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Fly ash-based water dispersible powder formulation of *Bacillus thuringiensis* var. *israelensis*: Development & laboratory evaluation against mosquito immatures

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***Background & objectives*: *Bacillus thuringiensis* var. *israelensis* (*Bti*) formulations are presently being used for insect control. In this study, a water dispersible powder (WDP) formulation using fly ash (FA) as a carrier material was developed and studied for its activity against the larval stages of major mosquito vector species.**

***Methods*: An indigenous isolate *Bti* (Vector Control Research Centre B17) was mass produced using a 100 l fermentor in soya-based medium. The bacterial biomass was mixed with lignite FA and made into WDP formulations. The most effective formulation was used for determining 50 per cent lethal concentration (LC_{50}) against the larval stages of major mosquito vector species, effect on non-target organisms and mammalian systems using standard protocols.**

***Results*: Sixteen types of WDP formulations were prepared, of which the formulation containing bacterial biomass, FA and carboxymethyl cellulose was found to be the most effective. The LC_{50} values of the formulation against *Culex quinquefasciatus, Aedes aegypti* and *Anopheles stephensi* larvae were 0.0417, 0.0462 and 0.1091 mg/l, respectively. The formulation was found to be safe to non-target organisms found associated with the mosquito larval stages and also to mammalian systems.**

***Interpretation & conclusions*: The study shows that FA can be effectively used to replace commercially available carrier materials used in biopesticidal formulations.**

Key words *Bacillus thuringiensis var. israelensis* - fly ash - laboratory evaluation - mosquito biolarvicides - water dispersible powder formulation

Mosquitoes play a predominant role in the transmission of malaria, dengue fever, yellow fever, filariasis and several other diseases[1]. Effective control of aquatic mosquito larvae has been reported to be achieved using *Bacillus thuringiensis* var. *israelensis* (*Bti*)[2]. Although *Bti* has been in use for more than two decades, no resistance has been detected in target insects exposed to this biolarvicide. *Bti* is highly selective for use against members belonging to the family *Culicidae* and *Simuliidae*. It offers an additional

advantage of not affecting non-target species of vertebrates and invertebrates, thereby ensuring the safety of its prolonged use on a large scale, without damaging the environment[3]. The effectiveness of *Bti* is dependent on the bioavailability of the material in treated areas, which in turn depends on the design of the formulation. An ideal mosquito larvicidal formulation comprises the active ingredient (*Bti*), additives and carrier material. A number of additives such as carboxymethyl cellulose (CMC), gum Acacia, Xanthan gum, guar gum, pectin, starch, polyethylene glycol (PEG), sodium alginate, paraffin, gelatin and lignin have been used in biopesticidal formulations[4-6]. In India, coal and lignite are the most economic and easily available raw materials for power generation, but its utilization is faced with several environmental constraints, the main being production of fly ash (FA) in enormous quantities (120 million tonnes per annum)[7] which needs to be disposed of in an appropriate manner.

The present study reports the development of water dispersible powder (WDP) formulations using FA generated from the neighbouring Neyveli Lignite Corporation, Neyveli, Tamil Nadu, India, and an indigenous isolate of *Bti* (VCRC B17). The best formulation was taken up for the evaluation under laboratory conditions against the larval stages of *Culex quinquefasciatus, Anopheles stephensi* and *Aedes aegypti,* vectors of filariasis, malaria, chikungunya and dengue, respectively.

Material & Methods

Bacterial strain: *Bti* (VCRC B17), an indigenous isolate[8], was obtained from the microbial culture collection of ICMR-Vector Control Research Centre, Puducherry, India. The strain was revived from the lyophilized spores and streaked onto modified nutrient yeast salt medium (NYSM) agar slants[9]. The slants were incubated at 30°C for 48 h and then stored at 4°C for further use.

Inoculum preparation: The seed inoculum was produced using shake flasks. The first-stage seed was prepared by inoculating 10 ml of NYSM broth with one loop full of a slant culture and incubating at 30°C on a rotary shaker (200 rpm) for 6 h. The second-stage seed was prepared by inoculating 2 per cent v/v of first-stage seed into 600 ml of NYSM medium in a 2 l Erlenmeyer flask and incubating on a rotary shaker as done earlier.

Pilot sale fermentation: The second-stage seed in log phase was used to inoculate a 100 l bioreactor at 2 per cent (v/v). Fermentation experiments were conducted in a pilot scale bioreactor (Bioengineering, Wald, Switzerland) filled with 60 l production medium (2.0% soya powder) as described earlier[10], except for the stirrer speed which was adjusted to 200 rpm. Fermentation was terminated after completion of spore-crystal complex formation as confirmed by microscope (Motic Microscope Model DM143, Germany). The fermentation was repeated on three different days.

Continuous flow centrifugation: The sporulated culture broth was harvested by centrifugation using a continuous flow centrifuge (CEPA Z41, Germany) as described elsewhere[11]. The bacterial biomass deposited in the form of a cake on the rotor was scooped out and used for the preparation of WDP formulation.

Processing of carrier material: The lignite FA was obtained from Neyveli Lignite Corporation Limited, Neyveli, Tamil Nadu, India. It was powdered using a ball mill and the FA powder was sieved through 25-µ mesh, to obtain particles of size ≤25 µ to suit the feeding size range of mosquito larvae. This FA material was sterilized and used in the preparation of formulations. The elemental analysis of FA was done using scanning electron microscope (SEM) (Model JSM-6510LV, JEOL, USA) and energy dispersive X-ray spectroscopy (EDS) (INCAPentaFETx3 Model 8129, Oxford Instruments, England).

Development of formulation: Sixteen types of WDP formulations were prepared using a mixture of bacterial biomass: FA (4:5) and various quantities of organic, plant-based and synthetic polymer additives, namely CMC or Acacia gum or soluble starch or PEG or Xanthan gum purchased from HiMedia, India, respectively (Table I). The formulations were dried at 40°C, ground to a fine powder, sieved to a size of ≤25 µ and stored after confirming the final moisture content to be 5 per cent. The final product of these formulations was greyish fine-sized powder that dispersed readily when mixed with water.

Laboratory evaluation: The WHO standard procedure[12] was followed to determine the efficacy of the WDP formulations against late third instars of *Cx. quinquefasciatus*, obtained from the rearing and colonization facilities of our institute. Suspensions of the WDP formulations were made by suspending 10 mg of each formulation in 10 ml of sterile distilled

Table I. Composition of fly ash-based water dispersible powder formulations

WDP formulation code	Active ingredient (g)	Carrier material (g)	Adjuvants
WDPAA	*Bti* (4)	FA (5)	-
WDPA1	*Bti* (4)	FA (5)	0.5% CMC
WDPA2	*Bti* (4)	FA (5)	1% CMC
WDPA3	*Bti* (4)	FA (5)	2% CMC
WDPB1	*Bti* (4)	FA (5)	0.5% PEG
WDPB2	*Bti* (4)	FA (5)	1% PEG
WDPB3	*Bti* (4)	FA (5)	2% PEG
WDPC1	*Bti* (4)	FA (5)	0.5% starch
WDPC2	*Bti* (4)	FA (5)	1% starch
WDPC3	*Bti* (4)	FA (5)	2% starch
WDPD1	*Bti* (4)	FA (5)	0.5% gum Acacia
WDPD2	*Bti* (4)	FA (5)	1% gum Acacia
WDPD3	*Bti* (4)	FA (5)	2% gum Acacia
WDPE1	*Bti* (4)	FA (5)	0.5% Xanthan gum
WDPE2	*Bti* (4)	FA (5)	1% Xanthan gum
WDPE3	*Bti* (4)	FA (5)	2% Xanthan gum

WDP, water dispersible powder formulation; CMC, carboxymethyl cellulose; PEG, polyethylene glycol; *Bti*, *Bacillus thuringiensis* var. *israelensis*; FA, fly ash

water and mixed well using glass homogenizer. Range-finding bioassays were performed using a wide range of concentrations ranging from 1 to 10 µg. Each concentration had four replicates each, along with appropriate number of controls which contained only plain water. Larval mortality was scored after 24 h. The experiment was done at least three times on different days with freshly prepared suspensions.

Statistical analysis: Larval mortality in control (5-20%) was corrected according to the Abbott's formula[13]. The corrected mortality was subjected to mortality-concentration regression analysis[14] to calculate 50 and 90 per cent lethal concentration (LC_{50} and LC_{90}) values as well as their 95 per cent fiducial limits (95% FL) using log-probit analysis software (SPSS Statistics ver. 21, IBM Corporation, NY, USA). The LC_{50} and LC_{90} values obtained for WDP formulations were subjected to one-way ANOVA followed by Tukey's honest significant difference (HSD) multiple comparison test to determine the differences in formulations.

Susceptibility of Anopheles stephensi and Aedes aegypti: The most effective WDP formulation from the test with *Cx. quinquefasciatus* was selected and the dose required for inciting LC_{50} and LC_{90} for the larvae of other two major mosquito vectors, namely, *An. stephensi* and *Ae. aegypti*, were determined using the WHO procedure[12]. The LC_{50} values obtained for different mosquito species were compared using one-way ANOVA and *post hoc* tests (Tukey HSD) were performed to determine the difference in the susceptibility among species.

Toxicity against non-target organisms: The safety of the selected WDP formulation to non-target organisms that are commonly found in association with mosquito larvae in aquatic habitats, namely *Ostracods*, *Cyclops*, *Daphnia* sp., *Notonecta* sp., *Diplonychus* sp. and fish, was studied[15]. These organisms were collected from the aquatic environments where the biopesticides are applied and tested in laboratory at the dose of 1.54 mg/l (10 times the LC_{90} value obtained for *An. stephensi* which was the species found to be most tolerant to the formulation) in quadruplicate. The fauna was observed for one week for mortality if any.

Mammalian toxicological studies: The safety of this WDP formulation on mammalian systems was done at the International Institute of Biotechnology and Toxicology (IIBAT), Padappai, Tamil Nadu, India. The tests done were acute oral toxicity/pathogenicity in Wistar rats and acute dermal toxicity/pathogenicity and

primary skin irritation in New Zealand white rabbits, respectively.

Results

Nature and properties of fly ash (FA): The SEM observation of FA sample revealed greater number of hollow glass spheres called cenospheres (Fig. 1), which are hard, rigid, lightweight and inert largely made of silica and alumina. This property prompted us to use FA as carrier in our formulation. The size of the FA particles used in the formulation ranged between 1 and 25 µ. The SEM-EDS analysis of FA revealed the presence of only macro- and micro-nutrients such as Si, Al, Ca, Fe together with Mg, S, Na and Cu. It did not contain any toxic heavy metals such as Pb, Cd, Ni, As, Hg, Se and Cr and radionuclides such as U, Ra and Th. (Fig. 2).

Evaluation of the water dispersible powder (WDP) formulations: Larvicidal efficacy of the various WDP formulations is given in Table II. Among the 16 formulations tested against *Cx. quinquefasciatus*, WDPA2 was found to be the most active, with an LC_{50} value of 0.0419 mg/l and LC_{90} of 0.0753 mg/l. This formulation contained FA and technical grade *Bti* in the ratio 4:5 with one per cent CMC as a binding agent. The ANOVA test showed that the LC_{50} and LC_{90} values among the WDP formulations were significantly different at $P<0.001$. The *post hoc* tests indicated that the activity of WDPA2 formulation was significantly different and required lesser concentration when comparing its LC_{50} and LC_{90} values with that of all the other formulations ($P<0.01$). The formulation WDPAA without binder showed less activity than WDPA2 with the LC_{50} and LC_{90} of 0.0646 mg/l (0.0606-0.0686) and 0.1062 mg/l (0.0999-0.1142), respectively (Table II). The formulation which showed the least activity was WDPB3 with LC_{50} of 0.0830 mg/l (0.0779-0.0883) and LC_{90} of 0.1378 mg/l (0.1287-0.1496).

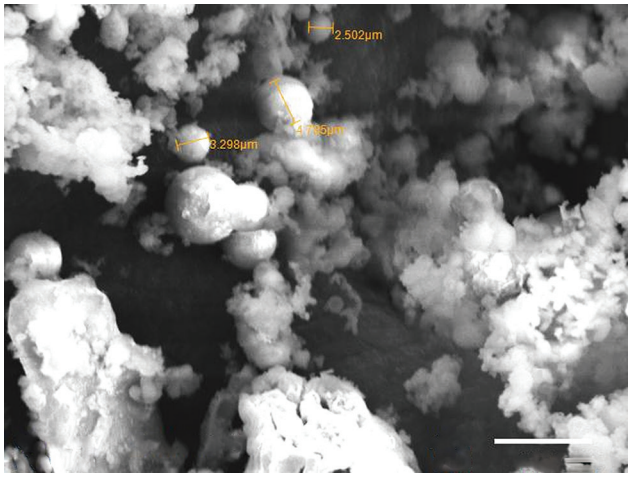

Fig. 1. Scanning electron micrograph of fly ash showing cenospheres in different sizes (×2000).

Susceptibility of different species of mosquito larva: Among the three species of mosquito larvae tested, the LC_{50} and LC_{90} values of the WDPA2 formulation against *Cx. quinquefasciatus, Ae. aegypti* and *An. stephensi* larvae were 0.0417, 0.0462 and 0.1091 mg/l and 0.0755, 0.0928 and 0.1541 mg/l, respectively (Table III). The ANOVA test showed that the LC_{50} and LC_{90} values among the mosquito larvae of different species were significantly different at $P<0.05$. The *post hoc* tests indicated that the LC_{50} and LC_{90} values were significantly lower at $P<0.06$ and $P<0.05$ for *Cx. quinquefasciatus* compared to that of *Ae. aegypti* and *An. stephensi* ($P<0.05$), respectively. The test further indicated that the LC_{50} and LC_{90} values for *Ae. aegypti* larvae were significantly ($P<0.05$) lower than that for *An. stephensi.*

Effect of ingredients (individual/combined) of the WDPA2 formulation on mosquito-larvicidal activity: Among the components tested, the *Bti* with FA+CMC

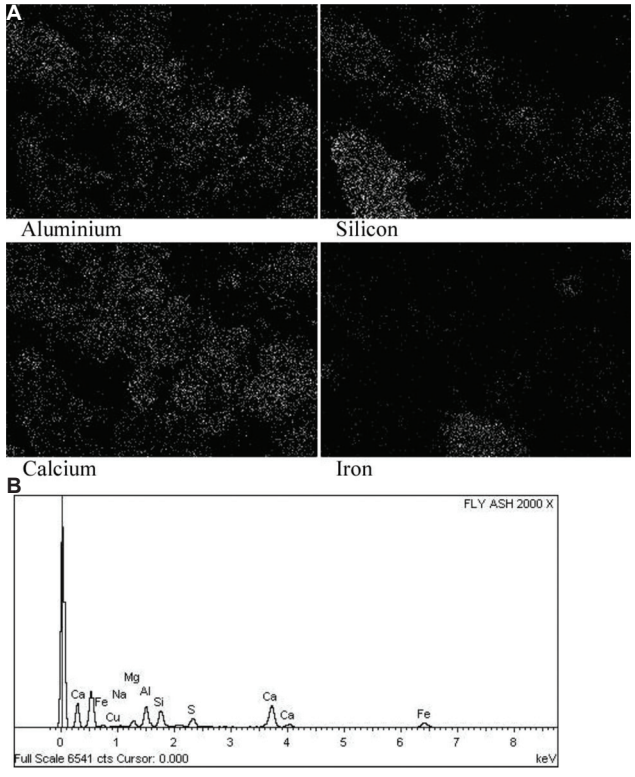

Fig. 2. (**A**) Energy dispersive X-ray spectroscopy image showing the major elements in the fly ash. (**B**) Scanning Electron Micrograph (Energy dispersive X-ray spectroscopy) showing the peaks of major and minor elements and its actual proportion in fly ash.

Table II. Toxicity of fly ash-based water dispersible powder formulations against late third instar *Culex quinquefasciatus* larvae

Formulation	Mean toxicity* (mg/l)		χ^{2+}	$P^{\#}$
	LC_{50} (95% FL)	LC_{90} (95% FL)		
WDPAA	$0.0646^{c,d,e}$ (0.0606-0.0686)	$0.1062^{b,c,d}$ (0.0999-0.1142)	37.118	1.000
WDPA1	$0.0574^{b,c}$ (0.0537-0.0612)	0.0960^{b} (0.0900-0.1035)	50.776	0.960
WDPA2	0.0419^{a} (0.0384-0.0453)	0.0753^{a} (0.0705-0.0812)	80.275	0.188
WDPA3	$0.0656^{c,d,e}$ (0.0617-0.0698)	$0.1069^{b,c,d}$ (0.1000-0.1157)	36.541	1.000
WDPB1	$0.0753^{f,g}$ (0.0704-0.0804)	$0.1286^{e,f,g}$ (0.1202-0.1393)	62.013	0.741
WDPB2	$0.0658^{c,d,e}$ (0.0590-0.0724)	$0.1212^{d,e,f,g}$ (0.1110-0.1354)	115.463	0.001
WDPB3	0.0830^{g} (0.0779-0.0883)	0.1378^{g} (0.1287-0.1496)	54.563	0.913
WDPC1	$0.0649^{c,d,e}$ (0.0604-0.0695)	$0.1142^{c,d,e}$ (0.1064-0.1242)	56.872	0.871
WDPC2	0.0550^{b} (0.0501-0.0598)	$0.1021^{b,e}$ (0.0948-0.1116)	82.237	0.150
WDPC3	$0.0706^{e,f}$ (0.0663-0.0751)	$0.1173^{c,d,e,f}$ (0.1097-0.1271)	54.822	0.909
WDPD1	$0.0699^{d,e,f}$ (0.0652-0.0748)	$0.1208^{d,e,f,g}$ (0.1125-0.1316)	51.746	0.950
WDPD2	$0.0614^{b,c,d}$ (0.0569-0.0658)	$0.1082^{b,c,d}$ (0.1012-0.1170)	68.431	0.531
WDPD3	$0.0660^{c,d,e}$ (0.0614-0.0708)	$0.1168^{c,d,e,f}$ (0.1087-0.1270)	70.633	0.456
WDPE1	$0.0769^{f,g}$ (0.0716-0.0823)	$0.1340^{f,g}$ (0.1249-0.1459)	75.407	0.308
WDPE2	$0.0756^{f,g}$ (0.0702-0.0811)	$0.1224^{d,e,f,g}$ (0.1139-0.1337)	107.956	0.002
WDPE3	$0.0781^{f,g}$ (0.0729-0.0835)	$0.1287^{e,f,g}$ (0.1199-0.1402)	86.559	0.087

*Means within a given column followed by the same alphabet letter are not significantly different, Tukey's multiple range test (α=0.05), $P<0.001$; +Pearson χ^2 goodness of fit test; #If the significance level is <0.150, a heterogeneity factor is used in the calculation of confidence limits. LC_{50}, lethal concentration that kills 50% of the exposed larvae; LC_{90}, lethal concentration that kills 90% of the exposed larvae; 95% FL, 95% upper and lower fiducial limits; WDP, water dispersible powder formulation

Table III. Susceptibility of III instar larvae of different mosquito species to water dispersible powder A2 formulation

Mosquito species	Toxicity* (mg/l)		χ^{2+}	$P^{\#}$
	LC_{50} (95% FL)	LC_{90} (95% FL)		
Culex quinquefasciatus	0.0417^{a} (0.0382-0.0451)	0.0755^{a} (0.0706-0.0814)	78.009	0.239
Aedes aegypti	0.0462^{a} (0.0412-0.0510)	0.0928^{b} (0.0852-0.1029)	98.400	0.014
Anopheles stephensi	0.1091^{b} (0.1012-0.1162)	0.1541^{c} (0.1430-0.1727)	166.083	0.001

*Means within a given column followed by the same alphabet letter are not significantly different, Tukey's multiple range test (α = 0.05); +Pearson Chi-square goodness of fit test; # If the significance level is less than 0.150, a heterogeneity factor is used in the calculation of confidence limits; LC_{50}, lethal concentration that kills 50% of the exposed larvae; LC_{90}, lethal concentration that kills 90% of the exposed larvae; 95% FL, 95% upper and lower fiducial limits

resulted in mortality rate of 51.7 per cent. The percentage mortality due to FA and CMC was only 1.3 and 2.3 per cent, which was well below the allowed mortality levels in control experiments. The ANOVA results showed that there was significant difference among percentage mortality due to ingredients ($P<0.001$). The *post hoc* tests further indicated that the percentage mortality in *Bti*+FA+CMC (WDPA2) was significantly different from FA and CMC tested alone ($P<0.001$). However, the mortality in FA alone did not differ significantly from CMC. Hence, FA and CMC as carrier and additive did not have any significant effect on the larval population and the larvicidal activity of the WDPA2 formulation was only due to the presence of *Bti*.

Tests against non-target organisms and mammalian systems: When tested at 10 times the concentration of WDPA2 formulation used for obtaining 90 per cent kill in the least susceptible mosquito larvae of species *An. stephensi*, the WDPA2 formulation was found to be safe to Crustaceans, namely *Ostrocods*, *Cyclops* and *Daphnia,* insects, namely *Notonectids* and *Diplonychus*, and fish, namely *Poecilia* (Table IV).

Table IV. Toxicity of water dispersible powder A2 formulation to non-target organisms

Organisms	Laboratory environment						Percentage mortality after one week
	Control			Treated*			
	R_1	R_2	R_3	R_1	R_2	R_3	
Ostracoda	10	10	10	10	10	10	0
Cyclops	15	15	15	15	15	15	0
Daphnia spp.	10	10	10	10	10	10	0
Notonecta spp.	5	5	5	5	5	5	0
Diplonychus spp.+		10			10		0
Poecilia spp.	10	10	10	10	10	10	0

*10 times (1.54 mg/l) the concentration of LC_{90} value of *Anopheles stephensi* larvae; +As there was cannibalism among *Diplonychus* spp., safety testing was done having one individual in each testing cup. Ten replicates each were kept for control and treatment. R, replicate; LC_{90}, lethal concentration that kills 90% of the exposed larvae

Acute oral toxicity conducted on rats and acute dermal toxicity and primary skin irritation tests conducted on rabbits showed that the WDPA2 formulation was non-toxic and non-irritant on mammalian systems.

Discussion

The trend to use biological control agents in mosquito control programmes has gained widespread importance in recent years due to detrimental effects of chemical insecticides on the environment and human health. One of our indigenously isolated mosquito-larvicidal agents *Bti* (VCRC B17) was found to be highly effective against larvae of different mosquito species in various aquatic habitats[16,17]. Mosquito larvae (especially late instars) being filter feeders are reported to selectively feed on particles of colloidal to 50 µ in size[18-20]. Hence, incorporation of FA as a carrier material has enhanced the chances of ingestion of this formulation by the mosquito larvae. The SEM analysis of FA used for making WDP formulations contains only macro- and micro-nutrients which is beneficial when applied in freshwater bodies such as paddy fields. Dutta *et al*[21] revealed that the leaching of toxic elements/heavy metals from FA was negligible when the *p*H of the water body was alkaline or nearly neutral and mosquito larval-breeding habitats are always reported to be alkaline in nature.

This study showed that the LC_{50} values were lower for *Culicines* than for *Anophelines*. The susceptibility pattern of the larval stages of the three vector species to this biopesticide was of the following order: *Cx. quinquefasciatus*<*Ae. aegypti*<*An. stephensi*. This was in agreement with many other reports with this bacterial species[22,23]. The relative lower efficacy of *Bti* formulation against *Anopheles* species might be due to their reduced filtration rates as has been reported by Aly *et al*[24]. Further, this variation in the susceptibility has been attributed to variation in the gut *p*H of these insect species which is known to play a major role in the activation of the endotoxins of this bacterium[25]. Differences in the feeding behaviour of these three species are also known to be responsible for this varied susceptibility[26].

The formulation was found to be safe to non-target organisms found in association with mosquito larvae in the aquatic habitats. This observation reinforces the results of several earlier studies conducted with *Bti*[15,27,28]. This study showed that the carrier material and additive, namely FA and CMC used in this formulation, as well as the active ingredient, *Bti*, were safe on mammalian systems. Hence, FA can be effectively used to replace commercially available carrier materials in the preparation of biopesticidal formulations. FA has earlier been successfully used as such or as carriers for the production of biofertilizers and biopesticides for agriculture[7,29]. Furthermore, powder formulations are reputed for their long shelf life, miscibility in water compared to technical grade materials, microgels and aqueous suspensions[30].

In conclusion, with biopesticides gaining wide importance in recent times in the wake of maintaining a safe environment, use of FA will help not only in adding to its utilization as almost half of the formulated material contains FA but also in ensuring safety to the environment where it is applied as it has proved to be safe to non-target organisms and mammalian systems.

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***Conflicts of Interest*:** None.

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