Evaluations of serological test in the diagnosis of 2019 novel coronavirus (SARS-CoV-2) infections during the COVID-19 outbreak

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Abstract

The ongoing SARS-CoV-2 outbreak has killed over twenty-one thousand and sickened over four

hundred thousand people worldwide, posing a great challenge to global public health. A sensitive and

accurate diagnosis method will substantially help to control disease expansion. Here, we developed a

chemiluminescence-immunoassay method based on the recombinant nucleocapsid antigen and the

magnetic beads for diagnosis of SARS-CoV-2 infections and surveillance of antibody changing pattern.

25   Serums from 29 healthy individuals, 51 tuberculosis patients, and 79 SARS-CoV-2 confirmed patients

26   were employed to evaluate the performance of this approach. Compared to the IgM testing, the IgG

27   testing was more reliable in which it identified 65 SARS-CoV-2 infections from the 79 confirmed

28   patients and only two false-positive cases from the 80 control group with a sensitivity and specificity

29   reaching 82.28% and 97.5%, respectively. However, only a slight difference (not statistically

30   significant) in the detected cases of SARS-CoV-2 infections was observed between the IgM and IgG

31   testing manner in patients at a different time of onset of disease. A performance comparison

32   between an ELISA kit using the same nucleocapsid antigen and our chemiluminescence method was

33   undertaken. The same false-positive cases were seen in both methods from the paired control group,

34   while ELISA kit can only detect half of the SARS-CoV-2 infections from paired SARS-CoV-2 confirmed

35   patients group than that of the chemiluminescence method, indicating a higher performance for the

36   chemiluminescence-immunoassay approach. Together, our studies provide a useful and valuable

37   serological testing tool for the diagnosis of SARS-CoV-2 infections in the community.

38   **Keywords:** SARS-CoV-2, serological testing, chemiluminescence immunoassay, IgM and IgG

39   ## Introduction

40   *Coronavirus*, belonging to the family of *Coronavirdiae* and order of *Nidovirales*, is a group of

41   enveloped, non-segmented positive-sense RNA virus that has been reported to be able to infect

42   humans and a wide range of animals including cattle, swine, chicken, cat, horse, camels, rodent, bats

43   and snakes and so forth (1-3). Based on the genetic properties, coronavirus was further divided into

44 four genera, namely *Alphacoronavirus* , *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*

45 *(4).* Prior to December 2019, a total of six coronaviruses have been documented to be capable of

46 causing disease in humans. These include two strains from *Alphacoronavirus* (HCoV-229E and HKU-

47 NL63) and four from *Betacoronavirus* subfamily (HCoV-OC43, HCoV-HKU1, SARS-CoV and MERS-CoV)

48 (5-10). Among them, the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and Middle East

49 Respiratory Syndrome coronavirus (MERS-CoV) are the most well-described as they directly led to two

50 deadly large-scale outbreaks globally, with 8,096 cases infections and roughly 10 percent mortality

51 and 2,494 cases and 34.4 percent mortality, respectively(9, 10).

52 Recently, the outbreak of a severe pneumonia COVID-19 was confirmed to be caused by the 2019

53 novel coronavirus infections (SARS-CoV-2) that was originated from a seafood wholesale market in

54 Wuhan, China(11). So far, this novel coronavirus has spread throughout the whole of China and over

55 198 countries globally, causing over468,905 laboratory-confirmed cases of infections with 21,200

56 people dead posing a great threat to the global public health (http://2019ncov.chinacdc.cn/2019-nCov/).

57 Besides, there are still numerous suspected cases and a myriad of medical monitoring people who

58 were quarantined in specialized hospitals or at homes because of their previous epidemiological link

59 to confirmed SARS-CoV-2 patients. All of these put an extreme burden on the emergency, hospital

60 and public health system particularly the epidemic zone worldwide. Therefore, a timely, sensitive and

61 accurate diagnosis approach is urgently needed and of pivotal importance for surveillances of disease

62 dissemination and the prevention of further expansions. Conventional diagnosis methods such as

63 virus culture and microscopic analysis are generally time-consuming and labor-intensive with limited

64 sensitivity (12, 13). In contrast, the last decade emerged molecular biologic and serologic approaches,

65   such as TaqMan Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR), Enzyme-linked

66   immunosorbent assay, colloidal gold immunochromatography and direct chemiluminescence

67   immunoassay(CLIA), can be developed into a rapid and effective tool for detections of respiratory

68   pathogens infections, even though in certain circumstances molecular biologic method like RT-PCR

69   had a low sensitivity for specimens from upper respiratory tract (14-17).

70   In this study, we developed a chemiluminescence immunoassay method to specifically detect the

71   induced antibody IgM and IgG by SARS-CoV-2 using the recombinant nucleocapsid (YP_009724397.2)

72   and evaluate its sensitivity and specificity in detections of SARS-CoV-2 infected patients. High

73   sensitivity and specificity results indicate this chemiluminescence immunoassay method in

74   combination with RT- PCR method can serve as highly sensitive and accurate tools for the diagnosis

75   and screen of SARS-CoV-2 infections in the community.

Material and methods

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Participants and specimens

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78   In Shenzhen city, China, patients infected by SARS-CoV-2 were all eventually admitted into a

79   specialized hospital (the third people's hospital of Shenzhen) for quarantines and treatments. In this

80   study, a total of 29 healthy individuals, 51 tuberculosis patients and 79 SARS-CoV-2 patients were

81   enrolled for serological testing. Twenty-nine healthy people were recruited from the Shenzhen

82   University aging from 16 to 72 years old. Fifty-one tuberculosis patients were enrolled from the

83   Shenzhen Baoan hospital, and their mycobacterium tuberculosis infections were previously confirmed

84   by sputum smear acid-fast-bacilli analysis, chest radiograph and the QuantiFERON®–TB Gold test.

85   COVID-19 patients were randomly enrolled from the third people's hospital of Shenzhen, and their

86   SARS-CoV-2 infections were confirmed by combinations of epidemiological risk, clinical features and

87   positive detections of SARS-CoV-2 RNA in respiratory specimens using the National Medical

88   Production Administration authorized GeneoDX kit according to the official instruction for diagnosis

89   and treatment of 2019 novel coronavirus infections issued by the National Health Commission of the

90   People's Republic of China. All healthy cohorts and tuberculosis patients had no epidemiological risks,

91   and they were persistently negative for SARS-CoV-2 RNA detections in at least three respiratory

92   specimens' tests using above mentioned GeneoDX kit. Peripheral blood samples were collected into

93   EDTA and sodium heparin containing tubes, and then the serum was separated by centrifugations

94   (800g ×10 minutes) for immediate testing or stored at −80°C until used. Verbal Informed consent was

95   obtained from all individual participants.

96   **SARS-CoV-2 Nucleic acid**

97   Total nucleic acid for collected respiratory specimens was extracted in BSL-3 laboratory using the

98   nucleic acid extraction and purification kit from Huayin Bio-Tech (Shenzhen, China) according to the

99   manufacturer protocol. Detections of SARS-CoV-2 RNA were performed using the National Medical

100   Production Administration authorized GeneoDX kit (Taqman RT-PCR method, targeting the ORF1ab

101   and N genes) according to the manufacturer instructions.

102   **Development of chemiluminescence immunoassay and test procedures**

103   After the transcription of extracted SARS-CoV-2 genome RNA into the cDNA, the coding regions

104   (YP_009724397.2) were then amplified and cloned into the pET30a vector. The recombinant full-

105   length nucleocapsid antigen was expressed in engineering *E*. coli BL21 (DE3) strains and purified using

106   the Ni-NTA resin (Darui Biotech, China). Magnetic beads Magnosphere™ MS300 used in this study are

107   commercially available in the JSR Corporation, Tokyo, Japan. Recombinant nucleocapsid antigens

108   were coupled to these tosyl magnetic beads using the catalytic reagent solution (3M Ammonium

109   sulfate / 0.1M Borate buffer, pH9.5) according to the manufacture's instruction, and the resultant

110   beads were further blocked by 0.05% BSA for six hours at 37 °C. The following testing and detection

111   procedure was automated on a chemical immuno-luminescence analyzer ACCRE6 (Tianshen Tech,

112   Shenzhen, China). It was comprised of those following steps. 50 microliter pure serum was firstly

113   incubated with the magnetic beads that were coupled with antigens for 5 minutes at 37 °C.

114   Subsequently, the unbound substance was gently removed and then washed by Tris buffer for three

115   times. Alkaline phosphatase labeled anti-human immunoglobulin (50μg/ml) was added and further

116   incubated for 5 minutes at 37 °C in the Mes Buffer. After three times washing to remove unbound

117   materials, the lumigen APS-5 substrate (50ug/ml) was eventually added. Ultimately, the light signal

118   was measured by the photomultiplier in ACCRE6 (Tianshen Tech, Shenzhen, China) as relative light

119   units, and the whole testing can be completed in 23 minutes. Confirmed SARS-CoV-2 positive-serum

120   and negative-serum were used as controls in each set test.

121   **Detections of IgG and IgM by a commercial ELISA kit**

122   In parallel testing, the commercial enzyme-linked immunosorbent assay kit (Darui Biotech, CHINA) for

123   detections of the anti-SARS-CoV-2 IgG and IgM antibody was used to measure the SARS-CoV-2

124   antibody level in above mentioned COVID-19 patients and control individuals. For the principle of this

125   ELISA kit, the specific SARS-CoV-2 nucleocapsid protein and anti-human IgM monoclonal antibody

126   were firstly coated on the plates, respectively. Subsequently, the 100 µl of 100- fold diluted serum

127   was added and then incubated for 60 minutes at 37 °C. After five times washing by PBST buffer, the

128   horseradish peroxidase (HRP) labeled mouse anti-human IgG antibody or HRP-labeled SARS-CoV-2

129   nucleocapsid antigen was added for 30 minutes incubation at 37 °C. Fifty microliter TMB substrate

130   was then added for 15 minutes incubation after the second time washing by PBST buffer. The

131   stopping solution was eventually added to suspend the reaction, and OD 450/630 values were

132   immediately measured using the Varioskan LUX™ Multimode Microplate Reader. The cutoff values for

133   positive were set based on the manufacturer's recommendations.

134   **Ethical statement**

135   The internal use of collected samples for diagnoses of etiological agents and serological research was

136   approved by the Ethical Committee in the third people's hospital of Shenzhen (SZTHEC2016001).

137   **Statistical analysis**

138   All statistical analysis was performed in GraphPad Prism 7 software. The One-way ANOVA test was

139   used to analyze the average RLU values difference between different participant groups. The chip-

140   square and Fisher's exact test was used for comparing the difference between the analyzed groups.

141   The difference was considered significant when a p-value is < 0.05.

142

143   # Results

144   **Detections of IgG and IgM antibodies induced by SARS-CoV-2 in serum and overall specificity and**

145   **sensitivity assessments**

146 To assess the specificity and sensitivity of the chemiluminescence immunoassay method developed

147 based on the recombinant nucleocapsid antigen, serum from 29 healthy individuals, 51 tuberculosis

148 patients and 79 confirmed SARS-CoV-2 patients were employed and tested. More than six-fold and

149 eight folds higher average RLU (relative light units) values were observed in the SARS-CoV-2 patients

150 group in the IgM testing compared to that of the healthy cohort and tuberculosis patients (Figure 1A).

151 This average RLU difference is more dramatic when it comes to the IgG testing reaching 60 and 70

152 fold increase in SARS-CoV-2 patients in comparison with the healthy and tuberculosis group,

153 respectively (Figure 1B). A Receiver Operating Characteristic curve was then obtained based on these

154 RLUs for the SARS-CoV-2 patients group and control group that consists of healthy cohort and

155 tuberculosis patients. According to the ROC curve and analysis results, we recommend a cutoff setting

156 for IgM (RLU 162296) and IgG (336697), in which the calculated sensitivity and specificity for IgM were

157 82.28% and 81.25%, and 82.28% and 97.5% for IgG, respectively.

158 Based on this cutoff and using the IgM testing, we identified 15 cases and 65 cases as SARS-CoV-2

159 positive from the control group (80 cases) and the SARS-CoV-2 confirmed group (79 cases),

160 respectively (Table 1). In contrast, using the IgG testing, we only detected two false-positive cases

161 from the control group, which is in line with the higher specificity for IgG (97.5%) compared to that of

162 the IgM testing (81.25%) as above described.

163

164 **The links between disease onset time, ages and IgM and IgG productions and detection efficiency**

165   To explore whether the onset time was significantly linked with the detection sensitivity by this

166   serological chemiluminescence method, comparison and statistical analysis of the sensitivity rates

167   between different onset time patient categories was undertaken. No statistically significant

168   difference was observed between the IgM and IgG testing results in the patients with the same onset

169   time, although two more cases from 12 cases were detected by IgM testing compared to that of IgG

170   testing in patients less than the one-week onset of disease (Table 2, p-value >0.05). In stark contrast,

171   two more cases SARS-CoV-2 patients were identified by IgG testing than that of IgM testing in patients

172   with more than two weeks onset of disease (p-value >0.05). In addition, we also compared the

173   detection rates between the different age groups people, and we found that a significantly lower

174   detection rate in both IgM and IgG testing manner for individual group younger than 18 years old was

175   observed compared to that of people aging from 18 to 65 (p-value < 0.01) or over 65 years old (p-

176   value >0.05) (Supplementary Table S1). No statistically significant differences were observed for male

177   and female groups as well.

178

179   **Comparisons with other ELISA kit**

180   To further characterize the patient's immune response to the SARS-CoV-2 antigens and to prove the

181   feasibility of the practical application of this serological testing kit in clinical diagnosis, 64 paired

182   serum from the above-mentioned control cohorts and 65 COVID-19 patients were also examined

183   using a recently developed commercial available ELISA Kit. A total of 14 false-positive cases (21%)

184   were identified by IgM testing in both methods. A very lower false-positive rate was observed in IgG

185   testing in both methods. Compared to the ELISA kit, a significantly higher detection rate for SARS-CoV-

186   2 in both IgM and IgG testing manners was seen in our chemiluminescence method, suggesting a

187   higher sensitivity of our approach compared to the tested ELISA kit (Table 3, p-value < 0.001).

188   **Discussion**

189   Compared with the conventional virological methods, the molecular biologic TaqMan RT-PCR method

190   has been widely used for clinical diagnosis of respiratory pathogens infections particularly in the initial

191   phase of disease because of its high specificity property (18, 19). Nevertheless, a relatively low

192   sensitivity (30%- 50%) in single upper respiratory specimen testing has been commonly reported

193   including the well-appreciated methods for SARS-CoV detections (20). Furthermore, since the SARS-

194   CoV-2 expansion from 2020 January, several cases reported that consecutive negative detections of

195   SARS-CoV-2 RNA were observed for upper respiratory specimens testing like throat swabs in patients

196   with apparent clinical symptoms, and the positive results can only be achieved by collecting the

197   bronchoalveolar lavage fluid for re-testing. Hence, a sensitive serological diagnosis method can serve

198   as a very useful compensation tool for current clinical diagnosis situations. Our results demonstrated

199   that a single IgG testing is feasible in the clinical diagnosis for SARS-CoV-2, as a higher specificity and

200   sensitivity were observed in our chemiluminescence method. In the humoral immune response, the

201   antibody IgM was generally produced earlier than the IgG isotype as the IgM can be expressed

202   without the isotype switching. Unexpectedly, we only observed a slight detection rate difference (not

203   statistically significant) between these two antibody isotype testing manner in patients in the first

204   week or more than two weeks after onset of disease. However, compared to the IgG approach, our

205   IgM method showed a lower specificity (higher false-positive cases) in our testing. As the IgM and IgG

206   using the same pure recombinant antigen and coupling condition (supplementary figure S1),   the

207   detection specificity difference is more likely linked to intrinsic antibody traits and concentration

208   differences in the patients' blood.

209   We noted that four patients with clinical symptoms less than four days were simultaneously detected

210   by both IgM and IgG testing. A close examination of their medical record reveals that all of them had

211   previous contact with confirmed SARS-CoV-2 individuals in at least 16 days ago, pointing to the

212   possibility that they were probably asymptomatically infected by SARS-CoV-2 for certain time already.

213   Fourteen cases (17.7%) from 79 SARS-CoV-2 confirmed patients were not identified by our serological

214   testing method (both the IgM and IgG manner). Interestingly, of them, seven people were younger

215   than 8-year-old or over 70-year-old. These people generally have low immunity in which a clinical

216   symptom may occur rapidly upon exposure to the SARS-CoV-2, and we speculate that the antibodies

217   in these people may not develop well yet when testing. More investigations are warranted to

218   uncover the real situations. When comparing the detection rates in different age groups by our

219   method, we noted that a significantly lower detection rate in both IgM and IgG testing manner for the

220   individual group younger than 18 years old was observed compared to that of people aging from 18 to

221   65. An in-depth look at the days after onset for these 12 individuals younger than 18-years-old, the

222   symptom onset time for all the 12 people are less than 14 days with six people even less seven days

223   (Supplementary Table S2). The lower detection rate for these 12 people younger than 18-years-old

224   was likely associated with no or less production of antibodies in them yet when we collected the

225   serums.

226   In our parallel performance testing, the same antigen of nucleocapsid protein was used in both the

227   commercially available ELISA kit and our chemiluminescence immunoassay. Unexpectedly, a

228   significantly higher sensitivity was observed in our method compared to the ELISA kit. This sensitivity

229   difference may be partially attributed to the difference in the serum amount for the first incubation

230   step. On the other side, the intrinsic method difference including the aspect of binding surface

231   interaction and mode of separations of the unbound material can also contribute to the sensitivity

232   difference in the chemiluminescence immunoassay and ELISA method.

233   In conclusion, in this study, we developed and evaluated a serological chemiluminescence

234   immunoassay testing technique for clinical diagnosis of SARS-CoV-2 infections using the recombinant

235   nucleocapsid antigen. This high sensitivity and specificity chemiluminescence immunoassay method

236   combined with the RT-PCR method can doubtless significantly improve the clinical diagnosis for SARS-

237   CoV-2 and contribute to the control of COVID-19 expansion globally.

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245

246   Figure 1. Detections and measurements of the SARS-CoV-2 IgM and IgG antibody in healthy people,

247   tuberculosis patients and SARS-CoV-2 confirmed patients using the chemiluminescence immunoassay method

248   (A and B). The average results were expressed as mean ± SEM of all individuals. Receiver Operating

249   Characteristic curves for IgM (C) and IgG(D) were obtained based on the RLU for the SARS-CoV-2 patient group

250   and the control group consisting of healthy cohorts and tuberculosis patients.

251   Supplementary figure S1. SDS-PAGE of the purified recombinant nucleocapsid antigen, M band indicates the

252   marker, 1-3 band are the purified recombinant nucleocapsid protein.

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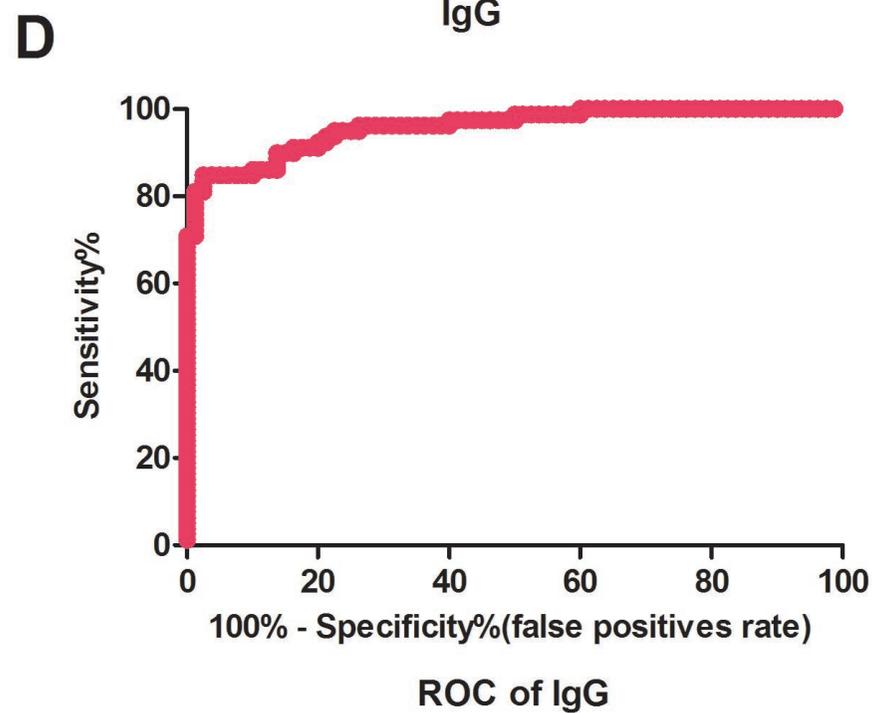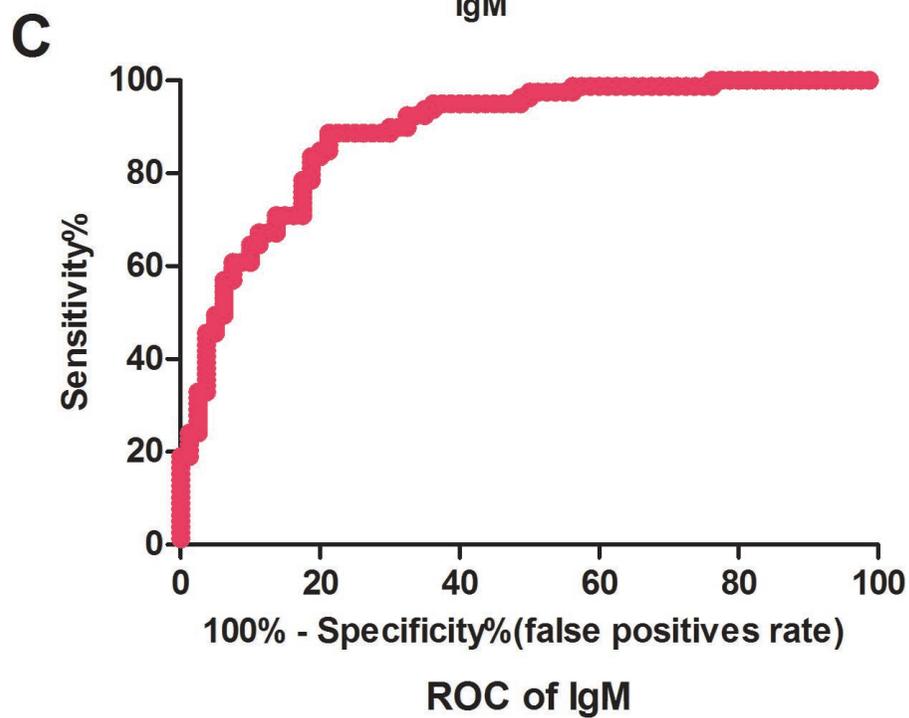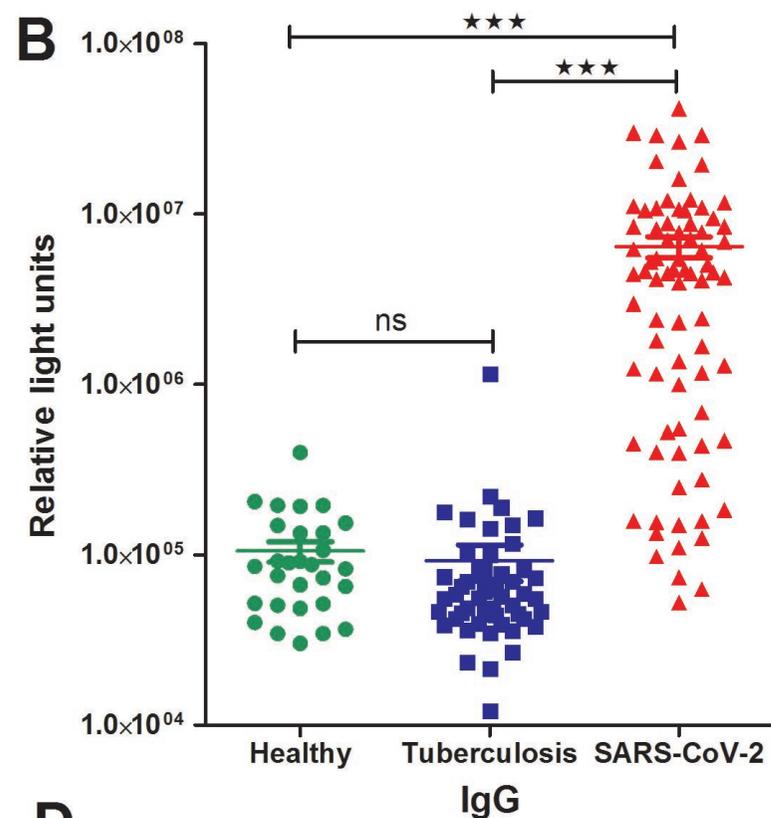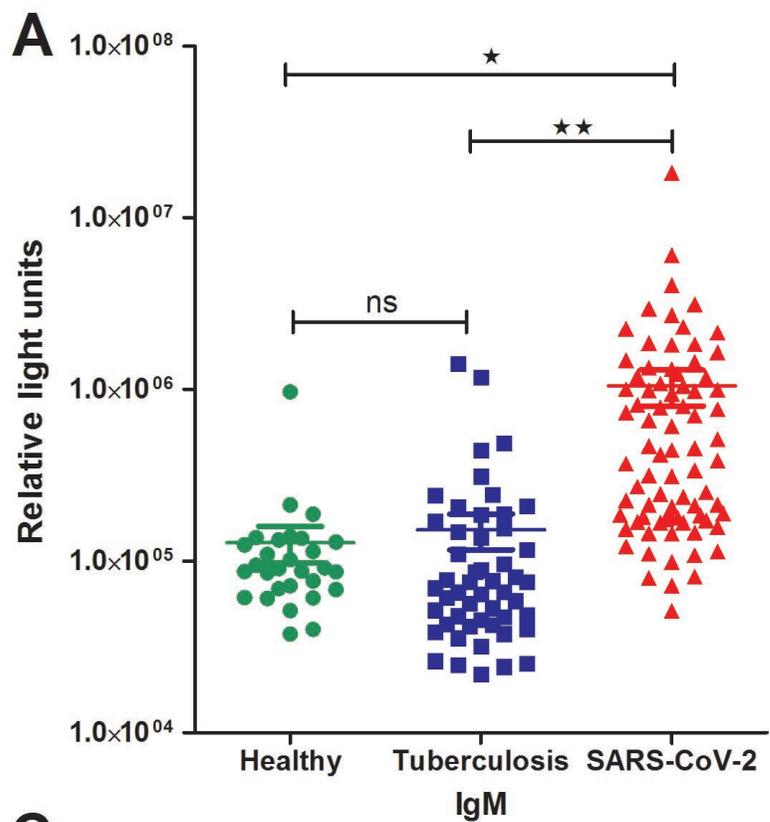
Table 1 Evaluations of a chemiluminescence immunoassay method for diagnosis of SARS-CoV-2 by detections of specific IgM and IgG in the patient's serum.

Participants category /total cases	IgM positive cases / (%)	IgG positive/ Cases / (%)	IgM or IgG positive cases/ (%)	IgM and IgG positive cases (%)
Healthy cohorts and tuberculosis patients/**80 cases**	15 /18.75%	2 /2.50%	16 /20.00%	1 /1.25%
SARS-CoV-2 confirmed patients /**79 cases**	65 /82.28%	65 /82.28%	72 /91.14%	58/73.42%

Table 2 Comparison of SARS-CoV-2 detections results in patients with different onset time between the IgM and IgG approach

Days after onset	Total cases	IgM positive cases / (%)	IgG positive/ cases / (%)	IgM or IgG positive cases/ (%)	IgM and IgG positive cases (%)
0-3	4	4/100%	4/100%	4/100%	4/100%
4-7	8	6/75%	4/50%	6/75%	4/50%
8-14	33	24/72.73%	24/72.73%	29/87.88%	19/57.58%
>14	34	31/91.18%	33/97.06%	33/97.06%	31/91.18%

Table 3 Detection differences between the chemiluminescence and ELISA method

Methods	Control group (total 64 cases)		SARS-CoV-2 confirmed patients (total 65 cases)	
	IgM/false-positive /%	IgG/ false-positive /%	IgM positive /%	IgG positive /%
ELISA	14 /21.8%	0 / 0%	30 / 46.1%	15 / 23%
chemiluminescence	14 /21.8%	2 / 3.1%	55 / 84.6%	53 / 81.5%
Identified in both	3 / 4.6%	0 / 0%	28 / 43%	15 / 23%