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Swiss Expert Committee for Biosafety SECB

# Monitoring the emergence of antibiotic resistance associated with the use of streptomycin to fight fire blight

## Summary

### 1 Introduction

In January 2008, the Federal Office for Agriculture (FOAG) authorised the use of streptomycin to fight fire blight under controlled conditions, with the proviso to monitor the development of antibiotic resistance in the plots treated with the substance. Agroscope Changins-Wädenswil Research Station (ACW) started a monitoring programme, focusing on the cultivable bacterial flora in soil and plants, in particular on *Erwinia amylovora*, the agent of fire blight. On 18 December 2008, the FOAG renewed the authorisation for 2009, together with the requirement to continue the monitoring programme. A similar authorisation is now granted each year until an efficient alternative solution can be found.

The Swiss Expert Committee for Biosafety (SECB) underlined the need for a multidisciplinary monitoring programme, encompassing not only the plant and the soil aspects but also the potential development of antibiotic resistance and multiresistance in humans and animals. The SECB therefore launched an interdisciplinary project in cooperation with various federal agencies, research institutions and other experts[1]. Considering the recent advances made both in genomics and metagenomics, the expert group proposed a 3-year project divided into several modules. The purpose of the programme was primarily to apply state-of-the-art scientific methods in order to obtain an objective assessment of the real risks involved by the use of streptomycin to fight fire blight, rather than to find arguments against this treatment.

The programme was financed by several federal offices and other organisations[2].

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[1] Swiss Expert Committee for Biosafety (SECB), Federal Office for the Environment (FOEN), Federal Office for Agriculture (FOAG), Agroscope Changins-Wädenswil Research Station (ACW), Agroscope Reckenholz-Tänikon Research Station (ART), Federal veterinary Office (FVO), State Secretariat for Economic Affairs (SECO).

[2] Financial support was received from: SECB, FOAG, FOEN, FVO, ACW, AWEL (Amt für Abfall, Wasser, Energie und Luft - Kanton ZH). In addition, ACW and IVB (Institute of Veterinary Bacteriology – University of Bern) provided laboratory space and equipment, overheads and working power.
The programme was coordinated by Jean-Claude Piffaretti, Interlifescience, Massagno.

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# 2 Experimental assays and results

## 2.1 Module A: Evaluating the impact of streptomycin applications on environmental bacteria (plants and soil) and on resistance genes

This module was carried out by the Swiss National Fire Blight Competence Centre at ACW. Part of the research was performed within COST frameworks (European Cooperation in Science and Technology). The main experiments and results obtained are outlined below.

### 2.1.1 Perfecting the molecular tools needed to monitor the resistance genes present in the environment

In order to monitor the quantitative evolution of the resistance genes in cultivable and non cultivable microorganisms present in the environment, methods to extract DNA from different ecosystems (vegetal matrixes, soil) as well as methods to detect and quantify these sequences had to be optimised and validated. The resistance genes considered were *aph3 (strA)*, *aph6 (strB)* and *aadA* for streptomycin, and *tetB*, *tetM* and *tetW* for tetracycline. The data generated were normalised using the sequences coding for ribosomal RNA (16S rRNA).

### 2.1.2 Efficiency of streptomycin application to fight fire blight

A comparative analysis was performed in two orchards. In the first one (Neukirch, TG), 67% of the trees and 17% of theinflorescences were found to be infected with *E. amylovora* in the absence of streptomycin treatment, whereas the infection rates were only 20% and 1%, respectively, with 3 streptomycin applications. The results were even better in the second orchard (Lömmenschwil, SG), since the infection rate of the trees decreased from 100% to 13%, and that of the inflorescences from 14% to <1%. Thus, these assays, although on a limited scale, confirmed that timely application of the antibiotic greatly reduces fire blight.

### 2.1.3 Establishing a baseline before streptomycin treatments, monitoring resistance after the application of the antibiotic and evaluating the impact on bacterial communities

The assays were carried out in plots of several commercial and experimental orchards treated with streptomycin (according to the directives laid out in the authorisation) or with alternatives (the yeast *Aureobasidium pullulans* or water). Samples from plants and soil were analysed.

The main observations were as follows:

a. No streptomycin-resistant clones of the pathogen *E. amylovora* were found, either before or after antibiotic application. However, resistant clones were detected in the natural bacterial communities even in the absence of streptomycin treatments (e.g., *Agrobacterium, Pseudomonas, Aeromicrobium*, etc.).

b. Successive streptomycin applications had no impact on native bacterial diversity (cultivable and not cultivable microorganisms) in flowers, fruit, leaves or soil, at any site. Even worst-case exposure (application of 100 times the authorised dosage) had no impact on biodiversity in native bacterial soil communities.

### 2.1.4 Evolution of the streptomycin and tetracycline resistance genes after streptomycin applications

The assays performed during three successive years (2010-2012) covered three experimental apple orchards in the cantons of Thurgau and Zurich. Genes encoding streptomycin and tetracycline resistance were monitored in cultivable and non cultivable bacteria from flowers, leaves and soil. Each year, there were 3 applications of streptomycin or water (control). Samples were collected one day before the first application, one day and two weeks after the third application, and at harvest.

The main results obtained were as follows:

a. Most resistance genes were detected in the first samples (soil, flowers, leaves) of the three sites prior to any treatment, at high levels.

b. On the three sites, the relative increases of the abundance of resistance genes after the streptomycin applications, when observed, were occasional, inconsistent, and not reproducible from one year to the other.

c. The observed relative increases of the abundance of resistance genes after the streptomycin applications were transient and not detected in the initial samples of the following year.

d. In conclusion, in the precise experimental conditions of the trials, no permanent effects on the levels of streptomycin and tetracycline resistance genes were observed.

### 2.2 Module B: Evaluating the impact of streptomycin applications on the commensal flora of animals living in the vicinity of treated plots

This module was carried out by the Institute of Veterinary Bacteriology at the veterinary hospital of the Vetsuisse Faculty in Bern.

Two flocks (test and control) of five lambs and two ewes each were placed on two distinct pastures of approximately 2,000 $m^2$ each. Four applications were made by spraying streptomycin on the test pasture directly on the grass of two adjacent surfaces of 180 $m^2$ and 120 $m^2$. The antibiotic concentrations were 17.5 $mg/m^2$ and 12.0 $mg/m^2$, respectively, so as to mimic the gradient of streptomycin contamination resulting from the treatment of orchards. After the application, the sheep were confined to the sprayed area for 12 hours and thereafter were released into the whole pasture. Anal and nasal swabs were taken from each sheep of both flocks on day 0, 3 days after the first streptomycin application, 6 days after the third application, 2 days after fourth application, and at days 67 and 90. Two indicator bacteria were looked for in the samples collected: *Escherichia coli* in the anal swabs and *Staphylococcus* spp. in the nasal swabs. Susceptibility testing to several antibiotics was performed on the colonies isolated. The genetic nature of the streptomycin resistance was analysed by searching for the sequences *aph3* (*strA*), *aph6* (*strB*), *aadA*-, *ant(6)-1a*, and by sequencing the *rpsL* gene encoding the ribosomal protein S12.

The main results obtained were as follows:

a. Overall, 455 *E. coli* strains were tested. Before the streptomycin application, 16% of the strains originating from the control group and 15% of those from the test group, were resistant to the antibiotic.

b. On day 90, the stools of the sheep exposed to streptomycin contained 44% of resistant *E. coli*, whereas the rate was 17% in the control group. If the total number of the *E. coli* isolated since the first application was considered, then the exposed sheep harboured 40% of resistant *E. coli* and the control group 22% (p= 0.0001).

c. Multiresistance (mainly sulfamethoxazole, ampicillin, tetracycline, chloramphenicol) was found to be significantly higher in the exposed sheep (40%) than in the control group (24%, p= 0.0002). A few strains showed resistance to third-generation cephalosporins.

d. 184 *Staphylococcus* spp. strains were tested. Overall, from the first antibiotic application to the last sampling, only 8 streptomycin resistant strains were detected in the nasal cavities of the exposed sheep and none from the control group. When analysed time point by time point, the difference in occurrence of resistant *Staphylococcus* spp. was not significant. Only when the data of all time points were pooled together did the difference in strain occurrence become significant (*p* = 0.001). The 8 streptomycin resistant strains were also resistant to tetracycline, fusidic acid and tiamulin.

## 3 General discussion

The results obtained by these two projects are apparently divergent. In the orchards, in the experimental conditions of the assays, occasional and transient increases of the abundance resistance genes could be observed, however these increases could not be associated with the streptomycin applications. Above all, an enduring increase of the resistance could not be detected 6 to 7 months after the end of the annual treatments. These reassuring results can be related to the severe directives accompanying the authorisations to use streptomycin to fight fire blight. Thus, it is essential that this policy be pursued. Yet, one cannot exclude that, in the long term, streptomycin application will lead to a durable increase of resistance. This is why monitoring the soils of the treated orchards should also be continued.

Concerning the assay with the sheep, the results are difficult to extrapolate. To be sure, a significant increase of resistance was observed in the *E. coli* strains of the intestinal flora of the animals having pastured in the streptomycin-treated fields. However, four antibiotic applications were made, not three or only two, as specified in the yearly authorisations. In addition, the sheep were allowed to pasture on the sprayed grass for 12 hours, which is not allowed by the directives released with the authorisations. Thus, very likely, the sheep were exposed to larger streptomycin amounts than those permitted by the authorisations. Nevertheless, this increase of resistance should not be ignored, it should be read as a clear signal for caution.

## 4 Conclusions and statement

The results obtained by this research programme, as well as the monitoring carried out by ACW since 2008, have shown that, in the experimental conditions applied, the streptomycin treatment had an occasional and reversible impact on the level of antibiotic resistance in the microorganisms present in the soil, the leaves, the flowers and the fruits of the orchards treated. Although transient, this increase cannot be ignored, nor can the indicative signal provided by the emergence of resistance in the *E. coli* strains isolated from the stools of the sheep exposed to streptomycin. These observations, together with the streptomycin residues occasionally found in samples of honey and apples, clearly suggest that vigilance must be maintained. Should further authorisations to use streptomycin to fight fire blight be granted, it will be essential:

a. to maintain the present severe control measures accompanying the authorisations to use the antibiotic;

b. to continue monitoring resistance levels in the soil of the orchards treated.

In addition, it will be also important to evaluate the risks associated with the method and conditions of the streptomycin applications, with the aim of possibly reducing them. When spraying streptomycin, it would be wise to protect nearby surfaces very carefully, in order to avoid contamination by drift. Finally, particular attention should be paid to the protection of the workers spraying the antibiotic, who should be informed correctly about the risks involved in case of overexposure to the drug.

Nevertheless, the best way to alleviate concerns about the use of streptomycin to fight fire blight resulting in increased bacterial resistance to antibiotics would be to find an efficient alternative. This underscores the need to actively promote scientific research in this field, ,and to provide adequate funding for it. Encouraging results have already been obtained with the bacterium *Pantoea agglomerans* at ACW, and with inorganic substances, such as aluminium potassium sulphate [$KAl(SO_4)_2$], which is being introduced in Baden-Württemberg. Another interesting perspective would be the selection of varieties of fruit-trees with decreased susceptibility to *E. amylovora*. This may be achieved by classic hybridisation procedures or significantly accelerated using genetic engineering techniques.

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[†] Unfortunately, Kathrin Mühlemann passed away in November 2012.

Note: Many of the results of the programme have been published in international scientific journals or are in press.