JOURNAL OF INVERTEBRATE PATHOLOGY 69, 46–50 (1997)
ARTICLE NO. IN964620

Effects of Diet-Age and Streptomycin on Virulence of *Autographa californica* M Nucleopolyhedrovirus against the Tobacco Budworm

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Received May 2, 1996; accepted August 8, 1996

Addition of the antibiotic streptomycin to two artificial diets routinely used in bioassays of neonate larvae of *Heliothis virescens* (tobacco budworm) infected with *Autographa californica* M nucleopolyhedrovirus (AcMNPV) increased lethal times of the virus. After storage of diets for 3 weeks at 4°C, lethal times of infected larvae were significantly slower compared to those for larvae bioassayed using diets stored for 2 weeks or less. The effect of diet-age on rate of mortality was not the result of a change in total protein content or pH of the diet, but was apparently the result of some other alteration in the quality of the diet (e.g. microbial spoilage, palatibility, and/or nutritional value unrelated to total protein). Although we did not determine why lethal times were slower in response to streptomycin concentration or diet-age, we did find that slower lethal times were correlated with slower relative growth rates (RGR) of infected larvae. In addition, RGR of infected larvae decreased as a function of increasing streptomycin concentration, diet-age, and the interaction of the two factors. These results demonstrate that it is difficult to obtain consistent and comparable bioassay results if antibiotic composition and diet-age are not controlled. We suggest a standardized diet or highly standardized procedures for a given diet be developed that permits comparison of bioassays among and within laboratories. © 1997 Academic Press

KEY WORDS: ***Autographa californica* nuclear polyhedrosis virus; *Heliothis virescens*; antibiotics; streptomycin; diet-age; virulence; relative growth rate.**

INTRODUCTION

Bioassays using artificial diets are commonly employed to compare the efficacies of insecticides or entomopathogens against insects. For example, the time to death after ingestion of viral inoculum is often used as an indication of the effectiveness of wild-type baculovirus isolates and recombinant viruses against noctuid larvae (Bonning and Hammock, 1992). Artificial diets traditionally used for these bioassays contain antibiotics (e.g., streptomycin or chlorotetracycline) to suppress microbial spoiling of the diet. Such antibiotics can also exert detrimental effects on the rate of growth and development of insects (Singh and House, 1970a) and, thus, may inadvertently affect bioassay results. However, insect diets lacking antimicrobial agents may become contaminated with bacteria and/or fungi which then cause biochemical changes in the diet leading to alteration of the nutritional value of diet. In addition, bacteria and fungi contaminating diet can produce toxins that poison the insect (Childress and Williams, 1973; Bell *et al.*, 1981). These alterations in diet can strongly bias the bioassay.

During the routine screening of recombinant baculoviruses, we became aware that the use of diets stored at 4°C for more than a few weeks did not produce consistent bioassay results despite the addition of antibiotics. In addition, a series of bioassays were conducted that produced significantly faster killing times than we had previously obtained with the standard wild-type virus. We discovered that the BioServ diet for tobacco budworm (BioServ, Inc., Frenchtown, NJ) normally used in our bioassays had been prepared without the usual addition of streptomycin. (The BioServ dry mix contains a constant 0.1% aureomycin.) Thus, we investigated the effects of streptomycin concentration in artificial diet and the age of the diet on the virulence of the baculovirus, AcMNPV, against *Heliothis virescens.*

MATERIALS AND METHODS

Diets. Four concentrations (0–0.5%) of streptomycin (Sigma, Inc.) were added to two different artificial diets commonly used to rear *H. virescens.* The wheat germ casein diet was prepared according to Vanderzant

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0022-2011/97 $25.00

et al. (1962) and the commercially available tobacco budworm diet was prepared according to the packaging instructions (Hunter, 1984; BioServ, Inc.). Both diets contained 0.1% aureomycin with correction for activity. The BioServ diet bulk mix contains aureomycin; thus it was added to the Vanderzant diet to be consistent with the BioServ diet. Diets were stored at 4°C until used for bioassays, which were conducted weekly starting from the day diets were made (Week 0) for a total of five bioassays over a 4-week period.

Bioassays. A stock solution of polyhedra (PIBS) of wild-type AcMNPV clone C6 (Ayres *et al.,* 1994) was derived from larvae of tobacco budworm and partially purified as described in Hoover *et al.* (1995). Forty neonate larvae of *H. virescens* (USDA Agricultural Research Station, Stoneville, MI) were infected at 2000 PIBS/μl for each treatment using the droplet feeding technique (Hughes *et al.,* 1986) and transferred to individual cups containing one of the above diets. Since all larvae received a lethal dose, only lethal times were monitored. Larvae were maintained at 26°C and 16L:8D and monitored every 4–8 hr until all had died. Median survival times (LT_{50}) and their 95% confidence intervals were obtained using the Kaplan Meier Estimator to give nonparametric estimates of the survival functions for the insects under each treatment (Kalbfleisch and Prentice, 1980; Collett, 1994). The data consisted of records containing information on each insect's (1) survival time t, (2) censor status, and (3) treatment group. Since none of the insects were lost, all censor statuses were equal. Also, since survival times were known only up to an interval of time, the survival times of the insects were estimated as the midpoints of the intervals in which they died.

Examination for changes in diet pH or total protein concentration. We hypothesized that contamination of diet by microorganisms may alter pH and/or the total protein concentration of the diet. Thus, we examined these two variables as a function of age of the diet at the beginning of each bioassay (BioServ diet only). Diet pH was determined by blending 200 mg of each diet in 2 ml of deionized water and measuring each solution with a pH meter. Protein concentration was determined by blending 200 mg of each diet in 2 ml of 0.5 M NaOH followed by centrifugation for 25 min at 5000*g*. The supernatant was tested for protein level using the Bradford Assay and bovine serum albumin as a standard (Bradford, 1986). Multiple regression was used to determine if pH or total protein content among diets varied as a function of streptomycin concentration or diet-age (Steel and Torrie, 1980).

Relationship between time to death and growth rate of infected larvae. To determine if the rate of mortality caused by the virus was correlated with RGR of the larvae, infected neonate larvae were maintained for 3 days on their respective experimental diets described above (BioServ diets only). Larvae were weighed after 3 days to the nearest microgram (Cahn 29 automatic electrobalance). RGR was calculated according to the equation described in Schroeder (1986). Twenty neonate larvae were used to obtain the mean starting weight for the equation and then discarded. Twenty larvae were used for each diet and the experiment was replicated once per week side by side with each bioassay. To ensure that infected insects were not responding atypically to these diets, RGRs of uninfected insects were measured also. Simple linear regression analysis of pooled means was used to determine if LT_{50}s were dependent upon RGR (Steel and Torrie, 1980). Multiple regression analysis was performed on the pooled data to determine if streptomycin concentration and/or diet-age were predictive of RGR, after determining that there was no significant treatment/replicate interaction.

RESULTS

Streptomycin concentration and diet-age affected the rate of mortality of larvae infected with the baculovirus AcMNPV. The median lethal time (LT_{50}) in bioassays performed on the same day that diets were prepared was significantly slower using the diet containing the highest concentration of streptomycin (0.5%) compared to all other diets (Figs. 1 and 2; Table 1). In addition, as diets aged, LT_{50}s for all diets became progressively slower. Bioassays using diets stored for 3 weeks at 4°C

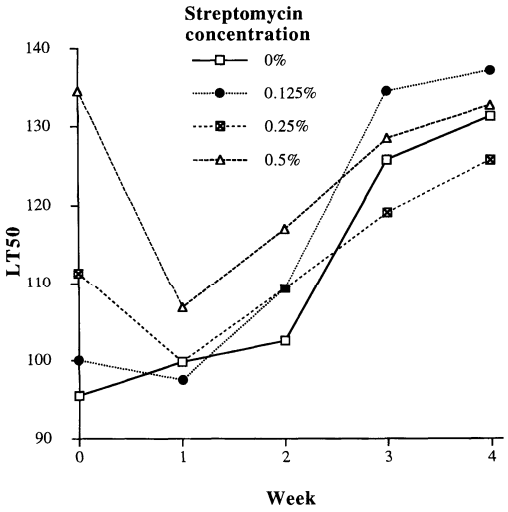

FIG. 1. The effect of aging and addition of increasing concentration of streptomycin to BioServ diet on rate of mortality of larvae of *H. virescens* infected with AcMNPV. Following infection by droplet feeding, larvae were fed on one of four diets containing different concentrations of streptomycin (0, 0.125, 0.25, and 0.5%) and a constant level of 0.1% aureomycin and maintained at 26°C. LT_{50}s were determined by the Kaplan Meier Estimator.

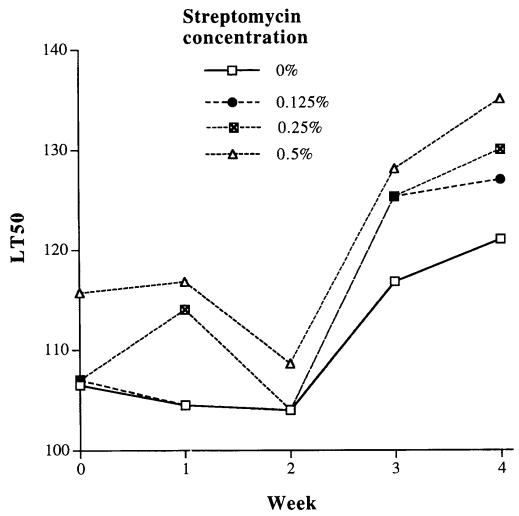

FIG. 2. The effect of aging and addition of increasing concentration of streptomycin to Vanderzant diet on rate of mortality of larvae of *H. virescens* infected with AcMNPV. See Fig. 1 for methods.

resulted in significantly slower LT_{50}s than those in bioassays performed on the day the diets were prepared (Figs. 1 and 2, Table 1). At 4 weeks of diet age, the LT_{50}s for all diets were significantly slower than those of all bioassays using diets stored for 2 weeks or less (Figs. 1 and 2, Table 1).

Relative growth rates of infected insects were slower the higher the concentration of streptomycin and the longer the diets were stored (Table 2). There was also a significant interaction between streptomycin concentration and diet-age in their influence on RGRs (Table 2; multiple regression on pooled data: RGR = 1.16–0.75 (strep) − 0.014 (week)^2 − 0.04(strep*week^2); $F = 199.4$; $df = 3, 389$; $P < 0.0001$). This relationship did not change when the analysis was performed on pooled means or pooled observations. RGR of uninfected insects followed the same trend in that the higher the streptomycin concentration, the more slowly larvae grew and the older the diet, the more slowly the insects grew (data not shown).

Median lethal times of infected larvae decreased as a function of increasing RGR of infected larvae (Fig. 3; $LT_{50} = -44.6(RGR) + 150, R^2 = 0.668, F = 36.2, df = 1, 18, P = 0.0001$). In other words, the faster larvae grew, the fewer hours postinfection were required to kill 50% of infected larvae.

TABLE 1

The Effect of Diet-Age and Addition of Increasing Concentrations of Streptomycin to Two Different Diets on LT_{50}s of Larvae of *H. virescens* Infected with AcMNPV

	Streptomycin concentration (%)			
	0	0.125	0.25	0.5
Bioserv diet				
Week 0	95.5a	100a	111a	135a[a]
Week 1	99.8ab	97.5ab	99.8ab	107a
Week 2	103ab	109b	109ab	117a
Week 3	126bc	135c	119bc	129b
Week 4	131c	137c	126c	133b
Vanderzant diet				
Week 0	106a	107a	107a	116a[a]
Week 1	104a	105a	114a	117ab
Week 2	104a	104ab	105a	109a
Week 3	117b	125bc	125b	128bc
Week 4	121b	127c	130b	135c

Note. LT_{50}s within columns with a different letter were significantly different at the 5% level. LT_{50}s and their 95% confidence intervals were computed using the Kaplan Meier Estimator.

[a] For bioassays in Week 0, the LT_{50} using the diet with the highest concentration of streptomycin (0.5%) was significantly slower at the 5% level than LT_{50}s using all other diets.

TABLE 2

Multiple Regression of RGR as a Function of Streptomycin Concentration and Diet-age

Variable	Slope	Partial *F*-statistic	*P*-value	95% fiduciary limits
Streptomycin	−0.750	81.601	0.0001	−0.914 to −0.587
Week^2 [a]	−0.014	6.669	0.0001	−0.019 to −0.009
(Strep*week^2)	−0.039	18.452	0.0001	−0.057 to −0.021

Note. Multiple regression on pooled data: R^2adj = 0.66; $F = 199.4$; $df = 3, 389$; $P < 0.0001$.

[a] Week was transformed to week^2 to linearize the quadratic function.

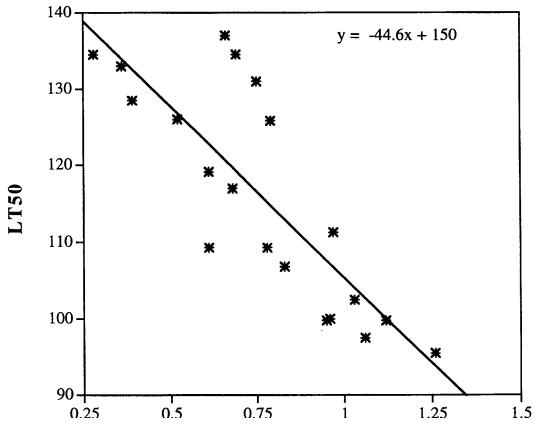

FIG. 3. Influence of relative growth rate (RGR) of neonate larvae of *H. virescens* infected with AcMNPV on LT_{50}s of the virus. Following infection by droplet feeding AcMNPV at a concentration of 2000 PIBS/µl, larvae were maintained for 3 days on their respective experimental diets described in Fig. 1. Larvae were weighed after 3 days to the nearest microgram. RGR was calculated according to the equation described in Schroeder (1986). Twenty larvae were used for each diet and the experiment was replicated once per week for a total of five bioassays (i.e., Weeks 0–4). $R^2 = 0.668$, $F = 36.2$, $df = 1, 18$, $P = 0.0001$.

Diet pH was not significantly affected by either streptomycin concentration or diet-age (R^2adj = 0.18, $F = 3.06$, $df = 2, 17$, $P = 0.07$). The mean pH for all diets over the course of the experiment was 6.15 ± 0.06. Mean total protein content decreased from 1.84 ± 0.06% to 1.47 ± 0.19% from Weeks 0 to 4, but protein content was not significantly dependent upon streptomycin concentration or diet-age (R^2adj = 0.10, $F = 2.10$, $df = 2, 17, P = 0.15$). In addition, LT_{50}s were not related to protein concentration or diet pH (R^2adj = −0.003, $F = 0.98, df = 2, 17, P = 0.40$).

DISCUSSION

Our findings show that bioassays of baculoviruses can be significantly affected by both addition of streptomycin and diet-age (Figs. 1 and 2, Table 1). In diets that already contain 0.1% aureomycin, the addition of 0.5% streptomycin altered bioassay results. In addition, insects that were infected and fed on diets older than 2 weeks died more slowly than insects that were infected and fed on diets used on the same day they were prepared. Moreover, bioassay results using diets older than 3 weeks gave significantly slower LT_{50}s than diets stored for 2 weeks or less. These findings suggest that diet quality can be affected with addition of antibiotics and/or storage time and can, consequently, result in aberrant bioassay results of baculoviruses and perhaps of other entomopathogens as well.

Our findings concur with other studies that show that the addition of antibiotics to diet can influence the virulence of entomopathogens such as baculoviruses and *Bacillus thuringiensis* (Ignoffo, 1963; Vail *et al.,* 1968; Ignoffo *et al.,* 1977; Beegle *et al.,* 1981; Bell *et al.,* 1981). For example, inclusion of formaldehyde in artificial diets markedly increased the LD_{50} and LT_{50} of a baculovirus in the cabbage looper *Trichoplusia ni* (Vail *et al.,* 1968). In this instance, however, larvae were infected by surface contamination of the diet with polyhedra, and formaldehyde may have caused chemical denaturation of the polyhedra. A subsequent study showed that a granulovirus of *Pieris brassicae* failed to produce infection in larvae when the virus was applied to the surface of a synthetic diet containing formaldehyde (David *et al.,* 1969). However, if larvae were fed diet containing formaldehyde after they were fed the virus on a formaldehyde-free diet or cabbage leaf, there was no effect on infectivity. In our study, direct interaction between polyhedra and diet constituents was avoided by infecting larvae by droplet feeding and subsequently placing them on diets.

The influence of diet-age and streptomycin concentration on the bioassay was not the result of a change in diet pH or total protein concentration of the diets. Although it is not clear why these factors affected the bioassay, it is clear that LT_{50} values were strongly negatively correlated with RGR of infected larvae that fed simultaneously on these same diets (Fig. 3). The faster larvae grew, the faster they succumbed to infection, suggesting that the ability of these factors to affect lethal times may be related to the nutritional value of the diet, either in the nutritional integrity of the diet (diet-age) and/or the ability of the insects to obtain supplemental nutrition from endosymbionts (antibiotics). We also show that there is an interaction between the factors of antibiotic concentration and diet-age in their ability to influence larval growth rates. When one factor is fixed, the other factor influences larval growth rate by a greater degree (i.e., the slope is more negative as a result of their interaction). It appears that streptomycin concentration functions as a positive influence on growth with increasing time, probably due to the degradation of antibiotics over time reducing their toxicity. At the same time, diet-age has a negative influence on growth with increasing time, possibly due to degradation of nutrients over time. The interaction of these factors will, therefore, influence growth differently (in magnitude and sign) at different points in time. Alternatively, diet quality may be impaired with prolonged storage by microbial spoilage or palatibility of the diet resulting in lower feeding rates.

Interacting effects such as described above are a common phenomenon in biology. For example, streptomycin and potassium sorbate have been shown to interact with nutrient levels in diets to adversely affect growth and development of the fly larva, *Agrinis affinis,* which has no gut symbionts (Singh and House, 1970). These authors found that larval toxicity was proportionately reduced by increasing the level of nutrients in the diet.

One cannot assume that faster larval growth rates will translate into faster speed of kill in other systems. The way nutrients and insect growth rates influence disease is poorly understood and experimental results are often contradictory (reviewed by Duffey *et al.,* 1995). For example, deficits or surfeits of protein have marked detrimental effects on many larval insects, including growth rates (Broadway and Duffey, 1986; Mattson and Scriber, 1987; Broadway and Duffey, 1988; Bloem and Duffey, 1989). However, it is currently impossible to determine why an excess of protein can enhance susceptibility to virus in some insects (e.g., *H. virescens,* Hoover *et al.,* unpublished), attentuate susceptibility in others (e.g., *T. ni,* Hoover *et al.,* unpublished), or have no effect in others (reviewed by Duffey *et al.,* 1995) despite enhanced growth rates as protein content in diet is increased. Thus, an assumption that rate of mortality of infected insects is always dependent upon RGR cannot be made and must be examined on a case by case basis.

In conclusion, it is clear that maximum viral efficacy can only be ensured if antibiotics are kept to a minimum and diets are not stored longer than 2 weeks.

These results demonstrate that it is difficult to obtain consistent and comparable bioassay results if antibiotic composition and diet-age are not controlled. Our results strongly suggest that a standardized diet, or at least highly standardized procedures for a given diet, be developed that permits comparison of bioassays among and within laboratories so that comparison of different viral isolates or genetic constructs can be made without the confounding influence of variable diets.

ACKNOWLEDGMENTS

This project was supported in part by grants from the National Science Foundation (DCB-91-19332), USDA Competitive Research Grants Program (94-37302-0567), National Institute of Environmental Health Sciences Center (P30 ESO5707), US/Israel BARD (IS-2139-92), UC Systemwide Biotechnology Research and Education Program, Interregional Research Project No. 4 (95-06746V), NSF DMS 95-10511 Center for Statistics in Science and Technology Group Infrastructure Grant, USDA Forest Service NAPIAP G-5-95-20-062, and E.I. DuPont De Nemours & Co. K.H. was supported by a fellowship from Novo Nordisk, Entotech. B. D. Hammock is a Burroughs Wellcome Scholar in Toxicology.

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