Electronic Supplementary Material (ESI) for Lab on a Chip.

Supplementary Figures

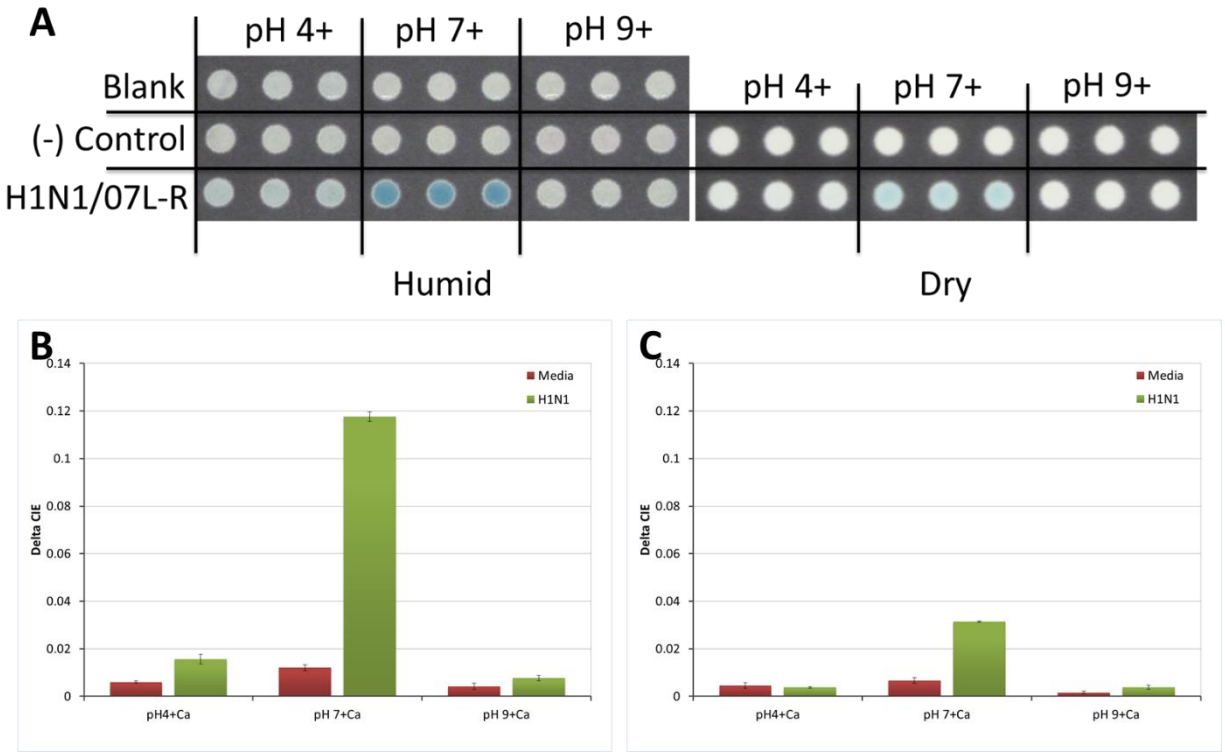

Figure S1. Comparison of Influenza Detection on Paper under Humid and Dry Assay Conditions. (A) Image of influenza neuraminidase activity assay under humidified and non-humidified (dry) assay conditions. Image analysis results (ΔCIE) of the influenza neuraminidase activity assay under (B) humid and(C) non-humid (dry) assay conditions. Specific H1N1/07L-R neuraminidase activity is observed at pH of 7 with 0.1 mM Ca+ present (pH7+).

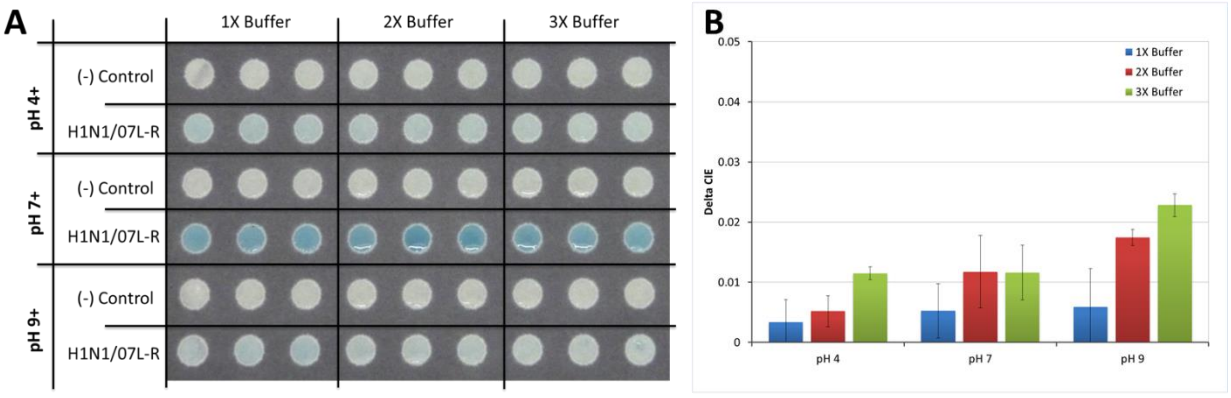

Figure S2. Comparison of Influenza Detection on Paper with Increasing Buffer Strength. (A) Image of influenza neuraminidase activity assay under 1X, 2X, and 3X buffer conditions. (B) Image analysis results (ΔCIE) of the H1N1/07L-R influenza neuraminidase activity assay with 1X, 2X, and 3X buffers at pH of 4, 7, and 9. All contained 0.1 mM Ca^{+2}.

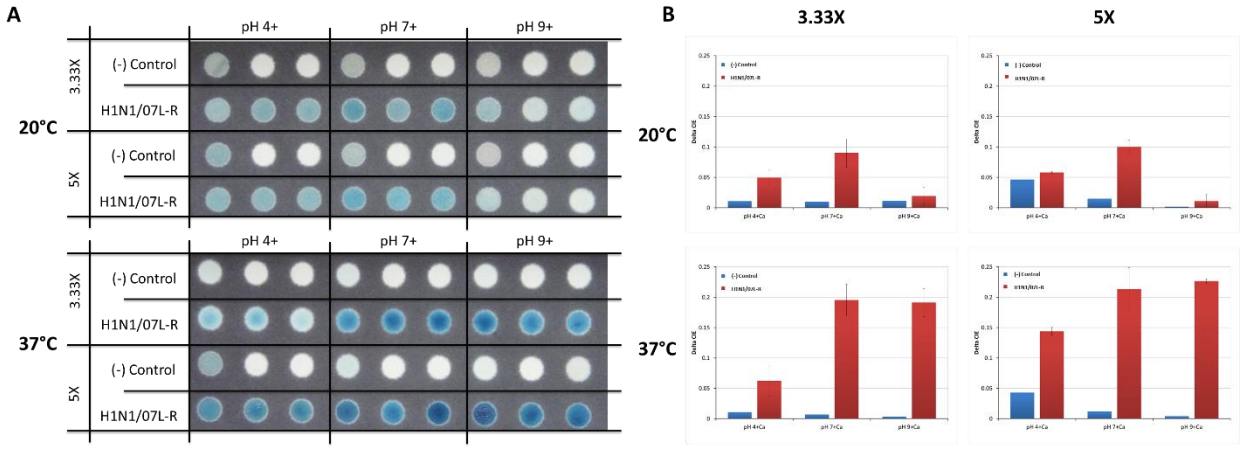

Figure S3. Comparison of Influenza Detection on Paper with Increasing Substrate and Increased Incubation Temperature. (A) Image of influenza neuraminidase activity assay after incubation at 20°C and 37°C, with substrate concentrations of 3.33X and 5X. (B) Image analysis results (ΔCIE) of the H1N1/07L-R influenza neuraminidase activity assay after incubation at 20°C and 37°C, with substrate concentrations of 3.33X and 5X and buffer pH of 4, 7, and 9. All buffers contained 0.1 mM Ca^{+2}.

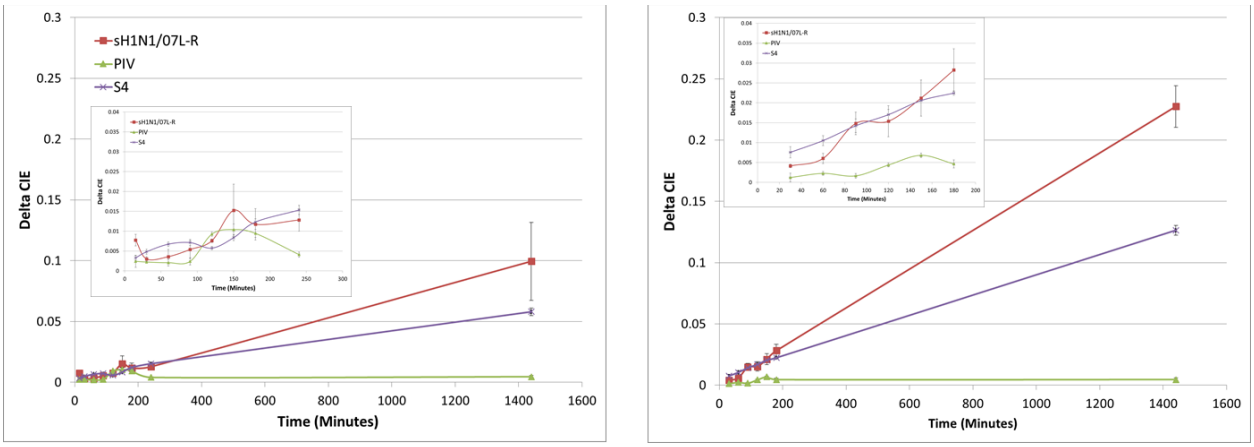

Figure S4. Time Course Studies for Determination of Minimum Assay Time at Different Incubation Temperatures. Neuraminidase activity of H1N1/07L-R, HPIV, and *S. pneumoniae* at (A) 20°C and (B) 37°C. All samples were tested in pH 7 buffer with 0.1 mM Ca^{+2}.

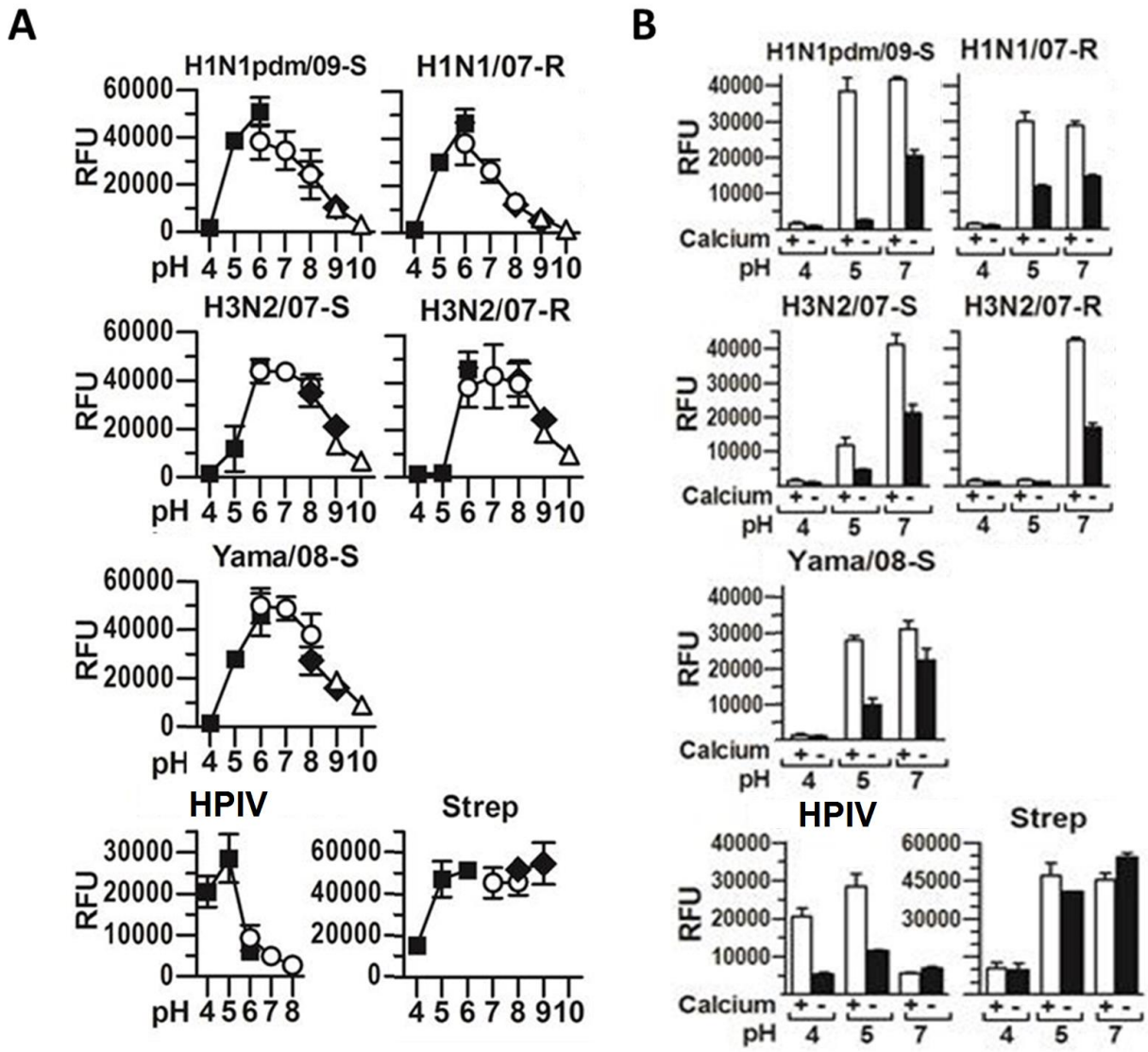

Figure S5. Characterization of the Influence of pH and presence of Ca^{+2} on NA Activity. (A) NA activity was measured by incubating virus with fluorogenic substrate at different pH values. NA activity in acetate buffer pH 4, 5 and 6 is shown as closed squares (■), in PBS pH 6, 7 and 8 is shown as open circles (O), in Tris buffer pH 8 and 9 is shown as closed diamonds (♦), in CHES buffer pH 9 and 10 is shown as open triangles. (B) Calcium (+) indicates NA activity measured in the presence of 0.1 mM Ca^{2+}; calcium (-) indicates NA activity measures in the presence of 25 mM of EDTA chelator. All plots show results of at least 3 independent experiments and the error bars indicate SD.

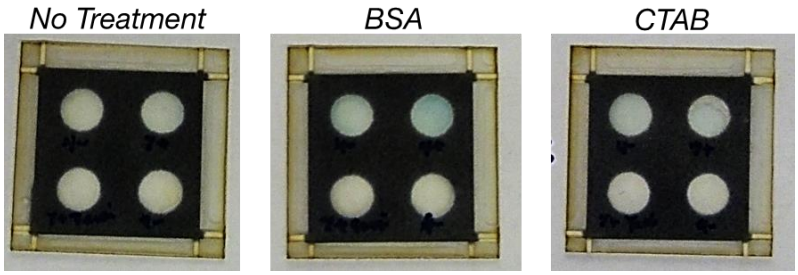

Figure S6. Effect of Pre-Treatment of Distribution Layer on Influenza µPAD Operation. Treatment was done to prevent non-specific adherence of influenza particles to cellulose fibers. (A) No Treatment. (B) Treatment with 0.5% w/v bovine serum albumin (BSA). (C) Treatment with 20 mM Cetyltrimethylammonium bromide (CTAB).

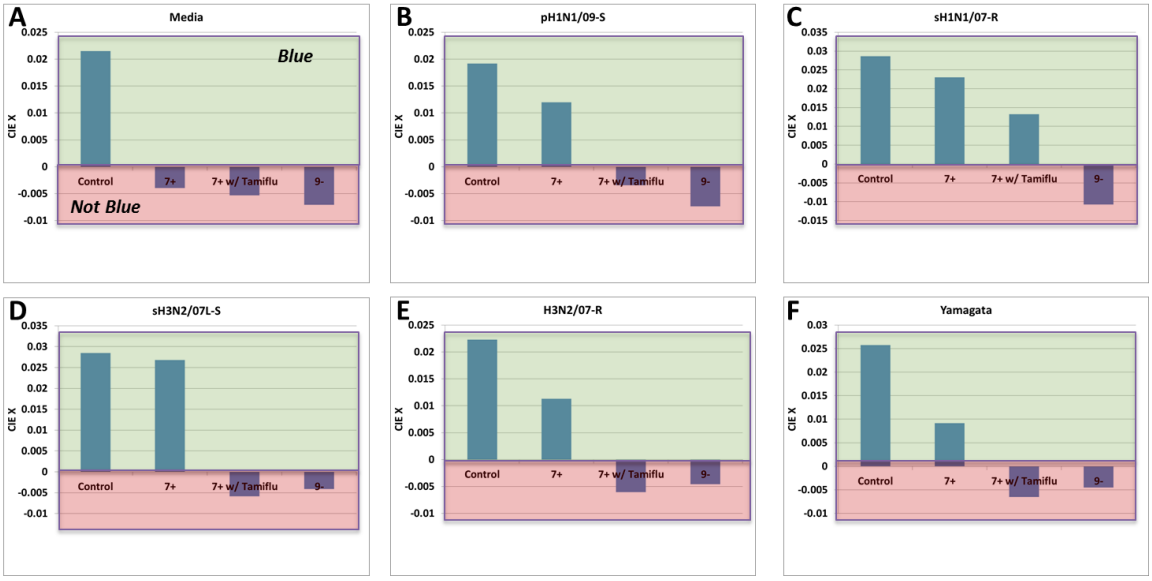

Figure S7. Image Analysis of the Influenza µPADs Tested with Various Influenza Strains. See Figure 6. In this case, the CIE X-coordinate change is being monitored for determination of a blue or non-blue result for each test spot.

Supplementary table

Table S1. Characterization of virus types and subtypes, as well as *S. pneumoniae* strains which are evaluated in this study.

Virus strain	HA Titer	FFU per mL[a]	NA act. RFU[b]	% of max NA act. +EDTA (pH)[c]	Osel[d] IC_{50} (nM)
H1N1 strains					
A/California/07/09 (H1N1pdm/09-S)	320	7×10^8	6600	44% (pH 6-7)	0.9
A/Brisbane/59/07-Like (H1N1/07-R)	160	3×10^7	5000	54% (pH 6)	2220
H3N2 strains					
A/Brisbane/10/07-Like (H3N2/07-S)	40	3×10^6	6700	50% (pH 7)	0.6
A/Texas/12/07 (H3N2/07-R)	160	4×10^6	6800	61% (pH 7)	15
Type B strains					
B/Florida/04/06 (Yama/08-S)	320	9×10^5	5000	88% (pH 7)	19
Parainfluenza virus Type 2 (HPIV)	ND	ND	4800	27% (pH 5)	ND
***S. pneumoniae* ATCC 49619** (Strep)	n/a	n/a	5000	108% (pH 6-9)	ND

[a] Fluorescent Focus units were determined using the fluorescent focus-forming assay. [b]: Rounded average of RFU/h/µL in NA assay in PBS, pH 7 for all influenza and *S. pneumoniae* strains, and sodium acetate buffer, pH 5 for Parainfluenza type 2.. [c]: % of maximal NA activity obtained in presence of 25 mM EDTA. All influenza strains tested at pH 7. Parainfluenza and *S. pneumoniae* tested at pH 5 and pH 7, respectively. [d]: Osel: Oseltamivir n/a: Not applicable; ND: Not determined

Supplementary Methods

Hemagglutinin (HA) titer

HA titration of influenza isolates grown in cell culture was performed as previously described (WHO, 2011). Briefly, serial 2-fold dilutions of influenza virus in phosphate buffered saline (PBS) buffer (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 128 mM NaCl, 2.7 mM KCl; pH 7.4) were incubated with a standardized turkey red blood cell (RBC; ViroMed) suspension (0.5% erythrocytes) in the wells of a microtiter plate. The plates were mixed and the RBCs were allowed to settle for 30 min at room temperature. The plate was tilted and read for complete agglutination. The inverse of the last dilution of virus that causes hemagglutination is considered the HA titer.

Influenza Fluorescent Focus forming Assay (FFA)

The influenza FFA was used to detect and quantify infectious influenza particles in cells (WHO, 2011). Briefly, MDCK cells were grown in 96-well plates to confluency. Serially diluted virus preparations were added to the cells and incubated for 2 h at room temperature. The infected cultures were washed, overlaid with media, and incubated for 20 h at 33°C, 5% CO_2. Cells were fixed with acetone, and virus was detected using monoclonal antibody against influenza A or influenza B nucleoproteins (Millipore) along with fluorescent secondary conjugated antibody (Millipore). The fixed cell monolayers were viewed using UV light microscopy and influenza positive cells, visible as fluorescent foci, were counted. The titer was calculated by multiplying the number of foci by the inverse of the dilution and dividing by the volume of inoculum. Final fluorescent focus forming units (FFU) were determined using the average of duplicate wells.

NA assay

NA activity was examined using the fluorescent substrate, 2-(4-Methylumbelliferyl)-a-D-N-acetylneuraminic acid, (MUNANA, Sigma-Aldrich, Inc.). Fluorescent-methylumbelliferone was measured with FLX-800 fluorimeter (BioTek) using excitation and emission wavelengths of 350 and 460 nm, respectively. Relative fluorescent units (RFU) were used to quantify NA activity. Dose response studies demonstrate that substrate is not limiting at viral dilutions of 1/30, and all subsequent assays were performed under conditions where substrate is not limiting. All reported results were collected from at least three independent experiments. The limit of detection of influenza virus was determined by measuring NA activity in PBS containing 10 μM MUNANA and 0.1 mM calcium at pH 6.5, while pH 7 was used to test *S. pneumoniae* NA activity. Dilutions

of virus or bacteria were incubated at 37°C for 3 h and read as described above. The results are collected from at least three independent experiments.

For pH studies, viruses were diluted 50-fold in five different reaction buffers, all containing 10 μM MUNANA, 200 mM NaCl and 0.1 mM calcium. Sodium acetate buffer (50 mM) was used for studies at pH 4, 5 and 6. PBS was used for studies at pH 6, 7 and 8. Tris(hydroxymethyl)aminomethane (Tris) buffer (50 mM) was used for studies at pH 8 and 9. N-cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer (50 mM) was used for studies at pH 9 and 10. 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer (50 mM) was used for studies at pH 10 and 11. Similarly, colonies of *S. pneumoniae* were suspended to an optical density of 1 at 600 nm in water and 50-fold dilutions were made in each of the reaction buffers. All reactions were incubated at 37 °C for 3 h. The pH optimum for detecting fluorescence was determined to be pH 8-9. All samples were adjusted to pH 8 using 1 M Tris buffer before fluorescence was determined.

In studies examining calcium dependence, both influenza and parainfluenza viruses were diluted 20-fold in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (50 mM HEPES, 200 mM NaCl; pH 7 for influenza virus and pH 6 for parainfluenza virus) containing 10 μM MUNANA, with different concentrations of $CaCl_2$, and 0 mM of $CaCl_2$ was designated when 25 mM ethylenediaminetetraacetic acid (EDTA) was added. RFUs were used to quantify NA activity and percentages to NA activity were calculated, defining 100% as the largest value in each data set.

NAI susceptibility assay

Drug susceptibility testing was performed by the 50% inhibitory concentration method, using two different procedures. First, we co-incubated the virus with the MUNANA substrate and NAI. Second, we pre-incubated the virus with NAI at 37°C for 30 min, followed by the addition of MUNANA. NA activity was measured in the presence of increasing concentrations of active oseltamivir (oseltamivir carboxylate, Toronto Research Chemicals Inc., Canada) and zanamivir (GlaxoSmithKline, USA). All experiments were performed at the optimal pH of each strain with 0.1 mM of $CaCl_2$. The half maximal inhibitory concentration (IC_{50}) was calculated using Prism 5.0 (GraphPad Software). The results are collected from at least three independent experiments.

Supplementary Reference

WHO (2011). Manual for the laboratory diagnosis and virological surveillance of influenza. Geneva, Switzerland: World Health Organization Press.