May a Strain Chlamydia Isolated From SARS Patient's Autopsy Issues Inhibit the Proliferation of SARS-CoV? An Early Observation in Vitro.

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Abstract We found that a strain chlamydia isolated from SARS patient's autopsy issues could decrease the proliferation of SARS-CoV in vitro; The inhibitory factors distribute both in the extracellular and intracellular cultures.

Key words: SARS-CoV; Chlamydia; Decrease of proliferation; In vitro

0 Introduction

Interspecific competition is common in a ecosystem^[1, 2]. The closer their ecological niche is, the more intense the competition is^[3, 4]. Some species compete for resources through inhibiting the growth and reproduction of other species by releasing secondary metabolites or synthetic chemicals. For example, sage kills surrounding species by releasing volatile chemicals for competing surrounding nutritional resources, as well as walnuts (release juglone), we call it allelopathy^[5, 6]; Another example is that penicillium secretes penicillin to inhibit the proliferation of Gram-positive bacteria^[7, 8]. In our daily life, 70% - 80% of natural antibiotics are produced by actinomycetes^[9-13]. Therefore, in the ecosystem, interspecific competition is very common to kill other competing species by releasing some chemicals.

We note that scrub-typhus can significantly inhibit the proliferation of human immunodeficiency virus type 1 (HIV-1, CXCR4) in HIV-1 patients. The HIV-1 viral load of two patients co-infected with Orientia Tsutsugamushi decreased below to the detection threshold^[14].

Prof. OTOOLE,C found that the viral load of HIV-1 patients with mycoplasma infection also

24 shown a decrease^[15]; Other cases included that mycoplasma contamination inhibited the
 25 proliferation of Adenovirus and Mengovirus in their cell cultures^[16]. These studies show that both
 26 rickettsia and mycoplasma can inhibit the proliferation of related different viruses under certain
 27 conditions. We note that rickettsia, mycoplasma and chlamydia all belong to the relationship
 28 between virus and bacteria, and because the ecological niche of chlamydia is closer to viruses
 29 compared to rickettsia and mycoplasma, it is doubtful whether chlamydia can inhibit the
 30 proliferation of viruses under certain conditions. A study conducted by Prof. Hong T et al showed
 31 that no Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) or few SARS-CoV were
 32 found in the tissue samples of some Severe Acute Respiratory Syndrome (SARS) patients, but a
 33 large number of chlamydia particles were found in their tissue samples^[17]. Therefore, in order to
 34 investigate the relationship between SARS-CoV and the related strain chlamydia, We carried out
 35 the following series of experiments. This article is divided into four sections, the first section is
 36 materials and methods, the second section is results, the third section is discussions, and the final
 37 section is conclusions.

38 **1 Materials and methods**

39 **1.1 Virus, chlamydia and cells**

40 SARS-CoV (BJ01 strain), the strain Chlamydia-1 (isolated from the autopsy specimens of
 41 SARS-CoV patients), and Human Embryo Kidney 293 Cells were kindly provided by Prof. Hong
 42 T (Chinese Center for Disease Control and Prevention, Beijing, China); the strain *Chlamydia*
 43 *psittaci* (*C. psittaci*) was purchased from the China veterinary Culture Collection Center
 44 (No.CVCC2410). Virus and chlamydia stocks were prepared after virus inoculation into 293 cells
 45 respectively. Following incubation at 37°C for 48 h, cultured fluids were collected, clarified by

46 low speed centrifugation and titrated for virus and chlamydia contents respectively.

47 For virus titration, serial dilutions of virus suspension were inoculated into cell culture plates
48 and their fifty-percent Tissue Culture Infectious Dose (TCID₅₀) titers determined using the method
49 of Reed-Muench (1938). Finally, the viral titers were determined to 10^{5.67}TCID₅₀/ml. For
50 chlamydia titration, 50 uL chlamydia suspension were inoculated in 293 cells with 1×10⁵ cells
51 each well (a 13 mm round coverslip was paved in each well in advance). After incubation at 37°C
52 for 48 h, the cells were fixed in absolute methanol for 10 min, and then stained by Giemsa method
53 for 45 min, the number of inclusion-forming units (IFUs) of chlamydia were calculated through
54 inverted microscope (Olympus, Japan). The final titration of the strain Chlamydia-1 were 1.2×10⁵
55 IFUs/mL, and the final titration of the strain *C. psittaci* were 3.0×10⁵ IFUs/mL.

56 1.2 Co-infection of SARS-CoV and chlamydia in 293 cells

57 For the co-infection of SARS-CoV and the strain Chlamydia-1: 293 cells were cultured in 200 uL
58 dulbecco's modified eagle's medium (DMKM) supplemented with 10% fetal bovine serum (FBS)
59 and 1% antibiotics/antimycotic, seeded in a 96-well cell plate with 1×10⁵ cells per well (a 13 mm
60 round coverslip was paved in each well in advance), and then kept in 5% CO₂, saturated humidity,
61 and 37°C. While cell density grown to about 80%, it began to inoculate virus and chlamydia.
62 Infection groups were divided into six groups, including SARS-CoV single infection group (A₁
63 group), the strain Chlamydia-1 single infection group (B₁ group), SARS-CoV and the strain
64 Chlamydia-1 simultaneous infection group (C₁ group), SARS-CoV infection first, then the strain
65 Chlamydia-1 infection group (D₁ group, the latter inoculation in 2 days later), the strain
66 Chlamydia-1infection first, then SARS-CoV infection group (E₁ group, as above) and control
67 group (F₁ group). when each inoculation, gently shake the cell plate every 20min for 1 hour, and

68 then discard inoculated fluids, wash with 10% phosphate buffered saline (PBS). Finally, add 200
69 uL 10% FBS DMKM to continue to incubate those inoculated cells. Both the inoculation volume
70 of virus and chlamydia were 50 uL. The viral load of SARS-CoV [by quantitative real-time
71 polymerase chain reaction (qRT-PCR)] and the number of IFUs (by Giemsa method and inverted
72 microscope) would be detected on the 3rd, 4th, 5th, 6th, 7th, 8th and 9th day after the first
73 inoculation. The concentration of SARS-CoV would be diluted to $10^{3.67}$ TCID₅₀/mL for the
74 inoculation and the concentration of the chlamydia-1 strain were diluted 6.0×10^4 IFUs/mL for the
75 infection.

76 For the co-infection of SARS-CoV and the strain *C. psittaci* : The same as above, except
77 replacing the strain Chlamydia-1 as the strain *C. psittaci*. The corresponding groups were
78 numbered as A₂, B₂, C₂, D₂, E₂, F₂ respectively.

79 1.3 Effects of chlamydia cell cultures on the proliferation of SARS-CoV in 293 cells

80 For the strain Chlamydia-1: four SARS-CoV single infection groups, two the strain
81 Chlamydia-1 single infection groups and one control group were cultured in the cell plate until the
82 6th day, then the cell cultures of two the strain Chlamydia-1 single infection groups and control
83 group were collected. For two the strain Chlamydia -1 single infection groups, one groups' culture
84 mediums were collected, centrifuged at 12000 rpm for 1 min, and then its supernatants were
85 collected, filtered with 0.22 um membrane, ready for use (extracellular cultures), while for the
86 other group, both its culture mediums and cultured cells (by cell scraper) were collected, broken
87 by ultrasonic wave on ice for ten times, 10 s/time, then centrifuged at 12000 rpm for 1 min, finally,
88 the supernatants were collected, filtered with 0.22 um membrane, ready for use (extracellular +
89 intracellular cultures). For the control group, its cell culture medium were collected, centrifuged at

12000 rpm for 1 min, and then the supernatant were collected, filtered with 0.22 um membrane, ready for use. Three SARS-CoV single infection groups were taken out and its growth mediums were discarded respectively, then, the above treated supernatants were added to them respectively. The above three groups were defined as extracellular group, extracellular + intracellular group and control group respectively, numbered as A₁, B₁, and C₁. Finally, 200 uL 10% FBS DMKM were added to them and the remaining one SARS-CoV single infection group (numbered as D₁) for continuing to incubate those groups until each detection. The viral load of each groups would be detected on the 6th, 7th, 8th and 9th day.

For the strain *C. psittaci*: the same as above, except replacing the strain Chlamydia-1 with the strain *C. psittaci*. The corresponding groups were number as A₂, B₂, C₂ and D₂, respectively.

1.4 Determination of IFUs

On the 3rd, 4th, 5th, 6th 7th, 8th and 9th day, the round coverslips of each group were collected respectively, fixed in absolute methanol for 10 min, stained by Giemsa method for 45 min, then the number of IFUs would be calculated by inverted microscope under high power microscopic view. The number of IFUs was calculated by the following formula.

$$\text{IFUs} = \text{infectious rate} \times 100\% \times 1 \times 10^5$$

The meaning of the formula: the number of IFUs in cell cultures. Infectious rate is the number of IFUs divided by the number of cells. 1×10^5 is the total number of cells.

1.5 Determination of viral load

Viral ribonucleic acid (RNA) was extracted by viral RNA kit (Qiagen, Germany), reversed by reverse transcription kit (Promega, United States). The sequence primers of SARS-CoV were synthesized by bio-engineering Co., Ltd. China (F:5'-GAAGCTATTCGTCACGTTTCG-3';

111 R:5'-TAACCAGTAGGTACAGCTAC-3'). The viral complementary deoxyribonucleic acid
112 (cDNA) was amplified and detected by SYBR Green PCR Master Mix kit (bao bio-engineering
113 Co., Ltd, China) on the ABI7500 instrument (ABI, USA). For qRT-PCR reactions, the 20 uL
114 reaction mixture included 2 uL cDNA, 10 uL 2×SYBR Green qPCR Mix, 0.4 uL of forward
115 primer and 0.4 uL of reverse primer, and 7.2 uL RNAase-free water. Conditions for reactions were
116 55°C for 10 min and 95°C for 3 min, 45 cycles of amplification at 95°C for 10 s and 72 °C for 10
117 s.

118 1.6 Statistical Analysis

119 The data are presented as averages $\pm$ standard deviations (SDs), as indicated. Statistical
120 comparisons were analyzed with two-tailed Student t-test and one-way analysis of variance
121 (ANOVA) method. All of the statistical analyses were performed with statistical product and
122 service solutions (SPSS) version 25.0. When $p > 0.05$, the results were not significant; when $p <$
123 0.05 , the results were significantly different; when $p < 0.01$, the results were extremely
124 significantly different.

125 2 Results

126 2.1 Results of co-infection of SARS-CoV and chlamydia in vitro

127 From figure 1A, we can see that the viral load of C₁ group (from day 3 to day 9) and D₁ group
128 (form day 4 to day 9) has a extreme significant reduction compared to A₁ group ($P < 0.01$). From
129 figure 1B, we can see that the viral load of C₂ group (from day 3 to day 9) and D₂ group (from day
130 6 to day 9) has a significant reduction compared to A₂ group ($P < 0.05$). From figure 1C, we can
131 see that the viral load of C₁ group (from day 3 to day 9), D₁ group (from day 4 to day 9) and E₁
132 group (from day 4 to day 9) has a extreme significant difference from that of C₂ group, D₂ group

133 and E₂ group respectively ($P < 0.01$).

134 From figure 2A, we can see that the number of IFUs has no significant difference among B₁
135 group, C₁ group and E₁ group ($P > 0.05$); It can be seen from figure 2B that the number of IFUs of
136 C₂ group (from day 3 to day 5 and day 7 to day 9) and E₂ group (from day 4 to day 6) has a
137 significant difference compared to B₂ group ($P < 0.05$). Statistical significance between
138 experimental groups was determined by the student's t-test, as follows: *: $p < 0.05$, **: $p < 0.01$.

139 Therefore, the strain Chlamydia-1 has a extreme significant effect in decreasing the
140 proliferation of SARS-CoV in vitro. while the strain *C. psittaci* seems also can significantly
141 decrease the proliferation of SARS-CoV in vitro, its effect is significantly inferior to the strain
142 Chlamydia-1. The proliferation of the strain Chlamydia-1 could not be affected by the
143 proliferation of SARS-COV in vitro, while the proliferation of the strain *C. psittaci* seems to be
144 affected by the proliferation of SARS-CoV in vitro.

145 2.2 Effects of chlamydia cell cultures on the proliferation of SARS-CoV in vitro.

146 It can be seen from figure 3A that the viral load of A₁ group (from day 7 to day 9) and B₁ group
147 (from day 7 to day 9) has a extreme significant reduction compared to C₁ and D₁ group ($P < 0.01$),
148 and there is a extreme significant difference in reducing the viral load of SARS-CoV between A₁
149 and B₁ group (from day 7 to day 9) ($P < 0.01$). From figure 3B, we can see that the viral load of A₂
150 group (from day 7 to day 8) and B₂ group (from day 7 to day 8) has a significant reduction
151 compared to C₂ group and D₂ group ($P < 0.05$). However, there is no significant difference in
152 decreasing the viral load of SARS-CoV between A₂ group and B₂ group (from day 7 to day 8) ($P >$
153 0.05). From figure 3C, we can see that the viral load of A₁ and B₁ group has a extreme significant
154 difference from that of A₂ and B₂ group respectively (from day 7 to day 9) ($p < 0.01$). Statistical

155 significance among groups was determined by one-way ANOVA method.

156 Therefore, for the strain Chlamydia-1, it contains anti-SARS-CoV factors in its cell cultures,
157 distributing both in the extracellular and intracellular cultures. While the strain *C. psittaci* cell
158 cultures seems also has anti-SARS-CoV factor, its result is extremely significantly inferior to that
159 of the strain Chlamydia-1 cell cultures. And for the strain *C. psittaci*, there is no significant
160 difference between the extracellular + intracellular and extracellular cell cultures in decreasing the
161 virus load of SARS-CoV.

162 **3 Discussions**

163 Because there was no SARS-CoV or few SARS-CoV was found in some tissue samples of SARS
164 patients, but a large number of chlamydia particles were found in their tissue samples, in order to
165 investigate the relationship between SARS-CoV and the related strain chlamydia in SARS
166 patient's autopsy issues, we conducted an experiment which involved the co-cultures of
167 SARS-CoV and the related strain chlamydia^[17]. We found that the strain chlamydia isolated from
168 SARS patient's autopsy issues could extremely significantly decrease the proliferation of
169 SARS-CoV in vitro. It means that the strain chlamydia isolated from SARS-CoV patient's autopsy
170 issues may inhibit the proliferation of SARS-CoV, which benefits for exploring anti-SARS-CoV
171 methods and drugs. Because there was no study that reported that some chlamydias could decrease
172 the proliferation of related virus in vitro or vivo before that, and this work is only our preliminary
173 observations, the specific mechanism that can explain the strain chlamydia isolated from
174 SARS-CoV patient's autopsy issues in decreasing the proliferation of SARS-CoV in vitro is still
175 unknown. Therefore, in future works we will explore this aspects. Moreover, because our
176 observed results indicated that the inhibitory factors distribute both in the extracellular and

intracellular cultures, we will still try to identify and separate the inhibitory factors from the strain
Chlamydia-1 cell cultures in future works. Finally, we noted that a recent study indicated that
there was a lower likelihood of intensive care unit admission with bacterial co-infection in
Coronavirus Disease-2019 (COVID-19) patients ($r = -0.28$, $p=0.04$), and *Chlamydia pneumoniae*
was the most prevalent co-infecting bacteria found in 13 patients (28% in total 48 COVID-19
patients). Co-infection with *Chlamydia pneumoniae* has a negative correlation with mortality in
COVID-19 patients ($r=-0.17$).^[18] Combined with our study to analyze, we suggest that it should
further explore the relationship between Severe acute respiratory syndrome coronavirus 2 and the
strain *Chlamydia pneumoniae* in COVID-19 patients with the widely spread of COVID-19 across
the world^[19].

4 Conclusions

We found that a strain chlamydia isolated from SARS patient's autopsy issues could decrease the
proliferation of SARS-CoV in vitro and the inhibitory factors distribute both in the extracellular
and intracellular cultures, which benefits for us to further exploring the relationship between
SARS-CoV and the related strain chlamydia in SARS patients, and even may provide helps for
finding anti-SARS-CoV methods and drugs.

Abbreviations

SARS-CoV: Severe acute respiratory syndrome coronavirus; SARS: Severe acute respiratory
syndrome; IFUs: Inclusion-forming units; HIV-1: Human immunodeficiency virus type 1; *C.*
Psittaci: *Chlamydia psittaci*; TCID₅₀: Fifty-percent tissue culture infectious dose; FBS: Fetal
bovine serum; DMKM: Dulbecco's modified Eagle's medium; PBS: Phosphate buffered saline;
qRT-PCR: Quantitative real-time polymerase chain reaction; RNA: Ribonucleic acid; cDNA:

199 Complementary deoxyribonucleic acid; SPSS: Statistical product and service solutions; ANOVA:

200 Analysis of variance; COVID-19: Coronavirus disease-2019.

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204 **Authors' contributions**

205 Conceived and designed the experiments: X.Q. Performed the experiments: X.Q., W.L. Analyzed

206 the data: X.Q. W.L. Wrote the paper: X.Q. All authors read and approved the final manuscript.

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210 **Availability of data and materials**

211 The datasets used and/or analyzed during the current study are available from the corresponding

212 author on reasonable request.

213 **Declarations**

214 **Ethics approval and consent to participate**

215 No applicable.

216 **Consent to publication**

217 The authors declare they are consent for the manuscript publication.

218 **Competing interests**

219 The authors declare that they have no competing interests.

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Figures

278 **Figure 2.** Time series changes of IFUs in the co-infection of SARS-CoV and chlamydia in
279 vitro. (A) Changes of IFUs in the co-infection of SARS-CoV and the strain Chlamydia-1. (B)
280 Changes of IFUs in the co-infection of SARS-CoV and the strain *C. psittaci*.
281

Figure 3. Effects of chlamydia cell cultures on the proliferation of SARS-CoV in vitro. (A) Effects of the strain Chlamydia-1 cell cultures on the viral load of SARS-CoV. (B) Effects of the strain *C. psittaci* cell cultures on the viral load of SARS-CoV. (C) Comparison of the effects between the Chlamydia-1 strain cell cultures and the strain *C. psittaci* cell cultures on the viral load of SARS-CoV. Bars with the same lower-case letters in two different columns show no significant difference ($p > 0.05$), and bars with different lower-case letters in two different columns show a significant difference ($p < 0.05$ or $p < 0.01$).