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IDENTIFICATION AND DETECTION OF BLOOD DISEASE BACTERIUM IN THE ISOLATES DERIVED FROM INSECTS' BODY PARTS

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Abstract

The purpose of this study was to identify and detect BDB derived from insects' body parts. The study was conducted in Plant Bacteriology Laboratory, Department of Plant Protection,Faculty of Agriculture, Bogor Agricultural University. Identification and detection of BDB were done through isolation from the outside and the inside of insects' body parts. After that, the isolates were identified to prove that the bacteria isolated from the insects' body are really BDB through stages: gram reaction test, hypersensitivity test and pathogenicity test. To get more accurate results, BDB performed molecular detection through PCR test. Research results indicate that the BDB has been identified and detected from isolates derived from the body parts of insects.

Key words: isolates, Drosophilidae, PCR

Introduction

Blood disease in banana plants is one of the important diseases in Indonesia (Supriadi 2005). This disease is caused by blood disease bacterium previously known as *Pseudomonas solanacearum*or*Ralstoniasolanacearum* (EF.Smith) Yabuuchi et al. Race which causes bacterial wilt disease, but because of differences in culture and biochemical reactions between BDB and *Ralstoniasolanacearum*, the name BDB is more appropriate to be used as a cause of diseases in banana plants that show symptoms of blood disease (CPC 2005). BDB is included in the *R. solanacearum* species complex, member of division 2, phylotype IV and sequever 10 (Fegan& Prior 2005). BDB ranks first in the priority list of banana plant diseases in Indonesia (Valmayor et al. 1991) and is lethal by infecting systemic vessel networks (Eden-Green 1992). BDB infection in banana plantations can cause death or produce fruit that cannot be consumed. Insects as one of the factors that support the spread of pathogens that cause blood diseases (Montong et al, 2019). Insects from the family Drosophilidae, Tephritidae and Muscidae are most commonly found in Sigli district, Banda Aceh (Sahetapy

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et al, 2020). The insects that were captured were then isolated for the purpose of identifying BDB deriviedfrom the insects' body parts. In addition to identify isolates derived insects, isolates derived from bananas were also isolated. This was done as a control in the identification and detection of BDB on a molecular basis.

Material and Method

The study was conducted at the Bacteria Laboratory of the Plant Protection Department, Bogor Agricultural University. Each type of insect that was caught was separated immediately after leaving the field by placing it in a small 10 ml bottle containing sterile water. This separation aimed to facilitate the isolation of bacteria that cause banana blood disease from the insects' body. Then the samples of these insects are put in ice boxes and taken to the laboratory for identification of bacteria that cause banana blood disease. The isolation to see the presence of bacteria that cause banana blood disease (BDB) in the insects' body was carried out on each type of insects that was caught by using a modified method of isolating blood diseases according to Cahyaniati et al. (1997).

Isolation of the bacteria that cause blood diseases (BDB) from the outside (surface) of the insects' body

The insects' body washing water was taken as much as 100 µl and added with sterile water as much as 900 µl, then it was diluted as many as five levels (10^1, 10^2, 10^3, 10^4, 10^5) of chosen concentrations that are 10^1, 10^3 and 10^5. This solution was then dripped on TZC media. By using glass bead, bacteria that grow regularly on TZC (tryphenyltetrazolium chloride) mediawere caught. Observation of the characteristics of the BDB colony was carried out after culture was 48-72 hours old, then incubated at 28 ºC. The purified bacterial colony was transferred to SPA media for 1-2 days at 28 ºC and stored in the sterile water.

Isolation of Bacteria that Cause Blood Disease (BDB) from the Inside of Insects' body

The insects' body tissue stored in a small bottle was disinfected with sodium hypochlorite solution 3-4 times for 5 minutes then rinsed 3-4 times with sterile water to remove the remnants of sodium hypochlorite. This tissue wascrushed and 10 ml of sterile water was added, then it was diluted incrementally as many as 5 times. Then, the bacterial suspension was etched on the prepared TZC media. Observation of the characteristics of the

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BDB colony was carried out after 48-72 hours of culture, then incubated at 28ºC, the purified bacterial colony was transferred to SPA media (sucrose peptone agar) for 1-2 days at 28ºC and stored in sterile water.

BDB identification

Identification was done to prove that the bacteria isolated from the insects' body wasreally BDB. The morphological and physiological characteristics of BDB isolates from insect origin are as follows: the colonies are small (2-3 mm), non fluidal, viscid and grow slowly. Gram negative, negative fluorescence pigment, negative arginine hydrolysis, positive hypersensitive reaction, negative bacteriophage production and positive pathogenicity. These isolates were grown on TZC media then proceed with purification of isolates on SPA media. Further identification was carried out to ensure that the bacteria isolated from the insects' body was really BDB. Identification of BDB bacteria was carried out using 3 stages namely:

Gram Stain Test

This testwas an initial step in identifying an unknown bacterial species. This test was carried out using a 3% KOH solution and if in this test there was a (sticky) reaction then it was classified as a gram negative reaction. Conversely, if there was no reaction, then the bacteria were gram-positive. BDB bacterial colonies were included in the gram negative group. 3% KOH dropped of 1-2 drops on the slide, using a loop, the bacterial colony was taken and mixed with KOH. After 5-10 seconds, the mixture was lifted using a loop. Gram-negativebacteriawas marked by the formation of mucus and in there were kind of threads in the raised loops. On the contrary,Gram-positive bacteria wasmarked by no formation of mucus and there were no kind of threads in the raised loops (Schaad et al 2001).

Hypersensitivity Reaction Test

Hypersensitivity reaction tests were carried out to determine whether the isolates that were successfully isolated were classified as pathogenic or non pathogenic. Most pathogenic bacteria will cause hypersensitive reactions when they are inoculated in non-host plants, while non-pathogenic bacteria will not cause hypersensitive reactions. The test was carried out using the Lelliott and Stead (1987) method. BDB isolates were bred in SPA media for 3

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days, then culture was suspended with sterile water until a population density of 10^8 (OD600 = 0.1) was obtained. A bacterial suspension was injected into the tobacco leaf through the secondary leaf bone. Pathogenic isolates showed transparent white symptoms, leaf tissue death (collapse) around the injection site within 24-48 hours after injection and finally the leaf tissue dries.

Pathogenicity Test

This test aimed to determine the ability of BDB isolates to cause symptoms of disease in the host. In this method, the injection inoculation method was used. The pathogen is directly injected into the banana hump tissue so that it does not go through the stages of the natural infection process whilethere are also methods of root-opening inoculation in which the pathogen suspension is splashed around the plant roots so that the pathogen is still outside the plant surface and must go through all stages of the infection process in order to cause symptoms of the disease (Goodman et al. 1986). This test aimed to determine the virulence of BDB isolates derived from insects. This test was carried out using 4-week-old Cavendish banana varieties derived from tissue culture (Appendix 4). Observations were carried out starting from theplants inoculated to becomewithered. Observation of the symptoms of witheringwas done by using the scale of the development of bacterial wilt according to Winstead and Kelman (1952) as follows:

Scoring Assessment withered Symptoms

Score	Description
0	There are no symptoms of wilt
1	1 withered leaf
2	2 - 3 withered leaves
3	All leaves withered. Except for 2 or 3 shoots
4	All leaves withered
5	Dead plants

While determining the virulence of each BTB derived from insects was carried out using the virulence scale as follows:

ScoreDescription	
0	Avirulent

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1 – 2	Low virulence (+)
3 – 4	Moderate virulence(++)
5	High virulence (+++)

DNA extraction and BDB detection on a molecular basis

Detection of BDB. The presence of BDB infection in the inoculated plants, was detected suing PCR method.The bacteria was isolated from flower, because it is the location of the transmission treatments. The infective insects for transmission were also isolated and then detected using the PCR method.

DNA isolation procedure.

Isolation of bacterial DNA was carried out using the Genomic DNA Mini Kit Geneaid ™ method for isolates of bananas, Drosophilidae and flowers that had been purified from the transmission test results. Bacterial isolates were taken in amount of 5 µl plus RBC Lysis Buffer in amount of 900 µl and stirred until homogeneous. Then, it was incubated at room temperature for 10 minutes. Put in a Centrifuge at 12,000 rpm for 5 minutes. Afterwards, the supernatant is removed. 100 µl RBC Lysis Buffer is added to the sample, and re-suspended. 200 µl GB of Lysis Buffer was added and gently turned to homogeneous. Incubated at 600 C for 10 minutes. During incubation, once every 3 minutes the tubes are stirred until homogeneous. 200 µl absolute ethanol are added and vortexed for 10 seconds. The sample (supernatant) is moved to the GD column (filter). Place the GD column in the collection tube. Centrifuge at 12,000 rpm for 5 minutes, then move the GD column to the new collection tube. Washed, by adding 400 µl W1 buffer.Centrifuged at 12,000 rpm for 30 seconds. Washed again, by adding 600 µl wash buffer. Centrifuged at 12,000 rpm for 30 seconds.Centrifuged at 12,000 rpm for 3 minutes. The GD column was transferred to a 1.5

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ml tube. Add an elution buffer in amount of 100 µl. Incubated at room temperature for 3 minutes. Centrifuged at 10,000 rpm for 30 seconds. Samples are stored at -20°C.

DNA amplification using PCR. Gene fragments were amplified by PCR using primers 759 R(5'-GTCGCCGTCAACTCACTTTCC-3') and 760 F (5'-GTCGCCGTCAGCAATGCGGAAT CG-3') (Sagar et al, 2014) The DNA amplification process was carried out with an initial denaturation process at a temperature of 94°C for 3 minutes. Denaturation in the cycle is carried out at a temperature of 94°C for 15 seconds, annealing at 57°C for 30 seconds, polymerization of the cycle at 72°C for 30 seconds, final polymerization at 72°C for 10 minutes to avoid the presence of DNA that has not been fully amplified. The cycle was done 35 times. PCR results can be stored at 40°C.

Electrophoresis. The results of DNA amplification were separated based on the size of the base pair by electrophoresis technique, which used 1.5% agarose gel in a TAE buffer 1X and added 5 µl Red Gel per each 50 ml agarose. Agarose gel solution is poured into a mold to make a well of a certain size. A total of 20 µl of PCR results were taken carefully, placed in a gel pit. As a standard molecule, a 1 kb ladder marker is used. Electrophoresis is run with a potential difference of 100 volts for about 30 minutes. Observation of DNA bands was carried out under UV light and documented.

DNA isolation of BDB bacteria was carried out using extraction kits (Geneaid) according to the instructions in the manual. Furthermore, amplification is done to 14 DNA samples from insects, 3 DNA samples from plants and 3 samples from Drosophilidae insects resulted from transmission tests using *Ralstoniasolanacearum* 759F primers and 760R (Opina et al. 1997). PCR used materials: DreamTaq Master Mix (Fermentas) 10 µl that consisted of 1 µl of primer, 1 µl of DNA, and 7 µl of ddH2O. The stage of PCR program are initial denaturation of 95 ° C for 2 minutes that was followed by denaturation of 94 ° C for 30 seconds, annealing (primary attachment) at 55 ° C for 30 seconds, extension 72 ° C for 30 seconds (all the three processes were repeated for 30 times) and ending with the final extension 72 ° C for 5

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minutes. The PCR results were electrophoresed using 1% agarose gel (0.3 g in 30 ml TAE buffer 0.5x) and stained with EtBr 1 µl. Then the results of the ectrophoresis were visualized with a UV transluminator and documented with a digital camera.

Results and Discussion

The isolation Blood Disease Bacterium (BDB) from insects' body parts

The results of BDB isolation from the body parts of captured insects were obtained by bacterial colonies with characteristics: slow growing (4-5 days), round, small in size (diameter <1 mm), white, pink at the central part, slightly sticky on the media, and gram negative based on KOH reaction test (Figure 1). The character of BDB isolates mentioned was in accordance with what was reported by Mairawita et al, 2012 that BDB colonies grow slowly, are round in size (0.5 - 3 mm), non motile, and slightly sticky (viscid).

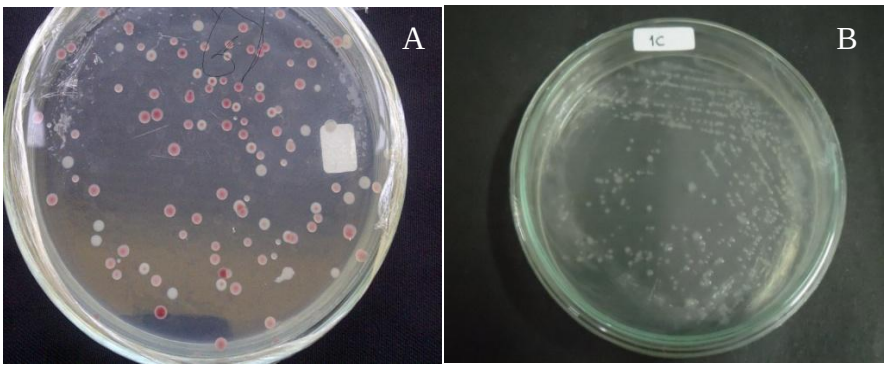

Figure 1. Characteristics of BDB colonies isolated from insects' body parts on TZC (A) and SPA (B) media.

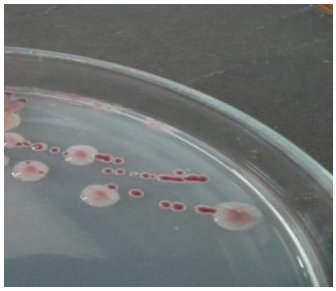

Figure 2. Characteristics of BDB colonies isolated from bananas on TZC media

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Before being tested for pathogenicity,hypersensitive reactions were tested to BDB isolates to quickly detect a bacterium as a pathogen in plants. In this test, the two months old tobacco indicator plants were tested. The total concentration of bacterial inoculum injected into plants was 0.2 ml. Meanwhile, the initial population number of bacterial cells inoculated to the test plant was 10^8CFU / ml. Observation of symptoms after 48 hours was marked by the appearance of hypersensitive symptoms on tobacco leaves in the form of necrotic symptoms

The initial symptoms were leaf weakening (flaccid) starting on the 7th day after the BDB suspension was injected into the banana weevil, the leaves wither, turn yellow and necrosis (Figure 4). Then, the plant died at 21-28 days after inoculation. This is consistent with the results of Rustam's research (2007) that the development of BDB symptoms begins with shrinking or weakening of banana plant leaves that occur starting on the 6th day with BDB suspense injection inoculation method on hump and 9th day with root scratches inoculation methods and watering suspension BDB.

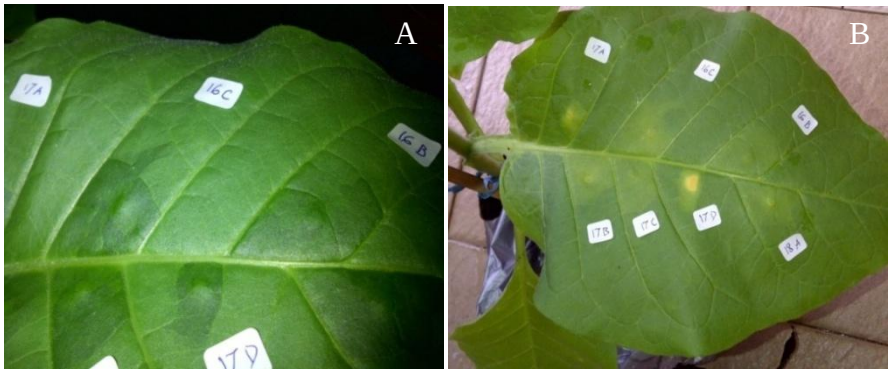

Figure 3. Hypersensitivity test, shortly after injection (A), after 48 hours necrotic symptoms (B)

Figure 4. Development of symptoms of blood diseases in Cavendish bananas: (A) Healthy banana plants (before being injected), (B) Leaves of banana

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plants that start to weaken, (C) Leaves of banana plants that begin to wilt.

Potential Insects in the Spread of Banana Blood Disease

Based on several tests of BDB isolates obtained from the body surface of insects and isolates from the insides of the insects' body, there are several potential insects in the spread of banana blood disease, which are presented in Table 1.

BDB isolates from insects that were tested for hypersensitive reactions were 38 isolates, 25 isolates showed positive reactions while 13 isolates showed negative reactions (non pathogenic). The results of the pathogenicity test showed that banana plants that were inoculated with BDB bacteria showed symptoms of withering on the average age of 14 days after inoculation with the highest disease scoring was 5. Among the isolates that had the highest virulence, there were 13 isolates consisting of 3 isolates derived from the inside of insects'bodyand 9 isolates derived from the surface of the insects' body. Whereas,there were 5 isolates which have medium virulenceand 7 isolates which have low virulence.

The types of insects that have the potential as vectors are those of the Diptera order, especially the Drosophilidae, Tephritidae, and Muscidae families. They are potential because of the BDB bacteria found in the body in all three orders. Whereas,for insects from other orders, BDB bacteria are found on the outside of the insects' body. This is in accordance with the opinion of Mairawita et al (2012), who said that the order Diptera (family Drosophillidae) has the potential as a vector of bacterial wilt disease in the banana studied.

Hypersensitivity testing to some BDB isolates showed positive reactions and some of them showed negative reaction but the isolates did not show symptoms of witheringat the time of the pathogenicity test. The incubation period of banana blood disease ranges from 7 to 14 days for high virulence, 21 days for moderate virulence and 28 days for low virulence. This is consistent with Siege's (1993) opinion that high virulence *Ralstoniasolanacearum* strains can cause symptoms of rapid wilt as a result of increased enzyme activity in the form of extracellular polysaccharides. The initial symptoms of young banana plants that are stricken with blood disease is when the edges of the leaves change color to yellow and these leaves will curl. After that, the midribs of banana leaves become wrinkled, then on some young leaves occur necrotic and eventually wither.

Based on the results of pathogenicity testing, it turns out that BDB isolates derived from netted insects were able to cause wilting symptoms in the test varieties. Based on the

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explanation above, it is known that several types of insects have the potential to spread blood diseases in banana plants. This information is very important considering that insects that have brought pathogens (outside the body and inside the insects' body) can support the process of development and expansion of disease events.

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Table 1. Hypersensitivity and Pathogenicity Testing Results for Some Bacteria Isolates Derived from Insects

No isolate	Origin of isolate		Reaction hypersensitivity	Pathogenicity Testing		
	Order	Family		Incubation period (Day)	Disease scoring	Virulence rate
BDBSB1131	Diptera	Muscidae(a)	+	14	5	***
BDBSB1132	Diptera	Muscidae(b)	+	7	5	***
BDBSB1133	Diptera	Calliphoridae(a)	+	0	0	A
BDBSB1134	Diptera	Micropetidae(a)	-	0	0	A
BDBSB1135	Diptera	Micropetidae(b)	+	14	5	***
BDBSB1136	Diptera	Micropetidae(a)	+	28	2	*
BDBSB1137	Diptera	Drosophilidae(b)	+	14	5	***
BDBSB21310	Diptera	Cypselasomatidae(a)	+	28	2	*
BDBSB21311	Diptera	Cypselasomatidae(b)	-	0	0	A
BDBSB21312	Diptera	Drosophilidae(b)	+	7	5	***
BDBSB21313	Diptera	Tephritidae(b)	+	14	5	***
BDBSB21314	Diptera	Platypezidae(b)	+	14	5	***
BDBSB21316	Diptera	Asilidae(a)	+	0	0	A
BDBSB21317	Diptera	Bombylidae(b)	-	0	0	A
BDBCP11318	Diptera	Micropezidae(a)	+	28	2	*
BDBCP11319	Diptera	Drosophilidae(b)	+	14	5	***
BDBCP11320	Diptera	Tephritidae(a)	+	14	5	***
BDBCP21324	Diptera	Tephritidae(a)	+	28	2	*
BDBCP21325	Diptera	Tephritidae(b)	+	14	5	***
BDBCP21326	Diptera	Micropezidae(a)	+	14	2	*
BDBCP21327	Diptera	Micropezidae(b)	+	21	3	**
BDBCP21328	Diptera	Neriidae(a)	-	0	0	A
BDBCP21329	Diptera	Neriidae(b)	+	28	2	*
BDBCP21330	Diptera	Dryomizidae(b)	+	21	3	**
BDBCP21331	Diptera	Drosophilidae(a)	+	7	5	***
BDBCP21332	Diptera	Drosophilidae(b)	+	14	5	***
BDBCP21333	Diptera	Muscidae(a)	+	21	3	**
BDBCP21334	Diptera	Muscidae(b)	+	7	5	***
BDBCP21335	Diptera	Muscidae(b)	-	0	0	A
BDBPC1336	Diptera	Phoridae(a)	+	28	2	*
BDBPC1337	Diptera	Phoridae(b)	+	21	3	**
BDBPC1338	Diptera	Muscidae(a)	-	0	0	A
BDBSB1138	Hymenoptera	Vespidae(a)	+	0	0	A
BDBSB1139	Hymenoptera	Vespidae(b)	+	0	0	A
BDBSB21315	Hymenoptera	Vespidae(b)	+	21	3	**
BDBCP11321	Hymenoptera	Apidae(a)	-	0	0	A
BDBCP11322	Hymenoptera	Vespidae(a)	-	0	0	A
BDBCP11323	Hymenoptera	Vespidae(b)	+	0	0	A

Note:

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a = BDB isolation from the body surface of insects
b = Isolation from the insects' body tissues
*** = degree of virulence

Molecular detection of BDB

positive PCR results was marked with the formation of DNA bands measuring 281 bp which was a DNA fragment from BDB that caused blood diseases in banana plants through electrophoresis with agarose gel 1%.

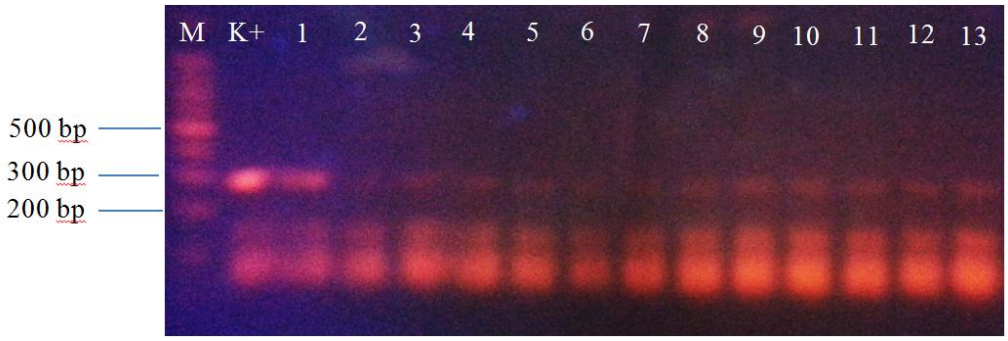

Figure 5. Visualization of DNA from PCR using primers 759F and 760R (M = marker 100 bp, + = positive control *Ralstoniasolanacearum*, 1 = BDBSB21314, 2 = BDBSB1137, 3 = BDBSB1132, 4 = BDBSB1134, 5 = BDBSB21313, 6 = BDBSP21325, 2 = BDBSB1137, 3 = BDBSB1132, 4 = BDBSB1134, 5 = BDBSB21313, 6 = BDBCP21325, 3 = BDBSB1132, 4 = BDBSB1134, 7 = BDBCP11319, 8 = BDBPC1337, 9 = BDBSB1131, 10 = BDBCP21333, 11 = BDBSB1136, 12 = BDBCP11320, 13 = BDBCP21331,

Table 2. PCR Test Results with Primers 759F and 760R on several isolates derived from insects.

No.	Isolate Code	Origin of Isolate		PCR results	Note
		Order	Family		
1	BDBSB21314	Diptera	Platypezidae	+	Inside the body
2	BDBSB1137	Diptera	Drosophilidae	+	Inside the body
3	BDBSB1132	Diptera	Muscidae	+	Inside the body
4	BDBSB1135	Diptera	Micropetidae	+	Inside the body
5	BDBSB21313	Diptera	Tephritidae	+	Inside the body
6	BDBCP21325	Diptera	Tephritidae	+	Inside the body
7	BDBCP11319	Diptera	Drosophilidae	+	Inside the body
8	BDBPC1337	Diptera	Phoridae	+	Outside the body

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9	BDBSB1131	Diptera	Muscidae	+	Outside the body
10	BDBCP21333	Diptera	Muscidae	+	Outside the body
11	BDBSB1136	Diptera	Micropezidae	+	Outside the body
12	BDBCP11320	Diptera	Tephritidae	+	Outside the body
13	BDBCP21331	Diptera	Drosophilidae	+	Outside the body

Conclusion

Diptera order insects (family Drosophilidae, Tephritidae and Muscidae) have potential as vectors of banana blood disease (BDB), the cause of this disease was found in insects' body tissues.

Diptera order insects (family Muscidae, Micropetidae, Cypselasomatidae, Tephritidae, Drosophilidae and Phoridae) were thought to be carriers of virulent BDB bacteria, which are contaminated on the outside of insects' body tissues.

Molecular detection of DHF of isolates derived from insects both from the surface and the tissues of the insects' body showed positive results.

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