ISOLATION AND IDENTIFICATION OF THE *SERRATIA MARCESCENS* CAUSING SEPTICEMIA IN THE SILKWORMS FROM YEN BAI PROVINCE AND PREVENTION MEASURES

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ABSTRACT

In Yen Bai (Northern Viet Nam), sericulture is an important part of agriculture. However, bacterial diseases often cause severe economic loss in sericulture. The results of this research are the first to use a combination of biochemical identification methods and sequencing of the 16S rRNA gene region to diagnose and determine the origin of the pathogen causing septicemia in silkworms collected from Yen Bai province. From the collected silkworm samples with septicemia, a strain of *Serratia marcescens* bacteria was isolated with morphological characteristics, and typical basic biochemical test. Additionally, the 16S rRNA gene region of the bacterial strain *S. marcescens* was sequenced. Research results showed that the MD2-16-27F bacterial strain has 99.63% similarity with the *S. marcescens* strain JW-CZ2. Research on preventing this bacterium shows that the disinfection solution, which contains the active ingredients of trichloroisocyanuric acid (0.0008 mol), formaldehyde (0.0059 mol), calcium hypochloride (0.0031 mol) and alcohol (0.137 mol), effectively prevented the growth of this bacterium after exposed to it for 30 minutes at a temperature of 24-25ºC. Antibiotic active ingredients, Norfloxacin (10 µg/l) and Doxycycline (30 µg/l), were effective in preventing *S. marcescens* bacterium with high sensitivity level; the antibacterial ring diameter of Doxycycline was 31 mm and that of Norfloxacin was 25 mm. The results of this study can be applied to the general prevention of septicemia in silkworms caused by *S. marcescens* bacteria in the Northern part of Vietnam, particularly in Yen Bai in particular and the North of our country in general.

Keywords: mulberry silkworm blood infection, prevention measures, *Serratia marcescens*, silkworm disease.

INTRODUCTION

Septicemia in silkworms is a disease caused by septic bacterial invasion and multiplication in the silkworm body leading to the dead of the silkworms. The majority of silkworms affected by this disease can be found to expel the gut fluid through vomiting. The body of the diseased silkworm becomes soft, shrunken and the

thoracic region is swollen. A large number of bacteria are observed in the haemolymph. The body wall ruptures easily, liberating foul smelling fluid containing large number of bacterial cells. The larvae die in a short span of infection. Several bacteria, viz., *Serratia marcescens*, *Pseudomonas* sp., *Bacillus proteus*, *Bacillus aergenes*, *Bacillus prodigiousus*, *Bacillus pyocyanes* and *Streptococcus* sp., have been isolated from silkworms affected with septicemia. But the most common are *S. marcescens* and *Bacillus* sp. In case of septicemia caused by *S. marcescens*, which infected silkworm plasma through mechanical wounding on the skin. The dead larvae develop dark brown spots and a reddish tinge, which extends to the whole body (Zhang *et al*. 2006).

Yiling Zhang *et al.* (2020) isolated two strains of *S. marcescens*, ZJ-1 and ZJ-2, from dead silkworms. When the environmental temperature changed, the ability to synthesize pigments of these two bacterial strains, ZJ-1 and ZJ-2, was affected. Kenichi Ishii *et al*. (2014) also showed that when infecting the body of silkworms, *S. marcescens* inhibited the wound healing process and blood coagulation, leading to infected blood loss.

The invasion by *Serratia piscotorium* into haemocoel in silkworm is observed to be enhanced by the presence of *Streptococcus faecalis/Streptococcus faecium* in the larval midgut. The lowering of pH by these bacteria favours the *S. piscotorium* to multiply and invade the hemocoel and produce fatal septicemia. The pathogenicity of orally inoculated *Serratia* causes a high mortality at high humidity, although the mortality varies with the number of bacteria added to the feed. Bacterial involvement in septicemia of pre-pupal or pupal stages leads to the spoilage of cocoon quality and a reduction in grainage productivity.

In Vietnam, sericulture has tended to expand the mulberry area and improve the level and efficiency of intensive mulberry cultivation and sericulture. However, this industry is also facing other serious problems, including environmental pollution and epidemics. Silkworm disease is one of the main causes of damage to the silkworm farming industry. However, research work on silkworm disease is still limited, and the results are not in-depth, complete, and methodical. Through investigation and isolation of a number of bacterial agents causing silkworm disease in the North, Nguyen Thi Dam (2007) concluded that silkworm diseases mainly arise and thrive in the summer, when the weather is continually hot and humid. Silkworm diseases are caused by viruses, bacteria, fungi, parasitic flies, etc, with bacterial diseases causing about 40 - 50% of the damage. At the same time, the authors also said that the level of harm caused by the agent *S. marcescens* is second only to *Streptococcus sp*.

Field investigation results of the Ministerial-level Potential Science and Technology Project "Research on measures to manage bacterial silkworm disease in the Northern provinces" for the period 2020-2022 also showed the harmful level of the septicemia was 28.17% of all silkworm diseases. The three main agents that caused septicemia have been pointed out: *Bacillus* sp. (abbreviated as: B.sp), *S. marcescens* (abbreviated as: S.m) and *Aeromona*s sp. (abbreviated as: A.m). In which, *S. marcescens* caused harm to both the silkworm races (Univoltine, Biovoltine and Multivoltine silkworm races).

In general, studies on bacterial pathogens causing septicemia in silkworms have clarified the diversity of bacterial pathogens, including S.m. Previous domestic research results have only stopped at monitoring the harmful development patterns of the disease through preliminary investigations of the epidemic situation. The research methods are still simple, not yet in-depth, so there are no detailed or specific conclusions about the causative agents of the disease. Therefore, isolating and identifying new strains of pathogenic bacteria is very useful for developing appropriate technical measures to prevent and control various types of bacterial septicemia.

MATERIALS AND METHODS

Materials

S. marcescens bacterial strain was isolated from days 4-5 of the 5th instar silkworm sample (Bivoltine races) collected in Yen Bai (symbol: MD2-16-27F).

Media: Prepared according to the methods of Nguyen Lan Dung *et al.* (2014), Dam Sao Mai *et al.* (2011), Tao (2011) and Bergey. D.H. Breed. R.S (2014).

LB medium was prepared by dissolving a mixture containing 10 g Peptone; 5 g Yeast Extract; 10 g NaCl in 1 liter of water. The solution was adjusted to a pH of 7.0 ± 0.2 then autoclaved at 121^{o}C for 20 minutes. Bacteria collected from silkworm blood were inoculated into culture in LB nutrient medium, incubated at 37^{o}C for 48 hours. Other mediums, including citrate medium, sugar fermentation medium, nitrate culture-medium, nutrient gelatin, glucose peptone water medium etc., were prepared according to Bergey. D.H. and Breed. R.S (2014).

Identification of *Serratia marcescens* bacteria

Isolation and morphological identification

Bacteria collection follows the method of LiuJiPing (2011).

Symptoms of septicemia caused by *S. marcescens*: Applied method of LiuJiPing (2011) and Tao (2011).

Silkworms infected with septicemia caused by *Serratia marcescens* bacteria were collected (followed methods of Tao (2011) and Jing (2000)), then washed with 70% alcohol before collecting bacteria from the silkworm's blood.

Isolation of bacteria: followed methods of Nguyen Lan Dung *et al.* (2014), Dam Sao Mai *et al.* (2011) and Tao (2011).

A naturally infected silkworm was collected on a farm in Yen Bai Province. The corpse was disinfected with 70% (v/v) ethanol for 1 to 2 min and washed twice with sterile distilled water after it had just begun to soften. Then, we used sterilized insect needles to pierce the body wall and withdraw body fluid with an inoculation loop. The body fluid was then inoculated onto nutrient agar and cultured at 30°C for 48 h. Dominant single clones were purified by streaking and re-streaking the same agar plates (Tao *et al.,* 2011).

Biochemical identification

Applying the method of Nguyen Lan Dung *et al.* (2014) Dam Sao Mai *et al.* (2011); Bergey. D.H. and Breed. R.S (2014) and F. Sharmin and M. Rahman (2007).

In order to identify bacteria belonging to the genus *Serratia marcescens*, a series of procedure including isolation, morphology

observation, Gram staining, and biochemical tests (catalase and oxidase tests) were conducted.

PCR and sequencing of the 16S rRNA gene region

Primer pair used for bacterial classification (Tran Van Dung *et al.,* 2021): 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGCTACCTTGTTACGACTT-3').

The PCR reaction was performed as follows (Applying the method of F. Sharmin, M. Rahman (2007) with appropriate adjustments)**:** Denaturation (94^{o}C, 5 minutes); pairing (62^{o}C, 30 seconds); prolongation (72^{o}C, 5.5 minutes); Steps 2 - 4 repeated 30 times, Marker: DL 2000. The PCR product was stored at 4°C. Following the completion of the reaction, 5 µL of the reaction solution and the stored PCR product (45 µL) were subjected to 1% agarose gel electrophoresis.

Sequencing method: Sanger method.

Identification method: use BLAST software to compare the gene sequence of the isolated bacterial strain with the reference sequence from the 16S rRNA database. The phylogenetic tree was built based on the Distance Method, ClustalX 1.83 and MEGA 7 software.

Prevention of *Serratia marcescens* bacteria with antiseptics and disinfectants

Protocol: Apply according to Le Thi Linh Lan's method with appropriate adjustments.

One mL of *S. marcescens* suspension (10^7 CFU/mL) was transferred into a test tube containing 9 mL of disinfectant solutions: 0.137 mol alcohol, 0.0060 mol lime water, 0.0035 mol Pavidon Iodine, 0.0031 mol calcium hypochloride, 0.0008 mol Trichloroisocyanuric acid, 0.0059 mol formaldehyde, stored at 24-26^{o}C for 30 minutes and centrifuged for 15 minutes (5000 rpm). Post centrifugation, the supernatant was discarded. The pellet was rinsed with distilled water for 2-3 times and, centrifuged remove the supernatant. Bacteria from the bottom of test tube was transferred to petri dishes containing LB medium by a culture rod, and incubated at 35-37^{o}C, 85% humidity for 48 hours, before the survival rate of *S. marcescens* bacteria was determined.

The experiment has 6 formulas: CT1: A. Lime water 0.006 mol - (CaOH)2; CT2: Alcohol 0.137 mol - C_2H_5OH; CT3: Povidon iod 0.0035 mol (I_2); CT4: calcium hypochloride ($Ca(OCl)_2$) 0.0031 mol; CT5: Trichloroisocyanuric acid ($C_3N_3O_3Cl_3$) 0.0008mol; CT6: formaldehyde (CH_2O) 0.0059 mol; CT7: Povidon Iod 0.0035 mol + Alcohol 0.137 mol.

Monitoring indicator: Density of *S. marcescens* bacteria.

Prevention of *S. marcescens* bacteria with antibiotics

Method: Followed method of Dam Sao Mai *et al.* (2011).

The bacterial strain *S. marcescens* was grown in LB medium and adjusted to a McFarland turbidity of 0.5. Then, spread the suspension onto the LB agar plate using a sterilized cotton swab. The antibiotic disc was placed on the bacterial inoculated plate and incubated at 35°C for 48 hours. The experiment had 5 formulas, each formula was 1 type of antibiotic.

CT1: Flophenicol (Fl, 20 μg); CT2: Norfloxacin (No, 10 μg); CT3: Neomycin (Ne, 30 μg); CT4: Oxytetracycline (Te, 30 μg); CT5: Erythromycin (Er, 30 μg).

Monitoring indicator: Diameter of antibacterial ring (mm).

Statistic methods: Descriptive statistics to compare antibiotic sensitivity tests

RESULTS AND DISSCUSSION

Based on the typical symptoms of the disease, silkworm individuals with typical symptoms of septicemia were collected and stored at the Silkworm Disease Research Laboratory Vietseri (Table 1 and Figure 2). Samples collected were 5-year-old silkworms on days 4-5.

Isolation and identification of bacterial strain *MD2 - 16-27F*

Silkworms with the disease have a sluggish body, poor appetite, the body is stretched, the thoracic segments are swollen, the abdominal segments shrink, vomiting occurs, and the stool is soft and granular. When a silkworm dies, its head and chest were straightened, its body became soft and discolored, and its fragile skin exudes an unpleasant stench. This is a common acute disease, the time from death to infection is about 10 hours at 28^{o}C and 1 day at 25^{o}C. The dead bodies of silkworms infected with *MD2 - 16-27F* have dark brown spots, the entire body was soft, their color changed and turned light red.

Figure 1. Symptoms of silkworm blood infection caused by the bacteria *MD2 - 16-27F.*

Table 1. Results of isolation of *MD2 - 16-27F* bacterial strain.

Indicator	***MD2 - 16-27F* Bacterium**
Bacterial Morphology	Rod
Size	1.1μm x 0.7 μm
Gram	-ve
Colony	The colonies are round, convex, shiny, and red in color
Colony size	2.4 - 3.1 mm

Figure 2. Colony and morphology of *MD2 - 16-27F* bacterium causing septicemia in silkworms.

Under a 600x optical microscope, *MD2 - 16-27F* bacterium has a short rod shape, is 1.1 µm x 0.7 µm in size and have a pink color when stained with Gram dye.

The results of morphological and biological characteristics research show that the MD2-16-27F bacterial strain belongs to the *Serratia marcescens* genus, consistent with the observations of Yiling Zhang *et al.* (2020) and Bergey D.H., Breed R.S. (2014), and Holt *et al.* (1994). Additionally, when isolating *S. marcescens* bacteria on MacConkey agar, Nguyen Thi Dam (2007) also found similar colony morphology. This suggests that *Serratia marcescens* bacteria can grow in diverse environments.

Table 2. Results of biochemical tests.

No	**Criteria**	***MD2 - 16-27F*** **Bacterium**
1	Glucose reaction	+
2	Sucrose reaction	+
3	Citrate reaction	+
4	Urea reaction	+
5	Calatase reaction	+
6	Gelatin reaction	+

The bacterial strain *MD2 - 16-27F* is an aerobic bacterium that is capable of fermenting sugar and decomposing citrate and urea products; it is also a calatase-positive aerobic bacterium that is capable of liquefying gelatin. The physiological and biochemical characteristic study results show that the MD2-16-27F bacterial strain aligns with Yiling Zhang *et al.* (2020),

Bergey D.H., Breed R.S. (2014), and Holt *et al*. (1994).

Sequencing of 16S rRNA gene region of *MD2 - 16-27F* bacterial strain

The electrophoresis result of DNA extraction products from a *MD2 - 16-27F* sample is shown in Figure 3, showing that the PCR product obtained a very specific band. The PCR product amplified the gene region encoding 16S rRNA has a size of about 1200 bp with a clear and single the signal.

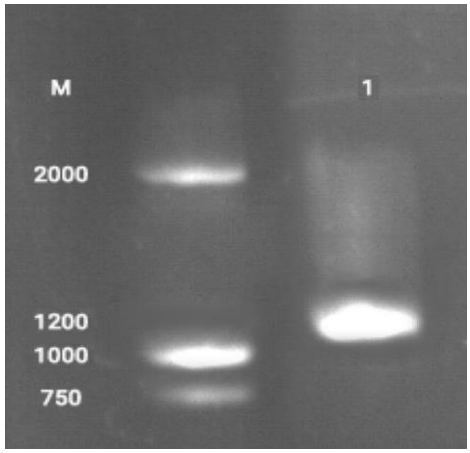

Figure 3. Gel electrophoresis analysis of the 16s RNA region of the bacterial strain *MD2 - 16-27F*. M:DL 2000 DNA; well 1: extraction product from *MD2 - 16-27F* strain.

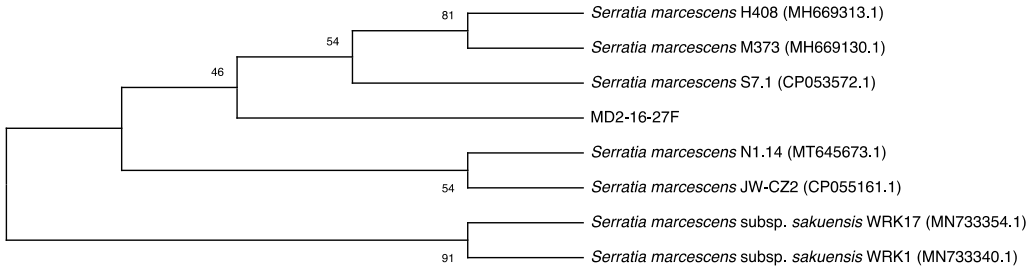

Figure 4. Phylogenetic trees of of the *MD2 - 16-27F* strain.

Table 3. Similarity coefficient.

Scientific name	Max score (bp)	Query cover (%)	Per. Ident. (%)	Accession
Serratia marcescens H408	1469	100	99.63	MH669313.1

Serratia marcescens M373	1469	100	99.63	MH669130.1
Serratia marcescens S7.1	1469	100	99.63	CP053572.1
Serratia marcescens N1.14	1469	100	99.63	MT645673.1
Serratia marcescens JW-CZ2	1469	100	99.63	CP055161.1
Serratia marcescens sub sp. Sakuensis WRK17	1469	100	99.63	MN733354.1
Serratia marcescens sub sp. Sakuensis WRK1	1469	100	99.63	MN733340.1
MD2 - 16-27F	1469	100	99.63	

The results of Figure 4 and Table 3 showed that MD2-16-27F strain is 99.63% similar to *S. marcescens* strain JW-CZ2 (Genebank accession number: CP055161.1). These results are in accordance with the report of *S. marcescens* in Bergey's Manual of Determinative Bacteriology (Bergey. D.H., Breed. R.S, 2014; Holt *et al*., 1994). Nguyen Thi Dam (2007) only isolated the bacteria but did not study the biochemical characteristics nor identify the bacteria.

Prevention of MD2-16-27F bacterium

Prevention with antiseptics and disinfectants

Results in Table 3 showed that the MD2-16-27F bacterial strain was completely inactivated when exposed for 30 minutes at 24-25^{o}C with the disinfectants Trichloroisocyanuric acid 0.0008 mol, formaldehyde 0,0059 mol, calcium hypochloride 0.0040 mol and alcohol 0.137 mol.

MD2-16-27F bacteria were not completely inactivated after exposure to lime water 0.0060 mol and pavidon Iodine 0.0035 mol for 30 minutes at 24-25^{o}C.

Table 3. Effects of some antiseptic active ingredients on *MD2 - 16-27F* bacteria.

Method	Disinfectant	Bacterial density after 24 hours (CFU/mL)
1	Lime water 0.0060 mol	1,9 x 10 ^{7}
2	Alcohol 0.137 mol	-
3	Povidone Iodine 0.0035 mol	1,2 x 10^{3}
4	Canxi hypochloride 0.0031 mol	-
5	Trichloroisocyanuric acid 0.0008 mol	-
6	Formaldehyde 0.0059 mol	-
7	Pavidon Iodine 0.0035 mol + Alcohol 0.137 mol	-

(Note: Initial density is 2.1 x 10 ^{7} CFU/mL; Sterilization time is 30 minutes at 24-25^{o}C).

Prevention with antibiotics

The results of studying the sensitivity of MD2-16-27F bacteria to some antibiotics in Table 4 and Figure 5 show that MD2-16-27F bacteria are sensitive to Doxycycline and Norfloxacin. Antibiotic active ingredients Norfloxacin (10µg/l) and Doxycycline (30µg/l) are effective in preventing MD2-16-27F bacteria with sensitivity level, the antibacterial ring diameter of Doxycycline is 31 mm and Norfloxacin is 25 mm. The results showed that the *Serratia marcescens* bacterial strain was not resistant to Doxycycline and Norfloxacin. The results are consistent with Nguyen Thi Dam (2007) findings regarding the effects of Doxycycline (Dx), Erythromycin (Er), and Norfloxacin (No) on *Serratia marcescens* bacteria. Our research adds experimental results on the effects of the antibiotics Flophenicol (FL) and Neomycin (Ne) on the bacteria. The results of antibiotic sensitivity testing have opened up a solution to treat blood infections in silkworms with antibiotics. Doxycycline can be used aloneor in combination with Doxycycline and Norfloxacin to treat the septicemia disease.

Table 4. Sensitivity of *MD2 - 16-27F* bacteria to some antibiotics.

No	Disinfectant	Antibacterial ring (mm)	Result
1	Flophenicol (FL)	17	Sensitive
2	Norfloxacin (No)	25	Sensitive
3	Neomycin (Ne)	19	Sensitive
4	Doxycycline (Dx)	31	Sensitive
5	Erythromycin (Er)	14	Intermediate

Figure 5. Sensitivity of *MD2 - 16-27F* bacteria to some antibiotics.

CONCLUSION

The MD2-16-27F bacterial strain collected from Yen Bai province showed 99.63% similar to the *Serratia marcescens* JW-CZ2 strain (genebank symbol: CP 055161.1) in terms of 16S rRNA gene, with positive biochemical reactions to glucose, sucrose, citrate urea, calatase and gelatin. This bacterial strain is sensitive to the antibiotics Norfloxacin 10 µg/L (sterile zone diameter is 31 mm), Doxycycline 30 µg/L (sterile zone diameter is 23 mm) and is inactivated with the antiseptics Trichloroisocyanuric acid 0,0008 mol, formaldehyde 0,0059 mol, calcium hypochloride 0.0031 mol and alcohol 0.137 mol after exposure for 30 minutes at 24-25^{o}C.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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