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# LOCAL PRODUCTION OF PRIMARY POWDER OF *BACILLUS THURINGIENSIS* SEROTYPE H-14 IN IRAN AND DETERMINATION OF ITS INSECTICIDAL PROPERTIES AGAINST *CULEX PIPIENS* AND *ANOPHELES STEPHENSI*

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**ABSTRACT**

Three media formulated from molasses and cornsteep liquor and another media based on the basal medium formulation of IPS-82 were assessed for the growth and production of insecticidal properties of *B.thuringiensis* H-14. Bacterial powders prepared from the broth cultures were assayed against the larvae of *Culex pipiens* and *Anopheles stephensi*. A standard powder of IPS-82 was included in the assay for comparison. Good growth was obtained in all the media and all powders were effective against the two types of mosquito larvae. Media with the basal medium formulation of Institute Pasteur Paris were the most effective. Molasses III media was also very favorable. The concentrations required to kill 50% of the larvae of *Culex pipiens* and *Anopheles stephensi* for these two were as follows:

- Media with the basal medium formulation of Institute Pasteur Paris:
24hr. Lc50 and it's 95% confidence limits of *Culex pipiens* and *Anopheles stephensi* = 0.0025,0.0031,0.0018 mg/lit
0.1792,0.2603,0.1508 mg/lit
- Molasses III Media:
24hr. Lc50 and it's 95% confidence limits of *Culex pipiens* and *Anopheles stephensi* = 0.0103,0.0115,0.0092 mg/lit
0.1994,0.2223,0.1769 mg/lit

The investigation shows that these media can be used for the production of *B.thuringiensis* H-14 primary powder.

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## INTRODUCTION

Malaria still takes a heavy toll on human life and suffering. Soon after the second world war the WHO recognised that malaria not only killed more people than any other disease but also interfered with the development of agriculture and industry, especially in the new independent countries. However, during the past decade there has been a considerable increase of malaria in several tropical areas.[1] Malaria is still prevalent in the south eastern parts of Iran. In recent years, chloroquine-resistant strains of *Plasmodium falcipar-*



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*um* in south east Asia have countinued to spread westward and have been officially reported up to the southern border of Iran.[2] Some of the resistant cases in Iran were observed among Afghan immigrants or Pakistani individuals or Iranian Baluch tribes.[3] Establishment of the resistant strain in the south eastern parts of Iran can play an important role in spreading the infection between the uninfected vectors.

Mosquito vectors of diseases such as malaria are increasingly difficult to control, due in particular to the development of resistance to chemical insecticides.[4] It has become increasingly evident that control of disease vectors cant not be based solely on chemical insecticides in view of the problem presented by the insects' resistance to insecticides, rising cost which limits adequate application in many countries, polluting the environment and destroying both harmful and harmless insects.[5,6] As mentioned, resistance to antimalarial drugs and the resistance of vectors to chemical insecticides are the important problems which indicate the importance of vector control.

Because of the above said reasons, scientists turned their attention to biological control mechanisms. Today biological control is regarded as a desirable technique for controlling insects, due to its minimal environmental impact and preventing the development of resistance in vectors. The entomopathogenic microorganism *Bacillus thuringiensis* is of the most promising biological control agent of pest and insect management since many strains are toxic specifically for lepidopterans and strain *Bacillus thuringiensis* serotype H-14 is highly toxic to dipterans. The first data on the existance and activity of the new strain *B. thuringiensis* H-14, appeared in 1977.[7]

*B.thuringiensis* H-14 is a spore-forming bacterium that produces one or more crystals of toxic protein (delta endotoxin) with each spore. The entomocidal properties of this protein crystal of *B.thuringiensis* H-14 have been shown to hold great promise as highly selective for mosquito and simulium vectors of major parasitic diseases such as malaria and filariasis.[6] In many countries in which disease borne by mosquitoes are endemic, local production of *B. thuringiensis* H-14 has an economic advantage.[4] *B. thuringiensis* H-14 is highly host specific and practically harmless to non-target fauna, including the natural enemies of vectors which survive and augment its impacts. *B.thuringiensis* H-14 is also very safe to man and other vertebrates.[5]

In this experiment therefore, three media with different formulations based predominantly on locally available substrates in Iran such as molasses and corn-steep liquor were assessed as media for the local production of primary powders of *B.thuringiensis* H-14. Another media based on the basal medium formulation of Institute Pasteur Paris was used for the production of a standard to be quite similar up to 90% to IPS-82 (International Pasteur Standard-82). These productions are then bioassayed against the larvae of *Culex pipiens* (one of the filariasis vectors) and *Anopheles stephensi* (malaria vector). This project is still continuing and the next results will be published in the future.

## MATERIALS AND METHODS

*Source of organism* Three flasks of standard powder IPS-82 were kindly granted by Professor H.DeBarjac, Institute Pasteur Paris. *B.thuringiensis* H-14 was isolated from this standard powder and was grown on brain heart infusion agar plate at 37°C for 24hr and stored at 4°C until used.

*Media* Three media designated Molasses I,II,III[8] and a medium based on basal medium formulation of IPS-82 were used for growth of *B.thuringiensis* H-14 in shake flask cultures. The pH of each medium was adjusted to 7-7.5 with %20 NaOH. Media were dispensed into 500ml Erlenmeyer flasks and sterilized at 121°C for 20 min (Table I).

*Seed Culture* Two loopfulls of *B.thuringiensis* H-14 from the BHIA plate was used to inoculate 50ml of TSB (tripticase soy broth) in 500ml flasks. TSB was used as a seed culture which was placed on the shaker (IRC-IU-Adult Kuhner AG, Co. Ltd.)

*Production flasks* Production flasks (500ml) containing 100ml of medium Molasses I, Molasses II, Molasses III each were inoculated with 100ml of the seed culture and incubated until 72 to 90% sporulation was obtained (after 72hr). Cultures were examined microscopically for sporulation at 24hr. intervals by staining spores with Schaeffer and Fulto's Method. Production flasks (500ml) containing 130ml of medium similar to standard 82 were inoculated with a full grown agar of the same agar media and was treated as Molasses media.

*Recovery* Four production media were recovered as bacterial primary powders at the end of fermentation by the freeze-drying technique (Lyovac GT3, Leyboldhareus Ltd.) and were examined with electron microscope (E.M.10c, Zeiss) for the detection of crystals.

*Total viable cell count and spore counts* Total viable cell and spore counts were determined in the final whole culture by the pour plate method. Serial decimal dilutions of the final whole cultures were made in sterile %1 pepton water (oxoid) and 0.5ml of each dilution in triplicate was added to a petridish, followed by the addition of 10ml of plate count agar (oxoid) at 45°C. The culture and agar were mixed thoroughly and allowed to set. Plates were incubated at 32±2°C for 24 to 48hr, then counted with a colony counter. For spore



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**Table I. Formulation of three molasses media[a] and media with the basal medium of Institute Pasteur Paris.**

| Medium | Formulation |
|---|---|
| Molasses I | molasses: $1^{ml}$<br>cornsteep: $10^{ml}$<br>$PO_4H_2K$: $2^{gr}$<br>made up to $100^{ml}$ with D.W. |
| Molasses II | molasses: $0.5^{ml}$<br>cornsteep: $5^{ml}$<br>$PO_4H_2K$: $2^{gr}$<br>made up to $100^{ml}$ with D.W. |
| Molasses III | molasses: $0.5^{ml}$<br>$SO_4Mg.\ 7H_2O$: $1.23^{gr}$<br>$SO_4Mn.\ 1H_2O$: $0.016^{gr}$<br>$SO_4Zn.\ 7H_2O$: $0.14^{gr}$<br>$Cl_2Ca.\ 2H_2O$: $1.47^{gr}$<br>$PO_4H_2K$: $3.4^{gr}$<br>Bactopepton: $0.7^{gr}$<br>made up to $100^{ml}$ with D.W. |
| Basal medium of Institute Pasteur Paris<br>Solution 1 | $PO_4H_2K$: $68^{gr}$<br>made up to $1000^{ml}$ with D.W. |
| Solution 2 | $SO_4Mg.\ 7H_2O$: $12.3^{gr}$<br>$SO_4Mn.\ 4H_2O$: $0.223^{gr}$<br>$SO_4Zn.\ 7H_2O$: $1.4^{gr}$<br>made up to $1000^{ml}$ with D.W. |
| Solution 3 | $(SO_4)_3Fe_2$: $2^{gr}$<br>$SO_4H_2N$: $100^{ml}$<br>made up to $1000^{ml}$ with D.W. |
| Solution 4 | $Cl_2Ca.\ 4H_2O$: $18.3^{gr}$ with D.W.<br>made up tp $1000^{ml}$ with D.W. |
| Preparation of the Basal medium | 100ml of Solution 1 + 10 ml of Solution 2,3,4<br>7.5gr Bactopepton<br>%30 glucose |

(a) 100ml evaporated cornsteep was equal to 8.5gr solid material. Total molasses carbohyrates were measured by «Somogy Micro Copper-Method» and was equal to %47.

**Table II. Degree of sporulation, pH, total viable cell count and spore count in final whole cultures of three Molasses media[a] & media [b] with the basal medium formulation of Institute Pasteur Paris.**

| Medium | % Sporulation | pH | T.V.C.C.[c] /ml | S.C.[d] /ml |
|---|---|---|---|---|
| Molasses I | 88-91 | 8-8.5 | $1.5\times10^9$ | $1.3\times10^9$ |
| Molasses II | 72-80 | 7-7.5 | $1\times10^9$ | $7.5\times10^8$ |
| Molasses III | 92-95 | 7-7.5 | $10\times10^9$ | $9.3\times10^9$ |
| media with the basal medium formulation of Institute Pasteur Paris | 97-100 | 7-7.5 | $36\times10^{15}$ | $35.3\times10^{15}$ |

a = after 72hr.
b = after 48hr.
c = T.V.C.C. (total viable cell count)
d = S.C. (spore count)

counts, cultures were pasteurized at 65°C for 20min before serial dilutions were made (Table II).

*Biossay of B.thuringiensis H-14 primary powders against mosquito larvae* Primary powders of *B.thuringiensis* H-14 produced from the four media were assayed against laboratory-rared third instar larvae of *Culex pipiens* and *Anopheles stephensi*. A standard powder of IPS-82 was included in the assay for comparison. The bioassay principle technique is exactly based on the bioassay method for the titration of *B.thuringiensis* H-14 preparations with IPS-82 standard. Plastic cups of $200^{cc}$ were prepared with 150ml free chlorine water with 25 $L_3$ larvae of *Culex pipiens* or *Anopheles stephensi* in each cup. 50mg of each powder was suspended in distilled water and exact dilution of the suspension



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**Table III. Larvicidal activities of B.thuringiensis H-14 powders produced from local materials and basal medium formulation of Pasteur Institute standard in contrast with IPS-82 against Culex pipiens larvae after 24hr.**

| Powder from medium and conc. mg/lit | Average % mortality at 24hr. | $L_c50$ mg/lit | 95% confidence limit | | $X^{2(a)}$ |
|---|---|---|---|---|---|
| | | | upper | lower | |
| Molasses I | | | | | |
| 0.001 | 0 | 0.195 | 0.0215 | 0.0179 | 4.090 |
| 0.003 | 1 | | | | |
| 0.005 | 5 | | | | |
| 0.008 | 10.7 | | | | |
| 0.01 | 15.7 | | | | |
| 0.02 | 57.3 | | | | |
| 0.03 | 73 | | | | |
| 0.04 | 80.3 | | | | |
| Molasses II | | | | | |
| 0.001 | 0 | 0.0171 | 0.0188 | 0.0155 | 9.641 |
| 0.003 | 2.7 | | | | |
| 0.005 | 13 | | | | |
| 0.008 | 16 | | | | |
| 0.01 | 19 | | | | |
| 0.02 | 65.7 | | | | |
| 0.03 | 74 | | | | |
| 0.04 | 83.7 | | | | |
| Molasses III | | | | | |
| 0.001 | 0 | 0.0103 | 0.0115 | 0.0092 | 27.611* |
| 0.003 | 5 | | | | |
| 0.005 | 23 | | | | |
| 0.008 | 47.3 | | | | |
| 0.01 | 66.3 | | | | |
| 0.02 | 75 | | | | |
| 0.03 | 79.3 | | | | |
| 0.04 | 84.3 | | | | |
| Media with the basal medium formulation of Institute Pasteur Paris | | | | | |
| 0.001 | 37 | 0.0025 | 0.0031 | 0.0018 | 3.524 |
| 0.003 | 48 | | | | |
| 0.005 | 61.3 | | | | |
| 0.008 | 72.7 | | | | |
| 0.01 | 79.7 | | | | |
| 0.02 | 86.3 | | | | |
| 0.03 | 91 | | | | |
| 0.04 | 92 | | | | |
| IPS-82 | | | | | |
| 0.001 | 41 | 0.0017 | 0.0021 | 0.0013 | 22.561 |
| 0.003 | 61.7 | | | | |
| 0.005 | 74.7 | | | | |
| 0.008 | 81 | | | | |
| 0.01 | 98.3 | | | | |
| 0.02 | 99.7 | | | | |
| 0.03 | 100 | | | | |
| 0.04 | 100 | | | | |

(a): df = 6
* critical value = 12.6

were added to the cups in order to obtain final concentrations of 0.04, 0.03, 0.02, 0.01, 0.008, 0.003, 0.001 mg/lit powders for *Culex pipiens* and concentrations of 0.4, 0.3, 0.2, 0.1 mg/lit of powders for *Anopheles stephensi* tests. 4 cups were used per dilution. Controls consisted of 4 cups each containing 150ml D.W. and 25 larvae for each powder assayed. No food was added. Each experiment was incubated at 25±2°C and each assay was repeated three times. Mortality counts were made at 24 and 48hr.



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**Table VI. Larvicidal activities of B.thuringiensis H-14 powders preduced from local materials and basal medium formulation of PasteurInstitute standard in contrast with IPS-82 against Anopheles stephensi larvae after 48hr.**

| powder from medium and conc. mg/lit | Average % mortality at 24hr | $L_c50$ mg/lit | 95% confidence limit | | $X^{2(a)}$ |
|---|---|---|---|---|---|
| | | | upper | lower | |
| Molasses I | | | | | |
| 0.001 | 0 | 0.0105 | 0.0114 | 0.0097 | 4.533 |
| 0.003 | 2 | | | | |
| 0,005 | 19 | | | | |
| 0.008 | 33.3 | | | | |
| 0.01 | 41.3 | | | | |
| 0.02 | 85 | | | | |
| 0.03 | 94.3 | | | | |
| 0.04 | 98.7 | | | | |
| Molasses II | | | | | |
| 0.001 | 0 | 0.0091 | 0.0099 | 0.0083 | 3.146 |
| 0.003 | 4 | | | | |
| 0.005 | 23 | | | | |
| 0.008 | 44.3 | | | | |
| 0.01 | 56.7 | | | | |
| 0.02 | 86.7 | | | | |
| 0.03 | 93 | | | | |
| 0.04 | 99 | | | | |
| Molasses III | | | | | |
| 0.001 | 0 | 0.0056 | 0.0061 | 0.0051 | 38.726* |
| 0.003 | 6.7 | | | | |
| 0.005 | 56.3 | | | | |
| 0.008 | 78 | | | | |
| 0.01 | 86 | | | | |
| 0.02 | 95.3 | | | | |
| 0.03 | 97.7 | | | | |
| 0.04 | 99 | | | | |
| Media with the basal medium formulaton of Institute Pasteur Paris | | | | | |
| 0.001 | 40.3 | 0.0020 | 0.0025 | 0.0016 | 14.077 |
| 0.003 | 50.7 | | | | |
| 0.005 | 67.7 | | | | |
| 0.008 | 76 | | | | |
| 0.01 | 91.7 | | | | |
| 0.02 | 95 | | | | |
| 0.03 | 96.3 | | | | |
| 0.04 | 97 | | | | |
| IPS-82 | | | | | |
| 0.001 | 51.7 | 0.0012 | 0.0015 | 0.0008 | 23.537 |
| 0.003 | 70.3 | | | | |
| 0.005 | 78.7 | | | | |
| 0.008 | 85 | | | | |
| 0.01 | 99.7 | | | | |
| 0.02 | 100 | | | | |
| 0.03 | 100 | | | | |
| 0.04 | 100 | | | | |

(a): df = 6
* critical value = 12.6

*Calculation of results* The average percentage of mortality at 24 and 48hr for each dilution of each powder was converted to probits with the probit transformation table and the regression line relating probits to log dose was calculated with a desk-calculator (Sx-30 cannon canula) to obtain $L_c50$ results and its confidence limits. Mortality among control larvae was very rare and not more than 4% (Table III,IV,V,VI).



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**Table V. Larvicidal activities of B.thuringiensis H-14 powders produced from local materials and basal medium formulation of Pasteur Institute standard in contrast with IPS-82 against Anopheles stephensi larvae after 24hr.**

| Powder from medium and conc. mg/lit | Average % mortality at 24hr | Lc50 mg/lit | %95 confidence limit upper | %95 confidence limit lower | $X^{2(a)}$ |
|---|---|---|---|---|---|
| Molasses I | | | | | |
| 0.1 | 0 | 0.5637 | 0.8712 | 0.4659 | 1.775 |
| 0.2 | 5.6 | | | | |
| 0.3 | 11.3 | | | | |
| 0.4 | 30.6 | | | | |
| Molasses II | | | | | |
| 0.1 | 1.3 | 1.834 | 62.37 | 1.1721 | 0.528 |
| 0.2 | 2.3 | | | | |
| 0.3 | 6.3 | | | | |
| 0.4 | 10.3 | | | | |
| Molasses III | | | | | |
| 0.1 | 20.6 | 0.1994 | 0.2223 | 0.1769 | 1.369 |
| 0.2 | 51 | | | | |
| 0.3 | 65.3 | | | | |
| 0.4 | 83.3 | | | | |
| Media with the basal medium formulation of Institute Pasteur Paris | | | | | |
| 0.1 | 33.3 | 0.1792 | 0.2063 | 0.1508 | 3.361 |
| 0.5 | 48 | | | | |
| 0.3 | 66.6 | | | | |
| 0.4 | 81.3 | | | | |
| IPS-82[b] | | | | | |
| 0.001 | 45 | 0.0016 | 0.0019 | 0.0012 | 24.128 |
| 0.003 | 52 | | | | |
| 0.005 | 79 | | | | |
| 0.008 | 95 | | | | |
| 0.01 | 97 | | | | |
| 0.02 | 98 | | | | |
| 0.03 | 99 | | | | |
| 0.04 | 100 | | | | |

(a): df = 2
critical value = 6
(b): df = 6
* critical value = 12.6

## RESULTS AND DISCUSSION

*B.thuringiensis* H-14 produced an appreciable amount of spores and delta endotoxin crystals in the four media between 48 and 72hr of fermentations. The degree of sporulation ranged from 72% in medium Molasses II to 95% in medium Molasses III. The T.V.C.C. were varied from $1\times10^9$ in Molasses II media to $10\times10^9$ in Molasses III media, and T.S.C. varied from $7.5\times10^8$ in Molasses II to $9.3\times10^9$ in Molasses III media. T.V.C.C. of 48hr medium with the basal medium formulation of Institute Pasteur was $36\times10^{15}$/ml with the degree of 97-100% sporulation after 48hr. Produced powders of *B.thuringiensis* H-14 were more effective against *Culex pipiens* than *Anopheles stephensi*; similar observations have been reported by various workers.[9-12] The bioassay results at 24 and 48hr are summarized in Tables III for the two mosquito species. Maximum numbers of dead mosquito larvae were recorded in all *B.thuringiensis* H-14 powders at 48hr. Increase in larvae death after this period was very small. The insecticidal activities of the *B. thuringiensis* H-14 powders produced from local media did not correlate to the degree of sporulation observed in the final whole culture, and this has also been previously reported.[13]

The most effective locally produced *B.thuringiensis* H-14 primary powder was from medium Molassess III with the $Lc^{50}$ of 0.0103 and 0.0056 mg/lit after 24 and 48hr for *Culex pipiens* and 0.1994 and 0.1939 mg/lit for



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**Table VI. Larvicidal activities of *B.thuringiensis* H-14 powders produced from local materials and basal medium formulation of Pasteur Institute standard in contrast with IPS-82 against *Anopheles stephensi* larvae after 48hr.**

| Powder from medium and concn. mg/lit | Average %mortality at 24hr | Lc50 mg/lit | 95% confidence limit | | $X^{2(a)}$ |
|---|---|---|---|---|---|
| | | | upper | lower | |
| Molasses I | | | | | |
| 0.1 | 4 | 0.3474 | 0.4419 | 0.3327 | 0.457 |
| 0.2 | 20.6 | | | | |
| 0.3 | 36 | | | | |
| 0.4 | 55.6 | | | | |
| Molasses II | | | | | |
| 0.1 | 2 | 0.6301 | 0.9917 | 0.4968 | 0.667 |
| 0.2 | 7 | | | | |
| 0.3 | 19.6 | | | | |
| 0.4 | 30 | | | | |
| Molasses III | | | | | |
| 0.1 | 23.3 | 0.1754 | 0.1939 | 0.1561 | 1.607 |
| 0.2 | 56.3 | | | | |
| 0.3 | 74.3 | | | | |
| 0.4 | 90 | | | | |
| Media with the basal medium formulation of Institute Pasteur Paris | | | | | |
| 0.1 | 34.6 | 0.1561 | 0.1790 | 0.1306 | 1.420 |
| 0.2 | 58 | | | | |
| 0.3 | 72.3 | | | | |
| 0.4 | 86.3 | | | | |
| IPS-82 | | | | | |
| 0.001 | 45 | 0.0014 | 0.0018 | 0.009 | 6.816 |
| 0.003 | 58 | | | | |
| 0.005 | 84 | | | | |
| 0.008 | 100 | | | | |
| 0.01 | 100 | | | | |
| 0.02 | 100 | | | | |
| 0.03 | 100 | | | | |
| 0.04 | 100 | | | | |

(a): df = 2
critical value = 6

*Anopheles stephensi*. Primary powder of medium with the basal formulation of Institute Pasteur can be compared favorably with IPS-82 ($Lc^{50}$ of 24 and 48hr IPS-82 for Culex and Anopheles were 0.0017, 0.0012, 0.0016, 0.0014 and $Lc^{50}$ of 24 and 48hr primary powder of medium with basal formulation of Institute Pasteur were 0.0025, 0.0020, 0.1792, 0.1561 mg/lit).

Although the three molasses media are different only in amount of molasses and cornsteep, the extent to which they encouraged production of delta endotoxin crystal and its effectiveness against the two types of larvae was marked and quite favorable. This experiment shows that local or byproduct materials such as molasses and cornsteep in Iran or other countries can be used to produce *B.thuringiensis* H-14 powders with good activities aganist important mosquito species, especially vectors of malaria in endemic areas.

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