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Short communication

Efficient fecal-oral and possible vertical, but not respiratory, transmission of emerging *Chlamydia gallinacea* in broilers

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ABSTRACT

Chlamydia gallinacea is an endemic *Chlamydia* agent in poultry with a worldwide distribution. The aim of this study was to investigate whether *C. gallinacea* can be transmitted via fecal-oral, respiratory and vertical routes. After co-housing with *C. gallinacea*-inoculated broilers (n = 10) for 15 days, over 90.0% of SPF broilers (n = 10) became *C. gallinacea*-positive in their oropharyngeal and cloacal swabs. Connection of isolators with ventilation tubing resulted in transmission of infectious bronchitis virus, but not of *C. gallinacea*, from infected broilers in one isolator to uninfected ones in the other isolator. *Chlamydia*-qPCR determined that 97.6% of shells of embryonated eggs (287/294) from a breeding farm were positive for *C. gallinacea*. *C. gallinacea* positivity in egg albumen increased significantly from 7.6% (10/128) before incubating to 44.4% (8/18) of 7-day incubation, and from 5.5% (7/128) to 38.9% (7/18) in egg yolk. After incubating for 19 days, *C. gallinacea* DNA was detected in heart (5/55, 9.1%), liver (3/55, 5.5%), spleen (7/55, 12.7%), lung (6/55, 10.1%), kidney (8/55; 14.5%) and intestine (4/55, 7.3%) of chicken embryos. Taken together, our data indicate that *C. gallinacea* can be efficiently transmitted by the fecal-oral route, but not via aerosol. Additionally, vertical transmission can occur via penetration of *C. gallinacea* from eggshell to albumen, yolk, and the growing embryo. Our findings provide essential information for the control of *C. gallinacea* in poultry farms.

1. Introduction

Chlamydia spp. are obligate intracellular bacterial pathogens that cause a number of important diseases in wild and domestic birds, mammals and humans. Two novel species of this genus, *C. gallinacea* and *C. avium*, have been recently described (Sachse et al., 2014; Szymańska-Czerwińska and Niemczuk, 2016; Laroucau et al., 2015; Vorimore et al., 2013; Taylor-Brown et al., 2016). *C. gallinacea* was found primarily in chickens, turkeys and guinea fowl, but was also shown to infect cattle (Li et al., 2016). Experimentally infected chickens with *C. gallinacea* exhibited no clinical disease symptoms but grew more slowly and had significantly reduced gains in body weight (6.5–11.4%) (Guo et al., 2016). *C. gallinacea* appears to be widely distributed and has been reported to be the predominant *Chlamydia* agent in poultry of Argentina, China, Holland, Italy, Poland and USA (Sachse et al., 2014; Hulin et al., 2015; Guo et al., 2016; Li et al., 2016; Li et al., 2017; Szymańska-Czerwińska et al., 2017; Donati et al., 2018; Heijne et al., 2018)

It was reported that *C. gallinacea* was the predominant chlamydial agent representing 63.8% of all positives (384/602) and 81.2% of positive chickens (359/442) in China (Guo et al., 2016). Similarly, Szymańska-Czerwińska et al. reported that *C. gallinacea* occurred in 65.5% of all positive poultry flocks and in 73.0% of positive chicken flocks in Poland (Szymańska-Czerwińska et al., 2017). As a widespread and predominant *Chlamydia* species with a potential public health significance, *C. gallinacea* is poorly understood in its mode of transmission. In this study, experimental models were performed to determine if *C. gallinacea* could be transmitted via fecal-oral, aerosol, or vertical routes.

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2. Materials and methods

2.1. C. Gallinacea and infectious bronchitis virus

The *C. gallinacea* strain JX-1 used in this study (GenBank Accession #: NZ_CP019792.1) was isolated from a cloacal swab of an asymptomatic chicken in Jiangxi province of China (Guo et al., 2016, 2017). The infectious bronchitis virus (IBV) strain JS/2010/12 (GenBank accession no. JQ900122.1) (Yu et al., 2017) was kindly provided by Dr. Yantao Wu from Yangzhou University College of Veterinary Medicine, China.

2.2. C. Gallinacea transmission by direct contact

All work in this study was reviewed and approved by the Institutional Animal Care and Use Committee of the Yangzhou University College of Veterinary Medicine, China. The experiments were performed in accordance with the approved IACUC protocol.

One-day-old SPF AA broiler chickens (n = 20) were obtained from Sandeli Animal Husbandry Development Co., Ltd (Zhenjiang, Jiangsu, China), and individually tagged and housed in a level-two containment facility with free access to antibiotic-free food and water. Prior to the commencement of the experiment, both of the oropharyngeal and cloacal swabs were collected from each SPF chicken for *C. gallinacea* detection by qPCR. After one week, the chickens were randomly separated into two groups. Ten chickens in group-1 were intranasally inoculated with 20 μL of 2×10^6 genomes of *C. gallinacea* (n = 10). Chickens in group-2 (n = 10) received 20 μL 1 × PBS, and were co-housed with the *C. gallinacea*-infected chickens (Fig. 1).

Every five days post inoculation (pi), oropharyngeal and cloacal swabs were collected from chickens in both groups for detection of chlamydial DNA. On day 26 pi, five chickens from each group were randomly chosen and euthanized. Eleven organs (heart, lung, proventriculus, duodenum, rectum, cloaca, kidney, ovary, testicle, liver and spleen) were collected for detection of *C. gallinacea* DNA by a qPCR as described before (Guo et al., 2016) (Fig. 1).

2.3. C. gallinacea transmission by aerosol

Two chicken GJ-1 isolators (Fengshi Group, Suzhou, China) were connected with ventilation tubing. The isolator has a size of 2200 × 850 × 1800 mm^3, and the airflow has a speed of 0.1 to 0.2 m/s from isolator-1 to isolator-2 through the ventilation pipe (Fig. 2).

Ten seven-day-old SPF AA broiler chickens were put in each of these two isolators. Each chicken in isolator-1 received 10^4 EID_{50} IBV by an intranasal inoculation while chickens in isolator-2 received 20 μL of 1 × PBS. The oropharyngeal swabs were collected from each chicken of both isolators from day 1 to day 5 pi for IBV detection by PCR (Callison et al., 2006).

Similarly, two GJ-1 isolators were used to study the transmission of *C. gallinacea* by aerosol. Chickens in isolator-1 (n = 10) received intranasal inoculation of 2×10^6 *C. gallinacea*, and the chickens in isolator-2 (n = 10) were given 20 μL 1 × PBS. The oropharyngeal and cloacal swabs were collected from each chicken in both isolators on days 5, 10, 15, 20 and 25 pi. On day 26 pi, five chickens from each isolator were randomly selected and euthanized while heart, lung, proventriculus, duodenum, rectum, cloaca, kidney, ovary, testicle, liver and spleen were collected for detection of *C. gallinacea* DNA by qPCR.

2.4. C. gallinacea-contaminated chicken embryos

A previous surveillance study by qPCR and *Chlamydia* culture determined that the Shaobo Breeding Chicken Farm in Jiangsu province of China was positive for *C. gallinacea* (Guo et al., 2016). In this study, cloacal swabs and semen from rooster (n = 28) and cloacal samples from layers (n = 100) were obtained for *C. gallinacea* detection. In the meantime, 294 freshly laid eggs from these 100 layers fertilized by these 28 roosters were collected for this study.

Eggshell swabs were taken from these 294 freshly laid eggs while albumen and yolk was obtained from 128 of these 294 eggs. Subsequently, the remaining 166 eggs were incubated while the unfertilized eggs and the dead embryos were recorded during the incubating process. On day 7 after start of incubation, 18 chicken embryos were randomly chosen to obtain albumen and yolk for *Chlamydia* DNA detection. On day 19 of incubation, the remaining chicken embryos were put in 4 °C for four hours, followed by collecting six organs (heart, liver, spleen, lung, kidney and intestine) from chicken embryos (n = 55) for *Chlamydia* DNA detection.

2.5. Extraction of nucleic acids and qPCRs for C. gallinacea and IBV

High-Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) was used to extract total nucleic acids from oropharyngeal and cloacal swabs, organs from chickens and chicken embryos, egg shell swabs and egg albumen and yolk according to the manufacturer's instructions and described before (Li et al., 2016). The extracted DNA was eluted in 200 μL elution buffer. Quantitative PCRs were performed as described to quantify *C. gallinacea* (Guo et al., 2016) and IBV (Callison et al., 2006).

2.6. Statistical analysis

Chi-squared Test was used to compare the positivity of *C. gallinacea* DNA detected in chicken embryos before and after hatching.

3. Results

3.1. Efficient transmission of C. gallinacea via fecal-oral route

All SPF chickens were confirmed to be free of *C. gallinacea* before the commencement of the experiment. In this study, ten *C. gallinacea*-inoculated broilers were co-housed with ten SPF chickens while oropharyngeal and cloacal swabs were collected every five days for *Chlamydia* DNA detection (Fig. 1). For the *C. gallinacea*-inoculated broilers, all were positive in their oropharyngeal swabs from day 5 to day 25 pi while five cloacal swabs were negative for *C. gallinacea* on day 5 pi and one cloacal swab did not become positive until day 25 pi. In comparison, for the PBS-inoculated broilers, 70% of the oropharyngeal swabs were positive on day 5 pi and all oropharyngeal swabs became positive from day 15 pi. None of the cloacal swabs from the PBS-inoculated broilers was positive on day 5 pi and *Chlamydia* DNA was detected in 90% of the cloacal swabs on day 15 pi. Nevertheless, all 20 broilers were positive for both oropharyngeal and cloacal swabs at the end of the observation (day 25 pi) (Fig. 1A).

On day 26 pi, ten organs were obtained from five chickens of each group for *Chlamydia* detection. The highest *C. gallinacea* burdens were found in the cloaca and rectum. *Chlamydia* DNA was found also in heart, duodenum, kidney, ovary and spleen, but not in testicle and liver (Fig. 1B).

3.2. Failure of C. gallinacea transmission via aerosol

Two sets of two chicken GJ-1 isolators were connected with a ventilation pipe, and the chickens in isolator-1 were inoculated with IBV (set 1) or *C. gallinacea* (set 2) while chickens in isolator-2 received 1 × PBS. Periodically, the swabs were collected from chickens for detection of IBV or *C. gallinacea* (Fig. 2).

While all ten infected chickens in isolators-1 were confirmed to be IBV positive from day 1 to day 5 pi, three of ten PBS-isolated chickens in isolator-2 were verified to be IBV positive day 4 pi, and the fourth became positive day 5 pi. This suggested that this set-up with airflow between isolators could be used to investigate the possible transmission

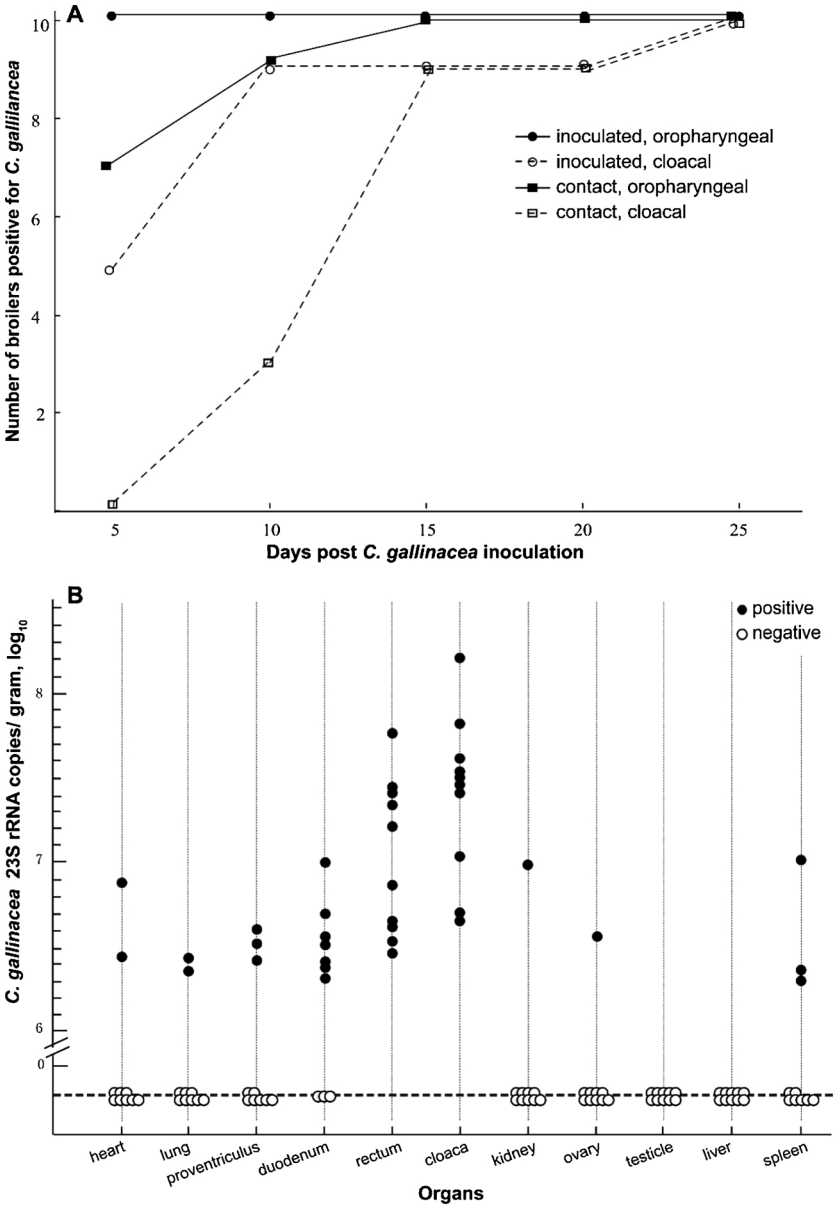

Fig. 1. Oral-fecal transmission of *Chlamydia gallinacea* in broilers. Ten SPF AA broiler chickens intranasally inoculated with 20 μL of 2×10^6 genomes of *C. gallinacea* were co-housed with 10 SPF broilers received 1 × PBS. **A**: *Chlamydia*-qPCR showed that all ten oropharyngeal swabs from *C. gallinacea*-inoculated broilers were positive until the end of the experiment (day 25 post inoculation). Whereas, 5 of 10 cloacal swab samples from inoculated boilers were positive at day 5 PI and all cloacal swabs became positive at the end of the observation. In comparison, the co-housed broilers without *C. gallinacea* inoculation showed increasing positivity over the course of observation. **B**: On day 26 pi, five chickens from each group were randomly chosen from each group and euthanized while ten organs were tested for *C. gallinacea* DNA by qPCR. The *C. gallinacea* DNA was not detected in testicle and liver, and was positive in other eight organs (heart, lung, proventriculus, duodenum, rectum, cloaca, kidney, ovary and spleen) with highest copy numbers in cloaca and rectum.

of *C. gallinacea* via aerosol.

All *C. gallinacea*-inoculated chickens in isolator-1 and the PBS-inoculated chickens in isolator-2 were monitored for the presence of *C. gallinacea* DNA for 25 days. While all chickens in isolator-1 were confirmed to be positive on day 15 pi, *C. gallinacea* DNA was not detected in oropharyngeal and cloacal swabs of chickens and organs of the euthanized chickens in isolator-2. The difference in IBV and *C. gallinacea* transmission ($p = 0.025$; Chi square test) demonstrated that *C. gallinacea* was not readily aerosolized from infected chickens and thus could not be transmitted via aerosol.

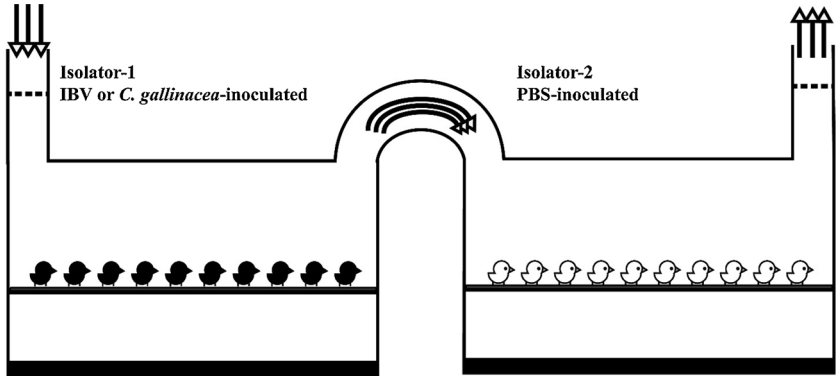

Fig. 2. Investigation of the possibility of IBV and *C. gallinacea* transmissions by aerosol between isolators. Two chicken GJ-1 isolators were connected with a ventilation pipe. The chickens in isolator-1 were inoculated with IBV (set 1) or *C. gallinacea* (set 2) while chickens in isolator-2 received 1 × PBS. Periodically, the swabs were collected from chickens of both for detection of IBV or *C. gallinacea*.

3.3. Possible vertical C. gallinacea transmission via eggshell penetration

Chlamydia qPCR determined that 75.0% (75/100) of cloacal swabs from layers in the Shaobo Breeding Chicken Farm in Jiangsu province were positive for *C. gallinacea* DNA. Surprisingly, the cloacal samples and semen of 28 roosters from the same breeding farm were all negative for *C. gallinacea*.

A total of 294 eggs were collected from the breeding farms, and qPCR was performed to determine *Chlamydia* DNA in eggs before incubation (shell, albumen, yolk), incubation for 7 days (albumen, yolk), and incubation for 19 days (heart, liver, spleen, lung, kidney, intestine). Up to day 7, 20 unfertilized eggs were excluded from this study. Between day 8 to day 19 after start of incubation, 37 fertilized eggs were found dead, and were eliminated from this study as well. On day 19 after incubation, 55 of 91 fertilized eggs were randomly chosen for *C. gallinacea* detection.

From before incubation to 7 days of incubation, *C. gallinacea* positivity increased significantly in albumen (day 0: 7.8%, 10/128; day 7: 44.4%, 8/18; $p = 0.001$; Chi square test) and in yolk (day 0: 5.5%, 7/128; day 7: 38.9%, 7/18; $p < 0.001$; Chi square test). After the chicken embryos were incubated for 19 days, *C. gallinacea* DNA was detected in all assayed organs with the positivity ranging from 5.5% to 14.5% (Fig. 3). Overall, the *C. gallinacea* positivity in eggshell was significantly higher than in albumen, yolk and chicken embryo (Fig. 3).

4. Discussion

In an epidemiological study of *Chlamydia* spp. on apparently healthy chickens in China, it was determined that *C. gallinacea* was the predominant *Chlamydia* agent, and the chickens contained high numbers of *Chlamydia*: up to 966,977 genomes/ cloacal swab, and 447,141 genomes/ oropharyngeal swab (Guo et al., 2016). The *C. gallinacea*-infected chickens shed large quantities of pathogens in their fecal droppings, facilitating the oral-fecal transmission of *C. gallinacea*. Consistent with this observation, this study showed that after co-housing with *C. gallinacea*-inoculated broilers for 15 days, over 90% of the mock-inoculated broilers became *C. gallinacea*-positive in their oropharyngeal and cloacal swabs. Caged chickens may show lower infection rates with *C. gallinacea* than cage-free chickens, as illustrated in the fecal-oral transmission of avian hepatitis E virus (Liu et al., 2017). In that study, cloacal swabs were collected from cage-free layers and caged rooster which were housed in the same room and there was a distance of four meters between layers and rooster. Interestingly, 70% of cage-free layers were positive in cloacal swabs while 26 caged roosters housed in the same room were totally negative in their cloacal swabs and semen. This may further suggest the efficient fecal-oral transmission of *C. gallinacea*, but not via aerosol.

It has been shown that the *C. gallinacea*-infected chickens were asymptomatic and did not show any respiratory signs, suggesting the low possibility of respiratory transmission of this pathogen. A set-up with two connected isolators verified the successful transmission of IBV from infected chickens in isolator 1 to uninfected chickens in isolator 2. However, the same set up did not identify respiratory transmission of *C. gallinacea* up to 25 days pi. The inability of respiratory transmission of this organism can be further suggested by the phenomenon that roosters housed in the same room with *C. gallinacea*-positive layers stayed *C. gallinacea* negative.

As described above, the *C. gallinacea*-infected chickens shed a large amount of pathogen through their fecal droppings, and this inevitably results in contamination of the eggshell of the embryonated eggs. The high load of pathogens in contaminated eggs may support horizontal transmission of the pathogen to other eggs (Ahmed et al., 2017). On the other hand, the pathogen on the eggshell can penetrate and get in egg albumen, yolk and growing embryos even with the protection of egg membrane. Consistent with this hypothesis, the freshly collected eggs in this study showed 97.6% *C. gallinacea* positivity in their eggshell, and the positivity was lower in albumen (7.6%), and the lowest in yolk (5.5%). However, after 7 days of incubation, the positivity increased by 6.6-fold in albumen (7.6%–50.0%) and by 7.1-fold in yolk (5.5%–38.7%). It seems also that the penetration of *C. gallinacea* can further reach the growing embryos. After incubation for 19 days, *C. gallinacea* DNA was detected in heart (5/55, 9.1%), liver (3/55, 5.5%), spleen (7/55, 12.7%), lung (6/55, 10.1%), kidney (8/55; 14.5%) and intestine (4/55, 7.3%) of chicken embryos.

Taken together, our data indicate that *C. gallinacea* is efficiently transmitted via the fecal-oral route, but not via aerosol. Additionally, vertical transmission can occur via eggshell penetration of *C. gallinacea* from eggshell to albumen, yolk, and then the growing embryo. Our findings provide essential information for the control of *C. gallinacea* in poultry farms.

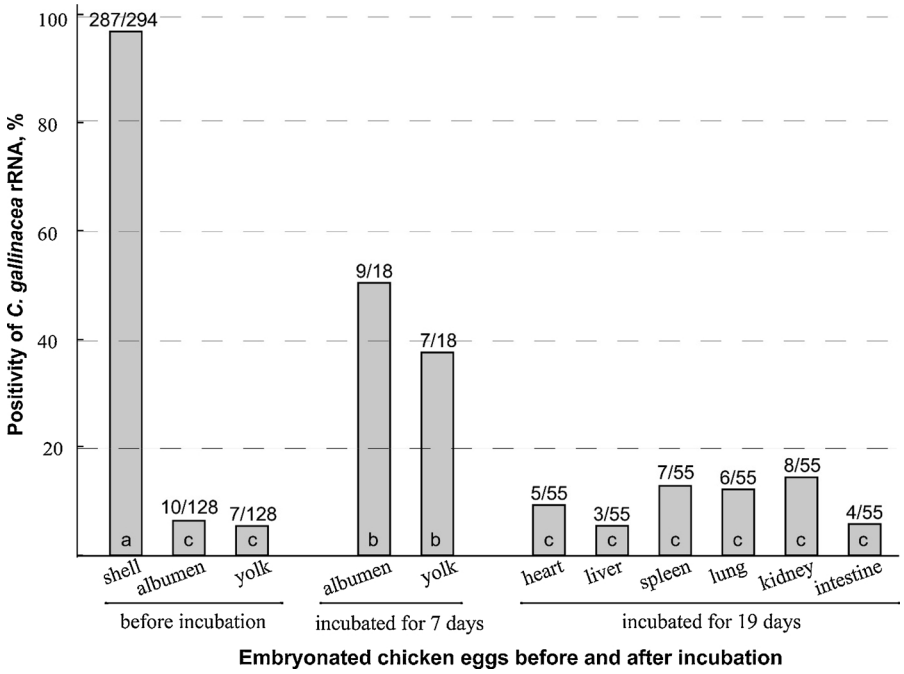

Fig. 3. *C. gallinacea* DNA detected in chicken embryos before and after incubation. The *Chlamydia*-qPCR determined that 97.6% of chicken embryos (287/294) was positive for *C. gallinacea*. The *C. gallinacea* positivity significantly increased after incubation for 7 days in albumen (7.6%, 10/128; 50.0%, 8/18) and yolk (5.5%, 7/128; 38.9%, 7/18). After the chicken embryos were incubated for 19 days, *C. gallinacea* DNA was detected in all assayed organs with the positivity from 5.5% in liver (3/55) to 14.5% in kidney (8/55). Chi-squared Test was used to compare the positivity of *C. gallinacea* DNA detected in chicken embryos before and after incubation. Different letters (a, b, c) indicate significant difference.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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