

## Selected Laboratory Aspects of Influenza Surveillance

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The importance of virologically documented infections in influenza surveillance is well recognized and has been reaffirmed in recent reviews. The large number of specimens tested in surveillance make efficiency and low cost of virologic methods important. Based on observations made by others and our work with reisolation of stored specimens we have used the continuous line tissue cultures MDCK and LLC-MK2 for virus isolation in large-scale influenza surveillance studies for three years. Both cell lines were equally successful in detecting influenza A viruses in 77 fresh, virus-positive specimens. However, during the influenza B outbreak of 1979-80, of 473 specimens positive in either or both tissue cultures, 54 were positive only in MDCK and just six in LLC-MK2 only. For parainfluenza viruses, LLC-MK2 was much superior to MDCK. The most promising alternative to tissue culture at this time, based on a review of the literature, appears to be enzyme immunoassay. Sensitivity sufficient for direct detection of viral antigen in routine specimens currently requires fluorescent or radioactive substrates. Identification of early virus growth in continuous cell line cultures by enzyme immunoassay is practical now and can be considered.

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Community- and family-based surveillance for the presence of influenza viruses has been performed in Houston, Texas, since 1974 [1]. Some of the issues addressed in this work have been the impact of influenza-associated illness in the community and family [2,3,4], the predictability of epidemics [5], antigenic variation in influenza viruses [6], the frequency and consequences of reinfection [7], and co-circulation of different influenza A subtypes.<sup>1</sup> The importance of surveillance based directly on documented infections was recently reviewed [2].

For community surveillance in Houston influenza infections have been identified by virus isolation. Even in family surveillance where sera are obtained before and after each winter season, association of illness with specific infection and identification of the infecting organism has required virus isolation. In both circumstances, the virus must be available for antigenic and genetic analysis of virus variants. A large number of specimens are tested in surveillance due to the large number of persons sampled in the community and the intensive frequent sampling in the families. Thus, improvements in the cost, rapidity, reliability, and labor required in the techniques used to detect and identify influenza viruses present in respiratory secretions of infected persons are of great importance.

The standard tissue culture method of identification of influenza viruses in the

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respiratory tract has been growth of the agents in primary rhesus monkey kidney tissue cultures [8]. Inoculation of eggs is an alternative method [8]. Primary monkey kidney tissue is expensive, of uncertain availability in quantity, cannot be passaged in the laboratory to a useful extent, may show variation in sensitivity between primary lots, and may contain adventitious simian agents. It may still be best suited for virus isolation in a diagnostic laboratory if a wide range of agents is being sought. However, the disadvantages noted led us to consider alternate techniques for use in influenza surveillance. The first option we considered was substitution of continuous line tissue cultures for primary monkey kidney.

The Madin-Darby canine kidney cell line (MDCK) [9] was shown to be susceptible to infection with influenza viruses if trypsin was present in the medium [10,11]; this was followed by reports that this tissue culture was an adequate substitute for rhesus monkey kidney for isolation of influenza from clinical specimens [12,13]. In addition, the rhesus monkey kidney derived continuous line, LLC-MK2, was found to be sensitive to sendai virus when the medium was supplemented with trypsin [14,15]. We compared these two continuous line tissue cultures to rhesus monkey kidney tissue for reisolation of stored clinical specimens [16] (Table 1) and also found that MDCK was an adequate substitute for primary tissue. LLC-MK2 looked useful as well.

Encouraged by our reisolation data and results from other laboratories, we implemented use of continuous cell lines for influenza surveillance in 1979 and have continued this approach through the 1981-82 season. Table 2 compares the isolation rates for MDCK and LLC-MK2 cells with primary inoculation of nasal wash or throat swab specimens collected and refrigerated as previously described [17]. Only specimens yielding virus are included. During 1979-80, all specimens were placed on both tissues but after that season only specimens from the family-based surveillance were tested on both cell lines. As can be seen in Table 2, there was a significant advantage to the use of MDCK cells, as compared to LLC-MK2, only with influenza B in 1979-80. Although the records of the specific specimens that were negative in LLC-MK2 were reviewed and they appeared to represent adequate tests, it should be noted that definite problems with quality of LLC-MK2 tissue were noted during the season and there may have been some unrecognized deficiencies as well. The difference between MDCK and LLC-MK2 has not been evident in subsequent years. It is possible that the findings indicate differences in the virus strains themselves.

Thus it would appear that for reliable influenza surveillance, MDCK cells are the better choice as a continuous line substitute for primary monkey kidney cells. LLC-MK2 is a good alternative and may actually be identical in sensitivity under

TABLE 1  
Reisolation of Influenza Viruses from Stored Clinical Specimens<sup>a</sup>

Influenza virus	No. positive/no. tested <sup>b</sup> (%) in		
	Rhesus monkey kidney	MDCK	LLC-MK2
A/Texas (H3N2)	35/53 (66)	32/38 (79)	39/52 (75)
A/USSR (H1N1)	36/77 (47)	39/64 (61)	36/78 (46)
B/Hong Kong	52/82 (63)	45/62 (73)	48/77 (62)

<sup>a</sup>Adapted from [16]

<sup>b</sup>Combined data from several experiments with each virus

TABLE 2  
Primary Isolation of Respiratory Viruses in MDCK or LLC-MK2 Tissue Cultures

Virus	Season(s)	No. specimens with virus isolate	No. (%) positive on:		
			MDCK only	LLC-MK2 only	Both
Influenza B	1979-80	473	54 (11) <sup>a</sup>	6 (1)	413 (87)
	<u>1981-82</u>	<u>40</u>	<u>8 (20)</u>	<u>5 (13)</u>	<u>27 (68)</u>
	1979-82	513	62 (12) <sup>a</sup>	11 (2)	440 (86)
Influenza A (H3N2)	1980-81	56	4 (7)	4 (7)	48 (86)
Influenza A (H1N1)	1980-81	12	1 (8)	0 (0)	11 (92)
	<u>1981-82</u>	<u>9</u>	<u>1 (11)</u>	<u>1 (11)</u>	<u>7 (77)</u>
	1980-82	21	2 (10)	1 (5)	18 (86)
Parainfluenza					
Type 1	1979-82	81	1 (1)	72 (89)	8 (10)
Type 2	1979-82	47	1 (2)	46 (98)	0 (0)
Type 3	1979-82	121	4 (3)	42 (35)	75 (62)
Respiratory syncytial	1979-82	85 <sup>b</sup>	2 (2)	5 (6)	0 (0)

<sup>a</sup>Significantly higher isolation rate on MDCK;  $p < .001$  by chi-square testing and analysis of proportions

<sup>b</sup>All positive on Hep-2 cells

ideal conditions. LLC-MK2 cells are more sensitive for detection of parainfluenza viruses (Table 2), but both MDCK and LLC-MK2 are far less sensitive than Hep-2 cells for detection of respiratory syncytial virus.

The use of MDCK cells in surveillance necessitates some attention to the early appearance and rapid progress of cytopathic effect (CPE) in this tissue and the early detachment of cells from the glass [12,13]. Supernatant fluids can be tested for virus by hemagglutination [12] or considered positive because of CPE only [13]. Identification can be accomplished by hemagglutination-inhibition directly on first passage supernatant fluids [13] if there is sufficient hemagglutinin present. We have used early hemadsorption and identification by indirect immunofluorescence [17] but frequently there are too few remaining cells even at two to three days and passage is required for fluorescence identification. This is an area in which enzyme-linked immunosorbent assay (ELISA) technology could contribute, as will be discussed below.

Isolation of virus, even with newer methods, is a costly and time-consuming task and alternative methods for surveillance would be welcome. The most promising methods at this time are direct detection of viral antigen in immunoassays, especially enzyme-linked immunosorbent assays or ELISA. We have not utilized ELISA in surveillance in Houston to date, but there is information in the literature relevant to future possibilities; there is some experience with direct detection of influenza viruses in respiratory secretions. Berg et al. [18] used three variants of ELISA based on colorigenic, fluorescent, and radioactive substrates in studying 126 nasal wash specimens from volunteers experimentally infected with influenza A/Victoria/3/75 (H3N2). The method using colorigenic substrate was not sensitive enough but the combination of the other two methods was similar in sensitivity to virus isolation for the first five days after infection and better on days six to eight. Each method alone,

however, was less sensitive than isolation until days six to eight when each was more sensitive than isolation. Sarkkinen et al. [19] compared direct immunofluorescence of cells in the clinical specimen (considered equivalent to isolation based on their previous data) with radioimmunoassay (RIA) and ELISA with colorigenic substrate in nasopharyngeal suction specimens from persons with influenza A/Texas/79-like (H3N2) infections. Of 41 positive for influenza A by direct immunofluorescence, all were positive by RIA, and all of the "selected" specimens tested by ELISA were also positive. These authors attribute their success with colorigenic substrate-based ELISA to the use of nasopharyngeal suction specimens and refer to unpublished results indicating lower sensitivity of their technique using nasal washes or throat swabs. Harmon and Pawlick [20] tested throat swab and nasal wash specimens isolation-positive for influenza A/Brazil/79-like (H1N1) virus from two seasons in Houston. Of 40 tested, 21 or 53 percent were positive in ELISA tests with colorigenic substrate.

Thus it appears that ELISA technology is not yet available for practical application to large-scale surveillance. Nasopharyngeal suction specimens present difficulties for collection by untrained persons at multiple surveillance sites and under differing conditions and the fluorescent and radioactive substrate techniques are still under development. In fact, because of the need to isolate the virus in at least a subset of cases to allow for antigenic and genetic analysis when indicated, it is likely that some component of virus isolation will always be needed for adequate influenza surveillance. When ELISA techniques are used, residual specimen will have to be preserved for later isolation.

While awaiting full implementation of newer techniques, hybrid systems can be considered. Under development in our laboratory [Harmon M, unpublished data] is a system of identification of isolates by ELISA similar to the quantitative immunofluorescence technique recently reported by Phillips et al. [21]. All specimens will be inoculated into MDCK tissue cultures (or LLC-MK2) but supernatant fluids from specimens positive by CPE or hemadsorption will be identified by ELISA. An aliquot of this same supernatant will also be stored for further antigenic or genetic study if needed. One of the advantages of a hybrid system may be a higher frequency of virus reisolation from high-titer tissue harvests than from primary specimens that usually contain lower quantities of virus. So far, the ELISA has been as sensitive as indirect immunofluorescence for detecting and identifying virus in tissue cultures inoculated with actual clinical specimens. The problems of insufficient hemagglutinin and need for standardization of supernatants for hemagglutination inhibition identification and of obtaining intact cells for identification by fluorescence are eliminated. Results are available promptly and handling of virus-positive supernatants in the tissue culture areas are minimized.

In summary, the clear need for virologically based influenza surveillance programs [2] has stimulated consideration of efficient newer methods for isolation and identification of virus in respiratory secretions. The combination of continuous line tissue cultures and ELISA methods promises to provide the needed information more rapidly and at less cost than earlier techniques.

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