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BACTERIAL CONTAMINATION TEST IN POWDER-FORMULATED *Helicoverpa armigera* NUCLEAR POLYHEDROSIS VIRUS (*Ha*NPV$_1$) SUBCULTURE

Uji Bakteri Kontaminan dalam Formulasi Serbuk *Helicoverpa armigera* Nuclear Polyhedrosis Virus (*Ha*NPV$_1$) Subkultur

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ABSTRACT

The Helicoverpa armigera Nuclear Polyhedrosis Virus (HaNPV1) is a subculture derived from the original HaNPV, and it has been cultivated in Spodoptera litura larvae as an alternative host. HaNPV1 was subsequently formulated using gypsum and talcum as carrier media. Following this formulation, a bacterial contamination test was conducted to assess the quality of the viral formulation. The experiment was arranged in the randomized factorial block design (RFBD) with 2 replications. The viral formulations was stored for 16 weeks and the samples were taken every two weeks for contamination analysis. The data was then analyzed with the analysis of variance (ANOVA) and a post-hoc using Duncan's Multiple Range test. The variable observed was the number of the bacterial colonies cultivated on the specific media i.e., Nutrient Agar (NA), Salmonella Shigella Agar (SSA) and Eosin Methilen Blue Agar (EMB). The results showed that the bacterial contaminants was detected from 0 to 12 weeks of storage time. However, the highest contamination was found in viral formulation after 8 weeks of storage time and the highest bacterial contaminations were recorded from all viral formulation tested in NA. The results indicated that the bacterial contamination were found around 1.45×10^9 cfu/gram and 1.97×10^9 cfu/gram in gypsum and talcum formulations, respectively. On SSA and EMB media, the bacteria contaminants from all formulation found in 8 weeks of storage time, but Salmonella, Shigella, or Escherichia coli (aspathogenic bacteria) were not found. After 12 weeks storage time, there was no indication of contamination found in all media. Furthermore, Bacillus species was found as a most dominant contaminant in all samples. In conclusion, although the viral formulations using gypsum and talc were not contaminated by pathogenic bacteria such Salmonella, Shigella, or E. coli. Nevetherless, the viral formulation was still easily contaminated by other non-pathogenic bacterial species. Thus, a more standardized and stricted strategy needs to be developed for a better viral formulation product.

Keywords: *Bacterial contaminants, biocontrol agent, carrier medium, gypsum, talcum*

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ABSTRAK

Helicoverpa armigera Nuclear Polyhedrosis Virus (*Ha*NPV$_1$) adalah *Ha*NPV yang dipropagasi pada larva *Spodoptera litura* sebagai inang pengganti. Sediaan *Ha*NPV$_1$ ini dibuat formulasi dalam bahan pembawa gypsum dan talk. Keberadaan kontaminan bakteri ini diteliti untuk mengetahui kualitas dari formulasi virus tersebut. Metode penelitian adalah eksperimental menggunakan Rancangan Acak Pola Faktorial dengan perlakuan 1 adalah waktu dengan 9 taraf dan perlakuan 2 adalah jenis medium pertumbuhan bakteri dengan 3 taraf yaitu *Nutrient Agar* (NA), *Salmonella Shigella Agar* (SSA) dan *Eosin Methylene Blue Agar* (EMB), dengan 2 ulangan. Formulasi virus disimpan selama 16 minggu dan sampel diambil setiap 2 minggu untuk analisis kontaminan bakteri. Data yang diperoleh dianalisis dengan analisis variansi (ANAVA) dan bila berbeda nyata dilanjutkan dengan Uji Jarak Berganda Duncan. Hasil penelitian menunjukkan bahwa bakteri kontaminan terdeteksi mulai minggu ke-0 hingga ke-12 waktu penyimpanan. Kontaminan bakteri tertinggi hanya tumbuh di medium NA ditemukan pada formulasi virus saat penyimpanan di minggu ke-8 yaitu sebesar rata-rata 1.45×10^9 cfu/gram dan 1.97×10^9 cfu/gram masing-masing diformulasi dalam gipsum dan talk. Pada media SSA dan EMB, bakteri kontaminan hanya ditemukan di minggu ke-8 waktu penyimpanan tetapi bukan merupakan *Salmonella*, *Shigella*, atau *Escherichia coli* (sebagai bakteri patogen). Setelah penyimpanan 12 minggu, tidak terindikasi ditemukan bakteri kontaminan pada seluruh media. Namun demikian, spesies *Bacillus* ditemukan sebagai bakteri kontaminan yang dominan di semua sampel formula virus. Pada penelitian ini, meskipun pada formulasi virus dalam bahan pembawa gipsum dan talk tidak ditemukan bakteri kontaminan yang patogen seperti *Salmonella*, *Shigella*, atau *E. coli*, formulasi virus ini masih mudah terkontaminasi oleh bakteri non-patogen. Perlu standarisasi dan strategi yang lebih ketat untuk pengembangan produk formulasi virus agar lebih berkualitas.

Kata Kunci: agensia biokontrol, bahan pembawa, bakteri kontaminan, gipsum, talk.

INTRODUCTION

Chemical insecticides were heavily used for controlling insect pests worldwide in the past four decades, which led to contamination of soil and water as well as an increase in pest resistance towards chemical insecticides (Monjane et al., 2019; Bass et al., 2015). For instance, insect pests have the ability to develop resistance mechanisms when they are constantly exposed to synthetic insecticides (Monjane et al., 2019). Thus, an eco-friendly and more sustainable approach must be taken in order to replace the toxic chemical insecticides (Mari et al., 2019). One alternative to chemical pesticides involves employing biocontrol agents that function as predators and pathogens against insect pests. (Thangavel & Sridevi, 2015; Raghunandan et al., 2019).

Microbial-based biocontrol agents are commonly used to control the population of insect pests due to their beneficial characterisics, such as being nontoxic and not harmful to non-target organisms (Thangavel & Sridevi, 2015). The microbial-based biocontrol agents consist of entomopathogenic fungi, bacteria, and viruses (Arthurs & Dara, 2018). Raghunandan et al. (2019) have found that biocontrol agents based on microorganism are more effective in controlling control most insect pests. Some reports showed that the baculovirus was highly effective in controlling *Spodoptera exigua* infestations on tomatoes. The percentage of damaged fruit was reduced from over 35% in the untreated control to 10%, resulting in an efficacy of 72% (Landwehr, 2021). From these examples, we assumed that viruses are still a promising biocontrol agent (Wilson, et al., 2020).

A group of potential biocontrol viruses, baculoviruses, have a longer persistence capacity in the environment (Wilson et al., 2020). Moreover, baculovirus have occlusion bodies (OBs) or polyhedra, that make them more resistant to changes in their environment and can keep insects from damaging plants (Jakubowicz et al., 2019; Raghunandan et al., 2019). Polyhedra,

which are structures formed by baculovirus, are intentionally chosen as a product for commercial microbial agents (Jakubowicz et al., 2019). The baculovirus product is formulated as liquid, concentrate, wettable powder, and granule (Cakmak et al., 2021). Baculovirus for plant protection is widely used in organic farming as a substitute for synthetic insecticides (Afolami & Oladunmoye, 2017). Entomopathogenic viruses specifically infect only target insect pests (Mondal & Kumar, 2021) and leave no harmful residues on plant products.

Nuclear Polyhedrosis Virus (NPV) is one of the most potential members of the baculovirus family which is widely used as a biological agent for controlling pest insects (Wilson et al., 2020). The NPV was isolated from the cadaver of *Helicoverpa armigera*, specifically as *H. armigera* Nuclear Polyhedrosis Virus (*Ha*NPV). *Ha*NPV can be subcultured on *Spodoptera litura* larvae as an alternate host, which is *Ha*NPV_1 (Belda et al., 2019). Previously, we had applied *Ha*NPV_1 in the form of a liquid formulation to protect cabbage plant to control the population of destructive cabbage pests such as *Crocidolomia pavonana* and *Plutella xylostella*. However, the results of this preceding study indicated that the liquid formulation could not attach to the cabbage leaves (Miranti et al., 2015). To solve that problem, *Ha*NPV_1 may be formulated in powdery form using gypsum and talcum as delivery carriers (Abid et al., 2019; Grzywacz, 2017). The potential carrier media used are gypsum and talcum. Gypsum is a hydrous mineral with the chemical formula ($CaSO_4 \cdot 2H_2O$), and it is non-toxic. Additionally, gypsum can serve as a source of nutrients for plants. Talcum is a fine powder of magnesium silicate ($3MgO.4SIO_2.H_2O$) and supports the formation of chlorophyll in plants.

However, one of the drawbacks of formulating bacterial agents in powdery form is the contamination, especially from bacteria (Tariq et al., 2022). According to Indonesian Minister of Agriculture regulation in 2019, biological products such as biofertilizer and biopesticides should not be contaminated with pathogenic bacteria (*Salmonella* and Coliform) that exceeded 10^3 cells mL^{-1} (Ministry of Agriculture, Republic Indonesia, 2019). Bacterial contamination in a product can be dangerous if it causes disease (Fox et al., 2018). One of the ways to reduce bacterial contamination in biopesticide products is by using delivery carriers or media with minimal water content (Fox et al., 2018). Stephane et al. (2017) found that spore-forming bacteria are usually the main bacterial contaminants in a bioproduct. This is because spores are a form of bacterial defense against dry environmental conditions.

In this study, the presence of bacterial contaminants in *Ha*NPV_1 formulated in gypsum and talcum as carrier media was evaluated for 16 weeks in order to determine the quality and safety of the biopesticide products. The outcomes of this study will contribute to an enhanced understanding of the potential for minimizing bacterial contamination in biopesticide products based on viruses.

MATERIALS AND METHODS

The experiment was arranged in a randomized factorial block design (RFBD), which consisted of two factors 9 × 3 and two replications. The experiment was conducted to assess the presence of bacterial contaminants in the virus formulation with gypsum and talcum as carriers in 16 weeks storages. The first treatment factor was bacterial contamination, with three levels:

m_1 = Bacteria cultured in nutrient agar (NA) medium

m_2 = *Salmonella* and *Shigella* bacteria cultured in Salmonella Shigella (SS) agar medium

m_3 = *Escherichia coli* grown in eosin methylene blue (EMB) agar medium

As for the second treatment factor was storage time (T) with nine levels: t_1 = 0 week, t_2 = 2 weeks….

t_9 = 16 weeks (with 2 weeks interval).

The virus formulations in gypsum and talcum were stored for 16 weeks. The sample was taken every 2 weeks and cultured on Nutrient Agar (NA) for *Bacillus* culture., Salmonella Shigella (SS) Agar for culturing *Salmonella* sp. and *Shigella* sp., and Eosin Methylene Blue (EMB) Agar for culturing *Escherichia coli* as specific media (Antony et al., 2016; Lamboro et al., 2016). Each treatment was replicated two times. The total

samples were 54. The number of single cell colonies of the bacterial contaminant that grew on the specific medium was counted. The total plate count method was used to count the number of bacterial colonies.

The samples were suspended in NaCl 0.85% solution with serial dilution techniques. The last three dilutions (10^{-6}, 10^{-7}, and 10^{-8}) were taken as much as 1 ml each and dropped on a petri dish. Each specific medium was poured into the petri dish, and the petri dish was carefully and lightly shaken. Furthermore, the petri dishes with the media were incubated at 37 ^{o}C for 24 hours. The number of single-cell bacterial colonies that grew was counted using the colonies counter. Acceptable bacterial colonies measured ranged from 30 to 300 colonies. The number of bacteria in each medium of each sample was calculated by the formula:

$$\text{Number of colonies} = \frac{(a\times10^{6})+(b\times10^{7})+(c\times10^{8})}{3}$$

Where: a,b, and c is a number of bacterial colonies (Cappuccino & Sherman, 2014).

The data of the colony number were arranged to a certain number times 10^{9} cfu/g sample.

The Virus Stock Preparation

The *Ha*NPV$_1$ was produced by propagating the *Ha*NPV in *Spodoptera litura* larvae and isolating only 1 passage. The third instar *S. litura* larvae were infected with 4 × 10^{5} polyhedra mL^{-1} viral suspension. The cadavers of infected larvae were collected in a glass container and stored at 4°C. Further, the cadavers of 40 larvaes were crushed by mortar and mixed with 20 mL of Tris buffer (1 mM, pH 7.6) solution and 20 mL of 0.1% sodium dodecyl sulfate (SDS) solution. This viral concentrate was stored at 4°C for 24 hours. After incubation, the viral concentrates were filtered through two layers of muslin cloth.

The viral suspension was centrifuged at 3.500 rpm for 15 min at 4°C. The first supernatant fraction was discarded, and the polyhedra were accumulated at the bottom of the tube as pellet. The pellet was resuspended in 5 mL of the mixture of Tris buffer (1 mM, pH 7.6) and 5 mL of 0.1% SDS solution, and centrifuged at 3.500 rpm for 15 min at 4°C. The steps of washing to purify the virus were replicated three times. The pellet obtained in the last washing step was resuspended in a mixture of Tris buffer (1 mM, pH 7.6) and 0.1% SDS solution and added to 0.2% sodium azide.

Polyhedral virus numbers were counted by resuspended of 0.1 mL of viral pellet and mixed by adding 0.9 mL of a mixed solution of Tris buffer (1 mM, pH 7.6) and 0.1% SDS in a 1:1 ratio. The polyhedral number in the virus suspension stock was counted with a Neubauer hemocytometer by a light microscope (magnification 400×). The polyhedra in the suspension were cuboidal in shape and green in color.

The final viral formulation was transformed into a powdery form by 1 ml of viral suspension in 99 g of gypsum gypsum and talcum powder. The concentration of the viral powder formulation was adjusted to 4 × 10^{7} polyhedra g^{-1} solution or gram powder. The powdered viral formulation was dried up in a plastic container for 48 hours. Then, the mixture was filtered through a wire sieve.

Bacterial Contaminant Identification

The bacterial contaminants that grew in Nutrient Agar were then identified by Gram staining and biochemical test media (lactose, glucose, maltose, sucrose, mannitol, catalase test, Voges Proskauer, and Simmon's Citrate media). The results were compared to the identification according to "Bergey's manual of determinative bacteriology, 2nd edition" (Antony et al., 2016; Brenner et al., 2001).

Statistical Analysis

The experiments were conducted in a randomized factorial block design (RFBD) with two replications. An analysis of variance (ANOVA) was used to analyze the effect of different treatments. Duncan's multiple range test was applied when ANOVA found significant differences between treatments. The statistical significance was set at $P<0.05$. All data are presented as mean standard deviation, and data analysis was performed using statistical software programs (SPSS 20, SPSS Inc.).

RESULT AND DISCUSSION

The *Ha*NPV$_1$ formulation in gypsum or talcum as a carrier was stored for 16 weeks. Every two weeks, samples were taken and cultivated in specific media, i.e., NA, SS, and EMB as explained above. The results of the cultivation of contamining bacteria in these media are shown in **Figure 1**.

Figure 1. shows that the bacterial contaminants growth in nutrient agar were found from virus formutation in gypsum and talcum. In Salmonella Shigella agar and Eosin Methylene Blue Agar, the growth of bacterial contaminants was only found after eight weeks of storage. The Salmonella Shigella agar and Eosin Methylene Blue agar are the specific media for *Salmonella, Shigella,* and fecal coliform bacteria growth. In this study, there was no genus of *Salmonella*, *Shigella*, or fecal coliform found in the two media, respectively. This shows that the virus formulation in gypsum and talcum as carrier was not contaminated with *salmonella, shigella*, and fecal coliform.

Salmonella spp., *Shigella* spp., and fecal coliform are known to be pathogenic bacterial contaminants (Fox et al., 2018). In biological products, these bacterial presence are not acceptable due to the risks to human health (Thangavel & Sridevi, 2015). In this study, in the viral powdery formulation, the presence of *Salmonella* spp., *Shigella* spp., and fecal coliform was not detected. This subsequently showed that the biological agents in powdered form are not easily contaminated with pathogenic bacteria. The presence of the pathogenic bacteria also severely compromises insecticidal efficacy (Wijayaratne et al., 2018). Similar results were reported by Cakmak et al., (2021) who found that the wettable powder formulation was not easily contaminated with bacteria and protected the virus from environment factors.

The fluctuations in the presence of bacterial contaminants in viral formulations of gypsum and talcum stored for 16 weeks are shown in **Figure 1**. The bacterial contaminants in the virus formulation in gypsum as a carrier have been detected since the first week, and the number of bacteria has increase up to 8-week storage. The bacterial contaminants in the virus formulation in talcum as a carrier were detected in the second week and showed the highest colony number in 8 week of storage. After 10 week of storage, the number of bacterial contaminants decreased and did not appear after 14 week of storage. Theoretically, both gypsum and talcum do not provide nutrition (Sepehrnia et al., 2018). However, in this study the bacterial contaminants were found since the first until the 12th week of storage in the virus formulation in both gypsum and talcum. The results of this study showed that the bacterial contaminants were able to grow without nutrition codition. Stephane et al. (2017), reported that only spore-forming bacteria can live in extreme conditions. Although, both gypsum and talcum contain less organic material that can support bacterial growth (Sepehrnia et al., 2018). However, in this research, the contaminant bacteria can be detected in the viral formulation up to 16-week storage period.

The analysis of variance (ANOVA) test for the fluctuation in growth of contaminant bacteria in virus formulation in gypsum carriers, shows that there was an interaction effect between time and media on the number of bacterial contaminants. In the virus formulation in gypsum, the bacterial contaminant growth was influenced by time and the type of medium. The storage time and type of medium affected the number of bacterial contaminants. The results of Duncan's Multiple Range Tests (DMRT) is shown in **Table 1**.

As seen from **Table 1**, the result confirmed that storage time significantly affected the number of bacterial contaminants. In the 8th week of storage, the highest number of bacterial contaminants was 1.45 × 10^9 cfu per gram of gypsum powder. However, bacterial contaminant were still found up to the 16th week of storage. The presence of bacterial contaminants proved that they survived in gypsum, which had no nutrients for bacterial growth. The presence of bacteria in a virus formulation was considered to originate from the air during the storage period. This indicates that gypsum is suitable for maintaining bacterial growth and viability. This finding was in agreement with Ashfaq et al., (2020) which showed the ability of gypsum to form a binding interaction with

bacteria as long as maintaining bacterial growth.

The type of media also affected the growth of bacterial contaminants. Most bacteria grow in nutrient agar. The bacteria that grew in Salmonella Shigella Agar and Eosin Methylene Blue Agar, they did not show the characteristics of *Salmonella*, *Shigella*, or coliform. The results of this study indicated that the bacterial contaminants were not salmonella, shigella, and coliform. ANOVA was applied to the data on the number of bacterial contaminants that appeared in virus formulation in talcum as a carrier shows that there was no interaction effect between time and media on the number of bacterial contaminants. Thus, further analysis with DMRT was done by separating storage time and growth media, as shown in **Table 2** and **Table 3**.

Table 2. shows that in the *Ha*NPV$_1$ formulation in talcum, the bacterial contaminants started to grow in the second week and did not grow after 14th week storage. The longer the storage time, the highest number of bacterial contaminants was detected in 8th week, it was 1.97 × 10^9 cfu per gram talcum powder. Bacterial contamination is thought to originate from the air during the storage period of the virus formulation. The bacteria found in the virus formulation should not be able to use talcum as a nutrient. Talcum is due to its poor nutrition content and high silicon dioxide, as explained previously in Alam et al.,(2019). So, these bacteria were regarded as bacteria that able to survive in conditions without nutrition.

Table 3. showed that the number of bacterial contaminants in virus with talcum formulation was higher in the nutrient agar medium. In the medium of salmonella shigella agar, some bacterial colonies that grew but did not show the characteristics of *Salmonella* and *Shigella*. The bacterial contaminants in the formulation were identified based on the results of Gram's staining and biochemical tests. They were identified as bacteria of the genus *Bacillus* spp (**Figure 2)**.

In this study, it was revealed that the bacterial contaminants contained in the virus formulation with gypsum and talcum were bacteria that survive without nutrients from gypsum and talcum. The spore-forming bacteria are usually found as the main bacterial contaminants in a bioproduct (Stephane et al., 2017). Spores of bacteria are a form of bacterial defense mechanism against dry environmental conditions. The similar result found that the bacterial contaminants in the virus formulation in both gypsum and talcum were the genus *Bacillus*. The bacteria was Gram-positive rod-shape and produced endospores. The biochemical tests showed positive catalase test, a negative Voges-Proskauer test, a positive citrate test, and a negative sucrose, mannitol, and lactose test, as well as positive in glucose and maltose test (Rajashekhar & Kalia, 2017). The spores are a form of bacterial defense against dry environmental condition and are usually the main bacterial contaminants in a bioproduct (Stephane et al., 2017).

Overall, *Salmonella* spp., *Shigella* spp., and Coliform bacteria were not found, which indicates that the viral formulation was not contaminated with pathogenic bacteria that is harmful to humans (Lamboro et al., 2016). Although no pathogenic bacteria were found, the presence of other bacterial contaminants can affect the quality of the viral formulation, as the presence of bacteria in some cases can damage viral agents (Afolami & Oladunmoye, 2017).

Another study showed that a fungi-based biopesticide, *Beauveria bassiana*, isolated from a major honey bee pest, *Varroa destructor*, showed a good result in a lab experiment. However, in a field experiment, the biopesticide was found to be contaminated with *Pseudomonas fluorescens*. This contamination had a negative impact on the growth of the bee colonies, such as a higher mortality rate, a loss of weight, and a higher *V. destructor* mite density compared to the control. This bacterial contamination was shown to suppress *B. bassiana* growth on the nutritive media (Meikle, et al. 2012). The contamination presents specific hazards to the manufacture and implementation of biopesticides.

A recent study showed that the recycled virus inoculum obtained from dead larvae of fall army worm (FAW) treated with a commercial baculovirus (Littovir) has the potential to function as an environmentally friendly biopesticide. In controlled laboratory

conditions, the recycled inoculum exhibited the highest mortality rate for 1st-3rd instar stage. In field conditions, the absence of significant differences in the maize grain yield among the chemical insecticide, baculovirus insecticide (Littovir), and the recycled inoculum highlighted a sustainable and cost-effective approach to FAW management (Mweke, et al. 2023).

From this study, it is known that the bacterial pathogen contaminants are absent in these formulations. Although it is crucial to include bacteriostatic and fungistatic agents into the formulation. In addition to that, baculovirus formulations should include chemicals like benzoates, sorbates, and parabens that act as common preservative, both bacteriostatic and fungistatic. However, researchers have paid little attention to the use of biostatic compounds to eliminate the chance of contaminant agents (Presa-Parra, et al. 2020). Furthermore, engaging in direct research within the fields is necessary to create intricate combinations of baculoviruses that can effectively manage multiple pests at the same time (Haase, et al., 2015).

CONCLUSION

The gypsum and talcum-based *Ha*NPV$_1$ viral formulation as a bioinsectide agent in powdery form was found to be slightly vulnerable to bacterial contamination. Bacterial contaminants from the product *Ha*NPV$_1$, formulated in gypsum and talcum as carrier mediums were found until the maximum (16-weeks) storage period. In gypsum as a carrier medium, it is significant that bacterial contaminants grow because of it influenced by storage time and type of medium. As for the talcum as a carrier medium, the presence of bacterial contaminants is influenced only by time and type of medium. The bacteria, *Bacillus* spp was found in both carrier media in the form of dormant spores. No pathogenic bacteria i.e., *Salmonella* sp., *Shigella* sp. And coliform detected, indicating that gypsum and talcum were safe for use as promising formulation methods. From this study, however, there is no indication of pathogenic bacteria detected in viral formulation. The presence of bacterial contamination during storage of the virus formula is not known for its effectiveness when applied for controlling pest insects in the field. Further tests need to be carried out to determine the quality of the virus formula contaminated with bacteria. More research need to be done to create a biopesticide with a long-lasting formula that includes ingredients that kill bacteria and fungi.

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Table 1. Duncan's Multiple Range Test of number of bacterial contaminants in *Ha*NPV$_1$ formulation in gypsum as a carrier medium

Time (week)	The total number of bacteria contaminant in specific medium ($\times 10^9$ cfu/g Powder)		
	Nutrient Agar	Salmonella Shigella Agar	Eosin Methylene Blue Agar
0	0.24±0.0006 A c	0 B D	0 B D
2	0.32±0.11 A bc	0 B D	0 B D
4	0.30±0.016 A bc	0 B D	0 B D
6	0.45±0.013 A B	0 B d	0 B D
8	1.46±0.12A A	0.0005±0.00006 B D	0.0002±0.00002 B D
10	0.39±0.042 A b	0 B d	0 B D
12	0.27±0.16 A b	0 B d	0 B D
14	0.0075±0 A b	0 B d	0 B D
16	0.0041±0 A d	0 B d	0 B D

Mean with different capital letters within rows and letters within a column indicate significant differences (P<0.05)

Table 2. Duncan's Multiple Range Test of number of bacterial contaminants in *Ha*NPV$_1$ formulation in talcum as a carrier medium in different storage periods

Threatment (time/week)	Notation
0	0 d
2	0.43±0.17 b
4	0.28±0.08 b
6	0.22±0.09 b
8	1.96±1.46 a
10	0.03±0 c
12	0.23±0.014 b
14	0 d
16	0 d

Means followed by different letters are significant different. (P<0.05)

Table 3. Duncan's Multiple Range Test of the number of bacterial contaminants in *Ha*NPV$_1$ formulation in talcum as a carrier medium in threatment medium.

Threatment (medium)	Notation
Nutrient Agar	3.16± 0.62a
Salmonella Shigella Agar	0.0006± 0.0001b
Eosin Methylene Blue Agar	0±0c

Means followed by different letters are significant different. (P<0.05)

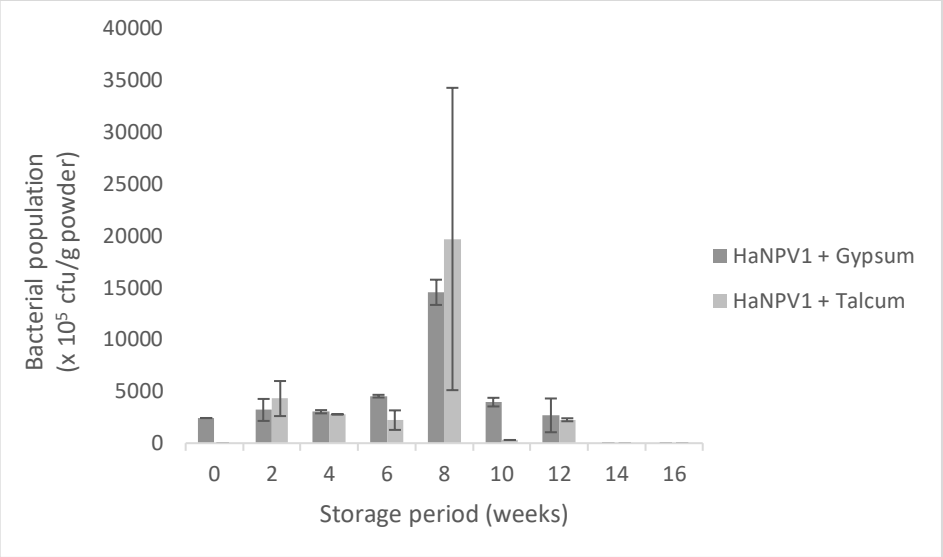

Figure 1. The presence of bacterial contaminants in virus powdered formulation in gypsum and talcum stored for 16 weeks

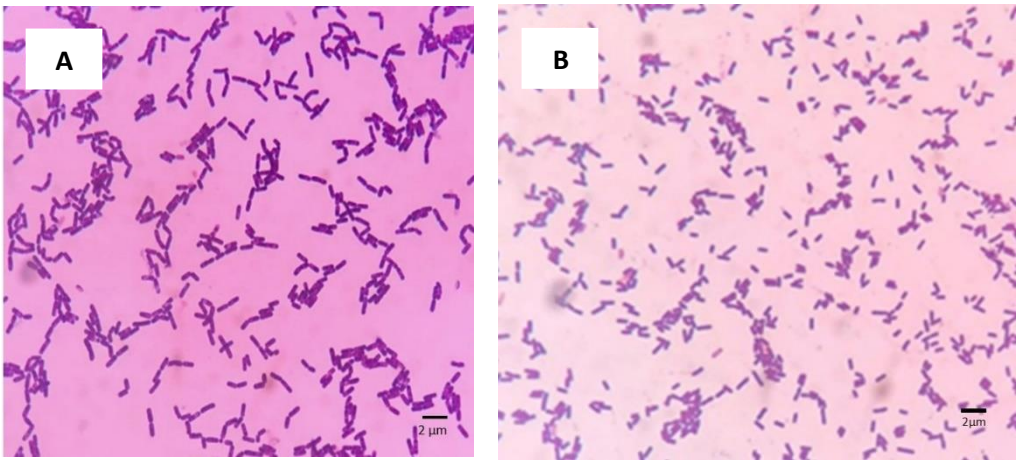

Figure 2. Gram Stain Results, A. *Bacillus* sp. 1 and B. *Bacillus* sp. 2