Homogeneity of *Pasteurella avicida* Bacterin

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Abstract □ A simple rapid procedure is described for determining whether an emulsified bacterin is homogeneous by measuring the UV absorbances of chloroform-washed centrifugates. These absorbances appear to depend on nucleic acid content.

Keyphrases □ *Pasteurella avicida*—determination of homogeneity of emulsified suspension, UV measurement of extracted cell contents □ Bacterial vaccine (bacterin)—determination of homogeneity of emulsified suspension of *Pasteurella avicida*, UV measurement of extracted cell contents □ Vaccine, bacterial—determination of homogeneity of emulsified suspension of *Pasteurella avicida*, UV measurement of extracted cell contents

Methods are available for determining either the number of microorganisms or the amount of cellular material in a suspension (1–4). However, a simple method is needed to determine the homogeneity of a bacterin (5), which is typically an inactivated aqueous suspension of bacterial cells emulsified with mineral oil. The bacterin is administered to chickens or turkeys from a multiple-dose container without shaking during the dosing period. Settling in the opaque emulsion during this time could change the bacterin concentration without visual detection, leading to the administration of different doses.

The procedure of Chalenko *et al.* (6) for quantitating all organic constituents in a typhus vaccine involves adsorption on aluminum hydroxide, oxidation with dichromate in sulfuric acid, and colorimetry at 580 nm; the reproducibility is ±7%. The method proposed here permits a simple, rapid determination of the homogeneity of a bacterial vaccine (bacterin) by measuring the UV absorbance of the washed extracted contents of cells.

EXPERIMENTAL

Shake thoroughly a 500-ml bottle of *Pasteurella avicida* bacterin[1] 30 times and then open the bottle. With a Cornwall syringe adjusted to deliver 1 ml, remove gently two 1-ml aliquots from approximately 1 cm below the surface. The syringe is fitted with a 12.7-cm (5-in.),, 14-gauge cannula[2] to which is attached a 5-cm length of tubing[3]. Transfer each aliquot to a different 60-ml separator.

Gently take two aliquots from the middle (half-full mark, 250 ml) of the bottle and discard them into a waste container. Then take two more aliquots from the middle of the bottle and transfer each aliquot to one of two additional separators. Gently remove two aliquots from within 1 cm of the bottom and discard them. Then remove two more aliquots and transfer each to a fifth and sixth separator.

Wipe both the needle and the extension after drawing each aliquot. Do not disturb the bottle during the period in which the homogeneity of the emulsion is being determined, except for the removal of samples at 1, 2, and 4 hr after the shaking. The sampling period was deliberately made longer than the time needed for a veterinarian to administer the contents from the container.

Add 15 ml of 6.7% sodium chloride solution to each funnel and shake the funnels intermittently for 15 min. Wash the aqueous suspension three times with 15-ml portions of chloroform to remove the oil and such inactivating agents as phenol and β-propiolactone. Discard the lower chloroform layer each time.

Transfer the aqueous layer from each separator through a narrow stem funnel into a different 25-ml wide mouth, screw-capped volumetric flask. Rinse each separator with water and use the washings to fill the appropriate flask to the mark. Cover the opening of each flask with aluminum foil, cap, shake each flask vigorously, and allow it to stand for 10 min. Then transfer approximately 5 ml from the flask with a pasteur pipet into a centrifuge tube and centrifuge for 30 min. Measure the absorbance[4] at the 257-nm maximum of the clear aqueous solution in 1-cm cells against water. Figure 1 shows the UV absorbance for the spectrum of a washed centrifuged extract between 230 and 350 nm.

RESULTS

Table I summarizes the UV absorbances of chloroform-washed, centrifuged 1-ml aliquots taken in duplicate from three regions of the container during a typical analysis. The data indicate that the emulsified suspension remains homogeneous for at least 4 hr after being shaken, allowing the administration of uniform doses during this period. The absorbances did not vary by more than the 5% expected to occur by chance. The coefficient of variation was 3%.

Four other lots from the same manufacturer were homogeneous, with coefficients of variation ranging from 1.9 to 3%. The average absorbance for different batches varied from 0.32 to 0.60, indicating variation in either cell number or cell contents.

DISCUSSION

This method appears to measure the UV absorbance of nucleic acids in the aqueous centrifugate, since there is a peak in the spectrum at 257 nm (Fig. 1) and the absorbance at 260 nm, when divided by the absorbance at 280 nm, yields a ratio of 1.95. This ratio is 1.94 for deoxyribonucleic acid (7) and 2.0 for ribonucleic acid (8), with peak absorbances at approximately 259 (9) and 258 (10) nm, respectively. The absorbance maximum depends greatly on nucleic acid composition (11).

Saline solution denatures some proteins and solubilizes nucleic acids (8). Chloroform, in addition to removing the oil and such inactivating agents as phenol and β-propiolactone from the bacterin, washes out some low molecular weight compounds, lipids, and proteins (4). Saline solution also helps to prevent the formation of an emulsion when the oil is extracted into chloroform.

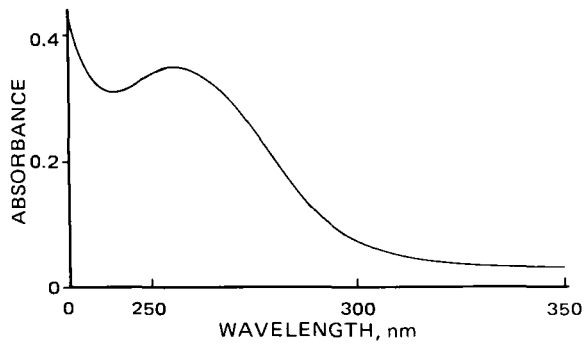

Figure 1—*UV spectrum of chloroform-washed centrifugate of* P. avicida *bacterin between 230 and 350 nm. Absorbance was determined against water. For details, see* Experimental.

[1] E. R. Squibb and Sons.
[2] Becton, Dickinson and Co.
[3] Teflon.

[4] Cary model 15 or other suitable spectrophotometer.

Table I—Absorbance of Chloroform-Washed Centrifugates from *P. avicida* Bacterin[a]

Hours after Shaking Bacterin	Region of Bottle	Peak Absorbance	
		Sample	Average
0	Top	0.329	0.327
	Top	0.324	
	Middle	0.324	0.330
	Middle	0.336	
	Bottom	0.330	0.328
	Bottom	0.326	
1	Top	0.320	0.318
	Top	0.315	
	Middle	0.303	0.315
	Middle	0.326	
	Bottom	0.331	0.318
	Bottom	0.305	
2	Top	0.323	0.329
	Top	0.335	
	Middle	0.315	0.322
	Middle	0.328	
	Bottom	0.318	0.322
	Bottom	0.325	
4	Top	0.316	0.319
	Top	0.321	
	Middle	0.320	0.326
	Middle	0.332	
	Bottom	0.316	0.321
	Bottom	0.326	

[a] See *Experimental* for details of sampling and handling.

Additional experiments demonstrated that only bacterial cell contents contribute significantly to the UV absorbance. A placebo mixture of oil, emulsifying agent, and water carried through the recommended procedure had an absorbance of 0.003 at 257 nm. Batches of the bacterin were allowed to stand undisturbed at 4° for about 10 days to allow the cellular material to settle approximately 2 cm below the surface. One-milliliter samples of clear liquid were removed from bottles of these batches within 1 cm of the surface and were carried through the analytical procedure. Samples of clear liquid taken from five batches obtained from four different vendors all gave final solutions with absorbances at 257 nm of less than 0.010. Neither 1% phenol nor 0.15% β-propiolactone (used as bacterial inactivating agents) interfered in the assay. In separate experiments, they were added to mineral oil which was carried through the procedure. The final absorbance at 257 nm in each case was 0.006. The preservative thimerosal used in some bacterins at a concentration of 1:10,000 does interfere. The absorbance at 257 nm for this concentration of thimerosal in water is 0.046. When this amount of thimerosal was added to mineral oil and carried through the procedure, the final absorbance at 257 nm was 0.027. Should thimerosal be present in a formulation, this slight increase in absorbance would be the same for samples from each time period. This would not change the conclusions about the homogeneity of the samples.

Weight variance of six 1-ml samples of cell suspension obtained with the Cornwall syringe was 3 mg, and the standard deviation was 2.4 mg. Other variables investigated that had no effect on absorbance were the concentration of saline solution (5–8%), the time of contact of the bacterin with chloroform (10–120 min), the number of chloroform washes (three to five), the time before centrifugation (10–50 min), and the duration of centrifugation (10–30 min). Higher absorbances may be obtained by using 2 or 3 ml of bacterin and extracting five times with chloroform.

Four samples of *P. avicida* bacterin from other vendors gave spectra similar to Fig. 1, with peak absorbances between 256 and 258 nm.

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