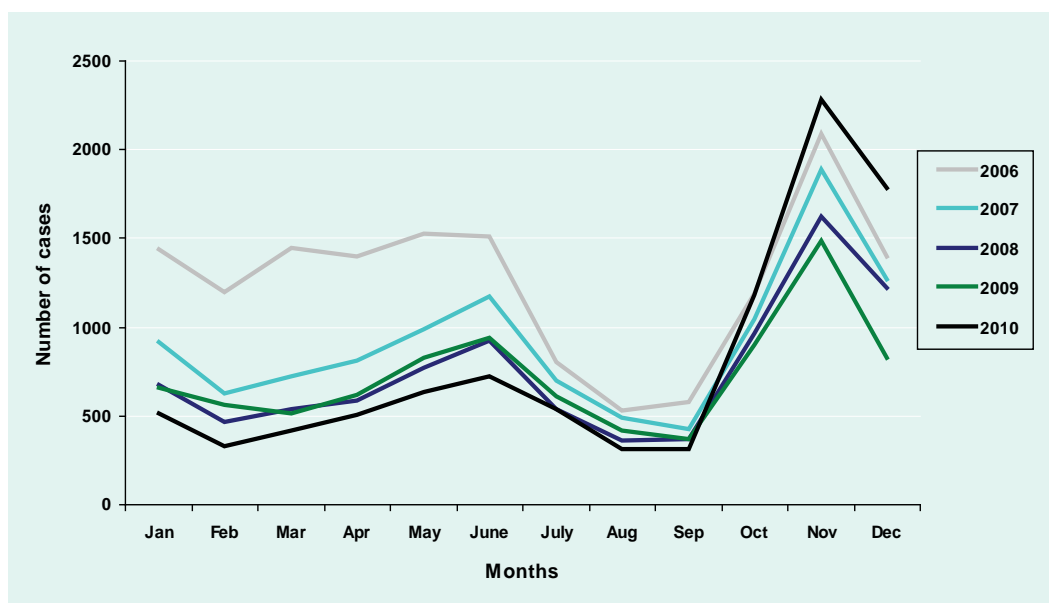


Figure 4. Haemorrhagic fever with renal syndrome seasonal distribution, China, 2006 – 2010



differential in gender distribution was similar among all five years examined. The male-to-female ratio of HFRS cases was similar in the cases reported by provinces (data not shown).

When cases were evaluated by employment status, it was determined that the majority of cases from 2006 to 2010 were represented by farmers (66.83%, **Table 1**). The employment type of HFRS cases was similar in the cases reported by provinces (data not shown).

DISCUSSION

Our data showed that the number of reported HFRS cases has declined remarkably after peaking in 1986. By 2009, the annual reported cases fell below 10 000. The changing trend of HFRS cases number during these years is consistent with other studies.^{9,14} However, some HFRS high risk areas still exist² and have occasionally experienced an increase in cases in recent years. From 2006 to 2009, the downward trend slowed, while

Figure 5. Haemorrhagic fever with renal syndrome age distribution, China, 2006 – 2010

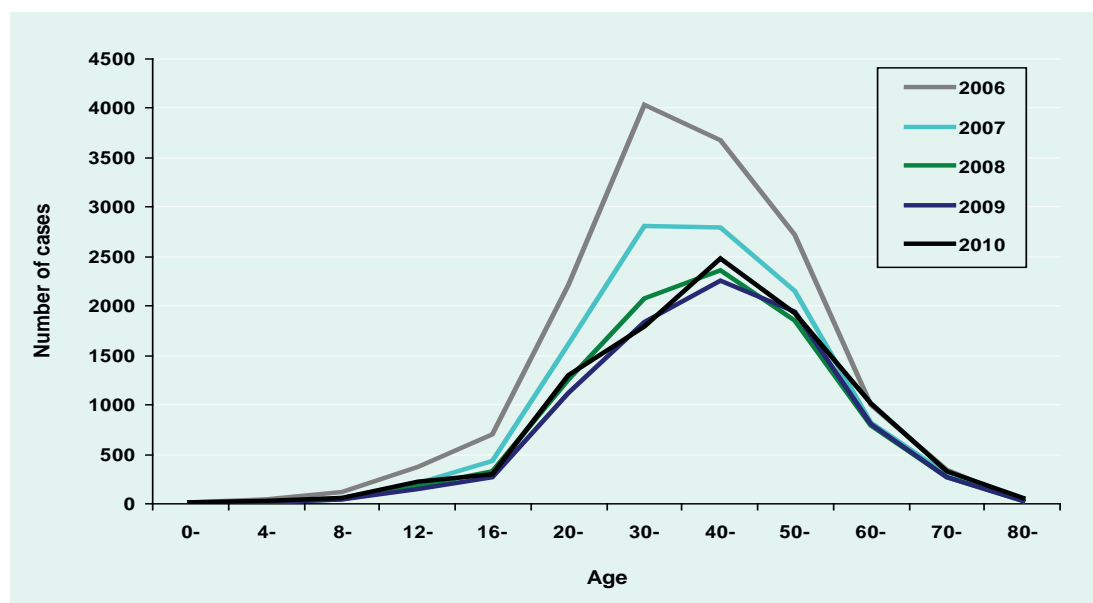


Table 1. Haemorrhagic fever with renal syndrome cases by employment status and sex, China, 2006–2010

Employment status	Number of cases (%)		Males (%)		Females (%)	
Farmers	35 732	(66.83)	26 840	(75.11)	8 892	(24.89)
Industrial labour or manual workers	3 914	(7.32)	3 442	(87.94)	472	(12.06)
Household workers and unemployed	2 811	(5.26)	1 509	(53.68)	1 302	(46.32)
Students	2 465	(4.61)	1 870	(75.86)	595	(24.14)
Migrant workers	1 905	(3.56)	1 677	(88.03)	228	(11.97)
Functionaries	1 161	(2.17)	1 009	(86.91)	152	(13.09)
Retirees	1 016	(1.90)	759	(74.70)	257	(25.30)
Business service sector	538	(1.01)	423	(78.62)	115	(21.38)
Teachers	452	(0.85)	344	(76.11)	108	(23.89)
Catering food industry workers	308	(0.58)	251	(81.49)	57	(18.51)
Herdsmen	141	(0.26)	116	(82.27)	25	(17.73)
Medical workers	133	(0.25)	101	(75.94)	32	(24.06)
Preschool children	131	(0.24)	83	(63.36)	48	(36.64)
Seafarers and long-distance drivers	66	(0.12)	66	(100.00)	0	(0.00)
Fishermen	59	(0.11)	54	(91.53)	5	(8.47)
Public service workers	25	(0.05)	14	(56.00)	11	(44.00)
Nurses and nannies	4	(0.007)	1	(25.00)	3	(75.00)
Others	2 028	(3.79)	1 685	(83.09)	343	(16.91)
Unknown	582	(1.09)	455	(78.18)	127	(21.82)
Total	53 471		40 699	(76.11)	12 771	(23.89)

the reported cases in 2010 were slightly higher than 2008 and 2009. This may be related to the outbreak in Shaanxi province in 2010 when HFERS cases were about twice that of 2009. Of those eight regions with a high incidence between 2006 and 2010, Heilongjiang, Jilin, Liaoning, Shandong, Shaanxi and Hebei had previously been identified high incidence regions in 2004 and 2005.¹⁴ A good example of the decline in cases is Hebei province, which was once

considered among the high-incidence provinces, but it experienced a significant decrease in cases in 2007 (<400; a 64.99% decrease from 2006), and has remained low. In 2010, Hebei was ranked as the 12th most endemic HFERS region in China, as compared to 5th in 2006. In contrast, Shaanxi represents the most intriguing and alarming region since it reported significantly higher numbers of HFERS cases in 2010.

HFRS seasonality in China is characterized by a bimodal distribution pattern throughout the year. The spring peak often lasts for about three months from March to May. The winter peak is relatively rapid and short-lasting. The reasons for the peak occurring in winter and in spring may be related to several factors:¹⁵⁻²⁰ viral types, hosts' reproduction and activities and natural or social factors e.g. flooding.

As in previous years, the late adolescent-to-adult age group accounted for the most cases of HFRS reported in the years from 2006 to 2010. Since 2008, seven of the particularly affected provinces (including five of the highest epidemic provinces and Hebei and Zhejiang provinces) have conducted immunization through the Expanded Programmes on Immunization (EPI) targeting the high-risk age group of 16–60 year olds; another 10 provinces were included since 2009. We noticed that the overall number of cases in this broad age group did decrease annually, and the decrease occurred for the entire study period both before and after the introduction of EPI, indicating that other factors may have contributed to the decline in cases.

The occupational and sex distribution in our study was consistent with the findings of an analysis of HFRS cases from 2004 and 2005.¹⁴ Young males, especially farmers, still are the focus for prevention and control. Since this is a rodent-borne infection, farmers are more likely to spend significant amounts of time in rodent-infested areas (barns, fields). In China, a farmer's living and working environment can lead to more exposure to rodents' feces, urine and saliva. In all occupations, except nurses and nannies, the number of male cases was more than the number of female cases. However, in other studies the male-to-female ratios of HFRS cases varies for the different species of virus.^{15,21} The different male-to-female ratio by virus type may be related to the different hosts of HFRS.

Unfortunately, we could not analyse the epidemiological characteristics of different viral types without the serum data of HFRS cases. Another limitation of the reported data is that with the development of monitoring and diagnostic technology, the detection of HFRS cases may be more accurate than that in the past. Moreover, with a deeper focus on HFRS by the Government, we saw that the laboratory-confirmed cases increased in 2010. The consistency of the surveillance data should be evaluated regularly.

Integrated intervention measures involving rodent control, environment management and vaccination have been implemented and may have played an important role in controlling HFRS in China. China still experiences significantly higher numbers of HFRS cases than other countries worldwide and an increase in cases was experienced in some areas in 2010. Our study has shown the recent epidemiologic characteristics of HFRS including regional, seasonal and population distribution.

Conflicts of interest

None declared.

Funding

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Molecular characterization of NDM-1 producing Enterobacteriaceae isolates in Singapore hospitals

Jeanette Teo,^a Grace Ngan,^a Michelle Balm,^a Roland Jureen,^a Prabha Krishnan^b and Raymond Lin^a

Correspondences to Jeanette Teo (e-mail: jeanette_teo@nuhs.edu.sg).

Objective: In this study, we molecularly characterized 12 NDM-1 producing clinical Enterobacteriaceae (*Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter cloacae*) isolates that were part of a collection of non-carbapenem susceptible isolates obtained during a one-year period. These isolates were obtained from four local general hospitals in Singapore.

Methods: Polymerase chain reaction (PCR) assays and sequencing was used to determine the presence of β -lactamase encoding genes (*bla*) including *bla*_{NDM-1} and plasmid-mediated quinolone and aminoglycoside resistance determinants. Conjugation experiments were performed to determine the transferability of *bla*_{NDM-1}. Isolate relatedness was determined by multilocus sequence typing (MLST).

Results: The isolates were completely resistant to the second- and third-generation cephalosporins tested as well as carbapenems. Susceptibility profiling of the isolates indicated that 100% retained susceptibility to tigecycline while 11/12 (91.7%) were susceptible to colistin. The *bla*_{NDM-1} gene was encoded on plasmids that were easily transferable. None of the patients had a travel history to countries where NDM-1 has been reported. The isolates appear clonally unrelated with MLST, revealing a diversity of clonal types among the *Klebsiella pneumoniae* and *Escherichia coli* isolates.

Conclusion: The ease of NDM-1 plasmid transmissibility may help their dissemination among the Enterobacteriaceae. Although it appears that the isolates are clonally unrelated, epidemiological links cannot be fully excluded without further research.

The discovery of a novel carbapenemase, the New Delhi metallo- β -lactamase-1 (NDM-1), generated much global alarm. These NDM-1 producing isolates gained media notoriety being labelled as superbugs which had the reputation of being impossible to treat. The first carbapenem-resistant NDM-1 isolates characterized in 2009 were *Klebsiella pneumoniae* and *Escherichia coli* isolated from a Swedish patient who had sought medical care in New Delhi, India. The strains were resistant to all antibiotics tested except colistin.¹ The ease of β -lactamase encoding genes (*bla*_{NDM-1}) dissemination has become apparent with the worldwide detection of NDM-1 producers.²⁻⁶

In this study, we provide the molecular characterization and epidemiology for 12 NDM-1 positive clinical isolates. These isolates were obtained as part of a hospital surveillance programme for carbapenem non-susceptible Enterobacteriaceae.

METHODS

Clinical isolates

During the period of August 2010–August 2011, 52 non-duplicate carbapenem non-susceptible clinical isolates from local hospitals were analysed. The isolates were submitted from four hospitals that represented 40% of the general hospitals in Singapore. The 52 isolates comprised the following species: 31 *Klebsiella pneumoniae*, 13 *Escherichia coli*, seven *Enterobacter cloacae* and one *Enterobacter aerogenes*. Two of these isolates (594 and 693) were obtained from the same patient but from different collection sites (**Table 1**). The identification and initial susceptibility testing of the isolates was performed with VITEK 2 automated system (bioMérieux Vitek, Inc., Hazelwood, MO, USA). The Etest MBL (bioMérieux, Marcy l'Etoile, France) was used for the phenotypic detection of metallo- β -lactamases.

^a Department of Laboratory Medicine, National University Hospital, Singapore 119074. Singapore.

^b Department of Laboratory Medicine, Tan Tock Seng Hospital, Singapore 308433. Singapore.

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Table 1. Characterization of clinical Enterobacteriaceae isolates harbouring bla_{NDM-1} and their transconjugants

Isolate	Patient age	Gender	Date of isolate collection	Origin	Hospital/ward	ST	β-lactamases	qnr gene	16S rRNA methylase gene
<i>Klebsiella pneumoniae</i>									
547	78	Female	8/9/2010	Urine	TTSH/7B	437	TEM-1, SHV-12, CTX-M-15, CMY-2	-	-
547T							CTX-M-15		
594	86	Female	13/10/2010	Urine	TTSH/ 11C	437	TEM-1, SHV-11, CTX-M-15	-	-
594T							CTX-M-15	-	-
693	86	Female	13/10/2010	Rectal swab	TTSH/ 11C	48	TEM-1, SHV-11, CTX-M-15, DHA-1	-	-
693T							TEM-1, SHV-11, CTX-M-15, DHA-1	-	-
509	35	Male	16/1/2011	Urine	NUH/ 42	237	TEM-1, SHV-1, CTX-M-15	-	-
509T							TEM-1	-	-
380	NA	NA	NA	Urine	PH	273	TEM-1, SHV-11, CTX-M-15	qnrB	-
380T							TEM-1	-	-
205	NA	NA	NA	Urine	PH	15	SHV-1	-	-
205T							NEG	-	-
<i>Escherichia coli</i>									
424	94	Male	22/9/2010	Urine	TTSH/7B	471	TEM-1, CTX-M-15, DHA-1	-	-
424T							TEM-1	-	-
N12	59	Male	26/10/2010	Urine	TTSH/ TWR2C	267	TEM-1, CTX-M-15	-	-
N12T							CTX-M-15	-	-
722	28	Male	2/12/2010	Rectal swab	TTSH/12C	43	TEM-1, CTX-M-15	-	-
722T							TEM-1, CTX-M-15	-	-
510	46	Female	27/10/2010	Blood	NUH/ 86	501	TEM-1, CTX-M-15, CMY-2, DHA-1	-	-
510T							TEM-1, CTX-M-15, CMY-2, DHA-1	-	-
<i>Enterobacter cloacae</i>									
459	79	Male	6/9/2010	Urine	TTSH/9C	ND	TEM-1, SHV-12, CTX-M-15, DHA-1	qnrB	-
459T							TEM-1, SHV-12, CTX-M-15, DHA-1	-	-
241	77	Male	8/2/2011	Urine	NUH/ 52	ND	TEM-1, SHV-1, CTX-M-15	-	rmtC, armA
241T							TEM-1, SHV-1, CTX-M-15	-	rmtC

Note: NEG – PCR assays were negative for the bla genes screened; NUH – National University Hospital, Singapore; PH – Private hospital, ward information was unavailable; ST – Sequence type determined by multilocus sequence typing (MLST); TTSH – Tan Tock Seng Hospital, Singapore; NA – Not available; and ND – Not done.

Antimicrobial susceptibility testing

Carbapenem resistance and resistance to other classes of antibiotics were confirmed by the Etest (bioMérieux) method with minimum inhibitory concentrations (MICs) determined after a 24-hour incubation at 37°C. Susceptibility was defined according to the breakpoints of the European Committee on Antimicrobial Susceptibility Testing. *Escherichia coli* ATCC 25922 was used as the quality control strain for antimicrobial susceptibility testing.

Plasmid analysis

Plasmids were extracted using the Plasmid Mini Kit (Qiagen, Hilden, Germany). Plasmids were separated on 0.7% Megabase Agarose (Bio-Rad, Hercules, CA, USA) and their sizes estimated using BAC-Tracker™ Supercoiled DNA Ladder (Epicentre, Madison, WI, USA) as a reference. Southern hybridization analysis was performed using DIG DNA Labelling and Detection Kit (Roche Diagnostics, Mannheim, Germany) and digoxigenin-labelled 291 bp bla_{NDM-1} was used as the probe.

Table 2. Primers used in this study for the detection of antibiotic resistance determinants

Targets	Prime name	Sequence 5'-3'	Reference
β-lactamases			
IMP-type			
IMP-1, -4, -6	IMP-1F	GCGTTTATGTTTCATACTTCGTTT	This study
	IMP-1R	TCTATTCGCGCCCGTGCTGT	
IMP-8	IMP-8F	AGCGGCTTTGCCTGATT	This study
	IMP-8R	GACCGTCCGGTTTAAACAAA	
VIM-type			
VIM-1,-4,-5,-12,-19	VIM-1F	GACCGCGTCTGTCATGG	This study
	VIM-1R	GGCGACTGAGCGATTTTT	
VIM-2, -23, -24	VIM-2F	GTCTATTTGACCGGTCTATCATGGC	This study
	VIM-2R	CGTTGCGATATGCGACCAAAC	
KHM-1	KHM-FD	AGTGGATTGACGCGCAGGGC	This study
	KHM-RV	TTCCAGCAGCGATGCGTCGC	
NDM-1	NDM-FD	CAACTGGATCAAGCAGGAGA	This study
	NDM-RV	TCGATCCCAACGGTGATATT	
NDM-1, Full length	NDM-FF	ATGGAATTGCCAATATTATG	This study
	NDM-RR	TCAGCGCAGCTTGTCGGCC	
KPC-type	KPC-1F	CGTTGACGCCCAATCC	This study
	KPC-1R	ACCGCTGGCAGCTGG	
GES-type	GES-7F	ATCTTGAGAAGCTAGAGCGCG	This study
	GES-1 to 5 and -7	GTTTCCGATCAGCCACCTCT	
DHA-1	DHA-F	GTCGCGGCGGTGGTGGAC	This study
	DHA-R	CCGCACCCAGCACACCTGT	
CMY-1-type	CY1-GC1M-F	GCTGCTCAAGGAGCACAGGATCCCG	7
	CY1-GC1M-R	GGCACATTGACATAGGTGTGGTGCATG	
CMY-2-type	CY-GC2M-F	ACTGGCCAGAAGTACAGGCAAA	7
	CY-GC2M-R	GTTTTCTCCTGAACGTGGCTGGC	
OXA-48	OXA-48F	ATGCGTGTATTAGCCTTATCGGC	This study
	OXA-48R	ACTTCTTTTGTGATGGCTTGGCGCA	
TEM-type	TEM-F	ATAAAATTCTTGAAGACGAAA	8
	TEM-R	GACAGTTACCAATGCTTAATCA	
SHV-type	SHV-F	GGGTATTCTTATTTGTCGC	8
	SHV-R	TTAGCGTTGCCAGTGCTC	
CTX-type	CTX-F	AAAAATGATTGAAAGGTGGTTGT	8
	CTX-R	TTACAGCCCTTCGGCGATGA	
16S rRNA methylases			
<i>rmtA</i>	RMTA-F	TTCCCTCTGCCCGGATACCG	This study
	RMTA-R	CAACCCCTGATGGATGTCG	
<i>rmtB</i>	RMTB-F3	GAGCTGGATACCCTGTACGA	This study
	RMTB-R4	GGCAAAGGTAAAATCCCAAT	
<i>rmtC</i>	RMTC-F	TATGGTGCCTTATTTGGTGGG	This study
	RMTC-R	GCATGCCAGCCTCCGTAAG	
<i>rmtD</i>	RMTD-F	GCACGTGCGCCTCCATCCATTC	This study
	RMTD-R	CGTTTGGGCGATTTGCTGTGCG	
<i>armA</i>	ARMA-F1	ATTCTGCCTATCCTAATTGG	9
	ARMA-R2	ACCTATACTTTATCGTCGTC	
<i>npmA</i>	NPMA-F1	TTTGGGTACTGGAGACGGTA	This study
	NPMA-R2	TCAATGCGAAAACCTGAGTT	

PCR screening for *bla* and other antibiotic resistance determinants

Genomic DNA used for the polymerase chain reaction (PCR) assays was extracted from the isolates using the DNeasy Blood and Tissue Kit (Qiagen). The presence of

genes encoding carbapenemases and extended-spectrum β -lactamases was detected using various primers (Table 2). Full-gene sequencing of *bla*_{NDM-1} was carried out using NDM-FF and NDM-RR primers (Table 2). This allowed the amplification of the 815 bp NDM-1 gene. In addition to *bla*_{NDM-1} detection, *bla* genes for

acquired MBLs (VIM-type, IMP-type and KHM-1), serine carbapenamases (OXA-48, KPC-1, GES-1, -2, -3, -4, -5 and -7) and extended-spectrum β -lactamases (TEM-type, SHV-type, CTX-M-type, DHA-1, CMY-type) were also PCR screened (Table 2). National Collection of Type Cultures strain 13443 was used as the bla_{NDM-1} PCR positive control. Plasmid-mediated quinolone (*qnr* genes) and 16S rRNA methylase aminoglycoside resistance determinants were analysed by PCR (Table 1). The presence of *qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS* was determined using published screening protocols.^{10,11} PCR assays were performed using HotStar Taq Plus Master Mix Kit (Qiagen) and setup according to the manufacturer's instructions. All amplicons were sent for sequencing at a local company (1st BASE, Singapore).

Multilocus sequence typing (MLST)

MLST was carried out using the protocol developed by Institut Pasteur (<http://www.pasteur.fr/recherche/genopole/PF8/mlst/index.html>) for *Klebsiella pneumoniae* isolates. Internal fragments of seven housekeeping genes: *rpoB* (β -subunit of RNA polymerase); *gapA* (glyceraldehyde 3-phosphate dehydrogenase); *mdh* (malate dehydrogenase); *pgi* (phosphoglucose isomerase); *phoE* (phosphorine E); *infB* (translation initiation factor 2); and *tonB* (periplasmic energy transducer) were amplified and directly sequenced. For *Enterobacter coli* isolates, MLST was performed using eight housekeeping genes: *dinB* (DNA polymerase); *icdA* (isocitrate dehydrogenase); *pabB* (para-aminobenzoate synthase); *polB* (RNA polymerase Pol II); *putP* (proline permease); *trpA* (tryptophan synthase subunit A); *trpB* (tryptophan synthase subunit B); *uidA* (β -glucuronidase). Internal fragments of these genes were amplified and sequenced. The assignment of sequence types was carried out at <http://www.pasteur.fr/recherche/genopole/PF8/mlst/index.html>. *Enterobacter cloacae* isolates were left untyped as there is no established MLST protocol.

Conjugation assays

Conjugation experiments were performed between the clinical donor isolates and azide resistant *Escherichia coli* J53 as a recipient. Transconjugants were recovered from Luria-Bertani agar plates containing sodium azide (100 mg/L) and imipenem (5 mg/L) with PCR confirming the presence of bla_{NDM-1} .

RESULTS

Detection of NDM-1 producing clinical isolates and antibiotic susceptibility

Fifty-two carbapenem non-susceptible isolates were screened with NDM-1 specific primers (Table 2). Of these, 12 isolates (23%) yielded a 291 bp amplicon, which upon sequencing, showed 100% identity with bla_{NDM-1} (GenBank:HQ162469). The 12 isolates positive for bla_{NDM-1} PCR were: six *Klebsiella pneumoniae*, four *Escherichia coli* and two *Enterobacter cloacae*. Full gene sequencing also confirmed that the genes encoded NDM-1 (Table 1). The 12 isolates were clearly Etest MBL (bioMérieux) positive. All the isolates were resistant to second- and third-generation cephalosporins and carbapenems (Table 3). Gentamicin resistance was seen in 9/12 (75%) of the isolates, with two isolates (16.7%) displaying a high level of amikacin resistance (Table 3). Resistance to chloramphenicol was also noted in 9/12 (75%) of the NDM-1 clinical isolates. High-level resistance to ciprofloxacin (≥ 32 mg/L) was seen in seven (58.3%) of the isolates. Susceptibility to tigecycline was retained by the NDM-1 positive isolates. Only one strain, *E. cloacae* isolate 459, was resistant to colistin, while the rest of the isolates retained their susceptibility (Table 3).

The NDM-1 producers are genotypically unrelated

Five different sequence types were obtained for the six *Klebsiella pneumoniae* isolates. Two isolates with identical sequence type (ST 437) were isolated one month apart in separate wards. Isolate 547 and 594 derived from the same patient had dissimilar MLSTs. All the *Escherichia coli* isolates had differing sequence types. Hence, NDM-1 producing isolates comprised a variety of sequence types and were therefore genetically different (Table 1).

bla_{NDM-1} is plasmid borne and transferable

Conjugation experiments indicated that bla_{NDM-1} was transferable and likely via a plasmid-mediated event. A typical agarose gel electrophoretic profile of the plasmids analysed is shown in Figure 1. Plasmid content from clinical donor strains and transconjugants revealed that clinical NDM-1 isolates and their respective transconjugants carried a common band of covalently

Table 3. Susceptibility profile of the clinical NDM-1 producers and their respective transconjugants

Isolate	Antibiotic minimum inhibitory concentration (mg/l)													
	IMP	MEM	ETP	CXM	CTX	CAZ	GEN	AMK	TET	TGC	CIP	CHL	COL	PMB
<i>Klebsiella pneumoniae</i>														
547	16	4	12	> 256	> 256	> 256	128	4	32	0.25	2	512	0.25	3
547T	> 32	1.5	1	> 256	> 256	> 256	0.064	4	8	0.064	1	32	0.064	0.5
594	> 32	12	> 32	> 256	> 256	> 256	128	4	32	0.25	2	> 256	0.5	1.5
594T	> 32	12	4	> 256	> 256	> 256	0.064	1	0.75	0.14	0.004	3	0.125	0.75
693	> 32	12	6	> 256	> 256	> 256	> 256	4	96	0.25	0.047	> 256	0.064	0.25
693T	> 32	12	6	> 256	> 256	> 256	0.125	1	0.05	0.047	0.002	4	0.25	0.38
509	> 32	> 32	> 32	> 256	> 256	> 256	64	8	> 256	0.14	> 32	> 256	0.38	1.5
509T	> 32	> 32	8	> 256	> 256	> 256	0.19	1	0.25	0.125	0.008	> 256	0.094	0.38
380	> 32	32	32	> 256	> 256	> 256	> 256	16	64	2	> 32	> 256	0.25	0.5
380T	> 32	32	6	> 256	> 256	> 256	0.19	1	0.38	0.125	0.008	> 256	0.094	0.38
205	> 32	> 32	> 32	> 256	> 256	> 256	256	2	2	0.75	> 32	> 256	0.25	0.5
205T	> 32	> 32	6	> 256	> 256	> 256	0.19	1	0.38	0.125	0.008	> 256	0.094	0.5
<i>Escherichia coli</i>														
424	> 32	4	12	> 256	> 256	> 256	1.5	8	32	0.064	> 32	512	1.25	2
424T	> 32	2	8	> 256	> 256	> 256	0.125	8	8	0.064	1	32	0.125	0.38
510	> 32	> 32	12	> 256	> 256	> 256	> 256	> 256	1	0.064	32	4	0.75	1
510T	4	2	12	> 256	> 256	> 256	> 256	> 256	2	0.094	32	12	0.047	0.25
N12	> 32	> 32	32	> 256	> 256	> 256	1	2	32	0.094	32	2	0.25	1.5
N12T	> 32	> 32	4	> 256	> 256	> 256	0.25	1	32	0.094	0.004	1.5	0.125	0.012
722	> 32	> 32	32	> 256	> 256	> 256	1.5	4	24	0.094	32	2	1	0.5
722T	> 32	16	8	> 256	> 256	> 256	0.5	2	1.5	0.023	0.004	2	0.125	0.125
<i>Enterobacter cloacae</i>														
459	24	16	32	> 256	> 256	> 256	64	6	> 256	0.5	12	16	4	16
459T	> 32	3	8	> 256	> 256	> 256	0.125	4	8	0.064	1	32	0.38	0.75
241	> 32	> 32	> 32	> 256	> 256	> 256	> 256	> 256	4	1	> 32	12	1	2
241T	> 32	0.5	0.2	> 256	> 256	> 256	> 256	> 256	0.5	0.064	0.004	8	0.38	0.5
<i>Escherichia coli J53</i>														
	0.125	0.016	0.04	0.38	1	0.064	0.19	1	0.18	0.064	0.003	2	0.125	0.38

Note: AMK – amikacin; CAZ – ceftazidime; CHL – chloramphenicol; CIP – ciprofloxacin; COL – colistin; CTX – cefotaxime; CXM – cefuroxime; ETP – ertapenem; GEN – gentamicin; IMP – imipenem; MEM – meropenem; PMB – polymyxin B; T – transconjugant; TET – tetracycline; and TGC – tigecycline.

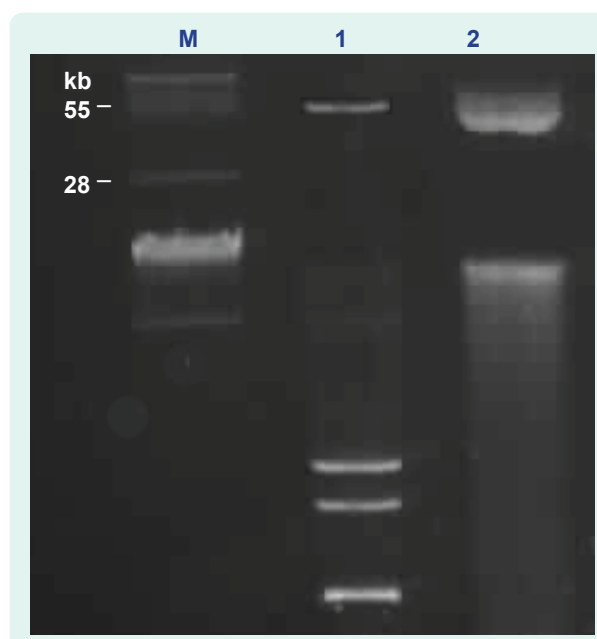
closed circular DNA larger than 28 kb in size (Figure 1). Southern hybridization analysis demonstrated localization of the NDM-1 gene to these plasmids (data not shown). We were unable to size the plasmids more accurately due to limitations by conventional gel electrophoresis.

PCR screening for *bla* and other drug resistance determinants

All the isolates were PCR negative for MBLs and serine carbapenemase genes (OXA-48, KPC-1, GES-1, -2, -3, -4, -5 and -7). PCR results showed that it was not uncommon for the clinical NDM-1 producers to carry *bla* genes for more than one type of extended spectrum β -lactamases as well as plasmid-encoded AmpC β -lactamases (Table 1). These *bla* genes were transferable and conferred high levels of resistance to second- and third-generation cephalosporins and carbapenems to their recipient strain (Table 3).

Plasmid-mediated quinolone (*qnr* genes) and aminoglycoside resistance determinants (*armA*, *rmtA*,

Figure 1. Typical agarose gel (0.7%) analysis of the plasmid content of clinical isolates and their transconjugants



M - BAC-Tracker Supercoiled DNA Ladder (Epicentre); 1 - plasmid DNA from clinical isolate 380; and 2 - plasmid DNA from 380 transconjugant.

rmtB, *rmtC*, *rmtD* and *npmA*) were analysed by PCR. *Enterobacter cloacae* 459 was the only isolate found to be positive for *qnrB*. This determinant was not transferred to the transconjugant (Table 1). 16S rRNA methylase genes were detected in only one isolate, *Enterobacter cloacae* 241. In this isolate, *armA* was co-detected with *rmtC* (Table 1).

DISCUSSION

In this study, we observed differences from an initial report from Singapore regarding NDM-1 producers in which the NDM-1 positive isolates were likely to have been imported from India and Bangladesh.² In contrast, the patients in our study had no recent travel history to countries where NDM-1 producers have been reported. The investigated isolates were obtained from four local hospitals and did not include the hospital of the initial report. Isolates from Tan Tock Seng Hospital were isolated over a four-month period, while the isolates at the National University Hospital were isolated over five months. No patient information was available for the isolates from the private hospital. Since the isolates originated from patients in differing wards and hospitals with their emergence being detected over a period of several months, we believe they were unlikely to have an epidemiological link. Epidemiological investigations done so far do not suggest nosocomial transmission of NDM-1 producing Enterobacteriaceae in these hospitals. However, we cannot completely rule out nosocomial transmission and further research is required to determine whether NDM-1 producing Enterobacteriaceae are endemic in Singapore.

The diversity of MLST strain types also indicates that the clones were genetically unrelated. Clonal diversity appears to be a characteristic of NDM-1 producers.¹² This was reflected in a study looking at isolates of global origin, which revealed a large variety of strain types.¹² The ease of dissemination of plasmid-bearing *bla*_{NDM-1} was apparent by the ability to obtain transconjugants for all the clinical donor strains. Similar findings on the ease of *bla*_{NDM-1} plasmid transmissibility have been noted.^{12,13} Investigations into the genetic context of *bla*_{NDM-1} reveal that the gene is frequently associated with insertion elements and often present on promiscuous plasmids bearing plasmid incompatibility groups Inc.A/C or non-typeable replicons.^{12,13} Due to the limited research capacity at our laboratory, we could perform only basic plasmid characterization. However, we acknowledge

that PCR-based replicon typing¹⁴ is an important tool for epidemiologically tracing these *bla*_{NDM-1} plasmids.

Susceptibility profiling indicates low rates of colistin and tigecycline resistance in the NDM-1 producers and this appears to be a fairly typical observation among NDM-1 positive isolates.^{1,3} We note that aminoglycoside susceptibility is retained by most of the isolates. This finding differed from those of the Health Protection Agency, United Kingdom¹⁵ and Kumarasamy *et al.*,³ i.e. that most NDM-1 producers are typically aminoglycoside resistant and carry a 16S rRNA methylase gene. Only one of the isolates possessed 16S rRNA methylase genes (*rmtC* and *armA*), suggesting that aminoglycoside resistance arising in these NDM-1 positive isolates is mostly likely due to the more commonly encountered mechanisms of enzymatic inactivation mediated by acetyltransferases, nucleotidyltransferases and phosphotransferases.¹⁶ High-level quinolone resistance is mediated primarily by chromosomal mutations to the quinolone-resistance determining region in DNA gyrase.¹⁷ It is likely that the high levels of quinolone resistance seen in our isolates is mediated via this mechanism. Although we did not sequence the mutations of DNA gyrase gene of isolates, we did find plasmid-mediated quinolone resistance determinants, *qnrB*, in two isolates.

While it appears that there is no clonal outbreak of NDM-1 producing isolates in Singapore, the detection and dissemination of *bla*_{NDM-1} in the Asia Pacific region highlights the importance of surveillance efforts to understand more about these carbapenemase producers.

Conflicts of interest

None declared.

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