
Different diagnostic methods for detection of influenza epidemics

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SUMMARY

Linking continuous community-based morbidity recording of influenza-like illness (ILI) with virological sampling has consistently proved its value as one of the earliest indicators of circulating influenza activity. The clinical morbidity recording in the Portuguese national surveillance network, during a 7-year period, and the contribution of different diagnostic techniques, including virus isolation, multiplex RT-PCR, immunocapture enzyme linked immunoassay (EIA) and complement fixation tests (CFTs) for the detection of influenza in such a community-based setting is described and evaluated in this study. There was good correlation between the increase of morbidity, total samples taken and the detection of influenza virus by all the methods although this was less evident for virus isolation and EIA than for RT-PCR or serology. From a total of 1685 throat swabs collected from cases of ILI, 43.6% were RT-PCR positive, 17.5% were positive by capture EIA and in 5% virus isolates were made. The detection of influenza by RT-PCR occurred earlier than by any other method and showed the best correlation with epidemic patterns of morbidity registration. We conclude that in surveillance systems where virus culture is sub-optimal, RT-PCR provides a rapid, sensitive, specific method for detecting influenza viruses from community-based sampling.

INTRODUCTION

Influenza infections are an important cause of respiratory disease worldwide. In the northern hemisphere influenza viruses circulate predominantly through the winter months from October to March. The period of influenza virus circulation is associated with increased consultation with medical practitioners [1, 2], hospitalizations [3] and excess deaths [4, 5]. There is a significant economic and public health impact associated with each annual influenza epidemic. This emphasizes the requirements for rapid detection and evaluation of clinical, epidemiological and virological information about influenza epidemics as they unfold.

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The impact of influenza is seen initially in primary care as an increase in GP consultation rate, and subsequently as an increased demand for hospital admissions. Therefore, information from primary care settings can contribute significantly to understanding and predicting the impact of influenza epidemics within a short period of its onset. National sentinel surveillance systems have been established in a number of countries, including Portugal, and a significant improvement in the specificity of clinical morbidity recording has come from linking clinical recording with virological surveillance for influenza [6].

We describe here clinical morbidity recording in the national sentinel network of physicians in Portugal and evaluate the contribution of different diagnostic

techniques, including virus isolation, multiplex RT-PCR, immunocapture ELISA and complement fixation tests (CFTs) for the detection of influenza in such a community-based setting.

MATERIALS AND METHODS

Clinical data

Data on the cases of influenza and influenza-like illnesses (ILI) were collected throughout the year by 202 general practitioners (GPs), who formed part of a national network, distributed throughout the country, and covering approximately 170 000 people, representing 1.8% of Portuguese population. Sentinel GPs were recruited on a voluntary basis thus uneven distribution (urban/rural) of practices is a feature of the system. All new episodes of illnesses were recorded, indexed, and reported weekly to the General Directory of Health to provide incidence rates, of new episodes per 100 000 population. Case definition of influenza were based on those published in the International Classification for Health Problems in Primary Care. A subset of 50 (25%) GPs also take part in virological monitoring as previously described [7].

Clinical specimens

Nasopharyngeal swabs were taken within 4 days of onset of symptoms and whenever possible acute and convalescent blood samples were also collected. On arrival in the laboratory, the swabs were placed into 2.5 ml tryptose broth supplemented with 0.5% gelatin and treated with antibiotics (penicillin 1 IU, neomycin 50 µg/ml, streptomycin 1 IU, amphotericin 50 000 IU). This suspension was then used immediately for procedures described below, or aliquots stored for subsequent analysis at -70°C .

Virus isolation

Clinical specimens were inoculated into Madin-Darby Canine Kidney (MDCK) cells in 24-well plastic plates (Greiner) and centrifuged at 300 g for 30 min. Cells were maintained post-inoculation in serum-free Minimal Essential Medium (EMEM, Gibco) with 1.25 µg/ml of TPCK-treated trypsin. After an incubation period of 3–7 days, cell supernatants were evaluated for the presence of influenza virus by

haemagglutination (HA) assay using guinea-pig, turkey or chicken erythrocytes (0.5% v/v).

Chick embryos, 10–11 days old, were inoculated according to standard procedures [8, 9].

Immunocapture enzyme linked immunosorbent assay (Capture EIA)

An immunocapture EIA was used for detection of influenza A and B nucleoprotein (NP) antigens as described previously [10].

Detection of viral RNA

A reverse transcriptase multiplex PCR (RT-PCR) was used for typing and subtyping influenza virus in clinical specimens as described and validated previously [12]. Viral RNA was extracted from 150 µl of original sample using guanidinium thiocyanate. For detection of influenza A H1 and H3 or influenza B viruses we used nested primer sets (5 pmol in the first amplification and 25 pmol in the second amplification) and cycling conditions as previously described [11].

Serological diagnosis

Complement fixation tests were used for the serological diagnosis of influenza virus [9]. The test was performed only on paired samples (acute and convalescent). A positive case was defined as either demonstrating a seroconversion or a fourfold increase in antibody titre from an acute serum to a convalescent serum.

Statistical analysis

Sensitivity was evaluated for the three diagnostic methods (isolation, EIA and RT-PCR) by year and overall years using positive result by any test as the gold standard. Statistical analysis of the agreement between each pair of diagnostic methods was carried out using Cohen's Kappa statistic [12].

RESULTS

Epidemiology

The weekly clinical incidence rate of ILI during a 7-year period, derived from the Portuguese National

Sentinel Network, is shown in Figure 1. During this period the clinical morbidity registration, using the value approximated for baseline [7] indicated, elevation above baseline and epidemic peaks in 6 of the 7 years. Epidemics lasted between a minimum of 6 to a maximum of 14 weeks exemplified by 1994–5 and 1996–7, respectively. During the seven winters surveyed, circulation of influenza B virus exhibited a roughly biennial appearance, although not always as the predominant type (Fig. 1). Epidemic activity due to influenza A virus was observed in 5 of the 7 study seasons (1993–4, 1995–6, 1996–7, 1997–8 and 1998–9) and influenza B virus was prevalent in 1992–3 and 1994–5.

In general there was no consistent pattern between the predominant virus circulating and the duration or magnitude of each seasonal epidemic; however peaks of clinical incidence appeared to occur earlier in the winter when influenza A predominated.

Virological detection of influenza

A total of 1685 throat swabs were collected by participating GPs from cases of ILI between 1992 and 1999. All samples were tested for influenza virus by culture, capture EIA and by multiplex RT–PCR (Table 1). A total of 86 (5%) virus isolates were made from the 1685 samples taken over the course of the 7 winter seasons; the majority 79 (91.9%) of these were influenza B isolates, 6 (7%) were influenza A H3N2 and 1 (1.1%) H1N1. No virus isolates were obtained in 1993–4 and 1995–6, years when influenza B virus was not circulating (Table 1, Fig. 1), indicating that the culture systems in the laboratory were biased in favour of influenza B.

A total of 295 (17.5%) samples were positive by capture EIA and 734 (43.6%) were RT–PCR positive. The percentage of positive samples detected by EIA in each year was consistently within the 15–24% range, whereas detection by RT–PCR was higher and ranged between 27 and 55% year to year, with exception of 1992–3 where a very low rate of positive samples were detected by either method (Table 1).

Diagnosis during epidemic periods

When the three different diagnostic methods were used to detect influenza virus during the seven seasons, it could be seen that the best correlation of virus

detection with clinical activity and the total number of samples taken, was obtained by RT–PCR. For all winter seasons the first confirmed detection of circulating influenza was obtained by PCR, usually well ahead of the rise of clinical morbidity registration by several weeks (Fig. 1). The number of samples collected and virus detection by capture EIA and PCR were analysed for each year and differentiated according to whether clinical morbidity registration was considered to be above baseline or outside the peak periods (Table 2). As expected, there was a correlation between the increase of morbidity, total samples taken and the detection of influenza virus by all methods although this was less evident for EIA data than for RT–PCR. For all 7 years RT–PCR detected highest percentage of influenza virus during peak periods when compared with capture EIA; a maximum of 67.4% of influenza virus detection obtained in 1998–9 contrasting with the 24.8% obtained by EIA (Table 2).

Sensitivity and concordance of testing methods

Difficulties with virus culture data, means that it was only possible to evaluate the sensitivity of the three tests using positive result by any test as the gold standard. As expected, sensitivity of the three diagnostic methods (isolation, EIA, RT–PCR) were clearly different (Table 3). Overall sensitivity was 11.2% for isolation, 39.0% for EIA, and 96.9% for RT–PCR. These differences were consistent over the years (Table 3). The agreement between each pair of tests showed a highly statistically significant agreement; giving the strong concordance between EIA and RT–PCR ($\kappa = 0.376$, approx. P value $\ll 0.001$).

Serology

The application of CFT serology for the surveillance of influenza was also evaluated (Fig. 2). As can be observed for 1993/4 or 1996/7 the paired serum samples collected showed excellent correlation with the clinical indices of influenza activity demonstrated in seasons when either influenza A or B virus were predominant. However, diagnosis of influenza infections was always delayed by an average of 2 weeks (Fig. 2) since the test cannot be performed until the two samples can be analysed in parallel. The percentage of positive results varied from a maximum of

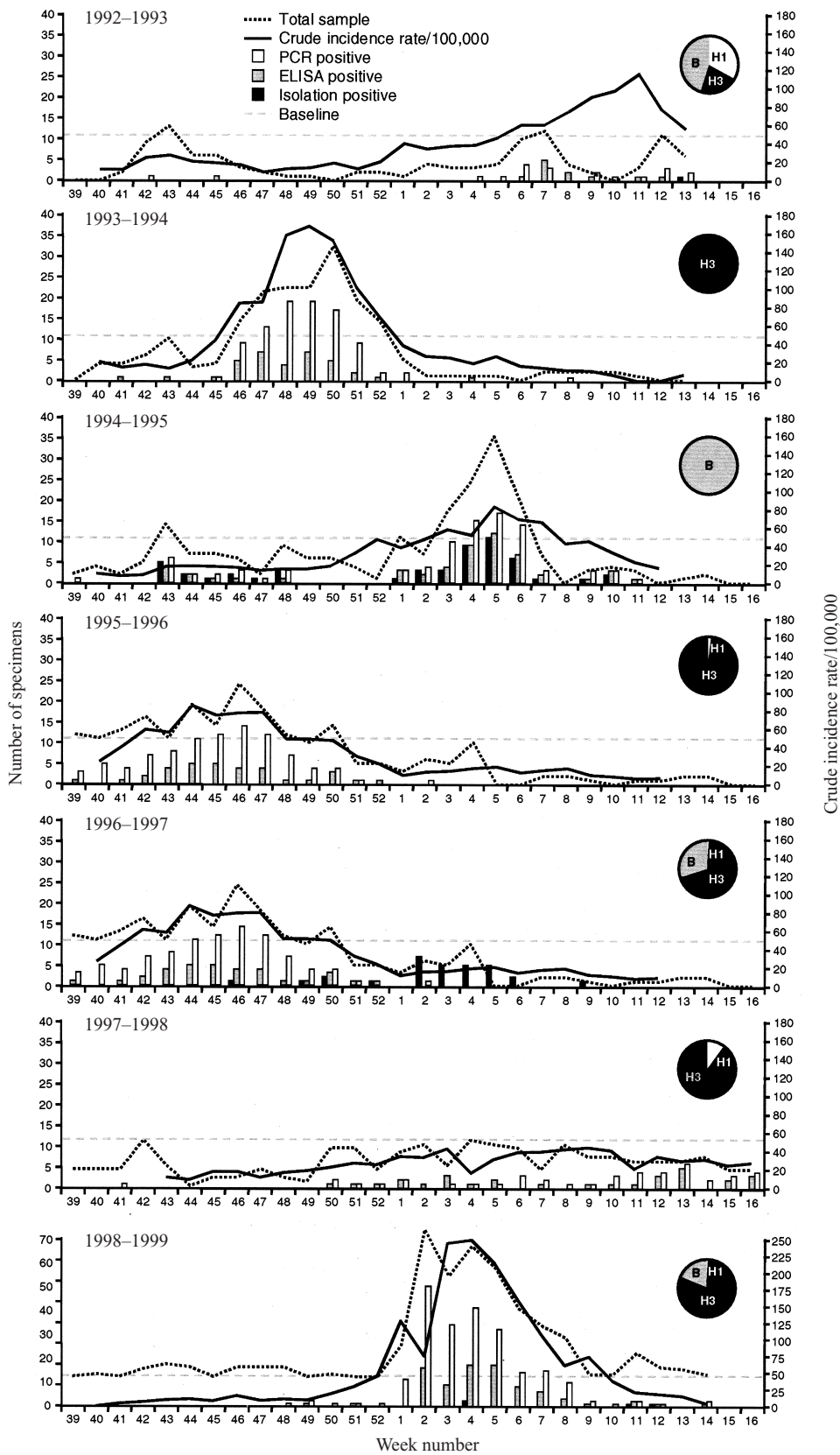


Fig. 1. Combined community-based clinical virological surveillance by winter season (1992/3 to 1998/9). The incidence rate for ILI per 100000 population is shown alongside the number of samples taken from individuals presenting with ILI in sentinel general practices and the number of influenza isolations, detections by EIA and RT-PCR. The proportion of different types and subtypes of influenza virus circulation in Portugal in each season is summarized by pie chart.

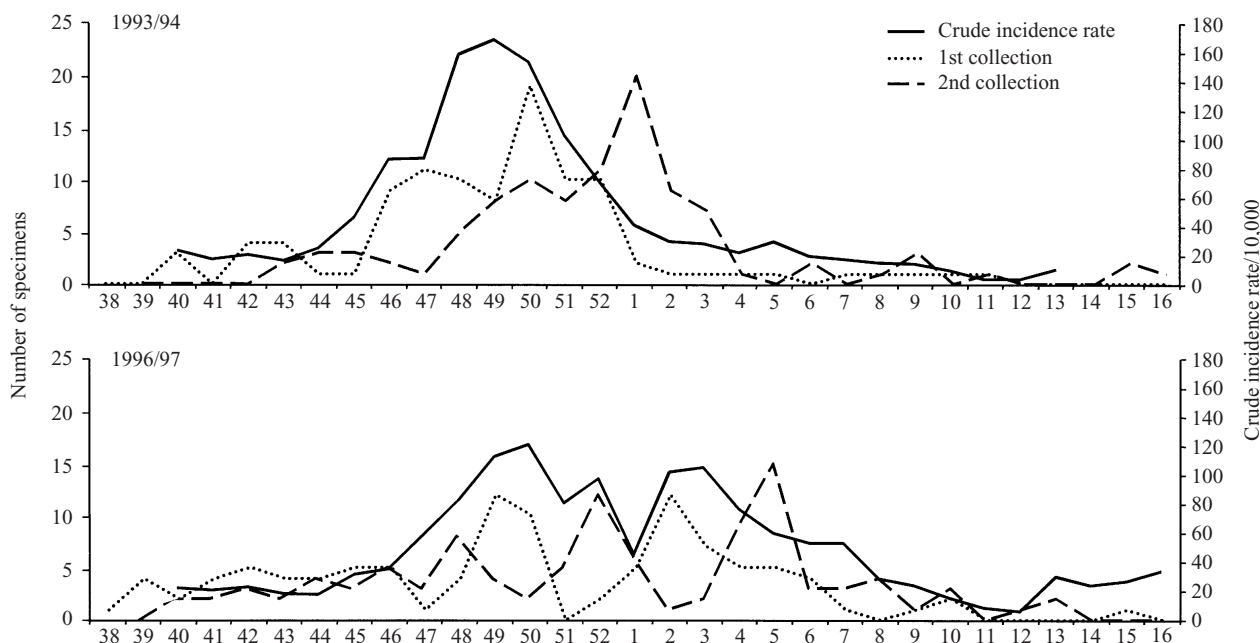


Fig. 2. Incidence rate of ILI by week and of collection of serum samples. Incidence rate of ILI per 100000 population is displayed against week number and shown alongside the paired blood samples displayed by the onset of the symptoms and the week of second blood collection.

Table 1. Number of cases of ILI with diagnostic sampling during winter seasons of 1992–9

Winter season	Total no. cases*	Predominant type	Isolation positive %	EIA positive %	PCR positive %	Serology positive %†
1992/3	111	B	0.9	8.1	10.8	26.3 (57)
1993/4	195	A(H3)	0	18.46	46.6	28.4 (102)
1994/5	213	B	23	24.8	44.1	37.5 (56)
1995/6	228	A(H3)	0	14.5	40.8	28.1 (57)
1996/7	297	A(H3)	10.1	16.1	54.5	56.6 (106)
1997/8	228	A(H3)	nd‡	14.5	26.8	12.8 (39)
1998/9	413	A(H3)	1	20.1	54	56.4 (94)
Total	1685		5	17.5	43.56	38.9 (511)

* Cases, number of patients with ILI who had a sample taken.
 † Total number of cases with paired samples for serology.
 ‡ nd, not done in that season.

56.6 in 1996–7 to a minimum of 12.8 in 1997–8 and did not correlate with the type of virus circulating (Table 1).

DISCUSSION

Combined community-based clinical-virological surveillance of influenza is a powerful tool for detailing the seasonality, duration and magnitude of annual influenza epidemics [13]. Linking continuous community-based morbidity recording of ILI with virological sampling has consistently proved its value in Portugal as one of the earliest indicators of circulating

influenza activity. Virological sampling ensures specificity of a surveillance system and the application of molecular diagnostic techniques such as RT-PCR for the detection of respiratory viruses may also have an impact on the quality of surveillance data [14]. The exact impact is likely to depend on the sensitivity of systems already in place. In countries where virus culture is suboptimal, RT-PCR is likely to be able to improve the speed of detection as well as the number of cases detected, whereas in countries with well developed culture-based surveillance, molecular approaches may seem rather poor value. The difficulties and problems of virus culture from primary clinical

Table 2. Comparison of positive results by RT-PCR and capture EIA according to epidemic periods

Winter season	Total no. cases*	No. of cases sampled at peak†	No. of cases sampled outside peak	PCR positive % sampled in peak	PCR positive % sampled off peak	EIA positive % sampled in peak	EIA positive % sampled off peak
1992-3	111	48	63	31.3	4.8	25	1.6
1993-4	195	144	51	61.1	7.8	21.5	5.9
1994-5	213	104	109	56.7	32.1	32.7	17.4
1995-6	228	138	90	57.2	15.5	21	4.4
1996-7	297	216	81	60.2	39.5	18.5	9.9
1997-8	228	0	228	0	26.2	0	14.5
1998-9	413	310	103	67.4	11.7	24.8	5.8

* Cases, number of patients with ILI who had a sample taken.

† Peak period defined by use of baseline [7].

Table 3. Sensitivity* of diagnostic tests (culture/EIA/RT-PCR)

Year	No. cases†	Positive result by any test	Sensitivity (%)		
			Isolation	EIA	RT-PCR
1992/3	111	27	3.7	44.4	66.7
1993/4	195	96	0	37.5	95.8
1994/5	213	97	52.6	54.6	96.9
1995/6	228	94	0	35.1	98.9
1996/7	297	165	18.2	29.1	98.2
1997/8	228	65	0	51.6	95.3
1998/9	413	222	1.8	37.4	99.5
Total	1685	766	11.2	39.0	96.9

* Using positive result by any test as gold standard.

† Cases, number of patients with ILI who had a sample taken.

material are amply demonstrated in this study. It is of some interest to note that the ability to perform virus isolation relates to the antigenic type of the circulating virus. Influenza B virus accounted for the vast majority of isolates made from nasopharyngeal swabs from community cases of ILI, either in tissue culture or in eggs. During the years represented in this study, circulating influenza B virus showed relatively little antigenic drift, however this was not the case for circulating influenza A epidemic variants. Factors which may have contributed to the low rate of virus isolation, include the type of swab collection device [15], the virus transport medium [16], the passage history of the mammalian cells used [17, 19] and the physiological stability of protease supplement to media. There are also several studies which have demonstrated alteration of receptor-binding properties of natural isolates of influenza A H3N2 and H1N1 virus since the late 1980s ([20-22], Zambon, unpublished data), although the impact of these changes on the ability of isolates to grow in either embryonated eggs or mammalian tissue is not de-

scribed, and it is possible that alteration in the receptor binding properties of influenza viruses circulating in Portugal during this period may have contributed to failure to grow [23-25], although this property of influenza virus is unlikely to account for the gross failure to detect influenza A virus isolates over several seasons. With poor virus isolation, comparative data in several different settings are needed to estimate the value and role of the different diagnostic techniques in surveillance, particularly at the beginning of each influenza season.

In this study, the detection of influenza by RT-PCR occurred earlier than by any other method in all the years studied, and consistently showed the best relation with epidemic patterns of morbidity registration. Positivity rates of samples during the peak weeks of epidemic season was between 56 and 67% (Table 2), which concurs with previous studies [11, 26]. Accurate typing and subtyping of influenza virus in clinical samples was available very rapidly and the products of the RT-PCR amplification reactions were suitable for further molecular analysis, including

sequencing and PCR restriction analysis [27]. The use of capture EIA has been described in surveillance work [28]. In the Portuguese surveillance system, the results of EIA showed a marked correlation with the clinical incidence data, although the relationship to total numbers of samples taken was less evident in some years (Fig. 1). Compared with RT-PCR, EIA is clearly less sensitive (Table 3), although we obtained good concordance between the two tests ($\kappa = 0.376$). The first EIA detection was between 2 and 12 weeks before the peak of clinical activity, and usually within 1–3 weeks of the first PCR detection. Thus EIA is a useful detection method for enhancing the specificity of clinical morbidity registration, although the sensitivity of this method for early warning of influenza strains may be less than optimum. In this instance, the monoclonal antibodies used in the EIA did not allow subtyping of the influenza A virus. However, such monoclonal antibodies have been described, and could potentially be used in subtyping EIA assays [29]. Despite these caveats, EIA is a cheap diagnostic method for surveillance, and is easily automatable, allowing a rapid throughput of nasopharyngeal samples.

The application of complement fixation (CFT) serology to influenza surveillance in a national surveillance system is demonstrated across 7 years. CFT titre rises in paired sera obtained from some of the cases presenting with ILI corresponded well temporally with the peaks of clinical morbidity registration. However, rises in CFT titre in individual patients lacks sufficient sensitivity to be used as an early warning indicator, as there is a requirement for paired sera taken at least 10 days apart, which inevitably delays the analytical antibody titre determination. Moreover, it is often difficult to obtain the second convalescent blood sample in adults as this requires an unnecessary physician visit. The use of paired CFT titres for surveillance is also heavily biased towards the adult population because invasive procedures in young children are hard to justify.

Overall, RT-PCR fulfils requirements for public health planning and early warning in community surveillance, and proved to be a powerful tool for large scale surveillance work when applied systematically to a national surveillance system. Nevertheless, virus isolation remains the cornerstone of global surveillance and is essential for ensuring the most appropriate match between circulating influenza strains and vaccine composition. PCR diagnosis cannot replace this essential surveillance function.

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