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Original Article

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Abstract

Introduction: In Africa, the burden of influenza is largely unknown since surveillance schemes exist in very few countries. The National Institute of Hygiene in Morocco implemented a sentinel network for influenza surveillance in 1996.

Methodology: Epidemiological and virological surveillances were established and influenza viruses circulating in Morocco were characterised. Four practice-specific indicators were collected during the 1996-1997 season and nasopharyngeal swabs were collected from patients with an influenza-like illness during a three-year period (between 1996 and1998). Laboratory diagnosis was done by viral isolation. The isolates were characterized by hemagglutination- and neuraminidase-inhibition assays and by sequencing the hemagglutinin gene and phylogenetic analysis.

Results: Among a total of 673 specimens, 107 (16%) were positive for influenza virus. Seasonal influenza strains were isolated from November to February. Antigenically, A(H1N1), A(H3N2) and B isolates were related to the vaccine strains. Genetically, one 1996/97 isolate A/Rabat/33/96 and the 1997/98 A(H3N2) isolates clustered with the new drift variant A/Sydney/5/97, a vaccine component of the 1998/99 season.

Conclusions: These results indicate a seasonal circulation of influenza in Morocco concentrated between November and February. Further, the results demonstrate the importance of including the maximum number of countries in influenza surveillance to contribute to the definition of the influenza vaccine composition.

Key words: Influenza A, Influenza B, virus isolation, molecular characterization, Morocco

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Introduction

Influenza is a common, highly transmittable viral illness having a short incubation period, high attack rate, and rapid spread within populations. It can cause serious illness, and is a major cause of morbidity and mortality worldwide. Rates of infection are highest among children, but severe illness and death from influenza are greater among the elderly and people with chronic medical conditions that place them at greater risk for influenza complications [1]. Current human influenza viruses (IV) belong to the subtypes A(H1N1) and A(H3N2), and between 2001 and 2005 subtype A(H1N2) viruses have been circulating widely [2-3]. Influenza B viruses are subdivided into the B/Victoria and B/Yamagata lineages [4-5].

Annual outbreaks or epidemics occur because viruses can mutate quickly, resulting in new variants with minor changes in their surface glycoproteins, against which humans have incomplete immunity [6]. Such antigenic drift requires the adaptation of the influenza vaccine composition based on data from the worldwide surveillance network coordinated by the World Health Organization (WHO) [7].

Due to the extreme heterogeneity of the clinical symptoms associated with influenza infections, it is not possible to confirm influenza on the sole basis of clinical symptoms. Therefore, the knowledge of the epidemiological situation prevailing locally, provided

Table 1. Location of sentinel network centres, number of sentinel physicians, virological results of influenza surveillance in Morocco from 1996 to 1998.

Surveillance				Practitioners			
Season	Cities	Data	Period (week/year)	General practitioners (GPs)	Paediatricians (PPs)	Number specimens	Viruses
1995/96	Rabat	virological	7/96-24/96	11	2	110	2A(H3N2) 6 (B)
1996/97	Rabat Kenitra Casablanca	clinical and virological	42/96-24/97	8	4	228	10A(H1N1) 24A(H3N2) 10 (B)
1997/98	Rabat Kenitra Casablanca Marrakech Agadir Fes Oujda Tangier	virological	42/97-24/98	20	12	335	55A(H3N2)

by surveillance networks of sentinel practitioners, is particularly valuable, especially when the surveillance is based on monitoring both IV circulation in the community and the evolution of acute respiratory infections (ARIs) or/and clinically defined influenza, often referred to as influenza like illnesses (ILI) [8-9].

In Africa, surveillance schemes have been implemented in a very small number of countries such as Senegal [10], Ivory Coast [11] and Madagascar [12-15]. In these countries, IV circulation and ILI incidence were demonstrated to vary according to the climate and geographical position of the particular country.

Until 1996, there was little data available about influenza activity in the community and the virological and epidemiological pattern of influenza in Morocco. Therefore, the National Institute of Hygiene in Morocco implemented a sentinel network for IV surveillance, inspired by the French sentinel system. The latter is based on the collection of both virological indicators that show the circulation of the virus, and indicators for health care system activities that show the impact of the epidemic on health. These indicators seem to be specifically related to influenza epidemics since they do not change when there is no such epidemic [8-9].

This paper describes the establishment of the sentinel network and provides the first report of influenza surveillance in Morocco over three seasons (1995-1998) when the surveillance was fully implemented.

Methodology

Sentinel system network study sites, data and specimens collection

The surveillance network began during the 1995/1996 influenza season in Rabat with volunteer private practitioners, specifically 11 general practitioners (GPs) and two paediatricians (PPs). It was expanded to Kenitra and Casablanca during the 1996/97 season. By 1997/98, it had grown to include eight sentinel sites and over 32 physicians in widely distributed locations providing coverage of the major population centers in Morocco (Table 1). During the 1996/97 season, both clinical and virological data were collected weekly by all physicians participating in the network. At the beginning of each week, all doctors were contacted by telephone and the following data related to their medical activities was collected for the past week (from Monday to Sunday): the number of days worked, the number of ARIs (defined as a person with sudden onset of respiratory signs, cough, difficulty breathing, rhinitis or coryza, accompanied by general symptoms such as fever, headache, asthenia and myalgia) diagnosed, the number of home visits, and the number of sick-leave prescriptions of less than 15 days. The indicators were based on the average number of ARIs diagnosed per day, the percentage of ARIs per consultation, the percentage of visits leading to 15

Table 2a. Antigenic characterization of the hemagglutinin and neuraminidase of influenza viruses type A(H3N2) isolated in Morocco during the 1996/97 and 1997/98 seasons. The A(H3N2) reference strains used were: A/Johannesburg/33/94 (JHB/33/94), A/Nanchang/933/95 (Nan/933/95) and A/Sydney/5/97 (Syd/5/97)

	Hemagglutination inhibition test (HI)				**Neuraminidase inhibition test (NAI)**		
Type A	**post-infection ferret sera**				**post-infection ferret sera**		
Reference strains	**JHB/33/94**	**Nan/933/95**	**Syd/5/97**		**JHB/33/94**	**Nan/933/95**	**Syd/5/97**
A/Johannesburg/33/94(H3N2	**640**	40	<20		**1280**	160	40
A/Nanchang/933/95(H3N2)	160	**2560**	40		320	**5120**	320
A/Sydney/5/97(H3N2)	<20	<20	**2560**		<40	640	**5120**
Clinical isolates							
1996-1997 season							
A/Kenitra/17/97	320	1280	ND[a]		ND[a]	ND[a]	ND[a]
A/Rabat/18/97	320	640	ND[a]		ND[a]	ND[a]	ND[a]
A/Rabat/33/97	320	1280	ND[a]		ND[a]	ND[a]	ND[a]
1997/1998 season							
A/Casa/78/98	80	320	80		NA[b]	NA[b]	NA[b]
A/Marrakech/90/98	40	320	80		NA[b]	NA[b]	NA[b]
A/Marrakech/ 92/98	80	320	80		<	160	640
A/Marrakech /95/98	80	320	80		<	640	1280
A/Marrakech/96/98	40	640	80		NA[b]	NA[b]	NA[b]
A/Marrakech/98/98	ND[a]	ND[a]	ND[a]		<	160	320
A/Marrakech/109/98	40	320	80		<	80	320
A/ Marrakech/111/98	40	320	20		<	640	1280
A/Marrakech/114/98	160	640	160		<	160	640
A/Marrakech /116/98	80	320	80		<	160	640
A/Marrakech/132/ 98	80	640	160		<	320	640
A/Fes/216/98	ND[a]	ND[a]	ND[a]		<	160	320
A/Rabat/227/98	40	640	160		<	160	320
A/Rabat/236/98	80	640	160		NA[b]	NA[b]	NA[b]
A/Marrakech/262/98	80	640	160		NA[b]	NA[b]	NA[b]
A/Fes/273/98	80	320	80		NAb	NA[b]	NA[b]
A/Fes/278/98	80	320	80		NA[b]	NA[b]	NA[b]
A/Fes/314//98	20	320	80		<	320	640

The A(H3N2) reference strains used were: A/Johannesburg/33/94 (JHB/33/94), A/Nanchang/933/95 (Nan/933/95) and A/Sydney/5/97 (Syd/5/97). The B reference strains uses were: B/Panama/45/93 (B/Pan/45/93), B/Beijing/184/93 (B/Beij/184/93), B/Nanchang/451/93 (B/Nan/451/93), B/Johannesburg/26/94 (B/JHB/26/94).
a: Not Done
b: Not applicable, the corresponding strains did not show any NA activity in our test making it impossible to further perform NA inhibition.

Table 2b. Antigenic characterization of the hemagglutinin of influenza viruses type B isolated in Morocco during the 1996/97 season. The B reference strains used were: B/Panama/45/93 (B/Pan/45/93), B/Beijing/184/93 (B/Beij/184/93), B/Nanchang/451/93 (B/Nan/451/93), B/Johannesburg/26/94 (B/JHB/26/94).

Type B	**Post-infectious ferret sera**			
Reference strains	**B/Pan/45/93**	**B/Beij/184/93**	**B/Nan/451/93**	**B/JHB/26/94**
B/Panama/45/93	160	40	20	80
B/Beijing/184/93	80	160	80	80
B/Nanchang/451/93	40	20	160	40
B/Johannesburg/26/94	160	320	80	320
Clinical isolates				
B/Rabat/41/97	80	160	160	160
B/Rabat/45/97	40	80	160	20
B/Rabat/61/97	40	160	40	40

days or less sick-leave prescription, and the average number of home visit per days.

In addition, throat and nasopharyngeal swabs were taken from patients presenting with ILI (defined as an outpatient with fever ≥ 38.5°C and cough or sore throat in absence of specific diagnosis with duration of illness of less than three days) for less than four days and were sent to the central laboratory along with patient-specific information (demographic data, medical history, clinical symptoms, and epidemiological context).

Virological analysis

Specimens were stored at 4°C and transported in a standard virus transport medium from the time of collection to final processing at the central laboratory. Samples were inoculated onto Madin-Darby Canine Kidney (MDCK) cells as previously described [16]. Culture supernatants were collected and evaluated for haemagglutination activity using 0.5% guinea pig red blood cells. Virus typing and sub-typing were performed by an haemagglutination inhibition test (HI) using post-infection ferret sera specific for type A(H1N1) and A(H3N2) subtypes or type B [17]. A neuraminidase inhibition test (NAI) adapted from the method described by Fiszon and Hannoun [18] was used.

Genetic analysis

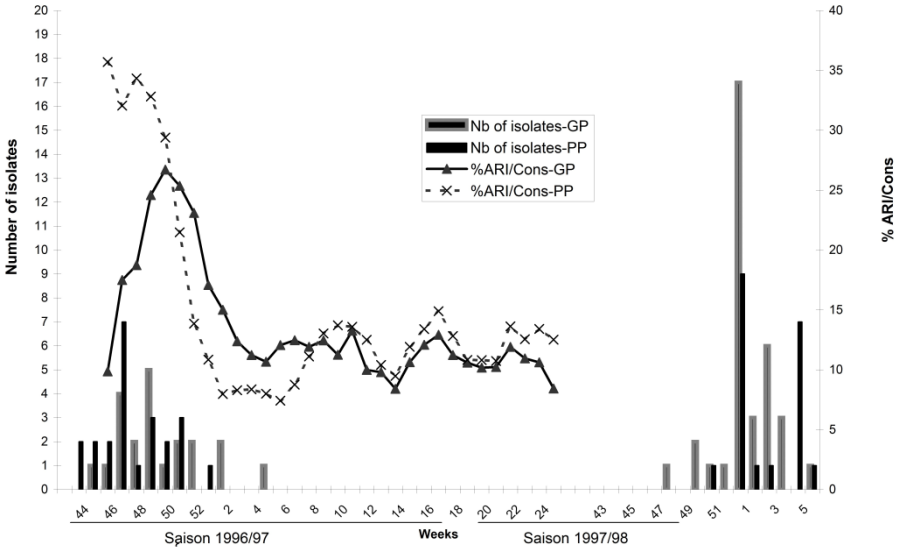
Viral RNA was extracted from 100 µl infected culture supernatant using the guanidium thiocyanate (Gibco BRL, Life Technologies, Grand Island, NY, USA), according to the manufacturer's recommendations, in the presence of 20 µg glycogen, molecular biology grade (Boehringer GmbH,. Mannheim, Germany). The RNA (5µl) was then used to prepare cDNA. The reaction mixture (7.1 µl) contained: 0.8 X PCR buffer (Promega, Madison, WI, USA); 8 mM DTT; 1 mM of each dNTP (Pharmacia Biotech, Uppsala, Sweden), 0.2 µM oligo Uni1 primer (5' AGCAAAGCAGG 3') (Oligo Express, Paris, France) [19], 0.3 U/µl Rnasin (Promega, Madison, WI, USA) and 0.5 U/µl AMV reverse transcriptase (Promega, Madison, WI, USA). The reaction mixture was incubated for 1 hour at 42°C and 15 minutes at 55°C. Samples were heated to 95°C for 5 minutes. The cDNA (2 µl) was then amplified in a 50 µl volume containing 1 x PCR buffer (Perkin Elmer Cetus, Norwalk, CT, USA), 0.2 mM 4 dNTP mix (Pharmacia biotech, Uppsala, Sweden), 1.5 mM $MgCl_2$ (Perkin Elmer, Cetus, Norwalk, CT, USA), 0.025 U/µl Taq polymerase (Perkin Elmer, Cetus, Norwalk, CT, USA), and 0.5 µM of each primer : for the H1 subtype, forward primer H1/5/+ (5'AAA GCAGGGGAAAATAAAAACAACC 3'), and reverse primer H1/1117/- (5' ATCATTCCAGTCCATCCCCCTTCAAT 3'), for the H3 subtype, forward primer H3/6B/+ (5'AAGCAGGGGATAATTCTATTAACC 3') and reverse primer H3/1197/- (5'CTGCTTGAGTGCTTTTAAGATCTG 3'), for type B, forward primer BHA/86/+ (5'GAAGGCAATAATTGTACTACTC 3') and reverse primer BHA/1235/- (CTCTTAAGGT CTGCTGCCAC TG) (Genset, Paris, France). Samples were subjected to one cycle for 5 minutes at 94°C, followed by 30 cycles of 45 seconds at 94°C, 1 minute 30 seconds at 72°C and 5 minutes at 72°C (Stratagene Robocycler Gradient 96, La Jolla, CA, USA). The HA1 PCR products that were generated were (1112 bp long) for H1 subtype, (1191 bp long) for H3 subtype and (1149 bp long) for B type. Nucleotide sequences were determined using the Big Dye Terminator sequencing kit (Perking Elmer, Cetus, Norwalk, CT, USA) and the ABI Prism 377 sequencing system (Perking Elmer Cetus, Norwalk, CT, USA). Primers were used at 0.5 µM per 25-50 ng template DNA. For H1 sequencing we used forward primers: H1/5/+, H1/360/+ (GGGTATTTCGCCGACTATGAGG), H1/682/+ (CTGTAGTGTCTTCACATTATAGC) and reverse primers: H1/793/- (GGTTCCAGCAGAGTCCAGTAGTA) and H1/1117/-. For H3 sequencing we used forward primers: H3/6B/+, H3/361/+ (GCAACTGTTACCCTTATGATGTG) and reverse primers H3/941/- (GCTTCCATTTGGAGTGATGC) and H3/1197/-. For type B we used forward primer: BHA/403/+ (AATCTTCTCAGAGGATATGAA) and reverse primer: BHA/961/- (GGCAATCTGCTTCACCAATTAAAGG) (Genset, Paris, France). Data analysis was done using the Mac Molly complign program (Softgene GmbH, Berlin, Germany). Phylogenetic analysis was performed based on the alignment produced by the clustal X program, using the neighbour-joining (NJ) method [20] and the tree was visualized with Tree-View (Roderic D.M. Page Institute of Biomedical and Life Sciences, University of Glasgow, Scotland, UK).

Results

Clinical and epidemiological data

Among the practice-specific indicators collected

Figure 1. Percentage of Acute Respiratory Infections per consultation (%ARI/cons) as seen by paediatricians (PPs) and general practitioners (GPs) during the 1996/97 season and influenza isolates during the 1996/97 and 1997/98 seasons.



by the sentinel practitioners, the percentage of ARIs per consultations (% of ARI/Cons) was most informative. As shown in Figure 1, the percentage of ARIs/Cons seen by the GPs increased sharply from week 45 in 1996 to peak at week 51 in 1996. The percentage of ARIs/Cons in children as observed by paediatricians was already high at the beginning of the reporting period (week 45 in 1996). In both cases, the percentage of ARI/Cons declined quickly after reaching its peak to "baseline-like" levels from weeks 2-3 in 1997 until the end of the reporting period.

During the 1995 through 1998 seasons, 673 nasopharyngeal swab specimens were collected from patients with ILI (110 during 1995/96, followed by 228 during 1996/07, and 335 during 1997/98 [Table 1]).The patients' mean age (and range) was 20 (0.08-80) years. During the 1996/97 season, 44 (19%) IVs were isolated from patients sampled between weeks 43 in 1996 and 4 in 1997, coinciding chronologically with the increased levels of ARI/Cons for both populations seen by GPs and PPs. During the 1997/98 season, 55 (16%) IVs were isolated between weeks 47 in 1997 and 5 in 1998, showing seasonality with peaks occurring between December and February.

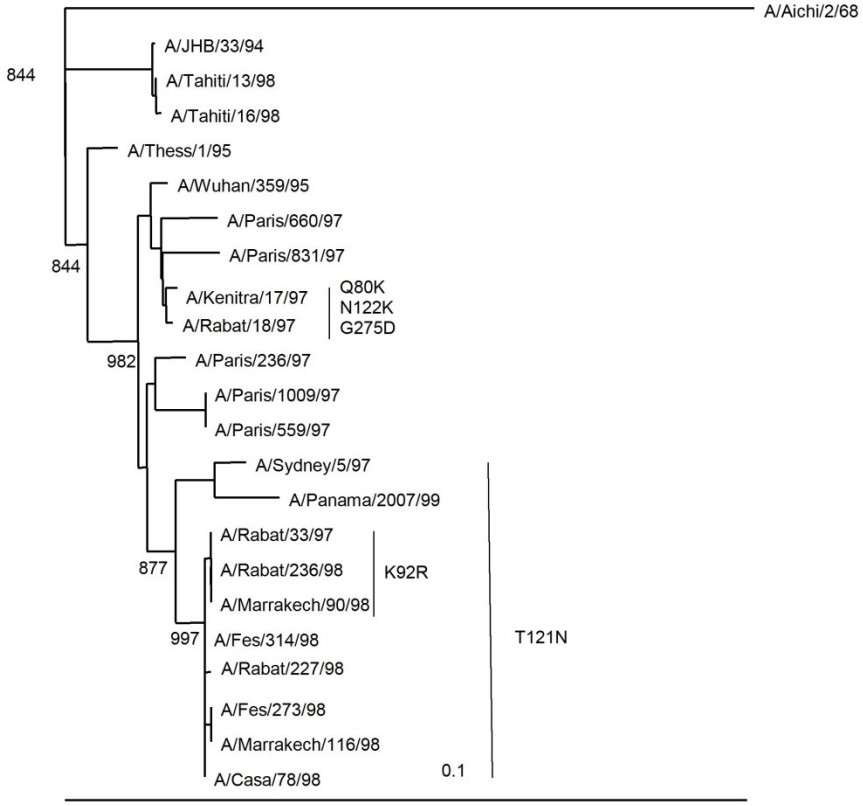
Antigenic and genetic characterization of Influenza virus isolates

From the 110 specimens collected during the 1995/96 season, two A(H3N2) and six influenza type B viruses were isolated (Table1). Of the 44 viruses isolated during the 1996/97 season, 34 (77%) were type A viruses (10 H1N1 and 24 H3N2) and 10 (23%) were type B viruses. During the 1997/98 season, 55 viruses were isolated, all of which belonged to the H3N2 subtype (Table 1).

For the antigenic and genomic studies, 29 viral strains were selected from different Moroccan cities based on the time of sample collection and the geographical location of the original sample: 11 strains in 1996/97 (3 H3N2, 5 H1N1 and 3 influenza type B) and 18 H3N2 in 1997/98.

Antigenic analysis of the H3N2 viruses isolated during the 1996/97 season showed that they were antigenically related to the A/Nanchang/933/95 vaccine strain [21] (Table 2a). Two of them, A/Kenitra/17/97 (Ken17) and A/Rabat/18/97 (Rab18), were genetically related to A/Wuhan/359/95 (H3N2), an A/Nanchang/933/95 (H3N2)-like strain according to phylogenetic analysis of the Hemeagglutinin (HA) gene (Figure 2). These Moroccan strains harbour three specific amino acids, K80, K122, and D275. Although the A/Rabat/33/97(Rab33) was sampled one week later (46/1996) than (Ken17) and (Rab18), its HA is genetically clustered in a group of viruses characterized by residues S124 and A196 that includes the A/Sydney/5/97 vaccine strain [22]. Among the 1997/1998 A(H3N2) isolates, sixteen were antigenically closely related to A/Nanchang/933/95 (H3N2) [23] and showed a 4- to 16-fold reduction in HI titer with the A/Sydney/5/97 (H3N2) as compared to the A/Nanchang/933/95

Figure 2. Phylogenetic tree of the HA1 domain of the HA gene of the Moroccan influenza A (H3N2) viruses, based on nucleotide sequences (Nt 1 to 980). The tree was constructed using the Clustal V program. Branch lengths (horizontal lines) are proportional to the number of nucleotide changes.



Reference and Genbank accession numbers for the Moroccan sequences used: A/Kenitra/17/97 (DQ534420), A/Rabat/18/97 (DQ534421),A/Rabat/33/97 (DQ534422), A/Rabat/236/98 (DQ534423), A/Marrakech/90/98 (DQ534424), A/Fes/314//98 (DQ534425), A/Rabat/227/98 (DQ534426), A/Fes/273/98 (DQ534427), A/Marrakech/116/98 (DQ534428), A/Casa/78/98 (DQ534429). The strains A/Paris/1009/97, A/Paris/559/97/, A/Paris/660/97, A/Paris/831/97, A/Paris/236/97, A/Paris/90/97, A/Paris/907/97 were isolated in Paris, A/Tahiti/013/98 and A/Tahiti/016/98 were isolated in Tahiti.

(H3N2) ferret serum. As shown in Figure 2, genetically, the HA1 sequences from all Moroccan 1997/98 H3N2 isolates as well as from Rab33 form a group clustered with A/Sydney/5/97 and A/Panama/2007/99, characterized by residue N121.

The Moroccan group is subdivided into two subgroups, one including Rab33 from the 1996/97 season, A/Marrakech/90/98 and A/Rabat/236/98 from the 1997/1998 seasons, and the other including most viruses. The difference between the two subgroups corresponds to a characteristic K92R substitution. The HI data do not support the genetic profile of the 1997/98 Moroccan isolates since their HA is antigenically closer to A/Nanchang/933/95, an A/Wuhan/359/95-like strain, than to A/Sydney/7/95.

Of the 18 strains tested for NA activity, seven (39%) did not display any activity on sheep erythrocytes, resulting in no antigenic data concerning their NA. In contrast to the HA, the NA was antigenically more closely related to A/Sydney/5/97 than to A/Nanchang/933/95, although results are significantly different between Sydney/5/97 and Nanchang/933/95 only for four strains (4-fold difference in titer). Among the eight type A viruses isolated in 1996/97, five were of the H1N1 subtype: A/Kenitra/15/97 (DQ534415), A/Rabat/10/97 (DQ534416), A/Kenitra/20/97 (DQ534417), A/Rabat/134/97 (DQ534418), and A/Rabat/137/97 (DQ534419), and were antigenically related to A/Singapore/6/86 (data not shown). Their HA1 sequences were genetically related to A/Bayern/7/95 (H1N1), the prototype vaccine strain for the corresponding season. They differ from A/Bayern/7/95 by a V57I mutation, similar to other strains isolated elsewhere in the world during the same period.

During the 1996/97 season, 10 influenza B viruses were isolated; in the HI test, B/Rabat/41/97 (DQ534413) reacted equally with ferret sera directed

towards B/Beijing/184/93, B/Nanchang/451/93, B/Johannesburg/26/94 and to a lesser extent with the serum directed towards B/Panama/45/93. Isolate B/Rabat/45/97 (DQ534414) was clearly antigenically distinct from B/Johannesburg/26/94 and B/Panama/45/93 and more closely related to B/Nanchang/451/93. As for isolate B/Rabat/61/97 (DQ534412), it was antigenically related to B/Beijing/184/93 (Table 2b). The phylogenetic analysis of their HA1 sequences showed that they were genetically clustered with B/Yamanashi/166/98, the vaccine prototype strain corresponding to the Yamagata lineage (data not shown). The HA1 of these isolates harboured the G183E substitution that distinguishes B/Yamanashi/166/98 from older strains such as B/Panama/45/90 and B/Harbin/7/94.

Discussion

A network of volunteer sentinel physicians based on laboratory influenza surveillance was established in Rabat city during the 1995/96 season. Given the success of this experiment, the network was expanded to other cities and the clinical data was integrated into the virological analysis during the 1996/97 season; epidemiological and clinical data were recorded by GPs and PPs. The percentage of ARI/Cons provided valuable information, showing that the peak of the percentage of ARI/Cons was reached in paediatricians' practices slightly earlier (about two to four weeks) than in GPs' practices and coincided with the isolation of IVs, as commonly observed in comparable networks in Europe. Although the number of paediatricians in the network is about half of that of GPs, there were more isolates from them than from GPs. Some indicators of medical activity such as ARI per home visit or the number of home visits (data not shown) did not give any interpretable data, most likely because home visits by GPs and PPs are rare in Morocco, unlike in some countries such as France. Unfortunately, data on medical activity could not be collected beyond the 1996/97 season because of fears from private doctors to provide information about their practice. Given the chronological coincidence between percentage of ARI/Cons and IVs, the raw number of IVs can be used not only for virological purposes but also for epidemiological surveillance as they indicate the start, peak and end of IV circulation in the respective cities. A sustained, well-organized community-based network focused only on virology, albeit not ideal, thus probably suffices for the surveillance of influenza as a default option.

The results of the present study suggest a seasonal circulation of IV in Morocco concentrated between November and February during the 1996/97 and 1997/98 seasons. As expected, ARIs were reported in all age groups, showing a peak of incidence during the winter season from December 1996 through to January 1997. The overall incidence of ILIs, shown by the specific clinical indicators recorded for each patient, which coincided with ARIs at the time samples were collected for IV isolation, shows an annual incidence of influenza, justifying the use of yearly vaccination as is routinely done in Europe and North America. In 1997, most viruses isolated were A(H1N1), whereas in the following season the vast majority were A(H3N2), most closely resembling A/Nanchang/933/95(H3N2) antigenically but genetically more related to A/Sydney/5/97(H3N2). Although Moroccan strains of influenza A and B viruses were genetically and antigenically comparable to those circulating in the rest of the world, their HA formed separate clusters of very closely related sequences. The Rab33 strain, although it matched antigenically (by HI test) with the vaccine strain, carried an HA genetically related to the prototype strain A/Sydney/5/97 (H3N2), which was only detected in Australia seven months later [22], leading to a mismatch for the H3N2 strain in the 1997/1998 vaccine. This particular example illustrates the importance of including the maximum number of countries in the active surveillance of influenza orchestrated by the WHO. This is even truer in Africa, especially northern and sub-Saharan Africa, where data is still limited and punctual.

The influenza surveillance system has provided valuable data on characteristics of influenza seasonality and circulating viruses. The data obtained in these first three years highlight the importance of the clinical, epidemiological and virological network of influenza surveillance as it permits the monitoring and the progression of the illness over time, as well as the rapid identification of potential epidemics and early isolation and characterization of new circulating virus strains. With further consolidation and accumulation of surveillance data, this network will have a valuable role in the prevention and control of influenza epidemics in Morocco.

Well-organized influenza surveillance networks are severely lacking in Africa. The results of this study could also help African countries develop and implement influenza surveillance networks.

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