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1 **A meta-analysis and experiment assessing phage-based FMDV vaccine**

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15 Abstract: Foot-and-mouth disease (FMD) is a pathological disease caused by the foot-

16 and-mouth disease virus (FMDV), which mainly affects cloven-hoofed animals. This

17 study was conducted to a meta-analysis and experiment on the effect of bacteriophages

18 used in the development of FMDV vaccines. A systematic search was conducted for

19 the collection of the protection effect for the phage-based FMDV vaccine using

20 sensitive search strategies. The extracted data were analyzed using Rev-Man 5.4

21 software. This experiment used the T7 phage to express the capsid protein VP1 of the

22 OHM-02 strain, and the recombinant VP1 phage was termed OHM-T7. Antibodies and

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23 cytokines levels were assessed after immunizing BALB/C mice with OHM-T7. The
24 results showed that a total of 115 articles were retrieved, and 4 of them met the inclusion
25 criteria. There was no heterogeneity with $I^2 = 0\%$, 20% or 43%. We used a fixed-effect
26 model for meta-analysis, and the results showed a protective effect on FMDV between
27 the phage group and control group ($P<0.01$) and between FMDV group and control
28 group ($P<0.01$). Furthermore, when the phage group was compared to the FMDV group,
29 there was also no significant difference ($P>0.05$). After successfully obtained the ohm-
30 t7 strain and immunized the mice, it could induce high levels of IFN-γ levels in mice
31 with little effect on IL-4 levels. OHM-T7 could be used to detect antibodies produced
32 by mice immunized with different FMDV antigens and produce high levels of anti-
33 FMD antibodies. In summary, these results showed the potential of phage-based FMDV
34 vaccines in FMDV prevention.

35 **Introduction**

36 Foot-and-mouth disease (FMD) is a pathological disease caused by the foot-and-mouth
37 disease virus (FMDV), which affects cloven-hoofed animals [1,2]. FMD causes serious
38 economic and social problems, and is listed as a legally reportable disease by the World
39 Organization for Animal Health (OIE) [3]. The virus has no capsule and has a diameter
40 of about 30 nm. FMDV can infect cloven-hoofed animals, including pigs, sheep, goats,
41 cattle and diverse wildlife species, and remains a major threat to the livestock industry
42 worldwide [4]. Cattle could spread FMDV to pigs [5]. O-type FMDV is the most
43 popular subtype around the globe. Currently, there is no vaccine that could protect
44 animals from all serotypes [6]. Therefore, new vaccines are urgently needed to prevent

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45 the spread of FMD [7]. The capsid protein VP1 of FMDV is a sequence-dependent
46 epitope, is the main antigenic site of FMDV, and can induce neutralizing antibodies.
47 Microphage (Phage) has been researched for decades [8]. Smith confirmed in 1985
48 that exogenous DNA could be inserted into the filamentous phage gene III and fused to
49 the pIII protein [9]. The phage display technology inserts a DNA sequence into the
50 phage coat protein's structural gene, allowing the foreign gene to be expressed along
51 with the coat protein [10-12]. Studies have shown that phages can be used to simulate
52 viral epitopes [13,14]. The asymmetry of phage can enhance the immune response of
53 helper T cell-1 and cause $CD4^{+}$T cells to secrete cytokines [15,16]. The current phage
54 display technology can insert DNA sequences into the structural genes of phage coat
55 proteins so that foreign genes and coat proteins can be expressed together [10,12,17].
56 Phage display technology has been shown to increase the stability and immunogenicity
57 of the antigen [18-20]. The phage display system is an ideal B cell epitope display
58 vector and recombinant virus-like particles (VLPs) [21]. Among them, VLPs were
59 found to be as immunogenic as the native virus, and the main reason for this is that the
60 capsids are more heat-labile [22]. Therefore, phage vaccine, as a particulate antigen,
61 can be quickly taken up by antigen presenting cells (APC). VLPs showed high
62 immunogenicity and are easily recognized by the immune system [23]. Phage particles
63 can also induce a strong cellular immune response [15]. Related studies have found that
64 the peptides displayed by T7 phage bind closely to FMD serum [24]. To develop an
65 effective FMD vaccine, we here conducted a meta-analysis and used a phage to display
66 the FMD VP1 protein.

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67 **Materials and Methods**

68 Literature search strategy

69 The literature retrieved in this meta-analysis was evaluated before March 2020 by two
70 researchers. The National Library of Medicine (Medline via PubMed), Embase, China
71 National Knowledge Infrastructure (CNKI), and Wan fang DATA were searched for
72 phage-based FMDV vaccines, using the keywords "FMDV," "phage," and "vaccine."

73 Inclusion and exclusion criteria

74 We used the following inclusion criteria: ① Published documents included Chinese
75 and English literature. ② FMDV was expressed by the phage vector. ③ The
76 protective effect of FMDV was evaluated in terms of the lethal dose. We used the
77 following exclusion criteria: ① Method dissimilar to FMDV vaccine. ② The carrier
78 was not a phage. ③ The results did not provide the necessary basic data.

79 Data extraction tired

80 The two researchers conducted a preliminary screening by reading the titles and
81 abstracts of the previous studies, then read the full text and made their selection
82 according to the inclusion and exclusion criteria. If there were differences of opinion,
83 we had already discussed and solved them. We independently extracted the data. The
84 data extracted included the first author, publication time, events, and total number of
85 animals in the trials.

86 Analysis of extracted data

87 The database was developed using Excel. RevMan 5.4 software was used in this meta-
88 analysis to perform the statistical analyses. The fixed-effect model was used for meta-

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89 analysis to calculate the odds ratios (OR), together with a 95% confidence interval (CI)
90 for dichotomous results. ORs were used to evaluate the difference in immunogenicity
91 between the two groups. A OR=1 indicates that data is of no worth. OR above 1.0
92 correspond to an effect favoring vaccination. Statistical heterogeneity between the
93 studies was assessed using the I^2 statistic and Q statistic. $P \geq 0.05$ or $I^2 \leq 50\%$ indicated
94 that the trials were free of heterogeneity, and a fixed-effect model was used to perform
95 the meta-analysis. $I^2 > 50\%$ led us to consider a random-effect model to perform the
96 meta-analysis. Where applicable, we presented results from individual trials and the
97 common effect estimate in a forest plot. Squares indicate individual study odds ratios
98 together with their 95% CI indicated as bars. Sensitivity analysis was performed using
99 the difference of the combined values of the model effects, and the funnel plot method
100 was used to evaluate the publication bias of the included works.
101 Evaluation of the quality of evidence
102 All of the included studies were animal experiments, and animal experiments may be
103 the highest level of evidence.
104 Construction of the recombinant vector
105 The VP1 gene plasmid of OMH-02 was doubles digested using *EcoR* I and *Hind* III
106 (TaKaRa, Dalian, China). In the control group, no enzyme was added; in the negative
107 control group, the enzyme was substituted by an equal volume of water. All samples
108 were incubated in a water bath at 37°C for 4 h. The digested product was subjected to
109 1% agarose gel electrophoresis. The target band was excised and purified with a DNA
110 gel recovery kit (TaKaRa, Dalian, China), according to the manufacturer's instructions.

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111 The extracted fragment of interest was ligated into the T7Select® vector in a reaction
112 (Merck KGaA, Darmstadt, Germany). The sample was added to a 1.5 mL EP tube,
113 gently pipetted up and down, incubated at 16°C for 16 h, and stored at 4°C.

114 Phage packaging and plaque assay

115 The T7Select® package extract was thawed on ice. Then, 5 μL of the extract was added
116 to 5 μL of the ligation reaction. The mixture was reacted at 22°C for 2 h, and 270 μL
117 of TB medium was added. The phage was supplemented to *E.coli* BLT5403 in the
118 logarithmic growth phase, and cultured at 37°C for 3 h. *E.coli* BLT5403 strain was
119 inoculated in M9TB medium and incubated at 37°C with shaking to OD_{600} =1.0. Molten
120 agarose was incubated in a water bath at 50°C. A 10^3-10^6 diluted sample was prepared
121 with sterile TB medium as a diluent. Next, 250 microliters of BLT5403 was added to
122 the EP tube, followed by addition of 100 μL of phage dilution and 3 mL of top agarose.
123 The mixture was poured into an agar plate, inverted and incubated at 37°C for 4 h. The
124 plaques were counted, and phage titers were determined.

125 Identification of OHM-T7

126 A single plaque was scraped off using a pipette tip and heated at 65°C for 10 min. The
127 sample was cooled to room temperature and centrifuged at 14,000 × g for 3 min.
128 Primers were: T7 Select-F 5′-GGAGCTGTCGTATTCCAGTC-3′and T7 Select-R 5′-
129 AACCCCTCAAGACCCGTTTA-3′, SDS-PAGE was run with 12% separation and 5%
130 stacking gels. Staining was performed with Coomassie Brilliant Blue staining solution.

131 OHM-T7 screening

132 A ninety-six-well ELISA plate was rinsed 3 times with deionized water. The Bovine

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133 FMD serum samples were diluted 30-fold with the ELISA coating solution, and 100 μL
134 was added per well of the ELISA plate, which was incubated overnight at 4°C. After
135 two washes with PBST, 200 μL/well of 5% skim milk powder was added and incubated
136 at 37°C for 2 h. The plates were washed 3 times with PBST, and OHM-T7 was added
137 for 2 h at 37°C. The cells were washed 3 times with PBST, and BLT5403 cells in the
138 logarithmic growth phase were added to the wells and cultured in a 37°C incubator for
139 1 h. The bacterial suspension was aspirated and added to 20 mL of BLT5403 culture in
140 the logarithmic growth phase, cultured at 37°C for 2 h. The library was preserved, and
141 the product was used for subsequent screening. The above experiment was repeated 3
142 times.

143 Immunization of mice

144 Twenty-four female BALB/C mice were purchased from Huaxing Laboratory Animal
145 Farm (Huiji Distract, Zhengzhou, China). All the experimental procedures involving
146 animals were approved by the Animal Experimental Ethical Committee Form of the
147 First Affiliated Hospital of Medical College, Shihezi University (No. A 280-163-01).
148 Female BALB/C mice were divided into the NaCl group, prokaryotic group and phage
149 group, with 8 mice in each group. In the NaCl group, 250 microliters of normal saline
150 were injected. The OHM-02 VP1 group was injected with prokaryote-expressed FMD
151 VP1 protein at 200 μg/mice in a volume of 250 microliters. The OHM-T7 group was
152 injected with 250 μL of OHM-T7 at a titer of 4×10^{11} Colony-Forming Units (CFU).
153 Serum was collected at 0, 14, 28, 42, 56, 70, 84 and 98 d, respectively, and antibody
154 levels were measured by ELISA. The OHM-T7 titer was 4×10^{11} CFU, and the sample

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155 was diluted 200-fold with the ELISA coating solution. IL-4 and IFN-γ were detected
156 using an ELISA kit (Solarbio, Beijing, China).
157 Statistical Analysis
158 The results of ELISA were analyzed with the SPSS 17.0 software (SPSS, Inc. Chicago,
159 IL, USA), and all other statistics were performed using the GraphPad Prism 6 software
160 package (Monrovia, CA, USA). A P-value of < 0.01 was considered greatly significant,
161 and a P-value of < 0.05 was considered significant. All experiments were independently
162 performed at least three times.
163 **Results**
164 Identified study reports
165 As shown in Fig. 1, document retrieval and filtering. A total of 115 articles were
166 searched from databases. After deleting 12 repeated articles and reading the title and
167 abstract, a total of 23 articles met the inclusion criteria. In the included literature, a total
168 of 4 articles were included for meta-analysis.
169
170 **Fig. 1. Flowchart of included and excluded trials.**
171 Characteristics of the reports
172 The characteristics of the included study are shown in Table 1. We also noted that all
173 the studies were conducted between 2007 and 2017.
174 **Table 1. Characteristics of eligible trials**

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Study		Phage Group		Control Group		FMDV Vaccine Group	
Author	Year	Events	Total	Events	Total	Events	Total
Dong	2015	3	5	0	5	4	5
Ren	2007	113	164	0	42	15	15
Ren	2015	110	128	0	37	6	6
Xu	2017	4	5	0	2	8	10

175

176 Meta-analysis

177 In order to solve the problem of poor test results caused by the small number of
178 documents, we here adopted a combination of statistical values and Q testing. The
179 comparison of immune effects between the control group and experimental group was
180 analyzed by fixed-effect model (Fig. 2).

181

182 **Fig. 2. Forest plot of meta-analysis for ORR.** There was no heterogeneity in this study
183 with $I^2 = 20\%$, $I^2 = 0\%$, and $I^2 = 43\%$. We used a fixed-effect model for meta-analysis,
184 and the results showed a protective effect on FMDV between the phage group and
185 control group (MH = 128.09, 95% CI = 18.95, 865.66, $P<0.01$) (A) and between FMDV
186 group and control group (MH = 160.16, 95% CI = 14.21, 1 804.51, $P<0.01$)(B).
187 Furthermore, when the phage group was compared to the FMDV group, there was also
188 no significant difference (MH = 0.34, 95% CI = 0.08, 1.40, $P> 0.05$) (C).

189 Publication bias

190 The funnel chart method was used to control the publication bias of meta-analysis
191 documents. By observing the funnel chart, we could see that although the pattern was
192 not completely symmetrical, the data were still within the acceptable range (Fig. 3).

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193 The results showed that the published literature had less publication bias and met the
194 requirements of this study.
195
196 **Fig. 3. Funnel plot.** (A) Phage group vs Control group; (B) Phage group vs FMDV
197 group; (C) FMDV group vs Control group.
198 Quality evaluation of evidence
199 All the experiments we selected evaluated the protective effect of the vaccine. All the
200 experiments used animals for evaluation. The outcome of the protective trial was only
201 survival or death, which was very different from the evaluation in humans.
202 Construct and identification of the phage
203 After double digestion with *EcoR* I and *Hind* III, two clear bands were detected by 1%
204 agarose gel electrophoresis. The target fragment was about 760 bp, which was
205 consistent with the expected size (Fig. 4 A). The OHM-T7 titer was determined (Fig. 4
206 B). O5, O6 and O7 plates were 10^6, 10^7 and 10^8 times diluted, respectively. Too many
207 plaques grew on plate 5, and plates 6 and 7 had 40 and 4 plaques, respectively,
208 indicating a titer of (4×108+4 ×108)/2=4×10^8. Protein gel electrophoresis showed that
209 OHM-T7 bands could be clearly observed at about 30 kDa, which proved that the phage
210 was successfully expressed and purified (Fig. 4 C).
211
212 **Fig. 4. Construction of OHM-T7.** (A) OHM-02 VP1 double restriction. M: DNA
213 Marker 1000; Lanes 1-4: Double enzyme digestion positive clones; Lane 5: control
214 group. (B) OHM-T7 plaque assessment; (C) OHM-T7 SDS-PAGE. Lanes 1-2: AKT-

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215 T7 control; Lanes 3-4: OHM-T7.

216 Screening

217 The first round of screening of OHM-T7 on the Figure 5 A-1 plate yielded a titer of
218 4×10^{10}. The titers of the second (Fig. 5 A-2) and third (Fig. 5 A-3) rounds for OHM-T7
219 plate were too high. The results showed that the OHM-T7 was enriched, and that with
220 fast passage ability was selected (Fig. 5 A). The OHM-T7 strain induced high levels of
221 IFN-γ levels ($P<0.01$) in mice with little effect on IL-4 levels ($P>0.05$).

222

223 **Fig. 5. Immunogenicity of OHM-T7.** (A) Reversal OHM-T7 reverse screening. (B)
224 IL-4 results analyzed by ELISA kits. The OHM-T7 strain induced little effect on IL-4
225 levels in mice($P>0.05$). (C) IFN-γ results analyzed by ELISA kits. The OHM-T7 strain
226 induced high levels of IFN-γ levels in mice ($P<0.01$).

227 Immunization of mice

228 The difference in anti-OHM-T7 antibody levels between 14 and 0 d was extremely
229 significant ($P<0.01$). The results showed that OHM-T7 could quickly induce high levels
230 of antibodies in mice. The difference in anti-OHM-T7 antibody levels between 98 and
231 0 d was also extremely significant ($P<0.01$). OHM-T7 antibody levels peaked at 98 d.
232 The results showed that OHM-T7 could stimulate mice to produce anti-FMDV
233 antibodies with high titers for a long time (Fig. 6 F). The level of IL-4 remained at a
234 low level with no significant changes. The type II helper T cells (Th2) cells were less
235 active after 14 d (Fig. 5B). Additionally, mice injected with the OHM-T7 strain had
236 greatly improved levels of IFN-γ (Fig. 5C). An increase in IFN-γ indicates that type I

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237 helper T cells (Th1) are activated and IFN-γ is a hallmark cytokine of Th1 cells, which
238 function is mainly to promote cellular immunity. This indicates that phage immunity
239 was mainly induced by Th1 cells after 14 d. OHM-T7 could be used to detect antibodies
240 produced by mice immunized with different FMDV antigens (Fig. 6).
241

242 **Figure 6. Detection of serum antibody levels in mice immunized with different**
243 **FMDV antigens by OHM-T7.** Blank, a blank control with nothing detected; NaCl,
244 mouse serum immunized with 0.9% NaCl; AKT-T7, mouse serum immunized with the
245 recombinant AKT-III VP1 phage; OHM-T7, mouse serum immunized with the
246 recombinant OHM-02 VP1 phage; AKT-III VP1, mouse serum immunized with the
247 prokaryotic expression the VP1 protein of the AKT-III virus; OHM-02, mouse serum
248 immunized with the prokaryotic expression VP1 protein of the OHM-02 virus; AKT-
249 III, mouse serum immunized with the AKT-III virus; OHM-02, mouse serum
250 immunized with the OHM-02 virus. The data were analyzed by T-Text using excel
251 software, * = P <0.05, ** = P <0.01.

252 **Discussion**

253 This meta-analysis showed the promise of phage-based FMD vaccines. Bacteriophages
254 have coexisted with humans and animals for a long time, and the safety of using them
255 medically is widely recognized [25]. Recent studies have shown that phages play an
256 important role in the mammalian immune system, and their interaction with mammalian
257 immune cells is of great significance [26].

258 OHM-T7 were screened using immunopositively bovine serum samples, showing

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259 that the phage could bind to positive sera. After repeated screening, a highly specific
260 OHM-T7 was obtained. The constructed phage library was reverse-screened, and
261 OHM-T7 with high binding ability was selected. While obtaining a phage with a strong
262 binding ability, phages with rapid propagation rates were also screened. Within 14 days,
263 the phage could rapidly induce high levels of specific antibodies in the body. The
264 current study only immunized the animals once, but after multiple blood collections the
265 antibody levels remained high, demonstrating that the phage could stimulate the mouse
266 body to maintain high antibody levels for a long time. Antibody levels were highest at
267 98 days, suggesting that antibody levels may increase. The antibody level of the AKT-
268 T7 group was not different from that of the OHM-T7 group. It showed that the phage
269 itself also caused the body to produce high levels of antibodies. The antibody level of
270 the OHM-02 strain was found to be higher than that of the AKT-III group. It showed
271 that OHM-T7 could specifically detect the O type antibody. It was very strange that the
272 test result of AKT-III VP1 was higher than in the OHM-02 VP1 group. We attributed
273 this to the instability of OHM-02 VP1 protein, and the results found in immunized
274 animals were not ideal. The antibody level produced by the protein of the phage carrier
275 was much higher than the prokaryotic expression. The phage vaccine produced much
276 higher levels of antibodies than the inactivated virus vaccine.

277 Although we did not evaluate FMDV in animal protection experiments, the
278 literature in the meta-analysis did include such experiments. In this way, our meta-
279 analysis suggested that our recombinant phages may protect animals from FMDV. In
280 2009, the World Influenza Conference in Europe focused on vaccines for phage-derived

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281 virus-like particles as delivery vehicles [20]. Phage particle vaccines can also be
282 administered via various immunization routes [26,27]. Under the conditions of large-
283 scale breeding, good oral immunization effects are of great significance for the
284 promotion and use of vaccines [28,29]. The use of a phage as a vector to display specific
285 epitopes of different pathogens can induce a strong immune response in the body [30-
286 33]. Therefore, the phage display technology has its unique value in the development
287 of vaccines.

288 **Conclusion**

289 The results of the present meta-analysis showed that the phage had protective effects
290 on FMDV, and no difference was detected between the FMDV group and control with
291 respect to this protective effect. The OHM-T7 was successfully constructed. OHM-T7
292 could be used to detect antibodies produced by mice immunized with different FMDV
293 antigens and produce high levels of anti-FMD antibodies. This meta-analysis and
294 experiment indicate the potential of phage-based FMDV vaccines in FMDV prevention.

295 **Acknowledgment**

296 This research was funded by CX Collaborative Innovation 2011 Collaborative
297 Innovation Special Project (Grant no. 0101-KC-0003).

298 **Author Contributions**

299 Conceptualization: CC.

300 Data curation: PW, NY, MX, YZ.

301 Formal analysis: PW. NY.

302 Funding acquisition: CC.

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303 Investigation: MX, YZ.

304 Methodology: PW, NY, YW.

305 Project administration: CC.

306 Resources: CC, PW.

307 Software: PW, NY.

308 Supervision: CC, PW.

309 Validation: CC.

310 Visualization: MX, YZ.

311 Writing – original draft: PW.

312 Writing – review & editing: PW, NY, Y.W.

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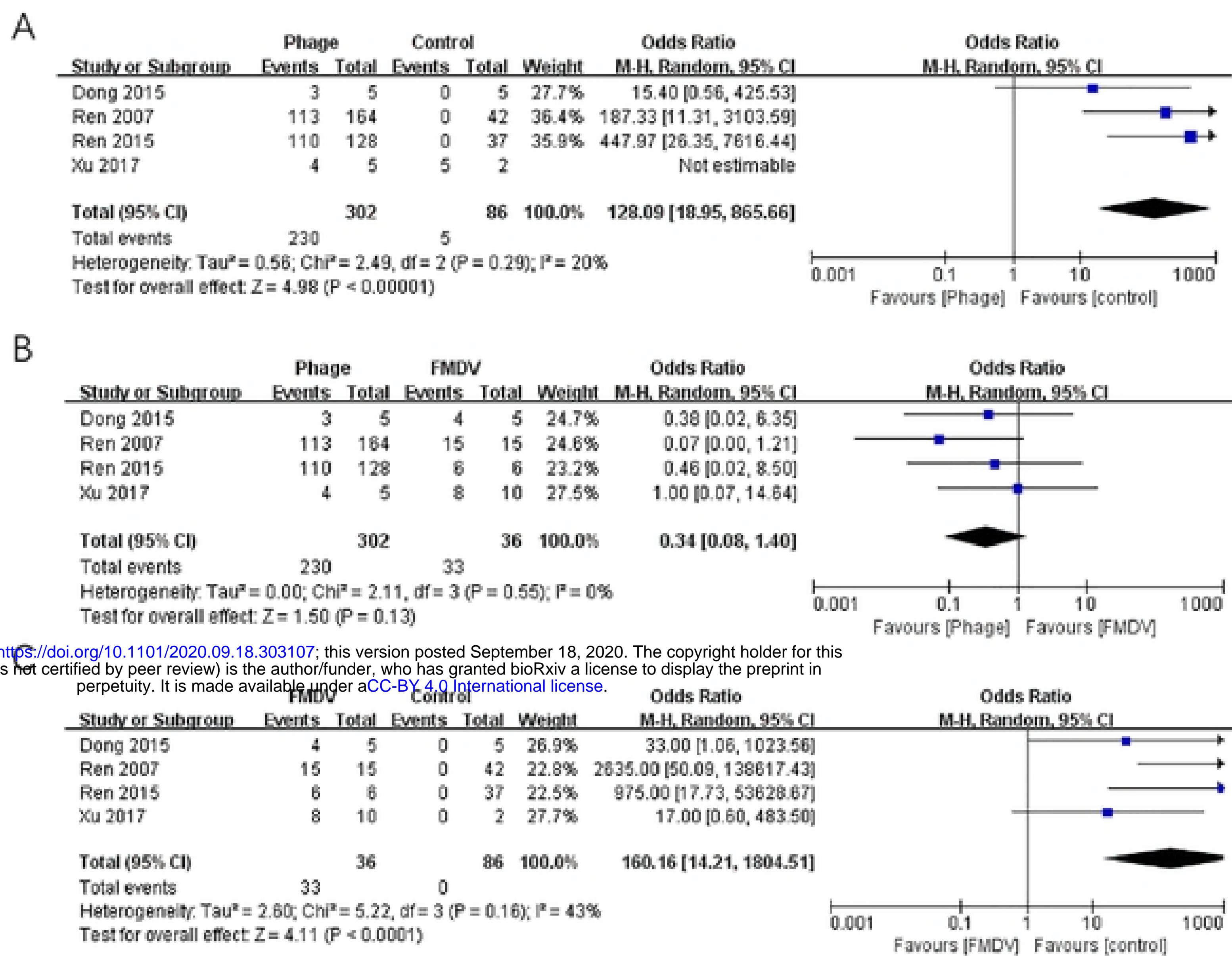

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Fig.2

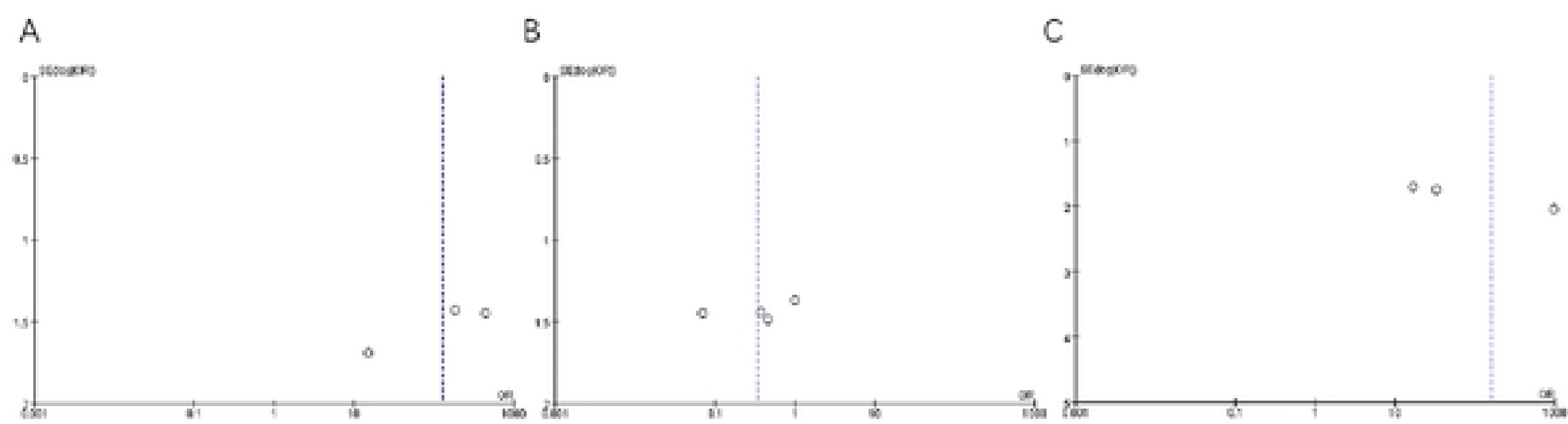

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Fig.3

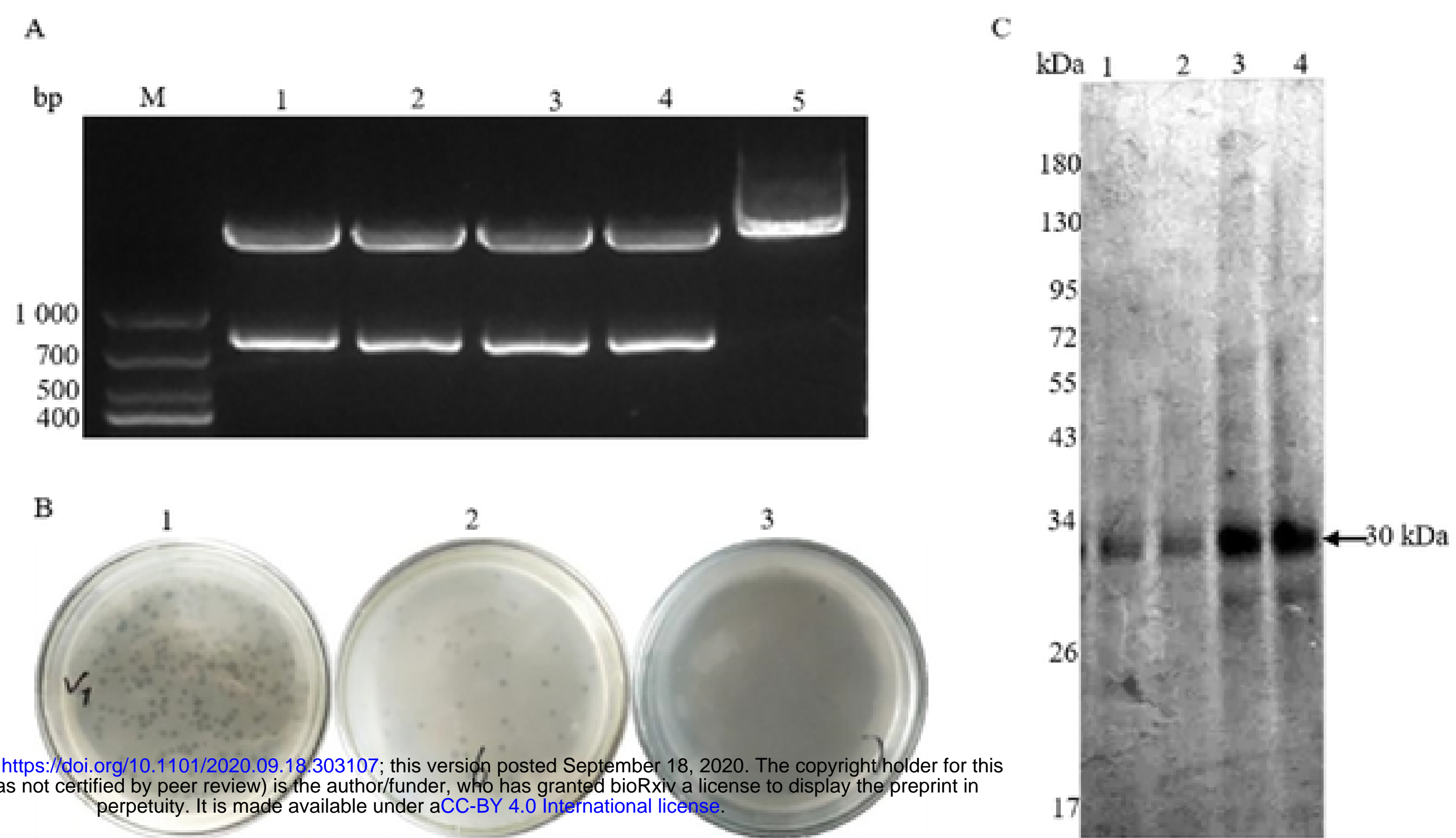

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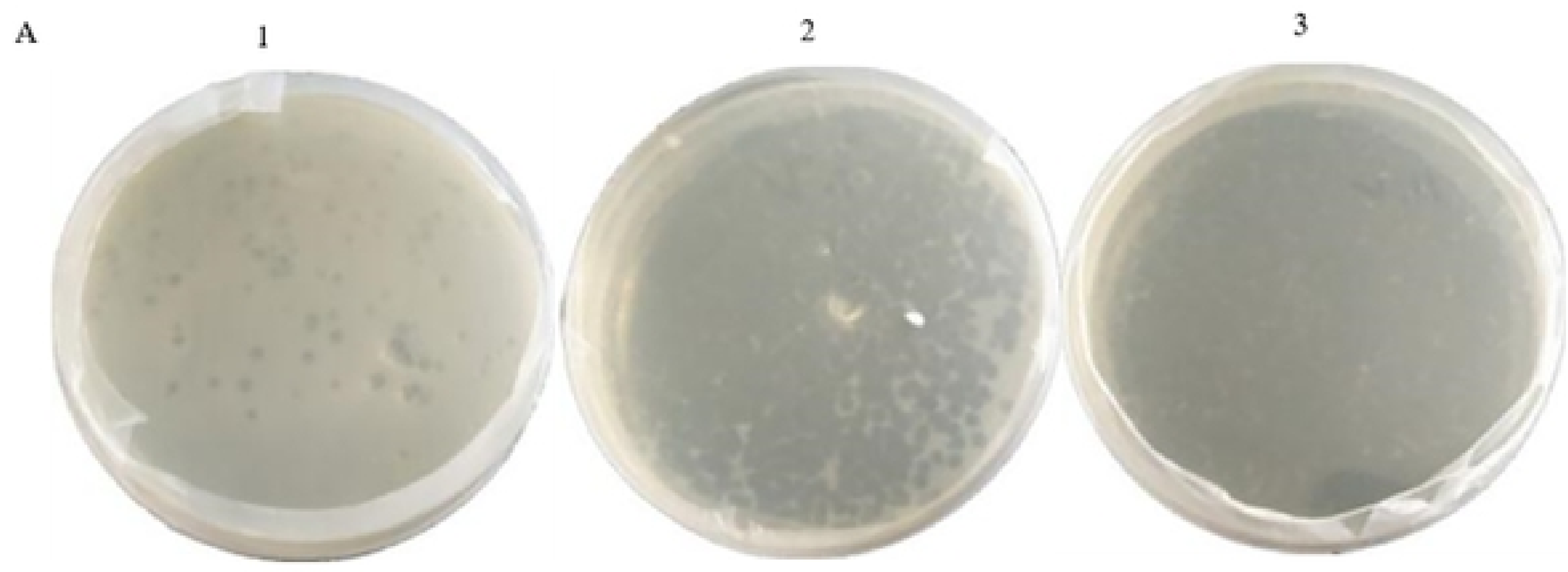
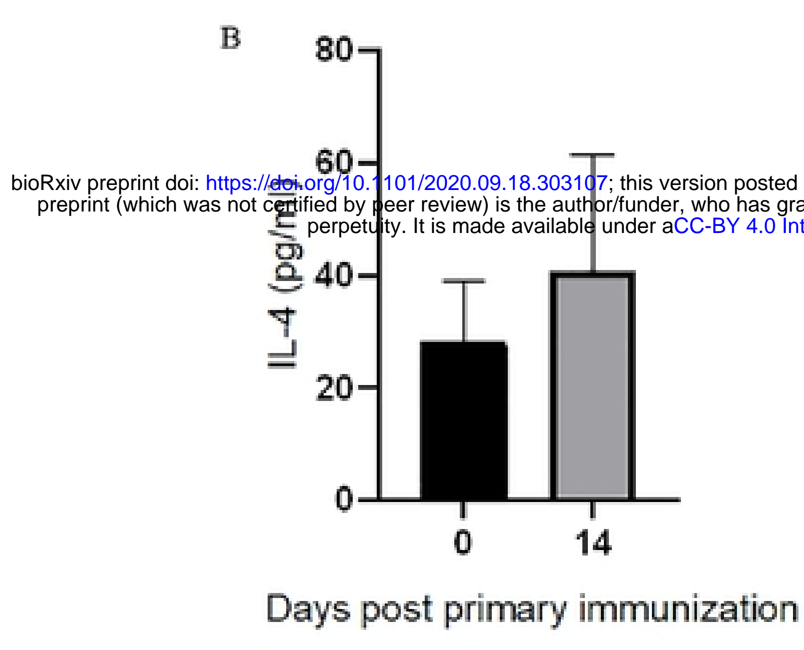
Fig.4





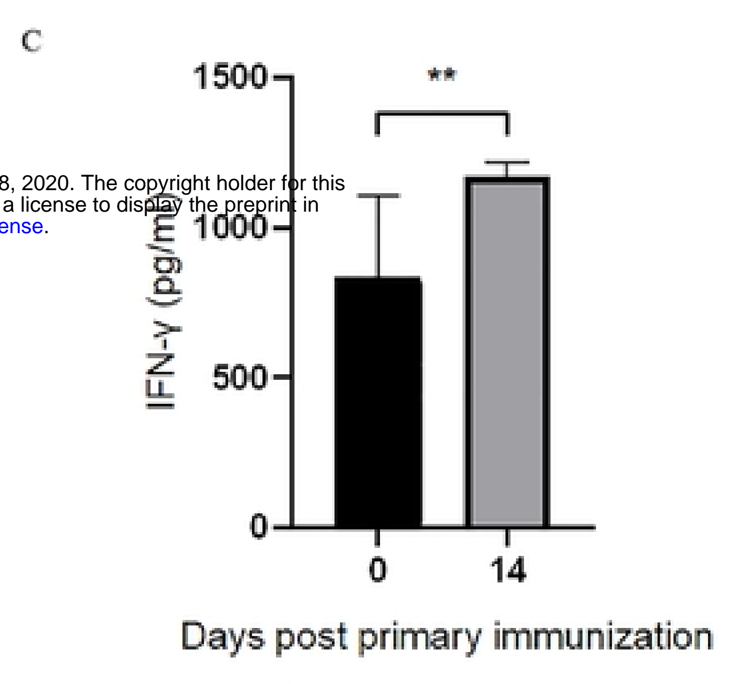


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Fig.5

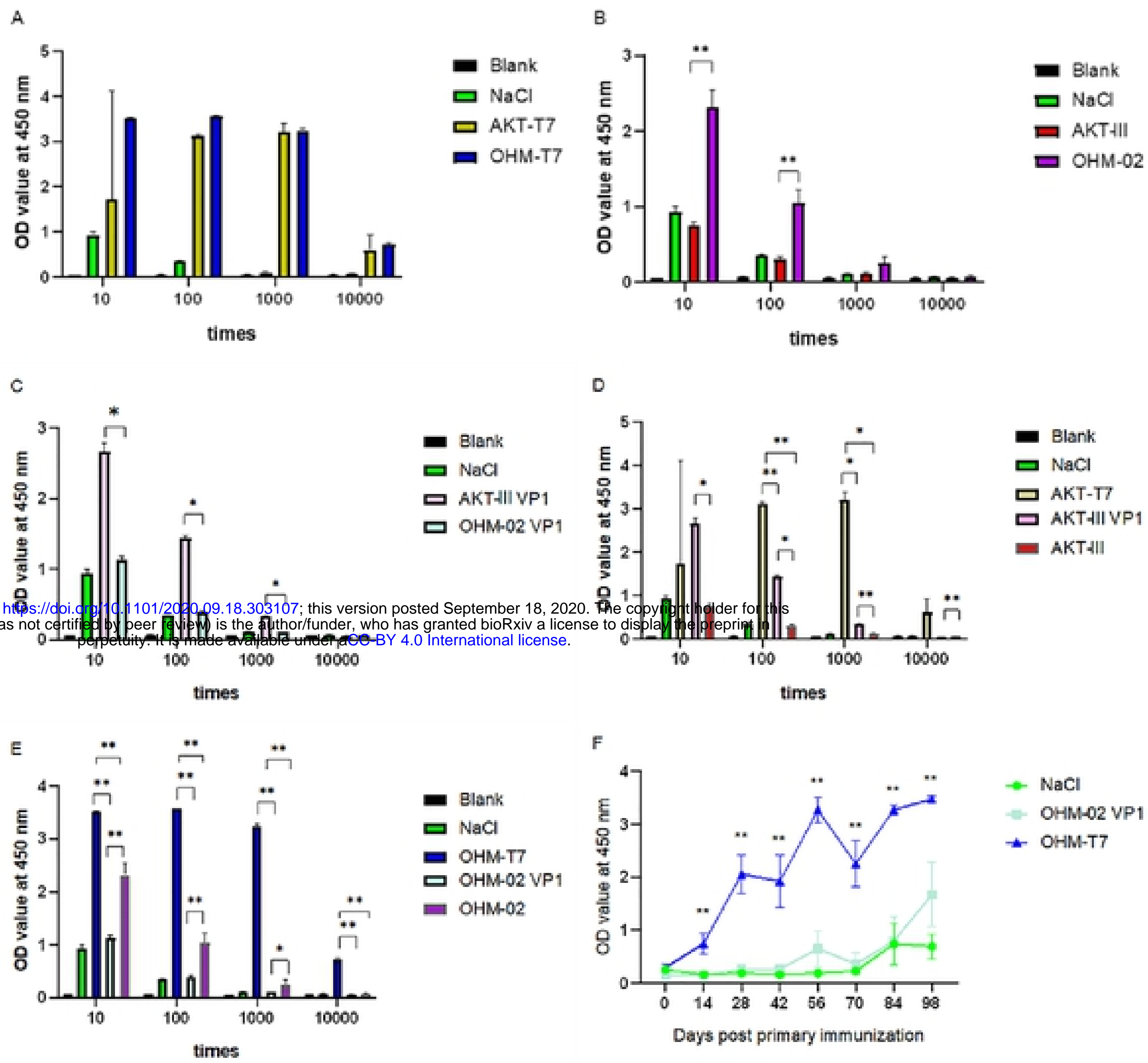

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Fig.6

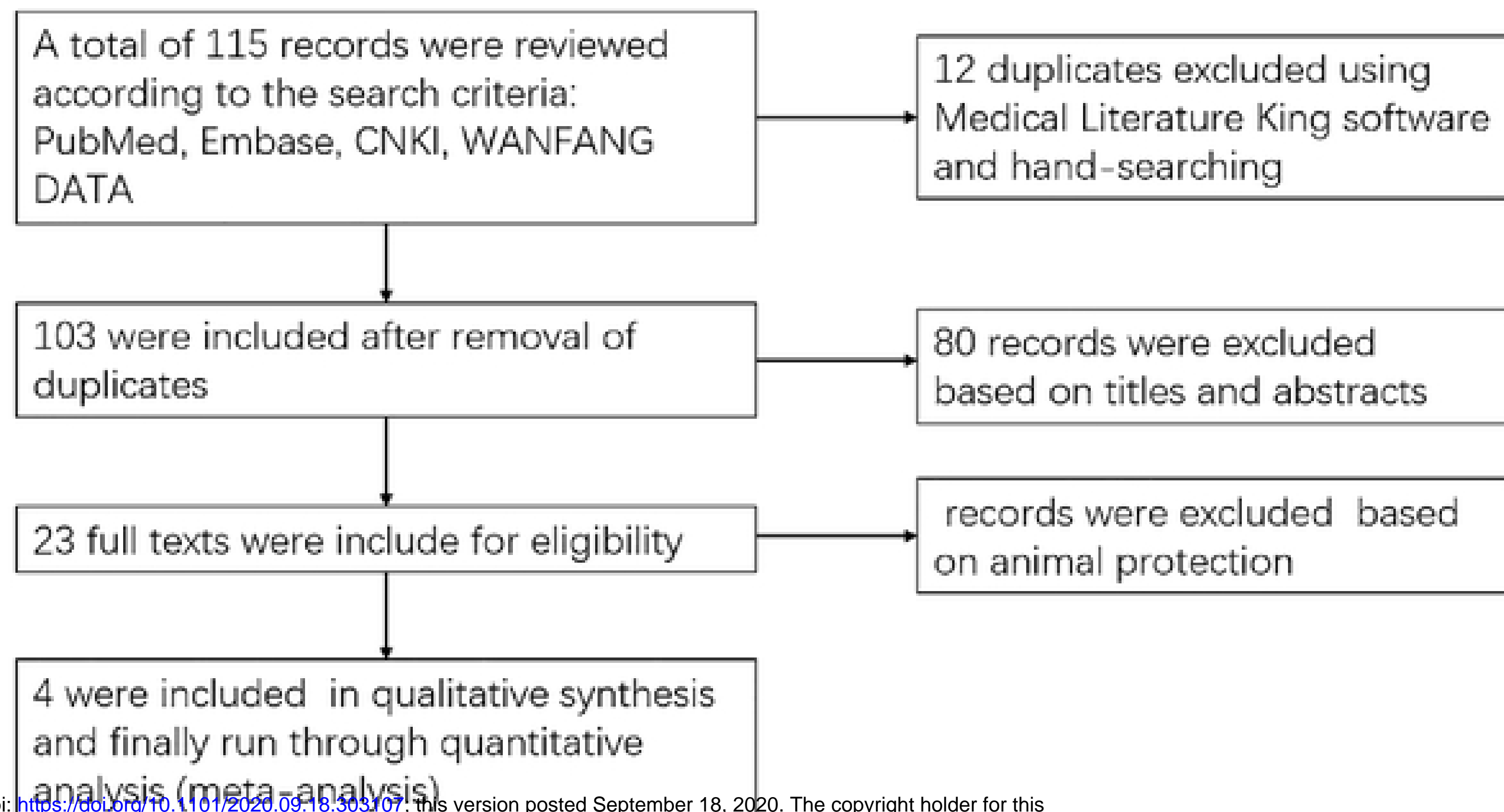

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Fig.1