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Original article

A strain of pathogenic *Bacillus subtilis* results in brain damage in ducklings when co-infected with *Riemerella anatipestifer*

T.F. Zhu, F.F. Chen, J.C. Li

Collage of Animal Science and Technology,
Anhui Agricultural University, Changjiang West Road 130, Hefei 230036, China

Abstract

Bacillus subtilis is a Gram-positive bacterium widely used in medicine and agriculture. So far, little is known about its pathogenicity in animals. In this study, a strain of *Bacillus subtilis*, HFBF_B11 isolated from brain tissue of ducklings co-infected with *Riemerella anatipestifer* was characterized. The strain demonstrated consistent characteristics of *B. subtilis* in staining and morphological, biochemical and physiological analyses. Moreover, its DNA sequence, which was obtained via PCR sequencing of 16S rRNA, exhibited 99% homology with the *B. subtilis* reference strain. In *in vitro* cultures HFBF_B11 exhibited β-hemolysis. The results of experiments showed that a single infection of HFBF_B11 in 9-day-old ducklings did not result in clear clinical symptoms. However, following co-infection with HFBF_B11 and *R. anatipestifer*, the animals demonstrated liver injury and blood-brain barrier disruption leading to infection and brain damage with a mortality rate of 100%. These results suggest that the HFBF_B11 strain of *B. subtilis* is an opportunistic pathogen of ducklings. This is the first report about the isolation of a *B. subtilis* strain with pathogenicity in ducklings.

Key words: *Bacillus subtilis*, co-infection, *Riemerella anatipestifer*, duckling, opportunistic pathogen

Introduction

The genus *Bacillus* comprises aerobic, Gram-positive bacteria of more than 200 known species. Most *Bacillus* species are not pathogenic, except for *B. anthracis* and *B. cereus*. Some species are even probiotics, for example, *B. subtilis* is widely used in medicine and agriculture. Various extracts from *B. subtilis* have been applied in the treatment of intestinal diseases (Selvam et al. 2009, Foligne et al. 2012, Gong Y et al. 2016), cancer (Chen et al. 2015), and asthma (Bang et al. 2015). In addition, the administration of medicinal CU1, a strain of *B. subtilis*, can improve the immunity of elderly people (Lefevre et al. 2015). Research in the field of agriculture has shown that various strains of *B. subtilis* can prevent tomato leaf fall disease (Maketon et al. 2008, Gao et al. 2013) and *Rhizopus* rot in peaches (Wang et al. 2013). *B. subtilis* has also been used in aquaculture and livestock breeding, and can improve growth, prevent

Correspondence to: J.C. Li, e-mail: 1979343589@qq.com

catfish pathogen disease (Ran et al. 2012), shrimp disease (Song et al. 2012, Zokaeifar et al. 2012) and juvenile sea cucumber disease (Zhao et al. 2012), and promote rumen fermentation (Sun et al. 2016). Moreover, *B. subtilis* can immunize the mucosa, thus it has been used as a carrier in recombinant vaccines, namely for foot-and-mouth disease and *Vibrio cholerae* infection (Hu et al. 2011).

However, little is known about the pathogenicity of *B. subtilis*. We isolated a strain of potentially pathogenic *B. subtilis* from a duckling co-infected with *R. anatipestifer*. In this study, the bacteriological characteristics and pathogenicity were demonstrated in a series of identification and animal experiments.

Materials and Methods

Bacterial isolation

Brains and liver tissues were obtained from 13-day-old dead ducklings with neurological symptoms. The bacteriological cultures were grown at 37°C in the presence of 5% CO_2 in tryptic soy broth (TSB) medium (Becton, Dickinson and company, MD, USA) for 36-48 h. Isolates were harvested on TSB medium with 10% DMSO and stored at -80°C until further use.

Morphological and biochemical identification of bacterial isolates

Colony morphology, size, shape and color of the isolated strains were recorded after 36-h culture. The bacteria were smeared on glass slides using an inoculating needle, and Gram staining was performed as described by Vincent and Humphrey (Vincent et al. 1970) using a commercially available kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The probiotic strain *B. subtilis* (Ehrenberg) Cohn (ATCC 23857), purchased from the China General Microbiological Culture Collection Center (Beijing, China), was used as the control strain.

A series of biochemical tests, including indole production, methyl red, Voges-Proskauer test, carbohydrate utilization test, catalase test, urease, hydrogen sulfide, Simmons' citrate reaction, nitrate reduction, arginine dihydrolase and gelatin test, were conducted to characterize the isolated bacteria using the criteria of Bergey's Manual of Systematic Bacteriology (Bergey et al. 1994). Bacterial hemolysis was evaluated using blood agar medium (with 10% rabbit whole blood) after 24-h culture at 37°C.

Molecular characterization of bacterial isolates

Bacteria were inoculated in TSB for 12 h using a needle and harvested by centrifuged at 12,000 × *g* at 4°C for 15 min to separate the mixtures. Genomic DNA was extracted using bacterial genomic DNA extraction kit (Guangzhou, China) according to the manufacturer's instructions. It was stored at -20°C until use as template DNA in PCR to amplify 16S rRNA for genetic analysis.

The 16S rRNA gene amplification was performed using the universal primers (Ludwing. 2007), BF 27f (5' AGAGTTTGATCCTGGCTCAG 3') and BF 1525r (5' AAGGAGGTGWTCCARCC 3'). PCR was performed in 20-µL volume reactions, including 10 µL of 2× premix *Taq* (Takara, Dalian, China), 1 µL each of forward and reverse primers (20 µM), and 1 ng of template DNA using the following condition: 30 cycles of denaturation for 10 s at 94°C, annealing for 30 s at 58°C, and extension for 60 s at 72°C, followed by final extension of 8 min at 72°C. The product was electrophoresed on a 1.0% agarose gel and the products were purified using a gel extraction kit (GBCBIO Technologies Inc, Guangzhou, China). The resulting PCR fragments were inserted into the pMD18-T vector (Takara) for sequencing using the direct-sequencing method by Sengon Bio Co. (Shanghai, China). The BLAST search program was used to identify nucleotide sequence homology of the 16S rRNA region.

Antibiotic susceptibility

Antibiotic susceptibility of the isolated bacterial strains was determined using the Kirby-Bauer disk diffusion method. The plates were then inoculated by the bacteria over the entire agar surface and the antimicrobial disks were placed using sterile forceps on the agar surface, incubated at 37°C for 24 h. The inhibition zone diameters were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guideline as susceptible (S), intermediate (I) or resistant (R) [Vandepitte J et al. 2003]. The following antimicrobial discs were used: clindamycin 2 µg (CC-2), vancomycin 30 µg (Va-30), kanamycin 30 µg (KAN-30), ampicillin 10 µg (AM-10), amikacin 30 µg (AN-30), gentamicin 10 µg (GM-10), penicillin 10 µg (P-10), erythromycin 15 µg (E-15), tobramycin 10 µg (NN-10) and neomycin 30 µg (N-30) (Binhe Microorganism Reagent Company, Hangzhou, China).

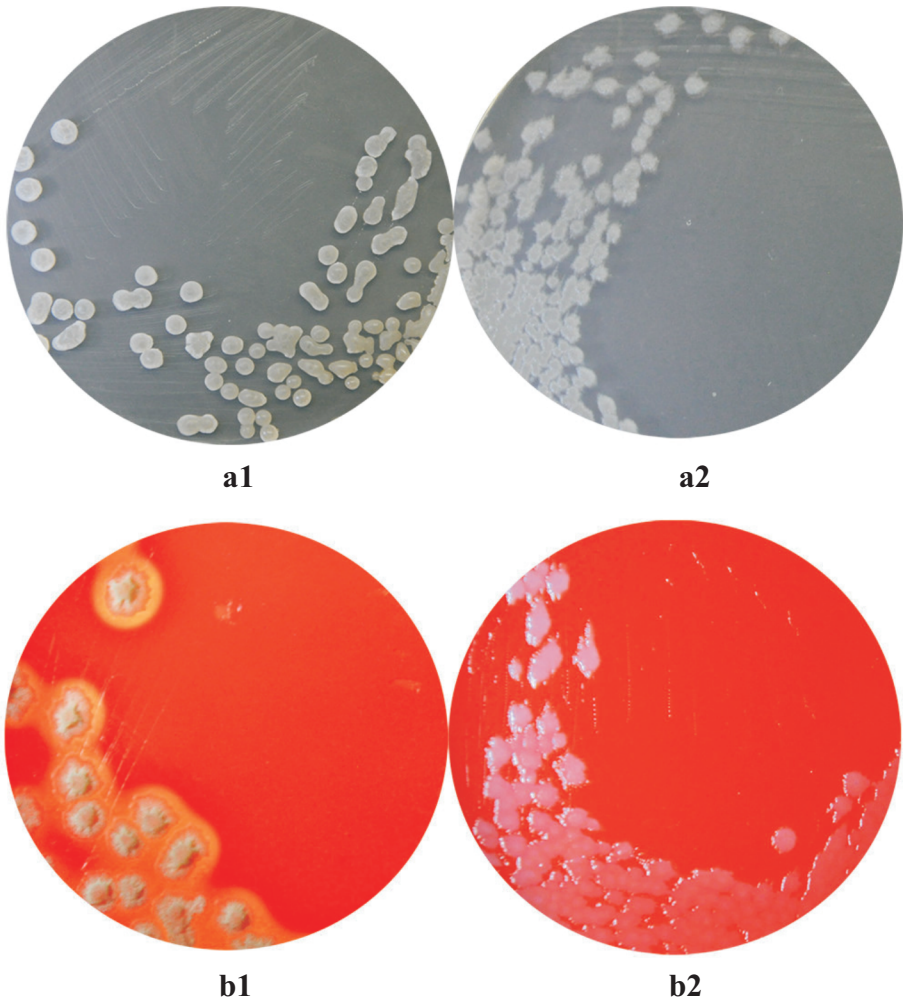


Fig. 1. The bacterial colony morphology and hemolytic activity of *Bacillus subtilis*. The isolated strain was cultured in the TSB plate (a1) or blood plate (b1) for 24h; it formed colonies with 1-1.5 cm in diameter and had β hemolytic activity, while the control strain (b1 and b2) formed relative larger in diameter colonies, showed smooth surface and edge, and had no hemolytic activity.

Animal experiments

Thirty healthy 9-day-old ducklings were obtained from a breeding farm in Anhui province. All procedures performed in studies involving animals were in accordance with the ethical standards of Anhui Medicine University at which the studies were conducted. The ducklings were fed food and water *ad libitum*, randomly assigned to five groups (n = 6 per group). The first group was the negative control (normal ducklings). The second group was infected with *R. anatipestifer* (5×10^9 CFU/ml), which was isolated and identified in our Lab, by intramuscular leg injection. The third group was infected with *B. subtilis* (HFBF_B11 strain, 5×10^9CFU/ml) by intraperitoneal injection. The fourth group was co-infected with *R. anatipestifer* (5×10^9 CFU/ml) and *B. subtilis* (HFBF_B11 strain, 5×10^9 CFU/ml) by intramuscular leg injection and intraperitoneal injection separately. The fifth group was co-infected with *R. anatipestifer* (5×10^9 CFU/ml) and the control strain (*B. subtilis*, ATCC 23857, 5×10^9 CFU/ml) by the same injection route as the fourth group. After infection, the ducklings were examined until death, and the remaining animals were sacrificed on postinfection day 5. Brain and liver tissues were collected and used for blood smears and Switzerland staining (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and bacterial isolation.

Results

The strain shared identical morphological, biochemical, and staining characteristics with *B. subtilis*

The strain, which was isolated from the brain and liver tissues of infected ducklings, was cultured for 36 h. It formed small colonies, which were white with

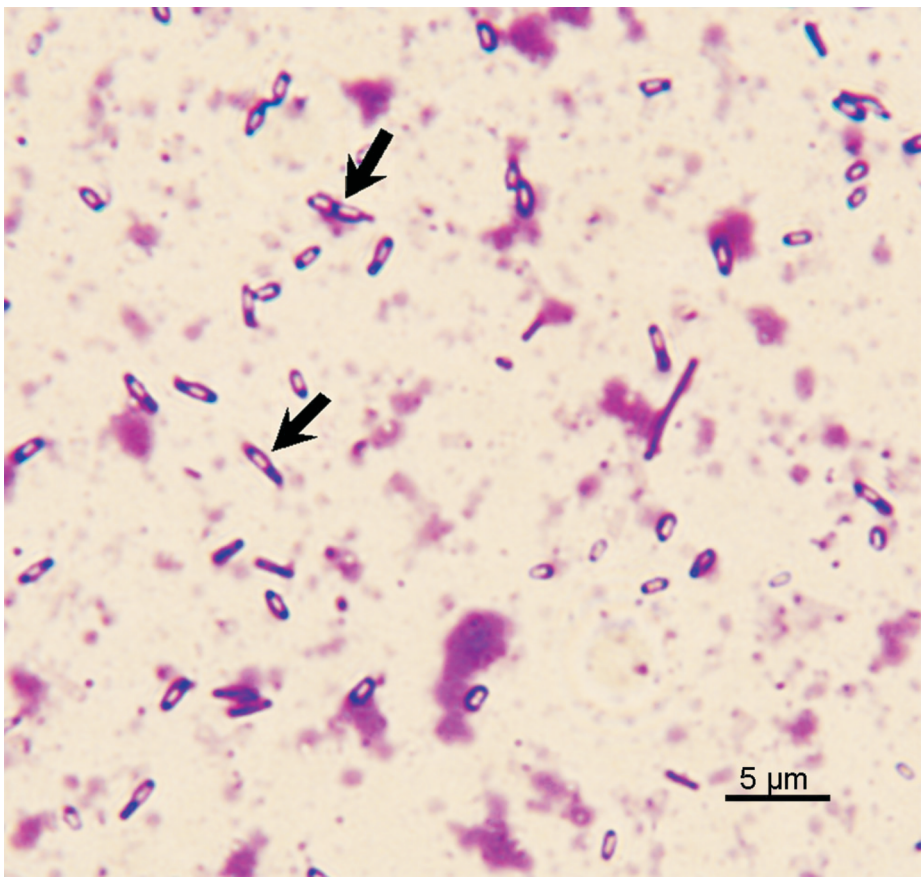


Fig. 2. The morphology on gram staining of *Bacillus subtilis* of bacteria. The isolated strain was cultured in the TSB plate for 24h and were stained with Gram reaction. Under microscope (Olympus IX53, 100X10) the bacteria showed gram-positive and had spore.

Table 1. Results of BLAST of *Bacillus subtilis* HFBF_B11 strain in GENBANK.

Description	Max score	Total score	Identify	Accession number
Bacillus subtilis subsp. subtilis strain BSD-2, complete genome	2837	28276	99%	CP013654
Bacillus subtilis strain TO-A JPC, complete genome	2837	28281	99%	CP011882
Bacillus subtilis strain UD1022, complete genome	2837	28228	99%	CP011534
Bacillus subtilis HJ5, complete genome	2937	19807	99%	CP007173

flat, smooth surfaces and regular edges (Fig. 1a1). The probiotic strain *B. subtilis* was gray-white with flat, rough surfaces and irregular edges (Fig. 1a2). The strain also showed β-hemolytic activity on blood agar medium cultured for 24 h (Fig. 1b1), whereas the probiotic strain did not (Fig. 1b2). After staining, the bacteria were observed under a microscope. As shown in Fig. 2, the bacteria were Gram-positive rods with rounded ends and spores located in the middle of the cells. Moreover, the results of carbohydrate utilization tests showed that the strain fermented glucose and sucrose, but not lactose, maltose, arabinose, raffinose, mannitol, trehalose, sorbitol or xylose. Voges-Proskauer, Simmons' citrate, nitrate reduction, urease, arginine dihydrolase, gelatin and catalase testing were positive, and indole production, methyl red and hydrogen sulfide testing were negative.

The 16S rRNA sequence of the isolated strain was identical to that of *B. subtilis*

We then performed PCR analysis using universal primers and the extracted genomic DNA of the isolated strain as the template. A 1561bp segment was amplified and sequenced, and a sequence alignment was performed (Table 1), which showed 99% homology with *B. subtilis* reference strains. We subsequently named the isolated strain *B. subtilis* (HFBF_B11) and the 16S rRNA sequence was deposited in GenBank (accession number KU644133).

Table 2. Results of bacteria antibiotic sensitivity tests for *Bacillus subtilis.*

Antibiotic	contents	Criterion			The inhibition zone diameters (mm)/ sensitivity
		Resistant (R)	Intermediate (I)	Susceptible (S)	
Clindamycin	2 μg (CC 2)	≤14	15-20	≥21	20/I
Erythromycin	15 μg (E15)	≤13	14-22	≥23	26/S
Vancomycin	30 μg (VA 30)	≤9	10-11	≥12	22/S
Kanamycin	30 μg (KAN 30)	≤13	14-17	≥18	24/S
Amikacin	30 μg (AMK 30)	≤14	15-16	≥17	26/S
Ampicillin	10 μg (AP 10)	≤13	14-16	≥17	22/S
Penicillin	10 μg (P 10)	≤28	–	≥29	30/S
Tobramycin	10 μg (TOB 10)	≤12	13-14	≥15	24/S
Gentamicin	10 μg (GM 10)	≤12	13-14	≥15	25/S
Neomycin	30 μg (N30)	≤12	13-16	≥17	24/S

The isolated strain was susceptible to commonly used antibiotics

Antibacterial susceptibility testing using the disk diffusion method demonstrated that the isolated strain was susceptible to most antibiotics used in veterinary science. However, the strain showed intermediate susceptibility to clindamycin (Table 2).

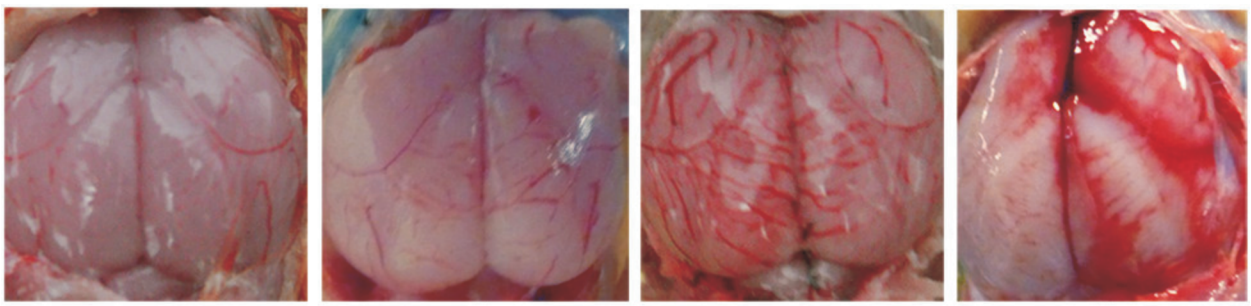
The isolated strain is a classic opportunistic pathogenic bacterium

In the animal experiment, the ducklings in the control group (infected with normal saline) showed no pathological symptoms and no bacteria were isolated from the tissues. The second group infected with *R. anatipestifer* exhibited decreased food and water intake. One duckling died on postinfection day 3, and *R. anatipestifer* was isolated from the live rather than from the brain. However, the other ducklings recovered and no bacteria were isolated. The third group infected with *B. subtilis* (HFBF_B11) exhibited no pathological symptoms, except for decreased food and drink intake, and no ducklings died. The ducklings subsequently recovered after postinfection day 3, and no bacteria were isolated from their tissues. The fourth group, co-infected with *R. anatipestifer* and *B. subtilis* (HFBF_B11), began to die on postinfection day 2. All ducklings died by postinfection day 5, and all ducklings displayed clear neurological symptoms. The fifth group, co-infected with *R. anatipestifer* and *B. subtilis* (ATCC 23857), exhibited the same results as the second group. *R. anatipestifer* was isolated from the brains and livers in the second, fourth and fifth groups, while *B. subtilis* (HFBF_B11) was isolated from the brains and livers in the third and fourth groups. Although all brain and liver tissues from the infected animals showed histological changes, such as congestion and swelling, the tissues from ducklings co-infected with *R. anatipestifer* and *B. subtilis* (HFBF_B11) showed obvious local pathological changes (Figs. 3 and 4) compared with the control group. Moreover, *R. anatipestifer* and *B. subtilis* (HFBF_B11) were found in the brains and livers of Switzerland-stained smears, and *B. subtilis* were determined in mononuclear phagocytes of the brain and liver (Fig. 5).

Discussion

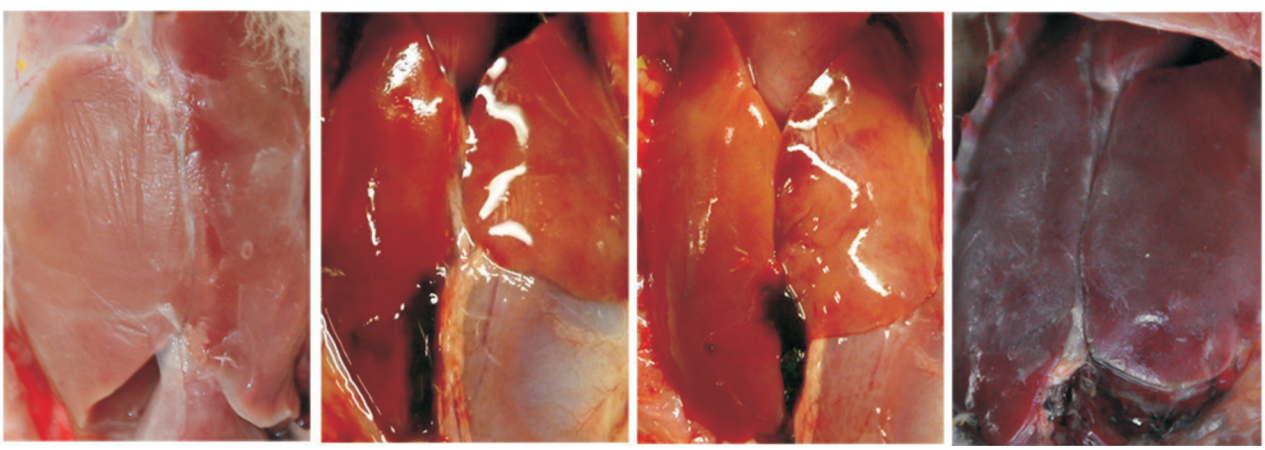
In our study, a bacterial strain isolated from a dead duckling co-infected with *R. anatipestifer* exhibited morphological and biochemical features common to *Bacillus* species (Figs. 1 and 2). We identified the isolated strain to be *B. subtilis* according to its 16S rRNA sequence (Table 2). Notably, this isolated strain appeared to be a specific pathogenic *B. subtilis* strain of ducklings, as demonstrated by β-hemolytic activity (Fig. 1), tissue damage (Figs. 3 and 4) and high fatality rate when co-infected with *R. anatipestifer*. Other *B. subtilis* isolates did not demonstrate such features.

Most pathogenic bacteria can cause septicemia; for example, *Streptococcus gallolyticus* causes fatal septicemia in goslings and turkeys (Droual et al. 1997, Barnett et al. 2008). Few cases about pathogenic brain injury, such as neonatal meningitis of ducklings, have been reported (Meixia et al. 2013). Most *Bacillus* species, including *B. subtilis* and *B. cereus*, have been shown to be beneficial and are used as probiotics. However, a few species can cause animal disease, although *B. subtilis* has not been reported to be pathogenic in animals. In our study, the isolated strain, HFBF_B11, was pathogenic in ducklings, but no animals died after a single infection; it exhibited pathogenic features and a high mortality rate only after co-infection with *R. anatipestifer* (Figs. 3 and 4). We speculate that infection with *R. anatipestifer* re-



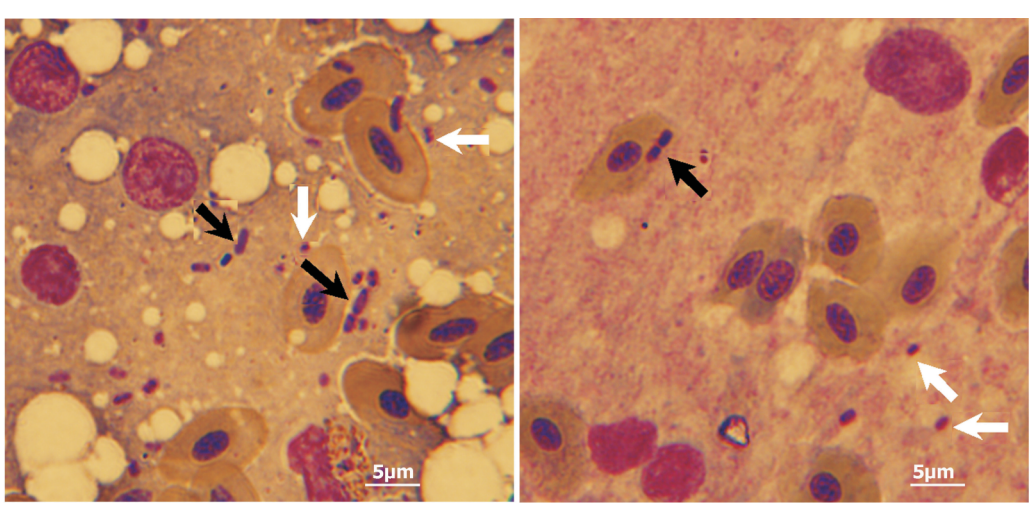
a b c d

Fig. 3. The brain damage of ducklings infected with *Bacillus subtilis*. a. the control without any bacteria; b. the animals were infected with HFBF_B11; c. the animals were infected with *R. anatipestifer*; d. the animals were infected with HFBF_B11 and *R.anatipestifer*.



a b c d

Fig. 4. The liver damage of ducklings infected with *Bacillus subtilis*. a. the animals were infected with *R. anatipestifer*, b. the animals were infected with HFBF_B11. c. the control without any bacteria. d. the animals were infected with HFBF_B11 and *R. anatipestifer.*



a b

Fig. 5. The smear of brain and liver of ducklings infected with *Bacillus subtilis* HFBF_B11 and *R. anatipestifer*. The brains and livers were collected from the died ducklings, which were infected with HFBF_B11 and *R. anatipestifer;* after preparation of brains and livers smears, the wright's staining was carried out. a. liver smear; b. brain smear. Arrows indicate bacteria.

sulted in histological and metabolic changes in the ducklings. Consequently, the HFBF_B11 strain could cause histological damage by disrupting the blood-brain barrier resulting in animal death.

In conclusion, the HFBF_B11 strain is an opportunistic pathogen that exhibits hemolytic activity and causes tissue damage according to metabolic disturbances or structural changes in ducklings after co-infection with *R. anatipestifer*.

Acknowledgments

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