Journal of the American Mosquito Control Association, 14(4):472–476, 1998

BACTERIAL CONTROL OF MOSQUITO LARVAE: INVESTIGATION OF STABILITY OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* AND *BACILLUS SPHAERICUS* STANDARD POWDERS

ISABELLE THIERY AND SYLVIANE HAMON

Unité des Bactéries Entomopathogènes, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris cedex 15, France

ABSTRACT. *Bacillus thuringiensis* var. *israelensis* and *Bacillus sphaericus* products were assayed against their respective reference powders IPS82 and SPH88. Since their production in 1982 and 1988, the potency and larvicidal activity of these standard powders have been regularly checked on their test insects *Aedes aegypti* (for IPS82) or *Culex pipiens* (for SPH88). Over the 16-year evaluation period of IPS82 and 10-year evaluation period of SPH88, their potencies were considered stable. The global mean of each year's mean showed a coefficient of variation of less than 20%. Larval rearing was the most important factor in the reproducibility of the bioassay, although some variation also originated from the person performing the bioassay. This study demonstrated that the SPH88 standard could be kept in a stock suspension at 4°C for 3 years without loss of potency. Moreover, after 9 years of storage in suspension, only a 2-fold decrease in the potency of SPH88 was detected.

KEY WORDS *Bacillus thuringiensis, Bacillus sphaericus,* mosquito larvae, standardization, bioassay, potency

The bacterium *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) de Barjac has been used since 1980 for the control of mosquito and blackfly larval populations. In a similar context, *Bacillus sphaericus* Neide has been used since 1987 to combat *Culex* Linnaeus and *Anopheles* Linnaeus mosquito larvae. Different preparations, such as liquids, powders, granules, pellets, micropellets, microgranules, and fizzy tablets, are applied to breeding sites depending on the mosquito species and their biotope (Thiéry et al. 1996). Before application, the potency of these bacterial preparations is evaluated and compared by titrating them against reference standard powders. These standard powders are dispatched by the Unit of Bactéries Entomopathogènes, Institut Pasteur, Paris, France, upon request. Standardization performance depends upon the reproducibility of bioassays and the stability of the larvicidal activity of these reference powders. In this note we describe the stability of the standard powders commonly used for *B.t.i.* and *B. sphaericus* products. To perform standardized bioassays, protocols for mosquito rearing as well as bioassay methods and *Aedes aegypti* L. (Bora-Bora strain) eggs are available upon request from the Unit of Bactéries Entomopathogènes, Institut Pasteur.

Bacillus thuringiensis var. *israelensis* (strain 1884) lyophilized powder (IPS82) is the international standard powder for *B.t.i.* preparations (de Barjac and Larget-Thiéry 1984). According to its relation with IPS78 (the first *B.t.i.* standard, which had a potency of 1,000 ITU/mg [de Barjac and Larget 1979]), IPS82 has a potency of 15,000 ITU/mg. The potency of a *B.t.i.* product is thus defined in ITU/mg on *Ae. aegypti* (Bora-Bora strain) young 4th-instar larvae according to the following formula:

$$LC_{50}\ (\text{IPS82}) \times 15{,}000/LC_{50}\ (\textit{B.t.i.}\ \text{product}),$$

in which LC_{50} represents the lethal concentration inducing 50% larval mortality after exposure of the larval population for 24 h.

Bacillus sphaericus products are comparatively bioassayed against *B. sphaericus* strain 2362 lyophilized standard powder, which is called SPH88. The potency is calculated according to the formula mentioned above, although the LC_{50} is recorded after 48 h of exposure of the larval population. The potency of this standard has been determined against the first *B. sphaericus* standard powder called RB80, which had a potency of 1,000 ITU/mg (Bourgouin et al. 1984) as 1,700 ITU/mg using *Culex pipiens* Linnaeus (Montpellier strain) young 4th-instar larvae (Thiéry, unpublished data). Likewise, another *B. sphaericus* (strain 2297) lyophilized standard powder, SPH84, was determined to contain 1,500 toxic units/mg on *Cx. pipiens* (Montpellier strain) in bioassays against RB80 (Thiéry, unpublished data). These 2 standard powders were produced to overcome problems with stability of the larvicidal activity and reproducibility when using the RB80 standard (Fig. 1). Since the preparation of SPH88 and SPH84, the RB80 standard has not been available. The SPH84 standard is usually not dispatched because most of the *B. sphaericus* products are made with strain 2362. Nevertheless, the potency of SPH84 is verified and it can also be used as an internal standard. As for IPS82 (de Barjac and Larget-Thiéry 1984), the heat-stabilities of SPH84 and SPH88 powders at 50°C for 4 wk have been checked and the mean of their LC_{50}s was identical to that of control powder kept at 5°C (Thiéry, unpublished data). Since 1988, SPH88 powder has been used as the acknowledged standard to rate the potency of *B. sphaericus* products based on strain 2362.

Ratings in ITU allow comparison of larvicidal

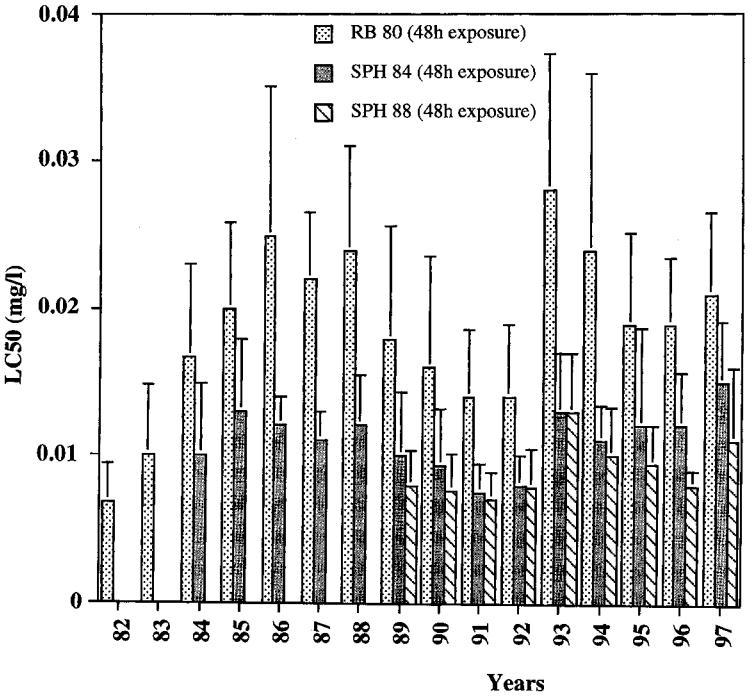

Fig. 1. Mean of the larvicidal activity of 3 *Bacillus sphaericus* standard powders (RB80, SPH84, and SPH88) from 1982 to 1997 against *Culex pipiens* 4th-instar larvae. The LC_{50} (mg/liter) represents the lethal concentration that kills 50% of the larval population exposed for 48 h. The bars represent the mean of LC_{50}s of at least 12 bioassays with the indication of the standard deviation (SD).

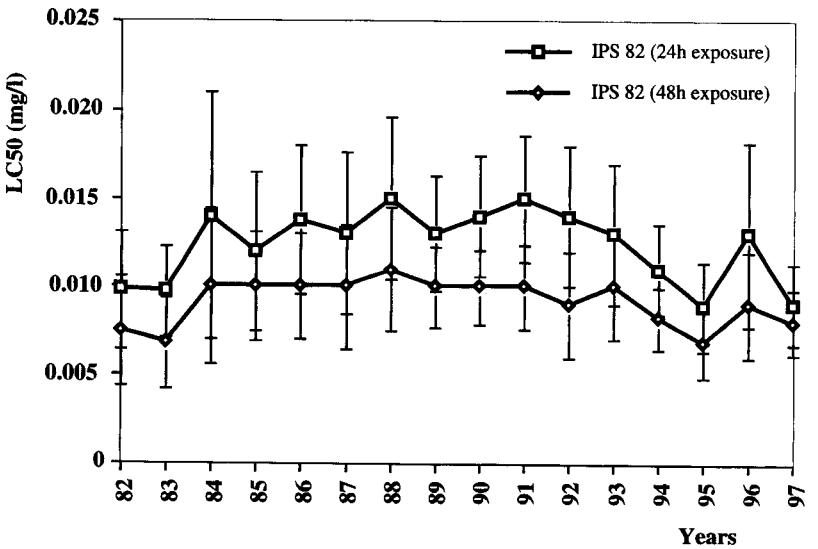

Fig. 2. Mean of the larvicidal activity of the *Bacillus thuringiensis* var. *israelensis* standard powder IPS82 from 1982 to 1997 against *Aedes aegypti* (Bora-Bora strain) 4th-instar larvae. The LC_{50} (mg/liter) represents the lethal concentration that kills 50% of the larval population exposed for 24 h or 48 h. The dots represent the mean of LC_{50}s of at least 12 bioassays with the indication of the standard deviation (SD).

Table 1. Comparison of the larvicidal activity of the SPH88 standard of *Bacillus sphaericus* kept in suspension for 8 years at 4°C to a fresh suspension of SPH88 powder.[1]

	SPH88 stock suspension (Oct. 1, 1989)[2]		SPH88 control (new flask)[3]		Mean of SPH88 over 1 year's time[4]	
Year	LC_{50} (mg/liter)	LC_{90} (mg/liter)	LC_{50} (mg/liter)	LC_{90} (mg/liter)	LC_{50} (mg/liter)	LC_{90} (mg/liter)
1989	0.0082 ± 0.0011	0.014 ± 0.0022	0.0082 ± 0.0011	0.014 ± 0.0022	0.008 ± 0.0024	0.015 ± 0.0043
1990	0.006 ± 0.0019	0.013 ± 0.005	0.0070 ± 0.0010	0.012 ± 0.0015	0.0076 ± 0.0025	0.016 ± 0.0058
1991	0.006 ± 0.001	0.009 ± 0.001	0.0075 ± 0.0025	0.012 ± 0.0055	0.0071 ± 0.0018	0.013 ± 0.0038
1992	0.012 ± 0.0038	0.023 ± 0.0074	0.0087 ± 0.0036	0.014 ± 0.0060	0.0079 ± 0.0026	0.016 ± 0.009
1997	0.018 ± 0.0041	0.042 ± 0.021	0.0076 ± 0.0047	0.015 ± 0.0081	0.011 ± 0.050	0.028 ± 0.020

[1] Bioassays of the stock suspension and control were performed on the same day; the results represent the mean of at least 3 experiments. Results are mean ± standard deviation. LC_{50}, median lethal concentration; LC_{90}, 90% lethal concentration.
[2] Stock suspensions contained 5,000 mg/liter and flasks were sealed after each sampling and kept at 4°C.
[3] For each bioassay a new flask was opened and powder was weighed in order to prepare a suspension containing 5,000 mg/liter; appropriate dilutions were made from this suspension.
[4] Results represent the mean of at least 12 bioassays during a 1-year period.

activity of bacterial formulations between producers and laboratories, thus avoiding variations due to intrinsic parameters of the method. Indeed, 2 main protocols are used for mosquito bioassays using different cups, volumes, water depth, dilutions, and numbers of larvae (de Barjac and Larget-Thiéry 1984, McLaughlin et al. 1984). Moreover, the species of *Culex* used are not the same. In some countries *Culex quinquefasciatus* Say are used instead of *Cx. pipiens*. Efficacy of standardization is valuable if standard bioassays are as reproducible as possible with little variation of the LC_{50}. To achieve this it is necessary to regularly check the larvicidal activity of the standard powders.

Since their production, IPS82, SPH84, and SPH88 as well as RB80 standards have been bioassayed monthly on their respective test insect (Figs. 1 and 2) and their LC_{50} and LC_{90} recorded. During the test period, all flasks were stored at 4°C and a new flask was opened for each bioassay. The 3 *B. sphaericus* standards were always bioassayed on the same day using the same larval mosquito population. At least 12 bioassays were performed each year, and all measurement values were included in order to observe the range of possible variations, despite the strict titration and rearing methods used. In all cases, rearing conditions were standardized as much as possible. Standardization of rearing *Culex* larvae was much more difficult than that of *Aedes* larvae. Indeed, *Aedes* eggs could be stored, dessicated, and hatched simultaneously. *Aedes* larvae reached the early 4th instar within 4–5 days at 25°C (when larval density was about 700 larvae per 2 liters with 3 cat food pellets (Friskies®, Friskies PetCare Company Inc., Glendale, CA) weighing ca. 0.3 g each). In contrast, *Cx. pipiens* rafts did not hatch simultaneously and larvae had to be separated during the first instars. Moreover, *Culex* larvae were more sensitive to the presence of food, which was needed when they were bioassayed. All of the results were influenced by the physiologic state of the larvae as well as by the person doing the bioassay. From 1984 to 1992, one person performed the bioassays and from 1993 to 1997, they were performed by another person. However, the most important source of variation in the bioassay was the way that the larvae were fed during the larval rearing (Skovmand et al. 1998).

In Figure 1, we show that the RB80 standard fluctuates greatly within each year and through the years; this fluctuation calls into question the utility of RB80 as a standard; in contrast the SPH84 and SPH88 standards showed less variation. Indeed, the LC_{50} mean ± SD over the 16-year evaluation of RB80 was 0.018 ± 0.0054 mg/liter (CV: 29% variation), the SPH84 14-year mean ± SD was 0.011 ± 0.0019 mg/liter (CV: 17% variation), and the SPH88 9-year mean ± SD was 0.0091 ± 0.0018 mg/liter (CV: 19% variation), where SD is the standard deviation and CV is the coefficient of variation. These numbers represent the average of the

Table 2. Larvicidal activity of SPH84 and RB80 kept in suspensions for 12 years at 4°C.[1]

Year	SPH84 stock suspension (July 1, 1985)[2]		SPH84 control[3]		RB80 stock suspension (July 1, 1985)[2]		RB80 control[3]	
	LC_{50} (mg/liter)	LC_{90} (mg/liter)	LC_{50} (mg/liter)	LC_{90} (mg/liter)	LC_{50} (mg/liter)	LC_{90} (mg/liter)	LC_{50} (mg/liter)	LC_{90} (mg/liter)
1985	0.010 ± 0.0043	0.021 ± 0.008	0.013 ± 0.0049	0.034 ± 0.026	0.011 ± 0.003	0.022 ± 0.006	0.020 ± 0.0059	0.051 ± 0.022
1997	0.040 ± 0.0057	0.12 ± 0.027	0.024 ± 0.0077	0.067 ± 0.025	0.041 ± 0.017	0.093 ± 0.054	0.022 ± 0.0057	0.045 ± 0.0067

[1] Bioassays of the stock suspension and control were performed on the same day; the results represent the mean of at least 3 experiments. Results are mean ± standard deviation. LC_{50}, median lethal concentration; LC_{90}, 90% lethal concentration.

[2] Stock suspensions contained 5,000 mg/liter and flasks were sealed after each sampling and kept at 4°C.

[3] For each bioassay a new flask was opened and powder was weighed in order to prepare a suspension containing 5,000 mg/liter; appropriate dilutions were made from this suspension.

means over each year's time. Based upon these results we considered SPH84 and SPH88 to be good standard powders. In the case of the IPS82 standard (Fig. 2), the LC_{50} mean ± SD at 24 h of larval exposure over the 16-year evaluation was 0.012 ± 0.0020 mg/liter given a 16% coefficient of variation. The lowest variation was observed after 48 h of larval exposure with the LC_{50} mean ± SD = 0.0091 ± 0.0012 mg/liter (CV: 13% variation). These results indicate that IPS82 is still a good standard powder. The variations in the larvicidal activities were mostly due to the physiologic state of the larvae and to the method of performing the bioassay, rather than to the loss of activity of the powders.

We tested the standard to determine whether, when in stock-suspension, it could be kept for years at 4°C. The SPH88 standard has been kept in stock suspension, containing 5,000 mg/liter, at 4°C since January 10, 1989. This suspension was the source for the serial dilutions and was sealed after each bioassay. The toxicity of this suspension was regularly checked on *Cx. pipiens* larvae until 1997 (Table 1). The lethal concentrations were compared with that of a fresh stock suspension bioassayed on the same day and with the mean of the LC_{50}s over the respective year. For 3 years no variation in the larvicidal activity of this suspension was noticed. After 4 years, a slight decrease in potency was noticed but it was not significantly different from the global mean, whereas after 8 years of storage, the suspension had a 2-fold reduction in potency. In 1997, a cell and spore count was performed from the January 10, 1989 stock suspension and a suspension used in October 1997 diluted to 1 mg powder/ml. The stock suspension contained 3.4 ± 0.1 × 10^8 cells/mg and 3 ± 0.3 × 10^8 spores/mg, whereas a fresh suspension contained 3.07 ± 0.06 × 10^8 cells/mg and 3.06 ± 0.8 × 10^8 spores/mg powder. Therefore, no increase had occurred in the number of spores and no contamination by other bacteria had occurred in the stock suspension that had been stored for years. This showed that SPH88 powder could be stored in aqueous suspension for 3 years without change in its potency. A similar experiment was performed with SPH84 and RB80 after 12 years of storage at 4°C (Table 2): at the end of the test period a 3- to 4-fold decrease in larvicidal activity of the stock suspension was observed. Nevertheless, these results indicated that the larvicidal activity of the SPH88 standard was not easily degraded after being in aqueous suspension for many years.

This survey showed that IPS82 and SPH88 standard powders could still be used as reference material, especially to rate *B.t.i.* and *B. sphaericus* powders, respectively. The efficacy of a standard product was questionable when we observed a variation in the standard bioassay (usually between 4 and 25%) and in product bioassays (usually more variation than the standard). This might enhance the

variations of the potency of a product. One must be aware of the limitations of a standardized assay due to the difficulty of standardizing living organisms and to the way larvae react to the product. Therefore, an assay with a final CV of 30% should be considered a reasonable result, based on our experience on bioassays of *B. thuringiensis* products. This is the case even when the assay is performed under high-quality, standardized conditions (e.g., rearing, mosquito populations, standardized methods), as defined in Skovmand et al. (1998) and by Thiéry et al. (1997).

Standardization is used less and less frequently for *B. thuringiensis* products on Lepidoptera larvae because of the variety of toxins commercially available and the high number of various target insects. To have accurate potency of bacterial products, one wonders whether one standard should be made for each toxin corresponding to one test insect and according to the formulation to be assessed. Indeed, Skovmand et al. (1997) have recently shown that the slopes of the bioassay curves of *B.t.i.* fluid products were steeper than those of *B.t.i.* powder products. This would suggest that IPS82 powder should only be used to assay *B.t.i.* powder products and not those in fluid forms. Presently, most of *B.t.i.* and *B. sphaericus* commercialized products are made with strain 1884 (Vectobac® and Bactimos® Abbott, Chicago, IL; Culinex®, Culinex Gmbh, Waldsee, Germany; Tecknar®, Thermo Trilogy Corporation, Columbia, MD) or 2362 (Vectolex® and Spherimos®, Abbott). Other commercially used strains, such as strain C3-41 (Wuhan, China) and B.101 (Sphericide®, Biotech International Ltd., New Delhi, India) of *B. sphaericus* or strain 197 (Wuhan Institute of Virology, Wuhan, China) and strain 164 (Bacticide®, Biotech International Ltd., New Delhi, India) of *B.t.i.* also contain the same toxins. But when new *B. thuringiensis* products that harbor mosquito toxins different from those of *B.t.i.*, such as *B. thuringiensis* var. *medellin* or var. *jegathesan,* will be commercialized, we will face a problem similar to that of the *B. thuringiensis* standard for Lepidoptera products. In such a case, the validity of the standardization assay, the aim of which is to check the quantity of active ingredient and the homogeneity and stability of a product, could be called into question.

We thank V. Patricio for help in mosquito rearing and bioassays performed since 1993.

REFERENCES CITED

Bourgouin, C., I. Larget-Thiéry and H. de Barjac. 1984. Efficacy of dry powders from *Bacillus sphaericus:* RB80, a potent reference preparation for biological titration. J. Invertebr. Pathol. 44:146–150.

de Barjac, H. and I. Larget. 1979. Proposals for the adoption for standardized bioassay method for the evaluation of insecticidal formulations derived from serotype H14 of *Bacillus thuringiensis* var. *israelensis.* Mimeographed Document. WHO/VBC/79-744. World Health Organization, Geneva, Switzerland.

de Barjac, H. and I. Larget-Thiéry. 1984. Characteristics of IPS82 as standard for biological assay of *Bacillus thuringiensis* H-14 preparations. Mimeographed Document. WHO/VBC/84.892. World Health Organization, Geneva, Switzerland.

McLaughlin, R. E., H. T. Dulmage, R. Alls, T. L. Couch, D. A. Dame, I. M. Hall, R. I. Rose, and P. L. Versoi. 1984. A U.S. standard bioassay for the potency assessment of *Bacillus thuringiensis* serotype H-14 against mosquito larvae. Bull. Entomol. Soc. Am. 30:26–29.

Skovmand, O., D. Hoegh, H. S. Pedersen and T. Rasmussen. 1997. Parameters influencing potency of *Bacillus thuringiensis* var. *israelensis* products. J. Econ. Entomol. 90:361–369.

Skovmand, O., I. Thiéry, G. L. Benzon, G. Sinègre, N. Monteny and N. Becker. 1998. Potency of products based on *Bacillus thuringiensis* var. *israelensis:* interlaboratory variations. J. Am. Mosq. Control. Assoc. 14: 298–304.

Thiéry, I., C. Back, P. Barbazan and G. Sinègre. 1996. Applications de *Bacillus thuringiensis* et de *Bacillus sphaericus* dans la démoustication et la lutte contre les vecteurs de maladies tropicales. Ann. Inst. Pasteur 7: 247–260.

Thiéry, I., T. Baldet, P. Barbazan, N. Becker, B. Junginger, J.-P. Mas, C. Moulinier, K. Nepstad, S. Orduz and G. Sinégre. 1997. International indoor and outdoor evaluation of *Bacillus sphaericus* products: complexity of standardizing outdoor protocols. J. Am. Mosq. Control Assoc. 13:218–226.