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Humidity as a non-pharmaceutical intervention for influenza A

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32 ## Abstract

33 Influenza is a global problem infecting 5-10 % of adults and 20-30 % of children annually. Non-
34 pharmaceutical interventions (NPIs) are attractive approaches to complement vaccination in the
35 prevention and reduction of influenza. Strong cyclical reduction of absolute humidity has been
36 associated with influenza outbreaks in temperate climates. This study tested the hypothesis that
37 raising absolute humidity above seasonal lows would impact influenza virus survival and
38 transmission in a key source of influenza distribution, a community school. Air samples and
39 objects handled by students (e.g. blocks and markers) were collected from preschool classrooms.
40 All samples were processed and PCR used to determine the presence of influenza and its amount.
41 Additionally samples were tested for their ability to infect cells in cultures. Deliberate classroom
42 humidification (with commercial steam humidifiers) resulted in a significant reduction of the
43 total number of influenza positive samples (air and fomite), viral copy number, and efficiency of
44 viral infectivity. This is the first prospective study suggesting that exogenous humidification
45 could serve as a scalable NPI for influenza or other viral outbreaks.

46 ## Author summary

47 Human influenza infections have a substantial impact on society (including lost productivity and
48 medical costs). Children, 3-4 years of age are the main introducers and spreaders of influenza
49 within a household and community. There is evidence from laboratory and epidemiological
50 studies that suggests that low humidity in winter (in temperate climates) may increase the ability
51 of influenza virus to survive and spread between individuals. We wanted to know if added in
52 humidity (through steam humidifiers) could reduce the amount of influenza present and its
53 spread within preschool classrooms (students aged 3-4 years)? Additionally, we looked at the
54 infectivity of the influenza isolated and if there were differences in the number of students with

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55 influenza-like illnesses during our study. We show that humidification can reduce the amount of
56 influenza present within samples from preschool classrooms and that there were fewer infectious
57 samples compared to non-humidified rooms. There were small numbers of students ill with
58 influenza like illnesses during our study so additional studies will need to look further at
59 humidification as a way to reduce influenza infection and transmission.

60

61 **Key Words: influenza, humidification, non-pharmaceutical intervention, preschool,**
62 **aerosols, fomites**

63

64 Introduction

65 Non-pharmaceutical interventions (NPIs) can complement traditional vaccination and anti-viral
66 medications for infectious disease. NPIs are particularly significant for diseases like influenza,
67 which have not shown to be well managed by vaccination alone. Worldwide, annual influenza
68 epidemics are estimated to infect 5-10 % of adults and 20-30 % of children resulting in 3 to 5
69 million cases of severe illness and 250,000 to 500,000 deaths[1]. These infections account for
70 10% of global respiratory hospitalizations in children under 18 years of age[2]. Direct medical
71 costs (2015) for influenza for inpatient care have been estimated at $3,650- $9,660 per case
72 (reviewed in[3]) and a total economic burden of influenza exceeding $87 billion per year in the
73 USA[4]. In Minnesota, USA, the 2014-15 influenza season was especially severe with 4,202
74 people hospitalized with laboratory-confirmed influenza infection and 10 confirmed pediatric
75 influenza-related deaths. This was the highest rate reported for the past six seasons including the
76 H1N1 influenza pandemic in 2009[5]. Influenza and respiratory syncytial virus (RSV) accounted
77 for over 50 % of hospitalizations (all ages) from respiratory infections with an additional 706

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78 outbreaks of influenza-like illness (ILI) reported in schools[5]. This increase in influenza was
79 attributed to antigenic drift, leaving the vaccine largely ineffective. The 2014-15 influenza
80 season illustrates why alternative approaches such as NPIs could prove to be valuable in disease
81 prevention.
82
83 The probability for influenza transmission increases in situations where many people are in
84 enclosed spaces and in close proximity such as airplanes and naval ships[6]. Children play a
85 critical role in the transmission of acute respiratory infections within a community[7]. Survival
86 and transmission of influenza may be impacted by the droplet size of airborne influenza. Larger
87 droplets settle out of air at a more rapid rate, and do not penetrate as deep into the respiratory
88 tract to seed infection[8]. Under laboratory conditions, others have shown that infectious
89 influenza virus can persist from hours to days (17 days on banknotes with respiratory
90 secretions[9]) in objects as varied as surface dust[10], cloth, pillow case, soft fabric toy, light
91 switch material, formica, vinyl, and stainless steel[11, 12]. Researchers have demonstrated
92 transfer of infectious influenza A from stainless steel surfaces (up to 24 hours) or from paper
93 tissues (15 minutes) to hands, while remaining infectious on hands for 5[13] - 60[12] minutes.
94
95 The mortality and transmission rates of influenza A have been associated with decreased
96 absolute humidity (AH) [14]. This epidemiological correlation suggests that deliberate increases
97 in AH could be a potential NPI to reduce the spread of influenza and other viruses. One
98 approach is to maintain relative humidity (RH) between 40-60 %, the proposed optimal range for
99 reducing growth opportunities for viruses, bacteria, and fungi[15]. Our previous study,
100 demonstrated that classroom humidification to RH of 40-60 % may be a feasible approach to

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101 increase indoor AH to levels with the potential to reduce influenza virus survival (a target of
102 10mb) and transmission as predicted by modeling analyses[16].
103
104 Community schools are promising locations for potential use of humidity as a NPI due to the role
105 of children as a key source of influenza transmission from the community into a household[4].
106 We developed a novel in-school sampling process (S1 Fig) sensitive enough to detect influenza
107 presence, quantity (viral RNA), and infectivity.
108
109 Outside of the laboratory, no prior studies have tested the potential for humidification to serve as
110 an NPI. A few groups have succeeded in collecting influenza (RNA) from air samples in health
111 care settings[17, 18]. One group also detected influenza (RNA) within air samples at a day-care
112 facility (babies' and toddlers' rooms) and on board airplanes across the USA[18]. However,
113 these studies did not detect and isolate infectious influenza from fomites in field conditions.
114 This study investigated the presence and infectivity of influenza A in active preschool
115 classrooms under control and humidified conditions.
116

117 **Results**

118 At the end of the 2015-2016 influenza season, an analysis of MN hospitalizations attributed to
119 influenza (data supplied by MN Department of Health via FluSurv-NET)[19] revealed three
120 troughs in atmospheric absolute humidity with the largest one (February 14, 2016)[20] preceding
121 the peak of the seasonal influenza outbreak (Fig. 1A). The peak for confirmed hospitalized
122 influenza cases (confirmed positive) was the week ending March 12, 2016.
123

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124 Elevated classroom humidification was maintained at an average of 9.89 mb in humidified rooms
125 compared to 6.33 mb in control rooms (January 25 through February 23). AH was targeted near
126 10 mb based on previously demonstrated achievable levels in classrooms and a calculated 1-hour
127 virus survival of 35 % (down from 75 % when AH ~3-4 mb). Samples positive for influenza A
128 virus are depicted in Table 1.

129 **Table 1. Influenza A positive samples by RT-PCR from preschool.**

Sample Type	Control # (%) positive	Control # samples	Humidified # (%) positive	Humidified # samples	OR, [95 % CI], P> |z|
Fomites	31 (22.1)	140	27 (18.0)	150	0.51, [0.33-0.78], 0.002
Air (total)	33 (18.3)	180	21 (11.7)	180	0.51, [0.29-0.89], 0.020
Air <1 μm	8 (13.3)	60	6 (10.0)	60	1.25, [0.91-1.72], 0.174
Air 1-4 μm	15 (25.0)	60	10 (16.7)	60	0.48, [0.16-1.42], 0.183
Air >4 μm	10 (16.7)	60	5 (8.3)	60	0.51, [0.23-1.10], 0.087

130 The number of positive samples in each sample type is indicated, the total number of samples
131 collected and the % positive (in parentheses) for control and humidified rooms. Statistical
132 analyses of OR, 95 % CI and P>|z| are indicated.

133

134 A total of 650 samples were collected (320 in control rooms, 330 in humidified rooms) of which
135 112 (17 %) were positive for influenza A virus by RT-PCR (S2A-2B Figs). There were fewer
136 influenza A positive samples in humidified rooms compared to control rooms for both fomites
137 and for total air. However, when individual sizes of air particles were examined, differences did
138 not achieve statistical significance. The distribution of influenza A positive samples within the
139 different sizes of air particles varied with the greatest percentage of positive samples within the
140 1-4 μm size for both control and humidified rooms (Table 1).

141

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142 Quantitative RT-PCR of influenza A virus for copy number revealed a significant reduction in
143 mean copy number in humidified rooms compared to controls for fomites (P<0.001) and air
144 (total) (P<0.001). Review of qRT-PCR data at individual particle sizes revealed that air <1 µm
145 (P=0.010), and air > 4 µm (P=0.011) experienced a significant reduction (Fig. 2A and Table S1).
146 Mean copy number for air between 1-4 µm (P=0.208) trended lower in humidified rooms but
147 was not statistically different from control rooms at a 95 % CI. Viral copy number varied among
148 different sample types with fomites having the highest copy number followed by air 1-4 µm
149 particles (Table S1).
150
151 Electrical impedance assay [21] revealed 30 % of RT-PCR positive classroom samples to be
152 infectious (Fig. 2B and Fig. S3). There were a smaller percentage of infectious samples from the
153 humidified rooms (19 %) than the control rooms (81 %). The corresponding average viral copy
154 number in the infectious samples in the humidified rooms was 30 and 40 in control rooms.
155
156 The majority of air particles were less than 1µm size for both humidified and control rooms.
157 Average particle numbers decreased with increasing size in both sets of rooms (<1 µm: 45500
158 control vs 70400 humidified), (1-4 µm: 2820 control vs 4060 humidified), (>4 µm: 139 control
159 vs 227 humidified). Particles generated from the humidifier and the average counts from 7
160 independent measurements over a 22-minute period showed more than 96% of the particles
161 provided by humidification were less than 1 µm. Yet, humidified rooms showed a near doubling
162 of both 1-4 µm, and >4 µm air particles. This indicates a likely combination of the small particles
163 added by humidification with present (and potentially influenza-bearing) particles. Larger
164 particles remain airborne for less time (4 µm takes 33 minutes to settle 1 meter in still air versus

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165 1 μm takes 8 hours)[17] and are unable to reach as deep within airways so are thought to be less
166 pathogenic[8].
167
168 From January 25- March 11, 2016, 10 influenza-like illnesses (ILI) from absent students were
169 recorded by school personnel. Seven of these absences were from control (non-humidified)
170 rooms and three were from humidified rooms. As shown in Table 2, in a per-room comparison of
171 humidified and control rooms the % positive (# PCR+ /total samples per room), % infective (#
172 positive by infectivity/ # PCR positive tested per room), and % of students with ILI absences (#
173 students with fever + cough or fever + sore throat / 32 days of classes) all were lower in
174 humidified rooms than control rooms.

175 **Table 2. Influenza A positive and infectious samples and ILI absences by room.**

	Control		Humidified	
Influenza A	Room A	Room B	Room C	Room D
% positive (# PCR+ / total samples per room)	33/160= 21 %	31/160= 19 %	27/165= 16 %	21/165= 13 %
% infective (# positive by infectivity/ # positive tested per room)	6/15= 40 %	7/12= 58 %	2/7= 29 %	1/11= 9 %
% students ILI* (# students ILI/ 32 days of classes)	3/32= 9 %	4/32= 13 %	2/32= 6 %	1/32= 3 %

176 Percentage of student absenteeism showed a logical flow from initial positive sampling. %
177 positive is number PCR+ divided by total samples per room. % infective is number positive by
178 electrical impedance assay divided by number PCR positive samples tested in electrical
179 impedance assay. ILI indicates influenza-like illness absence based on symptoms reported. % of
180 students with ILI absences is number of students out ill with fever plus cough at any point during

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181 the 32 days of classes (January 25- March 11, 2016) divided by the 32 classes. *Only absences
182 within the timeframe of sample collection (January 25- March 11, 2016) are included.
183

184 Discussion

185 This study monitored transmission of influenza A in preschool classrooms during the dry winter
186 months (low indoor humidity), which correspond with peak respiratory virus infections in
187 Minnesota. An increase in average AH from 6.33 mb in control rooms to 9.89 mb in humidified
188 rooms (RH ~42-45 %) was associated with a significant decrease in influenza A virus presence
189 in fomite and air samples in humidified rooms compared to control rooms. Additionally, PCR-
190 positive samples from humidified rooms exhibited lower infectivity than samples from control
191 rooms. The decrease in infectivity could be driven by a lower viral load of the PCR-positive
192 humidified room samples as compared to PCR-positive control room samples. This correlates
193 with the lower copy number of influenza A PCR positive samples observed in humidified rooms.
194 Enveloped viruses such as influenza are less stable in the environment than non-enveloped
195 viruses and are more sensitive to higher relative humidity[22-24]. The exact mechanism
196 underlying the action of humidity on the survival of the influenza virus is still not fully
197 understood[25]. Several studies have hypothesized that surface inactivation for viruses with
198 structural lipids may be due to denaturing of the lipoproteins found in enveloped viruses and
199 phase changes in the phospholipid bilayer leading to cross-linking of associated proteins[22].
200 Additionally, relative humidity impacts the salt concentration within droplets and at low relative
201 humidity (<50 %), the solutes crystalize and influenza viability was maintained[26].
202

203 Prior studies have largely focused on the influence of humidity (RH or AH) on influenza virus

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204 survival under laboratory conditions ([8, 24-27]). One study modeled influenza virus survival
205 across varied ranges of ambient indoor AH and humidification levels achievable in school
206 environments[16]. Based on these findings, we hypothesized that classroom humidification
207 might be a feasible approach to increase indoor AH to levels that could decrease influenza virus
208 survival and transmission. Taking classroom humidification the next step further we included
209 collection of samples from classroom environments and demonstrated that humidified rooms
210 exhibited fewer influenza A-positive samples and reduced copy number. Additionally, influenza
211 A-positive samples were less infectious in humidified rooms. Together, these outcomes strongly
212 support the hypothesis that deliberate humidification can mitigate influenza activity in a school
213 environment.
214
215 In this study, humidifiers significantly increased the overall amount of particles, especially by
216 increasing number of small air particles, finally leading to an increase in larger size particles,
217 thereby rendering a shift in distribution towards largest particles, >4 μm. This could be attributed
218 to the aggregation of particles in the humidified air, reducing the time of air suspension and
219 transmission via inhalation. These findings enable a better understanding of the impact of
220 humidity on influenza A virus survival (surfaces and objects) as well as virus transmissibility via
221 aerosols by measuring the size of aerosol particles and the distribution of viruses within different
222 sizes of particles.
223
224 Adding direct measurements of influenza (or other respiratory viruses) in a larger experimental
225 population may reveal higher resolution outcomes to this approach. Furthermore, the impact of
226 exogenous humidification on other viruses will need to be similarly measured to more fully

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227 understand the full potential impact of this NPI on other sources of communicable respiratory
228 infections.
229

230 Materials and Methods

231 Study site and absence data

232 The study was conducted at Aldrich Memorial Nursery School, Rochester, MN (a preschool with
233 students aged 2-5 years) from January 2016-March 2016. Classrooms of identical design (see S4
234 Fig) each with their own HVAC system for air handling were utilized. This study received prior
235 IRB review and has been approved as an IRB #15-000476, an exempt study (Not Human Subject
236 Research). Aldrich staff collected information on student absences (January 4, 2016- March 31,
237 2016) including ILI symptoms from students who were ill. ILI was defined as having fever plus
238 cough or sore throat.
239

240 Humidifiers, humidity measurements, and absolute humidity calculations

241 Model XTR (XTR003E1M) electrode steam humidifier with steam blower (SDU-003E)
242 (DriSteem, Eden Prairie, MN) was installed in two experimental classrooms. The boiling of the
243 softened tap water source provided decontamination of the steam distributed through the steam
244 blowers. Classroom temperature and relative humidity were recorded every 10 minutes during
245 the duration of the study with HOBO external data loggers, Model #U12-012 (Onset Computer
246 Corporation, Bourne, MA). Two data loggers were installed per classroom, placed on the interior
247 walls and set on top of the bulletin boards at a height of 2.032 meters. Data exported to Excel
248 using HOBOware software (Onset Computer Corporation, Bourne, MA). Outdoor temperature
249 and relative humidity was obtained from the North American Land Data Assimilation System

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(NLDAS) project[20]. Absolute humidity was calculated using Excel software using formulas as previously described[16].

Bioaerosol sampler

NIOSH two-stage bioaerosol cyclone samplers[28, 29] collected air samples and separated them into three size fractions (>4 µm, 1-4 µm, and <1 µm) at a flow rate of 3.5 L / minute. NIOSH samplers were connected to AirChek XR5000 personal air sampling pumps, Model 224-PCXR4 (SKC Inc., Eighty Four, PA). Flow rate was calibrated using a Mass Flowmeter 4140 (TSI, Shoreview, MN), prior to each run of 150 minutes. Falcon conical tubes (15mL) (Corning, Corning, NY), 1.5 mL Fisherbrand microcentrifuge (Fisher Scientific, Pittsburgh, PA) and 37 mm hydrophobic Fluoropore PTFE membrane with a 3.0 µm pore size (EMD Millipore, Billerica, MA) were used to collect 3 different size fractions. Air sampling pumps were placed inside plastic ammunition boxes lined with mattress topper material (donated by Rest Assured Mattress Co, Rochester, MN). Cyclone samplers were affixed to the outside box surface with Industrial Strength Tape Strips (Velcro, Manchester, NH).

Particle Counts

After class dismissal, particle counts were measured in the center of each classroom at a height of 91 cm for 1 minute using a Six Channel Handheld Particle Counter, Model 23v750 (Grainger, Lake Forest, IL). Sizes of particles measured were 0.3, 0.5, 1, 2.5, 5, and 10 µm. Particle size data was binned into sizes to match those collected by the NIOSH samplers such that <1 µm included 0.3 and 0.5 µm sizes, 1-4 µm included 1 and 2.5 µm and >4µm included 5

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272 and 10 μm. Particle counts were also measured before and after humidifier turned on in a pilot
273 test.
274

275 **Air samples**

276 NIOSH samplers were dissembled in a BSLII biosafety cabinet. Collection tubes received 1 mL
277 of infection media. Filters were retrieved from inside black polypropylene filter cassettes opened
278 with a Stainless Steel SureSeal Cassette Opener (SKC Inc., Eighty Four, PA) and placed inside a
279 15 mL conical tube containing 1 mL infection media. The air filter was pushed down into the
280 media using a sterile 1 mL serological pipette. Samples were placed on ice until ready for further
281 processing.
282

283 **Fomites**

284 25 % cotton linen paper (Southworth, Neenah, WI) wrapped objects were provided to students.
285 Objects included markers and a variety of wooden toys including blocks, rolling pins patterned
286 wheel press and stamping cubes (Melissa & Doug, Wilton, CT). Additional items present in the
287 classroom were also wrapped including rolling pins, hard rubber brayers, and plastic pizza
288 cutters. After play, paper was transported back to BSL2 laboratory.
289

290 **Processing of fomites and air samples**

291 Papers from classroom objects were lightly dusted with fingerprinting powder (Hi-Fi Volcano
292 Latent Print Powder, Sirchie Youngsville, NC). Once fingerprint was identified, a portion of
293 paper (~3.5-4 $cm^{2)}$ was removed and placed into a 15 mL conical tube containing 1 mL of
294 infection media. Samples were placed on ice until ready for further processing.

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295

296 Both fomites (paper) and air samples (in media) were vortexed briefly and incubated on ice for
297 15 minutes. Samples were vortexed again prior to centrifugation for 20 minutes at 4 °C at 3313g
298 (15 mL tubes) and centrifuged at room temperature at 1520g in a microcentrifuge (1.5 mL
299 tubes). Liquid was removed from 15 mL conical tubes and transferred to 1.5 mL conical tubes.
300 The supernatant from these samples was frozen at -80 °C and used for subsequent RNA isolation
301 and qRT-PCR.

302

303 **Influenza A virus controls and infectivity assay**

304 Viral stocks of influenza A (H3N2) were provided by the Clinical Virology Laboratory, Mayo
305 Clinic, Rochester, MN. Samples were thawed and used to infect bulk cultures of MDCK.
306 Influenza A infections followed published methods[30], except DMEM media used instead of
307 MEM. The contents of the flask were collected once cells became non-adherent and centrifuged
308 4 °C at 3313g for 10 minutes to pellet cellular debris. Aliquots of supernatant were stored at -80
309 °C. Samples were assayed for influenza A virus infectivity by electrical impedance assay[21]
310 run on an xCELLigence RTCA MP instrument (ACEA Biosciences, Inc., Sand Diego, CA).
311 After calibration with media, wells were seeded with MDCK cells. Twenty-four hours later,
312 media was removed and the cells were washed with PBS. Wells were inoculated with serially
313 diluted influenza A virus (positive controls), media (negative controls) and samples (tests).
314 Readings were taken every 15 min for 7 days post inoculation. Using influenza A virus stock,
315 dose dependent decline in cell index was demonstrated with 1:1000 dilutions, and gradual
316 decline at 1:10000 and 1:100000 dilutions, whereas further dilutions did not impact cell index
317 any differently than media controls (Fig. 2B and Fig. S3).

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318

319 **Viral RNA isolation and detection using quantitative reverse transcription PCR (qRT-**
320 **PCR)**

321 The supernatant from fomite and air samples was thawed and 140 µL used for viral RNA
322 isolation with the Viral RNA isolation kit (Qiagen) as per the manufacturer's instructions,
323 followed by RT-PCR analyses. The nonstructural (NS1 gene) sequence of influenza A was used
324 for virus detection. See S2 Table for list of primers, product sizes and annealing temperature
325 information. See S5 Figure for detection of viral RNA in multiplexed qRT-PCR. Briefly, SYBR
326 green qRT-PCR was performed as described by the manufacturer (Qiagen SYBR green
327 Quantitect kit). The PCR thermal profile consisted of an initial cDNA step of 30 minutes at 50
328 °C followed by 15 minutes at 95 °C and 30 cycles of 30 seconds at 95 °C, 30 seconds at 56.5 °C,
329 and 30 seconds at 72 °C. Detection, quantification and data analysis were performed in the CFX
330 manager real-time detection system (Bio-Rad).

331

332 **In-vitro transcription of influenza A viral RNA**

333 Though Influenza A and B and RSV were included in this analysis, low numbers of positive
334 samples for RSV and Influenza B moved the focus of the study to influenza A only.
335 Quantification of influenza A RNA was performed using in vitro transcription (IVT). The NS1
336 gene was amplified from a H3N2 influenza A virus isolate from infected MDCK cells using
337 primers containing T7 promoter sequence in the forward sites: Inf AF: 5′-
338 ACTGCTTAATACGACTCACTATAGGGAGATTTCACCGAGGAGGGAGCA -3′, Inf
339 AR: 5′- CCTCCGATGAGGACCCCAA -3′;. The amplified NS1 gene was in-vitro transcribed
340 with T7 RNA polymerase (Megascript T3 kit, Ambion, ABI) according to the manufacturer's

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341 instructions for synthesizing short transcripts (105 bp) with the following modifications:
342 incubation time increased to 8 hours and the enzyme mixture and template RNA was increased
343 by three-fold of its original concentration. The synthesized RNA pellet was suspended in 0.1%
344 diethylpyrocarbonate-treated water. RNA transcripts were purified, quantified and mixed with
345 nuclease free water for preparation of positive controls in the range from 10^1-10^7 copies for the
346 standard curve development. The amount of IVT-generated fragments was determined using the
347 NanoDrop ND2000 Spectrophotometer (NanoDrop Technologies, INc., Wilmington, DE) and
348 converted to molecular copies according to the formula:

$$Y \frac{molecules}{\mu L} = \frac{(\frac{Xg}{\mu L}\ IVT\ RNA\ x\ 6.02x10^{23})}{(340\ x\ transcript\ length\ (bp))}$$

349 The detection limit of the SYBR green real time RT-PCR assay was determined by testing serial
350 ten-fold dilutions of the in vitro transcribed influenza A viral RNA ranging from 10^1 to
351 10^7 copies/μL. Cycle-threshold (Ct) values were plotted against the RNA copy number to
352 construct the standard curve. The viral copy numbers of the processed samples were estimated
353 by plotting the respective Ct values on the standard curve. See S6 Figure for standard curve
354 example.
355
356 **Detection and quantification**
357 Using serially diluted in vitro transcribed influenza A RNA, viral particles were detectable
358 ranging from 10^1 - 10^7 RNA copies using the described assay. qRT-PCR sampling was
359 sufficiently sensitive to detect as few as 7 RNA copies.
360
361 **Statistical analyses**

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362 Percentage of samples positive for influenza A (Table 1): To account for within-room clustering
363 for class cohorts, generalized estimating equations were utilized with a binomial family and logit
364 link. Data are reported as odds ratio of positive test result for humidified rooms as compared to
365 control rooms, with a 95% confidence interval and p-value. Odds ratios below one (with
366 statistical significance) demonstrate protective effects of humidification in that positive influenza
367 was less likely to be obtained by the relevant capture system. Capture systems for influenza
368 included paper and air (total, >4 µm, 1-4 µm, <4 µm); results were treated as positive for any
369 capture within the room to minimize the effect of the different number of collectors per room for
370 the paper and air capture systems.

371

372 Mean copy number of influenza A positive samples (Table S1): To account for within-room
373 clustering for class cohorts, generalized estimating equations were utilized with a gaussian
374 family and identity link. Data are reported as the mean (standard deviation) of copy number for
375 humidified rooms as compared to control rooms, with a 95% confidence interval and p-value.
376 Capture systems for influenza included paper and air (total, >4 µm, 1-4 µm, <4 µm).

377

378 **Data Availability**

379 The data that support the findings of this study are available from the corresponding author upon
380 request.

381

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382 **Figure Legends**
383
384 **Fig. 1. Absolute Humidity and influenza hospitalized cases.** (**A**) Outdoor absolute
385 humidity (AH) values (n=65; one measurement per day) from Rochester, MN and influenza
386 hospitalized cases in MN (n=1070, week ending in January 16th- March 19th). Applying the
387 national trend model described by Shaman et al.[31] to the local humidity and illnesses,
388 onset of influenza followed the predicted delay of 10-16 days (grey box) after an absolute
389 humidity trough (blue box). Peak cases follow (pink box), as there is an incubation period
390 of 1-4 days with viral shedding up to 7 days after symptoms resolve. (**B**) AH in 4 preschool
391 classrooms (average of two sensors from 10 minute intervals over 150 minutes (n=16 per
392 sensor, room D) or (n=17 per sensor, rooms A,B, C) per class period per sensor). Center
393 values are mean of both sensors during class time and error bars are s.d. and
394 corresponding outdoor AH (n=15, 1 per day) on the 15 days of sample collection.
395 Humidifiers were running in humidified rooms through sample collection on February 23.
396
397 **Fig. 2. Influenza A (NS1) average copy number and infectivity of positive samples in**
398 **electrical impedance assay.** (**A**) Horizontal bars indicate mean copy number and error
399 bars are 95% CI. Fomites control, n= 31; Fomites humidified, n=27; Air (total) control,
400 n=33; Air (total) humidified, n=21. (**B**) Each line indicates a sample (well) for infectivity.
401 MDCK cells were added at time 0 hours and media changed to samples (n=27 in triplicate),
402 influenza A positive control dilutions (6) in duplicate or control media in triplicate at 24
403 hours. Cell indices that returned to 0 indicated cell death (infectious). Arrows indicate
404 individual samples. * indicates P<0.001.
405
406

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407 **Contributions of authors**

408 AG/GMS/HJF/KE/PL/WCH/SCE/CP designed the study. BD/JMR did the sample collection, all
409 experiments, analyses of results, and drafted the manuscript. GMS provided analysis of indoor
410 humidity data and procured air sampling equipment from NIOSH/CDC. GMS, KMS, MDU, and
411 HJF assisted with cell culture infectivity assays. BD and MDU performed RNA isolation.
412 MEMH directed UMR undergraduate work-study students in processing of paper wrapped
413 objects. HBL assisted with air particle analyses. JLG analyzed student absence/attendance data.
414 ARG conducted initial pilot study at Aldrich winter-spring 2015. THK provided outdoor
415 humidity data and assisted with humidity analyses. KE provided the student data, turned on/off
416 humidifiers and made adjustments to settings, and assisted with school management. PL
417 provided humidifiers. SCE helped with analyses and trouble-shooting and revised the
418 manuscript. WCH provided wording for collecting ILI school data and revised the manuscript.
419 HJF provided BSL-2 laboratory oversight, and assisted in analyses. CP conceived the study,
420 helped with study design, analyses and drafted the manuscript. FTE provided statistical analyses.
421

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531

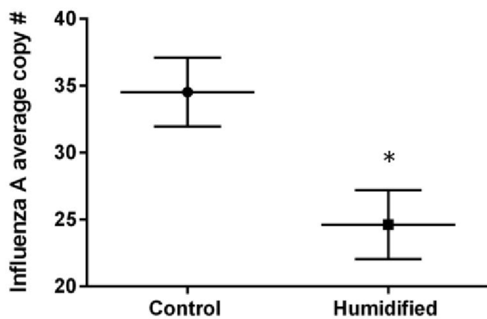
A Figure 1

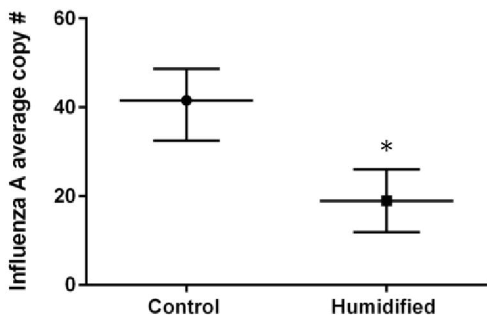
Predicted Flu outbreak onset
Peak cases
Absolute humidity (mb)
Absolute humidity trough
Influenza hospitalized cases/week
0
2
4
6
8
0
100
200
300
1/1
1/8
1/15
1/22
1/29
2/5
2/12
2/19
2/26
3/4
3/11
3/18

B
Humidified Room (D)
Humidified Room (C)
Outdoor
Control Room (B)
Control Room (A)
Text
Text
Text
Text
Absolute Humidity (mb)
0
4
8
12
16
1/25
2/1
2/8
2/15
2/22
2/29
3/7
Date

Figure 2

A





B

Cell Index

Time (hours)

Media controls

Influenza A positive controls

Positive samples