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ORIGINAL ARTICLE

Prevalence of asymptomatic Bordetella pertussis and Bordetella parapertussis infections among school children in China as determined by pooled real-time PCR: A cross-sectional study

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Abstract

Background: Studies have documented that older children and adolescents act as a reservoir of Bordetella pertussis infection for young infants who have not yet completed their primary immunization schedule. Asymptomatic pertussis infection has been reported during outbreaks. This cross-sectional study aimed to investigate whether B. pertussis and Bordetella parapertussis can colonize the nasopharynx of healthy school children, using culture and pooled real-time PCR with targets for insertion sequences *IS481* and *IS1001*. *Methods:* Nasopharyngeal (NP) swabs were taken from 629 asymptomatic school children aged 7 to 15 y in 4 counties of China during the period July–September 2011. The number of subjects included in each county ranged from 153 to 165. The 4 counties selected are located in the north, south, east, and southwest regions of China. NP swabs were inoculated onto Regan–Lowe agar for isolation of suspected Bordetella organisms. Pooled real-time PCRs were used to detect B. pertussis and B. parapertussis based on the *IS481* and *IS1001* targets separately. *Results:* Of the 629 subjects, 2 (0.3%) and 30 (4.8%) were confirmed to be culture-positive and PCR-positive, respectively, for B. pertussis, and 1 (0.2%) and 13 (2.1%) were confirmed to be culture-positive and PCR-positive, respectively, for B. parapertussis. All culture-positive samples were also PCR-positive. Furthermore, positive B. pertussis and B. parapertussis samples were found in all counties. *Conclusions:* Our results indicate that asymptomatic B. pertussis infections are common in school children in China, and asymptomatic B. parapertussis infections are more prevalent than previously documented.

Keywords: *Prevalence, asymptomatic infections, Bordetella pertussis, Bordetella parapertussis, pooled real-time PCR*

Introduction

The etiologic agent of whooping cough is Bordetella pertussis. Pertussis-like symptoms are also caused by the related species Bordetella parapertussis, although B. parapertussis infections are usually less severe [1]. Pertussis is a vaccine-preventable disease. The current vaccines against pertussis include both whole cell (wP) and acellular (aP) pertussis vaccines, which are in combination with diphtheria and tetanus toxoids (DTwP and DTaP). In China, the pertussis component of DTaP vaccines is mainly composed of pertussis toxin (PT) and filamentous hemagglutinin (FHA). Booster vaccinations against pertussis in older children and adolescents have not been applied in this country. Studies have documented that these older groups act as a reservoir of B. pertussis

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infection for young infants who have not yet completed their primary immunization schedule [2–7]. Asymptomatic pertussis infection has been reported during outbreaks in the USA and Finland [8,9].

The isolation of B. pertussis is the 'gold standard' for the diagnosis of pertussis. The success of culture is highly dependent upon proper sample collection and laboratory techniques, the age and immune status of the patient (success of culture is high with unimmunized infants, but low with older, immunized, and partially treated patients) and the onset of disease [10]. Compared to culture, nucleic acid amplification methods such as PCR have been proven to be more sensitive. Moreover, the use of PCR is highly suited to the detection of fastidious organisms, which are significant by their presence even in an asymptomatic individual. B. pertussis is such an organism, and many nucleic acid amplification-based tests have been developed over recent years. Real-time PCR is an efficient technique that can be applied to large numbers of samples. To increase testing efficiency and save costs, pooling of clinical specimens prior to testing for several infections by PCR has been used [11,12]. The size of the pools has differed among studies, and each sample in a positive pool has to be retested individually. Repetitive insertion sequences *IS481* (B. pertussis) and *IS1001* (B. parapertussis) were used as targets in our PCR assays, because of their high copy number per cell and species specificity. The detection of species-specific *IS481* and *IS1001* in nasopharyngeal (NP) swabs or aspirates by real-time PCR is recommended by the World Health Organization (WHO) for the diagnosis of B. pertussis and B. parapertussis [13].

In the present study, pooled real-time PCR assays targeting *IS481* and *IS1001* of B. pertussis and B. parapertussis were used to analyze 629 NP swabs collected from healthy school children in order to estimate the possible prevalence of asymptomatic infections with these pathogenic bacteria in China. The PCR results were also compared to culture.

Methods

Subjects and samples

We conducted a cross-sectional study on the 'carriage rate' of B. pertussis and B. parapertussis among 7–15-y-old healthy children in the provinces of Shandong, Guangdong, Zhejiang, and Yunnan, China, during the period July–September 2011. The 4 provinces are located in the north, south, east, and southwest regions of China, respectively (Figure 1).

Calculation of the number of subjects needed in this study in each province was based on the formula: $n = deff \frac{u_\alpha^z p(1-p)}{\delta^z}$. According to the results of a study on the pertussis seropositivity of the whole population of Guangdong Province, which had the lowest number of reported pertussis cases per year of the 4 provinces [14], P represented 10% of pertussis seropositivity and the relative permissible error (δ) was set at 5%. When a ratio of lost follow-up 10% was taken into account, 1.0 times the cluster sampling ratio (*deff*), it was determined that a total of 150 children needed to be enrolled in each province in this study. In terms of the sampling method, all children were selected randomly in a cluster of 1 school in 1 county affiliated to each of the 4 provinces. All children were asymptomatic when they entered the study. Children who reported symptoms of a respiratory infection such as cough, fever, or catarrh, currently present or having occurred in the past 3 months, were excluded.

Study questionnaire

A questionnaire was designed by the investigators of this study. The questionnaire was first validated and the reliability tested in the age group 7–9 y in 1 selected county. Following professional training, local Centers for Disease Control (CDC) staff served as investigators and assisted in filling out the questionnaires through face to face interviews with the children or parents. The questionnaire collected information including demographic details (age, gender, parents' education level, family income per year), history of pertussis and other respiratory infections, vaccination history, and pathogen exposure history.

Culture

NP swab samples were collected from the same subjects. All NP swabs were transported using Amies agar gel transport medium with charcoal to the local provincial laboratory within 24 h. NP swabs were inoculated onto Regan–Lowe agar (Oxoid, France) supplemented with 10% horse blood and cephalexin (40 mg/l) within 24 h in the 4 provincial laboratories. After inoculation, swabs were then placed into 600 µl of 1 × PBS (phosphate-buffered saline), swirled vigorously, and squeezed out to elute any bacterial cells. The elution liquid of swabs was stored at −80°C until transported to the China CDC laboratory. Plates were incubated at 35°C in a moist Perspex chamber containing air with adequate humidity for up to 7 days. Plates were examined daily for suspected colonies, which were identified phenotypically through observing the appearance, size, and gloss of colonies by naked eye, Gram stain, and the

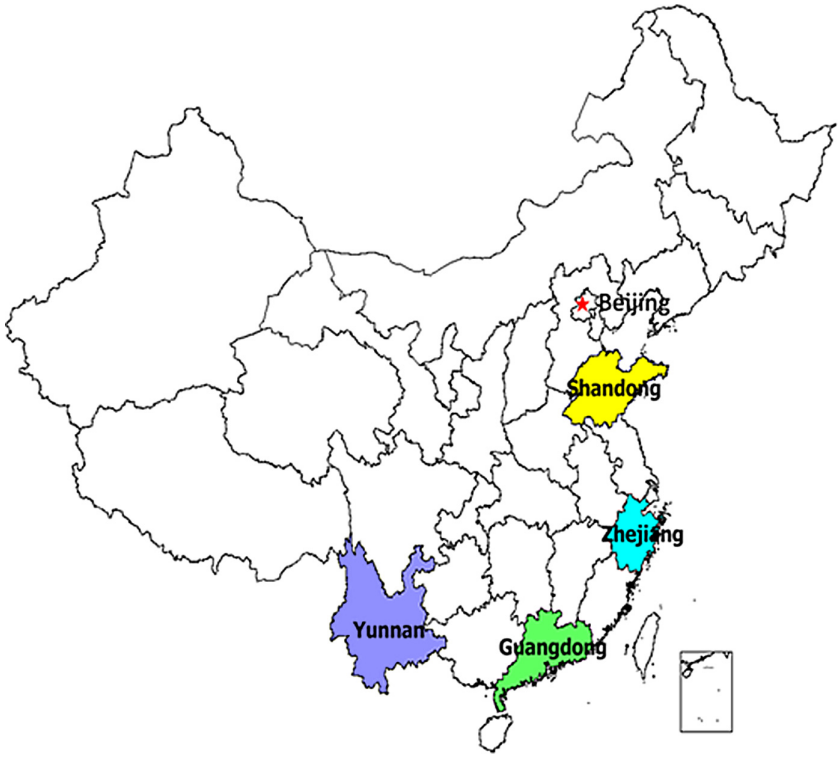

Figure 1. The location of the 4 provinces in China where the nasopharyngeal samples were collected.

form of the bacterial strain under an optical microscope. Suspected B. pertussis and B. parapertussis colonies were identified by specific agglutinating sera (Remel, Tokyo, Japan).

Pooling and DNA extraction

The elution liquid of swabs was transported in cold conditions to the laboratory of the China CDC, Beijing. Two hundred microliters of the individual elution samples were pooled in groups of 10, resulting in a pool volume of 2 ml. The process of pooling was conducted on ice. Altogether, 63 pools were constructed. For DNA extraction, 1 ml elution from each pool and 200 µl of supposed positive single specimen were centrifuged at 15,000 rpm for 30 min at 4°C. DNA extraction from the deposited pellets was performed as per the recommendations in the manual of the QIAamp DNA Mini and Blood Mini Kit (Qiagen, Germany). The extract was eluted into a 150-µl volume of Tris–ethylenediaminetetraacetic acid (EDTA) buffer. The DNA extracts were stored at − 80°C for future use.

Primers and probes for real-time PCR

The B. pertussis and B. parapertussis real-time PCR was based on the *IS481* and *IS1001* targets separately; the sequences of the primers (Table I) were adopted from a previously published work [15]. Briefly, the PCR was performed with a reaction mixture of 20 µl consisting of 10 µl of 2 × premix Ex Taq, 0.4 µl of 10 × ROX Reference Dye II (TaKaRa, Dalian, China), 0.25 µM each primer and probe, and 1 µl of DNA template. The PCR thermal profile consisted of an initial incubation of 2 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. Amplification, detection, and data analysis of *IS481* and *IS1001* were monoplex, performed with the Stratagene 3000P QPCR system

Table I. Primers for real-time PCR in monoplex assay.

Organism (insertion sequence) and primer	Sequence	Position	Length (bp)
B. pertussis (*IS481*)	GATTCAATAGGTTGTATGCATGGTT	12–36	25
	TTCAGGCACACAAACTTGATGGGCG	192–168	25
B. parapertussis (*IS1001*)	CACCGCCTACGAGTTGGAGAT	733–753	21
	CCTCGACAATGCTGGTGTTCA	1196–1176	21

(Agilent Technologies, Santa Clara, CA, USA). The primer and probe concentrations and the PCR thermal profile were the same as those used for the *IS481* real-time PCR.

Quality control

The quality of the NP swabs, the quality of the nucleic acid extraction step, and the presence of PCR inhibitors were controlled for by amplification of the human-2-microglobulin gene in each run. The primers and probe used for the amplification of the human-2-microglobulin gene are those described by Bidet et al. [16], with a minor difference in the molecular beacon labeled with FAM. The assay was performed as a monoplex real-time PCR. As well as the internal control, 1 blinded negative control and a positive control were included in each real-time PCR run in which 10 pooled samples or a single sample were tested.

Statistical analysis

For specimen PCR testing, under strict quality control, the specimen cycle threshold (Ct) values were determined using Stratagene MxPro QPCR analysis software. A Ct value ≤ 35 cycles of tested pools and single specimens was considered to be a positive test result. Data analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). For the statistical analysis, subjects were categorized into 3 age groups: 7–9 y, 10–12 y, and 13–15 y. The prevalence of asymptomatic infection between the age groups was examined by Chi-square test. A p-value of < 0.05 was considered statistically significant.

Ethical considerations

This study was approved by the Institutional Review Board (IRB) of the Chinese Center for Disease Control and Prevention, Beijing. Informed consent was received from all of the children's guardians before NP swabs were taken.

Results

Of the 629 NP swabs obtained, 153 were collected from Shandong, 158 from Zhejiang, 153 from Guangdong, and 165 from Yunnan. The number of children included in each of the 3 age groups was almost the same (Table II). Most of the children were asymptomatic during sample collection; only 3 children were excluded because of the symptom of cough.

Bordetella culture

There were only 3 positive cultures: 2 for B. pertussis and 1 for B. parapertussis (Table II). The 2 children who were B. pertussis-positive were 10 and 9 y old, and the child who was B. parapertussis-positive was 10 y old. They were all asymptomatic at the time of NP sampling. The 3 isolates were also confirmed by sequencing of their pertussis toxin A genes (data not shown).

Positivity rates determined by real-time PCR for IS481 and IS1001 assays

A total of 629 elution specimens from the same NP swabs were assembled into 63 pools, among which 1 pool consisted of 9 elution specimens. Several controls were included in the application of the real-time PCR for pools and single specimens in estimating the prevalence of asymptomatic infection. These controls included specific positive and negative PCR controls and negative DNA extraction controls, which went through the whole procedure of DNA extraction. In addition, inhibition of PCR amplification was also monitored. Only 1 potential positive elution specimen was negative for the human-2-microglobulin gene, which indicated the loss of nucleic acid extraction or inhibition of the PCR amplification. All other pools and potential positive specimens were positive for the human-2-microglobulin gene and the Ct values obtained were between 24 and 30 cycles. Sixteen pools were positive by B. pertussis *IS481* PCR and 10 pools were positive by

Table II. Positivity rates of Bordetella pertussis and Bordetella parapertussis among school children studied in 4 provinces of China.

		Number of positive RT-PCR and percentage		Number of positive cultures and percentage	
Group (y)	Number of children	B. pertussis	B. parapertussis	B. pertussis	B. parapertussis
7–9	215	7 (3.3)	2 (0.9)	1 (0.5)	0
10–12	205	13 (6.3)	9 (4.4)	1 (0.5)	1 (0.5)
13–15	208	10 (4.8)	2 (1.0)	0	0
Total	628	30 (4.8)	13 (2.1)	2 (0.3)	1 (0.2)

B. parapertussis *IS1001* PCR. There were 7 pools that were positive for both PCRs. When individual elution specimens were tested, a total of 30 NP samples (30/628) were positive by B. pertussis *IS481* PCR and 13 (13/628) were positive by B. parapertussis *IS1001* PCR. Further, all 3 culture-positive samples were also PCR-positive (Table II).

Prevalence of B. pertussis and B. parapertussis estimated by RT-PCR

According to the results of the real-time PCR for *IS481* and *IS1001*, the prevalences of asymptomatic infection with B. pertussis and B. parapertussis among the school children aged 7–15 y were 4.8% (95% confidence interval (CI) 3.1–6.5%) and 2.1% (95% CI 1.0–3.2%), respectively. The age group 10–12 y had a relatively higher prevalence of asymptomatic B. pertussis infection (6.3%, 95% CI 4.4–8.2%) than the other 2 age groups. However, the difference was not statistically significant ($p = 0.14$, Chi-square = 2.20). In addition, the prevalence of asymptomatic B. parapertussis infection in the age group 10–12 y was 4.4% (95% CI 3.6–5.2%), which was significantly different to that of the other 2 age groups ($p = 0.02$, Chi-square = 4.93). The distribution of positive specimens and prevalences of asymptomatic infection in the different age groups are shown in Table II. When the positivity rates of PCR for B. pertussis and B. parapertussis were compared among the 4 provinces, no statistically significant difference was found, although the highest positive rates for the PCR were observed in Zhejiang Province: 7.6% (12/158) (95% CI 6.5–8.7%) and 3.8% (6/158) (95% CI 3.0–4.6%), respectively (Table III).

Discussion

Pertussis is a reportable infectious disease and the number of reported cases has been decreasing in China since the introduction of DTwP vaccines in the 1980s. In China, pertussis is diagnosed clinically, and laboratory methods such as ELISA serology, PCR, and culture are not routinely used. According to the China CDC, 90% of reported pertussis cases have been in infants or young children who have suffered from classical 'whooping cough' symptoms. Studies have suggested that only 40–50% of pertussis cases show the classical clinical manifestation of a paroxysmal cough [17], often leading to misdiagnosis as a general respiratory infection and a failure to investigate for pertussis. The misdiagnosis of pertussis due to atypical pertussis symptoms has clearly contributed to the low incidence (under 1 per 100,000 since the 1990s) reported in China. When the reported pertussis cases in the 4 studied provinces were counted for the period 2004–2011, the total number of reported cases in Shandong Province was more than 2000 (estimated incidence (EI) 0.02‰), which was the highest. The number of cases in Yunnan and Zhejiang provinces ranged from 500 to 1000 cases (EI 0.01‰), ranked in second place, and there were relatively fewer cases in Guangdong Province, within the range of 100–500 cases (EI 0.004‰) [18]. According to official country estimates for China, the immunization coverage rates and districts achieving vaccination with 3 doses of DTaP in childhood have been more than 90% since 2002 [19]. The immunization coverage with 4 doses was over 99% in the y 2011.

The immunological reaction induced by injection of DTwP or DTaP vaccine provides significant protection against B. pertussis infection. Increasing evidence indicates that the protection offered by pertussis vaccination decreases with time [20–23]. Two studies have shown that the IgG anti-PT antibodies fall gradually to be around 18% from the peak value by the 6th y [24,25]. Therefore, school children who are partially immune to the bacterium become vulnerable to B. pertussis colonization or infection. In the present study, the detection of B. pertussis organisms by PCR among the asymptomatic school children was interpreted as asymptomatic infection with the pathogen.

In the present vaccine era, pertussis epidemics occur in a cyclic pattern, with peaks every 2 to 5 y [26]. The cyclic pattern is most likely due to the accumulation of vulnerable pools and the circulation of B. pertussis in the overall population. Data generated in previous studies suggest that the circulation of B. pertussis occurs in adolescents and adults

Table III. Number of subjects (%) positive for Bordetella pertussis and Bordetella parapertussis by RT-PCR.

Applied method (number of samples)	Shandong	Guangdong	Yunnan	Zhejiang
RT-PCR for B. pertussis	7 (4.6%)	2 (1.3%)	9 (5.5%)	12 (7.6%)
($n = 629$)[a] for B. parapertussis	1 (0.7%)	3 (2.0%)	3 (1.8%)	6 (3.8%)

[a]Of the 629 nasopharyngeal swabs, 153 were collected from Shandong, 153 from Guangdong, 165 from Yunnan, and 158 from Zhejiang.

[26,27]. Our finding of asymptomatic infections occurring in school children is in agreement with the earlier studies [28,29]. Because of passive surveillance of pertussis, only about 2000 cases were clinically diagnosed and reported in China in 2011 [18,30]. Although the cyclic pattern of epidemic pertussis has not been observed during the past 30 y, based on the present surveillance data, the reported pertussis cases increased 2-fold in 2012 compared with those reported in 2011 during the same period [18]. The results of this study also indicate that the true incidence of pertussis is underestimated in China. Our data showed that 10–12-y-old school children had the highest prevalence of B. pertussis (6.3%) compared to 7–9-y-old and 13–15-y-old school children. However, since the number of study subjects in each age group was not large enough, the possibility that the non-significant difference observed between the age groups was due to the sample size cannot be excluded. Moreover, the school children in this study were mainly sampled in county schools in the countryside. Higher prevalences of pertussis may be expected in the city. It appears that vaccination coverage in regions of the countryside may be lower than that in the cities. The true incidences in the city and countryside remain to be determined.

B. parapertussis infects humans and causes a pertussis-like syndrome, usually a milder illness than that caused by B. pertussis, although severe cases have been described [31]. In our study, more than 2% of study subjects were found to be PCR-positive for B. parapertussis. Moreover, subjects who were positive for B. parapertussis were found in all 4 provinces studied, suggesting that asymptomatic B. parapertussis infections are more prevalent than previously documented in this country.

The existence of asymptomatic pertussis infections has been a subject of debate for decades. Earlier studies on asymptomatic contacts of pertussis patients in which cultures were used for detection, gave a low rate of isolation of B. pertussis [32–34]. However, recent studies using the more sensitive PCR for the detection of B. pertussis have revealed higher positivity rates of asymptomatic pertussis infection [35,36]. In the present study, pertussis outbreaks and patients with typical pertussis symptoms were not reported in the schools of the 4 provinces during a period of 3 months before and after the start of the study. These children were on their school summer holiday when they were recruited. Therefore, it is unlikely that these samples were collected during school outbreaks of pertussis. The relatively lower prevalence of asymptomatic infection in this study (4.8%) compared to previous studies (25%, 4/16) [35] could be explained by the fact that a transient asymptomatic infection of pertussis probably existed, in which the bacteria were present in the nasopharynx in very small numbers that could be detected by PCR but not by culture. In addition, the possibility that the bacteria detected by PCR were already dead cannot be excluded. Our results also showed that the levels of IgG anti-PT of only 4 of the 30 PCR-positive subjects were more than 100 IU/ml, which indicated a recent or active infection with B. pertussis [37] (data not shown).

Colonization indicates the presence of an organism without clinical disease and is the carriage of the organism. In this study, we used asymptomatic infection instead of colonization for subjects with positive PCR results, because most of the subjects with positive PCR had low IgG anti-PT antibodies, indicating that this may have resulted from B. pertussis transient colonization and may not have elicited detectable anti-PT antibodies. Furthermore, it is known that the PCR positivity rate is usually higher in the early stage of infection [7], whereas higher antibody levels develop in the convalescent phase of the infection. In this study, the 2 subjects who were culture-positive for B. pertussis were in Shandong Province and were 9 and 11 y old. The subject who was culture-positive for B. parapertussis was in Guangdong Province and was 11 y old. All 3 subjects claimed that they had not had any symptoms (e.g., cough, sore throat, headache, or fever) during the 3 months before blood sampling. Further, they did not develop any respiratory symptoms during a 3-month follow-up. They had received all doses of the DTwP vaccine in childhood according to their vaccine history records. None of their household contacts had a history of cough in the 3 months before the study was started. The anti-PT IgG antibodies of these 3 children were 1.3 IU/ml, 14.1 IU/ml, and 4.9 IU/ml, respectively.

Although culture remains the gold standard for the diagnosis of pertussis, the low sensitivity due to the fastidious nature of the organism can hamper its use. Moreover, successful isolation and identification of B. pertussis by culture also depends on many factors, e.g. the type of transport system used, the time delay between specimen collection and inoculation, the skill of the technician, and the immune status of the patient. Since the culture of B. pertussis has not been performed for many years in the microbiology laboratories of the China CDC and provincial CDCs, the junior technicians performing culture lack experience and are not capable of discriminating Bordetella from other common respiratory bacteria. This might partly explain the low isolation rates observed in this study. In addition, we did not observe any epidemiological link among PCR-positive subjects in the 4 schools where the study was conducted.

The decision to implement a pooling protocol should be taken into account in the positivity rates

obtained. The benefit of a pooling protocol is reduced if every pool yields a positive result, which may occur during 'heavy' bacterial circulation in certain age groups, and thus demands subsequent testing of every specimen included in the pool. One strategy to circumvent this is to alter the size of the pool to account for the prevalence of the situation. For instance, if the positivity rate is near 10%, pools of 5 specimens may prove to be more practical than 10 specimens.

The advantage of this study was that the primers for *IS481* can amplify both B. pertussis and Bordetella holmesii, and primers for *IS1001* can amplify both B. parapertussis and B. holmesii. By performing both assays, discrimination between B. pertussis and B. parapertussis can be obtained. No sample was positive for both *IS481* and *IS1001* by monoplex real-time PCR, and carriage of B. holmesii was not identified in this study.

There are some limitations to the study. First, although the number of samples included in this study was more than 600, these samples were collected in 4 different provinces and the number of samples from each province was still limited. Second, of the 43 subjects who were PCR-positive for B. pertussis or B. parapertussis, only 3 culture-positive subjects were followed. Therefore, we do not know whether the rest of the subjects developed symptoms later.

Our results suggest that asymptomatic B. pertussis infections are common in school children in China, and asymptomatic B. parapertussis infections are more prevalent than previously documented. It is feasible to apply a pooled real-time PCR for *IS481* and *IS1001* targets to evaluate the prevalence of B. pertussis and B. parapertussis infections in immunized populations.

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