



agriculture,
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Department:
Agriculture, forestry & fisheries
REPUBLIC OF SOUTH AFRICA

AMERICAN FOULBROOD (AFB)

MANAGEMENT STRATEGY

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Compiled by: AFB Steering Committee

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A. Scope

This AFB management strategy is part of the South African honeybee bio-security protocol, which is a framework to ensure the protection of a healthy and viable honeybee population. The bio-security protocol will inform the establishment of an inspection and analytical capacity necessary to maintain a healthy honeybee population

B. Purpose

The purpose of this strategic plan is to manage AFB in South Africa and to give guidelines to the industry and government in this regard.

C. Background

American Foulbrood (AFB) is a notifiable disease under the requirements of the World Trade Organisation for Animal Health (OIE). In the relevant chapter of the OIE Terrestrial Animal Health Code, AFB is described as a disease of the larval and pupal stages of the honeybee (*Apis* species) that occurs in most countries where such bees are kept. The causative organism (*Paenibacillus larvae*) is a bacterium that can produce over one billion spores in each infected larva. The spores are very long-living and resistant to heat and chemical agents and only the spores are capable of inducing AFB. This is a serious disease which requires intensive management to prevent major colony losses. To prevent the spread of AFB early diagnosis and withdrawal of affected hives from the apiary are important together with other good apicultural practices and destruction or sterilization of infected items by means of appropriate bio-security and bio-containment measures.

Up to February 2009 AFB disease was not known to occur in Sub-Saharan Africa. Until early 2009 the beekeeping industry in South Africa has largely existed free from government involvement in the day to day management of this industry except for import control. Recent disease and pest incursions dictate a revision of this approach, and require a bio-security protocol to protect the critical resource provided by the indigenous honeybee population.

Consumers in South Africa as in most first world countries are also demanding a natural product that is traceable and without harmful substances. In order for government to comply with this mounting pressure, validation of honey and beehive products by government need to get the necessary attention

D. Status Overview

- a) AFB in South Africa is already too widespread to be eradicated.
- b) It should be accepted that it will be almost impossible to contain AFB in the current known affected areas in South Africa. Beekeepers move bees too widely and there are too many wild bees for any containment to be successful
- c) Similarly, it is very unlikely to be possible to stop the spread of AFB from the current known AFB-infected areas to the rest of South Africa, and from there to the rest of Africa.
- d) Unlike the AFB management plans developed in countries such as New Zealand, the South African plan needs to take into account the critical importance of the wild honeybee population. Unmanaged, indigenous honeybees in Africa are extremely important in conservation and biodiversity; in supplying the livelihoods for many people involved in small-scale beekeeping; and in replenishing the bees used in commercial beekeeping. Unlike the situation in almost all other countries dealing with AFB, it is not only about the effect of AFB on commercial beekeeping.
- e) Unlike the situation anywhere else in the world, the vast majority of beekeeping in Africa (except South Africa) is practiced using honeybee hives without movable frames. Traditional AFB mitigation procedures such as shook-swarm colonies or the application of antibiotics cannot be used in these non-movable frame colonies
- f) Unlike the situation in a country like New Zealand, South Africa is part of a much larger land mass (Africa) that as yet does not have AFB. The actions taken with response to AFB in South Africa are likely to have a direct bearing on honeybees and beekeeping in the rest of Africa in the near future.

- g) The AFB situation in South Africa is also not analogous to the situation in Europe, the USA, Canada, Australia or New Zealand, all of which have had AFB and AFB management strategies for more than a century. All of these countries have substantially changed their honeybee populations as a result of the AFB management strategies, and have mitigated the impact of AFB in these populations. Effectively, they have slowly eliminated the AFB susceptible honeybees and are now left with an essentially AFB tolerant population. Their current situation and strategies have little immediate bearing to the situation in South Africa, at the onset of AFB infection. It should also be borne in mind that all of these countries extensively used antibiotics to manage AFB at one time or another, even though many of them no longer do so.

E. Strategic Options and Recommendations

1. Option A: Zero interference

- a) Do nothing.
- b) Allow the infection to run its course.
- c) Assume that the susceptible colonies will die; that some colonies will get sick and then recover; and that some colonies will be tolerant to AFB.
- d) Assume that the percentage of susceptible colonies will be sufficiently low and that the honeybee population and beekeeping can withstand their loss.
- e) Creates a permanent solution as it eventually results in the development of an AFB tolerant population, with no further management needed.
- f) High risk, as there is no way to know what percentage of the population is indeed tolerant. There could be epidemic losses sufficient to collapse beekeeping and everything dependent on honeybees for pollination. Also could have a weakened, 'sick' honeybee population for a long time.
- g) Requires no inspection service, no analytical capacity, no management and no funding.
- h) Would have the same effect on all honeybees; on commercial bees, on small-scale beekeeping, on wild bees.
- i) Suitable for all of Africa; does not require funding, management or movable-frame hives.
- j) No need for beekeeper compliance or legislation.

- k) No need for the registration of beekeepers, for the registration of apiaries, or for any inspection program regarding AFB.
- l) Requires the development of a monitoring program for testing for residues in bee products, and legislation for this program, to ensure that no antibiotics are being used, as their use would hinder the development of tolerance.

2. Option B: Medication

- a) The preventative (prophylactic) treatment of the managed honeybee population with antibiotics.
- b) Compulsory treatment of all colonies, at the first sign of clinical symptoms in a bee keeper's operation according to the defined procedure.
- c) Probably one antibiotic treatment per annum.
- d) Prevents colonies becoming sick from AFB, as far as possible.
- e) Essentially the procedure followed in the USA, Canada and Argentina.
- f) In cases of antibiotic resistance or non response of colonies to treatment the colony must be eliminated (by burning).
- g) Where the beekeeper does not to treat with antibiotics, infected colonies must be destroyed by burning within 48 hours.
- h) Non-compliant beekeepers have their operations subject to sanction.
- i) Would result in no big losses to commercial honeybees.
- j) Does not promote the development of natural tolerance.
- k) Potential very large losses to wild honeybee populations; and permanent losses, as natural tolerance may not develop.
- l) Potential large losses to small-scale beekeeping, most of whom cannot apply shook-swarm procedures or antibiotics (because they don't use movable frame colonies). Potentially change the nature of beekeeping in the rest of Africa.
- m) Requires a reasonable inspection service, a small amount of laboratory testing, and a reasonable amount of management.
- n) Does not offer a permanent solution to the African scenario.
- o) Only suitable as a temporary strategy, to save commercial beekeeping and dependent agriculture from collapse.
- p) Results in problems of residues in honey; bee products less valuable and potentially denied markets.

- q) Requires the development of a monitoring program for testing for antibiotic residues in bee products, and MRL legislation for this program.
- r) Has the potential for the development of antibiotic resistance in the AFB.
- s) Requires a good level of beekeeping compliance.
- t) Requires all beekeepers to be registered, all apiaries to be registered and the compulsory inspection of all beekeeping operations.
- u) Requires a burning procedure, a sterilization procedure and a shook-swarm procedure.

3. Option C: Management without medication.

- a) A virulent management strategy.
- b) Allow shook-swarm control of lightly affected or non-clinical colonies.
- c) The destruction threshold is set at 50 clinically infected cells in a colony. This is based on Goodwin et al (1994), who report that colonies at this level of infection do not spread the disease in the apiary.
- d) Above the predetermined threshold of infection, colonies have to be immediately destroyed by burning according to procedure within 48 hours.
- e) Non-compliant beekeepers have their operations subject to sanction.
- f) The onus is on the beekeeper to keep infection levels in all colonies below this level; by shook swarm techniques; by other management techniques, other than the use of antibiotics; or by voluntary destruction. Colonies with infection levels above this threshold must be destroyed.
- g) Effectively a long-term abatement strategy, to eliminate the most virulent AFB strains and the most susceptible colonies.
- h) The emphasis is on self-management; beekeepers can choose their own level of quarantine, of sterilization, of shook-swarm management, of hygienic beekeeping. The system is self-regulating; if the measures applied by the beekeeper are insufficient, colonies will have above threshold clinical symptoms and have to be destroyed. The onus is therefore on each beekeeper to apply proper management to mitigate colony losses.
- i) Potentially substantial losses in commercial bees and wild bees.
- j) Cannot be implemented in small-scale beekeeping that does not use movable frame hives, and substantial losses in this sector can be expected.
- k) End result is a mostly tolerant population.

- l) Requires an extensive inspection service, laboratory testing for confirmation of clinical symptoms, and a high level of management.
- m) Requires the development of a monitoring program for testing for residues in bee products, and legislation for this program, to ensure that no antibiotics are being used, as their use would hinder the development of tolerance.
- n) Requires a high level of beekeeping compliance.
- o) Requires all beekeepers to be registered, all apiaries to be registered and the compulsory inspection of all beekeeping operations.
- p) Requires follow-up inspections of apiaries in which sub-threshold clinical AFB symptoms have been found, to ensure the application and success of management procedures. Colonies that are found still to show clinical symptoms in follow-up inspections are to be destroyed.
- q) Requires a burning procedure, a sterilization procedure and a shook-swarm procedure.

Recommended Strategy

The choice of which strategy is to be selected is based on the joint conclusions that bee diseases are basically ineradicable (Bailey 1999), and that the use of antibiotics can only ever be a temporary strategy, at best delaying the need for the development of long-term natural tolerance. Hence, the aim of any strategy should be to maintain the disease (and infectious agents) at tolerably low levels, below the **Economic Injury Level** (EIL) (Nasr & Kevan 1999). To do this, it is necessary to both mitigate the spread of the disease as far as possible, and to eliminate substantial sources of infection. It is clear that the most substantial source of infection in American Foulbrood is the horizontal transmission of the disease brought about by honeybee foragers robbing colonies that have collapsed from AFB. Numerous authors have shown that colonies near to an AFB-collapsed colony have a very great chance of contracting the disease (De Graaf et al 2001; Pernal & Melanopoulos 2006; Lindstrom et al 2008). 75% of colonies located 500m or less from an AFB-collapsed colony contracted AFB and died of the disease, 50% of colonies 1km from the AFB-collapsed colony died of AFB, while no colonies more than 2km from the source contracted the disease (De Graaf et al 2001). Conversely, Goodwin et al (1994) showed that the drift between colonies within an apiary does not transmit sufficient AFB spores to initiate infection in uninfected

colonies, even if there are colonies with low levels of AFB infection in the apiary. Hence, the focal point of any suggested AFB Management Strategy should be to ensure the efficient control or removal of any heavily infected AFB colonies, rather than trying to eradicate AFB from apiaries.

It is thus recommended that Option C is the strategy to be followed at present. In the long-term, should this strategy prove to be successful, it will be possible to slowly change the strategy to one more similar to Option A with less and less inspection and management necessary, as the level of infection in the population decreases.

The choice of Option C is based on the assumption that the bulk of the honeybee population in South Africa will turn out to be tolerant to AFB. As it is not known that this is the case, it is prudent and responsible to prepare for the implementation of option B, should a rapid switch-over from option C to option B become necessary. It should be noted that this switch-over will happen naturally, without permission and without a management plan, should beekeeping losses become such that individual beekeepers believe that they cannot sustain their operations without antibiotic treatment. In such instances, the use of antibiotics will be unregulated, and many types of antibiotics and all types of doses can be expected to be used. This is less desirable than having a controlled use of antibiotics. Hence, having a rapid-implementation antibiotic strategy (option B) is necessary, should its use become warranted.

The registration of OTC is therefore essential to ensure a probable treatment in case Option C is not effective.

Should AFB in South Africa be shown to be resistant to Terramycin other medication needs to be authorized. This should be the registration of the product only, its use to be contingent on DAFF deciding to switch from option C to option B, as a temporary measure. It is to be expected that DAFF would only take such a decision after extensive consultation with the beekeeping industry and other stakeholders.

F. AFB MANAGEMENT PLAN (For option C)

AFB is a highly contagious and transmittable disease of honeybees. It is also a disease that is not immediately showing symptoms after infection and can be sub clinical for a few years. It is spread predominantly and most rapidly by beekeepers through normal beekeeping practices. AFB spores are in all bee products, on adult bees, on and imbedded in all hive parts and other bee keeping equipment in infected apiaries. It can be spread by bee products such as honey thus by robbing of spilled honey or exposed hive parts and by adult bees swarming or by the division of colonies. It is spread from mother to daughter colonies. The spore count level is important to develop symptoms but is subject to the number of infectious introductions and the resistance of the colony to the specific AFB strain and the AFB strain's virulence. Early detection by beekeepers is vital to the success of managing the disease in apiaries. Samples can be taken to test for sub clinical symptoms on a regular basis.

The entire emphasis of the Management Plan is on the beekeeper to maintain the level of AFB in his/her apiaries below the danger (disease) level. To do so, beekeepers in South Africa will need to apply vastly improved hygienic beekeeping practices. While these are only recommendations and not legislated requirements, the onus is on the beekeeper to gauge the level of hygienic beekeeping procedures that are practical for his/her operation, and that are necessary to keep AFB levels at below threshold levels. Failure to do so can result in repeated inspections, repeated standstills and continuous destruction of colonies. Uncontrolled beekeeping operations can lead to high AFB infection levels which may cause big losses due to colonies that is unproductive or died out. Good beekeeping practices that are recommended to avoid AFB infection are included in Annexure 1.

1. Management of AFB in apiaries by beekeepers

- a) As part of good beekeeping practices, beekeepers are recommended to inspect their hives (all brood frames) for AFB on a regular basis.

- b) Once clinical symptoms are observed in a hive the nearest DAFF APIS office needs to be contacted and the disease presence reported indicating the registration number and current location of the hive, as per legislation.
- c) After clinical symptoms are observed the colony needs to be made free from AFB. . If there are less than 50 AFB cells in the colony, the beekeeper must institute management measures (shook swarm method; Annexure 3) to eliminate the clinical symptoms in the colony. If more than 50 AFB diseased cells are present in the colony, it must immediately be destroyed by burning (Annexure 4)
- d) Antibiotics should not be used to treat any colony can contaminate bee products, resistance can easily be built and it will mask the symptoms but will not cure the hive of AFB.
- e) It is important to note that when sub clinical symptoms are present AFB spores can still be spread via the same ways as with clinical symptoms. The difference is that the spore counts or levels are much lower and clinical symptoms development after spread to non resistant colonies will probably take longer to develop.
- f) When hives with clinical symptoms are detected in an apiary careful examination of all the hives in the apiary needs to be done.

2. Legislation

- a) Beekeepers are required to comply with the amended regulation No. R. 1674 of 24 December 1998 of the Agricultural Pests Act.
- b) Legislation will include:
 - annual registration with registration requirements
 - notification of AFB symptoms in an apiary
 - burning of hives which are showing AFB symptoms which are above the predetermined threshold
 - sterilization of beekeeping equipment
- c) The Executive Officer has the authority as described under section 4 and 6 of the Act and can therefore:
 - inspect hives at any time
 - inspect bulk and retail honey at any time

- draw samples, test and destroy hives if it is infected with AFB showing clinical symptoms and was not notified,
- destroy honey if antibiotic use is not authorized as it can mask the symptoms of AFB
- destroy hives when it is found to be unmarked and cannot be traced to any particular beekeeper.
- order specific practice by directive

3. Statutory Inspection

- a) The regular inspection of hives is firstly the responsibility of the beekeeper and forms part of good beekeeping practices. To support information gathering regarding AFB and the beekeeping industry in general official inspections will also be conducted. DAFF shall ensure that inspection services are instituted to give affect to the legislation and the Management Plan and Procedures.
- b) The objective of the inspection services is to:
 - monitor the severity and spread of AFB clinical symptoms in apiaries
 - advise beekeepers on effective AFB management actions when no symptoms are observed
 - ensure that beekeepers practise appropriate management options when the AFB symptoms are observed but is below threshold
 - enforce burning of hives which has an above threshold level of infection
 - ensure that beekeepers notify the occurrence of AFB symptoms in their apiaries
 - ensure that beekeepers register their operations and sites and that hives are properly marked
 - take the appropriate samples when necessary, i.e. to confirm clinical symptoms, etc
- c) Inspection will be conducted according to available capacity, budget and resources at any particular time at any particular place or area in the country

4. Awareness

AFB is a notifiable disease but also potentially a very serious disease and can influence the livelihoods of beekeepers in commercial as well as noncommercial beekeeping operations. As the management plan is directed to reach all beekeepers it is important that beekeepers are aware of the legislation and the content of the plan and good beekeeping principles. An awareness program should be instated to ensure that beekeepers are informed and educated regarding the management of AFB in their areas and apiaries.

This should be a joint effort by the Department, the Bee industry and the ARC.

5. Sampling and laboratory analysis

All official samples are handed in by APIS for testing through the Plant Health Diagnostic Services system. Sampling and laboratory analysis procedures are conducted according to Annexure 5 and 6

6. Documentation

All official reports regarding hives with AFB symptoms and official laboratory results will be submitted to Plant Health Early Warnings for analysis and documentation.

All information regarding surveillance data will be kept by Plant Health Early Warnings.

7. Procedures

The following procedural documents will be part of an annexure to the AFB management plan.

- a) Good beekeeping practices
- b) Hive destruction procedure
- c) Shook swarm procedure
- d) Sterilization procedure
- e) Sampling procedure

- f) Analysis procedure

8. Notification

As a member of the OIE the RSA should notify and have management measures in place regarding AFB. Regular reporting of the status of AFB in the affected country needs to be submitted to the OIE.

9. Contacts

Contact information of officers responsible for AFB matters will be posted on the AFB information page on the DAFF website.

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ANNEXURE 1

1. Good beekeeping practices to avoid AFB infection (hygienic beekeeping)

- a) Beekeepers should ensure they understand the disease, are knowledgeable with regards to the clinical symptoms of AFB, and are able to detect signs of AFB in their apiaries.
- b) Apply quarantine procedures that suit the logistics and practicalities of your operation. Separate your operation into functional units (be they colonies, apiaries or areas), mark all beekeeping equipment separately for each functional unit, and use all equipment in only one of the functional units.
- c) Practice hygienic beekeeping by thoroughly cleaning smoker and gloves with soapy water and a coarse brush before entering any apiary, and immediately after working with a hive with clinical AFB symptoms. All wax, propolis and honey to be removed from gloves and smoker. If practical, use disposable rubber gloves over your gloves and dispose of by burning after each apiary inspection.
- d) Thoroughly flame hive tools before entering any apiary and immediately after working with a hive with clinical AFB symptoms. Alternatively a clean set of tools need to be used for the next apiary.
- e) Never discard any hive products or components (wax, burr comb, brace comb, propolis, scrapings) in the apiary. Place all such material in a sealable bucket to be removed from the apiary, to be either rendered or burned.
- f) Always travel with a number of empty, sterilized colonies with sterilized combs with foundation, so that the shook swarm procedure can be immediately implemented (Annexures 3 and 5) or vandalism or other damage encountered

- g) Travel with a large, sealable container to place all frames removed from a colony that has been shook-swarmed. These frames are to be rendered or burned.
- h) Never buy old comb, or used frames, that have not been sterilized.
- i) Never feed honey or other bee products to hives.
- j) Never buy colonies without carefully checking them for AFB first.
- k) Always sterilize second hand equipment (Annexure 5).
- l) Try to prevent robbing.
- m) Never unnecessarily exchange brood or super frames.
- n) If honey supers and honey containers are to be fed back to bees, do this only at one designated site. At this designated site, maintain a small group of 'budgie' colonies that are inspected very regularly, to monitor the level of AFB in your operation.
- o) Inspect all apiary sites at least once every three months, checking all colonies and all brood frames in each colony.
- p) As far as possible, keep your apiary sites and colonies removed from other beekeepers.
- q) Ensure that all honey processing facilities are bee proof, and that no foraging bees have access to the facility.
- r) Replace a minimum of 3 frames in each colony per annum. Preferably remove old brood frames, and pollen bound frames. Removed frames should be rendered or burned.
- s) Use only movable frame hives

ANNEXURE 2

HIVE DESTRUCTION PROCEDURE

- a) The colony is killed and burned by the inspector or beekeeper on the same day as its discovery, whenever possible.
- b) The colony is killed late in the afternoon, or when few bees are foraging, whenever possible.
- c) 500ml of petrol or diesel is poured from the top into the colony to kill it, and the hive is immediately sealed with wrapping plastic.
- d) The hive is removed to the burn site under supervision of the inspector. Each region to identify a burn site.
- e) All frames (brood and super) and bees are burned.
- f) The hive parts (bottom board, queen excluder, hive body, supers, top cover) can be re-claimed by the beekeeper. The beekeeper must indicate to the inspector, before the release of the colony by an official order whether he/she wishes to re-claim the hive parts. If not, they are burned.
- g) No compensation is paid to beekeepers for the burning of colonies, or for the collection of hive material. No costs are applied to beekeepers for the burning of hives.
- h) Beekeepers may independently bring hives for burning to the burn site, with no costs being applied.
- i) If beekeepers burn colonies themselves, they must abide by all local fire regulations.

- j) If irradiation procedures on whole hives are shown to be effective in eliminating AFB spores from all areas of the hive, a beekeeper may choose to have the hives irradiated instead of being burned. In this case the beekeeper needs to inform the inspector accordingly. The colony will then be killed with petrol/diesel and sealed by the inspector, and tagged with an official label. The hive then needs to be delivered to the irradiation plant by the beekeeper within 3 days, and the irradiation plant has to document its arrival and irradiation, and this documentation transmitted to the Inspection Service. The cost will be for the beekeeper.

DRAFT

ANNEXURE 3

SHOOK SWARM PROCEDURE

The shook-swarm procedure is an ancient method practiced to control AFB. It is, in effect, artificial swarming, where the **bees only** from a diseased colony are transferred onto fresh combs in a fresh hive body, and the contaminated old combs and hive body are either destroyed or sanitized. In theory, any contaminated honey in the honey stomachs of the adult bees will be consumed while fresh comb is being built, and the new colony will be free of AFB. In practice, however, the disease may recur in the colony, but often only after a period of two years or more.

In a number of European countries the shook-swarm method is considered sufficient to control AFB. In South Africa, it is anticipated that shook-swarm procedures will be sufficient to manage AFB infections in lightly infected colonies, provided these procedures are thoroughly applied, inspection intervals are short enough to allow for early detection, and the honeybee populations of South Africa have some natural tolerance towards AFB. Hansen & Brodsgaard (2003) report that in strains of bees that are particularly susceptible to AFB, shook-swarm procedures are insufficient to arrest AFB infections and the bees have to be killed. In other honey bee populations, with some degree of AFB tolerance, shook swarming reduce the spore levels in the colonies by at least 1000 times, and prevents the recurring of clinical symptoms for two years or more (Hansen & Brodsgaard 2003).

The onus is on the beekeeper to determine how thorough a shook swarm procedure he or she wishes to apply. The beekeeper has numerous options that can be used, as follows:

- Use a single shook-swarm, or a double shook-swarm (where the procedure is repeated after 3 months).
- Transfer the bees into a new hive body with new frames; or into a sanitized hive body with fresh frames.
- Transfer the bees into a screened box for 3 days, before transferring to the new hive, to ensure all honey in the honey stomachs are consumed.

- Feed the bees with OTC according to the prescribed procedure while in the screened box, to eliminate any AFB present.(only when approved by DAFF)

As there is no clear evidence that the double shook-swarm method or the screened box method (with or without the use of OTC) offers any advantage over the single shook-swarm method, the following is recommended:

- It is generally best only to shook-swarm colonies on a honey flow, so that colonies can build comb, and to minimize the threat of absconding. However, in an effort to prevent the build up of AFB in apiaries, it is recommended that colonies be shook-swarmed as soon as clinical AFB is detected, without waiting for a honey flow.
- Hence, whenever a colony is found with a sub-threshold (for destruction) level of clinical AFB, this colony should be immediately shook-swarmed.
- A new or properly sanitized hive body should be placed on the position where the colony stands, and all the bees from the colony should be carefully shaken into the new hive body.
- One frame of brood and one frame of food should be removed from another colony in the apiary (a colony without clinical AFB) and placed in the new colony. The remaining 8 frames should be new frames with foundation wax. 2 new frames with foundation wax should be placed in the colony that yielded the brood and food frame.
- All the frames from the diseased colony should be placed in bee-proof containers and removed from the apiary. The contents of the frames can be rendered or burned. The frames should be burned.
- The hive body and the rest of the colony should be removed from the apiary and either burned, or thoroughly sanitized.
- The shook-swarmed hive must be properly marked for follow-up inspections.
- For better control hives with sub-threshold symptoms can be appropriately moved to a dedicated management and monitoring site before or after applying the shook-swarm method.

ANNEXURE 4

STERILIZATION PROCEDURE

It is recommended that frames from colonies with clinical AFB are not sanitized and re-used, but are burned. All the rest of the hive components can be sanitized and re-used by the beekeeper if he or she wishes. The reason why the sanitation of hive parts is allowed is because they contain relatively few AFB spores. Gochnauer (1981) found that for every AFB spore found on the hive body or in propolis, there are 27 spores in the wax, 13 spores in the pollen and 73 spores in the honey. There is sufficient likelihood, therefore, that hive parts can be successfully decontaminated.

Once again, the onus is on the beekeeper to determine the level of sanitization necessary for his or her operation. The intent is not to eliminate AFB spores from the equipment, but to reduce spores to below the Economic Injury Level. What is clear is that the Inspection Service **cannot** monitor or control the sanitization of hive parts. All the Inspection Service can do, and should do, is to monitor the outcome of the beekeepers efforts. If clinical AFB continually recurs in a beekeepers operation, then colonies will continually have to be destroyed, as the sanitization procedures used was clearly not effective.

Hence, all and any sanitization methods are to be allowed, and the onus will be on the individual beekeeper to reduce spore levels on his/her equipment and to ensure freedom from clinical AFB. While all methods are allowed, the following recommendations are made:

- All hive parts from colonies that have had clinical AFB must be very thoroughly cleaned. All wax, propolis and honey should be removed and all hive components thoroughly scrubbed with soapy water.
- If so desired, hive parts can then be additionally treated with irradiation, hot wax dipping (160° C for 10 minutes) or scorching.
- Sodium hypochlorite (3%) should only be used to treat foundation wax, or plastic hive components.

ANNEXURE 5

SAMPLING PROCEDURE

Many different types of sample can be collected for AFB analysis: adult bees (field bees, brood bees, super bees), healthy brood, diseased brood, brood honey, super honey, pollen, wax, propolis or hive scrapings. The sampling procedures followed in the survey are quite simple: when clinically-infected colonies are found, collect diseased larvae/pupae; and in all apiaries collect a composite honey sample from brood honey. This is done because AFB is viewed as an apiary disease, and hence if it is present in one colony in an apiary, spores of AFB are likely to be found in all colonies in the apiary. Hence, a composite apiary sample is the appropriate method to use. It would obviously be best to collect a sample from every colony in every apiary, but the suggested 5 colonies per apiary is an acceptable compromise, and should result in a positive AFB result in all but the most lightly infected apiary.

Recent literature suggests that these are perhaps not the best sampling procedures. The following points are salient:

- (a) While honey sampling has been widely used (Hansen 1984), in clinically-infected colonies, samples of adult bees are more likely to be AFB positive than is honey, pollen or propolis taken from these colonies (because this might have been collected and stored before the outbreak of the infection) (Hornitsky & Karlovskis 1989; Goodwin et al 1996; Nordstrom et al 2002; Lindstrom & Fries 2005; Gillard et al 2008). Lindstrom & Fries (2005) found that 100% of workers from clinically infected colonies tested positive for AFB. Only 86% of honey samples collected from clinically infected colonies were AFB positive (Gillard et al 2008), and honey sampling can always provide some false negatives for clinical colonies (Goodwin et al 1996; Pernal & Melanopoulos 2005; Gillard et al 2008). In this regard, super bees are as likely to be AFB positive as are brood bees.
- (b) In non-clinically infected hives, honey is a better indication of AFB spore levels than are adult bee samples, because adult bees are unlikely to have high spore loads if there is no clinical infection (Nordstrom et al 2002; Gillard et al 2008). It is

best to collect honey from a number of different areas of the colony, to best reflect the AFB spore load in the colony (as the honey will have been stored at different times). It is best to collect both open and sealed honey, to best reflect the honey storage history of the colony, and honey should always be collected from as near to the brood nest as possible (Genersch & Otten 2003).

- (c) As regards the hygienic collection of samples, it is impractical to be absolutely hygienic in the apiary. Nonetheless, very strict measures must be applied by the inspectors to limit any contamination that might occur. These measures are as follows.
- Before entering any apiary, and immediately after inspecting any hive with clinical AFB symptoms, the gloves and hands of the inspector must be extremely thoroughly scrubbed (using a brush) with soapy water (warm, if possible). All wax, honey and propolis must be removed. If possible, inspectors can also use disposable gloves over their own gloves, to dispose off after finishing any apiary or any clinical AFB colony.
 - If the inspector is using a hive tool, knife or any equipment to remove honey or brood samples (rather than using the equipment of the beekeeper, which is preferred), then it must be thoroughly flamed using a burner before entering any apiary, and immediately after inspecting any hive with clinical AFB symptoms. Alternatively inspectors should have more than one set of tools. Used equipment should then be sealed for sterilization at a later stage
 - All sample containers should be properly prepared **before** entering the field, with containers and sticks already placed in a sealed, labeled (blank) plastic bag. This bag should only be opened in the apiary, and when the apiary is finished the bag should be placed inside a second plastic bag, which is then sealed, before being returned to the carrying container.

With regard to the collecting of diseased brood from clinically infected colonies, the stick method is **completely correct and is the method used throughout the world**. This is the method recommended in the all-AFB-researchers review of de Graaf et al (2006). The OIE recommendation (and that of the USDA) to collect a piece of brood rather than directly removing the (presumed) diseased larvae is only in place to counter

the possible lack of knowledge of the collector. When samples of brood arrive for analysis at the USDA lab in Beltsville, for example, this brood is carefully examined and the most likely diseased brood is then removed using the stick method. Similarly, in York and everywhere else pieces of brood are submitted. Hence, the only change is that the diseased-larvae-selection takes place in the lab rather than in the field. This is acceptable if the laboratory staff has specialist knowledge of what to look for, but the submission of brood is generally not favored because diseased larvae are much easier to detect when fresh rather than old, and because pieces of comb can easily be overgrown with fungi. For this reason, many AFB labs (such as New Zealand) will not accept brood samples, and only use stick samples.

Hence, it is recommended that the following method of sampling be adopted.

- In clinically-infected colonies, a collection of diseased pupae and larvae, using the stick method; a collection of about 100 adult bees from the brood box or super, stored in 70% alcohol; and a collection of a composite honey sample from the apiary, with honey collected from multiple areas in each hive.
- In non clinically-infected apiaries: a collection of a composite honey sample from the apiary, with honey collected from multiple areas in each hive.

ANNEXURE 6

ANALYSIS PROCEDURE

1. Culture Media

The OIE recommends the use of BHIT (brain heart infusion, with thiamine), J agar, CBA (Columbia blood agar) or MYPGP agar (Mueller-Hinton broth with yeast extract, potassium phosphate, glucose and sodium pyruvate). To this list can be added SBA (sheep blood agar) and PLA (paenibacillus larva agar), both of which are confirmed to work for *P larvae*.

We are presently using J agar, and it is quite clear that J agar is fine (Hansen 2004; Piccini & Zunino 2001; Alippi 1995; Steinkraus & Morse 1992; Artunez et al 2007; Hansen et al 2003; Hornitsky 1990; Hornitsky & Nicholls 1993; Artunez et al 2004; Govan et al 1999). However, perhaps it is not the best? Hornitsky & Nicholls (1993) report that J agar is better than BHIT which is better than SBA – but all of Dingman & Stahly (1983), Ritter & Kiefer (1993) and Nordstrom & Fries (1995) report that MYPGP is better than J agar, especially at low spore levels, as does the review of de Graaf et al 2006. Steinkraus & Morse (1996) report that MYPGP is better than BHIT which in turn is better than J agar; but that if sodium pyruvate is added to J agar, then it is as effective as is MYPGP agar. We are not adding sodium pyruvate to our J agar.

Most recent studies have used MYPGP (Gende et al 2009a, Gende et al 2009b, Gende et al 2008a, Gende et al 2008b, Gillard et al 2008, Lindstyrom 2008, de Rycke et al 2002; de Graaf et al 2001; Fries & Raina 2003). To add to this, we should consider the PLA medium, used by Pernal & Melathopolous (2006), which has the great advantage of not needing a CO₂ incubator. The multi-author review of 2006 (de Graaf et al 2006, including all the world's AFB experts) suggests that MYPGP and PLA are the best to use, and maybe we should follow that advice?

Therefore, unless there is a good reason why J agar is used, we should switch to MYPGP or PLA media. And if we do use J agar, it should have pyruvate added.

2. The use of antibiotics

There are some errors in the publications with regards to the correct concentrations to use. Alippi (1995) has all concentrations three orders of magnitude out (mg/ml instead of $\mu\text{g/ml}$). This notwithstanding, an incomplete list of the range of usage of antibiotics in the media to inhibit other spore-forming bacteria in growth plates for *P larvae* is as follows:

Antibiotic	Concentration	References
nalidixic acid	3 $\mu\text{g/ml}$	(Alippi 1991; Hornitsky 1990; Hornitsky & Clark 1991; Hornitsky 1998; Piccini & Zunino 2001; Fries & Raina 2003; Hansen et al 2003; Gillard et al 2008; Lindstrom 2008)
	7 $\mu\text{g/ml}$	(Alippi 1995; de Ryke et al 2002; Govan et al 1999)
	9 $\mu\text{g/ml}$	(De Graaf et al 2001; Artunez et al 2004; Aliipe et al 2004; Gende et al 2008; Gende et al 2009)
	12 $\mu\text{g/ml}$	Lauro et al 2003)
	15 $\mu\text{g/ml}$	Alippi 1995; Pernal & Melathopolous 2006)
	30 $\mu\text{g/ml}$	Our survey
pipemedic acid	10 $\mu\text{g/ml}$	(Alippi 1995)
	20 $\mu\text{g/ml}$	(Alippi 1995; Pernal & Melathopolous 2006; Gende et al 2009; De Graaf et al 2001; Govan et al 1999; De Rycke et al 2002; our survey)

The OIE recommends 6-9 µg/ml for nalidixic and 10-20 µg/ml for pipimedic, and that seems to fit with most of the studies. It should be mentioned, however, that Alippi (1995) reports that *P larvae* can withstand 30 µg/ml of nalidixic acid and 20 µg/ml of pipimedic acid.

Unless there is a good reason otherwise, we should use 6 µg/ml for nalidixic acid and 20 µg/ml for pipimedic acid.

3. PCR primers

There are many listed methods for identifying AFB. Some of these are 'simple' methods like looking at the Brownian movement of AFB spores under a light microscope or using the Holst milk test; others are complicated like using fluorescent antibodies, biochemical tests (such as API50), or bacteriophage sensitivity. However, none of these methods are entirely reliable (de Graaf et al 2006), and increasing PCR testing is used to confirm the presence of AFB, as it is the cheapest and most reliable. Primers and techniques regarding PCR have improved to the level that Schuch et al (2001) report being able to detect as few as 10 spores per ml of honey, which is an extremely good level of detection.

There are a good number of AFB primers available. The first to be developed were those of Govan et al (1999), using 16D rDNA primers which produced a single amplicon, followed by those of Dobbelare et al (2001) that produced 4 amplicons. Both of these sets of primers are reported to be highly specific (they work for all strains and types of AFB, and not on any closely related species) (Dobbelare et al 2001; de Graaf et al 2006). However, neither set of these primers are able to distinguish between *Paenibacillus larvae larvae* and *Paenibacillus larvae pulvifasciens*. In light of the conclusions of Genersch et al (2005) and Genersch et al (2006) that the subspecies status should therefore be abandoned, leaving just a single species *Paenibacillus larvae*, this 'problem' is now moot. Before this conclusion was reached, however, a whole set of primers were developed that could distinguish between the two 'sub-species' (Alippi et al 2002; Piccini et al 2002; Kolwanski et al 2004; Alippi et al 2004; de Graaf et al 2006). It should now be regarded that these primers are capable of distinguishing between various strains or genotypes of AFB.

As the primers of Govan et al (1999) were found to be unable to detect AFB directly from honey (ie without culturing first) (Dobbelaere et al 2001), more and more sensitive primers have been developed in an effort to allow the direct testing of honey (without the laborious culturing). Alippi et al (2002) followed PCR with RFLP to improve sensitivity, Lauro et al (2003) used a nested PCR protocol, and all of Piccini et al (2002), Bakonyi et al (2003) and D'Alessandro et al (2007) developed primers reportedly sufficiently sensitive to allow for the direct testing of honey. In the case of the Bakonyi et al (2003) primers this increased sensitivity comes at the price of decreased specificity, with these primers amplifying the DNA of a range of similarly related species as well as that of *Paenibacillus larvae*.

As to which PCR primers we should use? The OIE list 3 sequences for PCR of AFB (Govan et al 1999, Dobbelaere et al 2001; Piccini et al 2002) and confirm that three of these are specific for AFB. All three have been used by used to confirm the presence of AFB in honey samples (e.g. Hansen et al 2003). These three are also listed by the review of De Graaf et al (2006) who also list a host of sequences used to distinguish *P larvae* 'subspecies' (or strains) (Alippi et al 2002, Alippi et al 2004, Piccini et al 2002, Kilwinski et al 2004, de Graaf et al 2006), as well as the novel method of Lauro et al (2003) for the PCR detection of AFB at very low levels. It should be noted that neither the OIE nor the multi-author review of de Graaf et al (2006) even mention Bakonyi et al (2003).

Therefore, unless there is a good reason otherwise, we should use either the primers of either Govan et al (1999) or Dobbelaere et al (2001) for the identification of **cultured** AFB colonies, and **not** the primers of Bakonyi et al (2003), **as is currently the case**. The reason is two-fold. Firstly, the primers of Govan et al (1999) and Dobbelaere et al (2001) appear to be the most general, and as we don't know what AFB strain we have, are mostly likely to respond to all AFB strains. Secondly, they are reported to be highly specific, and will not result in false positives, as the primers of Bakonyi et al (2003) are likely to do, by amplifying the DNA of related species.

4. Sample Preparation

A thorough review needs to be undertaken as to the laboratory procedures used for various types of samples to make sure that no mistakes are being made. What is important to consider is that entirely different procedures need to be used if the intent is to harvest and culture AFB spores (as from honey, pollen, wax, propolis, hive scrapings or adult bees); or if direct testing of these same samples is to be undertaken; or the direct testing of brood samples. It must be noted:

- There are no AFB vegetative cells in honey, pollen, wax, propolis, hive scrapings or adult bees; only AFB spores (Hornitsky 1988).
- If these spores are to be directly tested (rather than cultured, and the culture tested), then these spores have to be broken down before direct PCR analysis is possible.
- Larval remains are full of AFB vegetative cells and can hence be directly tested by PCR. They are often also full of AFB spores, which can be plated. However, if it is an 'early stage' diseased larvae, the infection might not have proceeded to the sporulating phase as yet, and there might not be any spores to culture (Dobbelaere et al 2001).

These are generalised procedures for sample preparation.

- a. Extraction from honey sample for plating (Alippi 1995; Schuch et al 2001; Piccini et al 2002).
 - Pre-heat to 45C
 - Mix with SDW or PBS
 - Centrifuge at 3000g for 30 minutes
 - Re-suspend pellet
 - 90C for 15 minutes
 - plate
- b. Preparation from cultured colony (Bakonyi et al 2003; Neuendorf et al 2004).
 - Mix in 0.5 ml PBS or SDW

- Centrifuge at 20 000g for 10 minutes
 - Re-suspend pellet
 - PCR
- c. Direct extraction from honey sample (Bakonyi et al 2003; Piccini et al 2002; Herman et al 1995).
- Pre-heat to 45C
 - 95C for 6 minutes
 - Centrifuge at 4000g for 30 minutes
 - Re-suspend pellet
 - DNA extraction with EDTA, microwaves or other method
 - PCR
- d. Direct extraction from a brood sample (Alippi et al 2002; Piccini et al 2002).
- Mix in SDW or PBS
 - 80C for 15 minutes
 - Mix thoroughly
 - Centrifuge at 825g for 5 minutes
 - Use the supernatant for PCR
 - Serial dilute to eliminate polymerase inhibitors (Piccini et al 2002).
- e. Extraction for plating from an adult bee sample (Lindstrom & Fries 2005; Gillard et al 2008).
- Crush
 - Sieve
 - Centrifuge at 27 000g for 10 minutes
 - Re-suspend pellet in SDW or PBS
 - 95C for 10 minutes
 - Serial dilution
 - Plate
- f. Extraction for plating from a wax sample.
- Place in water (1/10)
 - 90C for 10 minutes

- Add diethyl ether (1/9)
- Shake thoroughly
- Plate

As far as I can tell, the procedures that we are using are pretty much those given above (where appropriate), except for that of the brood sample. And this is potentially a critical distance. To the best of my knowledge, the brood sample procedure that we are following is from Bakonyi et al (2003), which is as follows:

- Pre-heat to 50C
- Shake
- Centrifuge
- Re-suspend pellet
- 80C
- Plate

Firstly, it should be completely unnecessary to plate a brood sample. It should always be directly analysed, so that results are available within hours. Any positive brood sample will have billions of vegetative AFB cells and there is no need for culturing. Secondly, if it a AFB infected larvae at the early stages of infection, it is quite possible that there will not yet be spores in the larvae. Hence, there are no spores to culture, and plating from this sample will result in empty plates, as we seem to be getting from some (presumed) AFB infected brood.

It is recommended that all procedures are checked against the above and the literature, and that the procedure for brood testing be changed so that direct PCR testing of these samples takes place.

5. Direct Testing

While larvae and other brood samples should **always** be directly tested with PCR, without culturing, efforts need to be made to eliminate the time-consuming, laborious and expensive culturing procedures for our large-scale honey screening as well. There is no reason why honey samples cannot also be

directly tested with results being available within a couple of hours at best (Piccini et al 2002; Lauro et al 2003; Bakonyi et al 2003; Alippi et al 2004), 24 hours at worst (D'Alessandro et al 2007).

There have been many attempts to detect AFB spores directly from honey samples, without plating. It was tested first by Alippi & Aguilar (1998) and then Dobbelaere et al (2001) without much success. Piccini et al (2002) then reported a method that could detect spores in honey in only a few hours, but this method has subsequently been shown to only work with very high spore loads and not to be practical for naturally contaminated honey (D'Alessandro et al 2007). All of Schuch et al (2001), Lauro et al (2003), Bakonyi et al (2003) and Alippi et al (2004) report success in detecting AFB spores directly from honey with a high degree of reliability in only a few hours. D'Alessandro et al (2007), however, express some concerns about these procedures when it comes to naturally contaminated samples, in that the DNA extraction procedures are considered insufficient, and have developed a very extensive spore de-coating, spore disruption and DNA purification process which they have shown to work on naturally contaminated honey and adult bee samples. The procedure is extensive and lengthy, but avoids polymerase inhibition problems, has conclusively been shown to work to a sensitivity of 20 spores per sample, and provides results in less than 24 hours.

It is recommended that the direct testing procedures of the above authors be tested and validated (by plating samples to see if positive results yield AFB colonies), and be incorporated in our programme.

Then, this procedure needs to be tested against known AFB positive honey that is irradiated to see if this honey also yields positive direct results.