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| Title | Quantitative analysis of four rapid antigen assays for detection of pandemic H1N1 2009 compared with seasonal H1N1 and H3N2 influenza A viruses on nasopharyngeal aspirates from patients with influenza |
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| Author(s) | Chan, KH; Chan, KM; Ho, YL; Lam, YP; Tong, HL; Poon, LLM; Cowling, BJ; Peiris, JSM |
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1   Quantitative analysis of four rapid antigen assays for detection of pandemic H1N1 2009

2   compared with seasonal H1N1 and H3N2 influenza A viruses on nasopharyngeal aspirates

3   from patients with influenza

4

5   Chan KH[a], Chan KM[b,c], Ho YL[b,c], Lam YP[b,c], Tong HL[b,c], Poon LLM[b,c], Cowling BJ[d] and Peiris JSM[c,d]

6   [a]Department of Microbiology, Queen Mary Hospital, Hong Kong

7   [b]Department of Microbiology, The University of Hong Kong; Hong Kong

8   [c]Centre of Influenza Research, The University of Hong Kong, Hong Kong

9   [d]School of Public Health, The University of Hong Kong, Hong Kong

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11   Running title: Rapid diagnosis of Influenza A

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13   Key words: Pandemic H1N1 2009, seasonal H1N1 and H3N2, rapid antigen detection kit, real time

14   quantitative RT-PCR

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16   Address correspondence: Dr KH Chan, Department of Microbiology, University Pathology Building, Queen

17   Mary Hospital Compound, Pokfulam, Hong Kong

18   E mail: chankh2@hkucc.hku.hk

19   Conflicts of interest: None

20

21    Abstract

22    Data on analytical sensitivity of rapid diagnostic assays are important for clinical management of influenza,

23    especially during a pandemic. Four rapid antigen detection assays were compared for detection of pandemic

24    influenza A H1N1 2009, seasonal H1N1 and H3N2 in 96 patients with influenza A infection confirmed by

25    real-time RT-PCR. These rapid antigen tests appeared to have lower sensitivity (55.8%) for the diagnosis of

26    pandemic influenza A H1N1 2009 as compared with seasonal H3N2 (71.0%) or H1N1 (69.4%) influenza

27    infections, a difference that was related to a lower viral load in patients infected with the pandemic influenza

28    A H1N1 2009 virus. The detection limit of these antigen detection tests in clinical specimens was an

29    influenza A M gene copy number of average $1.0 \times 10^{7}$ copies /ml. Significant variations between tests in

30    sensitivity for detection of pandemic influenza A H1N1 2009 (43.4-63.3%) were observed. The Directigen

31    EZ Influenza A+B and the Espline Influenza A+B had comparable sensitivity (63%) and were the most

32    sensitive among the four assays evaluated.

33

## 1. Introduction

Influenza A virus infection is a common cause of respiratory illness and contributes to morbidity and mortality annually, particularly in young children and in the elderly. Occasionally, animal influenza viruses transmit zoonotically to humans giving rise to severe clinical diseases such as avian H5N1 (de Jong et al., 2006). A novel influenza A (H1N1) 2009 (pH1N1 2009) virus of swine in origin was detected in Mexico and USA in April 2009 [Novel swine-origin influenza A (H1N1) virus investigation team, 2009]. The virus was efficient at transmitting from human to human and spread globally to cause a pandemic (Fraser et al., 2009). Rapid, simple and reliable diagnostic tests for confirming infection with influenza A can improve clinical management by guiding the appropriate use of antivirals and antibiotics. It has been previously demonstrated that the analytical sensitivity of many of these influenza A antigen detection tests for detection of avian H5N1 and pH1N1 2009 was comparable with that of seasonal influenza A infected cell lysates (Chan et al., 2007 and 2009). Recently, several studies reported that these rapid kits had clinical diagnostic sensitivity ranging from 10-80% for detection of pH1N1 2009 (Cowling et al., 2010; Ginocchio et al., 2009; Gordon et al., 2010; Kumar et al., 2010, Yang et al., 2011). However, the reasons for this variable and poor sensitivity of rapid antigen assays for clinical samples with pH1N1 2009 have not been investigated. The present study is to correlate clinical diagnostic sensitivity of four commercially available rapid antigen detection tests to viral load in the clinical specimens as determined by quantitative PCR methods in patients infected with seasonal H1N1 (sH1N1), seasonal H3N2 (sH3N2) and pH1N1 2009 influenza A. The performance of these rapid kits was also compared with direct immunofluorescence antigen detection kit and conventional virus culture.

## 2.	Materials and Methods

### 2.1	Clinical samples

Ninety seven nasopharyngeal aspirate specimens collected from hospitalized patients with suspected influenza from July 2009 to January 2010 sent to the virology laboratory at the Queen Mary Hospital, Hong Kong for routine diagnosis were used for this study. These specimens were routinely tested by direct immunofluorescence antigen test, RT-PCR for influenza A and culture for virus isolation as part of routine clinical care. The rapid antigen tests evaluated here (see below) were carried out on the residual specimen left over after routine tests were completed. Fifty-six of the patients were males and 41 were females with an age range of 9 months to 104 years. This study has been approved by the Institutional Review Board of the University of Hong Kong/ Hospital Authority Hong Kong West Cluster.

### 2.2	RT-PCR for H and M gene of influenza A

The diagnosis of pH1N1 2009 virus, sH1N1 and sH3N2 was performed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) using primers targeting the hemagglutinin gene of pandemic H1N1 virus according to method described earlier (To et al., 2010, CDC 2007). The quantitation of influenza A virus was performed by using real-time quantitative RT-PCR targeting influenza A virus M gene, as described previously (Li et al., 2010). Briefly, 12 µl of eluted RNA of Influenza A virus was used for cDNA using the Invitrogen Superscript II Kit with a random primer as described, and then, cDNA was amplified in a Lightcycler instrument with a FastStart DNA Master SYBR Green I Mix reagent kit (Roche

74   Diagnostics GmbH, Mannheim, Germany). In a typical reaction, 2 µl of cDNA was amplified in a 20 µl of

75   LC-PCR master mix containing 1X Fast-Start DNA master SYBR green I mix, 4.0 mM $MgCl_2$, 0.5 mM of

76   each primer. To determine the specificity of the assay, all the PCR products were subjected to a melting

77   curve analysis (65–95$^o$ C; 0.1$^o$ C per second) at the end of the assay. For quantitative assay, a reference

78   standard was prepared using pCRII-TOPO vector (Invitrogen, San Diego, CA) containing the corresponding

79   target viral sequences. A series of 6 log 10 dilution equivalent to 1 x 10$^1$ to 1 x 10$^6$ copies per reaction were

80   prepared to generate calibration curves and run in parallel with the test samples. If the specimen result was

81   outside the upper limit of the expected range, the extract of the sample was repeated with suitable dilution.

82   The detection limit of this assay was 900 copies of RNA per milliliter.

83

84     2.3   Rapid influenza antigen detection kits

85   Four rapid influenza antigen detection kits: QuickVue influenza A+B (Quidel Corpopration, CA, USA);

86   BinaxNow Influenza A+B (Binax, Maine, USA); Directigen EZ Flu A+B (Becton Dickinson and Company,

87   MD, USA); and Epsline influenza A+B ((Fujirebio, Tokyo, Japan) were evaluated. All these tests were

88   carried out according to the manufacturers' instructions (Chan et al., 2009). Briefly, for the QuickVue

89   influenza A + B test, 280 ul of sample was added to the extraction tube containing extraction powder. The

90   extraction tube was swirled gently to dissolve its content. A test strip was placed into the extraction tube. The

91   result was read at 10 minutes. The BinaxNow Influenza A + B test kit used 100 ul of specimen in a virus

92   transport medium to the test device and the result was read after 15 minutes. For the Directigen EZ Flu A +

93 B, 300 µl of sample was mixed with 4 drops of extraction reagent, 3 drops of the mixture was added to the

94 well. The result was read after 15 minutes. The Espline influenza A + B used 40 µl of sample added directly

95 to the well with the result being read after 10 minutes.

96

97 2.4 Viral culture

98 Madin-Darby canine kidney (MDCK) cell monolayers grown in culture tubes were inoculated with 200 µl of

99 sample and incubated at $35^o$C for 1 hour. The cells were fed with 1 ml of serum-free minimum essential

100 medium containing TPCK (tosylsulfonyl phenylalanylchloromethyl ketone)-treated trypsin (2µg/ml) (Sigma,

101 St. Louis, Mo) and antibiotics (Garamycin, 0.02 mg/ml, Schering-Plough Corporation, Heist-op-den-Berg,

102 Belgium; Penicillin-streptomycin, 100 units/ml, GibcoBRL, NY, USA; Nystatin, 20 units/ml, Sigma, St.

103 Louis, Mo). The cultures were harvested when cytopathic effect (CPE) was observed or after 10 days

104 incubation period for direct immunofluorescent antigen test as described previously (Chan et al., 2008).

105

106 2.5 Direct immunofluorescence antigen test

107 The direct immunofluorescence antigen  test was carried out with nasopharyngeal aspirate specimens

108 according to method described previously (Chan et al., 2008). Briefly, the nasopharyngeal aspirate was

109 centrifuged, and the cell pellet was washed in phosphate-buffered saline. The cell pellet was then spotted on

110 6-mm wells of Teflon-coated slides, air dried, and fixed in ice-cold acetone for 10 minutes. The smears were

111 stained with IMAGEN[TM] influenza A and B reagents (Oxoid Limited, Hampshire, UK) and then viewed at a

112  magnification of 400 under epifluorescent illumination using the fluorescein isothiocyanate (FITC) filter of a

113  Nikon fluorescent microscope.

114

115

116  **3.  Results**

117  3.1 Laboratory tests for diagnosis of influenza A infections

118  Ninety-seven nasopharyngeal aspirates collected were tested by RT-PCR for amplification of matrix (M) and

119  subtype-specific hemagglutinin (H) gene assays for diagnosis of influenza A infection. Direct

120  immunofluorescent antigen test and culture were done on all specimens for influenza A. Of these 97

121  influenza A positives, 30, 31 and 36 were identified as pH1N1 2009, sH1N1 and sH3N2 respectively.

122

123  3.2 Rapid influenza antigen detection kits performance

124  The data presented in Table 1 shows that the Directigen EZ Influenza A+B and the Espline Influenza A+B

125  exhibited apparently higher levels of sensitivity for detection of pH1N1 2009 (63.3%), compared with the

126  QuickVue A+B (53.3%) and the BinaxNow (43.3%) but this difference is not statistically significant

127  (Chi-squared test, p = 0.6). However, the four RDA tests have the similar levels of sensitivity for detection

128  of sH1N1 (71.0%) or sH3N2 (66.7%-72.2%).

129

130

131    3.3 Viral load of influenza A subtype in nasopharyngeal aspirate

132    The mean of viral load of Influenza A RNA subtype sH3N2 in nasopharyngeal aspirate samples was the

133    highest when compared with influenza A subtype sH1N1 or pH1N1 (Table 2) and these differences were

134    statistically significant (pH1N1 vs sH3N2 p=0.033; pH1N1 vs sH1N1 p=0.047). The limit of detection for

135    each rapid antigen test is defined as the viral load at which $\geq$ 95% of specimens were positive in that test.

136    Therefore the lower limit of viral load detection for each influenza A subtype (pH1N1, sH1N1 and sH3N2

137    by RDA was as follows: the Directigen EZ Influenza A+B ($1.1 \times 10^7$, $4.4 \times 10^6$ and $1.1 \times 10^7$); the Espline

138    Influenza A+B ($1.1 \times 10^7$, $4.4 \times 10^6$ and $1.1 \times 10^7$); the QuickVue Influenza A+B ($1.5 \times 10^7$, $4.4 \times 10^6$ and

139    $4.5 \times 10^6$) and the BinaxNOW Influenza A+B ($3.5 \times 10^7$, $4.4 \times 10^6$ and $4.5 \times 10^6$) (Fig 1). The average lower

140    limit for detection of each subtype by these rapid antigen assays is pH1N1 ($1.8 \times 10^7$), sH1N1 ($4.4 \times 10^6$)

141    and sH3N2 ($7.8 \times 10^6$) (Table 2).

142

143    3.4 Direct immunofluorescence antigen and culture performance

144    Sensitivity for detection of influenza A subtype by direct immunofluorescence antigen test for sH1N1,

145    pH1N1 and sH3N2 infections was 66.7%, 87.1% and 77.8% respectively (Table 2). Virus isolation from all

146    the samples was attempted on MDCK cells in the presence of TPCK treated trypsin. The isolation rate was

147    similar among the three influenza A subtypes (Table 2).

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149

## 4. Discussion

Previous report showed that these rapid antigen assays had comparable sensitivity to detect pH1N1 and sH1N1 using cell culture grown viruses (Chan et al., 2009). To further understand the analytical sensitivity of these rapid assays in clinical settings, the performance of rapid antigen assays on nasopharyngeal aspirate samples was assessed and compared with viral load by RT-PCR assays. The clinical diagnostic sensitivity of rapid antigen assays for detection of influenza depends on the quality, quantity, site and viral load of clinical specimens used in the assay method as well as its analytical sensitivity (Chan et al., 2007). The sensitivity of all these assays was comparable for the detection of sH1N1 and sH3N2 respectively. Any marginal difference in sensitivity between tests may be related to the volume of sample recommended for use in the assay methods. It was reported that larger test volumes gave rise to more sensitive methods (Chan et al., 2007). On the contrary, there was significant variation in the ability of these four assays to detect pH1N1 (Table 1). The Directigen EZ Influenza A+B and the Espline Influenza A+B were the most sensitive among the assays evaluated. These findings are also observed in previous study using culture infected cells (Chan et al., 2009; Hurt et al., 2009).

The influenza A M gene copy number in each clinical sample by RT-PCR have been determined. The highest RNA M gene copy number was found in patients with sH3N2 (2.5 to 5 folds higher) than for patients with sH1N1 or pH1N1 infection. The detection limits of the rapid antigen assays for determination of these subtypes are comparable (Fig. 1). The influenza A subtypes in the sample will generally not be detectable by

169  the rapid antigen assays if the viral load is below $1.0 \times 10^7$ copies per ml (Table 2). These clinical derived

170  detection limits are comparable with the detection limits using laboratory culture isolate (Chan et al., 2009).

171

172  The rapid antigen assays were shown to have better performance for the detection of human seasonal

173  influenza A than pH1N1 A in this study. Similarly, the direct immunofluorescence antigen test also shows

174  the highest sensitivity for detection of human seasonal influenza A than pH1N. Since their detection limits

175  for identification of these influenza A subtypes were comparable, the difference in clinical sensitivity is

176  likely to be related to the viral RNA load present in the sample. However, whether there are differences in

177  the affinity of the antibodies used in these different assays that may contribute to these differences in

178  performance cannot be excluded but such differences was not noted in the analytical sensitivity evaluation

179  using cultured virus (Chan et al., 2009).

180

181  Epidemiological and virological studies of the pH1N1 2009 have identified several risk factors for severe

182  infection, including host predisposing factors e.g. extremes of age, chronic underlying diseases, pregnancy,

183  obesity; viral factors and specific mutations of viral proteins such as the D222G mutation in the

184  hemagglutinin (Chen et al., 2010; Lapinsky et al., 2010; Louie et al., 2011). There are also differences in the

185  type of specimen used, with tracheal aspirates giving higher diagnostic yield that nasopharyngeal aspirates in

186  patients who are more seriously ill (Lee et al 2011).

187

188   Rapid point of care antigen detection tests continue to be used for clinical care, especially in out-patient

189   settings and for diagnosing and controlling influenza outbreaks in institutions. It is therefore important to

190   define the clinical diagnostic performance characteristics of these rapid antigen assays. The present studies

191   indicate that the lower clinical sensitivity of rapid antigen assays for pandemic influenza A H1N1 2009

192   infection is associated with lower viral load found in these patients.

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272 **Legend**

273 Table 1 Performance of the four rapid antigen assays for detection of different influenza A subtypes

274

275 Table 2 Compare different methods for diagnosis of influenza infections

276

277 Fig. 1 Viral RNA M gene copy number present in patient with pH1N1, sH1N1 and sH3N2 are denoted

278 together with the rapid antigen test results for each test kit. The limit of detection for each rapid antigen test

279 is denoted by a horizontal line as the viral load at which $\geq 95\%$ of specimens were positive in that test.

280

281    Table 1 Performance of the four rapid antigen assays for detection of different influenza A subtypes

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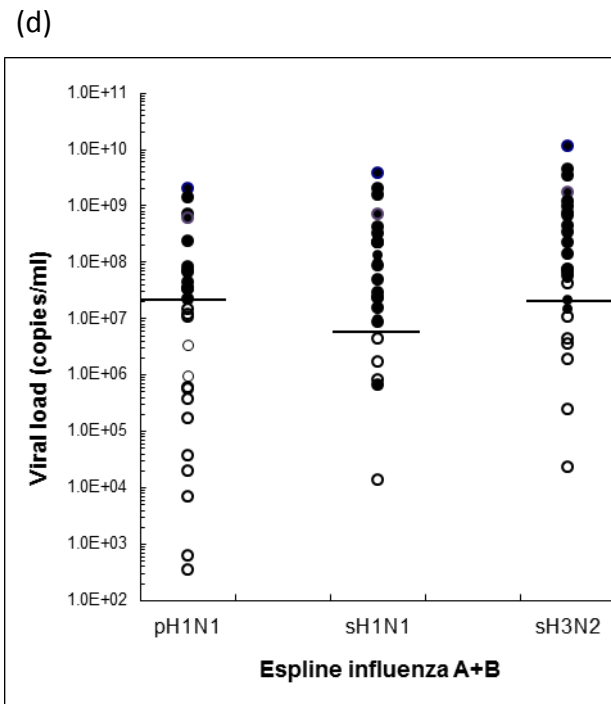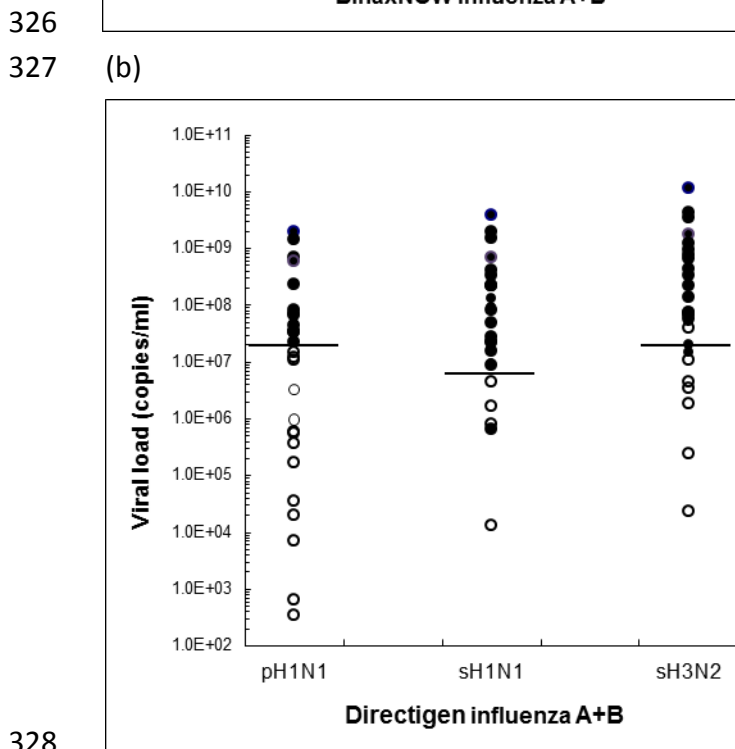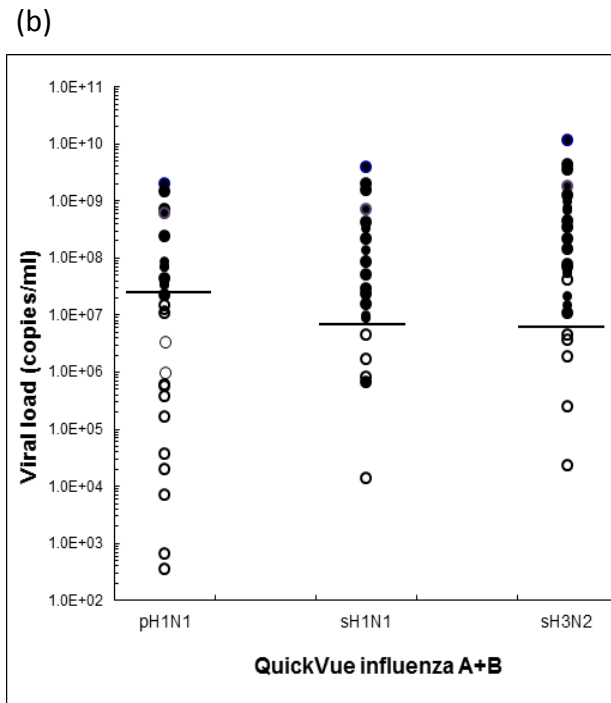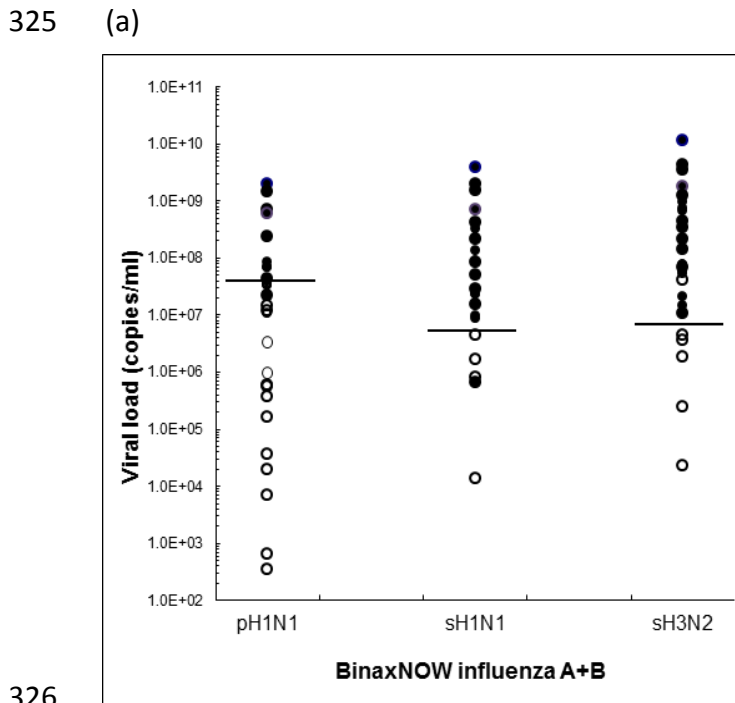| Influenza A subtypes (No. of patients) | BinaxNOW Influenza A+B | QuickVue Influenza A+B | Directigen EZ Influenza A+B | Espline Influenza A+B |
|---|---|---|---|---|
| pH1N1 2009 = 30 | 43.3% | 53.3% | 63.3% | 63.3% |
| sH1N1 =31 | 71.0% | 71.0% | 71.0% | 71.0% |
| sH3N2 =36 | 66.7% | 72.2% | 69.4% | 69.4% |

283
284

285 Table 2   Compare different methods for diagnosis of influenza infections

286

| Influenza A subtype = No. of patients | Mean of viral load (copies/ml) (Range) | IF | Culture | Rapid detection assays Mean of (Detection limit) | |
|---|---|---|---|---|---|
| pH1N1 2009 =30 | $1.83 \times 10^8$ $(6.37 \times 10^2 - 2.00 \times 10^9)$ | 66.7% | 96.7% | 55.8% $(1.8 \times 10^7)$ | 290<br>291<br>292 |
| sH1N1 =31 | $3.89 \times 10^8$ $(5.40 \times 10^4 - 3.40 \times 10^{10})$ | 87.1% | 97.0% | 71.0% $(4.4 \times 10^6)$ | 293<br>294<br>295 |
| sH3N2 =36 | $9.63 \times 10^8$ $(2.34 \times 10^4 - 1.17 \times 10^{10})$ | 77.8% | 97.2% | 69.4% $(7.8 \times 10^6)$ | 296<br>297<br>298 |
| Overall Mean | 5.12E+08 | 77.2% | 97.0% | 65.4% $(1.0 \times 10^7)$ | 299<br>300<br>301 |

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| Influenza A subtype | Mean of viral load | IF | Culture | Rapid detection assays |

321 Fig. 1 Viral RNA M gene copy number present in patient with pH1N1, sH1N1 and sH3N2 are denoted
322 together with the rapid antigen test results for each test kit. The limit of detection for each rapid antigen test
323 is denoted by a horizontal line as the viral load at which ≥ 95% of specimens were positive in that test.
324
325 (a)                                                              (b)



326
327 (b)                                                              (d)



328
329 ——— : Detection limit

330 ● Positive

331 o Negative

332