Environmental deposition of influenza virus from patients infected with influenza A(H1N1)pdm09: implications for infection prevention and control

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Supplementary Data

Table A: Study procedures conducted in Year1 and Year 2

Materials and **methods**	**Year 1**	**Year 2**
Timing	**Sept 2009 – Jan 2010**	**Dec 2010 – Jan 2011**
Recruitment • Community • Hospital	 Antiviral collection points, local media, letters via schools Nottingham, Leicester, Sheffield	 NHS walk in centres, A+E department, university campus GP Nottingham
PCR testing to confirm diagnosis performed?	No	Yes
Mean Follow up period[a]	8.7 days	4.8 days

Swabs[b]	Cotton tipped	Dacron tipped
Surface sampling[c]		
• Hospital	Table, window sill, bed button	Table, cup, bed button, door handle
• Community	Kettle, tap, door handle, bed table, TV remote, dining table	Kettle, tap, door handle, light switch, fridge, TV remote, computer
Frequency	Alternate days	Most days
Laboratory testing[d]	413 swabs collected, 402 samples processed (97%), 5 positive (1.2%)	485 swabs collected, 269 samples processed (55%), 28 positive (10.4%)
Air sampling[e]	Performed at both 3-7 and >7ft from subject and for 1 and 3 hours	Performed at >7 ft from subject for 3 hours
VTM volume[f]	750µl VTM was added to samples after collection	1.5mL VTM was added to samples after collection

Notes:

[a]Swabbing in Year 2 tended to be performed early in the course of illness. In Year 1 swabbing was evenly spaced over the duration of follow up (which averaged 8.7 days) compared to Year 2 where swabbing tended to be performed on most days of follow up (average 4.8 days).

[b]In Year 1 cotton tipped swabs (FB57835; Fisherbrand) were used and in Year 2 Dacron tipped swabs (FB57833; Fisherbrand) were used (in line with CDC advice to use synthetic fibre swabs for influenza diagnostics.

[c]The surfaces swabbed were slightly different – more commonly touched and more non-porous surfaces were selected in Year 2.

[d]In Year 1 most collected samples were analysed; in Year 2 samples were usually only tested when nose swabs were triplicate PCR positive on the same day. If Year 1 samples

had been subject to the same rules, then 201 (49%) would have been processed and the positivity rate would have been 4 out of 201 (2.0%). Overall this would have given a swab positive rate of 32/470 = 6.8%.

[e]In Year 1 collections took place at different distances (3-7 and ≥7ft) from the subject and for different periods of time (1, 2 or 3 hours). In Year 2 only one collection was made per sampling episode; sampling took place over 3 hours and the sampler was positioned in a convenient location in the same room as the subject (usually this meant that the sampler was ≥7ft from the subject).

[f] Volumes of VTM were increased in Year 2 to allow sufficient volume for testing.

Table B: Geometric mean viral loads compared between groups

Illness day	GM VL adults (95% CI)	GM VL children (95% CI)	Adult/Children GM ratio (95% CI)	P value
Day 3	121972 (26689, 557430)	132520 (16143,1087878)	0.92 (0.1, 10.4)	0.945
Day 4	98666 (26015, 374210)	20303 (5386, 76532)	4.86 (0.8, 29.1)	0.081
Day 5	31311 (12005, 81663)	26187 (4417, 155248)	1.20 (0.2, 8.1)	0.850
Illness day	**GM VL community (95% CI)**	**GM VL hospital (95% CI)**	**Hospital/Community GM ratio[†](95% CI)**	**P value**
Day 3	139051 (44444, 435047)	102360 (4271, 2452955)	0.74 (0.0, 14.7)	0.835

Day 4	36510 (10204, 130630)	104849 (19876, 553104)	2.87 (0.4, 21.3)	0.292
Day 5	16669 (5591, 49698)	56467 (12781, 249478)	3.39 (0.6, 19.7)	0.168

Note: GM = Geometric Mean, VL = Viral Load

Table C: Details of surfaces swabs that were PCR positive for A(H1N1)pdm09.

Subject	Surface	Material	Setting	Day of illness	VL surface (copies/ml x 10^4)	VL nose (copies/ml x 10^4)
Adult	Remote	Plastic	Home	3	0.07	27.2
	Tap	Metal		4	0.03	1.9
Adult	Tap	Metal	Home	3	1.2	70.2
	Games Console	Plastic			**1.8**	
Adult	Tap	Metal	Home	3	0.02	95.1
Adult	Fridge	Plastic	Home	4	1.07	773.5
	Tap	Metal			0.04	
Adult	Remote	Plastic	Home	3	0.11	99.9
	Door handle	Metal		4	0.11	316.6
	Laptop	Plastic			1.02	
	Fridge	Plastic			0.14	
	Remote	Plastic			1.1	
	Light switch	Plastic			0.16	
	Light switch	Plastic		7	0.02	2.1
	Tap	Metal			0.12	
Adult	Laptop	Plastic	Home	3	0.02	6.8
Adult	Laptop	Plastic	Home	6	0.07	55.4
Adult*	Kettle	Plastic	Home	4	**0.49**	72.2
	Tap	Metal		10	0.94	0.7
Adult	Table	Veneer	Hospital	3	0.06	0.3

Adult	Table	Veneer	Hospital	4	0.06	258.4
	Cup	Plastic			0.25	
	Cup	Plastic		5	0.1	39.8
	Bed Rail	Metal		9	0.02	2.6
Adult	Remote	Plastic	Home	4	0.01	0.2
Adult	Bed control	Plastic	Hospital	3	0.45	1120.5
	Table	Veneer			0.26	
Child*	Chair	Plastic	Home	5	0.04	0.6
Child*	Tap	Metal	Home	6	4.3	944.8
Child*	Light switch	Plastic	Home	2	0.65	15.0
	Tap	Metal			0.54	
	Cup	Ceramic			0.06	
Child	Bed control	Plastic	Hospital	3	0.16	286.5

*Note: * Other household members present who were exhibiting respiratory symptoms*
Culture positive

Table D: Viral loads and symptom scores compared between those with positive and those with negative air samples.

Illness day	**GM nasal VL air positive (95% CI)**	**GM nasal VL air negative (95% CI)**	**GM ratio (95% CI)**	**P value**
Day 4	120.9 (1.1, 12902.5)	42.1 (7.9, 225.1)	2.9 (0.1, 140.8)	0.565
Illness day	**Mean URT score air positive**	**Mean URT score air negative**	**Mean difference**	**P value**
Day 3	6.3	7.0	0.75	0.84
Day 4	4.6	7.1	2.5	0.34
Illness day	**Mean LRT score air positive**	**Mean LRT score air negative**	**Mean difference**	P value
Day 3	3.8	3.0	-0.8	0.60

Day 4	2.8	4.1	1.3	0.28

Note: GM = Geometric Mean, VL = Viral Load, URT = Upper Respiratory Tract, LRT = Lower Respiratory Tract

Laboratory methods

PCR

In year 2, a nasal swab was taken and sent to a local laboratory (Queens Medical Centre, Nottingham) where a PCR test against a panel of respiratory viruses was performed. This was done to aid recruitment only; the test result was not used to as a study outcome measure. Similarly, diagnostic information was sometimes obtained from clinical tests on hospitalised subjects. To give greater consistency across centres we only used results from the central (PHE) laboratory for study outcome measures.

HPA PCR methods:

Nucleic acid was extracted from the samples using the QIAsymphony SP instrument, QIAsymphony SP extractor mini kits, and a bacteriophage (MS2) internal control. Reactions were carried out on a RotorgeneTM 6000 (Corbett Research) real-time DNA detection system.

The primers and probes used were as follows:

Primers

Novel H1N1 influenza A (Metabion):

- H1FORSW: 5’-TCA ACA GAC ACT GTA GAC ACA GTA CT-3’
- H1REVSW: 5’-GTT TCC CGT TAT GCT TGT CTT CTA G-3’

Seasonal H1 influenza A (MWG Biotech):

- AH1 Forward: 5’-GGA ATA GCC CCC CTA CAA TTG-3’
- AH1 Reverse: 5’-AAT TCG CAT TCT GGG TTT CCT A-3’

Seasonal H3 influenza A (MWG Biotech):

- AH3 Forward: 5’-CCT TTT TGT TGA ACG CAG CAA-3’
- AH3 Reverse: 5’-CGG ATG AGG CAA CTA GTG ACC TA-3’

Influenza B (Metabion):

- BNP-F: 5’-GCA GCT CTG ATG TCC ATC AAG CT-3’
- BNP-R: 5’-CAG CTT GCT TGC TTA RAG CAA TAG GTC T-3’

MS2 control (MWG Biotech):

- MS2 Forward: 5’-TGG CAC TAC CCC TCT CCG TAT TCA CG -3’
- MS2 Reverse: 5’-GTA CGG GCG ACC CCA CGA TGT=A C-3’

Probes

Novel H1N1 influenza A (Metabion):

- H1SWp3: 5’-Cy5-AAT GTA ACA GTA ACA CAC CTG TTA ACC BHQ-3’

Seasonal H1 influenza A (ABI):

- AH1 Probe: 5’6FAM CGT TGC CGG ATG GA-MGBNFQ-3’

Seasonal H3 influenza A (ABI):

- AH3 Probe: 5’VIC-CCT ACA GCA ACT GTT ACC-MGBNFQ-3’

Influenza B (Biosearch Technologies):

- Flu-B Probe: 5’Quasar 705-CCA GAT CTG GTC ATT GGR GCC CAR AAC TG-BHQ-2-3’

MS2 control (Metabion):

- MS2 Probe: 5'ROX-CAC ATC GAT AGA TCA AGG TGC CTA CAA GC-BHQ-2-3'

RT-PCR protocol:

RT - PCR reactions comprised of 5µl of RNA and 20µl of mastermix (Table C). Primers were present at final concentrations of 1.25 mM (AH1), 0.25mM (Flu-B) and 0.1 mM (MS2) pmol/µl in the reaction mix. Probes were present at final concentrations of 0.05mM, 0.1 mM and 0.1 mM respectively. Cycles were performed as follows: reverse transcription at 50°C for 30 minutes, denaturation at 95°C for 2 minutes and then 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Table E: Composition of Mastermix

Stock concentration (pmol/µl)	**Volume of stock/reaction (µl)**
H1FORSW (20pmol/µl)	0.5
H1REVSW (20pmol/µl)	0.5
AH1 Forward (50pmol/µl)	0.45
AH1 Reverse (50pmol/µl)	0.45
AH3 Forward (50pmol/µl)	0.45
AH3 Reverse (50pmol/µl)	0.45
BNP-F (20pmol/µl)	0.25

BNP-R (20 pmol/µl)	0.25
MS2 Forward (20pmol/µl)	0.1
MS2 Reverse (20 pmol/µl)	0.1
H1SWp3 (10pmol/µl)	0.2
AH1 Probe (10pmol/µl)	0.1
AH3 Probe (10pmol/µl)	0.1
Flu-B Probe (10pmol/µl)	0.2
MS2 Probe (10pmol/µl)	0.2
2 x RT Platinum buffer (Invitrogen)	12.5
Superscript III Platinum enzyme	0.5
Water	2.7
Total volume:	20

Culture

Influenza A(H1N1)pdm09 did not readily form plaques on MDCK cells so an immunofluorescence (IF) assay was used to detect the influenza A/B nucleoprotein in order to demonstrate the presence of live replicating virus in the swab samples. Assays were performed on samples that were PCR positive. On occasions if a swab was IF positive on a given day (e.g. day 5) then an assumption was made that previous days (e.g.1-4) would also have been positive and no testing on these days was done.

Madin Darby Canine Kidney (MDCK) cells were used to propagate the virus. Initially, cells were plated onto 6 well tissue culture dishes at a concentration of 7.5 x 10^5/well and coverslips were added to each well; after 24 hours incubation the cells were washed x2 in serum free medium (SFM, Dulbecco's Modified Eagles Medium, DMEM) and 400µl of each sample applied to the respective well. After 30 minutes the cells were overlaid with 2mL serum free medium containing 0.14% bovine serum albumin (BSA) and 0.1% Worthington's Trypsin. 1:10 dilutions of influenza A (H1N1 human influenza virus A/PuertoRico/8/34) and a novel H1N1 influenza A isolate (A/Cambridge/AHO4/2009 H1N1) were also inoculated onto cells as positive controls. The cells were then incubated for 48 hours at 37°C. The virus was harvested and two dilutions were made in serum free medium, 1:2 and 1:10 (Yr1 only). 250 µl of each dilution was added to the appropriate well of a 24 well dish seeded with 1 x 105 MDCK cells per well.

Following 30 minutes incubation at 37°C, 1 mL of overlay (as before) was added to each well and the cells incubated overnight. After overnight incubation, the virus dilutions were aspirated off the cells. The cells were washed x 2 with phosphate buffered saline (PBS) and then fixed with 250 µl of 4% formaldehyde at room temperature for 20 minutes. The fix was aspirated off and the cells washed x3 with blocking solution (1% FCS in PBS). The cells were permeabilised in detergent (0.2% Triton x100 in PBS) and then washed x2 in block solution. 250 µl of a mouse monoclonal antibody (for influenza A = Abcam ab43821, 1:1000 dilution Yr1, 1:500 dilution Yr2 ; influenza B = Abcam ab54142, 1:1000 dilution) was added to each well and the plates incubated 60 minutes before washing x3 with blocking solution. The secondary antibody (goat anti-mouse 488 IgG2a, Molecular Probes) was diluted 1:1000 in blocking solution, and 4, 6 diamino-2-phenylindole (DAPI) diluted 1:2000. 250 µl of this mix

was added to the cells. Incubation was in the dark for 30 – 45 minutes. Cells were washed thoroughly with blocking solution, left in PBS and examined on the fluorescence microscope.

Figure

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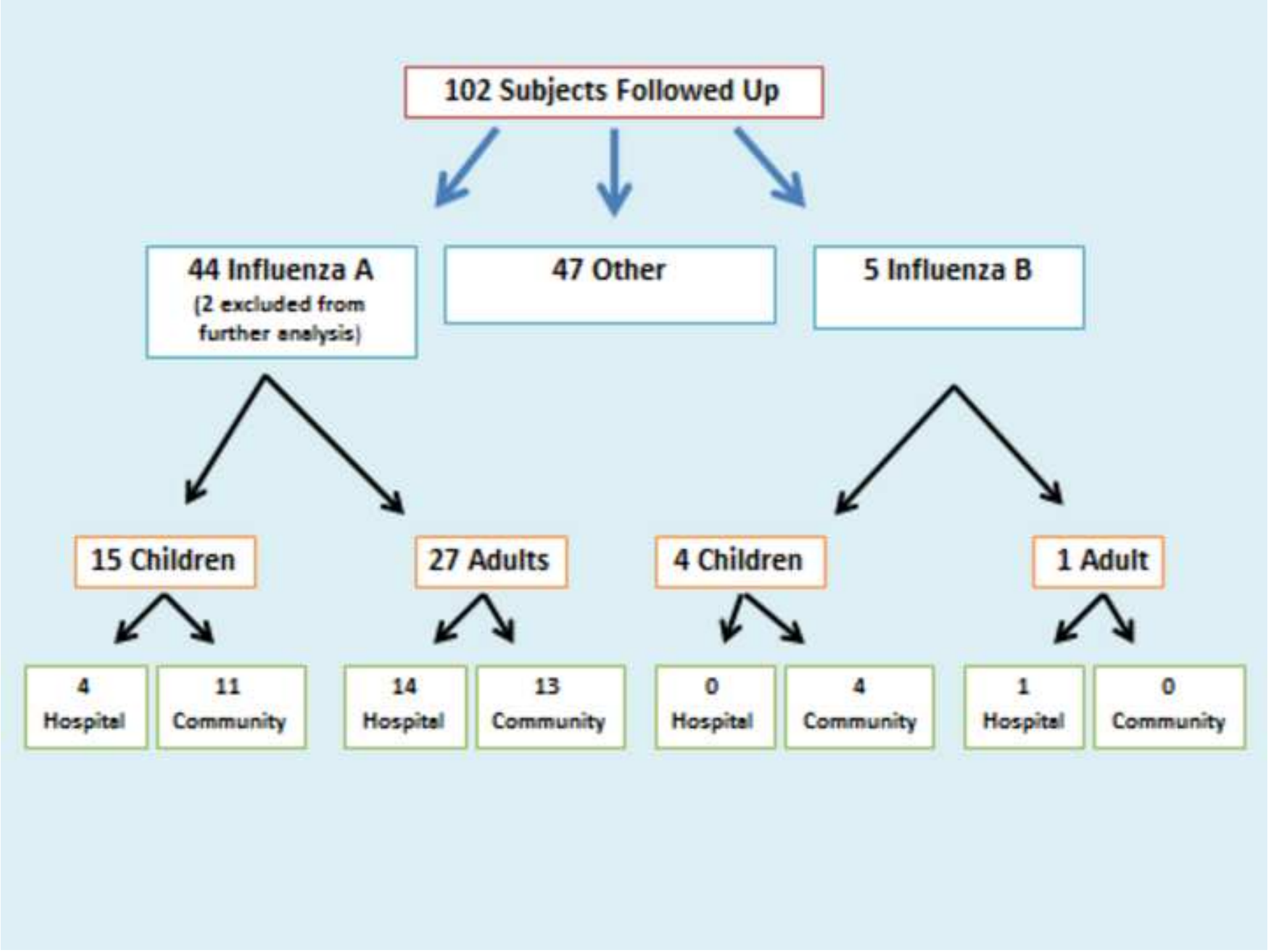

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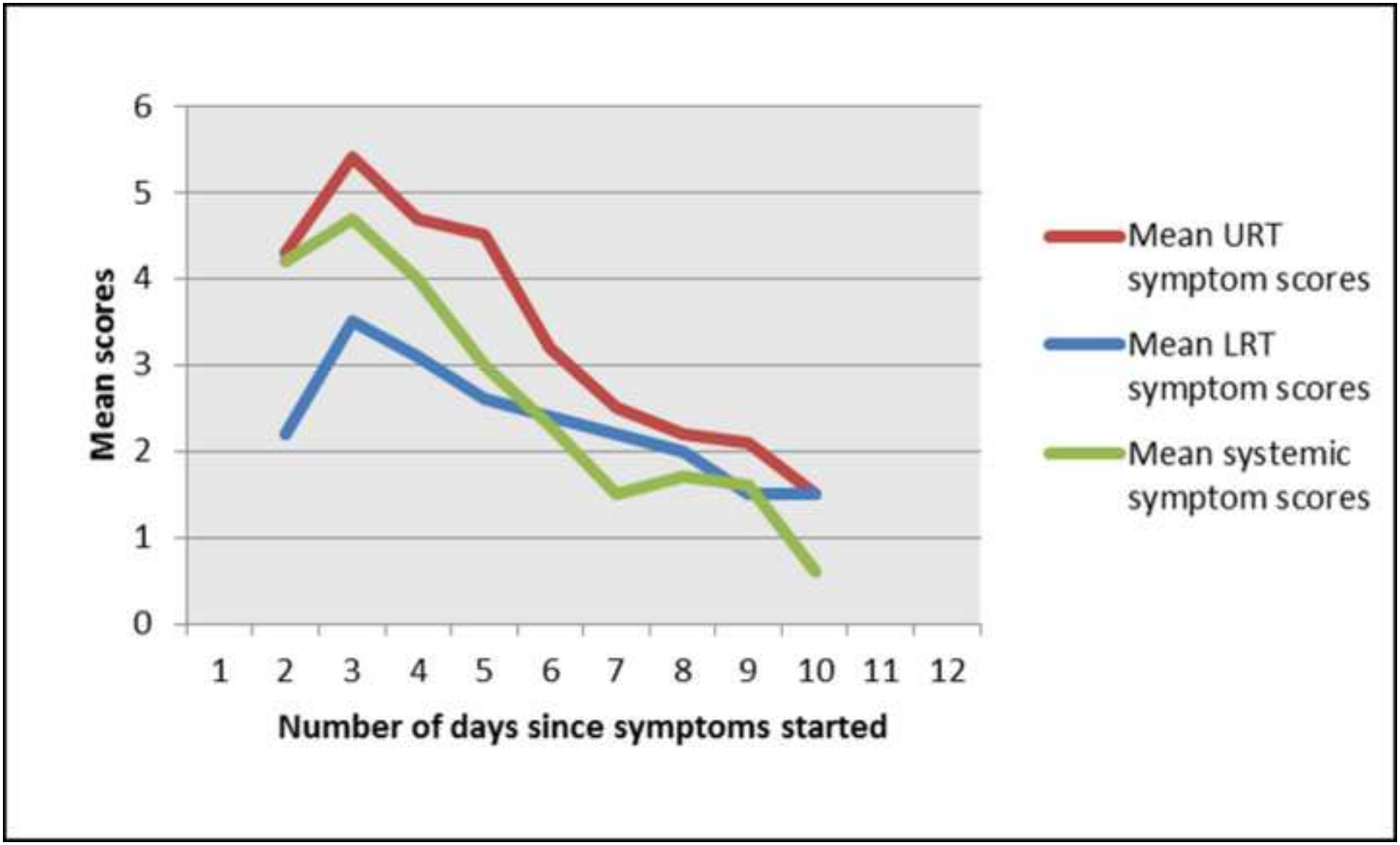

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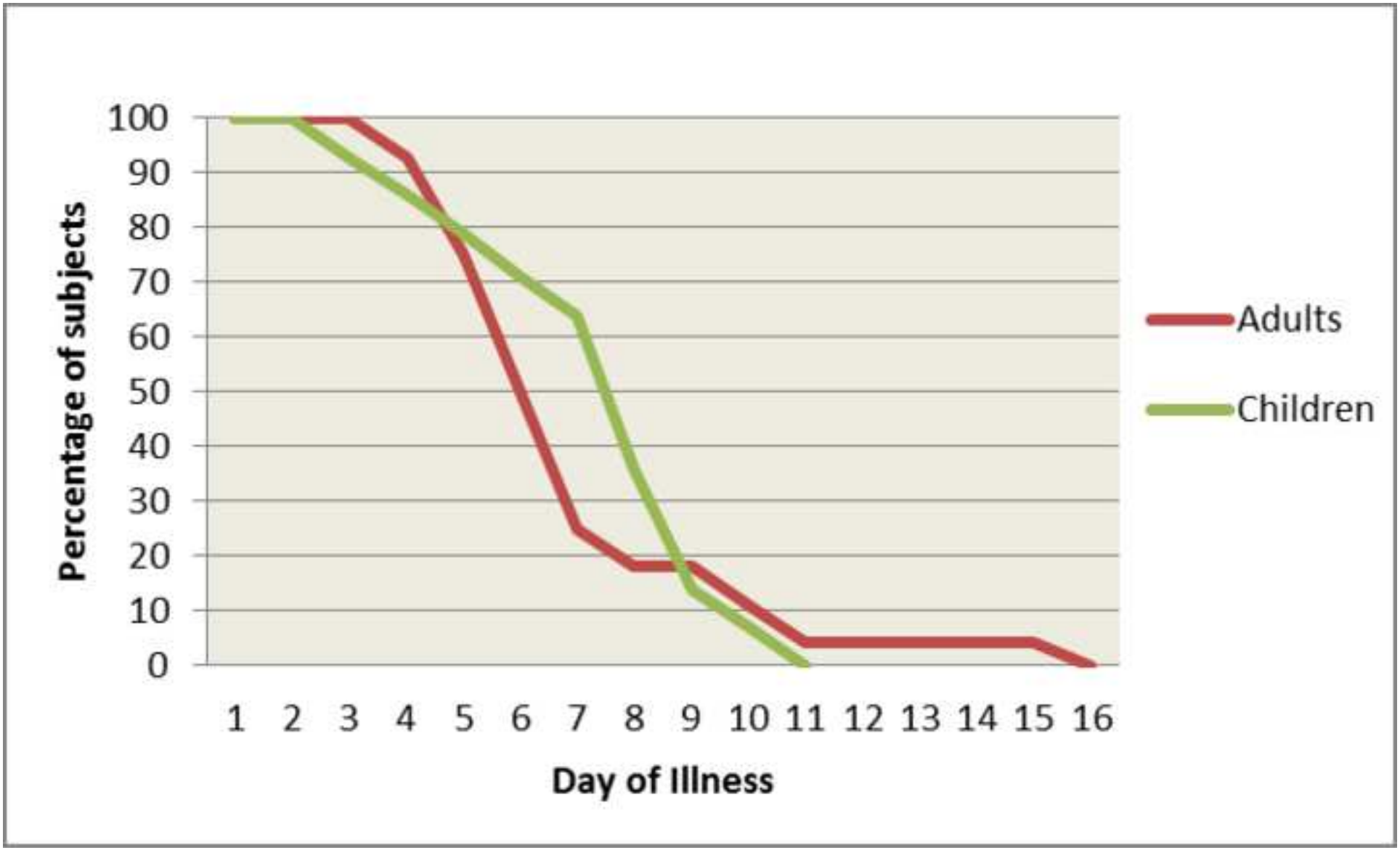

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