The developing role of microbiological agents in vector control[1]

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Introduction

The use of microbial pathogens and parasites of insect pests is well established in agriculture and forestry but is less well developed for vectors of human diseases. There are both economic and biological reasons for this disparity in development: far more money is invested into development of control methods for agricultural pests than the vectors of public health importance, especially in the developed countries; the tendency in agriculture toward monoculture is more suited for the augmentation of locally occurring pathogens and parasites of pest insects than it is against disease vector species in more heterogeneous environments; and most insect pathogens infect larval stages which are the primary agricultural pests whereas most medically important insects are vectors in the adult stage[2].

However, now that the use of conventional chemical pesticides in vector control is troubled by increased costs, resistance to established compounds, and concern with environmental pollution and effects on non-target species, more serious attention has been given to the use of biological agents in integrated vector control programmes. The purpose of this brief paper will be to describe some of the microbial agents and parasites that may play a role in the control of anopheline mosquitos in future malaria control programmes and to indicate the status of their development in the WHO testing scheme which has been recently developed[3]. This example has been chosen as malaria remains the most important global arthropod-borne disease.

The WHO scheme for screening and evaluating the efficacy and safety of biological agents for control of disease vectors

This scheme (Annex) was endorsed by the 21st WHO Expert Committee on Insecticides (WHO Tech. Rep. Ser. No. 561, 1975). It indicates the need for sequential testing of potential biological control agents in specialized laboratories to assure efficacy against target species and safety to man and non-target species in the general environment, and was developed when the Organization recognized that the current research

[1] Presented at the Annual Meeting of the Swiss Society for Microbiology in Geneva, June 1976.

[2] A. ARATA, Proc. 62nd Ann. Meeting N. J. Mosq. Exterm. Ass., Trenton (1976).

Annex. Preliminary scheme for screening and evaluating the efficacy and safety of biological agents for control of disease vectors

Stage I	Stage II		Stage III	Stage IV		Stage V
Laboratory	*Laboratory*		*Preliminary field trials*	*Laboratory*		*Large scale field trials*
A. Identification and characterization[a]	A. Mammalian infectivity tests to ensure safety to laboratory and field personnel[b]	Results of Review of stages I and II	Strictly regulated ponds tests under WHO supervision[c] to determine efficacy against disease vectors under natural conditions	More detailed tests on mammalian infectivity, using appropriate techniques	Review of stages I, II, III and IV by informal consultation group	To be conducted under WHO auspices. Not presently defined, and will vary according to target vector, habitats(s), mode of application, etc.
B. Assessment against selected target vectors				*Laboratory and field trials*		
C. Preliminary evaluation of ease of rearing in quantity	B. Preliminary assessment against certain nontarget species			Detailed studies on non-target range – especially other fauna in habitats where stage V trials may be conducted		
				Formulation		
				Studies on stability of suitable formulations and delivery systems		

[a] This study may vary from routine taxonomic determination (fish, nematodes, predatory insects) to the detailed serotyping necessary for microorganisms, especially viruses. [b] Not required in predator-prey situations, but more detailed tests on effects on non-target organisms could be substituted in trials of larvivorous fish, predatory insects, etc. [c] Especially where the biological control agent is not indigenous.

and development activities being conducted in various laboratories and institutes were not consistent and the results obtained often were not comparable. At present the scheme calls for the following stages of testing[2,3]:

I. Following isolation, an insect microbial pathogen or parasite must be properly identified by techniques appropriate to the taxon (i.e. bacteria, fungi, viruses, etc.); it must demonstrate efficacy against vector species in laboratory bioassay tests; and it must show promise for mass propagation[4,5].

II. The candidate agent will have to be tested for potential mammalian infectivity in laboratory animal tests appropriate to the nature of the pathogen group to assess the possible hazard to man of its use as a vector control agent. In addition, laboratory tests to determine the effect of the candidate agent on non-target species in the general environment (e.g. fish, crustaceans, and aquatic insect groups) must be conducted[6].

III. Candidate microbial and parasitic agents that have satisfactorily completed Stages I and II would be available for testing against vector species under field conditions. This would include initial laboratory bioassay tests against specific vector species and important non-target species at WHO field research units or Collaborating Centres, and if successful, limited field trials in well-controlled plot tests under natural conditions.

IV. Agents which are effective against target vectors in the previous trial will be considered as candidates for large-scale fields trials. However, before such trials are conducted it is necessary, depending on their nature (e.g. bacterial or viral), to conduct more detailed tests for safety to man and non-target organisms in the environment where their use is proposed. Such tests would be extensions of those carried out in Stage II and may require studies using mammalian cell lines and/or immunosuppressed laboratory animals. This stage (IV) will also allow time for development of formulations, testing stability of the product and appropriate means of dispersal.

V. Large scale field trials – to be designed according to the proposed application of the bio-insecticide (marsh, stream, rice fields, etc.).

In order to carry out these recommended testing stages, the Organization has strengthened during 1975 and 1976 the network of Collaborating Centres on Biological Control. When completed, these will include centres for:

a) Diagnosis of insect diseases – to identify insect pathogens received from around the world using the WHO insect pathogen kit which is freely distributed to scientists who wish to collaborate in the programme. The Centre has a large number of collaborating international specialists who provide identifications.

b) Insect parasites – to advise the Organization on developments in this field. Recently this Centre has taken on the added responsibility of testing candidate biological control agents for safety to selected non-target organisms.

c) Mammalian safety testing – to conduct appropriate infectivity tests using candidate biological control agents in laboratory animal (mammalian) models.

d) One or more new Centres will be established in other parts of the world to assist in the work being conducted by b) and c) mentioned above.

e) Insect viruses – to advise the Organization on the status of developments of these potential control agents, especially those associated with mosquitos and on advances in the means of identification of insect viruses.

f) Ecology and taxonomy of non-target species – to advise the Organization on protocols for study of such organisms and to assist in the identification of invertebrate species that might be secondary hosts of biological control agents, especially from areas where the fauna and flora are poorly known.

A specialist in invertebrate pathology and microbial control is stationed at the WHO *Anopheles* Control Research Unit in Kaduna, Nigeria, to carry out such field trials. Other WHO fields units are available to carry out trials under different ecological conditions and with different vectors and various governmental and non-governmental groups are being organized to assist in this work.

Tentatively we estimate that an insect pathogen identified at the WHO Collaborating Centre for Diagnosis of Insect Diseases and subsequently identified and cultured by a specialized collaborator, could pass through Stages I to III in approximately two years if proper cooperation between individual scientific groups is obtained. Experience indicates that industry will not invest substantial funds into research and development of biological control agents initially, and the scheme described herein (Stages I–III) should provide adequate information upon which industrial concerns may decide to carry out work on formulation and further development[7]. It has to be recognized that a pathogen as a biological species cannot be patented, and generally isolates are available for research and development. Only the formulation process can be patented, and it must be demonstrated to industry that a specific pathogen is active against target species and presents a minimal hazard to man and the general environment before it can be expected that major financial investments in research and development will be made.

As exchange of information is essential to encouragement of research in all fields, the Organization has commissioned an extensive annotated bibliography on pathogens of medically important pathogens to supplement an earlier WHO work published in 1964[8]. The manuscript (including over 1500 cross-referenced citations and complete through 31 December 1974) has

been finished and awaits publication in 1976[9]. In the future annual supplements will be released. In addition, the WHO Collaborating Centre on Biological Control for Diagnosis of Insect Diseases has computerized some 20,000 accessions, which are being augmented by material from other agencies working in this field, and is now able to provide computer searches by pathogen, vector host species and country[4].

Proposed operational use of microbial control agents in integrated control programmes

Adult insects are not readily susceptible to infection by pathogens, which operate against larvae and to some extent against pupae, and therefore must be considered as potential vector larvicides, not adulticides. Thus the development of bio-insecticides is expected to complement existing chemical larvicides in integrated control programmes and not as a unique means of vector control. The use of vector pathogens will be of particular interest in those areas where resistance to chemical insecticides and problems of environmental pollution exist.

As will be seen, certain bacteria and fungi are leading candidates for use in vector control. In agriculture, related bacteria have been successfully marketed and used for many years. Most producers in the developed countries who are engaged in industrial fermentation have experience in propagation of aerobic bacteria and fungi, and can produce large quantities at low unit cost to meet the needs of repeated applications. Government agencies of the countries producing these agents also have experience in the legal review and regulation of the use of microbiological agents for insect control in agriculture. Furthermore, the technology involved can easily be transferred to the developing countries, and the industrial competency and the raw materials for propagation of microbial agents are already available in most developing countries thereby making it possible for national programmes in microbial control of vectors to become self-sufficient.

Major groups of anopheline pathogens and the present status of their development

Although numerous pathogens of anopheline mosquitos have been isolated and several studied in detail, the present stage of development indicates that priority for work in the near future should be given: a) to the fungi and spore-forming bacteria; b) to the mermithid nematodes and microsporidan protozoa; and lastly c) the mosquito viruses. Representatives of each group are presented in relation to their known efficacy against target species (based upon both published and unpublished reports) and the stage they are judged to have reached in the WHO Screening and Evaluation Scheme. This scheme has been developed only within the past two years and the assessment is retrospective.

1. Viruses

To date, only a small number of viruses pathogenic to anophelines have been isolated and none of these is likely to be developed as a control agent in the foreseeable future. The various insect viruses have been isolated largely from species of *Aedes*, *Culex*, *Culiseta* and *Psorophora;* anophelines have been studied less[10]. Cytoplasmic polyhedrosis virus (CPV) has been reported from *An. bradleyi*, *An. crucians* (Louisiana, a USA), and *An. quadrimaculatus* (Florida, USA), and tetragonal virus has been reported from *An. crucians* and *An. freeborni*[11].

Potential viral control agents of anophelines remain in Stage I of the Screening and Evaluation Scheme and more work on their proper identification and structure(s) is required. In the further development and possible use of insect viruses, the potential hazards for man, other vertebrates, and non-target invertebrates must first be carefully assessed[12].

2. Spore-forming bacteria

The development of the crystalliferous spore-forming bacterium *Bacillus thuringiensis* as a control agent constitutes a major advance in the biological control of agriculture and forestry pests. The isolation of a strain of this bacillus from *Culex tarsalis* in 1968 indicated that *B. thuringiensis* might also have a potential for mosquito control[13]. Subsequently, an isolate of a related species, *B. sphaericus*, was made at the WHO Collaborating Centre on Biological Control for Diagnosis of Insect Diseases from *Culex pipiens fatigans* material provided by the WHO/ICMR Genetic Control Research Unit in New Delhi, India[14]. This pathogen has demonstrated its efficacy against *An. quadrimaculatus* in laboratory bioassay tests, although its activity against *Culex* spp. is greater. The same isolate, in different formulations, though it did not give encouraging results in tests against *An. gambiae* in Nigeria, did produce more than 90% larval mortality in recent bioassay tests against *An. albimanus*[15,16]. New strains have now been obtained from 11 countries (North America 1, Africa 2, Asia 5, and the Western Pacific area 3).

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[15] J. BRIGGS, unpublished WHO consultantship report (1974).
[16] J. BRIGGS, personal communication.

B. sphaericus is one of the most promising vector pathogens isolated to date. The experience gained in the development of *B. thuringiensis* for agricultural pest control improves the possibilities of developing *B. sphaericus* for vector control[7,13]. The Stage 1 tests are now complete; Stage II tests have been completed in part. This insect pathogen will be one of the first candidate agents to be examined by the new Collaborating Centre on Biological Control for Mammalian Safety Testing and then tested against non-target organisms. The initial Stage III trials will be repeated as new formulations become available.

The spore-forming bacteria are particularly promising agents for vector control since, in addition to their biological activity, they are suitable for production in developing countries, where local resources, including the technology associated with local fermentation industries, might be used. Problems exist in formulations and stability of shelf-life and further field trials against important vector species are soon to be initiated.

3. Entomogenous fungi

Five genera of insect-derived fungi have been suggested as possible biological control agents of anopheline mosquitos, where infection is fatal to the larvae. Each of these is considered below.

a) *Coelomomyces*. This genus was described from the larvae of *Aedes* (*Stegomyia*) *scutellaris* and has been the subject of many studies. Some are reported to infect at least 63 species of mosquitos belonging to 11 genera[17]. Despite early field trials against mosquito larvae, the life cycle of this fungus has only recently been elucidated. It is now evident that the minute crustacean *Cyclops* is an alternate host of *C. psorophorae*, and it may, therefore, have been their presence or absence which account for the diversity of results until now obtained in trials of this group of fungi as vector pathogens. *Cyclops* has also been shown to be the alternate host of both *C. punctatus* and *C. dodgae* using *An. quadrimaculatus*. Although the presence of an alternative host may increase the in vivo production of infective sporangia, it also creates possible environmental problems. Therefore, more work will be required to assess the feasibility of using this group of fungi for mosquito control[18,19].

From natural observations in the field, incidences of *Coelomomyces* as high as 95% have been reported in larvae of *An. gambiae;* 25% in *An. crucians;* and 80% in *An. quadrimaculatus*[11,20]. Most reported studies have used culicines rather than anophelines as target species[17,20]. Species of *Coelomomyces* may eventually prove to be useful in the biological control of anophelines, but further information on their life cycles is clearly necessary. As a group, the *Coelomomyces* have partially completed Stage I of the testing scheme; recent life-history studies indicate that Stage II is incomplete, and no Stage III tests have been made against anophelines.

b) *Beauveria. B. bassiana* has been used frequently for the microbial control of agricultural pests, but it has numerous hosts and reports on mosquito infection are rare. In addition, allergic reactions of humans on exposure to *Beauveria* have been reported and there are reports of honey-bee mortality[17,20].

Mortality of both larval and adult *An. albimanus* has followed their exposure to spores of *B. bassiana*, but there has been little recent work on this fungus. At present, work on *Beauveria* has completed the equivalent of most Stage I requirements, with limited success, but no Stage II trials have been reported[20].

c) *Lagenidium*. This genus was described in 1935 but research as a potential control agent has been recent, based on new isolates. Unfortunately, cultures have not been always available to laboratories interested in its development as a mosquito control agent. The most thoroughly studied isolate of *Lagenidium* was obtained from *Culex*, and despite its high laboratory and field infectivity for species of *Aedes* and *Culex*, little effect on anophelines (*An. albimanus, An. quadrimaculatus, An. stephensi, and An. sundaicus*) has been noted[11,21]. Development problems include those of mass production in artificial media, storage and safety testing. *Lagenidium* can be considered to have completed only part of the Stage I requirements and Stage II trials are partially completed.

d) *Culicinomyces*. This newly described genus has been reported from anophelines and culicines[22,23], but, as in the case of *Lagenidium*, cultures have not been made readily available for evaluation elsewhere. Reports on host ranges and possible mammalian infectivity are not known, and this candidate biological control agent must be considered only at the Stage I level.

e) *Metarrhizium*. Although mosquitos are known as 'natural' hosts of *M. anisopliae*, all tested species (including *An. stephensi, An. quadrimaculatus, An. funestus* and *An. gambiae* and a number of species of *Aedes* and *Culex*) have been found to be susceptible to infection by this fungus in the larval stages. This reflects its broad spectrum of infectivity for insects in general; from field and laboratory tests, it is known to infect more than 200 species from seven orders[17,20].

This fungus can at this time be considered as a model for development of microbial control of mosquitos. Among its advantages are 1) high infectivity rates in target species, and 2) ease of production on local materials in developing countries with modest technological facilities. Potential restrictions on its use would include 1) its broad range of host and therefore possible environmental effects, depending upon the manner of application envisaged; 2) its production of toxins and destruxins (which are depsipeptides) whose

safety to mammals has not been completely demonstrated; and 3) the need for studies on, and tests of, formulations for field trials. The safety aspect (Stage II) was considered in part before recent field trials at the WHO Anopheles Control Research Unit, No. 1, Kaduna, Nigeria (ACRU I), and there are no reports of this fungus being pathogenic for vertebrates or of allergic reactions in investigators experimenting with this fungus. However, more systematic studies are needed, and these commenced in 1976 at a newly established Collaborating Centre.

Mortality rates in *An. stephensi* larvae infected with *Metarrhizium* range from 60% (0.35 mg spores/1600 mm^2 water surface) to 75% (0.7 mg) and 100% (1.4 mg) in 10 days. The use of the destruxins (A and B) alone produce at 1 mg/20 ml water an LD_{50} of about 6–7 days with *An. stephensi* larvae[20].

Bioassay tests at ACRU I in late 1974 showed that *An. gambiae* larvae also were very susceptible to *Metarrhizium* conidia; more than 90% mortality was observed in 3 days (1 mg of *Metarrhizium* condidia in beakers of 24 cm^2 surface area). In most cases 100% mortality was obtained with both 0.5 mg and 1.0 mg of conidia per beaker[24].

At the present time, on the basis of available information, this is the most promising fungus for control of anophelines. Stage I is complete, Stage II is to be completely shortly, and initial Stage III field trials against *An. gambiae* have been moderately successful. Formulation and storage problems remain to be solved to develop a stable product giving predictable results.

4. Protozoa

Microsporidan epizootics have occurred throughout the world in mosquito colonies, principally in anophelines[11,25–27]. Important genera in these outbreaks have been *Nosema* and *Pleistophora*, but knowledge of the number of species of these and other microsporidan genera infecting mosquitos is inadequate. Fortunately, the classification based on electron microscopy rather than on spore size, other morphological features and host range, is currently being revised. In nature, microsporidan infection rates are often low: at ACRU I rates of 0.1% (*Nosema*) and 1.2% (*Parathelohania*) were recorded in *An. gambiae*. In *An. funestus* rates of 1.8% (*Pleistophora*) and 5.7% (*Parathelohania*) are reported[28]. There have been some reports of field infections by *Amblyospora* as high as 50% in *Aedes* and 80% in *Culiseta*. Assessment of the level of parasitism is difficult, however, because infected larvae often survive for several weeks longer than normal uninfected ones. A sample of a larval populations may therefore include a disproportionately high number of infected larvae due to the earlier pupation and emergence of uninfected adult mosquitos.

Laboratory trials of *Nosema algerae* against *An. stephensi* and *An. albimanus* have produced 100% larval infections[29]. Spores are producted in vivo in large lepdidopterous larvae. Larval mortality is usually not high at low spore concentrations, but emerging adults are infected and have shorter life expectancy (12.3 days for infected female *An. stephensi* against 33.0 days for uninfected control females). *An. albimanus* infected with *N. algerae* have reduced fecundity and usually do not live long enough to transmit malaria[29].

The microsporidan protozoans are promising microbial agents and their action is unlike that of other microbes such as, for example, bacteria and fungi. Transovarian infection, from adult to ova, is common in some forms and by this means the agents could be disseminated more widely in nature. However, microsporidan taxonomy remains a problem and in vivo production of spores is too costly for practical use in control. Thus certain aspects of Stage I are still incomplete. Stage II tests (conducted during late 1975 and early 1976 at the Collaborating Centres) are even more difficult as spores of the species selected for testing (*Nosema algerae*) can infect pig kidney cell cultures grown at lower than mammalian body temperatures in which they multiply and produce spores[30]. Safety for man and the general environment[31] must therefore be carefully tested in all future work with this class of pathogens.

5. Nematodes

Only one of the rhabditoid nematodes (*Neoaplectana*) is known to infect mosquitos and 42 species, principally of *Aedes* and *Anopheles*, were listed in 1964 as hosts for six genera of mermithid nematodes[8]. To date, nematodes are known as parasites of at least 63 mosquito species[32].

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The classification of the mermithids is still a subject of controversy, and reporting of distributions and local infection rates may be influenced by the presence and timing of specialists in a given area. For example, mermithid parasites of mosquitos are known from all continents except South America, yet half of the 36 host records for nematodes in the USA are from Louisiana, where there is an active and interested group of specialists[11].

Diximermis peterseni is specific to anophelines in laboratory and field studies, as well as in deliberate releases, whereas *Reesimermis nielseni* is known to infect 58 species of mosquitos in nine genera in both laboratory and field tests. There is no indication that mermithids would present a public health problem[33], but candidate species known to infect a broad range of hosts will have to be examined against selected non-target organisms.

High naturally-occurring rates of infection (70–80%) by nematodes have been reported in anopheline larvae, and in other mosquito genera, *Reesimermis* infection levels above 90% have been reported; 53% of *An. crucians* larvae have been reported infected with *R. nielseni*. In one test in Louisiana approximately 65% of all anopheline larvae present were parasitized when the water was treated by compressed air spraying (1000 preparasites/m^2 surface area)[11,25,26,34].

The results of field tests of mermithids against anophelines, or mosquito larvae in general, have not been consistent. Several genera, especially *Reesimermis*, have completed most Stage I requirements but problems of mass production for operational use for other forms are still unsolved. Stage II can be considered complete for *R. nielseni*, and a commercial bioinsecticide based on this species is now available in the United States, but information on susceptibility of important vector species is not yet available. In the USA, the Environmental Protection Agency has ruled that mermithid nematodes are out of their jurisdiction according to pesticide legislation in that country. The results of field tests that have been carried out do not satisfy the Stage 3 requirements against most major anopheline vector species, although almost 100 trials have been performed against a battery of mosquitos.

Conclusions

1. There are a large number of mosquito pathogens that lend themselves to development as potential biological control agents. To date, there has been little industrial interest in these agents as their operational use has yet to be demonstrated.

2. The Organization has developed a system for screening the potential utility of microbial agents in vector control (Annex), and is setting up a network of Collaborating Centres for this purpose to determine efficacy, human and general environmental safety of the use of such agents.

3. It is recognized, in general, that microbial agents will be used as larvicides, and their greatest potential use will be in integrated programmes, and where resistance to chemical insecticides exists.

4. The production of the most promising microbial pathogens as bioinsecticides would be within the means and capability of developing countries, and encouragement should be given to the development of these agents.

5. A concerted effort will be made by the Organization in the next five years to: a) stimulate the search for new microbial insect pathogens; b) complete Stage I–III trials on the most promising microbial agents especially for mosquito control; and c) provide the information that would be required to influence commercial development of microbial agents of vector species.

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