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Outbreak of pandemic influenza A/H1N1 2009 in Nepal

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Abstract

Background: The 2009 flu pandemic is a global outbreak of a new strain of H1N1 influenza virus. Pandemic influenza A (H1N1) 2009 has posed a serious public health challenge world-wide. Nepal has started Laboratory diagnosis of Pandemic influenza A/H1N1 from mid June 2009 though active screening of febrile travellers with respiratory symptoms was started from April 27, 2009.

Results: Out of 609 collected samples, 302 (49.6%) were Universal Influenza A positive. Among the influenza A positive samples, 172(28.3%) were positive for Pandemic influenza A/H1N1 and 130 (21.3%) were Seasonal influenza A. Most of the pandemic cases (53%) were found among young people with ≤ 20 years. Case Fatality Ratio for Pandemic influenza A/H1N1 in Nepal was 1.74%. Upon Molecular characterization, all the isolated pandemic influenza A/H1N1 2009 virus found in Nepal were antigenically and genetically related to the novel influenza A/CALIFORNIA/07/2009-LIKE (H1N1)v type.

Conclusion: The Pandemic 2009 influenza virus found in Nepal were antigenically and genetically related to the novel A/CALIFORNIA/07/2009-LIKE (H1N1)v type.

Background

The 2009 flu pandemic is a global outbreak of a new strain of H1N1 influenza virus, often referred to as swine flu [1]. The virus is a novel strain of influenza [2]. This new pandemic H1N1 influenza strain contained genes from five different flu viruses: North American swine influenza, North American avian influenza, human influenza, and two swine influenza viruses typically found in Asia and Europe [3]. Due to the genetic mutations in its hemagglutinin (HA) protein, the influenza viruses can escape from the host defense mechanisms and thus to be able to continuously infect human and other species [4,5].

On June 11, 2009, the ongoing outbreak of Influenza A/H1N1 was officially declared by the WHO to be the first influenza pandemic of the 21st century with new strain of Influenza A virus subtype H1N1 identified in April 2009 [6]. Till May 30, 2010 worldwide update by World Health Organization (WHO) more than 214

countries have reported laboratory confirmed cases of pandemic influenza H1N1 2009, including over 1,8,114 deaths [7].

Nepal has started screening febrile travelers with respiratory symptoms from affected countries for the Pandemic influenza A (H1N1) since April 27, 2009, and the first case was detected on June 21, 2009 and introduction of the disease to the country was declared on June 29. Community transmission of Pandemic Influenza A/H1N1 2009 was declared on 15 October onwards.

This study reflects the actual outbreak situation and its severity of Pandemic Influenza A/H1N1 in Nepal.

Methods

This is a Laboratory based prospective cross-sectional study carried out at National Public Health Laboratory from April 2009 to May 2010. Initially during the pandemic declaration of H1N1, samples were collected from patients of all age groups from both gender meeting the criteria of case definition of influenza like illness (ILI) with history of international travel from country of confirmed Pandemic H1N1 or close contact with

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confirmed H1N1 infected persons or with shortness of breathing or hospital admitted patients. As the community outbreak of pandemic influenza A/H1N1 was declared, sample collection was done by random sampling method from the patients meeting case definition of ILI. The patients already on antiviral treatment were excluded from the study. Influenza like illness (ILI) is defined as those who has fever $\geq 38^{\circ}\text{C}$ with at least one respiratory symptoms such as cough, rhinorrhoea or sore throat [8].

Posterior pharyngeal swabs were collected into Viral Transport Medium (VTM). For transportation from outside hospitals and outbreak area, Specimens were kept on ice box ($2-8^{\circ}\text{C}$) with bio-safety precaution and transported to the National Public Health Laboratory within 48 h after collection.

At NPHL, Laboratory diagnosis of Pandemic influenza A/H1N1 was made by one step probe-based Real Time-Polymerase Chain Reaction (PCR) on Posterior pharyngeal swabs. Initially, samples were tested for Universal influenza A, Once Universal influenza A was positive, it was further tested for Swine specific A followed by Swine specific H1. According to CDC guideline, Presumptive positive for Pandemic influenza A/H1N1 was declared if RT-PCR give positive results from either swine A or swine/H1 or positive from both tests [9]. All the specimens which give presumptive negative for Pandemic influenza A/H1N1 but universal influenza A positive were tested for seasonal influenza A (H1, H3 and H5) and those specimens negative for all swine A/swine/H1 and universal influenza A were tested for Influenza B.

Quality assurance and accreditation of Molecular Laboratory

five samples presumptive positive for Pandemic influenza A/H1N1 at our laboratory were retested at Microbiology Department of University of Hongkong, Hongkong and the concurrence was 100%. We are regularly participating in the WHO External Quality Assessment Programme for the detection of Influenza virus type A and type B by PCR from panel 8.

RT-PCR Technique

RNA was extracted from 140 μl aliquot of posterior pharyngeal swabs in Viral Transport Medium (VTM) by using viral RNA extraction kit (QIAmp, Qiagen) [10]. 5 μl of RNA extract was mixed with 5.5 μl of Nuclease free water, 0.5 μl of forward primer, 0.5 μl of Reverse primer, 0.5 μl of Probe, 0.5 μl of SuperscriptTMIII RT/Platinum *Taq* mix, 12.5 μl of 2 \times PCR Master Mix making total reaction volume 25 μl .

Three separate primer/probe sets for Universal influenza A(Inf A), Universal swine specific (swFluA) and

Swine specific H1 (swH1) were used. The internal positive control for human nucleic acid was RNase P primer/probe set which targets for human RNase P gene. The primers and probes used in the RT-PCR system were as described in Table 1.

RT-PCR system was performed as per protocol provided by Center for Disease Control, USA. The following RT-PCR program was used in this study: Reverse Transcription was at 50°C for 30 min, *Taq* inhibitor activation at 95°C for 2 min, PCR amplification cycle was at 95°C for 15 sec and 55°C for 30 sec with 45 times repetition. Fluorescence data (FAM) was collected during the 55°C incubation step [9].

Analysis in RT-PCR

When all controls i.e. Negative, Positive and human RNase P meet the requirements, a specimen is considered positive if the reaction growth curves cross the threshold line within 40 cycles. Similarly, a specimen is considered negative if growth curves do not cross the threshold within 40 cycles [9].

Virus typing

Blindly selected few positive samples of Pandemic influenza A/H1N1 from RT-PCR were sent to the WHO reference laboratory of Center for Disease Control and Prevention Division (CDC), Atlanta. All samples were isolated and antigenically characterized by Haemagglutination Inhibition Assay (HAI).

Statistical tools

Statistical tools like Probability were calculated by using Statistical Package for the Social Sciences (SPSS) programme.

Results

A total of 609 patients with suspected Pandemic influenza A/H1N1 were tested at National public health laboratory during the study period. All the samples were confirmed by Real-Time PCR. Out of these samples, 172 (28.3%) were Pandemic influenza A/H1N1 positive and 130 (21.34%) cases were seasonal influenza A as in Table 2. Due to heavy work load and priority for identification of pandemic H1N1, all the Seasonal influenza-A positive cases were not sub-typed. A sub-set of 88 seasonal-A positive samples, till November 2009 were sub-typed and found 70(79.5%) were seasonal H3, 8(9.09%) were seasonal H1 and 10(11.36%) were unsubtypeable seasonal influenza A. Among 219 negative cases for Universal Influenza A were tested for Influenza B and found only one positive case.

Ten randomly selected positive cases of Pandemic influenza A/H1N1 by RT-PCR were isolated and antigenically characterized by Haemagglutination Inhibition

Table 1 Primers and Probe sets used for the diagnosis of Pandemic Influenza A/H1N1

Primers and Probes	Sequence (5' > 3')	Working Concentration
Inf A Forward	GAC CRA TCC TGT CAC CTC TGA C	40 μ M
Inf A Reverse	AGG GCA TTY TGG ACA AAK CGT CTA	40 μ M
Inf A Probe	TGC AGT CCT CGC TCA CTG GGC ACG	10 μ M
SW Inf A Forward	GCA CGG TCA GCA CTT ATY CTR AG	40 μ M
SW Inf A Reverse	GTG RGC TGG GTT TTC ATT TGG TC	40 μ M
SW Inf A Probe	CYA CTG CAA GCC CA "T" ACA CAC AAG CAG GCA	10 μ M
SW H1 Forward	GTG CTA TAA ACA CCA GCC TYC CA	40 μ M
SW H1 Reverse	CGG GAT ATT CCT TAA TCC TGT RGC	40 μ M
SW H1 Probe	CA GAA TAT ACA "T"CC RGT CAC AAT TGG ARA A	10 μ M
RnaseP Forward	AGA TTT GGA CCT GCG AGC G	40 μ M
RnaseP Reverse	GAG CGG CTG TCT CCA CAA GT	40 μ M
RnaseP Probe	TTC TGA CCT GAA GGC TCT GCG CG	10 μ M

(HAI) Assay in WHO reference lab US CDC in October 2009. All the characterized virus found in Nepal were antigenically and genetically related to the novel influenza A/CALIFORNIA/07/2009-LIKE (H1N1)v type (Figure 1).

First case of Pandemic influenza A/H1N1 was detected in June 2009. Starting from the June, cases were increasing up to peak of the Pandemic Influenza outbreak in November (Figure 2).

Till May 2010, total number of confirmed positive cases of Pandemic influenza A/H1N1 was 172. Out of them, 36 cases were recorded before declaration of Community transmission among 29 Nepalese citizens residing within the country or from abroad, 2 foreigner, 5 close relatives of confirmed positive cases and remaining 136 cases were found after community transmission which was declared on October 15 2009.

Travelling history of pandemic influenza cases before community transmission was as shown in Figure 3. Among them, largest no of cases were found from US.

After community outbreak, most of the cases of Pandemic influenza were from Kathmandu district followed by Kaski and Chitwan (Figure 4).

All the confirmed cases of Pandemic influenza A/H1N1 were in the range of age group from 1-74 with mean age 21 years. Most of the cases were found in the age group 11-20 followed by 21-30 and 0-10. Among the positive cases 119(69.18%) were male and 28 (30.82%) were female (Figure 5 and 6).

Till May 2010, three death cases were reported due to Pandemic influenza A/H1N1. All were

Female patients with age 31, 29 and 23 (Figure 7).

Discussion

Since the middle of March 2009, infections with the new influenza A (H1N1) strain started to occur in Mexico, and the first two cases in the United States occurred in late March 2009, although they were not confirmed until April 15, 2009 [11]. The rapid global spread indicates towards the influence of international air travel on influenza [12]. According to the data from WHO till March 2010, this new influenza A (H1N1) was estimated to have a case-fatality rate (CFR) of 1.28%.

In this study, by May 28 2010, a total of 609 suspected patients were tested for Pandemic influenza A/H1N1 2009. Out of the collected samples, 130 (21.34%) were seasonal influenza A positive and 172 (28.3%) were Pandemic influenza A/H1N1 positive. The mean age of patients with confirmed Pandemic influenza A/H1N1 was 21, whereas the mean age of the positive cases of Seasonal influenza A was 28. Patients with pandemic (H1N1) 2009 were significantly younger ($p = 0.04$, $\chi^2 = 8.21$) than patients with Seasonal influenza A (< 20 years).

A characteristic feature of the H1N1 pandemic is that it disproportionately affected so far children and young adults [13]. Similar results were observed in different parts of the world: In Saudi Arabia, the age of the cases ranged between 1 and 56 years with mean (SD) of 24.2 (14.4) years [14]. One of the early studies from the USA showed that although the age of pandemic influenza A (H1N1) patients in the study ranged from 3 months to 81 years, 60% of patients were 18 years of age or younger. In most countries, the majority of Pandemic influenza A (H1N1) cases have been occurring in young

Table 2 Total cases of Suspected Pandemic influenza A/H1N1 (n = 609)

Cases	Universal Influenza A	Pandemic Influenza A/H1N1	Seasonal Influenza A
Positive	302(49.6%)	172(28.3%)	130(21.34%)
Negative	307(50.4%)	437(71.77%)	479(78.66%)

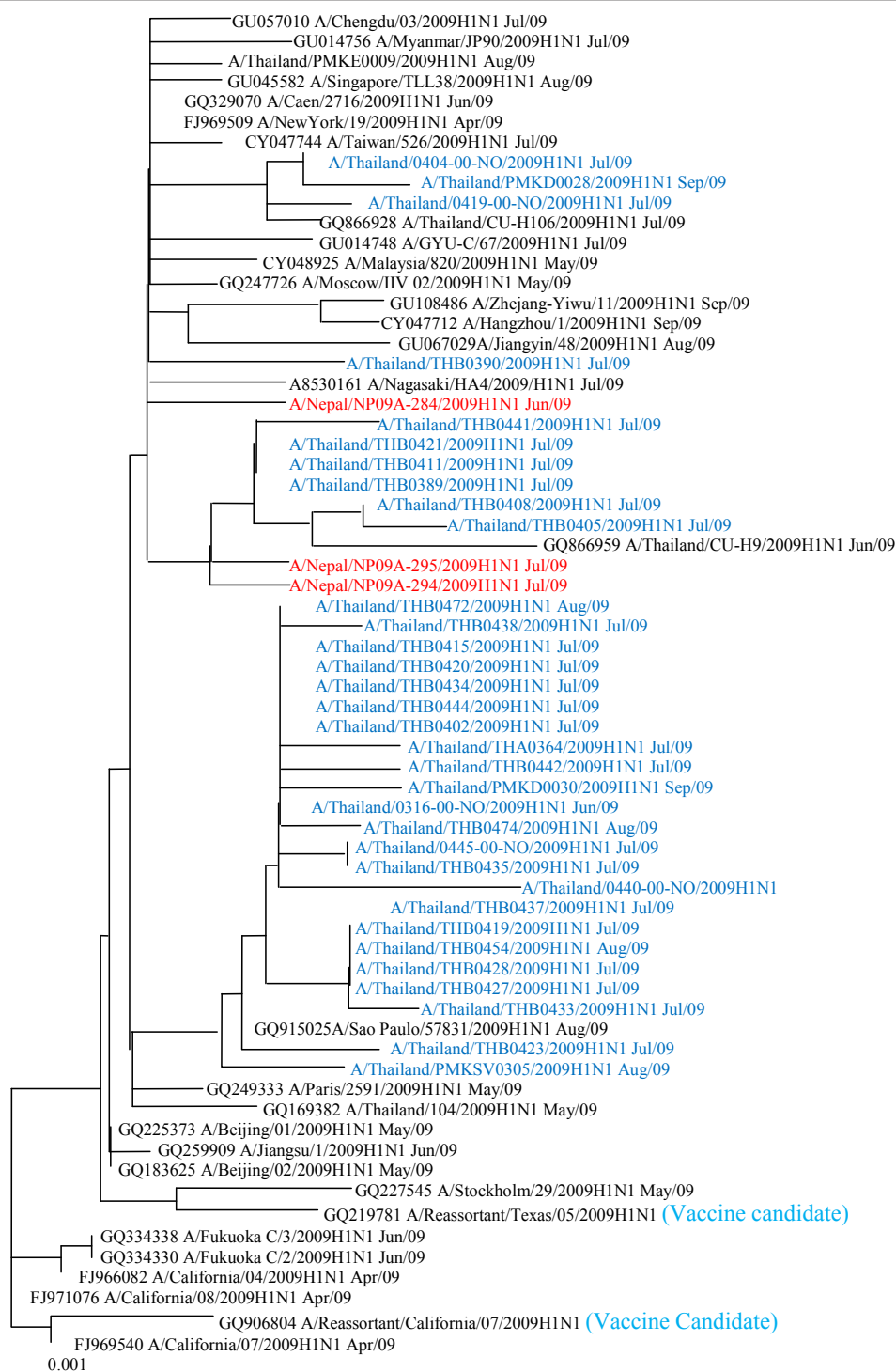


Figure 1 The neighbor joining tree of influenza A/H1N1 (Swine) 2009 complete HA gene (1,701 bp).

people, with the median age estimated to be 12 to 17 years in Canada, the USA, Chile, Japan, and the UK. Most of the pandemic influenza cases in young age people indicate towards partial immunity to the virus in the older population [15].

The overall case fatality ratio (CFR) found in this study was 1.74% as 3 death cases were reported till May 28, 2010. All the death cases were found in female with below 35 years of age. The data found in this study is similar with the other literatures. The overall case

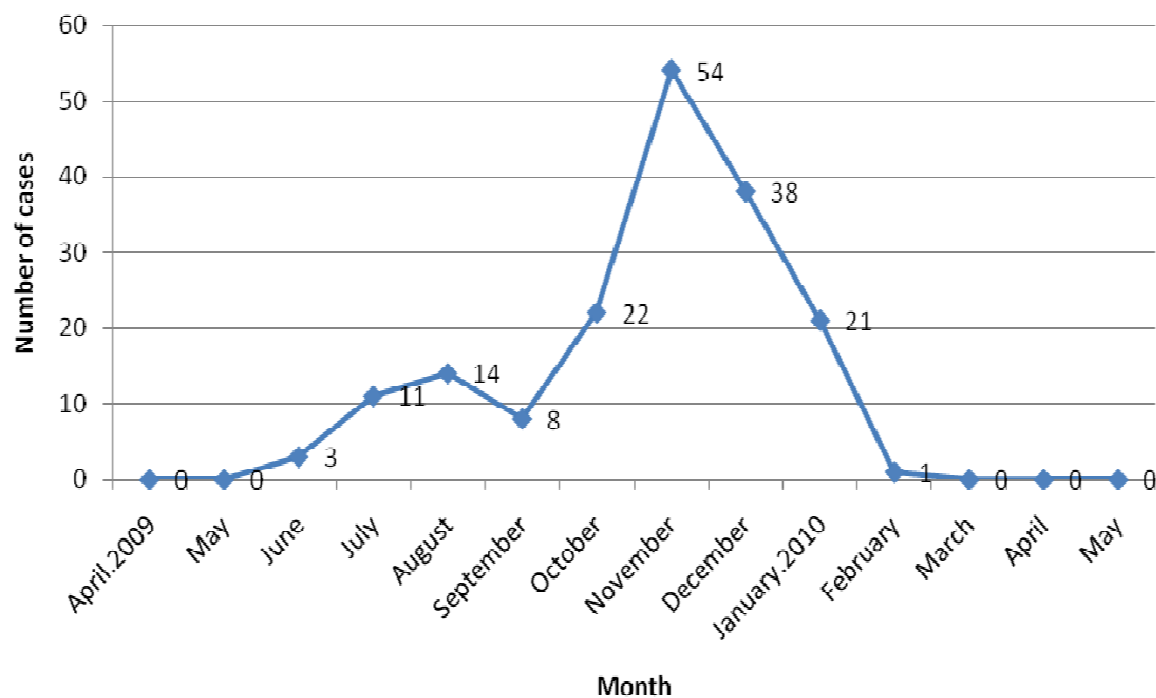


Figure 2 Epidemic curve of Confirmed cases of Pandemic influenza A/H1N1 2009 (n = 172).

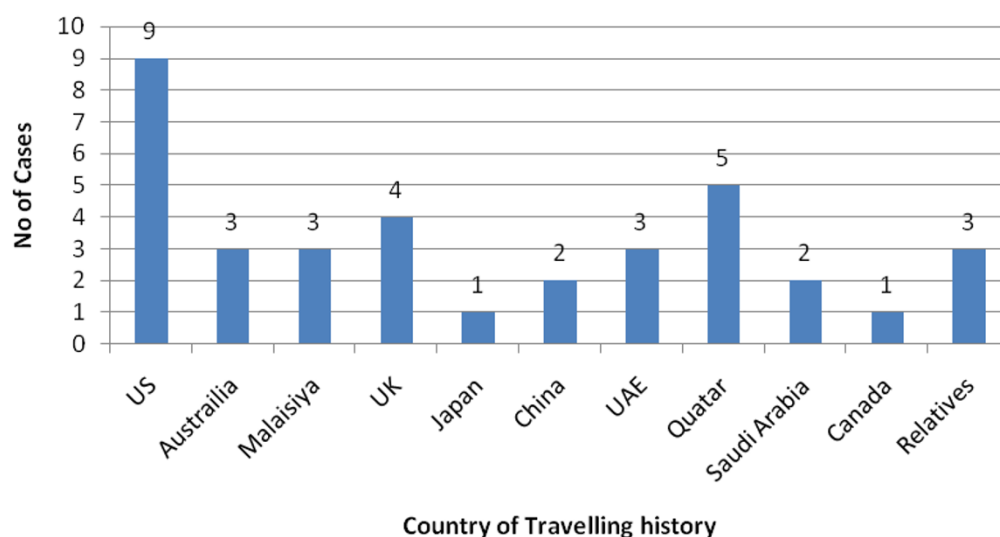


Figure 3 Travel history of confirmed Pandemic influenza A/H1N1 cases before community transmission (n = 36).

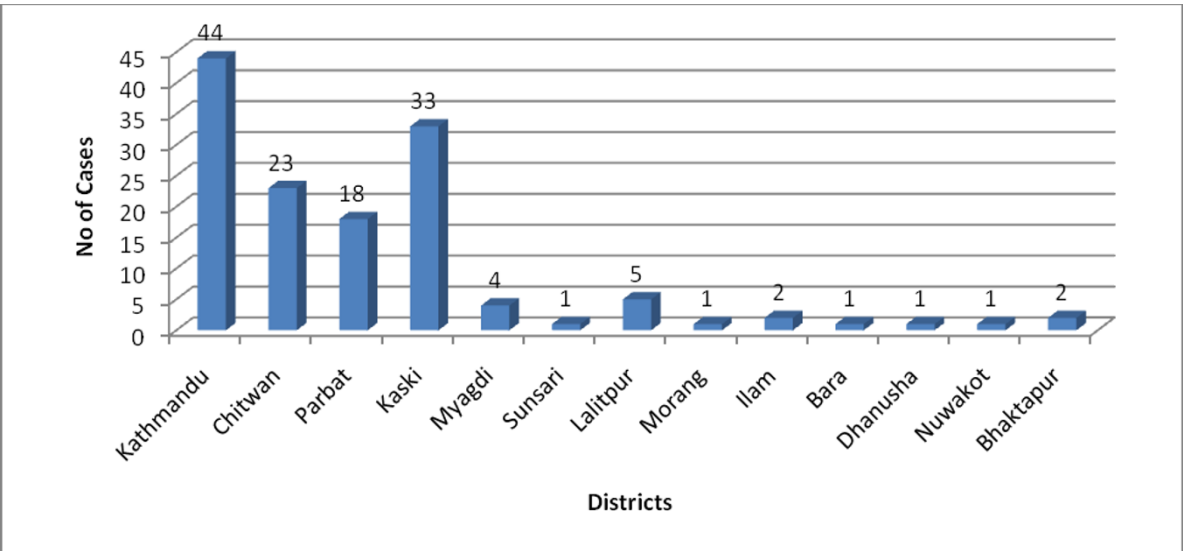


Figure 4 District wise distribution of total Pandemic influenza A/H1N1 cases after community transmission (n = 136).

fatality rate has been less than 0.5%, and the wide range of estimates (0.0004 to 1.47%) reflects uncertainty regarding case ascertainment and the number of infections [16-18]. The case fatality rate for symptomatic illness was estimated to be 0.048% in the United States [19] and 0.026% in the United Kingdom [20]. In contrast to seasonal influenza, most of the serious illnesses caused by the pandemic virus have occurred among children and

nonelderly adults, and approximately 90% of deaths have occurred in those under 65 years of age [21].

Conclusion

In Nepal, mostly young aged people were affected by the wave of this pandemic influenza and all the isolated pandemic influenza A/H1N1 2009 virus were antigenically

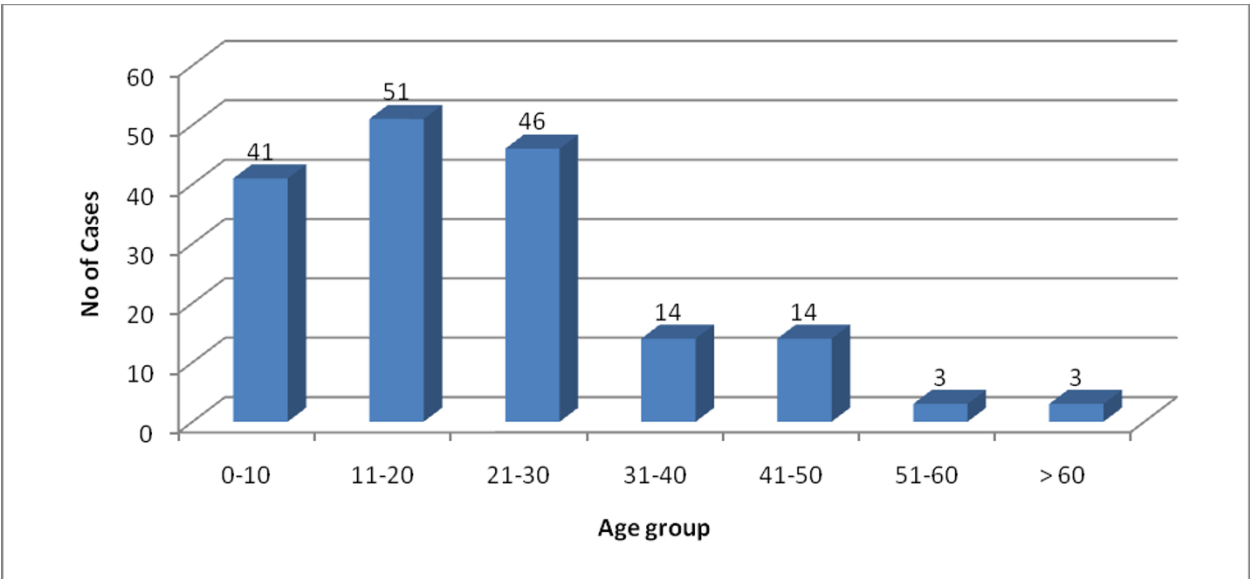


Figure 5 Age wise distribution of confirmed Pandemic influenza A/H1N1 cases (n = 172).

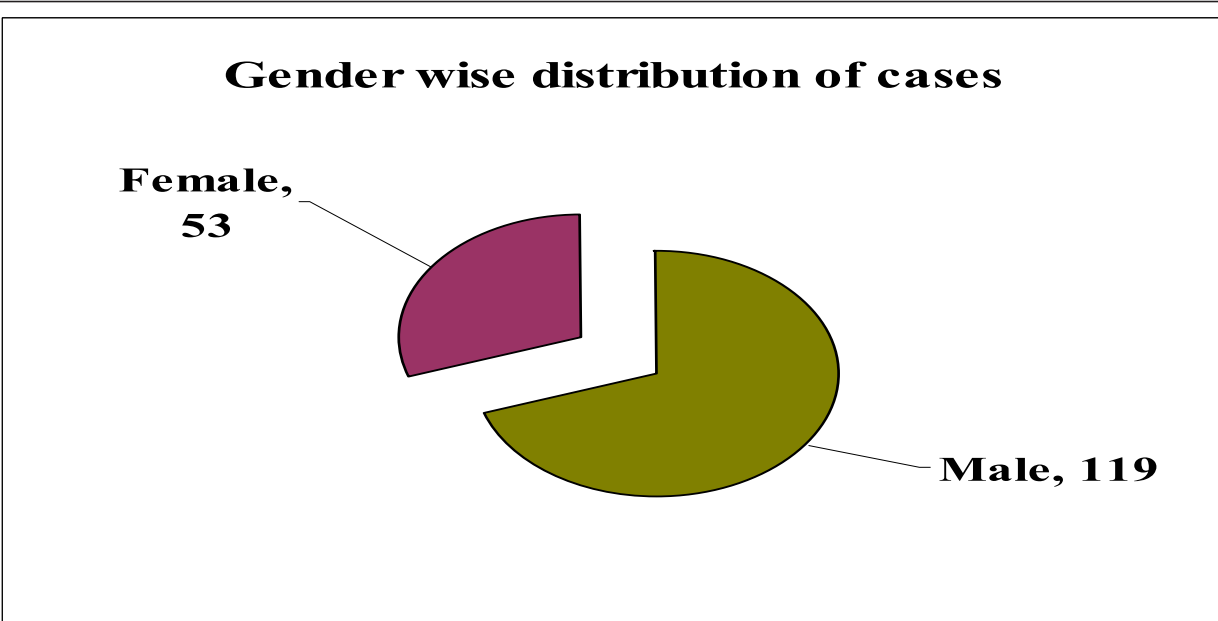


Figure 6 Sex wise distribution of confirmed Pandemic influenza A/H1N1 cases (n = 172).

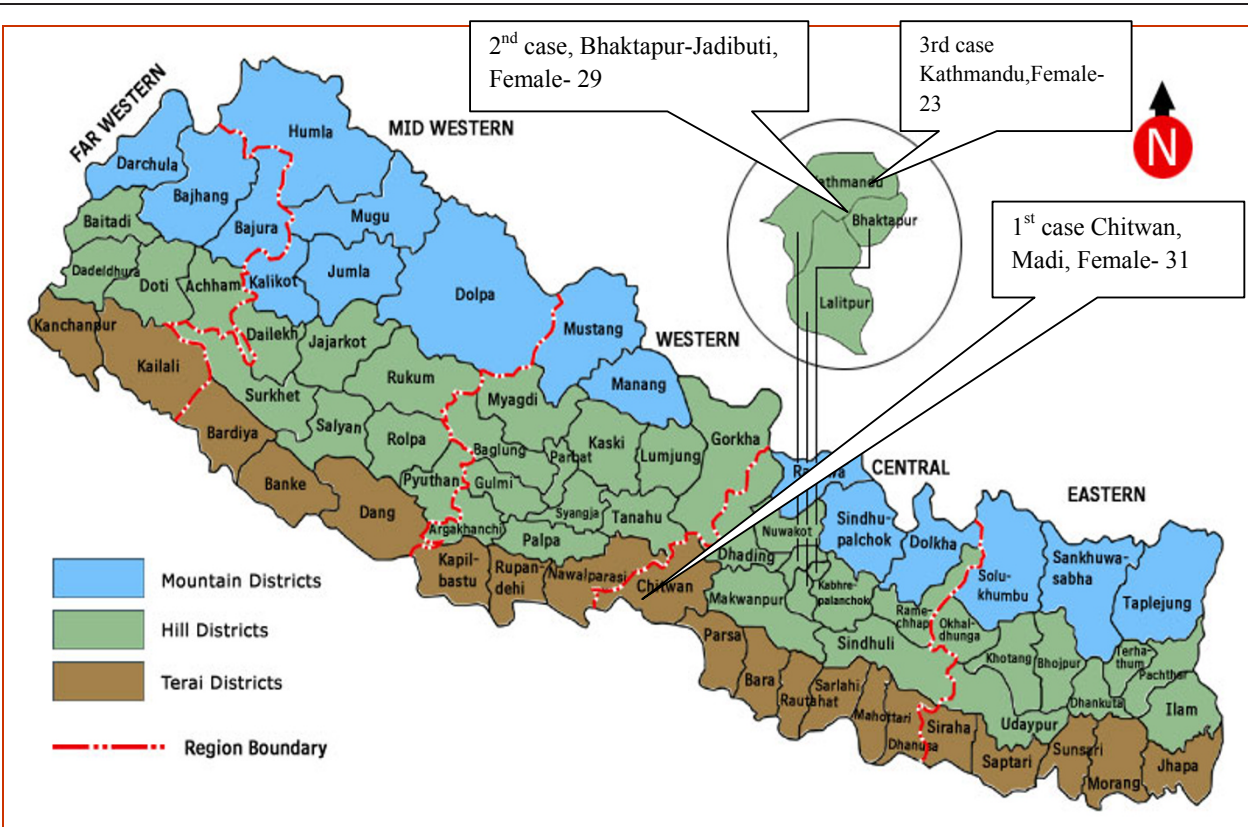


Figure 7 Information of death cases in Nepal by Pandemic influenza A/H1N1.

and genetically related to the novel A/CALIFORNIA/07/2009-LIKE (H1N1)v type.

Acknowledgements

We would like to acknowledge all the staff working in Virology Department of National Public Health Laboratory for their contribution during the study. We are grateful to WHO and CDC for providing primers/probes, enzymes, reagents and Viral RNA extraction kits.

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Authors' contributions

BA has made great contribution to design, acquisition, interpretation and analysis of data and overall frame out of this article. He has actively involved for the optimization of Real-Time PCR during beginning of this study. His contribution during the analysis of Real-Time PCR results made easy for drafting the article. GS has been involved for drafting the manuscript and given final approval for the publication. BU has actively participated in the laboratory work and co-ordinate overall activity of molecular diagnosis. KPK help for the sample management during Pandemic H1N1 outbreak and suggests some minor mistakes during preparation of this manuscript. SDS helped for the sample management during Pandemic H1N1 outbreak. She has contributed by proof reading of this manuscript. GRD has contributed in diagnostic part of this study. All authors read and approved the final manuscript.

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Competing interests

We have no competing interest to declare. This study is the property of National Public Health Laboratory though World Health Organization and Center for Disease Control and Prevention (CDC) supported by providing Primers/probes, reagents and kits for the study.

Received: 18 September 2010 Accepted: 23 March 2011
Published: 23 March 2011

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doi:10.1186/1743-422X-8-133

Cite this article as: Adhikari et al.: Outbreak of pandemic influenza A/ H1N1 2009 in Nepal. *Virology Journal* 2011 **8**:133.

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