Table 1. Seasonally integrated flux (SIF) of N_2O (g/ha)

Crop	Duration of cropping (days)	Soroda (SIF)	Tangibanta (SIF)
Green gram	72	8.652	14.75
Horse gram	105	11.835	14.005
Black gram	93	11.999	16.712

For GG, the maximum N_2O emission occurred during 20–40 days after sowing. N_2O emission was also higher in Tangibanta compared to Soroda. The soil N-content was less in Soroda compared to Tangibanta. Both places showed positive soil Eh. N_2O flux was also collected during HG cultivation in both places. In most of the cases, positive N_2O flux was observed. Maximum flux was observed after 56–70 days of sowing in both the places. pH of the soil was near neutral or slightly acidic throughout the cropping period. In all the cases, Eh of the soil was positive throughout the measuring period. The soil carbon content varied in the range 4–6 g/kg.

N_2O emission from agricultural activities depends on various factors such as N-content, moisture content, pH, Eh and temperature[2,3,7]. The NO_3^-, NO_2^- and NH_4^+ content in the soil at Soroda was found to be lower than that at Tangibanta. It was observed that in both the places, the highest N_2O emission was associated with the higher N-content of the soil. The increase in N-content may be associated with the biological N_2-fixation by leguminous crops, which might have increased the availability of NH_4^+ in the soil, to serve as the substrate for nitrification[8]. The seasonally integrated flux was observed to be different for different crops (Table 1). This variation may be attributed to nitrogen requirement and its management, the moisture regime, root type, pH, redox potential and temporal variation. The root can induce some microbial activity, including nitrification and denitrification processes through bacterial activities in the soil[8]. The pulses are cultivated during rabi season in Tangibanta and kharif season in Soroda. The temporal variability of N_2O emission during both the seasons might be due to variations in soil moisture content[9], soil N-content[10] and variable plant growth[11].

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Influenza virus infection during a pilgrimage at Pandharpur, Maharashtra, India

Influenza is an air-borne disease, and crowding during large gatherings or pilgrimages facilitates person-to-person transmission of influenza virus among highly susceptible population. Poor living conditions and stress during pilgrimages probably exacerbate susceptibility to infection. Many studies have been conducted on the role of influenza viral infection during the Haj[1–4]. Respiratory tract infection is the most common disease transmitted during this period, with influenza viruses identified as one of the most common etiological agents. India, as a confluence of different religions, has always attracted pilgrims from all over the world. The Kumbha Mela (the Great Fair) is a gathering of between 10 and 20 million Hindus upon the banks of the holy rivers, as periodically ordained in different parts of India, and is regarded as the largest gathering of humanity on earth. Many such small and large pilgrimages take place all over India all through the year. The present investigation is a pilot study conducted in 2005 during a pilgrimage to Pandharpur, Maharashtra, to investigate the extent of influenza during the pilgrimage.

The Pandharpur pilgrimage, a unique feature of Maharashtrian culture, is a 1000-year-old tradition followed by the warkaris (people who follow the wari, a fundamental ritual). People from different parts of Maharashtra gather at Alandi, a small town near Pune city and collectively go singing and dancing every year from Alandi to the holy town of Pandharpur in the months of June–July, cov-

ering a distance of about 250 km on foot. Many devotees join the procession on the way and the whole process lasts for 21–22 days with approximately 375,000 devotees arriving at Pandharpur on the last day of the pilgrimage. The Government of India establishes temporary clinics (approximately 300–350, pers. commun.) at major centres along the route and at Pandharpur during the entire period of the pilgrimage.

Samples were collected on 19 July 2005, the last day of the pilgrimage at two Corporation dispensaries in Pandharpur. Throat/nasal swabs were collected from 42 patients with symptoms of fever accompanied by sore throat, running nose and/or cough. The total attendance at these OPDs on 19 July 2005 was 1243, with 240 cases (19.3%) suffering from respiratory tract infection.

For virus isolation and identification, MDCK cells were grown in 25 cm^2 flasks and inoculated with 1.0 ml clinical specimen adsorbed for 1 h and incubated at 37°C in the presence of viral growth medium (minimal essential medium with 2 μg/ml TPCK trypsin). Cells were observed for 7 days and harvested when the cytopathic effect was evident. Supernatants from all the flasks were subjected to hemagglutination (HA) test using guinea pig and fowl red blood cells. Strain identification of HA-positive isolates was performed by HA inhibition (HI) test[5] using hyper-immune sera provided by WHO.

RT–PCR and *HA1* gene sequencing were conducted according to the CDC manual for influenza strain surveillance[6]. Briefly, RNA from six strains of A(H1N1) and two of A(H3N2) was extracted using QIAamp Viral RNA mini kit (Qiagen). cDNA was synthesized using 5 μl of RNA, 10 U AMV-RT (Promega) and 10 pmol of forward primer. cDNA was added to PCR mix containing 20 pmol each of forward and reverse primers. PCR products were subjected to electrophoresis on 2% agarose gel in TAE buffer. Amplicons (1200 bp) were purified using QIAquick PCR purification kit (Qaigen) and subjected to cycle sequencing using ABI Prism Big Dye terminator V3.1 cycle sequencing kit. Post-cycle sequencing purification was done using DyeEx2.0 spin kit (Qaigen). Sequencing was performed on ABI Prism 310 and sequence alignment and phylogenetic analyses were done using Mega version 3.1. The neighbour joining algorithm and Kumura 2 parameter distance model were utilized with 1000 bootstrap support.

A total of 42 samples (Table 1) were collected with mean age of 44.33 years (range 5–80 years) and males constituted 59.5% (25/42) of the cases. Among the samples collected, 34 were throat swabs (81%) and 8 (19%) nasal swabs. Clinical presentation consisted of fever in all cases along with cough (95.2%), nasal discharge (50%) and sore throat (19%). Other symptoms included breathlessness, headache, body ache, chills and diarrhoea in some patients.

Influenza viruses were isolated from nine samples (21.4%) and constituted patients of all age group (Figure 1) with mean age of influenza-positive patients being 35.33 years (range 5–70 years, Table 1). Mean post-infection period for cases with virus positivity was 1.5 days (SD ± 0.61), with 77.7 and 55.5% of the cases having cough and nasal discharge respectively, accompanying fever.

Among the nine isolates, seven were influenza A(H1N1) and two were A(H3N2). All the seven A(H1N1) strains were antigenically similar to A/New Caledonia/20/99 and the two A(H3N2) strains were similar to A/California/7/04, as identified by the HI test.

HA1 gene sequencing indicated that all the A(H1N1) strains formed a cluster which was genetically similar to A/New Caledonia/20/99 (Figure 2 *a*). This strain has been the vaccine-recommended strain since the year 2000 and continued to be the recommended strain[7] for the year 2006–07. The A(H3N2) strains were genetically similar to A/California/7/04, the vaccine-recommended strain for the 2005–06 influenza season for the northern hemisphere[8] and genetically distinct from A/Fujian/411/02 and A/Wellington/1/2004 strains that were vaccine-recommended strains for the 2004–05 northern[9] and 2005 southern[10] hemispheres respectively (Figure 2 *b*).

Pilgrimages are an integral part of the Indian culture, and numerous small and big pilgrimages are undertaken all the year round in various parts of the country. No report of influenza activity during any of these pilgrimages is available. Considering the fact that respiratory infections are common during these pil-

Table 1. Influenza virus isolation in relation to age, sex and clinical symptoms

	Total	Positive for influenza virus isolation
Number of samples collected	42	9 (21%)
Mean age (range)	44.33 (5–80)	35.33 (5–70)
Sex – male (%)	59.5	77.7
Mean duration of symptoms (SD*)	2 days (± 0.86)	1.5 days (± 0.61)
Fever (%)	100	100
Cough (%)	95.2	77.7
Nasal discharge (%)	50	55.5
Sore throat (%)	19	22.2

*SD, Standard deviation.

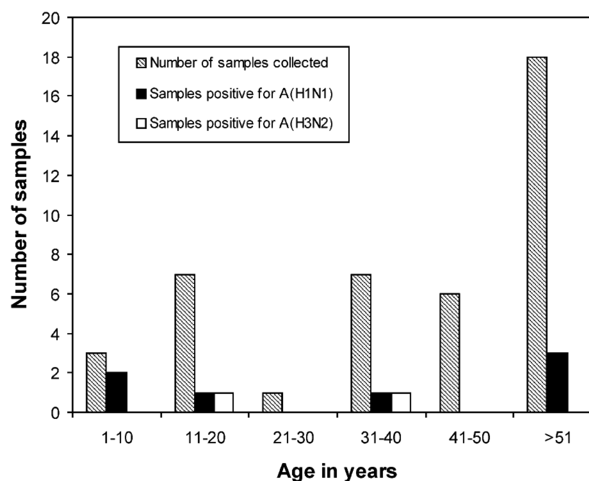

Figure 1. Age-wise distribution of cases and influenza virus positivity.

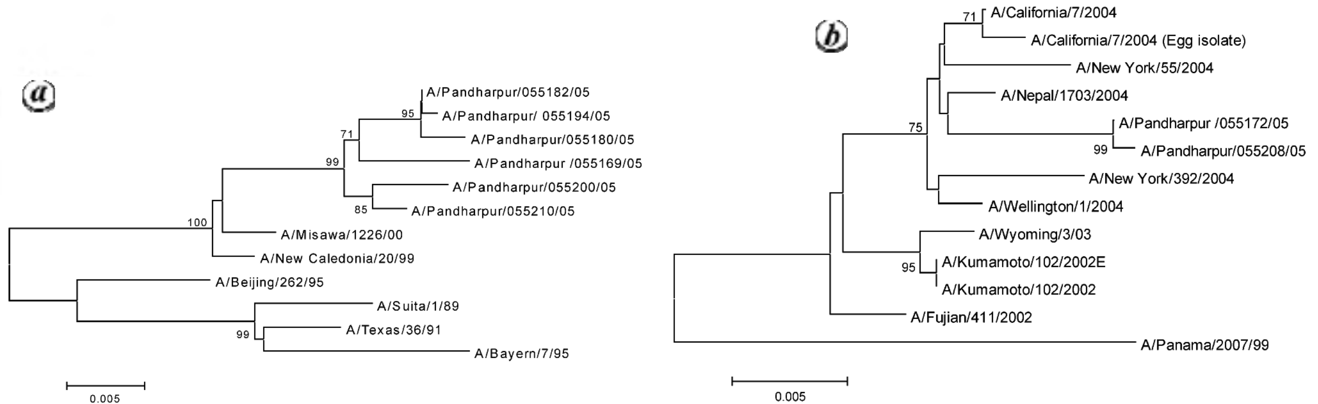

Figure 2. Phylogenetic analysis of influenza (***a***) A(H1N1) and (***b***) A(H3N2) strains using 1024 nucleotides of the *HA1* gene. Accession numbers of the sequences obtained from Genbank used in the analysis from top to bottom are as follows:
A(H1N1) – AY029292, AY289929, AY289928, D13573, CY009316, AJ457907, and of Pandharpur are EU032582, EU032583, EU032581, EU032580, EU032584, EU032585.
A(H3N2) – EF473341, EF473342, ISDN119760, AY945278, CY002064, EF566073, AY531033, ISDN69739, EF456797, ISDN38157, DQ487340, and of Pandharpur are EU032578, EU032579.

grimages, the present study was undertaken to investigate the role of influenza virus in these infections.

The major role of influenza virus causing respiratory infections was evident from high (21.4%) rate of influenza virus isolation. Influenza strain surveillance in the western region of India is being conducted for the past 30 years at the National Influenza Centre, National Institute of Virology, Pune, a place close to Pandharpur. The data from this centre indicate highest influenza activity during June–September every year, with isolation rate ranging from 2.9 to 9%[11,12]. The Pandharpur pilgrimage occurs in July, when this region receives monsoon rains and coinciding with peak influenza activity in this region. This could be one of the reasons for high positivity for influenza virus infection. Interestingly, though India lies in the northern hemisphere, peak influenza activity in its western region coincides with the influenza season in the southern hemisphere.

Both the subtypes, A(H1N1) and A(H3N2), were isolated from these cases. Devotees from all over Maharashtra participate in the pilgrimage and introduction of influenza viruses from different areas may decide the type and/or the subtypes causing infection. Overall, it appears that the influenza virus plays a major role in respiratory infection during the Pandharpur pilgrimage and vaccination against influenza can be considered a priority for those attending the pilgrimage. However, the decision regarding whether the vaccine recommended for the southern or the northern hemisphere should be used warrants larger and regular yearly surveillance studies during such pilgrimages, before proper recommendations can be issued.

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