ENTOMOPHAGA 28 (1), 1983, 9-16

MICROORGANISMS ASSOCIATED WITH PRODUCTION LOTS OF THE NUCLEOPOLYHEDROSIS VIRUS OF THE GYPSY MOTH, *LYMANTRIA DISPAR* [*LEP. : LYMANTRIIDAE*]

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Samples of a gypsy moth nucleopolyhedrosis virus product, Gypchek®, were taken each day during a 100-day production run and monitored for the presence of pathogenic bacteria and fungi. The standard plate count/g of product was $5.97 \pm 1.51 \times 10^8$ over the 100-day period, while the sporulating bacteria count was $3.81 \pm 1.21 \times 10^6$/g. We did not detect obligate anaerobic or fecal coliform bacteria in any of the samples. *Bacillus cereus, Staphylococcus epidermidis, B. licheniformis, Streptococcus faecalis, Serratia liquefaciens,* and *Aspergillus niger* were the most frequently isolated microorganisms. We did not detect primary pathogenic bacteria or fungi, but the presence of opportunistic pathogens indicated that assiduous monitoring of the virus production facility and rigorous quality control of production batches are necessary.

Microbial insecticides are being investigated as alternatives to hazardous chemicals for use in controlling agricultural and forest insect pests. A naturally occurring nucleopolyhedrosis virus (NPV) (*Baculovirus*) of the gypsy moth, *Lymantria dispar* (L.), was developed by the USDA Forest Service and registered with the U.S. Environmental Protection Agency (EPA) for use in gypsy moth management systems (**Lewis** *et al.*, 1979). The registered product, Gypchek®, was tested extensively to assure its safety for nontarget species (**Podgwaite**, 1980). The product must be free of human and other mammalian pathogens (United States Environmental Protection Agency, 1975). Pursuant to this requirement, a protocol was established for the microbiological examination of production lots of Gypchek (**Podgwaite & Bruen**, 1978). Because an efficient large-scale cell culture system is not available for producing gypsy moth NPV, Gypchek is produced at the U.S. Department of Agriculture's Otis Methods Development Center, Otis AFB, Massachusetts, using an *in vivo* system and clean-room technology developed by personnel at that facility. This is a report on microorganisms associated with Gypchek produced under these conditions.

MATERIALS AND METHODS

NPV PRODUCTION

Gypsy moth NPV was produced *in vivo* over a 100-day period by methods described in detail by **Shapiro** *et al.* (1980, 1981). Gypsy moth larvae (New Jersey strain) were reared to the 5th stage on artificial diet, infected with NPV, and harvested 14 days post infection. Larval cadavers were frozen at – 20 °C, dehaired, lyophilized, and ground to a coarse admixture of insect parts and virus.

BACTERIAL DETECTION, ENUMERATION, AND IDENTIFICATION

Microbiological examination of the NPV product was performed by modified methods of **Podgwaite & Bruen** (1978). A 50 mg sample of each daily NPV production lot was suspended in 5.0 ml of sterile phosphate buffer (Baltimore Biological Laboratories) (1) (BBL), pH 7.2, and triturated in a glass tissue homogenizer. The resulting stock virus suspension was used as inocula for all subsequent detection and enumeration procedures.

Standard pour plate counts (SPC) were determined on trypticase soy agar (TSA) (BBL) after incubation at 35 ± 0.5 °C for 24 ± 2 h. Sporulating bacteria counts were done on TSA at 35 ± 0.5 °C for 24 ± 2 h following incubation at 65 ± 1 °C for 30 mn. Anaerobic bacteria were detected and enumerated by techniques of **Sutter** *et al.* (1975). Samples were plated on both 5 % defibrinated sheep blood agar (BBL) and anaerobic agar (BBL), and also inoculated into thioglycollate broth (BBL). Cultures were incubated aerobically, under 10 % CO^2 and under anaerobic conditions (gas-pak system, BBL) at 35 ± 0.5 °C for 48 ± 2 h. Anaerobically incubated cultures were held for an additional 72 h to detect slow-growing species. Coliform bacteria counts were determined on eosin methylene blue agar (EMB) (BBL) incubated at 35 ± 0.5 °C for 24 ± 2 h. Suspect colonies on EMB, producing gas after culture in lauryl tryptose broth (BBL), were confirmed using API enteric diagnostic strips (Ayerst Laboratories, Inc.). Fecal coliforms were detected by inoculation of suspect colonies from EMB agar into EC broth (BBL) and incubating at 44.5 ± 0.2 °C for 24 ± 2 h. Gas producers were confirmed using the API system. Members of the genera *Salmonella* and *Shigella* were sought on Salmonella-Shigella agar (BBL) spread plates incubated at 35 ± 0.5 °C for 48 ± 2 h.

Fungi were detected by spot plate inoculation of Sabouraud dextrose agar (BBL) and Mycosel agar (BBL). Incubation was at 22 ± 2 °C for 5 days.

All bacterial and fungal isolates differing in colonial and morphological characteristics were subcultured on appropriate media and identified according to conventional microbiological methods described in a variety of manuals and texts (American Public Health Association, 1976 ; **Blair** *et al.*, 1970 ; **Buchanan & Gibbons**, 1974 ; **Edwards & Ewing**, 1972 ; **Gibbs & Shapton**, 1966 ; **Gibbs & Skinner**, 1966 ; **Gordon** *et al.*, 1973 ; **Larone**, 1976 ; **Skerman**, 1967 ; **Sutter** *et al.*, 1975).

RESULTS AND DISCUSSION

Standard plate counts and sporulating bacteria counts for daily production lots of NPV are shown in table 1. The SPC/g of product ranged from 1.3×10^7 to 4.6×10^9 and averaged $5.97 \pm 1.51 \times 10^8$ (x ± SE) over the 100-day production period. Sporulating bacteria counts

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TABLE 1

Enumeration of bacteria in daily production lots of the gypsy moth nucleopolyhedrosis virus product, Gypchek®

Production day (a)	Standard plate count/g	Sporulating bacteria/g	Production day	Standard plate count/g	Sporulating bacteria/g
01	2.2×10^8	3.6×10^5	52	1.3×10^9	5.0×10^6
02	3.1×10^8	0	53	2.5×10^9	0
03	1.0×10^8	0	54	1.8×10^9	1.8×10^5
04	2.0×10^9	2.8×10^5	55	3.1×10^9	0
06	2.4×10^7	4.0×10^3	56	1.7×10^9	6.3×10^6
07	1.1×10^9	0	57	2.3×10^9	2.1×10^5
08	1.2×10^9	0	58	1.1×10^9	1.1×10^3
09	1.4×10^8	4.4×10^6	59	9.9×10^8	3.6×10^5
10	4.0×10^7	8.0×10^3	60	3.2×10^8	4.9×10^5
11	1.5×10^8	1.6×10^6	61	9.5×10^8	7.1×10^6
13	4.2×10^8	8.0×10^4	62	1.0×10^8	4.0×10^6
14	3.7×10^7	1.2×10^4	63	3.3×10^8	2.9×10^6
15	2.5×10^8	7.3×10^6	64	3.1×10^8	1.7×10^5
16	2.9×10^7	3.5×10^5	65	3.2×10^8	3.5×10^5
17	1.1×10^8	1.6×10^7	66	1.1×10^8	8.5×10^3
18	1.1×10^8	2.9×10^6	67	5.6×10^8	8.6×10^5
19	1.1×10^9	6.1×10^6	68	3.1×10^8	3.7×10^6
20	3.0×10^8	6.4×10^6	69	7.8×10^8	1.3×10^6
21	1.7×10^8	1.3×10^7	70	2.5×10^8	2.9×10^4
22	1.4×10^9	1.3×10^6	71	2.8×10^8	3.3×10^5
23	3.5×10^8	1.5×10^6	72	9.0×10^8	7.0×10^6
24	2.6×10^8	7.7×10^6	73	3.4×10^8	1.3×10^7
25	1.3×10^9	1.0×10^6	74	2.1×10^8	1.1×10^6
26	3.5×10^8	4.7×10^5	75	1.4×10^7	2.7×10^5
27	8.0×10^8	5.9×10^4	76	1.5×10^8	3.0×10^4
28	1.6×10^8	8.0×10^4	77	7.1×10^8	1.5×10^6
29	1.1×10^8	7.3×10^5	78	1.9×10^9	4.9×10^6
30	1.5×10^8	8.2×10^4	79	7.0×10^7	2.2×10^5
31	9.5×10^7	1.2×10^5	80	8.3×10^8	0.9×10^5
32	1.4×10^8	1.2×10^6	81	5.6×10^8	6.3×10^6
33	5.2×10^8	1.1×10^5	82	2.6×10^8	4.7×10^5
34	5.4×10^8	4.1×10^5	83	1.2×10^8	3.8×10^5
35	2.5×10^8	1.8×10^6	84	2.6×10^8	3.5×10^5
36	1.6×10^8	3.3×10^5	85	6.0×10^8	1.7×10^6
37	4.7×10^8	2.0×10^5	86	1.0×10^8	3.4×10^5
38	3.1×10^8	4.4×10^7	87	5.5×10^8	1.2×10^5
39	7.3×10^8	1.5×10^7	88	1.7×10^8	3.2×10^5
40	4.6×10^9	5.7×10^4	89	8.3×10^8	2.0×10^5
41	6.8×10^8	3.7×10^5	90	4.0×10^7	1.6×10^5
42	3.7×10^8	5.6×10^5	91	2.4×10^8	1.6×10^6
43	2.0×10^8	5.7×10^4	92	3.1×10^8	5.7×10^6
44	5.3×10^7	4.1×10^6	93	1.8×10^8	1.0×10^6
45	7.9×10^8	2.6×10^6	94	9.9×10^7	3.1×10^5
46	1.2×10^9	7.3×10^6	95	2.4×10^8	1.3×10^6
47	8.1×10^8	2.5×10^6	96	8.3×10^8	4.6×10^7
48	3.4×10^8	1.1×10^6	97	1.2×10^8	2.2×10^7
49	5.5×10^8	1.2×10^6	98	8.4×10^8	1.0×10^7
50	1.3×10^7	1.3×10^7	99	2.0×10^8	1.5×10^6
51	5.5×10^8	5.0×10^4	100	8.3×10^8	4.6×10^7

(a) No sample taken on days 5 and 12.

ranged from zero to 4.6×10^7/g and averaged $3.81 \pm 1.21 \times 10^6$/g over the same period. We did not detect obligate anaerobes or coliform bacteria in any of the samples.

The microorganisms associated with production lots of NPV are shown in table 2. *Bacillus cereus, Staphylococcus epidermidis,* (phosphatase negative), *B. licheniformis, Streptococcus faecalis, Serratia liquefaciens,* and *Aspergillis niger* were the microorganisms most frequently isolated from production samples.

TABLE 2

Microorganisms and frequency of occurrence in production lots of Gypchek®

Microooorganism	Frequency of occurrence (a)
Bacillaceae	
B. cereus	.726
B. coagulans	.065
B. licheniformis	.484
B. macerans	.016
B. subtilis	.081
Enterobacteriaceae	
Enterobacter agglomerans	.048
Serratia liquefaciens	.355
Micrococcaceae	
Micrococcus luteus	.016
Staphylococcus epidermidis	.629
Streptococcaceae	
S. durans	.081
S. faecalis	.435
S. faecalis var. *liquefaciens*	.065
S. faecium	.032
Pseudomonadaceae	
Pseudomonas fluorescens grp.	.065
P. pseudomallei	.032
Pseudomonas spp.	.081
Actinomycetaceae	
Actinomyces naeslundii	.177
Neisseriaceae	
Acinetobacter calcoaceticus var *antitratum*	.032
Cryptococcaceae	
Rhodotorula sp.	.032
Moniliaceae	
Aspergillus niger	.339

(a) Based on the examination of samples through day 62 of Gypchek production.

The presence of *B. cereus* was of particular concern because it often occurred in NPV production samples at greater than 10^6 spores/g. Although this bacterium is not considered a primary human pathogen, it has been implicated, albeit rarely, in cases of food poisoning (**Mossel** *et al.*, 1967). More importantly, in the context of quality assurance, *B. cereus* is a mouse pathogen (**Lamanna & Jones**, 1963). Because one of the requirements for acceptance of the NPV product is that it causes no mortality when injected intraperitoneally into mice (**Podgwaite & Bruen**, 1978), the microorganism must not occur at levels exceeding 10^6 spores/g in the NPV product. Thus, the bacterium must be absent from the NPV inocula used to infect larvae, and further, the production facility must be monitored closely to minimize *B. cereus* introduction into the production line.

Other members of the genus *Bacillus* found in the product (*B. coagulans, B. licheniformis* and *B. macerans*) have been implicated in food spoilage but are not of particular public health concern in the context of the use patterns envisaged for the microbial insecticide.

The only 2 members of the *Enterobacteriaceae* found in NPV samples were *Enterobacter agglomerans* (*Erwinia herbicola*) and *Serratia* (*Enterobacter*) *liquefaciens.* Both of these may have entered the production line in one of the diet ingredients because both are associated with plants. Some strains of *Erwinia* are plant pathogens and may be opportunistic human pathogens (**Muraschi** *et al.*, 1965), however, there is no cause to expect that either *E. herbicola* or any of the other nonsporulating bacteria encountered in this study is resistant to sunlight and temperature-desiccation effects that would be encountered during the application of the NPV product.

The 2 species within the family *Micrococcaceae* isolated from NPV samples were *Micrococcus luteus* and *Staphylococcus epidermidis.* Of the 2, only *S. epidermidis* is of public health significance. Strains of the species were isolated from over 60 % of the NPV samples. However, all strains were phosphatase negative (**Baird-Parker**, 1963) and considered to be nonpathogenic. Because *S. epidermidis* is commonly found on the skin and mucous membranes of humans, it is likely that it entered the product through production worker handling of diet or insects, or both.

Three species of group D *Streptococcus* were found in NPV production batches, but only *S. faecalis* was isolated with any frequency (43 %). This bacterium is a gypsy moth pathogen (**Doane & Redys**, 1970, **Podgwaite & Campbell**, 1972) and its association with the product was not unexpected. Though *S. faecalis* is a fecal microorganism, the absence of associated coliform bacteria pointed to a nonfecal source of contamination.

Members of the *Pseudomonas fluorescens* group and other *Pseudomonas* spp. were isolated with low frequency. This group represents a variety of food spoilage microorganisms and opportunistic pathogens. There were 2 isolations of *P. pseudomallei,* an accidental pathogen that has been implicated as the causative agent of melioidosis (a Glanders-like infection) in rats, guinea pigs, rabbits, and man in Southeast Asia (**Redfearn** *et al.*, 1976). Its introduction into the NPV production line is unexplained.

Actinomyces naeslundii was isolated from 11 NPV samples. This microorganism is an opportunistic pathogen, but its normal habitat is the oral cavity of man (**Howell** *et al.*, 1962). Thus, its appearance in the products is likely from worker contamination, but it is of no particular concern.

Acinetobacter calcoaceticus var *antitratum* (*Herellea vaginicola*) was found in 2 samples. This organism is commonly found in soil and water, yet may be opportunistic in compromised individuals (**Graber** *et al.*, 1962).

Rhodotorula sp. and *Aspergillus niger* were the other microorganisms isolated from the virus product.

No primary mammalian pathogens were isolated from any NPV samples, and under the present *in vivo* production system their entry into the product is not likely. However, in light of the high bacterial counts and the diversity of microorganisms found, careful microbiological quality control must be performed on each production batch before its acceptance and use.

ACKNOWLEDGMENTS

We thank **H.K. Kaya**, University of California, Davis ; **J.A. Shadduck**, University of Illinois, Urbana ; **E.W. Davidson**, Arizona State University, Tempe ; and **F.B. Lewis**, USDA Forest Service, Hamden, Connecticut for their comments on the draft.

RÉSUMÉ

Micro-organismes associés à des lots de production du virus de la nucléopolyhédrose de *Lymantria dispar* [*Lep. : Lymantriidae*]

Des échantillons d'une préparation à base du virus de la nucléopolyhédrose de *Lymantria dispar* (L.), le Gypchek®, furent prélevés tous les jours durant un cycle de production de 100 jours, et ont été examinés afin d'y détecter des bactéries et champignons pathogénes. Le nombre moyen de colonies bactériennes par gramme du produit était de $5.97 \pm 1.51 \times 10^8$ durant la période de 100 jours, tandis que le nombre de bactéries sporulées était de $3.81 \pm 1.21 \times 10^6$/g. Nous n'avons trouvé ni bactéries anaérobies ni bactéries coliformes fécales dans aucun des échantillons. *Bacillus cereus, Staphylococcus epidermis, B. licheniformis, Streptococcus faecalis, Serratia liquefaciens* et *Aspergillus niger* furent les micro-organismes le plus souvent isolés. Nous n'avons pas trouvé de bactéries ou de champignons pathogénes primaires, mais la présence de pathogènes opportunistes indiquait qu'une surveillance assidue de l'équipement de production du virus et un contrôle rigoureux de la qualité des lots de production sont nécessaires.

REFERENCES

American Public Health Association – 1976. Standard methods for the examination of water and wastewater, 14th ed. – *Am. Public Health Association,* Washington, DC.

Baird-Parker, A.C. – 1963. A classification of micrococci and staphylococci based on physiological and biochemical tests. – *J. Gen. Microbiol.,* 30, 409-427.

Blair, J.E., Lennette, E.H. & Truant, J.P. (eds.). – 1970. Manual of Clinical Microbiology. – *Am. Soc. Microbiol.,* Bethesda, Md.

Buchanan, R.E. & Gibbons, N.E. (eds.). – 1974. Bergey's Manual of Determinative Bacteriology, 8th ed. – *Williams & Wilkins Co.,* Baltimore.

Doane, C.C. & Redys, J.J. – 1970. Characteristics of motile strains of *Streptococcus faecalis* pathogenic to larvae of the gypsy moth. – *J. Invertebr. Pathol.,* 15, 420-430.

Edwards, P.R. & Ewing, W.H. – 1972. Identification of *Enterobacteriaceae,* 3rd ed. – *Burgess Publishing Co.,* Minneapolis.

Gibbs, B.M. & Shapton, D.A. (eds.). – 1966. Identification Methods for Microbiologists, Part B. – *Academic Press,* New York.

Gibbs, B.M. & Skinner, F.A. (eds.) – 1966. Identification Methods for Microbiologists, Part A. – *Academic Press,* New York.

Gordon, R.E., Haynes, W.C. & Hor-Nay Pang, C. – 1973. The genus *Bacillus.* – *U.S. Dep. Agric. Agric. Handbook No. 427,* Washington, D.C.

Graber, C.D., Rabin, E.R., Mason, A.D. Jr., & Vogel, E.H. Jr., – 1962. Increasing incidence of nosocomial *Herellea vaginicola* infections in burned patients. – *Surg. Genecol. Obstet.,* 114, 109-112.

Howell, A., Stephan, R.M. & Paul, F. – 1962. Prevalence of *Actinomyces israelii, A. naeslundii, Bacteronema matruchetii* and *Candida albicans* in selected areas of the oral cavity. – *J. Dent. Res.*, 41, 1050-1059.

Lamanna, C. & Jones, L. – 1963. Lethality for mice of vegetative spore forms of *Bacillus cereus* and *Bacillus cereus*-like insect pathogens injected intraperitoneally and subcutaneously. – *J. Bacteriol.*, 85, 532-535.

Larone, D.H. – 1976. Medically Important Fungi, a Guide to Identification. – *Harper & Row Publishers*, Hagerstown.

Lewis, F.B., McManus, M.L. & Schneeberger, N.F. – 1979. Guidelines for the use of Gypchek to control the gypsy moth. – *U.S. Dep. Agric. For. Serv. Res. Pap.* Ne-441.

Mossel, D.A.A., Koopman, M.J. & Jongerius, E. – 1967. Enumeration of *Bacillus cereus* in foods. – *Appl. Microbiol.*, 15, 650-653.

Muraschi, T.F., Friend, M. & Bolles, D. – 1965. *Erwinia*-like microorganisms isolated from animal and human hosts. – *Appl. Microbiol.*, 13, 128-131.

Podgwaite, J.D. – 1980. Development and utilization of a nucleopolyhedrosis virus (Baculovirus) for control of the gypsy moth. – *Mem. 8th, Reunion Nac. Control Biol.*, Mexico.

Podgwaite, J.D. & Bruen, R.B. – 1978. Procedures for the microbiological examination of production batch preparations of the nuclear polyhedrosis virus (Baculovirus) of the gypsy moth, *Lymantria dispar* (L.) – *U.S. Dep. Agric. For. Serv. Gen. Tech. Rep.* NE-38.

Podgwaite, J.D. & Campbell, R.W. – 1972. The disease complex of the gypsy moth. II. Aerobic bacterial pathogens. – *J. Invertebr. Pathol.*, 20, 302-308.

Redfearn, M.S., Palleroni, N.J. & Stainer, R.Y. – 1966. A comparative study of *Pseudomonas pseudomallei* and *Bacillus mallei*. – *J. Gen. Microbiol.*, 43, 293-313.

Shapiro, M., Bell, R.A. & Owens, C.D. – 1981. *In vivo* mass production of gypsy moth nucleopolyhedrosis virus. In : The Gypsy Moth : Research Toward Integrated Pest Management. (**C.C. Doane & M.L. McManus**, ed.) – *U.S. Dep. Agric. Tech. Bull.* 1584, 633-655.

Shapiro, M., Owens, C.D., Bell, R.A. & Wood, M.A. – 1980. Simplified efficient system for *in vivo* mass production of gypsy moth nucleopolyhedrosis virus. – *J. Econ. Entomol.*, 74, 341-343.

Skerman, V.D.B. – 1967. A Guide to the Identification of the Genera of Bacteria, 2nd ed. – *Williams & Wilkins Co.*, Baltimore.

Sutter, V.L., Vargo, V.L. & Finegold, S.M. – 1975. Wadsworth Anaerobic Bacteriology Manual. – *Univ. California*, Los Angeles.

United States Environmental Protection Agency. – 1975. Guidance for safety testing baculoviruses. In : Baculoviruses for Insect Pest Control : Safety Considerations (**M. Summers, R. Engler, L. Falcon & P. Vail**, ed.) – *Am. Soc. Microbiol.*, Washington, D.C., 170-184.