FAO/WHO/OIE Round Table on the Use of Rev.1 Vaccine in Small Ruminants and Cattle

Organized by FAO and CNEVA
21-22 September 1995
CNEVA Alfort, France

B. Garin-Bastuji & A. Benkirane, Editors

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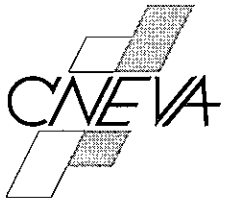

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Opening address

A. BENKIRANE
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First of all, I have pleasure conveying to you the best wishes from the part of Drs. Fujita and Cheneau, respectively Director of the Animal Production and Health Division and Chief of the Animal Health Service at the FAO of the UN. They are both convinced that the present FOA/WHO/OIE Round Table on the use of the Rev.1 vaccine in small ruminants and cattle is of paramount importance in that sense that it is expected to provide guidance with regard to future steps to be undertaken in the control of animal brucellosis worldwide and, especially in the 7 Near-East countries that have adhered to the FAO/WHO/OIE guidelines for the control of brucellosis in the Middle East established at Amman, in 1993. Hence, they are expecting with great interest the normative and practical implications of the recommendations of this meeting.

Among zoonotic diseases, brucellosis is universally recognized as the most important in the Near East. Although brucellosis due to *Brucella abortus* exists within the region, *B. melitensis* infection is more widespread, and hence has demanded an ever increasing attention by the national veterinary authorities. *B. melitensis* infection is the cause of repetitive outbreaks of abortion among sheep, goats, cattle and, to a lesser extent buffaloes and camels. Furthermore, the disease is widespread in the human population due to the fact that rural communities live in close contact with their livestock, drink and sell -in rural as well as urban areas- raw or slightly soured milk and milk products, which results in an alarming disease incidence rate among the human population of these countries.

Although the Rev.1 vaccine has been used for the control of brucellosis for more than 40 years, a lot of conflict, or at least discrepancies, are described in the literature with regard to both its safety and efficacy. Nevertheless, no alternative or safer vaccine has been found so far. Consequently, the use of Rev.1 vaccine is a central issue to the strategy for the regional brucellosis control programme for the Middle East. Accordingly, FAO, in collaboration with WHO and OIE, considers it imperative to seek an expert collective advice on the standardization of Rev.1 as a biological product. This is the fundamental objective of this Round Table meeting. So far, FAO has sought individual opinions from 7 leading experts in brucellosis and these can be summarized thus:

1. At present, there is no alternative to the mass vaccination using the Rev.1 vaccine if the aim is to control brucellosis in countries which still lack good veterinary services and a sound disease surveillance and monitoring system ;

2. The use of Rev.1 vaccine is associated with substantial risks that should be overcome through increasing public awareness, restricting adult vaccination to lactating and eventually late pregnant ewes (and goats ?) and the choice of a good quality vaccine to be certified by an internationally recognized vaccine quality control centre, and ;

3. There is a need for carrying out bacteriological diagnosis while implementing the project as well as a need for further applied research with regard to immunity and safety in vaccination of sheep, goats and cattle/buffaloes using Rev.1 vaccine or other potential vaccine candidates.

Therefore, the aim of this Round Table should be regarded as to debate the best ways of using the Rev.1 vaccine rather than a step back vis-à-vis our previous guidelines for brucellosis control. The Near East project, that we will have the opportunity to discuss tomorrow has now reached the final stage of its preparation and is being submitted to potential donors. Our hope is that this meeting will come up with some practical advice aiming at minimizing the potential risks associated with the use of such a live vaccine in the context of a major zoonotic disease.

It is unfortunate that neither of the two experts who drafted the above guidelines (namely Prof. Nicoletti and Dr. Mustafa) was able to attend this Round Table. However, others who were involved in the preparation of these guidelines are among us and, hopefully, their interaction with the group will be most fruitful and enable us to produce an up-to-date state of knowledge about the Rev.1 vaccine ; thus contributing to a better understanding of brucellosis epidemiology and control.

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Control programmes of *B. melitensis* infection in sheep and goats

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Summary
The prevention and control strategy of brucellosis in small ruminants is mainly based on the pathogenesis and the epidemiology of the infection. General non specific measures should be implemented, taking into account the long survival time of *Brucella* in the environment. Used exhaustively and on a long period in small ruminants, the Rev.1 vaccine induces a great decrease of the infection prevalence in animals and humans. Then, the level of prevalence may become compatible with the implementation of a control programme based on test-and-slaughter combined with vaccination of kids and lambs. The test-and-slaughter strategy (with no more vaccination) which implies an exhaustive identification of animals and flocks and a strict control of animal movements, requires also enough economical means to be implemented and to be lead to the eradication of the disease.

Due to its economic importance and the threat it poses to human health, it is necessary to take steps to fight brucellosis in animal populations.

The choice of a stratregy for the control and prevention of brucellosis in animals and humans is largely related to the pathogenesis and the epidemiology of this infection (the bacteria itself, the disease, host and farming conditions characteristics).

The control and prevention of brucellosis is achieved by respecting general rules of hygiene on the farms and the setting up, at a regional level, of a campaign against animal brucellosis which is based on sanitary measures (test and slaughter) and/or medical measures (vaccination). All these measures cannot be really effective without educating, training and mobilising the professional people and the general public in contaminated areas.

General preventive measures

In terms of general hygiene, various measures allow the risk of human contamination and the spreading of infection in and between the herds to be reduced.

On the farms, the principal sources of contamination are parturition and abortions issues. At the time of parturition it is essential that the animals be isolated and that all non-living matter and foetal tissues is destroyed. These steps are essential in limiting the spread of the infection to the rest of the herd, neighbouring farms and to the human population. It is recommended to disinfect buildings, equipment and materials on the farms, either by heat or by using various disinfectants including dilute hypochlorite solutions. The personnel must also disinfect any part of their body or clothing which has been in contact with infected animal tissue. Contaminated pasture should also be abandoned for a period of at least three months.

For field personnel, simple precautions (such as wearing protective clothing and, most important of all, disinfecting all equipment) must be taken in order to avoid contaminating people and the passive transmission of the infection from animal to animal, or to other production units.

Education, training and information

The control of brucellosis is of obvious interest to people involved in stockfarming and the general public - consumers of animal products. However, the inconvenience caused by vaccination and repeated testing campaigns can outweigh the medium and long-term advantages of control. It is therefore necessary to inform people involved in stockfarming of the advantages of the campaign (long lasting economic interest, elimination of the health risk to humans).

Among the other important functions of an information campaign there are:
- information on the different phases of the campaign
- motivating the people involved in stockfarming, workers in relevant industries and the general public in the different actions ;
- cooperation between physicians and veterinarians, public health and veterinary services ;
- training of qualified personnel ;
- informing professional people and the population at risk ;
- informing and motivating politicians and economic decision takers, in order to secure and maintain their support.

Table I gives the main subjects to be dealt with in an education campaign forming part of the fight against brucellosis.

Sanitary prophylaxis of the herds (Eradication by test-and-slaughter)

Faced with the relative ineffectiveness and the cost of treating livestock with antibiotics, eliminating the disease by slaughtering test-positive animals is one of the solutions used in the campaign against animal brucellosis. For this solution to be effective and economically feasible, the prevalence rate of herds, at the time that the campaign is set up, must be relatively low (<1% of the herds). The herds must also be of a reasonable size and the farms very closely supervised.

These plans are usually based on systematic and regular testing of the herds (individual testing or milk bulk-tank tesing , allergic skin-testing), notification of any cases of abortion and recording the movements of all animals. All the species exposed to the risk of infection must be tested. The existence of latent forms of

brucellosis means that the tests must be repeated before the herds can be certified free of brucellosis. Regular and continuous checking is then necessary to monitor the state of the herds which have been declared free of brucellosis. The free movement of animals (trade, transhumance, markets etc.) can only be allowed for those animals which have been individually tested and which come from herds certified free of brucellosis. Finally, these measures can only be envisaged, and hoped to be effective, in those cases where permanent identification of the animals and the livestock is employed.

Added to the above are the sanitary procedures which are applied when animals test positive or outbreaks appear, these require the immediate slaughtering of infected individuals or even whole herds which are infected. The herds should not be rebuilt until the buildings have undergone a rest period of at least 2 to 3 months, and no healthy animals should be allowed to graze on infected pasture during this period.

These steps are, of course, only applicable if adequate financial compensation is available to the farmers whose animals have been slaughtered. Subsidies should also be envisaged for farmers selling milk from herds certified free of brucellosis.

Table I. **Health Education Topics in the Control of Brucellosis**
(From Joint FAO/WHO Expert Comm. Brucellosis, 6th report, Tech. Report Series, WHO 1986)

GROUP	TOPIC	EXPECTED CONDUCT
Livestock breeders	· Concept of brucellosis · Characteristics of the disease · Damage done to human health · Damage done to animal production · Legislation backing the measures taken by the control agencies	Collaboration with the measures of prevention and control of brucellosis carried out by public health and animal health services
Personnel that work in direct contact with animals (shepherds, milkers,..)	· Concept of brucellosis · Characteristics of the disease · Damage done to human health · Affected species · Means of transmission to man · Preventive measures: - use of protective clothing - medical supervision - handling of live and dead animals - personal hygiene - environmental health	Application of the recommended measures in order to prevent the disease
Population	· Concept of brucellosis and its importance as a zoonosis · Ways of transmission to man · Symptomatology in man · Methods of prevention, especially related to milk or fresh cheese consumption	Positive attitude with respect to the care of their own health and aknowledgment of brucellosis as a human disease

Medical prophylaxis of herds (Immunization)

When the prevalence rate of infected herds is high (>5-10%), or when the dispersed nature of the farms does not allow sufficiently strict testing of the livestock and animals (extensive grazing, transhumance), medical prophylaxis based on mass vaccination is usually resorted to. At the present time it is the only way to reduce the rate of infection in areas with a high frequency of infected herds.

The most commonly used vaccines are S19 vaccine for cattle and Rev.1 for small ruminants. These vaccines have proved their effectiveness, for they considerably increase the resistance of animals to infection, then reduce the number of brucellosis related abortions. Thus these vaccines reduce in equal proportion the spread of the infection in and between the herds. They are cheaper to use and more effective than the inactivated vaccines. Wherever they are used, these vaccines can only achieve their maximum effectiveness as long as their immunogenicity characteristics have been verified and the continuity of the cold chain has been maintained between the production site and the vaccinated animal.

For the vaccination program to have a real and rapid impact on the frequency of infection (animal and human), it is necessary in the beginning, to vaccinate both young and adult animals.

The use of these vaccines does not generally, in itself, allow brucellosis to be "eradicated" at a regional level. Moreover, they often have serological after-effects which are more or less persistent, especially in adult animals. Several vaccine protocoles which reduce the duration of the after-effects are available, such as vaccinating at an early age, or vaccinating the adults with a reduced dose or even better via the conjunctiva. However, once the number of cases of brucellosis has been sufficiently reduced, due to an effective vaccination program (at least 80% of the animals), and only after a long period of generalised vaccination (5-10 years), is it possible to use a mixed medical/sanitary prophylaxis (vaccination of young animals, testing and slaughtering of adult animals).

The second phase of prophylaxis allows the rate of infection to be reduced and at the same time to maintain the minimum level of protection necessary relative to the pressure exerted by the infection.

Following this, when the frequency of infected herds is around 1%, which generally requires 5 to 10 years of systematic and generalized vaccination, eradication can usually only be achieved by the application of a strict sanitary prophylaxsis as described above.

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Live vaccines: virulence, immunogenicity/ protection[1] and safety: Historical, theoretical and practical considerations applied to the *Brucella melitensis* Rev1 vaccine.

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Summary

Natural immunity following a primary infectious disease is a medical observation of antiquity. It suggested that this immunity can be reproduced by artificial inoculation, in controlled conditions, of virulent material to susceptible naive people or animals, thus preventing secondary uncontrolled severe disease. These conditions, before Jenner, chosen to restrict extension and development of the disease were the inoculation site and the moment relatively to the natural host resistance. The low virulence for man of the Cowpox virus and the excellent immunity it induces against Smallpox (Variola) after a small restricted lesion to the inoculation site was the Jenner's seminal observation that had led to the vaccination concept. Other historical examples, Anthrax, Tuberculosis, Polio and Brucellosis illustrate the live vaccines problematic: a subtle and stable equilibrium between virulence and immunogenicity for a particular animal species.

In Brucellosis, *B. abortus* S19 and *B. suis* S2 have been obtained by spontaneous loss of virulence by *in vitro* transfers, while *B. melitensis* Rev1 was obtained by selective mutagenesis correlated with a loss in virulence while good immunogenicity was preserved. These strains and corresponding virulent standard strains, *B. melitensis* H 38 and *B. abortus* 544 have been studied in laboratory animal models, mostly mice and natural hosts, respectively goats and ewes, and cows.

Virulence, a genetically determinated strain characteristic, of which genes have not yet been identified, studied in mice is related to the ability to: multiply and persist (resist) *in vivo* and induce lesions. Vaccine strains can multiply at different rates (S19 > Rev1, S2) and persist for different times (Rev1 > S19 > S2). This parameter is conveniently expressed by the 50 % recovery time (RT50). The vaccine strains can also in certain circumstances induce lesions, *i.e.* placentitis.

Pathogenicity is the interaction between a particular strain and the host. It depends on the host species and circumstances of the contact: contaminating or inoculated doses, physiological status (pregnancy, immunodepression ...) and inoculation route. A peripheric route such as the conjunctival one that offers several lymph nodes

[1] In this paper, immunogenicity is used to express the ability to increase resistance to experimental inoculation, so to confer protection.

barriers before blood born dissemination helps restrict colonization to that nodes and so reduce pathogenicity.

Immunity in Brucellosis is for a large part not-host or strain specific. Against the high virulent *B. melitensis* strains in sheep and goats, a high and long lasting immunity requires a strong vaccine stimulation: from our INRA experimental data in mice and ewes, it can be estimated to be of at least one month. For this, in a particular system, the vaccine strain being supposed correct, we can adjust both parameters, route and dose, in order to optimize the immune response with no pathogenic effect. With strain Rev1 in ewes, the conjunctival route and dose 10^9 is the best combination in non pregnant ewes, whereas a lower dose, 10^8, may be preferred situation where pregnancy is not a controlled parameter.

Live vaccines story

From the beginning of the history of medicine, immunity following the course of some, if not all, contagious diseases was known, investigated and in the best circumstances used to confer - under medical control - a good and long lasting protection against relapses and recurrent epidemics.

The first well documented example was «Variolisation», the inoculation of the virulent variole (small pox) virus by the way of powdered old crusts harvested from infected humans on the skin of the arm. **On that site**, in normal good health people, the Pox lesion keeps localized while a life long immunity develops. However, in weak or "immunocompromized" people, extension to the face and the entire body may occur causing a severe disease with a high fatality rate. The discovery of Jenner (1796) that the Cowpox virus, an **attenuated poxvirus for man** was able to induce the same strong immunity without propensity to extend and/or provoke the variola disease was the historical landmark for the live vaccines birthday. Extensively used over the world, the original Jenner's virus was probably lost, substituted often unknowingly by other pox virus, evidencing the difficult problem of preservation of the vaccine strain, with its original properties, its subtle balance between virulence and immunogenicity.

A second well known example is Anthrax. Even before the Pasteur famous Pouilly-le-Fort demonstration (1881) of **the artificially attenuated** living vaccine wonderful efficiency, some audacious veterinarians knew how to immunize cows by inoculating virulent material from Anthrax lesions at the top of the tail: a necrotic lesion **restricted to that site** develops concomitantly with immunity. The Pasteur vaccine was obtained by culture at "dysgenic" temperature (42,5°C) which progressively selects a low virulent yet immunogenic bacterial population. This attenuated strain was considered stable. It was stable in fact in normal animal hosts, but can be reversed to virulence by passage on new born mice. Since the Keppie-Smith group research (1954) we know that virulence and immunogenicity are related to several exotoxins - one of which is a good protective antigen - so attenuation appears as the result of selection of a low secreting population in which some still toxigenic cells subsist. These cells can preferentially multiply in favourable circumstances, such as the newborn mice.

We can from this Anthrax example insist again on the two points: necessity for a living vaccine to be genetically stable ; advantage to prefer an inoculation site that restricts by itself and localizes the vaccine infection.

The antituberculous BCG vaccine - the worldwide most used vaccine - sets a third example. Obtained after hundred *in vitro* transfers (1910-1920) correlated with a progressive loss of virulence, the genetic basis of which are only now being studied. But modifications occured spontaneoulsy over the time in the strains preserved in official, international reference laboratories. After comparisons between these strains under the WHO auspices, it was again concluded that protection in man requires a low level of residual virulence - for the strain to survive long enough to stimulate immune protective mechanisms. Conversily, the intradermic route of inoculation should be preferred to avoid both systemic dissemination or granulomatous localized lesions. (i.v. inoculation may cause tuberculous type lesions in organs). In addition, this vaccine should not be administered - or only with great care - to immunocompromised people. Inactived, killed vaccines are not efficient at all against Tuberculosis.

The fourth example, vaccination against Polio, had taught us that live attenuated (Sabin, 1962) and inactivated (Salk, 1954) vaccines are both protective. But the live vaccine has the great advantage to be more efficient and easier to administer, by the oral route. The immunity is thus locally stimulated, on the proper site by which the virulent virus enters the body.

The brucellosis vaccines story

As soon as *Brucella* was recognized as the agent of the Malta Fever (Bruce, 1887) and the goat infection (Zammit, 1905), vaccines of different types have been tested, with usually poor results. It was soon evidenced however that the animal infection induces its own immunity: the acute infection revealed mainly by abortion does not usuallly relapse ; and, more important, that young animals exposed to contamination before sexual maturity are often "naturally" immunized, in that they would resist to abortive infection, but may remain infected and excretors. From this observation, premunition with virulent strains inoculated to calves was proposed and used for long at least in France, with the results that if abortion storms were prevented (at that time, *ca.*1950, up to 50 % of cows might abort in the first year of an epidemic), the disease was disseminated over the country.

So the introduction by Cotton, Buck and Smith (1930) of the *Brucella abortus* strain 19, a hypovirulent and good immunogenic strain, was the first and most important step in bovine Brucellosis vaccination. Because this vaccine has been, and still is, used worldwide, thousand of researches have been devoted to it, that can be summarized in five points:

- **Genetic stability:** no reversion to virulence has been observed, but loss of immunogenicity may occur (had occurred) without evidence from *in vitro* tests. Genetic backgrounds of virulence and immunogenicity being largely unknown (or are they in 1995 ?) great care, relevant to good bacteriological practices must be taken in preserving the original and master seeds.

- **Immunity** following natural infection or vaccination is only relative in that it may be overcome by high virulent strains or massive exposure.

- **Doses**: In calves (4-8 m. old), a normal "full" dose (60 x 10^9) induces a several years lasting immunity and a short lasting (6-12 m.) serological response. In adults, a « reduced » dose (5 x 10^9) is usually chosen to avoid a long lasting disturbing diagnostic tests response, with a somewhat lower but serviceable immunity.

- **Time and Route**. Pregnancy causing a state of higher susceptibility to both virulent and vaccinal strains of *Brucella* (the fetus which has by itself no immunity, is highly susceptible to blood born bacteria), vaccination during pregnancy is not recommended. If however this vaccination is required, it must be at reduced dose, or better by conjunctival administration.

- The **conjunctival** vaccination was indeed developed with in mind the aforementioned restricting effect of a peripheric site of inoculation. We observed incidentally that after a virulent conjunctival challenge, some cows may develop an immunity strong enough to get rid of the infection, without any serological response (Philippon, Plommet *et al.*, 1971). This vaccination procedure (route, dose, recall) was later on develop at INRA for cows with strain 19 (Plommet, 1976 ; Fensterbank *et al.*, 1979), then for ewes and goats with strain Rev.1 (Fensterbank *et al.*, 1982).

To summarize, the conjunctival vaccination induces a somewhat equivalent immunity than the classic subcutaneous one, with a limited serological response, and no - or a lower risk of abortion if administered during pregnancy. It can be then conveniently used in youngs and adults, even (if unavoidable) during pregnancy, and can be recalled as required.

While attenuation of virulence was naturally obtained for strain 19 by *in vitro* transfers, progresses in bacterial genetics in the fifties gave opportunities to select from a bacterial population particular strains cloned either at random or on selective characters (nutritional, antibiotic resistance etc.) expected to be linked by some traits to virulence.

Such was the *B. abortus* strain 45/20 (Mac Ewen, 1955): the rough mutant of the smooth virulent strain was expected to be both immunogenic and non agglutinogenic (by lack of the LPS-smooth antigen). The rough state however was non-stable and swiftly reverse *in vivo* to the S, virulent form. This same idea of a rough living strain has been and is still being developed in the USA with strain RB 51.

It is in this context that Elberg (Herzberg, Elberg, 1955) obtained the smooth Rev1 vaccine, by a triple mutation against streptomycin, of low virulence, good immunogenicity and good genetic stability - with no documented return to virulence.

Conclusions

From this brief historical survey of live vaccines, we should underline that in the fight between the agressor and the host - which leads to either disease or recovery and immunity - the subtle balance between both actors depends on five factors:
- genetic background of the agressor and its stability,
- animal host species. One cannot predict from one species to another one,
- host physiological situation: age, pregnancy, nutritional and/or immunological status,
- level of contamination or vaccine dose,
- route of contamination or vaccination.

We will see now how these points have been studied and can still be studied at the lab in the case of Brucellosis live vaccines.

The brucellosis problems

1. Pathogeny

- When a *Brucella* gets into contact with a susceptible host, it penetrates through a mucosa, reaches the regional lymph node where, depending on dose, virulence and immunity, it may be trapped then progressively destroyed (while inducing immunity) or it can escape to the blood circulation, colonize both professional scavengers organs, liver and spleen and can also reach, if highly virulent, all other organs of the body inducing corresponding symptoms.
- An equilibrium between both actors can result, leading progressively to cure, but particular events may also dramatically interfer with it.
- In human Brucellosis, an unstable equilibrium results in a periodic release of bacteria from the liver and the spleen, inducing the historical famous «undulant fever». This equilibrium can be broken experimentally (and therapeutically) by injection of an additional load of antigens or bacteria, the «rebound» phenomenon.

In animal and human Brucellosis bacteremia occuring during prengnancy can colonize the placenta thence the fetus, leading to abortion or to congenital infection of the newborn.

- In Brucellosis, immunity is mediated by both antibodies and T-cells. To oversimplify, one can say that antibodies are very active during the first stages of the infection, restricting its dissemination to lymph nodes and professional organs while the T-cells system is mainly assigned to the killing of intracellular bacteria. Vaccines must thus stimulate both arms, humoral and cellular and prepare the organism to promptly react to aggression. One should keep in mind that it takes at least one month for the vaccination or the infection itself to induce a significant, efficient immunity, a time long enough for the infection to spread causing severe lesions and disease. In contrast, standing immunity and/or the secondary (recall) immune response to infection or vaccination are active at once or after a very short period of time.

2. Virulence and models

Virulence is an intrinsic characteristic of a bacteria to multiply, survive and induce lesions (and symptoms) by direct (toxic) or indirect (host reponses) mechanisms in a particular host. It is a genetically determined character usually linked to one or several factors and genes, with a large scale of reponses from highly to low or no virulence. In contrast, pathogenicity is the result from the interaction between bacteria and host. In common language both terms are often confounded, when for example the animal species is implicit or stated, as in "*B. abortus* is not virulent (= pathogenic) for ewes". We should however keep in mind this difference: pathogenicity depends on the system, (1) the strain (with its virulence), the dose, the site of inoculation or contamination, and (2) the animal species and its particular susceptibility (pregnancy, intercurrent disease ...)
Generally speaking, *B. melitensis* strains are highly virulent (pathogenic) for goat, ewe, cow and man, while *B. abortus* are less virulent for man, goat, ewe but highly virulent for cow.
In the lab, Guinea pigs have been used for long as a good model for human and animal Brucellosis. They are still considered to study the disease (lesions...) and immunity, but a great variability in responses makes their use difficult, requiring a

great number per group to give valid results. Mice in contrast give very reproducible results (provided good care to experimental design were taken ... !), in particular for kinetic studies of virulent or vaccinal infection. I will show a few of our INRA 15,000 data of virulence and immunogenicity experiments to illustrate the Rev1 vaccine position.

3. Virulence in the mice model

The infection is determined and measured by counts of the *Brucella* inoculated in the meaningful organs, in particular the spleen, and/or liver, lymphnodes, placenta and fetus. Inoculation is done with an exactly calibrated challenge - eventually several spaced doses - by the appropriate route, i.v., i.p. or in the footpad, for the experiment. Counts performed on successive necropsied groupe give the kinetic of the infection ; counts performed at one particularly chosen time post challenge give an good index of virulence or of immunity.
Figures 1, 2, 3 and 4 show that infection, virulent or vaccinal, evolutes at short and long terms in a relatively constant manner, depending on the initial inoculation dose and virulence (Fig. 8). Clearly the ability for a strain to multiply *in vivo* is not the main factor of virulence, since the vaccinal strain S19 can multiply as much as the highly virulent strain H38. In contrast, the ability to resist to the immune mechanisms and to survive (and in the case of H38 to induce lesions and rebound) appears as the main factor of virulence. In addition, this survival parameter expressed for example by the 50 % recovery time (RT 50) is an important character for a vaccinal strain, since the multifactorial antibrucella immunity requires to be persistent at a high level a strong and long stimulation.

4. Immunity in the mice model

Immunity induced by vaccination reverses the kinetics of the infection comparatively to control (Fig.5), by reducing the growth of the virulent challenge strain and accelerating thereafter the decrease to recovery.
We used this model to study the mechanisms of immunity and to measure it. We have shown for example that in strictly standardized conditions, the difference between the two curves at day 15 is a good indicator of the immunogenicity - the value - of a vaccine.
From this observation, we developed our standard control method for *Brucella* vaccines (see Bosseray paper). The example in Figure7 shows a dose response curve to three doses of the Rev1 vaccine ; an excellent immunity was induced by the three doses with a slight but significative dose response. This result was expected since the recovery time of the vaccine is dose dependent (Fig.1), and that immunity depends on it (Fig.4).
This model had, in addition, evidenced the rebound phenomenon (Fig.6): a residual infection in the stage of recovery can be destabilized by administration of a new load of bacteria or antigen, *i.e.* brucelline. This point has evident implications for the control of vaccines (delay and differential count).

5. Immunity in the animal host model

Immunity induced by a vaccine can be, from a theoretical point of view, estimated in a laboratory or a natural host model by several methods, which depending of the stage - research, development or routine controls - have different objects and constraints. I will not discuss these points but simply recall that the variable parameters are the vaccine and the challenge dosis, and their combinations. Results

are the number (percent) of protected animals comparatively to controls, and/or the level of infection (as in the mice model) at a time appropriate after challenge.
We have at INRA used extensively this classic animal model in cows, ewes and goats (see the Verger paper) to compare the protection afforded by the vaccine at one or a few doses, by subcutaneous or conjunctival route, against a virulent challenge either *B. abortus* 544 or *B. melitensis* H 38 administered at mid pregnancy, that infects about 95 % of controls. Results, expressed by the percent of protected animals (no infection), usually in the range 50 - 60 % may look trivial. It corresponds yet to an increase of resistance of more than 100, as it can be computed from dose responses curves in goats and ewes (Renoux, 1957), and we know that such a vaccine if properly used in fields conditions confers a near total protection, since the level of natural contamination is usually lower than the experimental 95 % infecting challenge.

General conclusions

From the presented and our many other results, I can tentatively propose a general picture for *Brucella* strains and vaccines, that links virulence and immunogenicity on one hand (Table I) and virulence and pathogenic effects on the other. Because a good, strong and long lasting immunity against the highly virulent *Brucella melitensis* strains requires that the vaccine strain persists a time long enough in lymphoid organs, it should have a low but real residual virulence, linked to its ability to multiply and resist *in vivo*. As a consequence, pathogenic effects may result if the time, the site and the dose were not well chosen. This is why, concerning the Rev1 vaccine in ewes and goats, we proposed the conjunctival route at dose 10^9 in non pregnant animals, and 10^8 (if impossible to delay that vaccination) in pregnant ones.

References

- **PLOMMET M., PLOMMET A.M., 1981**. Ann. Rech. Vet. **12**, 345-351.
- **BOSSERAY N., PLOMMET M., DE RYCKE J., 1982.** Ann. Rech. Vet., **13**, 153-161.
- **PLOMMET M., PLOMMET A.M., 1988-1.** Ann. Rech. Vet, **19**, 65-67.
- **BOSSERAY N., PLOMMET M., 1990.** Vaccine, **8**, 462-468.
- **PLOMMET M., 1981**. Personal.
- **PLOMMET M., PLOMMET A.M., 1988-2.** Ann. Rech. Vet, **19**, 245-251.
- **PLOMMET M., PLOMMET A.M., 1976**. Personal.
- **VERGER J.M. *ET AL.*, 1985**. Vaccine, **13**, 191-196.
- **FENSTERBANK R. *ET Al.*, 1985.** Ann. Rech. Vet., **16**, 351-356.
- **FENSTERBANK R.*ET AL.*, 1982.** Ann. Rech. Vet., **13**, 295-301.
- **FENSTERBANK R; *ET AL.*, 1987.** Ann. Rech. Vet., **18**, 397-403.
- **HERZBERG M., ELBERG S.S., 1955.** J. Bact., **69**, 432-435.
- **ALTON G.G., 1985** in Verger J.M., Plommet M. (ed). *Brucella melitensis*. Nijhoff Pub. Dordrecht.
- **PHILIPPON A., PLOMMET M. *ET AL.*, 1971.** Ann. Rech. Vet., **2**, 147-157.
- **PLOMMET M., FENSTERBANK R., 1976.** Ann. Rech. Vet, **7**, 9-23.
- **FENSTERBANK R., PLOMMET M., 1979.** Ann. Rech. Vet., **10**, 131-139.
- **BOSSERAY N., 1978.** Br. J. exp. Path., **59**, 354-365.

Table I. **Relationship between *in vivo* behaviour of *Brucella* strains and virulence and immunogenicity**

Strains	*In vivo* ability to			Virulence	Immunogenicity
	Multiply	Resist	Cause lesion(1)		
H38	+++	+++	+++	+++	not relevant
544	+	++	++	++	"
Rev1	+	+	±	±	+++(2)
S19	+++	±	-	-	++(2)
S2	+	-	-	-	+

(1) depending on route and dose
(2) speculative since direct comparison between Rev1 and S19 against *B. melitensis* on ewes has not been done

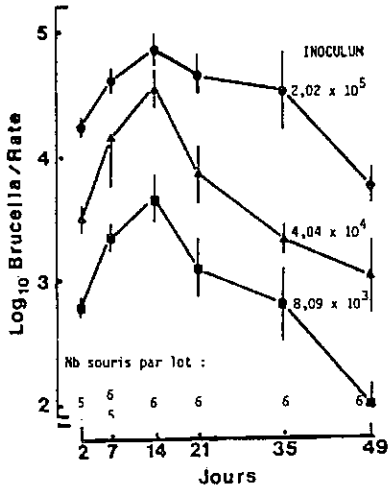


Fig. 1. **Short term kinectics of *Brucella* strain 544 infection in mice: relation to challenge dose (Plommet *et al.*, 1981).**

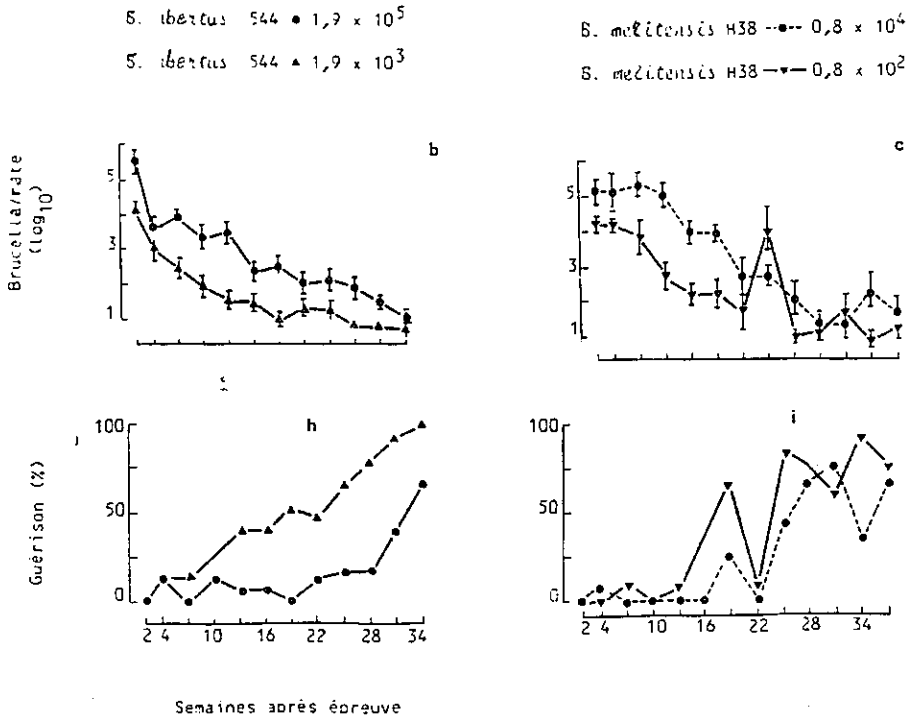


Fig. 2. **Long term kinetics of *Brucella* strain 544 and H38 infection in mice: spontaneous cure occurs progressively with time at different rate, reflecting the difference of virulence. Rebound can be observed with high virulent strain H38 (Bosseray *et al.*,1982).**

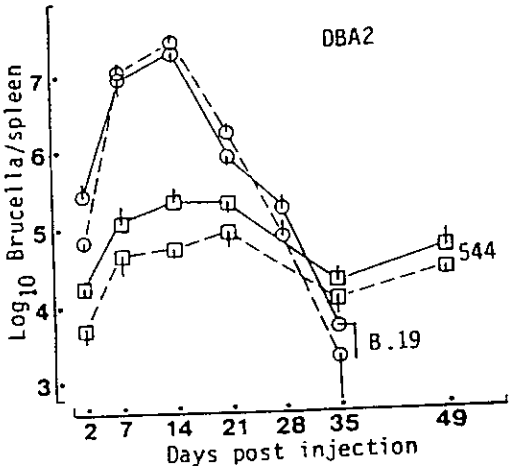


Fig. 3. **Comparative kinetics of *Brucella* virulent strain 544 and vaccinal strain 19: S19 multiplies first rapidly before being eliminated by the immune response while 544 multiplies only slowly but resists (Plommet *et al.*, 1988-1).**

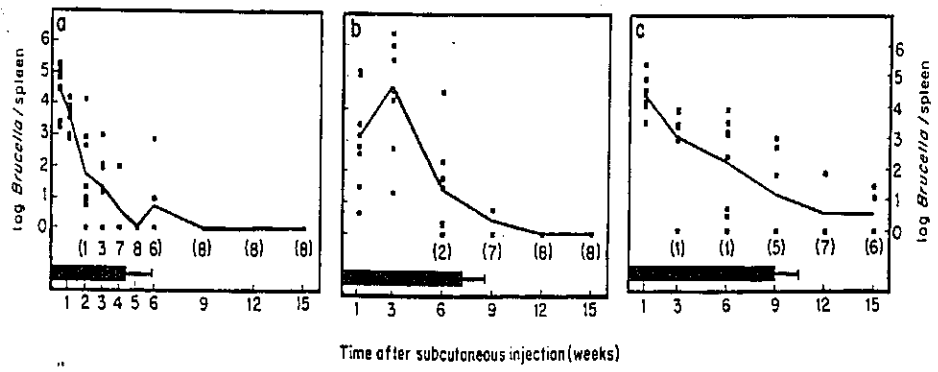


Fig. 4. **Long term comparative kinetics of the three vaccinal strains. S19 only multiplies notably the first two weeks. Resistance to the immune response, expressed by the survival or persistance of the strain in 50 % of mice (recovery time 50 %, RT 50) indicates large differences between the strain (S2 < S19 < Rev1). While short term immunity induced in mice by the three strains is about equal, long term immunity tested against the highly virulent strain H38 is lower with vaccine S2 (Bosseray *et al.*, 1990).**

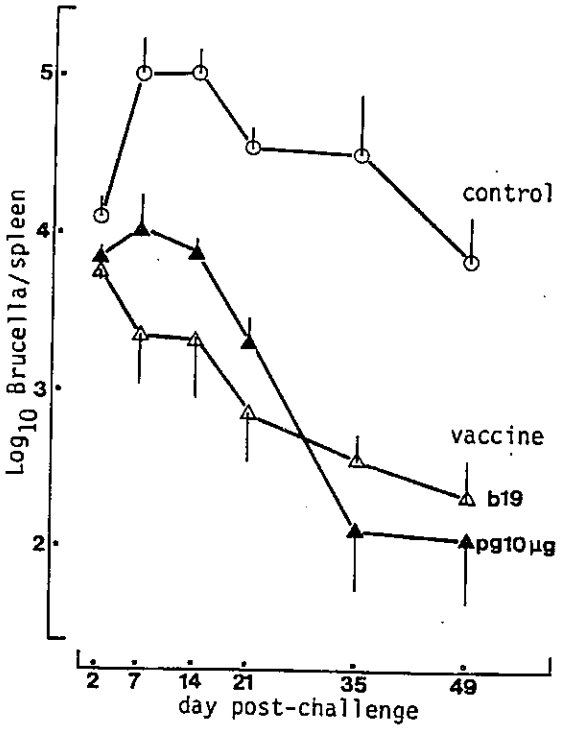
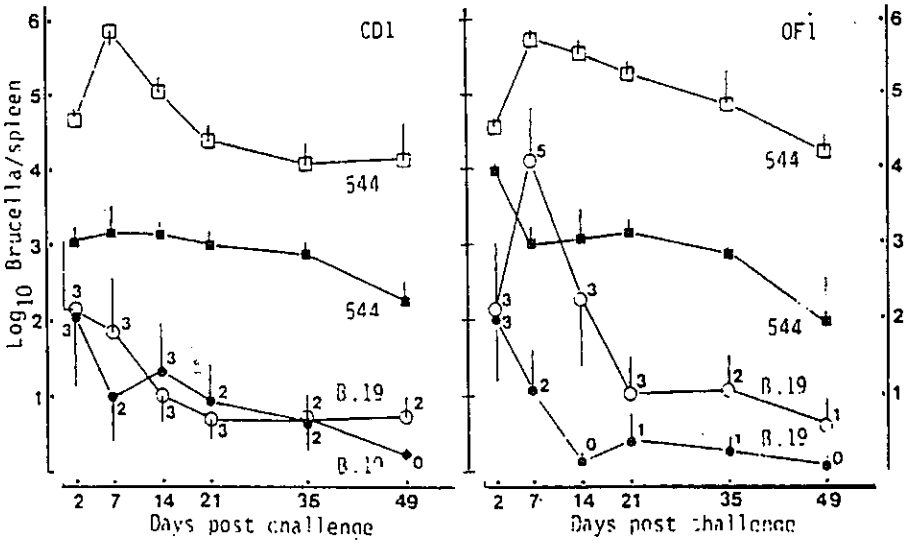


Fig. 5. **Kinetics of *Brucella* 544 infection in control and vaccinated mice; immunity reverses the evolution of infection and accelerates the recovery process (Plommet *et al.*, 1981).**



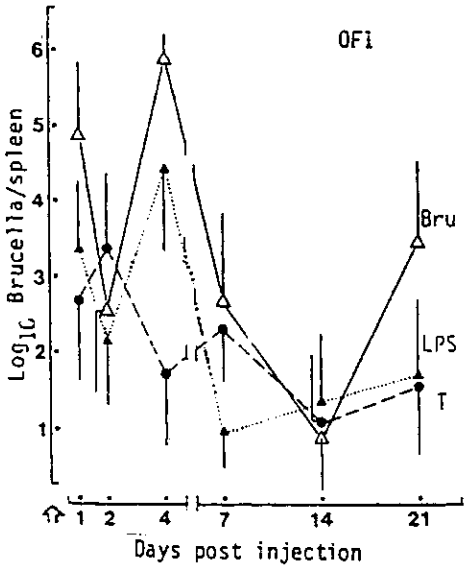


Fig. 6. **The rebound phenomenon: a stabilized infection in the process of cure can be destabilized by an additional administration of a live or a chemical antigen (*i.e.* Brucelline). In this figure, strain 19 on the way of cure was reactivated by the challenge strain 544. This has implications in the control of vaccines (Plommet *et al.*, 1988.2).**

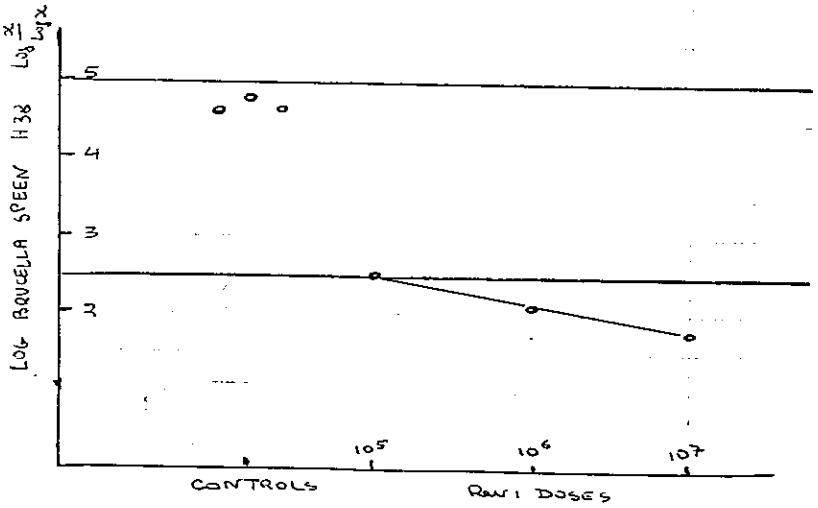


Fig. 7. **Immunity conferred by Rev.1 vaccine and tested in mice by a *Brucella* H38 challenge,1 x 10^4, is excellent and slightly dependent on the vaccine dose (Plommet, 1976).**

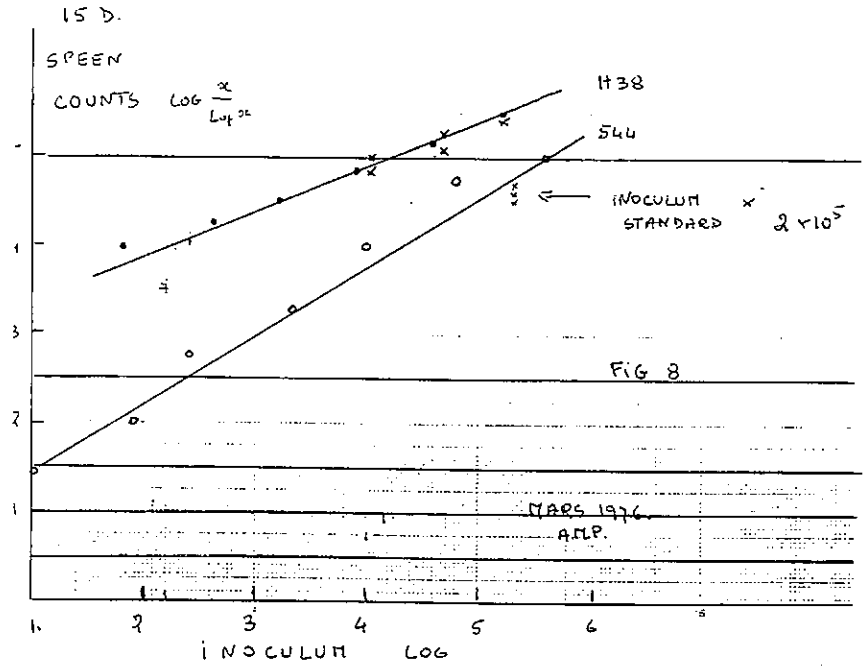


Fig. 8. **Challenge doses response curves in mice, 15 days post i.p. challenge, with strains *B. abortus* 544 and *B. melitensis* H38 (Plommet, 1976).**

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Efficacy and advantages of the Rev.1 conjunctival vaccine against *B. melitensis* infection, as evaluated in standard controlled conditions

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Summary
Brucella melitensis strain Rev.1 (Rev.1) is recognized all over the world as the reference vaccine for protecting sheep and goats against *B. melitensis* infection. The classical administration of the vaccine by the subcutaneous route unfortunately may cause some systematic disturbances (abortion) and long-lasting post-vaccinal serological responses. Conjunctival administration of Rev.1, which restricts colonization of the vaccinal strain to the head lymph nodes, induces immunity as good as by the s.c. route and limits the serological response to 4-6 months after vaccination. The conjunctival Rev.1 is moreover safer for the pregnant female, for man and the environment and, in practice, easier and faster to perform. These advantages of the conjunctival administration over the classical subcutaneous injection of the vaccine brings Rev.1 closer to the ideal vaccine and makes its use fully compatible with test and slaughter control programmes.

Brucella melitensis strain Rev.1 (Rev.1) has proved to be a very effective vaccine against sheep and goat brucellosis (Alton and Elberg, 1967; Elberg, 1981). However Rev.1 has 2 drawbacks when injected subcutaneously (s.c.) at full (standard) dose, *i.e.* 1-2 x 10^9 viable bacteria enumerated as colony forming units (c.f.u.): it may induce long-lasting serological responses (Fensterbank *et al*, 1982) which do not allow discrimination between infected and vaccinated animals, and, if injected during pregnancy, it may cause abortion in pregnant ewes and goats (Alton and Elberg, 1967; Elberg, 1981).
The conjunctival route was first developed in cows (Plommet and Plommet, 1976), then in ewes (Fensterbank *et al*, 1982 and 1985) and goats (Fensterbank *et al*, 1987), initially to limit the serological response, which on test and slaughter control programmes may disturb the diagnostic tests. Regarding Rev.1, it soon occurred that the conjunctival Rev.1 conferred as high and durable rates of protection against sheep and goat brucellosis as with the classical s.c. route, and moreover was safer for the pregnant female, for man and the environment and, in practice, easier and faster to perform. This presentation is aimed at emphasizing the efficacy and the advantages of the conjunctival administration of Rev.1 for the vaccination of sheep against *B. melitensis* infection. Correspondent data for goats, being very similar, will not be presented here.

1. The conjunctival Rev.1 confers to the ewe a high and durable immunity against *B. melitensis* infection

We recently compared the efficacy of Rev.1 and *Brucella suis* strain 2 live vaccines (Verger *et al*, 1995). The efficacy of both vaccines in protecting sheep against *Brucella melitensis* infection was evaluated by clinical and bacteriological examination of ewes vaccinated conjunctivally with a dose of 1×10^9 c.f.u. when four-month-old, and then challenged with 5×10^7 c.f.u. of the *B. melitensis* virulent strain 53H38 (H38) at the middle of the first or second pregnancy following vaccination . The results concerning Rev.1 are presented in Tables 1 and 2. Animals were considered as protected when no abortion, no excretion of the challenge strain and no infection at slaughter had occurred. The percentages of protection in Rev.1-vaccinated groups, challenged either during first (80%) or second (62%) pregnancy were significantly different ($p<0.001$ and $p<0.05$ respectively) when compared to the relevant unvaccinated control groups (In contrast no significant difference in protection was found between the S2-vaccinated and control groups). This experiment clearly confirms the excellent efficacy of the conjunctival Rev.1 against *B. melitensis* infection in sheep, which is quite comparable to that induced by the subcutaneous administration of the vaccine, as already demonstrated by Fensterbank *et al* (1982, 1985).

2. The administration of Rev.1 by the conjunctival route significantly limits the serological response which, in animals vaccinated at 4 months, is negative again in less than 4-6 months after vaccination.

The kinetic curve of Complement Fixation Test (CFT) responses to Rev.1 conjunctival vaccination (1×10^9 c.f.u. at 4 months) is presented in Figure 1 for a group of ewes that were then challenged with 5×10^7 c.f.u. of the *B. melitensis* virulent strain H38 at the middle of the second pregnancy following vaccination (Verger *et al*, 1995). Responses were considered as positive if 25% or more fixation occurs at 1:4 or more serum dilution. A similar curve (data not shown) was observed for Rose Bengale Plate Test (RBPT) responses. This figure clearly confirms the short-lasting serological response previously observed after conjunctival vaccination either with Rev.1 (Fensterbank *et al*, 1982, 1985, 1987) or *B. abortus* strain 19 (Plommet and Plommet, 1976) vaccines.

3. The administration of Rev.1 by the conjunctival route improves the safety of the vaccine for the pregnant ewe.

The safety is however not absolute and, as shown in standard controlled conditions, clearly depends on both time of pregnancy and dose of vaccine (Table III from Jiménez de Bagüès *et al*, 1989 and Table IV from Zundel *et al*, 1992). The placenta appeared to be particularly susceptible to Rev.1 infection when 1×10^9 c.f.u. of the vaccine was administered by the conjunctival route to ewes at mid-pregnancy (Table IV).

Rev.1 conjunctival vaccination of sheep in field conditions is however not known to produce such severe clinical effects. No abortion was recorded among 1039 pregnant ewes from 3 field flocks, conjunctivally vaccinated with 1×10^9 c.f.u. Rev.1 (Michel R., personal communication). Similar differences in susceptibility to a *Brucella* conjunctival challenge have already been reported between pregnant cows from an experimental station and others purchased from field herds (Goode *et al*, 1957) Although there is no satisfactory explanation for these differences, it may be assumed that in experimental and well-controlled conditions, animals are more "naive" towards bacterial infections and therefore *a priori* more susceptible to a

virulent challenge or a living vaccine. Whatever that may be, in the commercially available conjunctival Rev.1 ("Ovirev", Vétoquinol, Lure, France) each vaccinal dose comprises 1 x 10^8 c.f.u. Rev.1 which is much more safe for the pregnant ewe at mid-pregnancy (Table IV) and as effective as 1 x 10^9 c.f.u. (Fensterbank *et al*, 1985).

4. The risk of environmental contamination with Rev.1 due to the conjunctival administration of the vaccine is negligible.

To evaluate this risk, 22 pregnant ewes were kept in close contact in the same fold. When they were at mid-pregnancy, eleven of them were conjunctivally vaccinated with 1 x 10^9 c.f.u. Rev.1 and the others remained unvaccinated as "contact" controls. The local persistance and the diffusibility of Rev.1 in vaccinated ewes and the reaction of unvaccinated ewes to the conjunctival administration of Rev.1 in vaccinated ones were evaluated by clinical, bacteriological and serological examination (Zundel *et al*, 1992).

As shown in Table V, in the days following vaccination, Rev.1 was isolated, in very low quantities, from ocular, nasal and buccal swabs in about 50% of vaccinated ewes and disappearead in 7-15 days after vaccination. Rev.1 was never recovered from corresponding swabs in unvaccinated contact pregnant ewes which also remained serologically negative until the vaccinated animals began to abort (Fig. 2). During the abortion period, unvaccinated controls ewes were weakly contaminated in spite of the very close contact with vaccinated animals: no abortion occurred, no Rev.1 was recovered from the vagina or the milk (Table V) and only 1 ewe was found to be weakly positive to the CFT (Fig. 2). From these results it may be assumed that the diffusion of Rev.1 in the environment at the moment and in the days following the conjunctival administration of the vaccine is probably negligible..

5. The conjunctival vaccination is in practice easier, faster to perform and safer (no needle) than the subcutaneous administration.

All who experienced (with an objective view) the conjunctival vaccination in field conditions quite agree on these 3 practical advantages of the method. Safety is of particular importance since when Rev.1 is administered by the s.c. route, the risk for man of accidental injection and therefore infection is not negligible (Blasco and Diaz, 1993). In France, in areas where conjunctival Rev.1 vaccination was used on a large scale, cases of human contamination by the vaccine were no longer reported.

Conclusion

What should be an ideal vaccine ?

(1) effective but without inducing a long-lasting vaccinal infection

(2) independent of the usual tests for the immunological diagnostic of brucellosis

(3) with no limitation of its use, *e.g.* in pregnant animals

(4) safe for man when performing the vaccination

The conjunctival vaccination which induces immunity as good as by s.c. route, with no or less systematic disturbances (abortion) and more safety for man, and which limits the serological response, brings Rev.1 closer to the ideal vaccine as defined above and makes its use fully compatible with test and slaughter control programmes.

References

- **ALTON, G.G. AND ELBERG, S.S.** Rev.1 *Brucella melitensis* vaccine. A review of ten years of study. *Vet. Bull.* 1967, **37**,793-800

- **BLASCO, J.M. AND DIAZ, R.** *Brucella melitensis* Rev.1 vaccine as a cause of human contamination. *Lancet* 1993, **342**, 805

- **ELBERG, S.S.** Rev.1 *Brucella melitensis* vaccine. Part 2, 1968-1980. *Vet. Bull.* 1981, **51**, 67-73

- **FENSTERBANK, R., PARDON, P. AND MARLY, J.** Comparison between subcutaneous and conjunctival route of vaccination with Rev.1 strain against *Brucella melitensis* in ewes. *Ann. Rech. Vét.* 1982, **13**,295-301

- **FENSTERBANK, R., PARDON, P. AND MARLY, J.** Vaccination of ewes by a single conjunctival administration of *Brucella melitensis* Rev.1 vaccine. *Ann. Rech. Vét.* 1985, **16**: 351-356

- **FENSTERBANK, R., VERGER, J.M. AND GRAYON, M.** Conjunctival vaccination of young goats with *Brucella melitensis* strain Rev.1. *Ann. Rech. Vét.* 1987, **18**, 397-403

- **GOODE, E.R., MANTHEI, C.A. AND AMERAULT, T.E.** Relationship of age to susceptibility of non-vaccinated cattle to *Brucella abortus*. *Am. J. Vet. Res.* 1957, **18**, 279-282

- **JIMENEZ DE BAGUES, M.P., MARIN, C.M., BARBERAN, M. AND BLASCO, J.M.** Responses of ewes to *B. melitensis* Rev.1 vaccine administered by subcutaneous or conjunctival routes at different stages of pregnancy. *Ann. Rech. Vét.* 1989, **20**, 205-213

- **PLOMMET, M. AND PLOMMET, A.M.** Vaccination against bovine brucellosis with a low dose of strain 19 administered by conjunctival route. *Ann. Rech. Vét.* 1976, **7**, 1-8

- **VERGER, J.M., GRAYON, M., ZUNDEL, E., LECHOPIER, P. AND OLIVIER-BERNARDIN, V.** Comparison of the efficacy of *Brucella suis* strain 2 and *Brucella melitensis* Rev.1 live vaccines against a *Brucella melitensis* experimental infection in pregnant ewes. *Vaccine* 1995, **13**, 191-196

- **ZUNDEL, E., VERGER, J.M., GRAYON, M. AND MICHEL, R.** Conjunctival vaccination of pregnant ewes and goats with *Brucella melitensis* Rev.1 vaccine: safety and serological responses. *Ann. Rech. Vét.* 1992, **23**, 177-188

Table I. **Efficacy of the conjunctival Rev.1 vaccine against a *B. melitensis* experimental infection in pregnant ewes**
(from data in: Verger *et al*, 1995, Vaccine, 13, 191-196)

* Vaccination: 1 x 10^9 c.f.u. conjunctival Rev.1 at the age of 4 months
* Challenge: 5 x 10^7 c.f.u. H38 at 76 days of the first pregnancy following vaccination
* Slaughter: 4-6 weeks after delivery

	Rev.1 vaccinated	unvaccinated controls
No. of ewes	15	16
No. of abortions (%)	1 (7)	16 (100)
No. of excretors [a] (%)	3 (20)	16 (100)
No. of infected carcasses at slaughter (%)	2 (13)	16 (100)
No. of protected animals [b] (%)	12 (80)	0 (0)

[a] Positive cultures from milk and/or vaginal swabs and/or non-viable lambs

[b] Animals were considered to be protected when no abortion, no excretion of the challenge strain and no infection at slaughter occurred

Table II. **Duration of the immunity conferred by the conjunctival Rev.1 vaccine against a *B. melitensis* experimental infection in pregnant ewes** (from data in: Verger *et al*, 1995, Vaccine, 13, 191-196)

* Vaccination: 1 x 10^9 c.f.u. conjunctival Rev.1 at the age of 4 months
* Challenge: 5 x 10^7 c.f.u. H38 at 76 days of the first pregnancy or second pregnancy following vaccination
* Slaughter: 4-6 weeks after delivery

	Challenge during	
	first pregnancy	second pregnancy
No. of ewes	15	13
No. of abortions (%)	1 (7)	3 (23)
No. of excretors [a] (%)	3 (20)	4 (31)
No. of infected carcasses at Slaughter (%)	2 (13)	3 (23)
No. of protected animals [b] (%)	12 (80)	8 (62)

[a] Positive cultures from milk and/or vaginal swabs and/or non-viable lambs

[b] Animals were considered to be protected when no abortion, no excretion of the challenge strain and no infection at slaughter occurred

Table III. **Safety for the pregnant ewe of the Rev.1 vaccine administered by either the subcutaneous (sc) or the conjunctival (conj) route, in early and late pregnancy**(Jiménez de Bagüès *et al*, 1989, Ann. Rech. Vét., 20, 205-213)

	Vaccination during pregnancy at:			
	55 days		120 days	
Route	sc	conj	sc	conj
Dose	1-2 x 10^9	5 x 10^8-1.8 x 10^9	10^9	10^9
No of ewes	16	17	11	9
No of excretors of Rev.1 (%)	13 (81)	7 (41)	10 (91)	2 (22)
No of abortions (%)	11 (69)	2 (12)	1 (9)	0 (0)

Table IV. **Safety for the pregnant ewe of the Rev.1 conjunctival vaccine administered at mid-pregnancy. Influence of the dose**
(from data in: Zundel *et al*, 1992, Ann. Rech. Vét., 23, 177-188)

	Conjunctival vaccination at mid-pregnancy (75 days)	
Dose	1×10^8	1×10^9
No of ewes	5	11
No of excretors of Rev.1 (%)	1 (20)	10 (91)
No of abortions (%)	1 (20)	10 (91)

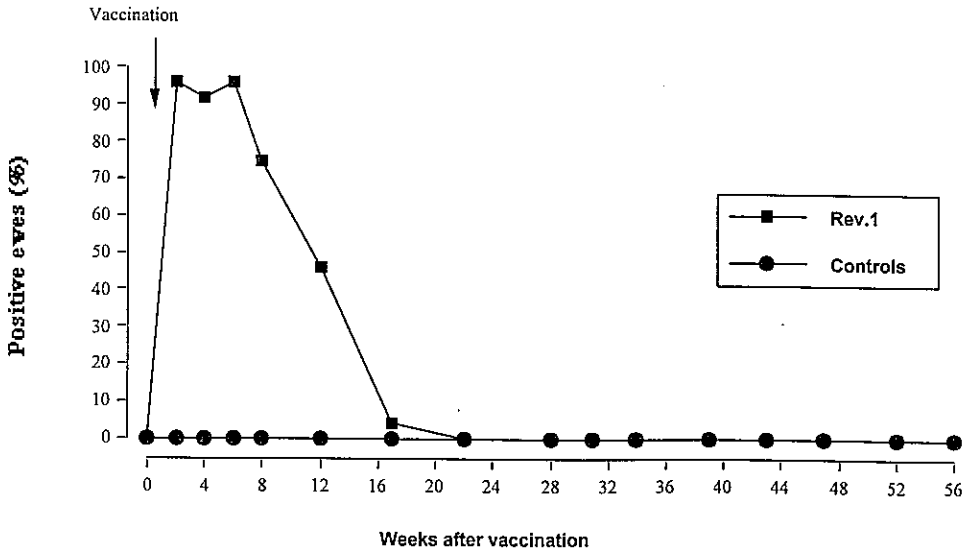

Fig. 1. **Post-vaccinal serology (CFT) of ewes conjunctivally vaccinated at 4 months with 1×10^9 c.f.u. Rev.1**

Table V. **Evaluation of the risk of environmental contamination by Rev.1 conjunctival vaccination**
(from data in: Zundel *et al*,1992, Ann. Rech. Vét., 23, 177-188)

	22 ewes at mid- pregnancy	
	11 conjunctivally vaccinated with 1 x 10^9 Rev.1	11 "contact" unvaccinated
Local persistence of Rev.1 [a]	+ (no more than 15 days) in about 50% of ewes	- (serology negative)
Mean duration of pregnancy	132 days	147 days
No of excretors of Rev.1 (%)	10 (91%)	0
No of abortions (%)	10 (91%)	0
Viable lambs (%)	6	100

[a] As evaluated by culture from ocular, nasal and buccal swabs in all animals, vaccinated and contact

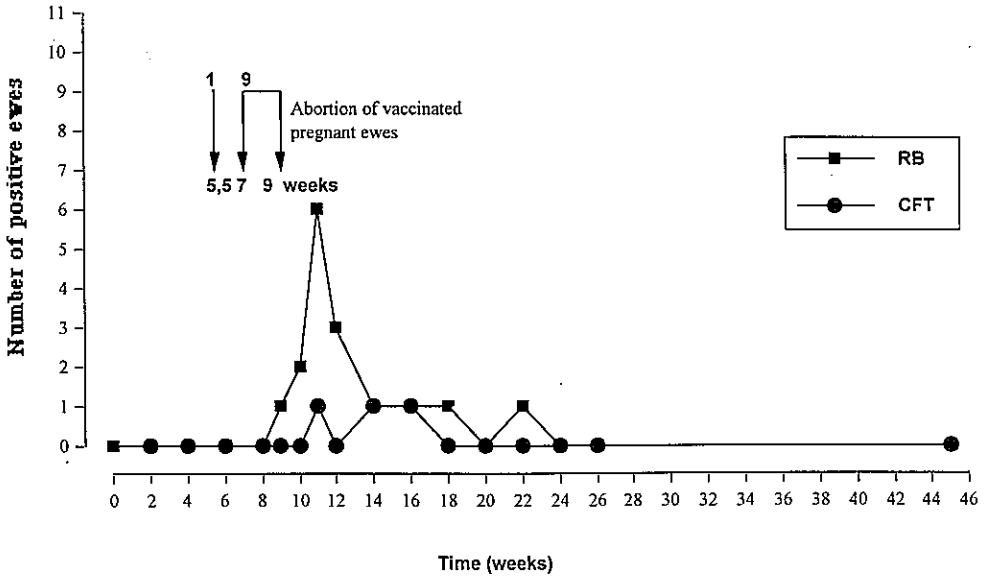

Fig. 2. **Serology (CFT and RBPT) of unvaccinated pregnant ewes kept in close contact with pregnant ones vaccinated at mid-pregnancy with 1x10^9 c.f.u. Rev.1**

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Brucella melitensis Rev.1 vaccine: stability of markers, residual virulence and immunogenicity in mice.

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Summary

Brucella melitensis Rev.1 (Rev.1) attenuated living vaccine is still the most efficient one against *B. melitensis* infection of sheep and goats. The strain was used in many countries with fluctuating practical results extending from excellent to doubtful protection without clear explanation. This laboratory developed and standardized mouse tests for control of living or killed anti*Brucella* vaccines that revealed that Rev.1 national strains from different countries may differ from the original Elberg strain. We extended the observation to commercial vaccines. As some vaccines were phase-dissociated at primary isolation, 16 characteristic substrains in different colonial phases were purified to analyse: (1) stability of the *in vitro* characteristics (size of colonies, phase, resistance to penicillin and streptomycin) and the *in vivo* behaviour in mouse models for residual virulence and for immunogenicity, after subculture, lyophilization or passage in mice, (2) the predictive significance of *in vitro* characteristics for *in vivo* behaviour.

Strains showed a rather good stability in *in vitro* tests after storage or passage in mice. Variations of the limits of resistance to penicillin and streptomycin after storage or passage did not generally exceed a two-fold dilution and may then be considered a stable characteristic for Rev.1 strains. *In vitro* tests had no clear-cut predictive value for residual virulence and immunogenicity, except for phase-dissociation which might be taken as a presumptive test for quality of vaccines. *In vivo* tests for residual virulence and immunogenicity considered together provided good discrimination of strains. They showed that a S-phase strain (normal state for Rev.1 strain) with a low residual virulence may induce a normal protection in mice.

Significance of *in vivo* mouse results for vaccinal activity in natural hosts is questionned. Recently published results in mice and ewes suggest that the residual virulence, that is the ability of the vaccine strain to survive in the host, is a simple test and a good indication of the vaccinal value of the strain. Thus to be efficient in the field against *B. melitensis* infection, Rev.1 vaccines must have both the immunogenicity and residual virulence of the reference strain. As the success of vaccination campaigns relies first on the quality of the vaccine used, Rev.1 production should be strictly controlled by independent official laboratories. The use of the reference strain delivered by national laboratory, systematic control of master seed lots before the first commercial production and random routine quality controls of commercial batches should deliver good Rev.1 vaccines in the field.

Introduction

Among the killed and live vaccines developed long ago against brucellosis, *Brucella melitensis* Rev.1 (Rev.1) attenuated living vaccine is still the most efficient one against *B. melitensis* infection of sheep and goats. Rev.1 vaccine was obtained by Elberg (1957) from the virulent strain 6056 by a two-step mutation with streptomycin for: (1) resistance + dependence and (2) reversion to independence. As genetic markers for attenuation are not yet identified, it still should be differenciated from wild *B. melitensis* strains by three characters: (1) slow growth and small smooth phase colonies ($\leq$ 1.2 mm) on agar medium, (2) resistance to 2.5 µg/ml streptomycin, (3) inhibition by 5 IU (3 µg/ml) penicillin (Alton et al, 1967). Millions doses of the strain have been used in many countries with practical results fluctuating from excellent to uncertain protection without clear explanation for these differences (Alton, Elberg 1967; Elberg 1981).

This laboratory has developed and standardized mouse tests for control of living or killed anti*Brucella* vaccines (Bosseray, Plommet 1976, 1983, 1990; Plommet, Bosseray 1977, 1984; Bosseray et al. 1984). These tests had revealed that some Rev.1 (national) strains from different countries might differ from the original Elberg strain (Bosseray 1985). We extended this observation to commercial vaccines. As some vaccines were phase-dissociated at primary isolation, characteristic substrains of different colonial phases were purified to analyse:
(1) the stability of purified strains controlled by *in vitro* characteristics and *in vivo* behaviour in mouse models for residual virulence, expressed by the recovery time 50% (RT50), and for immunogenicity, that is the ability of the vaccine to confer a good protection against a standardized challenge, after subculture, lyophilization or passage in mice,
(2) the predictive significance of the *in vitro* characteristics for the *in vivo* behaviour.

This work published in details elsewhere (Bosseray, 1991) is summarized here.

Materials and methods

Briefly, *B. melitensis* Rev.1 strain was obtained in lyophilized ampoules from Elberg (Berkeley, California) in 1981 and used to prepare a lyophilized substock in this laboratory (batch PR 1/81) as indicated in details previously (Bosseray 1991). A new tube of batch PR1/81 was used in each experiment as original strain.
Among normal Rev.1 colonies (diameter $\leq$ 1.2 mm), large colonies ("giant", diameter $\geq$ 1.5 mm) developed in some subcultures of the original strain at about 1 in 10^3 colonies. Both giant and normal types were cloned by three successive isolations. Resistance to penicillin and streptomycin and immunogenicity were then compared with the original strain.
Five commercial vaccines (Table I) from different sources were streaked across Petri plates of Blood Agar Base (BAB No. 2, Difco, France) and Trypcase Soya Agar medium (TSA, Biomérieux, France) supplemented with 0.1% of yeast extract to control the colonial morphology at first isolation by the oblic reflected light examination method before and after staining by crystal violet and by acriflavine agglutination according to Alton et al. (1988). Representative colonies of smooth (S), non-S or giant types were purified. The 16 purified substrains were controlled *in vitro* and *in vivo* either after purification (initial test) or after storage or passage in mice (Table I).

In vitro tests included:
(1) examination of colonial morphology,
(2) analysis of resistance to penicillin and streptomycin, comparatively with the original strain according to a quantitative test (Bosseray, 1991) summarized in Table II.

In vivo tests using CD1 female mice (Charles River, France) were:
(1) residual virulence, expressed by the recovery time 50% (RT50) in the spleen of mice inoculated by subcutaneous injection of 10^8 cells, and killed 3, 6, 9 and 12 weeks later,
(2) immunogenicity, expressed by the challenge strain spleen count, 15 days after intraperitoneal inoculation with $2x10^5$ bacteria of *B. abortus* virulent strain 544 on mice vaccinated subcutaneously with 10^5 *B. melitensis* Rev.1, 30 or 45 days before challenge.
To determine the normal response for Rev.1 vaccine, both tests were comparatively done with the original reference strain. Immunogenicity tests included non vaccinated controls to define the non-protection response zone (Bosseray, 1991 and Table IV).

Results

Colonial morphology at primary isolation

Vaccines A, B and C showed a smooth to non-S dissociation rate $\geq$ 20% at first culture while D and E developed non dissociated smooth colonies of normal size (D) or large diameter (E $\geq$ 1.5 mm). Among the 16 strains purified from these vaccines, 9 were in the S-phase, 3 non-S, 1 in rough phase (R) and 3 were S-giant colonies (Table III).

Colonial morphology and antibiotic resistance at the initial test, after purification and after storage or passage

All the S-strains remained in S-phase after storage or passage. All the non-S or R strains, except B3, reverted to typical S phase after lyophilization and subculture. The S-giant strain E3 had persistent S-giant type colonies.

Penicillin and streptomycin resistance of the original strain were observed in 4/16 vaccine strains at the initial test (Table III). These strains were in the S-phase and could then be considered as normal Rev.1 strains. The other strains had lower resistance to one antibiotic (11 strains) or to both (strain B1). Strains A6 and B1 had lost their resistance to streptomycin, strains E1, E2, E3 had a higher resistance to streptomycin, while G-variant of the original strain, used as control mutant, had normal antibiotic resistance.
Most strains (16/26) had their initial resistance levels preserved after storage by lyophilization or subculture or after passage in mice. Five increased their resistance to penicillin to normal after storage or passage. Five increased their resistance to streptomycin (A6, B1 strains), however, their response was still abnormally low.

Reverse mutation from non-S or R-phases to the S-phase did not restore the normal antibiotic resistance.

Residual virulence and immunogenicity

One strain only among 8 (E3) had a residual virulence similar to that of the original strain at the initial test (Table IV). The other strains had lower (D2) or no residual virulence at all (no recovery of the vaccine strain at any time: A6 and B3).
Residual virulence of the original strain was not modified by subculture or by lyophilization. All vaccine strains kept their initial residual virulence level after storage, except C2 and D2 that lost virulence after subculture and B2 that was comparatively more virulent after lyophilization. Reversion of A6 and B1 strains to smooth phase after storage did not restore their ability to persist in mice as normally expected for a smooth strain.

In the initial test for immunogenicity, 5 strains (G-variant, normal-control, B2, D2, E3) gave a response similar to that of the original strain; 2 strains were non protective at all (A2, B3) (Table IV). The other strains gave an intermediate response.
Immunogenicity of the original strain was not significantly modified by subculture or by lyophilization; the responses remained in the maximum protection zone. Five vaccine strains kept their initial immunogenicity after storage (A2, B3: no or poor protection; B2, D2, E3: maximum protection). Strains A6, B1 and C2 reached the maximum protection zone after subculture. Reversion of A6 and B1 strains to the S-phase might favour some selection of more immunogenic strains after subculture. It is interesting to note that reversion to the S–phase may restore some immunogenic activity without return to normal residual virulence.

When both *in vivo* activities were simultaneously considered (Table IV), only strain E3 had an activity similar to that of the original strain.

Relation between *in vitro* tests for phase and resistance to antibiotics and *in vivo* tests for residual virulence and immunogenicity

As shown on Table V, *in vitro* tests had no predictive significance for the *in vivo* activies, except for the non-S strains that never had normal *in vivo* activities. The S-phase, normal state for a Rev.1 vaccine strain, was not clearly correlated with normal *in vivo* activities: 9/16 S-strains had normal immunogenicity but decreased or nile residual virulence.

Discussion

Routine control of living attenuated vaccines must ensure conformity of the strain to bacteriological and biological characteristics of the original vaccine strain for which official authorization was delivered. When genetic markers for attenuated virulence and protective activity have been identified, routine control of vaccines may mainly rely on evidence of the functional genes, in addition to control of vaccine doses. However, as genetic basis of virulence and immmunogenicity of *Brucella* are still unknown, controls of the vaccines are by far more difficult to manage and interpret.

In vitro tests, for bacteriological identification of Rev.1 strains, showed a rather good stability of characters (either normal or abnormal) after storage or passage in mice. Most vaccine strains shared the original characteristics for resistance to penicillin and streptomycin as defined by Alton et al. (1967). Variations in the limits of resistance after storage or passage evidenced by quantitative tests did not generally exceed a two-fold dilution. They may thus be considered stable characteristics for Rev.1 strains.

In vitro tests had no clear-cut predictive value for residual virulence and immunogenicity. Phase-dissociation should however be taken as a presumptive test for poor quality of vaccines since non-S strains at initial isolation had poor *in vivo* activities, even if restoration of the S-phase occured after subculture. Consequently, commercial vaccines with dissociation (S to non-S) rate $\geq$ 5% must be discarded and a new seed lot prepared from the original strain delivered by the national laboratories. If normal S–phase is a prerequisite for good *in vivo* activity, we must keep in mind that it does not imply *per se* the right biological activities.

Both *in vivo* tests, residual virulence and immunogenicity, gave a better discrimination between conform and non conform strains: some strains may exhibit for example a normal protection in mice while having a lower residual virulence than the reference one (underlined responses, Table IV).
We considered previously (Bosseray, 1991) from our laboratory and field observations that a Rev.1 vaccine strain having an immunogenicity test in mice statistically equivalent to the reference strain but a lower RT50 ($\geq$ 4.5 weeks) could be acceptable. New informations gained from our analysis in mice with vaccine strains *B. suis* S2 comparatively to Rev.1 (Bosseray, Plommet 1990) and from the results of Verger et al. (1995) with the same vaccines in ewes, may modify our point of view. Vaccine S2 which has in mice a short RT50 (4.3 weeks) induced a good short-term immunity (challenge at 45 days) against *B. abortus*, *B. suis* or *B. melitensis* challenges. However, this immunity declined with time (challenge at 150 days) when tested with the highly virulent *B. melitensis* H38 strain, while immunity induced by reference Rev.1 strain did not decrease. In ewes, immunity induced by S2 and tested after 10 and 18 months was poor comparatively to the Rev.1 one at either short- or long-term. So, the predictive value of the usual short-term protection test in mice alone may be questionned. Because long-term tests either in mice or ewes are too long for routine control methods, the residual virulence by the RT50 test must have a reinforced significance.
Consequently, Rev.1 vaccines with good immunogenicity and a RT50 not statistically different from the reference strain (7.6 $\pm$ 1.3 weeks, Table IV) should be considered as potentially protective in natural hosts. Vaccines with good immunogenicity in mice but with RT50 between 4.5 and 6.2 weeks would be of potentially conflicting activity with either good, or low protection of natural hosts against *B. melitensis* infection. Such Rev.1 vaccines should not be used longer in the field.

Recommendations

General recommendations for production, control and acceptability of vaccines have been previously published in details (Plommet, Bosseray 1983 ; Bosseray et al. 1983 ; Bosseray 1991, 1992 a, 1992 b). The main ones for Rev.1 vaccine are:
(1) production of master seed lots from the original seed strain delivered by national laboratories,
(2) strict control of phase-dissociation in master seed lots (less than 1% non-S colonies) and every vaccine batch (less than 5% non-S colonies) ; control of number of viable bacteria per vaccinal dose,
(3) systematic control by official laboratories of markers, residual virulence and immunogenicity on the first commercial master seed lot, in rigourously managed experiments, comparatively with a reference vaccine strain,
(4) routine quality controls on commercial master seeds and vaccines when required by the official control service.

References

ALTON G.G., ELBERG S.S. (1967) Rev.1 *Brucella melitensis* vaccine. A review of ten years of study. Vet. Bull., 37, 793-800.

ALTON G.G., ELBERG S.S., CROUGH D. (1967) Rev.1 *Brucella melitensis* vaccine. The stability of the degree of attenuation. J. Comp. Pathol., 77, 293-300.

ALTON G.G., JONES L.M., ANGUS R.D., VERGER J.M. (1988) Techniques for the brucellosis laboratories, Alton G.G., Jones L.M., Angus R.D., Verger J.M. (eds) INRA, Paris.

BOSSERAY N. (1985) Quality control of four Rev1 anti-*Brucella* vaccines. In Verger J.M., Plommet M. (eds) *Brucella melitensis*, Martinus Nijhoff, Dordrecht, 229-236.

BOSSERAY N. (1991) *Brucella melitensis* Rev.1 living attenuated vaccine: stability of markers, residual virulence and immunogenicity in mice. Biologicals, 19, 355-363.

BOSSERAY N. (1992 a) Le vaccin Rev.1 : dérive des caractères d'immunogénicité et de virulence indépendante des marqueurs classiques. In M. Plommet (ed), Prevention of Brucellosis in Mediterranean Countries. CIHEAM publications, 1, 182-186.

BOSSERAY N. (1992 b) Control methods and thresholds of acceptability for anti*Brucella* vaccines. Dev. Biol. Standard., 79,121-128.

BOSSERAY N., PLOMMET M. (1976) Transformation normalisant la distribution du nombre de *Brucella* dans le rate de souris inoculées par voie intrapéritonéale. J. Biol. Standard., 4, 341-351.

BOSSERAY N., PLOMMET M. (1983) A laboratory reference vaccine to titrate immunogenic activity of anti*Brucella* vaccines in mice. Ann. Rech. vét., 14, 163-168.

BOSSERAY N., PLOMMET M. (1990) *Brucella suis* S2, *Brucella melitensis* Rev.1 and *Brucella abortus* S19 living vaccines: residual virulence and immunity induced against three *Brucella* species challenge strains in mice. Vaccine, 8, 462-468.

BOSSERAY N., PLOMMET A.M., PLOMMEt M. (1984) Theoretical, practical and statistical basis for a general control method of activity for anti-*Brucella* vaccines. Dev. Biol. Standard., 56, 257-270.

ELBERG SS. (1981) Rev.1 *Brucella melitensis* vaccine. Part II 1968-1980. Vet. Bull., 51, 67-73.

ELBERG SS., FAUNCE K.J.R., (1957) Immunization against *Brucella* infection.
VI. Immunity conferred on goats by a nondependent mutant from a streptomycin-dependent mutant strain of *Brucella melitensis*. J. Bacteriology, 73, 211-217.

PLOMMET M., BOSSERAY N. (1977) Le contrôle des vaccins antibrucelliques par dénombrement des *Brucella* dans la rate de souris, vaccinées ou non, inoculées par voie intrapéritonéale. J. Biol. Standard., 5, 261-274.

PLOMMET M., BOSSERAY N. (1984) Proposition pour une méthode générale de contrôle d'activité des vaccins antibrucelliques. Dev. Biol. Standard., 56, 247-255.

VERGER JM., GRAYON M., ZUNDEL E., LECHOPIER P., OLIVIER-BERNARDIN V. (1995) Comparison of the efficacy of *Brucella suis* strain 2 and *Brucella melitensis* Rev.1 live vaccines against a *Brucella melitensis* experimental infection in pregnant ewes. Vaccine, 13, 191-196.

Table I. **Experimental design. Number of tests performed *in vitro* or *in vivo* according to the origin of strains**

Origin of strains	Cloned or purified strains No.	Tests *in vitro* (a)				Tests *in vivo* (b)		
		Initial test	After storage or passage (c)			Initial test	After storage (c)	
			Subcult.	Lyophil.	Passage		Subcult.	Lyophil.
Original strain		3	2	1		3	2	2
	Giant variant	1				1 *		
	Normal control	1				1 *		
Vaccine								
A	A1 to A6	6	4	2	1(3 w)	2	2	2
B	B1 to B3	3	3	3	2(6 w)	3	3	3
C	C1, C2	2	1	1	1(6 w)	1	1	1
D	D1, D2	2	1	1	1(9 w)	1	1	1
E	E1 to E3	3	3	1	1(9 w)	1	1	1
Vaccine strains controlled		16	12	8	6	8	8	8

(a) Colonial morphology and resistance to penicillin and streptomycin.
(b) Tests for residual virulence and immunogenicity, except for *: immunogenicity test only.
(c) Storage: either by subculture (Subcult.) on BAB slopes 7 times every 8 weeks with storage at 4°C between subcultures, or by lyophilization (Lyophil.). Passage: strains isolated from mouse spleens after inoculation. In brackets: number of weeks after inoculation.

Table II. **Growth of Rev.1 original strain on penicillin and streptomycin in two experiments**

Resistance to penicillin or streptomycin is the highest dose (µg/ml) giving a growth index ≥ 2: penicillin = 0.37 µg/ml and streptomycin = 2.5 µg/ml for the original strain. Considering the high reproducibility of results, a difference of one dilution between limits of resistance of two strains may be considered significant.

No. experiment	Triplicate	Resistance to *							
		Penicillin (µg/ml)				Streptomycin (µg/ml)			
		0.09	0.18	0.37	0.75	1.2	2.5	5.0	10.0
1	a	4	4	2	0	4	2	0	0
	b	4	4	2	0	4	2	0	0
	c	4	4	2	0	4	2	0	0
2	a	4	4	3	0	4	2	0	0
	b	4	4	3	0	4	2	0	0
	c	4	4	3	0	4	3	0	0

* Growth index determined comparatively with growth on control plates (BAB medium): 4 = equal to control plates; 3 ≥ 50% control; 2 < 50% control; 1 = slight growth or more than 10 resistant colonies; 0 ≤ 10 resistant colonies or no growth.

Table III. **Stability of *in vitro* tests after subculture, lyophilization or passage in mice**

Strains	Initial tests *in vitro*			New response after storage or passsage in mice (c)		
	Colonial morphology (a)	Resistance to (b) Penic.	Strepto.	Subcult.	Lyoph.	Passage
Original strain	**S**	**0.37+**	**2.5+**	no !	no	...
G-variant	S-G	0.37	2.5	...	...	...
Normal control	**S**	**0.37**	**2.5**	...	...	...
Vaccine strains:						
A3	**S**	**0.37**	**2.5**	...	...	...
A5	**S**	**0.37**	**2.5**	...	...	...
C1	S	0.18	2.5	...	...	...
D1	S	0.18	2.5	...	...	...
A2*	**S**	**0.37**	**2.5**	no	no	no (3w)
A4*	**S**	**0.37**	**2.5**	no	...	...
C2*	S	0.18	2.5	no	no	no (6w)
E1	S-G	0.37	5.0	no	...	...
E2	S-G	0.37	5.0	no	...	...
E3*	S-G	0.37	5.0	no	no	no (9w)
B3*	Non-S	0.37	2.5	no	no	...
				Mutation to S phase, resistance to penicillin or stroptomycin unchanged		
A1	Non-S	0.18	2.5	S	...	...
				Enhanced resitance to penicillin		
B2*	S	0.18	2.5	no	0.37	0.37 (6w)
D2*	S	0.18	2.5	0.37	0.37	0.37 (9w)
				Mutation to S phase ± enhanced resistance to streptocycin		
A6*	R	0.37	<1.2	S ; sm: 1.2	S ; sm: 1.2	...
B1*	Non-S	0.18	<1.2	S ; sm: 1.2	S ; sm: 1.2	S ; sm: 1.2

(a) Phase aspect of colonies controlled by directly reflected light, before and after staining with cristal violet, and by acriflavine agglutination. S = smooth ; non-S = non-smooth (smooth intermediate or mucoid like) ; R = rough phase ; S-G = smooth strain developing giant-type colonies (diameter ≥ 1.5 mm).
(b) Resistance to penicillin or to streptomycin is the highest dose (µg/ml) giving a growth index ≥ 2
(c) Storage or passage: see Table I.
Bold: initial tests equal to that of the original strain;
underlined: new response after storage or passage;
...not done ; + 3 tests ; ! 2 tests ; * Strains analysed *in vivo*.

Table IV. **Results of in vivo tests for residual virulence and immunogenicity**

Strains	Residual virulence (a)			Immunogenicity (b)		
	Initial	Tests after storage		Initial	Tests after storage	
No.	test	subculture	lyophilization	test	subculture (c)	lyophilization (c)
Original strain	7.9	7.7	6.7	1.8 ± 0.5 (c)	2.0 ± 0.5	2.1 ± 0.9
"		8.2	7.7	2.1 ± 0.9	1.7 ± 0.7	2.0 ± 0.7
"				2.1 ± 0.9		
G-variant				2.3 ± 0.7		
Normal control				2.5 ± 0.9		
Vaccine strains:						
A2	2.6	0	0	3.9 ± 0.5*	2.9 ± 1.2	3.2 ± 1.0
A6	0	0	0	3.5 ± 0.7	2.7 ± 0.5	3.6 ± 0.9
B1	1.6	0	0	3.0 ± 0.6	2.5 ± 0.8	2.8 ± 1.0
B2	1.6	2.6	5.0	2.6 ± 0.8	2.4 ± 0.6	2.3 ± 0.5
B3	0	0	0	4.2 ± 0.2*	4.0 ± 0.2*	4.3 ± 0.6*
C2	3.5	1.8	3.0	2.8 ± 0.8	2.2 ± 1.0	3.1 ± 0.9
D2	5.0	3.6	4.3	2.5 ± 1.2	2.0 ± 1.2	2.4 ± 0.9
E3	**7.3**	**8.0**	**7.8**	**2.3 ± 0.5**	**2.3 ± 0.8**	**2.1 ± 0.7**
Control mice				4.9 ± 0.2	4.6 ± 0.2	4.5 ± 0.2
(non vaccination)				4.6 ± 0.3		
				4.7 ± 0.2		

(a) Recovery time 50% (week) with confidence limit for P = 0.05: 1.3 weeks.
0 = No recovery of Rev.1 in spleen at any time.
(b) Spleen count of *B. abortus* strain 544 after challenge: mean, log (x/log x) ± standard deviation.
(c) *B. abortus* strain 544 challenge injected 45 days after vaccination instead of 30 days.
Bold: residual virulence and immunogenicity not significantly different from those of the original strain: residual virulence = 7.6 ± 1.3 (recovery time 50%, week ± confidence limite for P = 0.05), immunogenicity = 2.00 ± 0.78 (spleen count of *B. abortus* strain 544 after challenge; mean ± least significant difference for P = 0.05).
Underlined: Immunogenicity not significantly different from that of the original strain.
* No protection = spleen count of *B. abortus* strain 544 after challenge not statistically different from that of control mice = 4.66 ± 0,72 (mean ± least significant difference for P = 0.05).

Table V. **Evidence that *in vitro* tests cannot predict *in vivo* residual virulence and immunogenicity. Pooled analysis of initial tests and tests after storage**

In vitro tests (a)	*In-vitro test* (b)			
	Normal virulence and immunogenicity	Decreased or nile residual virulence, immunogenicity:		
		normal	decreased	nile (c)
Colonial morphology				
S phase	0	9	6	1
S-G colonies	3	0	0	0
Non S	0	0	2	3
Total	3	9	8	4
Resistance to penicillin and streptomycin				
Normal	0	3	3	3
One resistance modified	3	4	5	0
Both resistances modified	0	2	1	0
Total	3	9	9	3

(a) See Table III
(b) Normal response determined with the original strain: normal residual virulence = 7.6 ± 1.3 weeks ; normal immunogenicity = 2.20 ± 0.78.
(c) No protection = spleen count of *B. abortus* strain 544 after challenge not statistically different from that of control mice = 4.66 ± 0.72.

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Experience on the use of the mouse model for the control of Spanish Rev.1 vaccines.

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Introduction

The live attenuated *B. melitensis* Rev1 vaccine has been used in Spain since mid seventies, being the first official vaccination campaign initiated in 1976.

The original strain was provided by Dr. Elberg when he went to Spain in the early sixties to carry out some vaccination experiments in the South of Spain. This strain was apparently maintained by Dr. Esteban-Velazquez (the former Director of the Laboratorio Nacional de Referencia para la Brucelosis -LNRB). However, it is possible that several Rev.1 strains of different origin were present in Spain at that time. As an example, the Ministry of Agriculture conducted several experiments using a Rev.1 strain obtained from the Instituto Zooprofilatico from Pisa (Italy).

The Rev.1 vaccine has been produced since then by private laboratories and purchased by the Ministry of Agriculture to be applied in control campaigns. The quality control was carried out by the LNRB on the basis of exclusive in vitro testing. This laboratory has been, since then, the responsible for the distribution of the original strain in a seed lot system to the different laboratories producing the Rev.1 and of the ensuing quality control of the final products obtained.

With the initiation of the Rev.1 mass vaccination programmes in Spain in the early eighties, it was evident that some Rev.1 vaccines induced abortions when vaccinating pregnant sheep while others did not (see JM Blasco, this meeting), indicating the existence of variability in the residual virulence of Spanish Rev.1 vaccines. Samples of the five commercial vaccines produced in Spain during 1.984-85 were sent by Dr JM Blasco to Dr. N Bosseray to be tested in the mouse model that had been proposed to control the activity of anti-*Brucella* vaccines (1). The results obtained by Dr. Bosseray confirmed the heterogeneous quality of Spanish vaccines at that time (see JM Blasco, this meeting).

To the best of our knowledge, is not until around 1988 when the standard original seed culture (batch named PR1/81) obtained from the collection held at the Institute National de la Recherche Agronomique (INRA, France) was introduced into the LNRB and distributed to different Spanish laboratories.

The current legislation in Spain demands that the original seed culture and seed lots should have a lack of contamination and dissociation (less than 0.1% in the original seed culture and less than 1%, in the seed lots). In the final products obtained by the different private laboratories, the dissociation should be less than 5% and the viable counting should be according to the standard dose (1-2 x 10^9/dose). Apart from the control carried out by the LNRB, a representative sample of each commercial batch sent to the different representative state departaments is checked again in the

corresponding departamental laboratories for control of contamination, dissociation and viable counting, before being finally distributed to the veterinarians and applied in the field. Accordingly, the official control of Rev.1 in Spain is carried out exclusively following *in vitro* characteristics.
Dr. N Bosseray and coworkers (1, 3), proposed an interesting control model having in mind not only the *in vitro* markers but also the behaviour of the vaccines in vivo, based on the interaction of the Residual Virulence and Immunogenicity evaluated in mice (3). This animal model has never been officially applied in Spain. A total of 2 Rev.1 seed lots and 4 commercial Rev.1 Spanish vaccines, as well as the reference original seed culture strain, have been tested in our laboratory during the last two years using this model. The aim of this contribution is to describe the results obtained and to comment our experience on the suitability of this control procedure.

Evaluation of spanish seed lots and vaccines

The Rev1 reference original seed culture was obtained from the INRA (batch PR1/81) and the two seed lots tested were obtained from Laboratorios Ovejero S.A., Avda. Peregrinos, s/n, Aptdo. 321, León, and Laboratorios Iven S.A., Polígono Industrial de Vallecas, C/ Luis I, s/n., 28031 Madrid). The four final products tested were produced by the Laboratorios Cyanamid S.A. (Ctra. Camprodon, s/n. 17813 Vall de Bianya, Gerona; 2 different batches) and Laboratorios Syva (Avda. Párroco Pablo Díez, 49-57, 24010 León; 2 batches).

The two seed lots and the original seed culture strain were rehydrated in 1 ml. of sterile Phosphate Buffered Saline (PBS) and the final products in 5 ml. of sterile distilled water. Samples of each rehydrated were smeared onto six BAB plates, incubated at 37°C for 24 -72 hours and the resulting growth (without purification) harvested in sterile milk-lactose vehicle and kept frozen in 1 ml aliquots. Moreover, the rehydrated were also seeded and incubated in the same conditions for 5 days to obtained isolated colonies.

Two stable substrains from the original seed culture (3), one smooth and the other rough, the original seed culture without purification, both seed lots and the four final product strains were tested for in vitro markers incubating in both air and 10% CO_2 atmospheres. The colonial size after 5 days of incubation, degree of dissociation, phage sensitivity, agglutination with monospecific sera, growth in Thionin, Fucshin, Safranin, penicillin and streptomycin were determined following standard procedures (2).

From the classical phenotypic markers of Rev.1 (2), the sensitivity to dyes and antibiotics, were modified by the incubation in 10% CO_2 atmosphere. When incubated in air, only differences in colonial size and dissociation were observed in some strains respect to that described for Rev1 (2). However, in these incubating conditions, the colonial size and phase did not modified the remaining in vitro characteristics described for Rev.1 (2, 3).

Aliquots from the original seed culture, the two seed lots and the four commercial vaccines were tested in CD1 mice according to the protocols described by Bosseray (3). The results of residual virulence and immunogenicity obtained are exposed in Table I and represented in Figure 1.
The mean RT_{50} value obtained in five consecutive experiments with the original seed culture was 8.30 ± 1.28 weeks (limit values between 7.02 and 9.58 weeks), quite similar to the values obtained by Bosseray (3, 6). Only one commercial strain

(strain L) and both seed lots (A and K) showed RT_{50} values inside the confidence limits (Fig. 1). However, the remaining strains tested showed decreased (strain T) or increased (strains W and O) virulence, being all of them statistically different from reference strain.
Concerning to the immunogenicity, the original seed culture strain gave similar results in successive experiments and closely related to that obtained by Bosseray (3, 6). Only two strains (seed lot A and K) were placed inside the immunogenicity confidence limits (Fig. 1).

Thus, only two Spanish strains (seed lot A and K) should be acceptable, according the conditions proposed by Bosseray.

Discussion and recommendations for future research
The method proposed by Bosseray (3) is the only described until now that has in consideration in vivo parameters to control the quality of anti-*Brucella* vaccines. The results of this method are repeatible and, has to be considered useful for the quality control of Rev.1 and strain 19 vaccines. However, some technical and practical aspects of this method should be clarified and improved, to be extended and applied with warranty, particularly in laboratories with limited experience in animal modeling. In our opinion, the more relevant aspects are:

A. Residual virulence aspects
1) Obtention of results.
Under our point of view, the assessment of the residual virulence following the manual Bonet-Maury method modified by Bosseray (3, and personal comunications) would need an unificated standard training to obtain repeatible, objective, and therefore, precise results.
Sometimes is difficult to assess the results obtained from a bioassay, for example, in the cases in which some point time gives equal or lower number of recovered mice than in the immediately previous point time, the values represented manually in the special modified Bonet-Maury paper are quiet dispersed and is complicated to draw an adequate line placed at equal distance of all values obtained and having an adequate slope. Following the protocols of Bosseray (3), these results are invalid and should be repeated.
Trying to faciliate the interpretation of this and other unclear aspects of residual virulence results, the data obtained with the Spanish vaccines were processed with statistical program by a regression method using the Probit transformation of the percentage of accumulated recovered mice and the time (expressed in weeks) as arithmetic scales. The Probit transformation was made using the statistical package of SAS (5) and the values obtained are exposed in Table II.
The RT_{50} values obtained by Probit-regression method are quite similar to that obtained by the method proposed by Bosseray. However, in our opinion, this computer method provides a more objective assessment of results than the manual method minimising the differences of each value respecting to the regression line and allowing a better asessment of results to untrained personnel. We would suggest the r^2 regression values close to 1.00 and never lower than 0.95.

2) To develop simplified protocols.
The protocol described by Bosseray is expensive and of long duration. An alternative method to simplify the RT_{50} assessment could be to slaughter an adequate number of mice at only one point time, as for example as described by Alton et al (2). This test propose to inoculate twenty four mice with 10^8 Rev.1 organisms subcutaneously

and to slaughther all animals 8 weeks later. When the Rev.1 strain be recovered from the spleen of 25-50 % of inoculated mice, the residual virulence will be considered as normal. According to our work (mean RT_{50} value obtained in Zaragoza: 8.30±1.28 weeks), we would suggest a range of 35-65% of recovered animals at 8 weeks post-inoculation. We are presently trying to develop a similar simplified procedure using the strain Balb/c mice, that gives a more homogeneous response than CD1strain.

B) Immunogenicity aspects

1) Statistical assessment of results.

The method described by Bosseray is based in the confidence limits (mean ± standard deviation) obtained after several repeated experiments with the reference strain. In our experiment, only two strains (seed lot A and K) were placed inside the confidence limits described by Bosseray and, accordingly, the Spanish commercial vaccines do not fulfil the requirements to be considered as acceptable vaccines. However, most of vaccines tested were placed (Fig. 1) closer to the protection zone than to the non protection zone. When comparing statistically (T test) the results of vaccines with respect to the values obtained with unvaccinated controls (Table III), all vaccines gave statistically significant protection. A more adequate approach could be to compare the results with respect to the values obtained with the reference strain (Table III). In this case, strains A, K and W should be considered to be as protective as the reference vaccine.

We have field evidences in vaccinated sheep correlated with the results obtained in mice. For example, the strain T that showed an RT_{50} decreased and gave significant diferences in immunogenicity with respect to the reference strain using both the Bosseray approach or our statistical comparison, did not induced antibodies in vaccinated sheep and did not induce abortions when vaccinating pregnant ewes. By contrast, the strain W, induced antibodies 15 days after vaccination in sheep and abortion in pregnant ewes. This W strain showed an increased virulence in mice and was not protective enough (Table I; Fig. 1). However, when using the T test comparison (Table III), the protection afforded by this strain was not statistically different from that of the reference strain.

2) Duration of immunity

The assessment of immunity in the model proposed is carried out too close (challenge 30 days post-vaccination) to the vaccination date. There is published information demonstrating that some vaccines, such the *B. suis* strain 2 (6) or the *B. abortus* RB51 (7), protecting mice when challenged 30 days after vaccination, resulted nonprotective in sheep (8, 9, 10). Moreover, it has been published that the *B. suis* strain 2 that induced protection in mice challenged with *B. melitensis* 45 days after vaccination, was not adequately immunogenic when the challenge was carried out 150 days after vaccination (6). Therefore, it would be desirable to increase the interval vaccination-challenge to determine not only the existence of immunity but also the duration of this immunity .

References

(1) **BOSSERAY ET AL. 1984.** Theorical, practical and statistical basis for a general control method of activity for antibrucella vaccines. Develop. Biol. Standard., 56, 257-270.

(2) **ALTON ET AL.** Techniques for the Brucellosis laboratories. 1988.

(3) **N. BOSSERAY.** *Brucella melitensis* Rev1 attenuated vaccine: Stability of markers, Residual Virulence and Immunogenicity in mice. Biologicals,19,355-363. 1991.

(4) **BONET-MAURY ET Al.** La mesure statistique de la virulence et de l'immunité: Application a l'etude de la virulence du bacille typhique et a la mesure du pouvoir immunisant des vaccins antityphoïdiques.Rev. Immun.,18, 21-49.1954.

(5) SAS Institute Inc. SAS(R). Language Guide for personal computers, Release 6.03 Edition.Cary, NC: SAS Institute Inc., 1988. 558 pp.

(6) **N. BOSSERAY AND M. PLOMMET.** *Brucella suis* S2, *Brucella melitensis* Rev1 and *Brucella abortus* S19 living vaccines: Residual virulence and immunity induced against three *Brucella* species challenge strains in mice. Vaccine, vol. 8, 1990.

(7) **M.P. JIMENEZ DE BAGÜES ET AL.** Vaccination with *Brucella abortus* rough mutant RB51 protects Balb/c mice against virulent strains of *Brucella abortus, Brucella melitensis,* and *Brucella ovis*. Infection and Immunity, vol. 62, nº 11. 1993.

(8) **VERGER ET AL.** Comparison of the efficacy *Brucella suis* strain 2 and *Brucella melitensis* Rev1 live vaccines against a *Brucella melitensis* experimentalinfection in pregnant ewes. Vaccine 13, 191-196. 1995.

(9) **J.M. BLASCO ET AL.** Efficacy of *Brucella suis* strain 2 vaccine against *Brucella ovis* in rams. Vaccine, vol. 11, nº13. 1993.

(10) **M.P. JIMENEZ DE BAGÜES ET AL.** The *Brucella abortus* RB51 vaccine does not confer protection against *Brucella ovis* in rams. Vaccine, vol. 13, nº3. 1995.

Table I. **Residual virulence (RT_{50}) and immunogenicity of the Rev.1 Spanish vaccines tested in CD1 mice according to the method of Bosseray (3).**

Rev.1 strains	Tests on mice	
	Residual virulence (RT_{50}) in weeks	Immunogenicity mean ± sd
Original seed culture J	8.30 ± 1.28	2.24±0.22
Seed lot A	7.29 ± 1.60	2.36 ± 0.33
Seed lot K	7.58 ± 1.75	2.27 ± 0.36
Strain O	14.5 ± 1.5	2.96 ± 0.55
Strain T	6.58 ± 1.49	2.67 ± 0.46
Strain W	10.82 ± 1.29	2.96 ± 1.05
Strain L	8.00 ± 1.37	3.23 ± 1.16
Unvaccinated controls		5.05 ± 0.62

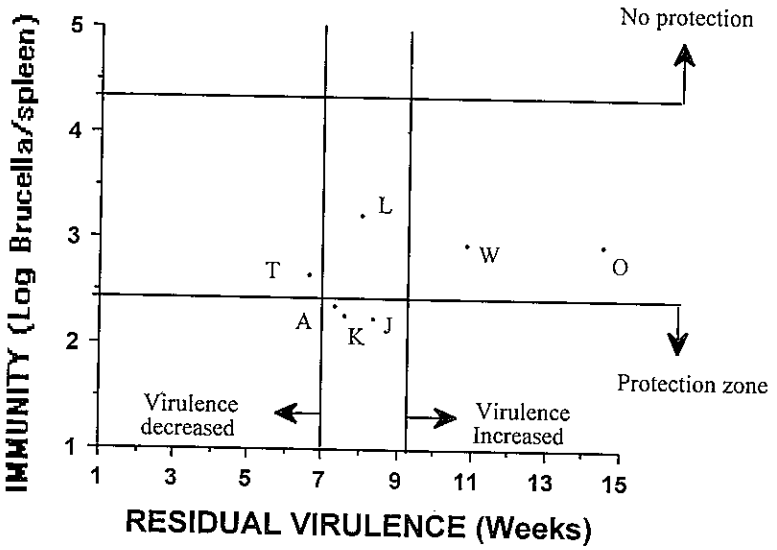

Fig. 1. **Residual virulence (RT_{50}) and immunogenicity of the Rev.1 Spanish vaccines represented according to the method of Bosseray (3).**

Table II. **Comparison of Residual Virulence (RT_{50}) values of Spanish Rev.1 strains obtained by the Probit-regression method or by the modified Bonet-Maury method (3).**

Rev.1 strains	Residual virulence values (in weeks)	
	Bonet-Maury method	Probit-regression method
Reference Strain J	8.30 ± 1.28	8.45 ± 1.38
Seed lot A	7.29 ± 1.6	7.06 ± 1.4
Seed lot K	7.58 ± 1.75	7.13 ± 1.59
Strain O	14.5 ± 1.50	14.98 ± 1.5
Strain T	6.58 ± 1.49	6.90 ± 1.3
Strain W	10.82 ± 1.29	10.66 ± 1.3
Strain L	8.00 ± 1.49	8.18 ± 1.7

Table III. **Statistical comparison (T test) of immunity results of Spanish Rev.1 strains with respect to the results of the reference seed culture and that of the unvaccinated controls.**

Rev.1 strains	Immunogenicity (p value respect to)	
	Reference strain	Unvaccinated control
Reference		0.0001
Sedd lot A	0.25	0.0001
Sedd lot K	0.25	0.0001
Strain O	0.013 *	0.0001
Strain L	0.048 *	0.002
Strain W	0.8	0.0001
Strain T	0.041 *	0.0001

* $p<0,05$

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The quality assurance of living *Brucella* vaccines, the French experience with the Rev.1 vaccine

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Summary
The author presents here the control methods and criteria used in France to assure the quality of living *Brucella* vaccines. The aim of this control is to provide vaccines with a sufficient quality in the present frame of the generalisation of Rev.1 vaccination in infected areas. A light and systematic control of production lots (including identity, purity, enumeration and phase dissociation controls) is associated with a thorough control, systematically applied to seed-lots and occasionally applied to production lots (including a control of residual virulence and immunogenicity on mice).

Introduction
In the European Union, the veterinary vaccines, as all drugs and other biological products used *in vivo* must be officially certified ("Autorisation de Mise sur le Marché" [AMM]). The certification procedure consists in, *(i)* examination by a national independent commission of a dossier including, the description of the product and manufacturing process, and an expert's report with regard to the analytical, clinical and pharmaco-toxicologic aspects of the product, and *(ii)* the laboratory control of the final product.
Up to 1990, *Brucella* living vaccines were following the same rules and the official control by the National Reference Laboratory was only applied on a final lot at the time of the dossier registration or renewal (every 5 years). However, in terms of cost/efficacy rate, the Rev.1 vaccine is considered as the most important tool used for the control of sheep and goats brucellosis in infected areas. Due to some vaccination failures observed in the field and the demonstration that some circulating lots could not evidence the required quality, authorities have wished that a thorough control should be applied systematically to seed-lots and at least occasionally to the final lots of *Brucella* vaccines. Then, all the procedures have been revised and the procedure used at the present time is that described in this paper. It has been proposed to be included as a monograph in the European Pharmacopoeia.

I. Control strategy

The properties of the Rev.1 strain of *Brucella melitensis* biovar 1, used with the normal dose (1-2 x 10^9) by the subcutaneous way or with a slightly reduced dose (1-2 x 10^8) by the conjunctival route in small ruminants is now well known and acknowledged in terms of innocuousness and efficacy. Therefore the efficacy and innocuousness controls applied to target species (*i.e.* sheep and goats) required for new vaccines (or new presentations or new way of inoculation) are not necessary for the Rev.1 vaccine. However, it is required to make sure that each seed-lot shows characteristics consistent with those of the initial seed, especially in terms of immunogenicity and residual virulence. Then, if no failure is observed in the field, an occasional control of final lots, limited to identity, purity, enumeration and phase dissociation, appears sufficient.

II. Control methods and norms

II.1 General measures

All the controls are performed by the National Reference Center for Animal Brucellosis, and all vaccine lots used in France are produced by the private industry.

Controls common to seed- and final lots are as follows:
(i) absence of biological contaminants,
(ii) identity of the vaccinal strain,
(iii) dissociation phase control (Methods of Henry and White & Wilson).

Each seed-lot is also submitted to the following controls:
(i) Residual virulence (persistence time 50% on mice),
(ii) immunogenicity (on mice)

Each final lot is also submitted to a control of enumeration and a control of the label and directions for use.
Then, a control including residual virulence and immunogenicity controls may be decided no matter when, especially if a vaccination failure is suspected in the field.

The seed-lot control is realised 4 months at least before the production of the first final lot. The final lot control is realised 1 month at least before the commercialisation and at any time before the expiry date.

Then, the samples for official control should be sent to the reference laboratory with the results of the internal quality control.

II.2 Label and directions for use

Each vaccine flask or vial (final lot) must be labelled (same label on the packing material). This label must present the informations required by the "Vaccinum ad usum veterinarium" monograph of the European Pharmacopoeia. The directions for use must respect the latter norm and must recommend the use of the vaccine as prescribed by the national regulations (route of inoculation, dose, animal species, age of vaccinated animals, biohazard for environment and human health).

II.3 Purity control

This assay (derived from the "Sterility monograph" of the European Pharmacopoeia should evidence the absence of contaminants (aerobic and anaerobic bacteria, fungi).

It is realised in conditions preventing the contaminations. The media used (AC medium, thioglycolate liquid medium, Sabouraud and Columbia media) are previously controlled with the following reference strains: *Staphylococcus aureus*, *Bacillus subtilis*, *Clostridium sporogenes* and *Candida albicans*.

The Gram staining method should have beforehand evidenced the sole presence of an homogenous population of Gram-negative coccobacilli without any other bacteria or fungi.

II.4 Identity control

Brucella should evidence the characteristics of the Rev.1 original strain, that is those of *Brucella melitensis* biovar 1 reference strain 16M (ATCC N°23456) (1, 4) except those specific for the strain Rev.1, *i.e.*:

i) the colony size is smaller for *Brucella melitensis* strain Rev.1 than for the biovar 1 reference strain 16M;

ii) no growth on media containing either thionin 20 µg/ml, either basic fuchsin 20 µg/ml, or benzylpenicillin 3 µg/ml;

iii) growth on media containing streptomycin 2.5 µg/ml.

II.5 Viability control (enumeration) (1, 5, 6)

This assay is performed by enumeration of living bacteria after plating dilutions on solid medium and incubation 5 days at 37°C.

Table I. **Common characteristics of *B. melitensis* 16M and Rev.1 strains**

Phase	Oxydase	CO_2	H_2S	Antiserum [a]			Phage [b]		
		requirement	production	A	M	R	Tb	Wb	Iz1
S	+-	-	-	+	-	-	-	+	

a: Agglutination by monospecific anti-A (A), -M (M) or -R (R) sera;
b: Lysis by phages Tbilisi (Tb), Weybridge (Wb) or Izatnagar$_1$ (Iz_1)

Table II. **Differential characteristics of *B. melitensis* 16M and Rev.1**

Strains	Colony size (mm)[a]	Growth in the presence of			
		Thionin 20µg/ml	Basic fuchsin 20µg/ml	Benzyl-penicillin 5UI/ml	Strepto-mycin 2.5µg/ml
16M	1-2	+	+	+	-
Rev.1	0.5-1	-	-	-	+

a: TSA ; incubation in normal atmosphere for 4 days at 37°C.

II.6 Control of dissociation rate (1)

B. melitensis strain Rev.1 should not undergo variations in the colonial morphology, smooth → rough (R) or smooth → intermediate (I), which are generally associated with losses in immunogenicity. It is then essential to pay attention to the colonial morphology to ensure that: *(i)* original seed culture and primary or secondary lots, then, *(ii)* final lots are in the S phase.

Vaccine is streaked across 6 agar plates (Serum-dextrose agar or Trypticase-soy agar added with serum 5% (v/v) or yeast extract 0.1% (w/v) in such a manner that the colonies will be close together in certain areas, semi-separated and separated in others. Slight differences in appearance are more obvious in adjacent than widely separated colonies. Plates are incubated at 37°C for 5 days. Plates are then examined by obliquely reflected light (method of Henry) before and after staining (3 plates) with crystal violet (coloration of White and Wilson).

The dissociation rate (R + I / S+R+I) must be lower than 1% for seed-lots and 5% for final lots.

II.7 Control of residual virulence (3, 4)

The aim of this assay is to control that the vaccinal strain has kept an acceptable level of virulence.

A batch of 32 female CD1 mice, aged 5-6 weeks, are inoculated subcutaneously with 10^8 CFU of the vaccine to be controlled. The exact number of injected bacteria is controlled *a posteriori* by plating 4 agar plates with 0.2 ml of an appropriate dilution. Animals are sacrificed (by randomized lots of 8) 3, 6, 9 and 12 weeks later. Spleens are individually sampled and ground in 10 volumes of PBS diluant (pH = 6.8). The suspension is spread on solid medium dishes, 0.4 ml per dish on 3 dishes at least (minimum level of detection of 5 bacteria by spleen). Persistence time 50% (PT50) is calculated using the probit transformation test of Bonet-Maury (8 mice per point, cumulative totals then graphic analysis on log-probit paper, arithmetic time-scale). The vaccine is conform to the Rev.1 strain when the PT50 is not significantly different from the PT50 of the original reference strain (7.9 ± 1.2 weeks).

II.8 Immunogenicity control (2, 3, 4, 7)

The model used for this control is the INRA mice model (2, 3, 4, 7). This control measures the ability of the vaccine to confer a good protection against a standardized challenge.

Six randomized lots of CD1 mice aged 5 to 7 weeks are used, 4 lots for the controlled vaccine, 1 unvaccinated control lot et 1 reference lot vaccinated with a S19 reference strain (availble at INRA).

Each vaccinated mouse is inoculated sub-cutaneously with 10^5 CFU of the vaccine to be controlled and each mouse of the reference lot with 10^5 CFU of the S19 reference strain. The exact number of bacteria is checked *a posteriori* by plating 4 agar plates with 0.2 ml of an appropriate dilution. At day 30 after vaccination, each mouse is inoculated intraperitoneally with 2 x 10^5 CFU of the CO2-dependent*B. abortus* 544 strain in 0.2 ml. Mice are starved 16 hours before to obtain an injection of good quality. The exact number of bacteria is checked *a posteriori* by plating 4 petri dishes of solid medium with 0.2 ml of a dilution of 10^{-3} of the inoculum.

At day 45 after vaccination (15 days post challenge), mice are sacrificed. Spleens are individually aseptically removed, defatted and weighed. They may be stored at -20°C from 24 hours to 7 weeks. Spleens are then ground in 9 times (or 19 times if their

weight is lower than 120mg) their weight of PBS diluant (pH = 6.8) to obtain after grinding a suspension of 1/10 (or 1/20). Two fractions of 0.2 ml of this suspension and its dilutions of 1/10, 1/100 and 1/1000 are spread on an agar plate. The medium is added with erythritol (1g/l) or benzylpenicilline (3mg/l) to inhibit the growth of vaccinal strain which may survive until killing (S19 and Rev.1 respectively). The enumeration of *B. abortus* 544 colonies is realised after incubation for 5 days at 37°C in atmosphere added with 10 % of CO_2, on plates giving at least 300 CFU.
Results are expressed as numbers of bacteria per spleen. If no colony is observed, the spleen is considered as infected by 5 CFU. The number X of *B. abortus* strain 544 is transformed in Y using the following formula:

$$Y = \log_{10}(X/\log_{10}X)$$

Mean and standard deviation of Y are then calculated for each lot of 6 mice. The control conditions are conform to the limits of the model if the response (mean of Y) of unvaccinated control mice is higher than 4.5 ; the response of mice vaccinated with the reference S19 is lower than 2.5 ; and if the residual standard deviation on these two responses (expressed per unit of 6 mice) is lower than the critical value of 0.8. The Rev.1 vaccine shows a good immunogenicity if the answer calculated on the 4 vaccinated lots is lower or equal to 2.5. In case of marginal response and if there is no objective reason to reject the batch (dissociation, insufficient residual virulence, etc.) the assay is performed again.

III. Consequences of the control

If a seed or final lot has not given good results to one of the controls described previously, the manufacturer is informed without delay. The lot has to be destroyed and in case of a final lot, the veterinary services are immediately informed.

Conclusion

The real quality (efficacy and innocuousness) -the insurance of which is one of the conditions of the efficiency of control programmes against sheep and goats brucellosis- of a Rev.1 vaccine should be logically evaluated by studies conducted on the target species. Such studies, necessary when a new product is to be commercialised, are long and expensive, and impracticable for the routine quality control of vaccines.
Nevertheless, controls are required to warrant a regular efficacy of the products used in the field. Laboratory controls are necessary. The control methods described here allow to warrant that the characteristics of the vaccines commercialised are conform to the original Rev.1 strain whose efficacy and innocuousness are admitted for a long time.

However, these controls are quite heavy, expensive and long. They require a well exercised personnel (bacteriology and animal experimentation). Adapted equipment is also needed to realise these controls in good conditions of security and quality. The 16M and Rev.1 reference strains, as well as the 544 challenge and the S19 reference vaccine strains should also be maintained in the control laboratory, without any modification of their characteristics.

References

1 - **ALTON G.G., JONES L.M., ANGUS R.D., and VERGER J.M.,** Techniques for the Brucellosis laboratory, INRA, Paris, 1988.

2 - **BOSSERAY N.,** Quality control of four Rev.1 vaccines, In *Brucella melitensis*, Verger J.M. and Plommet M. Eds, Martinus Nijhoff, Dordrecht, 1985, 229-236.

3 - **BOSSERAY N., and PLOMMET M.,** *Brucella suis* S2, *Brucella melitensis* Rev.1 and *Brucella abortus* S19 living vaccines: residual virulence and immunity induced against three *Brucella* species challenge strains in mice, Vaccine, 1990, 8, 462-468.

4 **BOSSERAY N.,** *Brucella melitensis* Rev.1 living attenuated vaccine: stability of markers, residual virulence and immunogenicity in mice. Biologicals, 1991, 19, 355-363

5 - **COMITE MIXTE D'EXPERTS FAO/OMS DE LA BRUCELLOSE,** 6ème rapport, Série de rapports techniques 740, 1986.

6 - **COMITE OMS D'EXPERTS DE LA STANDARDISATION BIOLOGIQUE,** 28ème rapport, Normes relatives au vaccin *Brucella melitensis* souche Rev.1, Norme N°25, 1977, N°610.

7 - **PLOMMET M. et BOSSERAY N.,** Propositions pour une méthode générale de contrôle d'activité des vaccins antibrucelliques, Dev. biol. Standard., 1984, 56, 247-255.

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Rev.1 Conjunctival Vaccine ; Quality assurance and quality control.

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Introduction

In order to assure and control the quality of a vaccines before releasing a batch, it is of primary importance for a vaccine producer to assure the quality, the safety and the efficacy of this vaccine at all stages starting from the constitution of the master seed to the shipment of the finished product. Quality assurance and quality control procedures must be implemented during the development phase (GLP and GCP) as well as during production (GMP). It is of importance that these control activities are performed by a department independent from production.

The two most crucial points in the quality assurance and quality control chain of the Rev.1 conjunctival vaccine will be addressed in this short paper. These two steps are:
1) seed lots and their controls
2) controls before, during and after production.

Seed lots and their controls

The Rev.1 strain used in this vaccine originated from the laboratory of Prof. Elberg (UC Berkeley, USA) and was obtained from Dr. Verger (INRA, Tours, France).

From this original strain a master seed and working seed were derived. These are stored lyophilised at two different places. Strict adherence to this seed lot system is necessary to avoid dissociation of the strain and potential loss of potency.

Dual controls are carried out independently by the company and by the CNEVA. These controls include the following: purity, absence of contaminants, identity, lack of dissociation, level of residual virulence and immunogenicity.

Production

Production is carried out under conditions of GMP. Each batch is produced according to a standardised protocol. This protocol includes controls before production, during production and final product testing. Again, final product testing is carried out in duplicate by the company and CNEVA.

The essential production steps include:
- Primary culture of working seed on agar
- Scale up steps of increasing size to a final 20 L fermenter
- Concentration / resuspension to a standard concentration

- Filling and lyophilisation
- Final product and packaging

Controls before production
All starting materials are controlled following the monographs of the European Pharmacopoeia with additional internal standards. As described, seeds are fully documented and validated. In addition, all equipment undergoes routine validation. To avoid possible risk of cross contamination, strict containment is used with the vaccine being produced in delineated areas equipped with independent air handling and under negative pressure.

Controls during production
At each stage of scale up, samples are taken for purity, dissociation rate, viable count and opacity. Viable counts are determined for the final concentrate which is then diluted to the standard concentration and recounted. Final diluted material also undergoes purity checks prior to filling.

Controls during filling and lyophilisation
Vials and stoppers are checked for sterility.
During filling 20 vials are removed at random for control of volume, viable count, dissociation rate, purity and absence of contaminants.
A standard cycle is used for optimal lyophilisation and this cycle is recorded by computer.

Final product and packaging
Fifty vials are removed at random and checked for: appearance, moisture content, viable count, purity, absence of contaminants, identity, dissociation rate.
Packaging is controlled for appearance, batch number and conformity of label text.

Final product is stored under quarantine at 4°C. A full batch processing record which contains all the above information is reviewed in detail by the independent QC department.
Additional samples are sent to CNEVA where they are routinely tested for purity, identity, absence of contaminants, dissociation rate and viable count. Occasional batches are also subjected to testing for residual virulence and immunogenicity.

Conclusion
This extensive QA/QC scheme can only be successful if all personnel receive the appropriate training and are fully committed to the standards of "total quality". This is only possible if the company provides a continuing program of personal development.

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Production and Quality Control of Vaccines for Brucellosis with a Specific Emphasis on Rev1 Vaccine

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Summary
The goal of an industrial production of vaccines is not only to make available to the user a product that meets a number of predetermined standards but also to insure that this level of quality is obtained batch after batch and remains stable for the prescribed period of stability.
This goal is reached through a thorough control of all production and control parameters: seed lots, fermentation equipment and media, freeze-drying, quality controls. Methods to protect personnel and environment against accidental contamination must be in place.
All steps must be performed under the guidelines of good laboratory and manufacturing practices (GLP, GMP).
Although these requirements apply to all vaccine production, they are especially important for Rev1 because of the particular nature of the strain (ease of dissociation, live vaccine...).
It must be emphasized that the cost of a vaccination program in a given region is only in a small part affected by the cost of the vaccine itself. However, the entire success of the program rests on the quality of the vaccine used. Accordingly, it must be recommended that the upmost care be taken in choice and quality of the Rev1 vaccine that is used.
A vaccine is not just a strain, grown on a defined culture media, and tested once by challenge in animals. A vaccine is a product that, put into the hands of the final user, will reliably and reproducibly provide the expected results. Indeed, a vaccine truly exists only when its production has been scaled up and shown to been reproducible.
The purpose of any industrial process for the production of vaccines is to carry out, in a large scale, methods and techniques that may often be well tested but generally suitable only for laboratory scale fermentation. This presentation will illustrate some of the major parameters that must be taken into a account to turn out, reliably, batches of vaccines of consistent quality, meeting prescribed standards and produced under conditions that protect the product, the personnel involved and the environment.

The basic bacteriological techniques as well as the standards that brucellosis vaccines for sheep and goats must meet, have been described in detail elsewhere (1, 2, 3).

Table I. **Basis characteristics of Rev1 vaccine used for the control of brucellosis in sheep and goats (4**

B. melitensis Rev1
Live vaccine (Elberg & Faunce, 1957)
Non streptomycin dependent revertant of *B. melitensis* biovar 1
Dose 10^9-2×10^9 live bacteria/dose
Freeze-dried

I. Vaccine production

I.1 Strains: Initial seed, master seed and seed lots

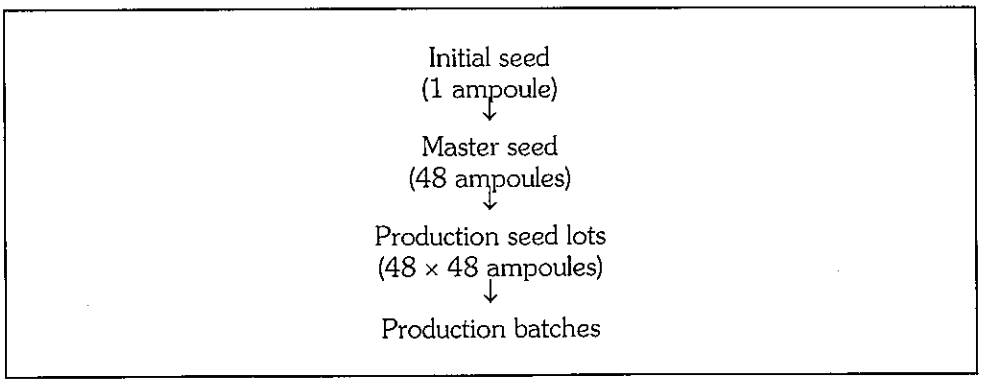
The initial strain used must be obtained from an international reference centre and have demonstrated a sufficient level of immunogenicity in challenge trials in susceptible sheep. This requirement is described in detail in the Rev1 monograph of the European Pharmacopoeia (8). An appropriate strain of *B. melitensis* Rev1 may be obtained from the Service Européen de la Qualité du Médicament/European Department for the quality of Medecines, Strasbourg, France (1, 2).

Table II. **Differential identification of the major strains of *Brucella* sp. used in vaccine production**

	B. melitensis		***B. abortus***				***B. suis***
	Rev1	**H 38**	**19**	**99**	**544**	**45/20**	
Colonial aspect	**S (±1 mm)**	S	S	S	S	R	S
H2S production	-	-	+	+	+	+	+
Urease (Christensen)	+	+	+	+	-	+	+ (1 h)
Penicillin (5 UI)	-	-	-	+	+	±	-
Streptomycin (2,5 µg)	+	-	-	-	-	+	-
Erythritol	+	+	-	+	+	+	+
Thionine 1/50 000	-	+	-	-	-	-	+
Fuchsine 1/50 000	-	+	+	+	+	+	-

Having obtained the suitable initial seed, the responsibility of the producing laboratory is then to prepare and maintain a master seed lot and production seed lots in such a way that the characteristics of the initial seed are completely preserved. By performing two passages from the initial strain, enough seed lots can be produced with identical characteristics for a large number of vaccine batches (Table III). This procedure ensures that each seed lot is not more than three passages removed from the initial seed.

Table III. **Description of a seed lot system enabling the production of a large number of seed lots with minimal passaging**



The methods used to maintain the strains and preserve both their viability and their characteristics will depend on equipment and facilities available. Many techniques have been described (5). We have found freeze-drying to be the easiest and safest. Strains are preserved in glass ampoules frozen and dried in a centrifuge type freeze-drier and stored at +5°C (5).

The stability is excellent and exceeds 5-10 years. Care must taken at all steps of this production to ensure batch homogeneity (culture, harvest, ampoule filling, freeze-drying).

Quality control of the production seed is critical to ensure that it has not derived from the original strain. It must be identical, by all techniques used (Table II), to the master and initial seed. The viability of the production seed lot must also be checked in order to provide a reliable initiation of each industrial production run. A further concern must be the risk of cross contamination. This is especially true in laboratories handling many strains of *Brucella*. This possibility must be avoided by a strict implementation of good laboratory and manufacturing procedures (GLP, GMP) with specific separation of steps involving different strains. An appropriate quality control must be put into place to demonstrate the absence of cross contamination. We have established a practical selection of various well described bacteriological methods to do this (Table II).

I.2 Bacterial fermentation

The choice of equipment for bacterial fermentation will depend on amounts to be produced and local conditions. Production on solid media is time proven. This

method is however labour intensive and will usually be limited to small batches. Large amounts of vaccine can be turned out from fermenters, thus minimizing the number of batches and the number of controls. Fewer personnel may be used and containment of the bacteria better controlled. This method will however require a larger initial investment and specialized technicians especially for maintenance.

Whatever the method, particular attention must be given to the choice of raw materials. A good quality medium is an essential element in overcoming one of the principal difficulties in Rev1 production: dissociation. Peptones must be of high quality. Unavoidable batch to batch variation of these can be overcome by stocking sufficient amount of one batch for a large number of vaccine lots. Quality control methods should be well standardized and include vaccine trial runs and sterility for bacteria, fungi and mycoplasma (6). Heat sterilization of animal origin ingredients will avoid controls for viral contaminants. Although the probability of transmitting bovine spongiforme encephalitis (BSE) by vaccination has not been assessed, some countries are now requiring that peptones originate from BSE free countries.

Scale up passages may be done in a number of ways: solid media, liquid media or a combination. Although highest yield is sought, the method chosen must maintain the properties of the vaccine strain. In-process controls will especially emphasize purity (absence of bacterial contamination) and dissociation (smooth to rough) (Table IV). A complete identity test will be carried out in the final fermenter culture prior to freeze-drying (Tables 3 and 4). It should be noted that most of the control methods used (excepting direct microscopic observation) will provide results after the culture has been passaged, and freeze-dried, thus making the cost of a non satisfactory test and the subsequent destruction of the serial more costly. This emphasizes the need for a quality assurance program that will prevent defects.

I.3 Vaccine formulation

Formulation of the vaccine will take the active ingredient (live bacterial suspension) and turn out a finished vaccine in its final "ready to use" format. Live vaccines such as Rev1 can be presented in a liquid or freeze-dried form. Freeze-drying will maintain viability for much longer periods of time (18 months versus 2-4 weeks) (3). A detailed description to the technology of freeze-drying is beyond the scope of this presentation. However the following parameters must be taken into account when optimizing a process: quality of the initial bacterial suspension, nature and quality of the substrata, the freezing and drying cycles, the atmosphere used to seal the container. Determination of residual moisture is an important quality control parameter that should be monitored. Water content should be between 2 and 3% to insure the best stabilization of viability (Fig. 1) (7). Freeze-dried vaccines should be stored in the cold (+5°C ±3°C) or may be frozen (-20°C).

Table IV. **Schematic of the scale up passages of a bacterial vaccine strain and quality control**

Seed lot ampoule			Media ingredients Other raw materials
⇙ QC	⇘ ⇙		⇘ QC
	Tube	-	Purity: Gram strain bacterioscopy
	↓	-	Dissociation
Open steps	Flasks	-	Purity: Gram strain bacterioscopy
(Hood)	↓	-	Dissociation
	Jug	-	Purity: Gram strain bacterioscopy
	↓	-	Dissociation
Closed step	Fermenter	-	Purity: Gram strain bacterioscopy-
(steam sterilized ducts)		-	Identity, dissociation

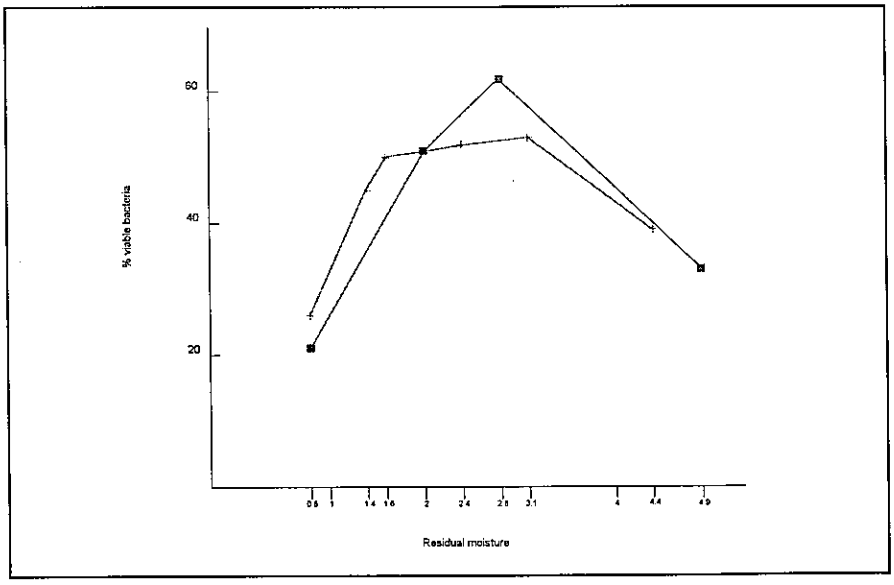


Fig. 1. **Stability of freeze-dried strain 19 vaccines with different levels of residual moisture**

II. Quality control

The standards applied to brucellosis vaccines have been well documented (2, 3, 4, 7). In all cases, control methods should conform to international and local authority requirements. Stringent standards are necessary to insure that the vaccines being used in long term control programs are of consistent quality.
Quality control methods can be broken down into the broad categories:

II.1 Physico-chemical controls (Aspect, residual moisture, pH, amount in final container)

A consistent appearance of a well formed freeze-dried pellet is important to insure that the batch is homogeneous. Defects in filling or adjustment of the freeze-dryer will be revealed by the appearance of the pellet.

Residual moisture is, as described above, a good indicator of the quality of the lyophilization steps and of the stability of the batch. It should be emphasized that high quality vials and plugs are also very important to guaranty stability and avoid rehydration of the pellet.

II.2 Biological controls *in vitro*

These methods have been described in detail (1)

Bacteriological purity

This test should insure that the batch contains only *B. melitensis* Rev1 and is free of any other bacterial and fungal contamination. Appropriate media must be used to check this and adapted where necessary to take into account a particular risk in a given production facility (for example sporulated bacteria).

Rev.1 identity and dissociation

These tests will be identical to those performed on the seed lots and will confirm that the production batch is identical in all its characteristics to the master seed (Table IV).

Viability

In most cases, this test which will provide the number of viable colony forming units (CFU) of Rev1 in each vial of the batch is the measure of the potency of the vaccine. It will determine the number of doses (10^9 to 2×10^9 CFU/dose) in each vial. The exact method has been described in detail (1). It must be performed with great care and sampling must ensure batch homogeneity.

II.3 Biological controls *in vivo*

These include controls for activity and safety: Rev1 vaccine is tested in laboratory animals for reactivity and immunogenicity as described (3, 7). The duration of the tests for reactivity and immunogenicity may be incompatible with the rhythm of production. In such a case the tests may be applied to each seed lot and the first batch of vaccine from a new seed lot. Particular care must be taken to insure that bacteria in the subsequent batches have maintained their cultural properties.

III. Security measures

Brucellosis is a disease affecting humans through consumption of animal products or professional contact with infected animals or the organism in a laboratory. In the

course of a control program, every precaution should be taken so that the laboratory producing the vaccine does not become a health hazard for its workers or environment.

Various parameters should be taken into account when designing a facility for production of vaccines for brucellosis (Fig. 2).

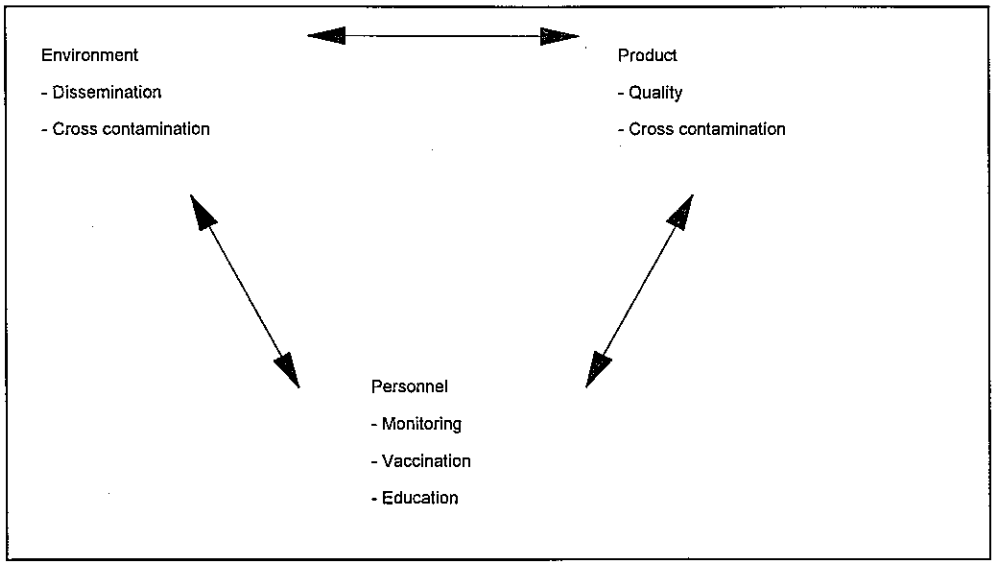


Fig. 2. **The factors involved in containment of *Brucella* during the industrial production of vaccines**

III.1 The product

Cross contamination of the product can be avoided by good manufacturing procedures: handling one strain at a time in a given laboratory, all open transfers performed under class II laminar flow hoods, prefer transfers through steam sterilized ducts.

III.2 The environment

Production laboratories must be under negative pressure. Liquid waste should be inactivated (heat or chemical inactivation). Air is expelled though high efficiency filters. Ideally solid waste should leave the laboratory through a double door autoclave. If unavailable, bags should be clearly marked for disposal and carry a biohazard warning. Adequate procedures should exist in writing to describe the complete disposal procedure (autoclaving or incineration).

III.3 The personnel

Technicians involved in the production of vaccines for brucellosis are at a high risk of acquiring an infection as well as causing a dissemination of the agent. All technicians are regularly tested for antibodies and skin hypersensitivity to *Brucella* antigens. A person with a generalized allergic reaction to *Brucella* antigen will be kept away from the production or control laboratories. Special care must be taken when handling infected animals. Finally, but most importantly personnel must be well trained and reminded of the requirements for handling a class III pathogen.

IV. Conclusion

Although all or most of the basic methods for the production and control of vaccines for brucellosis in sheep and goat have been published, their production at a large scale, requires additional expertise. At all levels, many factors must be taken into account to insure that the quality of the production output is homogeneous from batch to batch. Only strict implementation of good manufacturing practices and a quality assurance program will ensure this.

Batch quality control must be considered as a series of methods that will detect gross defects of a production run. They cannot be applied on all vials of a batch and certain tests (immunogenicity in animals) are cumbersome and costly.

All these factors must be taken into account by authorities in charge of implementing a brucellosis control program to guaranty that the vaccine they are using is of high quality. We should remember that although the cost of the vaccine is only a fraction of the total cost of a control program, its quality will directly and dramatically affect the outcome of the program.

References

1. **ALTON G.G., JONES L.M., ANGUS R.D. AND VERGER J.M.** Techniques for the brucellosis laboratory. INRA, Paris, 1988.

2. **Joint FAO/WHO Expert Committee on Brucellosis**. 6th Report. Technical Report Series 740, WHO, Geneva, 1986.

3. **WHO Expert Committee on Biological Standardization** 28th Report. Technical Report Series 610, WHO, Geneva, 1977.

4. **ELBERG S.S. AND FAUNCE K.J.** Bacteriol. 1957, 73: 211.

5. **KIRSOP B.E. AND SNELL J.J.S.** Maintenance of Microorganisms, 1st edition, Academic Press, London, 1984.

6. Code of Federal Regulations, 9 CFR 113.

7. **PRECAUSTA P., BENET G., LANGUET B. AND MILWARD F.** Microorganismes et fabrication des vaccins. Conservation des Microorganismes, 12ème Colloque de la Société de Microbiologie Industrielle, Lille, 1987.

8. *Vaccinum brucellosis (Brucella melitensis stirpe Rev1) vivum cryodesiccatum ad usum veterinarium*. European Pharmacopoeia Monograph, 1992.

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The Rev.1 vaccination plan for sheep in the Provence-Alpes-Côte d'Azur (PACA) Area A prerequisite for a successful eradiction of *Brucella melitensis*

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Introduction

My report will try to demonstrate the efficiency of the Rev.1 vaccine in the field, in situation in the PACA area, which has a real mediterranean character.
In spite of a complexe epidemiologic situation, the sanitary levels have been greatly improved, since it is reasonably thought that an almost total eradication should occur around 2005/2010. Following the beginning of the medico-sanitary scheme in 1980-1990,
The epidemiologic complexity comes from the fact that:
- 500,000 sheep transhume every summer to the mountain-pastures ;
- an endemic situation of "*Brucella melitensis*" does exist ;
- a permanent mixing of herds and flocks is not controlled ;
- the farming system in extensive (little productivity, little ancient) ;
- an inter-specific infection between unprotected and unvaccinated dairy producing cattle and goats does also exist;
- in certain situations the environment could be contaminated.

Presentation of PACA area

The PACA region is a rather mountainy area, bordering Italy and the Mediterranean sea, in the South-East of France, Marseille is the capitale (Fig. 1).
On the geographical, climatic, sociologic and economic points, it could be defined as heterogeneous. It also includes 6 "départements" (administrative subdivisions).

The livestock is quite small: 630,000 adult ewes of which 500,000 transhume every summer to the mountain-pastures in the North (Alpes) (Fig. 2).
Sixty thousand cattle of which forty thousands transhume, thirty thousands goats of which twenty thousand are sedentary; specialised in dairy and cheese production.

PACA is the greatest european area for interdepartemental transhumance, concerning more than 1,800 moutain-pastures units, regrouping 1,000 "communes", over the 6 "départements".

The movements and mixing between different herds and flocks are not always supervised as they should be and therefore goes uncontrolled ; 2,000 sheep and goat flocks and 1,000 cattle herds are transhumant, the pastures ground covers a fairly large area, but is rather under-exploited. The technical knowledge of the sheep-breeders is rather low, with an extensive production in the sheep "sector" (0.8 weaned lamb for one ewe in average).

The interspecific contacts between the 2 groups (sheep and cattle) are still very frequent and tensions are not rare, [the bovine breeders (with no *Brucella* and no vaccine) and the ovine breeders still infected (around 10% of their livestocks in 1995) and vaccinated].
The epidemiologic complexity of the region represents a "good" observation system.

• ***On the sheep level*** (very stongly infected with *Brucella* at the beginnig of the scheme, 100% of livestocks prevalence in 1972).

The prophylaxis programs have been realised in two phases and in four stages:
- a first phase of strict vaccination (medical)
i - H38 from 1972 to 1981
ii - Rev.1 systematic on young sheep 100,000 vaccines/year from 1981 to 1989.

- a second phase of medico-sanitary prophylaxis combining the Rev.1 vaccination plan on young sheep and the sero-diagnosis of the adults (more than 18 months / 2 years old) and subsidized slaughtering of sero-positive animals.

iii - Rev.1 subutaneous 1989 -> 1994 .

iv - Rev.1 conjunctival starting 1995.

• ***On the dairy producting bovine and caprine level*** (midly infected in 1974, 25% of prevalence in bovine livestocks).
The control of bovine brucellosis has started in 1973 with a community of breeders already "used" to collective operations of sanitary prophylaxis (foot and mouth disease, tuberculosis).

- Phase 1: medico-sanitary (S19 vaccine till 1984).

- Phase 2: strictly sanitary since 1984.

A precise chart showing the different prevalence levels of brucellosis in cattle, sheep and goats is given in Figure 3.

Results
The epidemiologic results let appear after more than 20 years of organised struggle that the vaccines and more precisely the Rev.1 has permitted a control of the *Brucella melitensis* infection in the small ruminants.
The Rev.1 gives a lasting immunity during the " economic life" of the ewes (4 pregnancies).
This immunity is indirectly measurable with regards to the statistics of the bacteriologic analysis of the abortions: still 40% of the abovtions were due to brucellosis in 1981 in spite of 9 years of H38 vaccinations, to less than 5% in 1989.
The animal prevalence of about 40% in 1972 fell down to 3 % thereabout in 1988.

The livestocks prevalence of almost 100% in 1972 fell to 60% in 1988 (before the start of the medico-sanitary prophylaxis) (Fig. 4).
Since 1981 more than 1,5 million young sheep have been immunised with the Rev.1 vaccine.

Operating conditions on site

The Rev.1 vaccine needing either an injection or an oculary instillation does not determine any local reaction nor stress. It is always well accepted by the breeders (where the H38 never was); after a phase of information which still is necessary.
It is in particular essential to identify the vaccinated animals (tags, tattoos,...) and to fill a certificate of vaccination to have a real knowledge of the epidemiological situation.

The sanitary veterinary practicians, at the beginning were feeling a little drawback by the use of the Rev.1 vaccine. They felt that some infections could be tied up with accidental injection during the prophylaxis operations in the flocks.

Thoses fears have been cleared up by the following facts: accidents of these types are in fact fairly rare if the animals are well hold. Infection is also very well under control owing to the immediate use of active antibiotics against the Rev.1 strain.
Only a few very rare cases of allergy have been described on people infected with *Brucella* beforehand and getting "spiked" during a vaccination session.

The practician should take a minimum of preventive measures during a vaccination session:

- Flocks or herd in good sanitary health and situation outside a stressfull periode (shearing, transhumance, climatic disorders).

- A previous history of "Malta fever " for the people handling the animals (a risk of allergy by accidental injections).

- Protective gloves and glasses for the veterinary practicians.

- The use of sterile equipment by heat or single use seringue

- The strict respect of the cold chain during the transport (5 degrees Celcius)

- The respect of the short time delay between the extemporaneous preparation and the injection of the vaccine.

- The environment protection by removing and destroying after disinfection with antisceptics of vaccine bottles, needlesand seringues...
The veterinary practician should take care not to injecte pregnant animals even if in the PACA region no abortion accidents have ever occurred following a Rev.1 vaccination. (It must be said that the vaccination in France only concerne the ewes under six months of age, rarely pregnant at this age).
No problem of contagion has ever been reported between a lot of sheep recently vaccinated and a lot of pregnant ewes mixed in the same flock.

To organise and to structure the operations

A structured organisation of the operation of prophylaxis is completly useful to attain a exhaustive vaccination, sole warranty of optimal efficiency to control the abortion and progressively decrease to "infection pressure" (excreting animals and contaminated surroundings).

The organisation in the PACA region has allowed:

- The census of all the herds and flocks in the area (3,100 in 1995) and the attribution of a breeding number.

-The individual identification of each animal (630,000 sheep in 1995) .

- An unfading tattoo of the Rev.1 vaccination act in the ear of the impubecent sheep.

- A proof of the vaccination act and a summary of numbers of animals vaccinated (vaccination certificate).

- The computerized records of all vaccinal or serodiagnosis data of the breedings and their certification.

- Control of trade and transhuming animals.

In adopting the french organisation towards the prophylaxis of reputed contagious illness, the PACA region has started a truly joint management venture in this eradiction scheme between the administration (DSV: Directorate of Veterinary Services) and the breeders associations (GDS: Sanitary Defense Group).
The turn of these GDS being that every breeders should take into account his own responsability in the out carrying of this sanitary program of collective interest and to associate henceforth **every** actor in the success of these objectives.

Conclusion

The exhaustive use of the Rev.1 vaccine on the almost entire young ovine population has been for the PACA region an absolute prerequisite for an engagement on the "Road of *Brucella melitensis* eradication". The Rev.1 has allowed us to control abortions, an essential factor in the environment and animals contamination, dividing by 10 their expression in 8 years of vaccination.
The epidemiological constraints of the PACA region (the mixing between herds and flocks, the massive transhumance, the low technical level of the breedings) lead as in all logic to proceed with the medico-sanitary prophylaxis until his final aim: eradication around 2005/2010. In his conjunctival conditionning, the Rev.1 allows us to diminish the frequency of "interference" in the sero-diagnosis of the "sanitary phase" of the prophylaxis.
During a phase where the sheep prevalence of animals becomes really low (0,3 %) any interference with vaccinal antibody is detrimental to the breeder and to the public finances.
In this hypothesis, a stop in the vaccination program could have been thought of with contempt for any epidemiologic reasoning. To avoid this error, the conjunctival vaccine becomes in our context an "almost ideal" tool, even if an ideal vaccine does not exist. May be never will...

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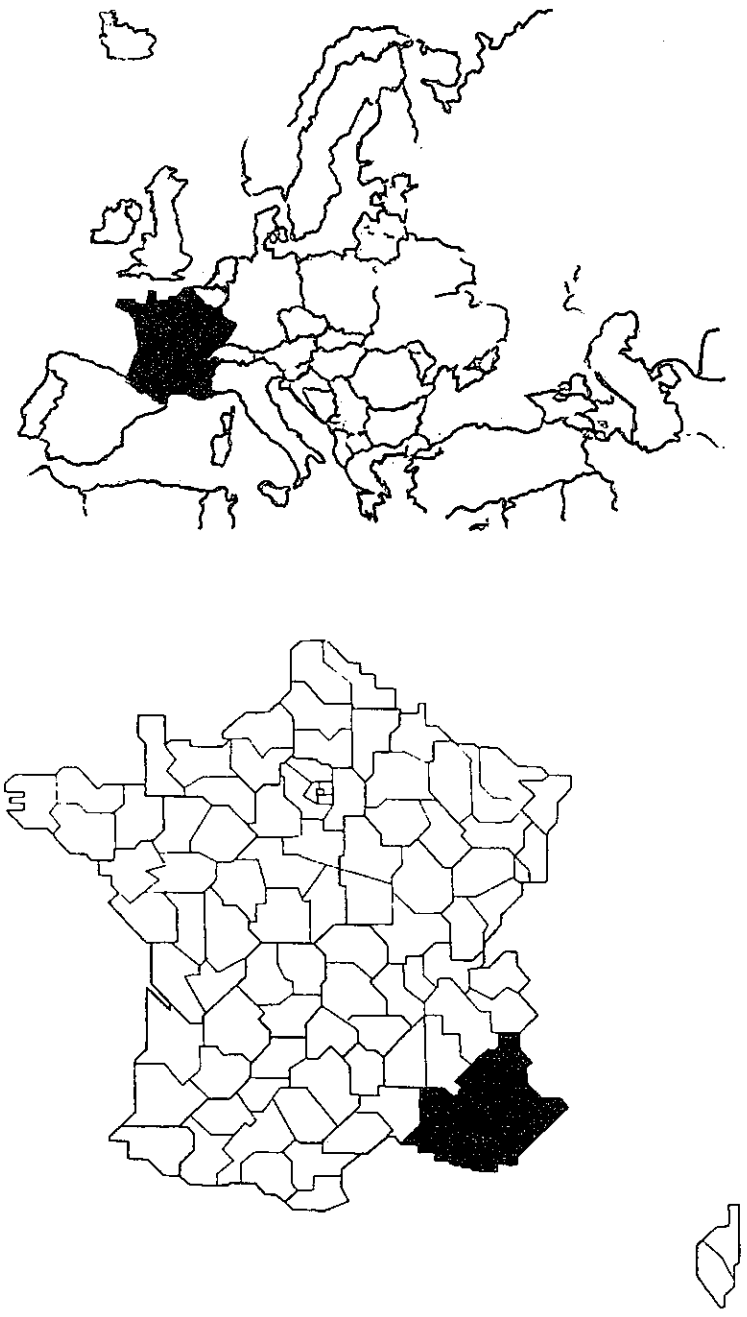


Fig. 1. **Geographical situation of France in Europe and of Provence-Alpes Côte d'Azur Region in France**

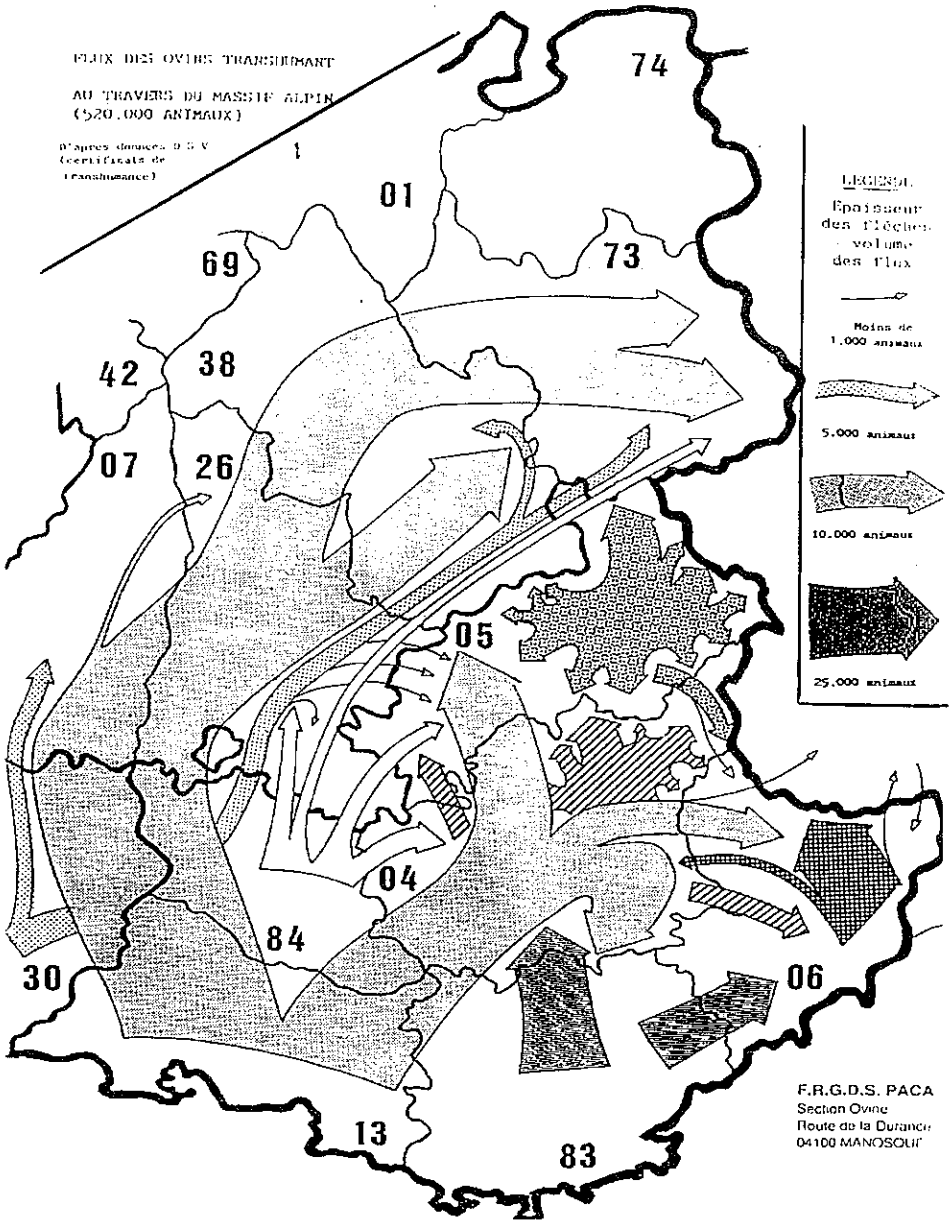
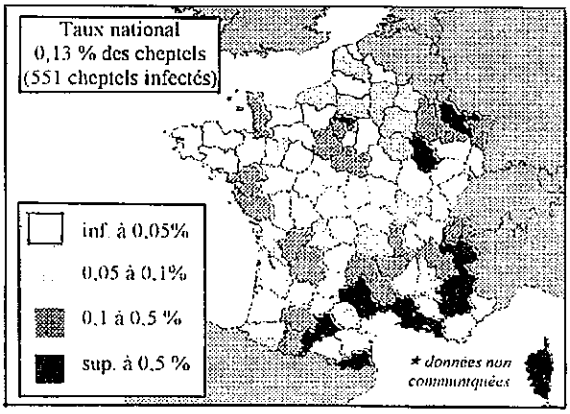
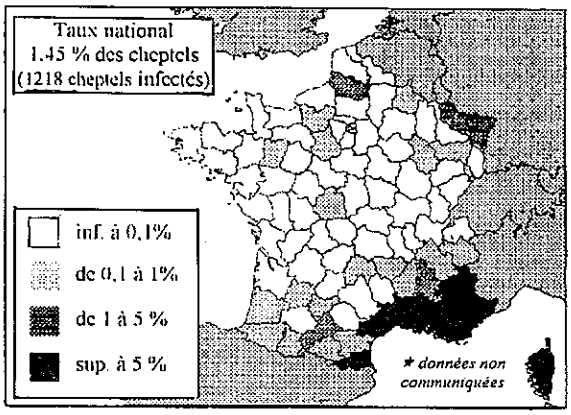


Fig. 2. **Transhumance of sheep during summer in the Southern French Alps**

Brucellose bovine



Brucellose ovine



Bilan des déclarations d'avortement

	Mars 1995	du 01/04/94 au 31/03/95	du 01/04/93 au 31/03/94
Avortements déclarés	3 414	50 239	52 677
dont brucelliques	20	219	202
% de brucelliques	0,59%	0,44%	0,38%

Incidence des maladies (en cheptels)

	Mars 1995	du 01/04/94 au 31/03/95	du 01/04/93 au 31/03/94
Tuberculose bovine	35	288	419
Leucose bovine	59	433	714
Brucellose bovine	88 (12 nre)	635 (141 nre)	861 (130 nre)
Brucellose ovine	60	501	495
Brucellose caprine	8	42	53

Bilan des abattages réalisés

	Mars 1995	du 01/04/94 au 31/03/95	du 01/04/93 au 31/03/94	du 01/04/92 au 31/03/93
BOVINS	2 562	24 008	28 260	46 741
Tuberculose	256	3 961	6 246	7 577
Leucose	240	2 755	4 293	8 527
Brucellose	2 066	17 292	17 721	30 637
OVINS	1 377	20 417	20 869	22 383
CAPRINS	27	459	972	716

Brucellose caprine

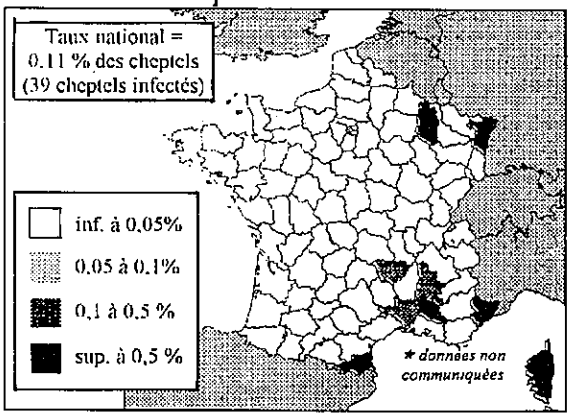


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Fig 3. **Epidemiological situation of Bovine, Ovine and Caprine brucellosis in France (March 1995)**

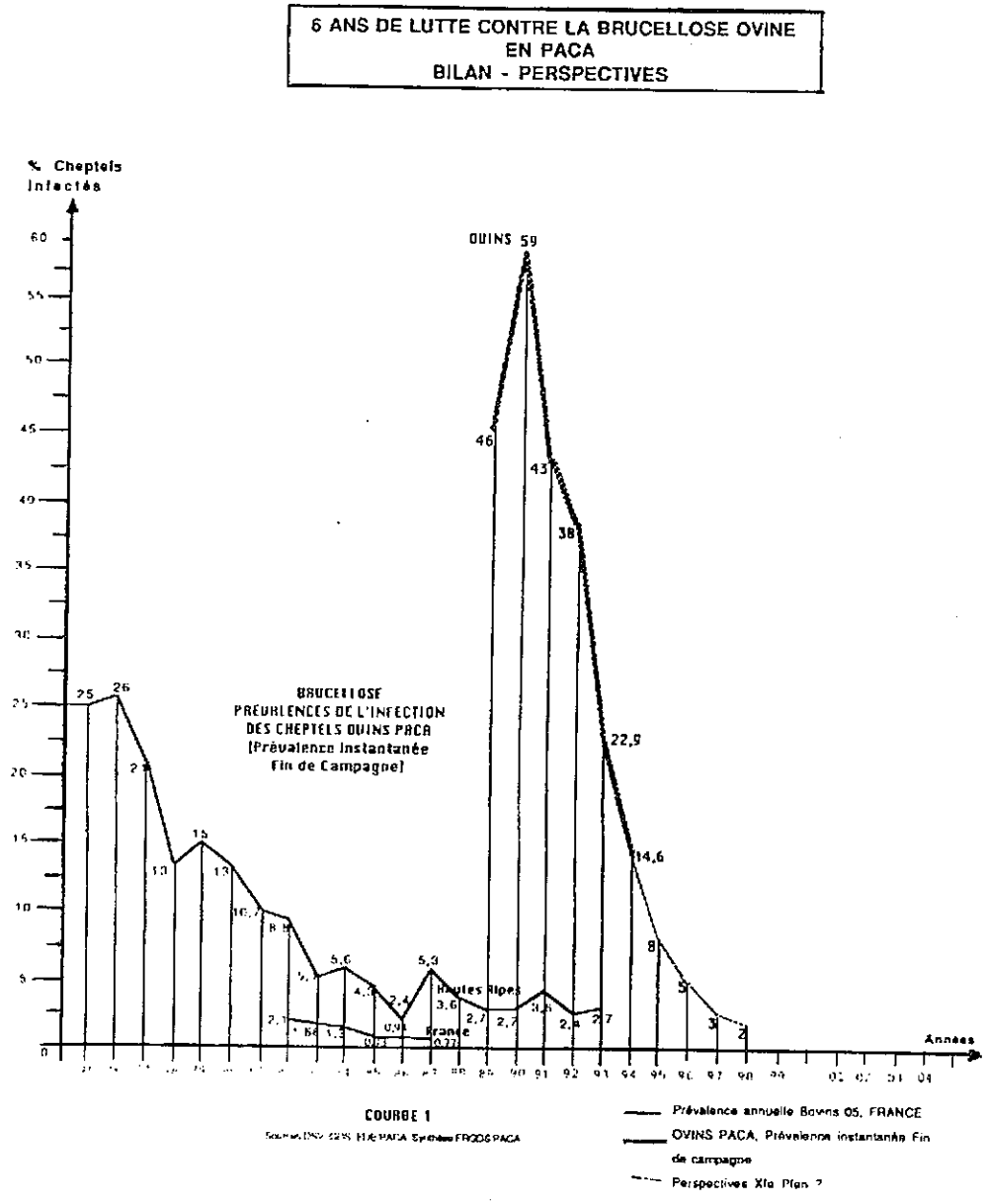

Fig. 4. **Evolution of point prevalence (31 December) of bovine and ovine brucellosis in France and Département of Hautes-Alpes (PACA Region) from 1975 to 1993.**

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Problems associated with the persistence and possible horizontal transfer of *Brucella melitensis* Rev.1 vaccine in connection with serological surveillance in Israel

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Summary
The control of *Brucella melitensis* requires that three elements will be simultaneously implemented. These are: serological surveillance of the animals using standardized diagnostic techniques, test and slaughter of the infected, animals and vaccination of the young progeny. A living attenuated vaccine strain *B. melitensis* Rev.1 has been developed and found superior to other available vaccines. However, its main application in Israel led to realization of several adverse effects that were encountered during the years. In top of all Rev.1 caused human infection in at least one recognized incident. Secondly, bacteriological persistence of the strain was demonstrated in several cases and in a few of them Rev.1 changed to a rough form while being transferred horizontally in the flock. Serological persistence of the antibody titer occurred as well predominately in intensively managed sheep and goat farms with a high vaccination cover. As a result, diagnosis was severly hampered and the need for diagnostic methods that can distinguish between serological and bacteriological responses to vaccination and infection was raised. The recent identification in Israel of an atypical *B. melitensis* biovar1 strain with characteristics resembling those of the Rev.1 vaccine strain best demonstrate our current problems in the diagnosis of the disease. Lastly, cattle is the other animal population at risk due to *B. melitensis*. Should Rev.1 be used as a multi-host vaccine?

Elberg and his colleagues in the University of California has first envisaged the idea that a living vaccine strain is necessary to achieve best immunization of the animals against *Brucella melitensis*. As a result the vaccine strain Rev.1 was developed in 1955 and since then it was established worldwide not only as the definitive best vaccine for small ruminants but also as a potential vaccine for other domestic farm animals.
The Rev.1 vaccine was evaluated in respect to two parameters (for review see ref. 1,2). The safety of use was tested in relation to possible loss of attenuation and reversion to virulence with no evidence to such events. Nevertheless Rev.1 was secreted in the milk of a few percentage of lactating ewes revealing the problem of bacteriological persistence of the strain. Similarly, it was demonstrated that serological persistence might occur in several animals up to about 2 years after the vaccination of the animals.

Was Rev.1 a good vaccine in respect to its capacity to confer protection on the vaccinated animals? This question was studied by challenging either vaccinated animals with no infection history or those that were vaccinated when being already infected with a virulent strain. Whereas most of the studies provided satisfactory evidence on the protective efficacy of the vaccine there was at least one report that had weakened this conclusion.

The Israeli experience on use of the Rev.1 vaccine started with the inclusion of Neeman in the teams that evaluated the vaccine (3,4,5). Soon after, full dose Rev.1 vaccination of ewe lambs and kid goats was implemented as part of the Israeli control program of *B. melitensis*. While sheep and goats were the target animals to this disease dairy cattle was soon realized as potential animal population at risk.

The results of 10 years surveillance, between 1970 to 1979, had been summarized by Feinhaken and Dafni (6). Besides bacteriological isolation and characterization of all 3 *B. melitensis* biovars in the sheep, goats and cattle population, and among human beings, Rev.1 isolates were obtained as well during these years. Thus, whereas the problem of Rev.1 persistence could be suggested from the small scale vaccination experiments the bacteriological isolation of this strain from field samples indicated that this in fact had become a major obstacle in surveillance of the disease in places where vaccination is complied.

Brucella abortus infection in cattle ceased to be a problem in Israel since 1985. In contrast the problem of occasional *B. melitensis* infection in beef cattle and several incidents of the disease in large dairy cattle farms (7,8,9) raised questions of the usefulness of B19 vaccination of the heifers, as has been applicated in Israel all over these years.

Because eradication program had not been incepted in Israel until recently, control of the disease basically relied on serological surveillance of the target animals followed by "test and slaughter" of infected flocks, bacteriological confirmation of the strain and Rev.1 vaccination of ewe lambs and kid goats and B19 vaccination of heifers. The annual rate of human infection was assessed in a collaborative work between the Israeli Health Ministry and serological and bacteriological confirmation of individual cases by the Central Laboratory for the disease in the Kimron Veterinary Institute. In July 1993, the Israeli Veterinary Services in collaboration with the Central Laboratory implemented an interim control program, aimed at reducing the level of *B. melitensis* infection in heavily infected foci. This project included several elements, as follows: a) Serological diagnosis of the flocks by CFT; b) Test and slaughter of animals in flocks where serological prevalence is less than 10% and the culling of the whole flock in places with incidence rate higher than 10%; c) intensification of Rev.1 vaccination of all the small ruminant population (full dose, single vaccination of ewe-lambs and female kid goats at the age between 3 to 6 months); and d) establishment of brucella free intensively managed sheep and goat farms for purposes of complying to import-export regulations and restocking of depopulated farms.

The interim project ended in April, 1995, when Israel allocated capital sufficient to carry out a national eradication program. This program includes CFT surveillance of all the small ruminant population which had accomplished first gestation (indicating that residual titers are not expected), test and slaughter or culling of the whole flock according to categories of less or over 10% infection rate, respectively, and bacteriological identification of the ethiological agent. The data collected during the years are described in this report focusing on the adverse effects of Rev.1 vaccination.

I. Rev.1 infection of sheep in an intensively managed farm which led to infection of the owner with the strain.

In 1987, a 3 year-old ewe aborted in an intensively manged sheep farm that had a record of over 90% Rev.1 vaccination. The farm was geographically isolated in the area.

The aborted fetus was sent to the Central Laboratory in the Kimron Veterinary Institute and *B. melitensis* Rev.1 vaccine strain was isolated from its organs. Further surveillance of the flock revealed many ewes that reacted positively to the serological tests. Although milk samples from many of these ewes were bacteriologically tested, and several of the ewes slaughtered for the purpose of bacteriological tests upon their reproductive organs and lymphatic nodes, none yielded brucella isolation.

About seven months after diagnosing the disease in the flock, the owner contracted the disease and a *B. melitensis* strain Rev.1 was cultured from his blood. The strain possessed biochemical characteristics similar to those of Rev.1: *e.g.*, susceptibility to penicillin and to the high concentrations of aniline dyes - basic fuchsin and thionin - and resistance to streptomycin and to the lower concentrations of the abovementioned dyes. However, this particular strain was shown to be more virulent than a normal vaccine strain to guinea pigs (10). Rev.1 infection in sheep and man had been similarly reported, in the same period, in South Africa (11-13).

II. Asymptomatic infection of an unvaccinated ewe with *B. melitensis* biovar 1 virulent strain with aberrant urease activity.

The abortion of a few young ewes in an intensively managed flock, with 100% vaccination of all the newborn, was reported. Subsequent serological tests led to diagnosis of more than 50% of the ewes which reacted positively in the CFT test. A test and slaughter program was initiated in the flock, but the bacteriological tests failed to reveal brucella.

It took about three years before a coincidental culling of a gestating ewe, which during the course of the survey tested negative serologically, led to a positive isolation of a *B. melitensis* field strain in two of four fetuses found in the uterus during post mortem. A retrospective study revealed the ewe - with several others - had been imported unvaccinated onto the farm as a means of restocking the genetic material of the flock. While the imported ewes had been left unvaccinated, all newborn were vaccinated in due time.

Interestingly, although the *B. melitensis* isolate had bacteriological features of a field strain, its urease activity was slow, resembling the characteristics of Rev.1. Originally, Rev.1 had been shown to be urease negative (1), but the currently used strain is a slow urease producer (Banai, unpublished).

Although it was difficult to draw a conclusive explanation to this incident, it was possible to suggest the following: Firstly, the intensive vaccination of the flock possibly prevented secretion of the brucella either through abortions (no subsequent abortion had occurred) or in the milk. Secondly, it was not possible to distinguish between serological responses of the ewes due to vaccination or infection. Finally, the isolation of the strain indicated beyond doubt that infection occurred in the flock. There were two questions, however: why didn't the ewe react serologically even though infected, and did the virulent strain originate from the vaccine strain?

III. Isolation of a rough *B. melitensis* Rev.1 like strain from unvaccinated sheep.

A flock of 114 sheep, of which about 70% were vaccinated, was tested by the CFT. About 83% and 68% of the vaccinated and unvaccinated sheep, respectively, reacted

at positive titers. Sixty-six milk samples were tested bacteriologically. A *B. melitensis* Rev.1 like organism was isolated from the milk samples of two unvaccinated sheep. The strain was in a rough form as shown by the acriflavine test and its resistance to *Brucella* bacteriophages, but it grew on agar plates similar to the growth pattern of a normal Rev.1 strain. Since the strain in the rough form was isolated from two different sheep it seems that the possibility of a laboratory artifact could be excluded. Therefore, this incident probably indicated that mutation of the vaccine strain had occurred which led to the regaining of its virulence property and horizontal transfer of the strain into the unvaccinated sheep.
A similar occasion of rough Rev.1 infection of sheep and horizontal transfer of the strain to the unvaccinated ones had been recorded in another sheep farm. Once again, most animals in the flock were vaccinated as ewe lambs and the management was intensive.

IV. Isolation of normal Rev.1 strains from the milk of vaccinated and unvaccinated sheep and occurrence of a mixed infection in the flock of Rev.1 and a field strain.

The data shown in Table I depicts the annual number of *B. melitensis* isolates obtained from milk samples or from lymphatic nodes and uterine samples of slaughtered animals. Because of encountering higher numbers of positive CFT reactors among sheep from intensively managed farms the possibility of Rev.1 infection was assessed by bacteriological culturing of milk samples form positive reactors. As can be seen in the table about 3 to 4% of the milk samples, mostly collected from intensively managed flocks, but some collected from extensive flocks, had yielded *B. melitensis* field strains. However, Rev.1 strain was isolated from about 0.8% of the milk samples. Interestingly, although brucella isolates were obtained at higher rates from lymph nodes and uterine samples, Rev.1 was not isolated from these organs probably indicating that the strain could not survive in this environment.
One should also note the fact that in at least three cases a mixed infection of Rev.1 and a field strain was identified in the flock. In the first case, milk samples from several sheep from an infected flock were sent to the laboratory. Whereas two milk samples yielded normal field strain isolates, the third sample yielded a Rev.1 isolate.
In the second problematic case, an extensively managed flock was diagnosed as infected with *B. melitensis* biotype 1 strain. After a test and slaughter program, the ewes were cleansed of the infective agent and newborn vaccination was continued. Nevertheless, a sudden wave of abortions commenced among ewes five months post-vaccination. While the virulent strain was isolated from a few ewes, *B. melitensis* strain Rev.1 was isolated from another ewe. Definitely, this incident demonstrated that a mixed infection of field and vaccine strains could occur.
Moreover, the infection of the recently vaccinated young ewes with a *B. melitensis* virulent strain raised questions about the efficacy of vaccination, at least in heavily infected flocks.

In a recent brucellosis outbreak in a sheep flock the Rev.1 vaccine strain has been co-isolated with a field strain from an aborted placentum.

V. The problem of serological surveillance of intensively managed flocks with high vaccination rate

The data presented in Table II depicts the annual rate of Rev.1 vaccination, as a percentage of the total sheep and goat population per veterinary district jurisdiction, from 1991 to 1994. Usually a value of about 30% vaccination rate of the total

animal population would indicate accomplishment of vaccination of the whole progeny. As can be seen in Table II this objective was achieved by at least four veterinary district jurisdictions while the others had also successfully intensified vaccination of the flocks. Nevertheless, in at least 3 places, in which most of the nomadic small ruminants reside (about half of the total small ruminant population in Israel), the vaccination rate is not satisfactory. No wonder, therefore, is the fact that *B. melitensis* is endemic in these places being the reservoir of reinfection of other flocks, and predominantly of dairy cattle infection.

Table III provides information on the achievements of the interim project carried out between July 1993 to March 1995, and some data on the beginning of the national eradication program since April 1995. The columns of ImmunoComb diagnosis and skin test diagnosis should obtain specific notation.

The immunoComb test is a solid phase ELISA, recently developed in Israel in a collaborative research study between the Central Laboratory in the Kimron Veterinary Institute and the Biogal company, Galed, Israel. This test was compared with conventional CFT and SAT and results suggested it was equal in sensitivity and specificity to CFT.

Therefore, it was decided to survey intensive sheep and goat flocks by this method, in order to reduce the size of serological samples sent to the laboratory. A total of 22573 animals, all had completed at least one gestation (indicating that they should not react in the CFT), from 112 flocks were tested. As can be seen in the table 1990 animals (8.2%) reacted in the test and about half of them were confirmed positive in the CFT as well.

In about 10% of the flocks the percentage of reactors ranged between 19 to 37%. If it was infection, such a figure would have meant that "test and slaughter" was not possible and all the animals should have been culled. On the other hand, if these animals reacted due to their vaccination the flock would have not been considered infected.

In an effort to identify the agent 334 milk samples were tested. Whereas Rev.1 was isolated in two flocks (one however in a rough form) another isolate was characterized as a normal field strain.

The low efficacy of bacteriological isolation definitely indicated that vaccination was important in reducing shedding of brucella from infected animals. Moreover, no abortion was recorded in these flocks supporting the working hypothesis that vaccination protects the animals form this clinical event. However, because infection with a field strain was not prevented two problems were realized: a) vaccination was not sufficiently effective in preventing the disease, and b) the currently available diagnostic methods could not distinguish between serological responses to vaccination or infection. Thus, without bacteriological confirmation (which was hampered due to vaccination) the vaccination complicated further diagnosis of the flocks.

VI. Identification in Israel of a *B. melitensis* biovar 1 atypical strain.

This strain resembles Rev.1 in its susceptibility to penicillin and to the dyes fuchsin and thionin. However, unlike Rev.1 it is also susceptible to streptomycin. The virulence of the strain is comparable to that of a normal strain (14).

Since the first report we continuously isolated this strain over the years and we could conclude that the strain has become endemic in Israel. Whether it evolved from a mutant that originated in a field strain or in the vaccine strain should be possible to indicate. Our latest data suggest that this strain did not evolve from the vaccine strain. Nevertheless, its occurrence in Israel complicates the diagnosis and requires additional bacteriological tests to facilitate the biotyping procedure.

Conclusions

The adverse effects of Rev.1 vaccination were shown. These include bacteriological persistence of the strain and as consequent persistence of the serological antibody titers in the vaccinated animals. The possibility of horizontal transfer of the strain to unvaccinated animals was raised. In a single case human Rev.1 infection has been confirmed.
Problems that require further elaboration are:

1) Has Rev.1 mutated and become more virulent than the originally developed strain?

2) Besides possible horizontal transfer of Rev.1 in the field, what would be its pathogenicity to human beings?

3) Is Rev.1 the ultimate vaccine also to target animals other than sheep and goats? This is specifically important when concerning protection of dairy cattle from *B. melitensis*.

4) There is continued need to develop new diagnostic techniques that will enable to identify infected animals in flocks where kidhood vaccination is practiced.
In the last year our laboratory had developed two important techniques that improve this specific diagnostic field. Firstly, an ELISA test was developed to distinguish between positive ovine, caprine and cattle reactors in the CFT whether their response is due to vaccination or due to infection. Secondly, a PCR method was developed that specifically identifies the Rev.1 strain. The both techniques are being employed in the Central Laboratory in the Kimron Veterinary Institute as part of the serological and bacteriological surveillance of the animal population in Israel.

References

1. **ALTON, G.G. AND ELBERG, S.S.** Rev.1 *Brucella melitensis* vaccine: a review of ten years of study. Vet. Bull. **11**:793-800, 1967.

2. **ELBERG, S.S.** Rev.1 *Brucella melitensis* vaccine. Part II 1968-1980. Vet. Bull. **51**:67-73, 1981.

3. **NEEMAN, L.** A comparative study of the efficacy of three vaccines in conferring resistance on sheep exposed to contact infection with *Brucella melitensis*. Refuah Veterinarith **20**:123-134, 1963.

4. **NEEMAN, L.** Virulence stability of the Rev.1 strain of *Brucella melitensis* on passage in pregnant sheep. Refuah Veterinarith **25**:188-202,1968.

5. **NEEMAN, L.** The safety of the Rev.1 strain of *Brucella melitensis* for pregnant sheep by natural contact. Refuah Veterinarith **25**:260-265.

6. **FEINHAKEN, D. AND DAFNi, I.** Identification of *Brucella* isolates in Israel, 1970-1979. Refuah Veterinarith **37**:117-123, 1980.

7. **DAVIDSON, M., SHIMSHONY, A., ADLER, H., BANAI, M. AND COHEN, A.** Protection of Brucellosis - free areas from reinfection. In: Adams, L.G. (ed.), Advances in *Brucellosis* Research, Texas A&M University Press, College Station, pp. 407-427. 1990.

8. **DAFNI, I., HOIDA, G., FEINHAKEN, D. AND BANAI, M.** Eradication of bovine brucellosis in Israel, 1970-1987. Israel J. Vet. Med. **45**:233-240, 1989.

9. **DAFNI, I., HOIDA, G., FEINHAKEN, D. AND BANAI, M.** Observation on *Brucella melitensis* infeciton in Israeli cattle herds. Israel J. Vet. Med. **46**:13-19, 1990.

10. **BANAI, M., HOIDA, G., RAPOPORT, E., ZAMIR, O., HAIMOVICH, M., KATZ, E., MAYER, I., COHEN, A. AND DAVIDSON, M.** Is *Brucella melitensis* undergoing evolutionary development or a change in the vaccinal Rev.1 strain? In: *Brucella* and brucellosis in man and animals (E. Tumbay, S. Hilmi, and O. Ang, eds.), Publication of the Turkish Microbiological Socieyt No. 16, 1991, Ege University Press. Izmir, Turkey.

11. **PIETERSON, P.M., GUMMOW, B., PEFANIS, S., VENTER, C.G. AND HERR, S.** The characteristics of a variant strain of *Brucella melitensis* Rev.1. Onderstepoort J. Vet. Res. **55**:15-17, 1988.

12. **PEFANIS, S.M., GOMMOW, B., PIETERSON, P.M., WILLIAMSON, C.C., VENTER, C.G. AND HERR, S.** The isolation and serology of the "FSA" *Brucella melitensis* Rev.1 mutant in a flock of sheep. Onderstepoort J. Vet. Res. **55**:181-183, 1988.

13. **HUNTER, P., PEFANIS, S.M., WILLIAMSON, C.C., BOTHA, W.J.S. AND SCHALKWYR, M. V.** Horizontal transmission in sheep and delayed clearance in guinea pigs and mice of a *Brucella melitensis* Rev.1 mutant. Jl. S. Afr. Vet. Ver. Ass. **60**:92-94, 1989.

14. **BANAI, M., MAYER, I. AND COHEN, A.** Isolation, identification, and characterization in Israel of *Brucella melitensis* biovar 1 atypical strain susceptible to dyes and penicillin, indicating the evolution of a new variant. J. Clin. Microbiol. **28**:1057-1059, 1990.

Table I. **Annual number of *Brucella* isolates in relation to total number of samples tested**

Year	No. of milk samples tested	No. of *Brucella* isolates		No. of organ samples	No. of *Brucella* isolates	
		Field Strain	Rev.1 isolate		Field Strain	Rev.1 isolate
1993	231	8 (3.4%)	1[a] (0.4%)	23	4 (17.4%)	None
1994	567	26 (4.6%)	5[b] (0.8%)	45	6 (13.3%)	None
1995	1092	36 (3.3%)	9 (0.8%)	16	0 (0.0%)	None

[a] Rough strain isolated from unvaccinated ewe
[b] Two Rev.1 isolates, one in a rough form and the other in a normal smooth form, were obtained from unvaccinated ewes in two separate flocks. The other Rev.1 isolates were all obtained from vaccinated ewes in a normal smooth form.

Table II. **Annual rate of Rev.1 vaccination, as a percentage of the total sheep and goat population per veterinary district jurisdiction, from 1991 to 1994.**

Year	Veterinary District Office									
	B.S.	Hd	Tb	Jr	Nz	Ac	Af	K.S.	Rv	Rn
1991	2.2	4.4	10.0	11.7	5.6	2.2	10.5	4.4	12.8	12.8
1992	4.4	8.9	16.7	13.9	10.5	5.0	15.3	9.4	10.5	17.8
1993	10.0	13.1	21.6	16.7	16.1	11.1	23.2	12.2	17.2	25.5
1994	8.9	18.9	26.1	27.8	16.6	14.4	33.3	18.8	18.9	31.1

Table III. **Evaluation of the interim project and the initial stages of the national Brucellosis control program**

Period	Method of diagnosis[a]						Total No. of tested flocks	Total No. of[b] slaughtered animals
	Serology		Immunocomb		Skin test[c]			
	No. of tests	No. of reactors	No. of tests	No. of reactors	No. of tests	No. of reactors		
July 1993 to March 1995	60,804 (1,540)	3,761 (6%)	25,573 (112)	1,990[d] (8.2%)	5,730 (26)	None	1,678	7,410 ($895,000)
April 1995 to July 1995	57,462[e] (1,358)		N.D.		N.D.			5,097 ($1,000,000)

[a] The number in brackets denotes the number of flocks. ND - Not Done.
[b] The number in brackets denotes compensation costs in US $.
[c] Intensively managed sheep fattening farms.
[d] In about 10% of the flocks the percentage of reactors ranged between 19 to 37%. Eight hundred and fifty four sera were also positive in CFT. Three *Brucella* isolates were obtained from 334 milk samples, two Rev.1 vaccine strains (one smooth and one rough) and one field strain. No isolate was obtained from lymph nodes and uterin samples of 17 slaughtered animals.
[e] In the first two months of the program 18,000 animals from 448 flocks were tested. Results were: 331 flocks (9546 animals) were brucellosis negative; 89 flocks (8984 animals) had less than 10% infection (273; 3%); and 27 flocks (813 animals) had higher than 10% infection (257; 31.6%).

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Some experience from brucellosis control with Rev.1 vaccine in a heavily infected country - Mongolia

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The informations presented here derived from the first and one of the most extent *Brucella melitensis* vaccination programme in sheep and goats in Mongolia. The WHO assisted program started in 1965 with epidemiological investigations and serological surveys, to determine the incidence and prevalence of brucellosis and its specific features in animal and human populations. On the base of results of several vaccine field trials in heavily infected areas, proposals were worked out for control of brucellosis in small ruminants with Rev.1 vaccine. In the second part of the program 1975-1978, a laboratory for local production of Rev.1 vaccine was established with a peak capacity of 5 million doses per year. Along with production started the extent Rev.1 vaccination, according to the control program, planned out for 10 years, with gradual involvement of the whole sheep and goats population in Mongolia. Over 30 million of animals were to be immunized. Later on, Rev.1 vaccine was used also in cattle. The information on this Rev.1 vaccination program in extremely harsh natural conditions, with very high prevalence of brucellosis might be considered now too old and somewhat outdated. The program, however, provided several new information on most practicable and effective application of vaccine, and optimal schemes of vaccination and on reasonable use of simple diagnostic methods, for which informations are valid up to now. The specific natural and ecological conditions, the countrywide high prevalence of brucellosis, and the local animal husbandry practices forced us to use procedures, which fairly differred from the valid concepts. After careful weighing the pros and cons, several deviations from conventional ways of *Brucella* immunization were adopted. As it turned out later, the changes were expedient and effective. Some of experience, *e.g.* vaccination of all suceptible animals, irrespective of their age, gravidity and serological status, were widely proven and generally accepted, but some others are still under discussion and not approved yet for common, practical use, *e.g.* vaccination of breeding males in infected flocks; the use of Rev.1 in cattle; the use of allergic skin test for surveys. Therefore, it might be useful to explain the points of view on such problems, often encountered, when immunization is to be introduced.

1. Control of brucellosis with the test and slaughter (T&S) of reactors system

In large flocks with high prevalence of brucellosis, the T&S, applied to individual reactors only, has proven to be entirely ineffective and unreliable. All attemps and efforts to control brucellosis in this way, were condemned to failure, due to limited reliability of the nowadays available diagnostic tests, which are unable to reveal all

infected animals, especially those in latent phase of disease. The elimination of reactors, detected in subsequent testings, result in considerable reduction of the tested flocks, but only very rarely to definite elimination of the disease from the flock. The very low effectiveness of the T&S method has proven also in Mongolia, where it was used country-wide for 3 subsequent years. Special veterinary diagnostic teams from COMECON states, equipped with mobile laboratories, tested all flocks of sheep and goats in allergic skin test, and partly in SAT and CFT, repeatedly in 3 subsequent years (12 million sheep and goats were tested). Although the permanently identified reactors were immediately eliminated and slaughtered, the next year, new reactors were found again. After total failure of this control method, the Government decided to abandon it and to switch to vaccination. In countrary to that, the T&S method might be highly effective, if the slaughter is applied to the whole flock, which proved to be infected. Such radical stamping out procedure is, as a fact, the most reliable, fast, and from long perspective, also the most economical method, how to get rid of brucellosis definitely. Unfortunately, in most situations it is not practicable due to high at once costs and difficult provision of replacement healthy animals. Nevertheless, it is the far most reliable way to get rid of brucellosis, when it is introduced to healthy areas (*e.g.* by imported animals), and when the disease should be immediately eradicated at any costs. In small flocks, with single reactors only, and where abortions did not occur yet, the T&S of reactors, may be worth of trying, if high quality tests are available. But even then, the final evidence of elimination of infection should be based on successful delivery of all sheep and goats in the flock.

2. Simple survey for detecting *Brucella* infected flocks

In conditions of economically underdevelopped countries, with nomadic or seminomadic animal husbandry, the *Brucella* infected flocks can be most easily detected in allergic skin test. The test is easy to perform, does not require identification of tested animals, the results can be read after 48 hours, in presence of owners, and the reactors immediately earmarked with permanent punching the ear. It is unnecessary to test all animals in the flock, but only a representative number of susceptible animals, preferably the adult females. The percentage of animals to be tested depends on the size of flock. If allergen is not available, the RBT can be used. In unclear, doubtful cases the examination should be repeated in SATT or better in CFT, if possible.

3. Advantages of vaccination of all sheep and goats in infected flock/area, regardless to the age, pregnancy and brucellosis status.

The conventional way of immunization with live *Brucella* vaccines, limited to young animals, is in conditions of extensive, nomadic animal husbandry, impracticable, and does not solve the problem of minimizing the sources of infection. Moreover, it prolongs the recovery period in flocks and complicates the process of control. The non-immunized adult animals remain unprotected, thus the infection can spread freely. At the same time the vaccination of adult animals has practically no adverse effect to their health status, as found by Alton (1962), and then proven in several field experiments in Mongolia. In spite of that, we tried to avoid vaccination of sheep and goats in second half of pregnancy, whenever possible, considering that it could provoke abortion in infected, sensitized animals. Therefore, the vaccination campaigns were carried out before the seasonal mating, or shortly after that (August-September). Thus the abortions were successfully prevented, except in few cases, when by mistake, highly pregnant, usually infected animals were vaccinated. In general, the Rev.1 vaccine proved to be fully safe in low pregnant, non-sensitized

sheep and goats, and did not influence adversely neither the oestrus not the fertility. In some infected, hypersensitized animals, the injection of full dose of Rev.1 vaccine may cause short increase of temperature, and swelling lymphrodes corresponding with region of infection. In most cases, however, the infected animals did not show any visible reaction nor fertility disorders after vaccination. The main objection against vaccination of adult animals, the long persistence of antibodies, have no importance in infected flocks, since transfer is permitted only to slaughterhouse, and the control is not based, at least not in the first 2-4 years, on T&S policy. Testing the adult animals prior to their vaccination and elimination of reactors was soon abandoned as impracticable, very labourious and unjustified measure, which cannot increase the efficacy and safety of vaccination an prevent the spread of infection. The total vaccination of the entire flocks helps to establish a relatively immune stock, helps to stop abortions, to reduce the number of excretors and the massivity of excretion, thus reducing and even eliminating the transmission to other animals or humans;

4. Immunization of breeding males

The original instructions did not advise to vaccinate the males with live *Brucella* vaccines. This requirement was, however, contraproductive in heavily infected areas in Mongolia, because the non immune healthy rams and bucks, kept in strictly separated male flocks, became soon infected when put into infected flocks for mating. Thousands of breeding males had to be slaughtered after breeding season, because of brucellosis, not rarely manifesting orchitis. The concern, that the Rev.1 strain may reside and multiply in tissues of testes and epididymis and cause orchitis, aspermia or oligospermia was not proven in experiments, when the young rams and bucks were given full doses of vaccine. The animals tolerated the vaccine without any local or general reaction and the antibodies disappeared from blood in 1-3 months. therefore vaccination of males was considered safe and introduced into routine practice in Mongolia.

5. The use of Rev.1 vaccine in cattle

After total failure of T&S method in control of bovine brucellosis in Mongolia, immunization with S19 vaccine was introduced in calves and later on in seronegative heifers and cows too. The results were in some herds satisfactory, in others abortions continued to occur as before. Isolations of *B. melitensis* in such herds led to consider the Rev.1 vaccination instead of S19. The proposal to test the Rev.1 vaccine in cattle, however was strongly opposed by soviet epidemiologists, because of their fears that Rev.1 may cause infection in cattle and consequent excretion in milk. The only available information from Drimmelen and Herwell (1964) who did not find any adverse effect of this vaccine in cattle, was not accepted as suffcient and it was required to check the Rev.1 in local bovine breeds and in yaks. Due to favourable results, a controlled comparative trial was carried out on 200, most low pregnant, heifers, divided in three groups: 1. Rev.1 vaccinated group (67), 2. S19 vaccinated group (61) and control group (70). Four months after vaccination, about half of heifers of each group were challenged conjunctivally with *B. abortus* 544 strain and the second half with *B. melitensis* H38 strain. The results can be briefly summarized in following points:

-no abortion occured during the first 6 weeks after vaccination in either of vaccinated groups.

- 50% of vaccinated animals were already seronegative after 120 days.

- The protection (measured by abortion rate, successful delivery and excretion of *Brucella*) was higher in animals vaccinated with Rev.1 vaccine, which proved to be

effective against both challenge strains. S19 provided significantly less protection against *B. melitensis*.
After above experiments, the Rev.1 vaccine was used instead of S19 in more and more herds and districts and since 1980 almost the entire cattle and yak population was imunized with Rev.1, without any negative effect.

6. Preparation and organization of the field vaccination program.

An approximative "time schedule" of immunization, indicating the territories and numbers of animals to be involved each year, was the base and starting point of the detailed planning. The following measures proved to be very important for successful realization of the program: Careful organization of the campaigns at all levels; Proper training of the personnel (field and laboratory); Wide publicity of the program; Establishing a veterinary committee (storage and distribution of vaccine); The well functioning, centralized state administration including veterinary services, and the discipline in executing the orders proved to be most helpful.

7. Performance of vaccination

The vaccination was carried out before the mating season (which enables better mobilization) when the cooler weather facilitated the transport and storage of vaccine. At first vaccination, all sheep and goats, without age limitations, were subjected to vaccination (except castrated males). In the following 6-10 years only the replacement animals were immunized. Breeding rams and bucks kept in special breeding stations were vaccinated only once, 2-3 months before mating. All vaccinated animals were immediately earmarked.

8. Postvaccination controls

The control was performed by serological testing of about 5% of vaccinated animals in about 2% of at random selected flocks, 2-3 weeks after vaccination. The control testing indicated that the percentage of reactors was in some flocks only 90-95% and even lower, supposedly owing to improper vaccination. Because some few animals may not respond with production of antibodies, it was often difficult to find out the percentage of real faults. The effect of vaccination depended much, beside the vaccine quality at the application, on the quality of vaccinator's work. Despite the strict control, cases of faulty vaccinations and even cheatings occured.

9. Impact of vaccination of small ruminants on brucellosis morbidity

The 2 ½ years since the start of vaccination program and the termination of WHO's assistance, was a relatively short period for making some exact, far reaching conclusions. Nevertheless, even the results, covering only that limited time, indicated that the vaccination had a significantly favourable effect. This was obvious from the reduction of the number of abortions (which were mostly due to *Brucella*). For that purpose data from 3 years prior to vaccination were compared with figures obtained after vaccination. In two aimaks where vaccination started in 1975, the abortion rate decreased from 4.6 to 2.1. Comparison of abortion rate in already vaccinated aimaks with still not vaccinated aimaks showed that abortion rate was 3.2 higher in non-vaccinated aimaks. The best evidence of the vaccination benefit seems to be the sharp decrease of human brucellosis incidence, which was 3-7 times higher in the first year after vaccination, and 30 times higher two years after vaccination. It is to say, however, that 2 ½ years period is not sufficient for final conclusions, and that longer observations and sampling of more factual information for analysis are needed for more definite and unambiguous evidence of effectiveness and benefit of

vaccination against brucellosis. After 1978, when the WHO project terminated, no further official information were available. According to some personal communications in 1982-1984, the program continued successfully. If true, it is an evidence of successful organization of the program and of the appreciation of the Government to the usefulness of the project.

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Current Knowledge on the Use of Rev.1 Vaccine in the State of Kuwait

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Summary

Brucellosis is an important disease of ruminant livestock and humans in the State of Kuwait. Prior to 1984, control policy was testing and slaughter of reactions and compensation. This policy was discontinued when it was found to be ineffective in reducing the disease incidence in dairy cattle.

The seroprevalence of brucellosis continued to increase both in ruminant livestock and humans from 1984 to 1986, until a widespread control project was undertaken from mid-December 1986 to mid-January 1987 in cattle, and between September 1987 and March 1988 in small ruminants. The effects of the vaccination campaign reduced the prevalence of brucellosis to 1.5% in cattle and 6.5% in sheep and goats. However, still some cases of abortions due to *Brucella melitensis* are diagnosed in dairy cows and small ruminants.

B. melitensis strain Rev.1 has been used only to immunise small ruminants. Neither the safety nor efficacy of this vaccine has been fully monitored among sheep and goats in the State of Kuwait. Abortions were reported by some farmers following the use of this vaccine. It is not certain whether abortions were induced by vaccination alone. As argued by some researchers, complaints of abortions are no longer received by the Animal Health Services since the government stopped compensation to farmers. Furthermore, the effects of vaccination project implemented so far need to be assessed.

Introduction

The State of Kuwait has estimated ruminant livestock population 8,000 cattle, 400,000 sheep, 50,000 goats and 6,000 camels. Besides these, a large number of sheep and goats are imported into the country for slaughter as well as breeding. The management of ruminant livestock varies with the type of species maintained. All dairy cattle are maintained under an intensive management system, while most of the small ruminant and camels are reared under extensive system.

Brucellosis is an endemic disease of cattle, sheep, goats and camels in the State of Kuwait and is a priority diseases for control, because it causes loss of production in ruminant livestock and serious zoonosis in humans.

Prevalence of brucellosis

The main signs of the disease, abortion and retention of the placenta, are seen in ruminant livestock, but only a few cases are confirmed by Laboratory diagnoses. As in other countries, in Kuwait too, brucellosis is mostly caused by *B. abortus* and sometimes by *B. melitensis*, in cattle and *B. melitensis* in small ruminants.

Table I. **Incidence of *Brucella* seropositive cows, abortions and positive cultures among 12,000 dairy cattle in Sulaibiya before and after vaccination with strain 19.**

		Abortions		Organism isolated	
Year	Seropositive (%)	Number	% culture +ve	*B. abortus*	*B. melitensis*
1984	3.0	25	16.0	3	1
1985	7.9	95	12.6	10	2
1986	9.6	199	13.1	24	2
1987	not tested	248	13.3	27	0
1988	not tested	180	3.9	7	0
1989	5.2	130	1.5	2	0

Source: Adapted from Al Khalaf *et al* (1991).

Brucellosis was first diagnosed in the State of Kuwait in 1963 (PAAF, 1990). Data on the prevalence of brucellosis are scarce, but limited ones available (Al Khalaf *et al.*, 1991) showed that, there has been a rise in seroprevalence of this disease in dairy cattle from 3.0% in 1984 to 9.6% in 1986 (See Table I). In few cases, abortions in cows were due to *B. melitensis*.

After a broad based vaccination campaign conducted during December 1986 and June 1987, the prevalence of brucellosis had declined to 5.2% in September, 1989 in Kuwait (Al Khalaf *et al.*, 1991).

Data on the prevalence of brucellosis in small ruminants are even more scanty. However, serological test done in 1993 on a flock of sheep (n=85) from Wafra had a seroprevalence of 9.4%. In 1994, laboratory diagnosis on limited specimens, most of the sera, gave prevalence rates of 14% in sheep and 7% in goats (Serology Laboratory, Unpublished report). Such specimens were submitted by clinicians from flock suspected to have the disease and, therefore, are highly biased, because they overestimated the level of the disease. Table II presents the limited data on the occurrence of brucellosis in sheep, goats and camels. A serological survey carried out on sheep by GRM from May to August 1993, revealed a seroprevalence rate of 12.9%. Out of few specimens submitted to the Bacteriology Laboratory in 1994, *B.melitensis* were isolated from 5 sheep, 2 goats and 2 cows (Animal Health Department, 1994). Now, neither the incidence of brucellosis nor its various effects on production are quantified.

Table II. **Seroprevalence of brucellosis in small ruminants, camels ans cases in humans in the state of Kuwait.**

Year	Small ruminants	Camels[2]	Human cases rate/100,000[1]
1985		14.6	68.9
1986			58.7
1987			39.9
1988	8.6[2]	14.8	20.1
1989	6.6[1]	7.7	15.3

Sources:
[1] Al Khalaf *et al.* (1991).
[2] Public Authority for Agriculture Affairs and Fish Resources (1990).

Human cases of the disease are not uncommon in Hospitals in the State of Kuwait.There was a decline in the incidence of the disease from 68.9 cases/100,000 persons to 15.3 cases/100,000 persons (Al Khalaf *et al.*, 1991)

Control policy and strategy

From 1974 to 1984, brucellosis control policy in dairy cattle was test and slaughter of reactors with compensation. However, this was abandoned because of the lack of finance to compensate and the ineffectiveness of the method (Al Khalaf *et al.*, 1991; Anon, 1990).

B. melitensis strain Rev.1 vaccine has been used only in small ruminants since September 1987. Lambs and kids between the age of 3 to 8 months are vaccinated with a standard dose (10^9), while older females receive a diluted Rev.1 (10^7 cells) subcutaneously. In small ruminants, Rev.1 was given simultaneously along with anthrax, pox and clostridial vaccines.

Safety of the Rev.1

No Rev.1 safety tests have been conducted in the laboratory or in the field, on adult ruminants in the State of Kuwait. However, the aforementioned vaccination campaign conducted on small ruminants in 1987 and 1988, and that pursued by the Animal Health Department since early 1993 gives some indications on the safety of the vaccine.

No adverse local tissue reactions were reported following vaccinations with Rev.1. Nonetheless, during a survey among 55 flock owners, some farmers reported of vaccination, induced abortions among ewes (Al Khalaf *et al.*, 1991; Shakir Al, Mahmud, personal comm.). There was no assessment of whether abortions were due to Rev.1 or due to other factors. Crowther *et al.* (1977) in Cyprus reported a similar finding with a reduced dose of 10^6 Rev.1 and attributed these responses to stress associated with simultaneous vaccination with pox. Al, Khalaf *et al.* (1991) consider farmers' desires to get compensation as a possible reason for abortions reported. Such complaints are no longer heard from farmers.

Efficacy of rev. 1 in small ruminants

The only work carried out to assess the efficacy of Rev.1 vaccine in small ruminants was the one done by Al Khalaf *et al.* (1991). They conducted a serological survey on a limited number of sheep and goats during January and November 1989 after the aforementioned brucellosis vaccination campaign and reported that the incidence of the disease had dropped from 11.1% pre-vaccination period to 6.6% after the campaign.

Future programmes

A study is planned by Animal Health Department initially in dairy herds and later in small ruminants.

References

Animal Health Department (1995) Annual Report of 1994. Animal Health Department.

CROWTHER, R. W., ORPANIDES, A. AND POLYDREOU, K. (1977). Vaccination of adult sheep with reduced doses of *B. melitensis* strain Rev.1: Safety and serological responses. Trop. Anim. Hlth. Prod. 9, 85, 91.

Public Authority for Agriculture Affairs and Fish Resources (1990). Status of brucellosis and other zoonotic diseases in Kuwait. Country report. Zoonotic Disease Seminar. 21-24 January 1990. Kuwait.

SULTAN A. SULTAN KHALAF, BAHSIR TAHA MOHAMAD AND PAUL NICOLETTI (1991). The control of brucellosis in Kuwait by vaccination of cattle, sheep and goats with *Brucella abortus* Strain 19 or *B. melitensis* Strain Rev.1. Trop. Anim. Hlth. Prod. 24, 45-49.

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A brief review of the National Project for the Control of Brucellosis in Saudi Arabia

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In Saudi Arabia, brucellosis is one of the most important zoonosis of both public and ecocomic signficance.
In Saudi Arabia, sheep are estimated over 10 million, goats are probably 6 million, cattle are about 300,000 and camels over 400,000. Sheep and goats are nearly always found in the same flock, and frequently raised together with camels.
During the last ten years, all *Brucella* species isolated from sheep, goats, cattle and camels were *Brucella melitensis* biovar 2.
With the occurence of this disease among animals in recent years, several cases of Malta fever among humans have beend reported and attributed to *Brucella* transmitted to humans through milk and dairy products, or through direct contact with infected animals.
The Department of Animal resources in the Ministry of Agriculture and Water in Saudi Arabia has initiated in 1990 a National Brucellosis Control Project Plan in all regions of Saudi Arabia, adopting a massive vaccination policy using Rev.1 vaccine. Twenty four million Saudi riyals (about 6.5 million US$) were allocated for this project for three years as a first stage.

Project objectives:
1. To immunize female sheep and goats using Rev.1 vaccine (10^6 CFU/dose) for animals over 8 months old (reduced dose).
2. To immunize younger female animals between 3-8 months of age at standard dose (10^9 CFU/dose).
3. Overall extensive periodic vaccination of replacement herds (new-born at three-month age) covering all regions.
4. Vaccination of sheep and goats imported for breeding purposes.
5. Vaccination of 3-8 month old female calves with Rev.1 vaccine at standard dose (10^9 CFU/dose).
6. Vaccination of individuals animals raised within cities and suburbs.
7. Utilizing the project to obtain data and statistics on animal herds of all species in Saudi Arabia.
8. To minimize financial losses arising from the treatment of *Brucella* infected persons and protect the society from the danger of this disease.

Project Strategy:
1. Reducing the rate of infection among animal herds as an inital stage.
2. Future acquisition of infection-free animal herds. This will only be possible after having continued vaccination for several years, particularly new born animals.
3. Reducing damages arising from animal abortion cases.
4. Reducing the rate of infection among citizens and residents.

The table below shows the number of animals (of different species) vaccinated against brucellosis during the period from the beginning of the project in 1990 up to 06/1995. This project is still in progress so as to cover more animals in addition to vaccination of new-born animals.

Number of animals vaccinated through the National Brucellosis Control Project in Saudi Arabia (1990-06/1995)

Species				
Cattle	Camels	Goats	Sheep	Total
162,430	72,265	7,237,724	12,246,049	19,718,468

It is noteworthy that, according to the results of a study published by a team of research workers in King Khalid Teaching Hospital of Medicine of King Saud University in Riyadh, the number of human *Brucella* cases diagnosed bacteriologically in this hospital has declined sharply from 8.6 to 1.4 cases in every 1,000 patients from 1985 to 1991. These research workers considered vaccination of domestic animals in Saudi Arabia against brucellosis as one of the most important reasons behind this decline.

In Saudi Arabia it is thought that Rev.1 is the ideal vaccine for many reasons.
1. Rev.1 was produced from *B. melitensis*, the strain prevailing in cattle, sheep and goats in this country
2. It gives a fine immunity
3. It gives immunity against all strains isolated in the whole country
4. While it is a living vaccine, it does not cause permanent infection in animals and does not infect humans.
5. It does not cause any side effect.

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The use of Rev.1 in adult vaccination programmes in small ruminants in Spain.

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Summary
The whole flock vaccination is the only feasible alternative to control *B. melitensis* infection in small ruminants under extensive management conditions. This contribution describes the practical problems found and the adquired experience after using this control strategy in the past decade in Spain. The differences in safety and immunity existing between the different Rev.1 vaccines could account for the discordant safety results obtained world-wide using Rev.1 in mass vaccination trials. We present field and experimental results supporting that because of the induction of abortion in pregnant animals and the low degree of immunity conferred, the reduced doses of Rev.1 should not be recommended as a prophylaxis alternative to the full doses. Due to the induction of abortion when vaccinating pregnant animals, an entirely safe method for the application of the Rev.1 vaccine in mass vaccination programmes does not exist. The conjunctival vaccination is safer than the subcutaneous vaccination but is not innocuous enough to be applied whatever the pregnancy status of animals, and only should be used in restrictive conditions. The conjunctival administration of full doses of Rev.1 during the late lambing season could be recommended as a whole flock vaccination strategy to minimise the risk of Rev.1 induced abortions.

Introduction

Despite the considerable increase in the knowledge of the epidemiology and diagnosis of *B. melitensis* infection produced in the last years, many aspects related to its control, particularly in developing countries, remain obscure. In these countries, and even in some of the so called developed, the classical test and slaughter policy alone is inapplicable, being vaccination the only suitable mean to control the disease. The vaccination of the whole animal population, repeated at regular intervals, is followed by a significant prevalence decrease in the following years. In our experience, this strategy is the only reasonable alternative to control *B. melitensis* infection in small ruminants in areas having high prevalence, extensive management systems and low socioeconomical level. Spain is probably one of the most experienced countries in the application of the Rev.1 vaccine in adult vaccination programmes. The aim of this contribution is to describe the practical problems found and the adquired experience after using this control strategy.

The preliminary experience

The control programme against *B. melitensis* infection in small ruminants in Spain, based on the compulsory vaccination of young female replacements (3-5 months old

sheep and goats) with the Rev.1 vaccine using the classical standard dose (10^9) and route (subcutaneously), was officially initiated in 1976. To facilitate vaccination, the Ministry of Agriculture financed the total costs of vaccination, that was entirely free for producers. Assuming that replacement rates for small ruminants were around 20% yearly, the programme aimed to reach the vaccination of the whole population during the following five to six years, decreasing the prevalence to levels compatible with the application of a test and slaughter policy to achieve eradication. However, after six years of application of this vaccination programme, *B. melitensis* was infecting 6.5% of sheep population, more than 64% of sheep flocks contained reactors and about 8,000 human cases were declared, indicating the failure of this vaccination strategy to control the disease. This failure was mainly due to the low vaccination coverage obtained. Considering the official census and the number of Rev.1 doses purchased by the Ministry of Agriculture, about 30-40% of the small ruminant population remained unvaccinated during this six year period (4). Therefore, to apply a more efficient vaccination strategy was a priority at that time.

If vaccination of only the young replacement stock was unsatisfactory, it seemed logical to extend vaccination to adults to increase the vaccination coverage of population in a faster way. Accordingly, the whole flock vaccination seemed the only alternative to be assayed, but.... what vaccine should be used?. Nonliving vaccines (of type H38 or similar bacterins) had practical inconveniences such as the need of revaccination and their use for the collective prophylaxis had been abandoned in other experienced countries like France. Among the two live vaccines available at that time (strain 19 and Rev.1), the *B. melitensis* Rev.1 vaccine seemed the more logical alternative to be applied in small ruminants (1, 10). However, the full doses (1×10^9) of Rev.1 administered subcutaneously induced abortions when vaccinating pregnant animals (10). The use of highly reduced doses (1×10^4) of Rev.1 seemed to resolve this safety problem in goats (2), but little and contradictory experience was available in sheep. Some authors reported that the reduced doses of Rev.1 (10^5 to 10^7) induced abortion when administered subcutaneously in sheep during the second or third month of pregnancy (9). However, others (12) did not report abortions after vaccinating sheep between the second and fourth month of pregnancy with full doses of Rev.1 by subcutaneous route.

Contradictory results were obtained also among veterinarians in the preliminary field trials in Spain and two adult vaccination procedures were mainly used: **1)** the emergency procedure based on the subcutaneous vaccination with half-dose (5×10^8) of Rev.1, that was reported as successful to control disease in flocks suffering abortion outbreaks by *B. melitensis* (11), and **2)** the reduced dose (1×10^6) of Rev.1 administered by subcutaneous route, that was adopted as the whole flock official method by the Ministry of Agriculture and several millions of sheep and goats vaccinated in Spain since 1983.

The half-dose emergency method was initially reported as satisfactory, particularly when vaccinating sheep during the last month of pregnancy in flocks suffering abortion outbreaks by *B. melitensis* (3). However, when this procedure was applied indiscriminately in all pregnancy periods, the abortion rate was increased after vaccination in most of cases, particularly when sheep were vaccinated between the second and third month of pregnancy. Moreover, safety differences among the different commercial vaccines used began to be reported by Spanish veterinarians at that time. The safety of three different commercial Rev.1 vaccines applied subcutaneously at half doses in 60 to 140 day pregnant sheep were compared. The

three vaccines were considered suitable after being tested in vitro for colony counting and dissociation. One of these vaccines increased significantly the abortion rate while the other two vaccines did not. The highest percentage of abortions took place between 40-60 days after vaccination (J.M. Blasco, unpublished results). A cause-effect relationship was evident since *B. melitensis* biovar 1 strain Rev.1 was consistently isolated from vaginal swabs, milk and fetuses from several abortion outbreaks happening after half-dose vaccination trials (4), and this vaccination procedure abandoned.
The official vaccination campaign based on the whole flock subcutaneous vaccination with reduced doses (1×10^6) of Rev.1 was followed by thousands of vaccine induced abortions. During the 1985 campaign we communicate to the veterinary authorities that the *B. melitensis* biovar 1 strain Rev.1 had been consistently isolated in our laboratory from vaginal swabs, milk and fetuses from over 150 abortion outbreaks taking place after vaccination in Aragon and Castilla La Mancha (4). The features of these abortion outbreaks closely resembled to those induced by half-dose vaccination, with the highest percentages of abortion taking place between 40 and 60 days after vaccination (4). While in some regions this method was immediately abandoned, the reduced doses continued being applied in other regions in Spain until recently.

Therefore, at that time, we had enough adquired experience and scientific basis supporting that differences in safety were evident among the vaccines produced by the different Spanish laboratories, and that the reduction of the Rev.1 dose was not a safe enough method to be applied whatever the pregnancy status of animals.

The research results
Two relevant research results obtained by our colleagues from the former Station de Pathologie de la Reproduction (INRA. Nouzilly. France) opened some lights to continue our research in Spain: **1)** the development of a mouse model to control the virulence and protective efficacy of anti-*Brucella* vaccines (7), and **2)** the demonstration of the protective efficacy against *B. melitensis* of conjunctival vaccination with Rev.1 in sheep (13).

The commercial vaccines elaborated by the five laboratories producing Rev.1 at that time in Spain were sent to Dr. Nicole Bosseray (INRA. Nouzilly. France) and tested in the mouse model. A lot of differences in the virulence and the protection parameters were obtained (8; and N. Bosseray, personal communications). A direct relationship was found between the mouse results and those obtained in sheep explaining, at least in part, the contradictory results in terms of safety obtained in the preliminary field trials. As a representative example among the published results, two of the commercial Spanish vaccines that did not induce abortions in pregnant sheep in field trials carried out in Spain (3) and Portugal (19), were showing low virulence and poor protection in mice. By contrast, the only Spanish vaccine showing adequate virulence and protection in mice, induced abortion in 60-70% of pregnant vaccinated sheep (15). The example may be indicative of the importance of performing an adequate quality control on the Rev.1 vaccine.

On the other hand, we were interested in testing if the conjunctival vaccination procedure developed by the INRA colleagues was safest than the subcutaneous method for vaccinating pregnant sheep. It was reported that reduced doses (1×10^6) of Rev.1 did not confer adequate immunity in vaccinated sheep even after revaccination (13). Therefore, the use of reduced doses by conjunctival route was

discarded and only full doses of Rev.1 were considered to be tested in pregnant sheep. In a comparative experiment it was demonstrated that the conjunctival method reduced the number of abortions induced by the subcutaneous vaccination but an important proportion of conjunctivally vaccinated sheep excreted Rev.1 and aborted (15). In a similar experiment conducted more recently, the conjunctival vaccination at mid pregnancy was followed by abortion in over 70% of vaccinated sheep and goats (22). Most of vaccine induced abortions took place between 40 and 60 days after vaccination, with variable intensity depending mainly on the month of pregnancy at vaccination. The lowest percentage of abortions was obtained when the animals were vaccinated during the last month of pregnancy (15).

Accordingly, the field and research results obtained in the last years support the following conclusions: **1)** differences in safety and immunity exist between the different Rev.1 vaccines produced world-wide (8), accounting for the discordant results in terms of safety obtained in field trials in different countries, **2)** because of the lack of safety and the low degree of immunity conferred, the reduced doses of Rev.1 should not be recommended as a prophylaxis alternative to the full doses, and **3)** the conjunctival vaccination with Rev.1 is safer than the subcutaneous vaccination, but is not innocuous enough to be recommended for whole flock vaccination programmes whatever the pregnancy status of animals.

The present situation

Alternative live vaccines to Rev.1 such as *B. suis* strain 2 or *B. abortus* RB51have not been found suitable for the prophylaxis of brucellosis in small ruminants (6, 16, 21). Therefore, while an alternative vaccine be developed and successfully tested, the Rev.1 has to be considered as the only suitable vaccine for the prophylaxis of brucellosis in small ruminants. Due to its lack of safety, the Rev.1 should be applied only when the animals were unpregnant, but this is unpractical under field conditions and some risk of induced abortions has to be considered as unavoidable when applying a Rev.1 based whole flock vaccination programme. However, two important factors influencing the safety of Rev.1 (at least in sheep) have to be considered: **i)** that conjunctival vaccination during the last month of pregnancy minimise the risk of abortions (15) and no vaccine excretion is produced in milk of conjunctivally vaccinated lactating ewes (18), and **ii)** that sheep reproduction under extensive management conditions has seasonal variations in most of countries with concentrated lambing seasons in determined months of the year, allowing the selection of an ideal period of time in which most animals be unpregnant, lactating or in the last month of pregnancy.

Considering these factors, and at least for sheep, a relatively safe Rev.1 mass vaccination programme could be proposed as follows:

1) *Vaccine*: a good quality batch of *B. melitensis* Rev.1 obtained from a laboratory with adequate quality control standards (seed lots regularly tested for stability of in vitro markers and virulence and immunity in mice), and administered at full dose ($0.5\text{-}1 \times 10^9$) by conjunctival route .

2) *Population coverage*: the whole population, young and mature, males and females.

3) *Period of time*: during the lambing-lactation-premating period (for example, February to March in the vast majority of extensively managed flocks in Spain and Tunisia) to minimise the vaccine induced abortions.

4) *Follow up*:
a) the ideal from the safety standpoint: vaccinating the whole population the first year and only young replacements the following. However, this may be unpractical in developing countries and, as indicated above, has been unsuccessful in Spain.
b) the expeditious: a whole flock vaccination coverage being repeated every two years on the whole population for at least 8-10 years.

These proposals would be also applicable to goats, but information is lacking concerning the safety of Rev.1 in lactating goats and billy goats.

While there is almost total agreement in considering the Rev.1 as the best vaccine available, the methods for its use in mass vaccination programmes are controversial. The guidelines for a regional brucellosis control programme for the middle east elaborated in Amman in 1993 are a good indicator of the existence of disagreement between the methods for the application of this vaccine in adults. The advantages and inconvenients of the two discrepant methods are summarised in Table I. The subcutaneous reduced dose method has been recommended by the Joint FAO/WHO Expert Committee (17) and considered by many as suitable for mass vaccination programmes in small ruminants (14, 20). By contrast, on the basis on the above experiences, we have seriously questioned this method and proposed the use of the conjunctival vaccination with full doses of Rev.1 during the late lambing season as the most reasonable whole flock vaccination programme, at least in sheep (5). This programme was applied in 1992 on around 1.6 million of sheep and goats in Tunisia under support from the FAO. The reults, at least from the safety standpoint, were successful (Said El Bahri, personal communication).

In conclusion, an entirely safe method for the application of the Rev.1 vaccine in mass vaccination programmes does not exist, but we have some possibilities to minimise the risks. If we will continue discussing during the next years about what should be the ideal method for using Rev.1 or, by the contrary, we will be able to recommend a given one to be immediately applied in interested countries, would depend from a fruitful discussion during this meeting.

Acknowledgements
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References

1. **ALTON GG, SS ELBERG, 1967.** Rev.1 *Brucella melitensis* vaccine. A review of ten years of study. *Vet Bull* 37, 793-800.

2. **ALTON GG, 1970.** Vaccination of goats with reduced doses of Rev.1 *Brucella melitensis* vaccine. *Res vet Sci* 2, 54-59.

3. **BLASCO JM, A ESTRADA, M MERCADAL , 1984.** A note on adult sheep vaccination with reduced dose of *Brucella melitensis* Rev.1. *Ann Rech Vet* 15, 553-556.

4. **BLASCO JM, 1990.** Control y profilaxis. Brucelosis ovina. *Ovis* 8, 65-69.

5. **BLASCO JM, 1993.** Diagnosis and control of *B. melitensis* infection. *FAO/WHO/OIE Regional workshop for the formulation of guidelines for a regional brucellosis control programme for the Middle East*. Amman, Jordan. February 1993.

6. **BLASCO JM, CM MARIN, MP JIMENEZ DE BAGUES, M BARBERAN, 1993.** Efficacy of *Brucella suis* strain 2 vaccine against *Brucella ovis* in rams. *Vaccine* 11, 1291-1294.

7. **BOSSERAY N, AM PLOMMET, M PLOMMET, 1984.** Theoretical, practical and statistical basis for a general control method of activity for anti-*Brucella* vaccines. *Develop Biol Standard* 56, 257-270.

8. **BOSSERAY N, 1991.** *Brucella melitensis* Rev.1 living attenuated vaccine: stability of marquers, residual virulence and immunogenicity in mice. *Biologicals*, 19,355-363.

9. **CROWTER RW, A ORPHANIDES, K POLYDOROU,1977.** Vaccination of adult sheep with reduced doses of *Brucella melitensis* strain Rev.1. *Trop Anim Hlth Prod* 9, 85-91.

10. **ELBERG SS, 1981.** Rev.1 *Brucella melitensis* vaccine. Part II 1968-1980. *Vet Bull* 51, 67-73.

11. **ESTEBAN-VELAZQUEZ E, 1982.** Brucelosis en la cabra y en la oveja. VII Jornadas de la Sociedad Española de Ovinotecnia, Murcia, 199-204.

12. **FALADE S, 1983.** Serological response of sheep to *Brucella melitensis* Rev.1 vaccine. *Zbl Vet Med B* 30, 546-551.

13. **FENSTERBANK R, P PARDON, J MARLY, 1982.** Comparison between subcutaneous and conjunctival route of vaccination with Rev.1 strain against *Brucella melitensis* infection in ewes. *Ann Rech Vet* 13, 295-301.

14. **GARRIDO F., 1989.** Profilaxis y erradicacion de la brucelosis en España. *I Congreso Internacional sobre Brucelosis*. Ciudad Rodrigo. 7 Octubre 1989, 91-118.

15. **JIMENEZ DE BAGUES MP, CM MARIN, M BARBERAN, JM BLASCO, 1989.** Responses of ewes to *B. melitensis* Rev.1 vaccine administered by subcutaneous or conjunctival routes at different stages of pregnancy. *Ann Rech Vet* 20, 205-213.

16. **JIMENEZ DE BAGUES MP, M BARBERAN, CM MARIN, JM BLASCO, 1995.** The *Brucella abortus* RB51 vaccine does not confer protection against *Brucella ovis* in rams. *Vaccine* 13, 301-304.

17. Joint FAO/WHO Expert Commitee on Brucellosis. 1986. Sixth report. *WHO Technical Report Series* 740. WHO. Geneva.

18. **PEREZ M, CM MARIN, MJ GRILLO, 1995.** Vacunación de ovejas lactantes con Rev.1 por via conjuntival. *ITEA* 16, 539-541.

19. **SALES HENRIQUES H, WD HUESTON, KH HOBLET, WP SHULAW, 1992.** Field trials evaluating the safety and serologic reactions of reduced dose *Brucella melitensis* Rev.1 vaccination in adult sheep. *Prev Vet Med* 13, 205-215.

20. **SULTAN AL-KHALAF SA, B. TAHA MOHAMAD, P NICOLETTI, 1992.** Control of brucellosis in Kuwait by vaccination of cattle, sheep and goats with *Brucella abortus* strain 19 or *Brucella melitensis* strain Rev.1. *Trop Anim Hlth Prod* 24, 45-49.

21. **VERGER JM, M GRAYON, E ZUNDEL, P LECHOPIER, V OLIVIER-BERNARDIN, 1995.** Comparison of the efficacy of *Brucella suis* strain 2 and *Brucella melitensis* Rev.1 live vaccines against a *Brucella melitensis* experimental infection in pregnant ewes. *Vaccine* 13, 191-196.

22. **ZUNDEL E, JM VERGER, M GRAYON, R MICHEL, 1992.** Conjunctival vaccination of pregnant ewes and goats with *Brucella melitensis* Rev.1 vaccine: safety and serological responses. *Ann Rech Vet* 23, 177-188.

Table I. **Advantages and inconvenients of the two controversial methods for the application of the Rev.1 vaccine in mass vaccination programmes in small ruminants.**

	Conjunctival method	Reduced dose method
Defined dose	Yes. 0.5-1 x 10^9	Not. 10^4-10^7 ??
Induced abortions	Yes	Yes
Milk excretion after abortion	Yes	Yes
Immunity	High	Unknown or low (13)
Duration of immunity	At least two pregnancies (21)	Unknown
Serological response	Low +	Low ++
Safety for manipulators	Safer ??	
Feasibility	Better ??	
Cost/animal		Lower ??

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Field experience with Rev.1 vaccine in Turkey

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Summary
Brucellosis has always been a threat to human being and animal health in Turkey. Control of brucellosis has been very important and difficult because of the geographical situation of Turkey. Until 1950's, *Brucella* agglutination test antigen and strains were prepared and used in the laboratories by their own methods. the serological studies and antigen production have been done by internationally accepted methods and standardized with the international standard serum since 1950. With the studies to determine the level of brucellosis in Turkey in 1952-1970, reactor percentages were found varying from 2.6, to 11.1 on sheep and goats. 16.13% of sheep serums were positive for *Brucella* in infected herds. A national survey performed by the Ministry of Agriculture, Forestry and Rural Affairs gave an overall rate of 1.26% positive reactors in 1989, 2.08% in 1990 and 1.83% in 1991 among sheep in different provinces. In a seroepidemiological study carried out in Kars province from 1071 sheep serum samples, 0.93% prevalence were determined. *B. melitensis* biotype 2 were reported as more prevalent type in sheep. biotype 1 and 2 were also found. The activities for the control and eradication of brucellosis in sheep and goats were started mainly in the state farms in 1952. in this plan, the animals were tested by agglutination tests and reactors were slaughtered. with the poor results, it was determined that vaccination would give better results. First studies for the application of *B. melitensis* rev1 vaccine were started in 1968. the results were 5 times less abortion rate and 6 times less reactor rate in the Rev.1 vaccinated lambs in the subsequent season. so, all the sheep, lambs, goats and kids were started to be vaccinated with *B. melitensis* Rev.1 vaccine in the state farms in the following year and some private farms were also added to the campaign. *Brucella* abortion rate fell down to 7.3% in 1968 and 0.57% in 1969 in the state farms. This rate was almost nil in the following years. Increase in the lambing rate also reached to about 15-25%. After the preliminary investigations, the vaccination of sheep herds in the private farms started in 1974 and 1.5 million sheep and goats were vaccinated in the first 5 years with good results but with the 60 million sheep population, it was evident to take long time to eradicate brucellosis. *B. melitensis* Rev.1 vaccine was produced on solid medium in Turkey until 1990's and then studies were performed to produce the vaccine in fermenters to supply high amount of vaccine demand. the studies for the production of *B. melitensis* Rev.1 vaccine with continuous culture system gave good results. With the implementation of a national brucellosis control and eradication project to be lasted for 26 years on a regional basis in 1983, a total of about 7.000.000 calves and 64.000.000 small ruminants have been vaccinated so far. An adult vaccination program was started in thrace region in 1991 which was selected as a pilot region and 884.000 adult

female sheep and goats were vaccinated with reduced dose of Rev.1 vaccine. following 1991, all the female adult animals in infected herds after laboratory diagnosis have been vaccinated with reduced dose of Rev.1 vaccine. as the end of 1995, a total of 4.000.000 female sheep and goats have been vaccinated. Currently applied *Brucella* eradication project requires not only vaccination of young and adult animals but also covers various other measures such as annual training activities for the veterinarians, regular radio programmes on pactical information and know-how on brucellosis, publication of simply and easily understandable booklets providing practical information on brucellosis to farmers and breeders. The Ministry of Agriculture and Rural Affairs has also been in close interrelation with Ministry of health at all levels for surveying and getting cooperative measures against brucellosis. The Pendik Veterinary Control and Research Institute which was built for the veterinary services in 1914 plays an important role in this program producing full and reduced dose *B. melitensis* Rev.1 and *B. abortus* S19 vaccine, *Brucella* Rose-Bengal Plate Test, Standard Tube Agglutination Test and Milk Ring Test antigens.

Brucellosis has always been a threat to human being and animal health in Turkey. The diagnosis of the first case of human brucellosis was from a patient in a military hospital in 1915. After this human case, *B. abortus* from cattle was first reported in 1931 and *B. melitensis* from sheep was reported in 1944. Control of brucellosis has also been very important and difficult because of the geographical situation of Turkey which is located as a bridge on the transit passageway between Asia and Europe.

Until 1950's, *Brucella* agglutination test antigen and strains were prepared and used in the bacteriology institutes and regional laboratories by their own methods. Since 1950, the serological studies have been carried on by using the standard antigen prepared with the international methods and standardized with the international standard serum (16).

According to the reports of Ministry of Health, 637 SAT positive reactions in human were obtained from 37 provinces in the 6 years period between 1958-1964. The highest infection rate was found in Diyarbakir (30.3%), followed by Balikesir (17.9%), Bursa (7.5%), Mardin (6.1%), Ankara (3.9%) and Istanbul (1.7%). A total of 65 human sera obtained from the hospitals in Balikesir was investigated for brucellosis in 1966-1967 and 87.7% gave + reaction with SAT at 160 iu/ml, 93.8% gave a positive CFT reaction at 1/4 whilst 92.3% gave a coombs titer of 1/10 or over. Of 1,240 samples from persons who came to hospital to get health certificate, 0.72% were positive to the coombs test, 0.4% to the CFT but none to the SAT (13).

A serological study mainly on sheep and goats in state farms was conducted with 198,116 blood serums in 1952-63 and showed 2.6% as reactors. 11.1% of reactor rate were found in the herd of Cifteler state sheep farm suffering acute brucellosis in 1957. Human rate in this farm was 23 %. Another study on sheep herds performed at Karacabey state farm in 1960-1970 showed 5% of them as reactors. In Merino sheep breeding farm, 9% of animals were found positive in 1965-1966.

For biotyping of *Brucella* strains, several studies were done. *B.abortus* biotype 3 in cattle and *B. melitensis* biotype 2 in sheep were reported as more prevalent than

other types of *Brucella* species in 1968 in Turkey. Other types determined were biotype 1, 2, 4 and 6 for *B. abortus* and biotype 1 for *B. melitensis* (11,21). All of 60 *B. abortus* field strains isolated from cattle in istanbul area were also reported as biotype 3 in 1983 (1). 69% of 13 *B. abortus* strains were biotype 3 and 31% were biotype 1 isolated from 13.4% of 97 aborted fetus of cattle and 86% of 29 *B. melitensis* strains were biotype 1, 10% biotype 2 and 3.4% were biotype 3 isolated from 20% of 145 aborted fetuses of sheep which were submitted to laboratory in Thrace region of Turkey between 1989-1992. 11% of 361 cattle and 16.13% of 1029 sheep serums were positive for *Brucella* taken from *Brucella* infected herds (7).

75 *B. melitensis* infected sheep serum were tested with rivanol test, rivanol plate test, RBPT and SAT. 69.3% of the serums were positive with RBPT, 70.6% with SAT, 85.2% with RT and 86.7% with RPT (3). *Brucella* bacteria were detected in 18.8% of 69 sheep and 4.5% of 22 goat aborted fetuses submitted to laboratory in Izmir province between 1971-1977. 20% of 65 sheep and 5.8 of 17 goat blood samples which all aborted were serologically positive to SAT for brucellosis (10).

A national survey performed by the Ministry of Agriculture, Forestry and Rural Affairs (MAFRA) in 1989 gave an overall rate of 1.26% positive reactors among sheep ranging from nil to 10% in different provinces and 3.56% positive reactors in cattle. In 1990, prevalence of sheep and goats was 2.08% (from 7,361 blood sera) and prevalence of cattle was 1.2% (from 5,701 blood sera). The national survey in 1991 showed 1.83% prevalence for *B. melitensis* (from 11,122 blood sera) and 1.01% prevalence for *B. abortus* (from 7,812 blood sera). In 1992, a seroepidemiologic study on 3580 cattle and 4417 sheep sera from four different geographical areas of Turkey showed a positivity aginst Brucellosis as 1,48 % in sheep and 0,6 % in cattle.

In a seroepidemiological study carried out in Kars province, 1,480 cattle and 1,071 sheep serum samples were taken and tested against brucellosis by serologic methods. The results showed 6.49% prevalence in cattle and 0.93% prevalence in sheep (5).

With the risk of brucellosis as the most important zoonosis in Turkey for the health of human beings, its negative effects on livestock activities and enormous economic losses, the *B. abortus* infection in cattle was subject to legislation in the 1930's. This was the first step for a National programme. A regulation enacted in 1960 made calf vaccination with strain 19 compulsory on state farms. Later, some private farms were added to the campaign.

The activities for the control and eradication of brucellosis in sheep and goats had been started mainly in the state farms in 1952. In this plan, the animals were tested by agglutination test and reactors were slaughtered leaving the non-reactors in the herd. As the years passed, it became clear that that kind of programme would not work because many animals remained in the herd were the source of the infection even if they were serologically non-reactors. Although other tests such as CFT and Coombs were superior to catch the chronic cases than agglutination tests, they were not found 100% successful. Despite the sheep and goats were serologically tested twice a year in these years, reactor percentage did not decrease. With these conclusions, it was understood that vaccination would give better results. By this way, while the herds would acquire immunity in 4 to 5 years, the owner would be protected from economical liability.

First studies for the application of *B. melitensis* Rev.1 vaccine were started in 1968. A heavily infected state sheep farm was selected first with a 15% reactor rate and 5% abortion rate. All the adult sheep and goats one month before the breeding season and lambs and kids between 4 to 6 months old were vaccinated with full dose *B. melitensis* Rev.1 vaccine ($2x10^9$). The results were very promising with a 5 times less abortion rate and 6 times less reactor rate in the subsequent season. After these results all the sheep, lambs, goats and kids were vaccinated with *B. melitensis* Rev.1 vaccine in the state farms in the following year and some private farms were also added to the campaign.

Brucella abortion rate fell down to 7.3% in 1968 and 0.57% in 1969 in the state farms. This rate was nil in the following years. Increase in the lambing rate also reached to about 15-25% in these farms.

A detailed study was performed in these years to determine the duration and level of protection of *B. melitensis* Rev.1 vaccination in the following three pregnancy period. Four groups of 10 to 12 ewes which had been vaccinated between 4 and 6 months of age with *B. melitensis* Rev.1 vaccine were challenged in their first pregnancy together with an equal number of unvaccinated ewes from the same *Brucella*-free herds. The vaccine gave almost complete protection against both the 10^6 and 10^5 doses of the H38 challenge which made all or nearly all unvaccinated controls abort. It gave also complete protection against the 10^5 dose of the Cifteler strain (12). The same group of animals was kept from the middle of their 2nd pregnancy with 10 donor ewes which had been earlier infected with the Turkish strain Cifteler of *B. melitensis* biotype 2 and were in most cases excreting *B. melitensis* continuosly. The vaccine gave protection against this natural exposure. 23 of 29 vaccinated ewes lambed normally. 3 of them were found sterile and 3 others died of septic metritis. In control animals, 2 of 18 unvaccinated control ewes aborted, 13 had abnormal lambs and 3 were sterile. Of the 13 normal lambs, 12 were infected (6). In the following study, two groups of sheep each consisting of 16 vaccinated and 17 unvaccinated all in their third pregnancy were challenged with 10^4 and 10^5 biotype 2 *B. melitensis* Cifteler strain, respectively. The vaccinated sheep in their third pregnancy still retained a degree of immunity (14).

In another study, lambs from infected farms were vaccinated between 4 to 6 months of age with *B. melitensis* Rev.1 vaccine and named group A. Antibody response disappeared at the end of six months. Some of the vaccinated lambs received a second dose of *B. melitensis* Rev.1 vaccine seven months after the the first vaccination and named group B. Some of them were revaccinated 11 months after the first vaccination and named group C. In this case antibody response disappeared after seven months. The three groups of 80 sheep and 34 unvaccinated sheep all in their first pregnancy and later second pregnancy were challenged with organisms of *B. melitensis* biotype 2 Turkish field strain Cifteler. In control group, 83.3% had generalized infection, 16.7% were infected, 17% had bacteria in the milk and 2.8% were culturally positive from vaginal swabs. In group A: 12.5% had generalized infection, 16.4% were infected, 17.2% had bacteria in the milk and 28% were culturally positive from vaginal swabs. In group B: all cultural results were negative with no abortions and no infected lambs. In group C: 0% had generalized infection, 18.2% were infected, 0% had bacteria in the milk and vaginal swabs with no abortions and no infected lambs. Second pregnancy challenge results of the control group were: 81.2% had generalized infection, 18.7% were infected, 100% had bacteria in the milk and 100% were culturally positive from vaginal swabs. In group

A: 6.25% had generalized infection, 6.25% were infected, 33.9% had bacteria in the milk and 39.2% were culturally positive from vaginal swabs. In group B: all cultural results were negative and in group C: 6.25% had generalized infection, 6.25% were infected, 0% had bacteria in the milk and 23.8% were culturally positive from vaginal swabs. The highest degree of protection was conferred in both first and second pregnancy ewes of group B (15).

Aerosol immunization of sheep against brucellosis was experienced by using 39 lambs and 26 ewes from a brucellosis free farm which were kept in a room and exposed to 300 ml of $20x10^9$/ml aerosol *B. melitensis* Rev.1 vaccine for 20 minutes and afterwards taken to pasture. It was given to the ewes one month before their mating. Blood samples of the vaccinated animals had been taken immediately before vaccination, 21 days later and monthly thereafter. The antibody titers increased uniformly on the first month after vaccination and disappeared in lambs four months after vaccination. 20 vaccinated and 10 unvaccinated pregnant ewes challenged with Turkish field strain of *B. melitensis* biotype 2 and $8x10^5$ organisms were inoculated via conjunctival sac. The antibody in all the controls was 100 %. The antibodies in vaccinated animals decreased 25% in SAT, 12.5% in CFT and 12.5% in RBPT in control animals, all the cultures of the offsprings were positive. 70% of their ewes were heavily and 30% were infected only. 10% of the lambs of vaccinated ewes were infected. 5% of their ewes were infected. No immunological difference was observed between the aerosolly vaccinated and the subcutaneously inoculated animals (17).

After these preliminary investigations, the vaccination of sheep herds in the private farms started in 1974 and 1.5 million sheep and goats were vaccinated in the first 5 years with good results but with the high sheep population of 60 millions, it was evident to take long time to eradicate brucellosis.

B. melitensis Rev.1 vaccine was produced in solid medium in Turkey until 1990's and then studies were performed to produce the vaccine in fermenters to supply high amount of vaccine demand. It was shown that production of *B. melitensis* Rev.1 vaccine in liquid medium with tryptone in fermentation tank gave better results. About 2.5% concentration was obtained with no dissociation in the batch system in the first ten days (4). A study was done for the production of *B. melitensis* Rev.1 vaccine with continuous culture system. Optimal culture conditions were investigated in 7 different production cycles each lasting 15 days. PCV of the culture using tripton liquid medium sterilized by autoclaving reached upto 2,5% and viable cell levels reached upto $71x10^9$/ml until the end of 15 days period. these figures were 3,5% and $80x10^9$/ml in the same period using tripton liquid medium sterilized by filtration. Dissociation percentage were in the acceptable levels (19). The stability of the lyophilized *Brucella* vaccines were also studied. Lyophilized full dose *B. melitensis* Rev.1, reduced dose *B. melitensis* Rev.1 and full dose *B. abortus* s19 vaccines produced in fermenters in liquid medium were maintained at five different storage temperatures of -20, -10, +4, +20 and +37°C for a period of 52 weeks and their aviability determinations were performed periodically. The dissociation percentages of the vaccines in all groups during the study did not change. No significant losses in the viability of all groups of vaccines stored in temperatures of -20, -10 and +2/+8°C were determined. Although *B. abortus* s19 vaccine, full dose *B. melitensis* Rev.1 vaccine and reduced dose *B. melitensis* Rev.1 vaccine decreased slightly during storage at 20°C, they were found in accepable protocol limits during the first 4 weeks, 2 weeks and 6 weeks, respectively. the viability of all

the vaccines stored at 37°C dropped dramatically to unacceptable levels in a few days (18). In a study in order to determine the effect of the quantity of vaccine diluent, three groups of lambs each consisting of 30 animals from *B. melitensis* Rev.1 vaccinated mothers with different doses and diluents ($2x10^9$/1ml., $2x10^9$/0.5ml. and $1x10^9$/0.5ml.) were used. Neither the immunity differences nor general reactions were observed between the groups. SAT agglutinins were detected in all the animals one month after vaccination and lost completely within two months. 36%, 6.6% and 0% were positive with mercapto-ethanol test, 96 %, 100% and 50% of the animals were positive with rivanol test, 83.3%, 90% and 0% were positive with RBPT, 40%, 20% and 6.4% were positive with AGT and 100%, 100% and 36.6% were positive with CFT, in one month, two months and three months after vaccination, respectively. it was concluded that same dose of vaccine in 0.5 ml. instead of 1 ml of diluent would be more economic and practical (2).

With the implementation of a National Brucellosis Control and Eradication Project on a regional basis in 1983 in which all the female calves between 4 and 8 months old to be vaccinated with *B. abortus* S19 vaccine and all the lamb and kids between 3 to 8 months old with *B. melitensis* Rev.1 vaccine in 26 years, a total of about 7,000,000 calves and 64,000,000 small ruminants have been vaccinated so far. An adult vaccination program has also been started in Thrace region in 1991 which was selected as a pilot region and 124,000 adult female cattle and 884,000 adult female sheep and goats were vaccinated with reduced dose of S19 and Rev.1 vaccine. following 1991, all the female adult animals in infected cattle and sheep herds after laboratory diagnosis have been vaccinated with reduced dose of S19 and Rev.1 vaccine, resp. as the end of 1995, a total of 520,000 female cattle and 4,000,000 female sheep and goats have been vaccinated.

In a study, 15 brucellosis free ewes in their lactation period were vaccinated subcutaneously between 15-45 days after lambing and 10 pregnant ewes were vaccinated conjunctivally with *B. melitensis* Rev.1 vaccine at 10^8, 10^7, 10^6, 10^5 and 10^4 doses. No abortion was detected in pregnant ewes vaccinated conjunctivally in all doses and no *Brucella* bacteria was isolated from the milk, vaginal fluid and tissues of the vaccinated animals in 100-200 days after vaccination but a decrease in milk production of up to 25% was declared (8). Serologic tests such as RBPT, SAT and CFT were done with the sera of 40 pregnant and non-pregnant adult Kivircik ewes 1, 2, 3 and 4 months after they were vaccinated with reduced dose ($5x10^4$/ml.) *B. melitensis* Rev.1 vaccine. It was determined that *B. melitensis* Rev.1 vaccine containing $5x10^4$ bacteria did not cause abortion in pregnant ewes. The antibodides did not persist any longer than 3 months and their levels with these tests were undetectable at the 4th and 5th months after vaccination (9).

The Ministry of Agriculture and rural affairs has been continuing efforts and activities for the protection project very strongly and success of this project will not only protect the human and animal population against brucellosis but also improve the culture breed cattle.
The most important points in a brucellosis project are: The supply of good quality vaccine and antigen produced in experienced labs in sufficient quantity; Keeping cold chain conditions on the vaccines from production to the use in the field; Training of the field staff, owner and veterinarians and to make them realize the benefit of vaccination and hygiene; Application of test and slaughter method when incidence falls to low levels; Establishing close contact and information system between Province Directories of MAFRA, Diagnostic Laboratories and Production

Laboratories; Establishing information exchange between MAFRA and Ministry ofHealth in every level; Strict control of animal movements; Surveillance of brucellosis in the regional bases; Application and determination of the most convenient control and protection methods in each region; Evaluation and change of these regional methods periodically when necessary.
Currently applied *Brucella* eradication project requires not only vaccination of young and adult animals but also covers various other measures such as:
- Annual training activities in the form of seminars and conferences for the veterinarians within the ministry organizations as carried out by specialists.
- Regular radio programmes on pactical information and know-how on brucellosis, mainly covering the state and contagious nature of brucellosis in animals and the chance of contamination to human beings through contaminated raw milk and its products.
- Publication of simply and easily understandable booklets providing practical information on brucellosis to farmers and breeders.

The MAFRA has also been in close interrelation with ministry of health at all levels for surveying and getting cooperative measures against brucellosis. The following items produced in Pendik Veterinary Control and Research Institute which was built for the veterinary services in 1914 are very important tools in the prevention and diagnoses of brucellosis ;
-Full dose *B. melitensis* Rev.1 vaccine ($1\text{-}3x10^9$),
-Reduced dose *B. melitensis* Rev.1 vaccine ($5\text{-}10x10^4$),
-Full dose *B. abortus* S19 vaccine ($40\text{-}120x10^9$),
-Reduced dose *B. abortus* S19 vaccine.($1\text{-}3x10^9$),
-*Brucella* Rose-Bengal Plate Test antigen,
-*Brucella* Slow Tube Agglutination Test antigen.
-*Brucella* Milk Ring Test antigen

References

1) **ALTINEL, C. (1983) :** A study on biotyping of *Brucella* strains isolated from cattle in istanbul area. The Journal of the Veterinary Control and Research Institute, Pendik, Istanbul, Turkey, 15, no.:1-2, june-dec., pp.12-21.

2) **COKER, A. and METE, K. (1990):** The difference in the immunobiologiC reactions after the inoculation of 3-8 months old female and male lambs and kids with a complete dose ($2x10^9$ germs) and a half dose ($1x10^9$ germs) of *B. melitensis* Rev.1 vaccine in 1 ml and 0.5 ml of diluent. The Journal of the Veterinary Control and Research Institute, Pendik, Istanbul, Turkey, 21, no.:2, dec., pp.10-16.

3) **COKER, A., METE, K. and KAYA, O. (1990):** A comparison of the results of rivanol plate test with the results of other serologic tests carried out on the sera obtained from *B. melitensis* infected sheep. The Journal of the Veterinary Control and Research Institute, Pendik, Istanbul, Turkey, 21, no.:2, dec., pp.17-22.

4) **COKER, A., METE, K. and KAYA O. (1990):** Preparation of *B. melitensis* Rev.1 vaccine in fermenters. The Journal of the Veterinary Control and Research Institute, Pendik, Istanbul, Turkey, 21, no.:2, dec., pp.5-9.

5) **DEMIROZU, K., CELIK, M. and IYISAN, A.S. (1993):** Seroepidemiology of brucellosis in Kars province. The Journal of the Veterinary Control and Research Institute, Pendik, Istanbul, Turkey, 24, no.:2, dec., pp.123-132.

6) **ERDEM, R., WILLIAMS, C.F. and STABLEFORTH, A.W. (1969):** *B. melitensis* Rev.1 vaccine. Experiment on the duration of immunity. Second pregnancy challenge. The Journal of the Veterinary Control and Research Institute, Pendik, Istanbul, Turkey, 2, no.:2, dec., pp.20-38.

7) **ERDOGAN, I., GUREL, A., TEKIN, C., UYANIK, F. and BITGEL, A. (1993):** The detection and distribution of bacterial abortions in sheep, goats and cattle in Thrace region. The Journal of the Veterinary Control and Research Institute, Pendik, Istanbul, Turkey, 24, no.:1, june, pp.23-35.

8) **EROGLU, M., COKER, A. and METE, K. (1988):** The result of vaccination of sheep with *B. melitensis* Rev.1 vaccine in lactation period. The Journal of the Veterinary Control and Research Institute, Pendik, Istanbul, Turkey, 19, no.:1-2, pp.14-27.

9) **IYISAN, A.S. (1992):** Determination of antibodies after vaccination of adult ewes with reduced dose *B. melitensis* Rev.1 vaccine by several methods. The Journal of the Veterinary Control and Research Institute, Pendik, Istanbul, Turkey, 23, no.:2, dec., pp.175-185.

10) **OZMEN, M., PIR, M. (1979):** Isolation of infective agents from the aborted fetuses of sheep and goats sent to the Bornova Veterinary Control and Research Institute through 1971-1977. The Journal of the Veterinary Control and Research Institute, Pendik, Istanbul, Turkey, 11, no.:2, dec., pp.5-10.

11) **SARISAYIN, F., EROGLU, M. and NADAS, U.G. (1968):** A work on detection of species and their biotypes of the *Brucella* strains which have been isolated in our country. The Journal of the Veterinary Control and Research Institute, Pendik, Istanbul, Turkey, 1, no.:2, pp.24-36.

12) **UNEL, S., ERDEM, R., WILLIAMS, C.F. and STABLEFORTH, A.W. (1969):** *B. melitensis* Rev.1 vaccine. Duration of immunity experiments. First pregnancy challenge. Research in Veterinary Science, no.3, may pp.254-259.

13) **UNEL, S., ERDEM, R., KAVUNOGLU, I., WILLIAMS, C.F. and STABLEFORTH, A.W. (1970):** Detection of *Brucella* antibodies in the blood samples of patients suffering brucellosis, thyphoid and infections with unknown origin. The Journal of the Veterinary Control and Research Institute, Pendik, Istanbul, Turkey, 3, no.:1, nov., pp.58-68.

14) **UNEL, S., ERDEM, R., WILLIAMS, C.F. and STABLEFORTH, A.W. (1972):** *B. melitensis* Rev.1 vaccine. Duration of immunity experiments. Third pregnancy challenge. The Journal of the Veterinary Control and Research Institute, Pendik, Istanbul, Turkey, 4, no.:2, dec., pp.3-19.

15) **UNEL, S. (1973):** The value of revaccination with *B. melitensis* Rev.1 vaccine in the field. Tubitak Temel Bilimler Arastirma Grubu Proje no.: TBAG-66. Veterinary Control and Research Institute, Pendik, Istanbul, Turkey.

16) **UNEL, S. (-):** Prevalence of brucellosis in Turkey. Institute Veterinary Bacteriology and Serology Pendik, Istanbul, Turkey. Cento unclassified. pp.1-5.

17) **UNEL, S. and DURUKAN, S. (-):** Aerosol immunization of sheep against brucellosis. Tubitak Veteriner Hayvancilik Arastirma Grubu Proje no.: VHAG-336. Veterinary Control and Research Institute, Pendik, Istanbul, Turkey.

18) **UYSAL, Y. (1995):** Detection of stability of lyophilized *B. melitensis* Rev.1 and *B. abortus* s19 vaccine in different temperatures. The summary of ph thesis. The Journal of the Veterinary Control and Research Institute, Pendik, Istanbul, Turkey, 26 no.:1, june, pp.45-54.

19) **UYSAL, Y. and OZKARA, A. (1995):** Preparation of *B. melitensis* Rev.1 vaccine from continuous culture produced cells. The Journal of the Veterinary Control and Research Institute, Pendik, Istanbul, Turkey, 26 no.:1, june, pp.35-44.

Results of epidemiologic studies on brucellosis

Year	Sheep (%)	Year	Cattle (%)
1952-63	2.6	1957-70	4.4
1960-70	5-9	1957-86	1.6
1989	1.26	1989	3.56
1990	2.08	1990	1.20
1991	1.83	1991	1.01
1992	1.48	1992	0.6

Human morbidity and mortality for brucellosis according to the years

Year	Population	Number of outbreaks	Morbidity (1/1 000,000)	Death	Mortality (1/1 000,000)
1970	35 321 000	37	0.10	2	0.05
1971	36 215 000	70	0.19	0	0.00
1972	37 132 000	63	0.17	1	0.03
1973	38 072 000	84	0.22	0	0.00
1974	39 038 000	70	0.16	0	0.00
1975	40 078 000	69	0.17	0	0.00
1976	40 915 000	69	0.17	0	0.00
1977	41 768 000	62	0.15	0	0.00
1978	42 640 000	72	0.17	0	0.00
1979	43 530 000	157	0.36	0	0.00
1980	44 438 000	186	0.42	0	0.00
1981	45 540 000	438	0.96	1	0.02
1982	46 688 000	676	1.45	1	0.02
1983	47 864 000	618	1.29	1	0.02
1984	48 070 000	1,135	2.31	0	0.00
1985	50 308 000	1,177	2.34	0	0.00
1986	51 546 000	1,563	3.03	1	0.02
1987	52 845 000	1,809	3.42	1	0.02
1988	54 176 000	2,356	4.35	1	0.02
1989	57 426 316	3,145	5.48	0	0.00
1990	57 582 446	5,003	8.69	2	0.03
1991	57 736 288	4,658	8.07	4	0.07
1992	58 088 101	5,197	10.48	0	0.00
1993	60 384 474	6,795	11.25	2	0.03
1994	60 384 474	8,383	13.88	0	0.00

Serum number and serological test results in state farms

Year	Total	Positive	Reactor-rate (%)
1957	25893	912	3.5
1958	35012	1434	4
1959	65138	1094	1.6
1960	49407	1091	2.2
1961	50183	2048	4
1962	38248	1636	4.2
1963	49660	2718	5.4
1964	59939	2830	5
1965	63960	3419	5
1966	51500	2601	5
1967	54610	2953	5.2
1968	40459	2133	5.2
1969	33037	2132	6
1970	13981	609	4.57
1975	15486	93	0.6
1976	17030	339	2
1977	22428	355	1.58
1978	18541	271	1.46
1979	18265	137	0.75
1980	17906	1292	7.2
1981	19305	271	1.4
1982	21876	183	0.8
1983	19583	278	0.8
1984	18903	189	0.9
1985	17356	72	0.4
1986	12208	122	0.9

The number of *Brucella* outbreaks according to the years

Year	*B. abortus*	*B. melitensis*	Total
1980	8	-	8
1981	16	-	16
1982	22	22	44
1983	35	129	164
1984	41	191	232
1985	32	98	130
1986	16	77	93
1989	15	97	112
1990	23	152	175
1991	16	185	201
1992	18	170	188
1993	6	237	243
1994	11	97	108
1995 (6 mn)	2	26	28

Doses of *Brucella* vaccines dispatched to the field

Year	*B. abortus* S19 full dose	*B. melitensis* Rev.1 full dose	*B. abortus* S19 reduced dose	*B. melitensis* Rev.1 reduced dose
1983	98,000	1 200,000		
1984	250,500	9 350,000		
1985	343,000	4 700,000		
1986	481,000	5 000,000		
1987	776,000	6 400,000		
1988	725000	5 700,000		
1989	783,000	6 900,000		
1990	809,000	6 600,000		
1991	707,000	6 000,000	124,000	884,000
1992	674,000	5 500,000	168,000	1,001,000
1993	391,000	3 500,000	50,700	715,700
1994	325,000	2 700,000	175,000	1,300,000
1995	420.000	3 000,000	2,000	600,000
TOTAL	6,782,500	63,550,000	517,700	3,900,700

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Trends in the Development of Diagnostic Tests

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Introduction

The successful eradication of brucellosis, particularly bovine brucellosis in developed countries, has usually been achieved using regular monitoring with the classical serological methods in conjunction with the slaughter of reactors. This methodology has proved successful under the conditions pertaining in these countries, but it has proved very costly. The control policy must be designed to suit the region in which it is to be applied, and it is almost certain that this type of test and slaughter campaign is entirely inappropriate for use in the Middle East. It is accepted that under the proposed programme a comprehensive vaccination programme will be used for a prolonged period of time and that serological monitoring will not be used for the identification of reactors for slaughter. It will however be valuable if used to monitor progress and assess vaccine coverage. The classical serological tests have proved effective in the past, but have associated problems and difficulties. For example, the serum agglutination test (SAT) has poor sensitivity and specificity, the complement fixation test (CFT) is complex and can only be conducted in a well equiped laboratory by well trained personnel. All the classical tests are badly affected by post-vaccinal antibody titres.

Speed

In some circumstances animals may be held confined pending the results of serological testing, or in others, rapid results may be required following a disease outbreak. Tests which can be carried out in the field, or certainly those which can be carried out in less well equipped local laboratories offer this advantage. The Rose Bengal plate test and the ELISA meet these criteria.

Automation

Automation may aid speed. If large numbers of samples are to be tested as part of monitoring, then some form of automation is desirable. Tests based on the microtitre format such as the CFT and ELISA are particularly easy to automate.

Convenience

Much equipment is available for use with the microtitre format to carry out tests and read results which can be computerised to aid automated data calculation, recording and result transmission. Tests such as the ELISA that are made available in kit format with all reagents contained within the kit are very easy to use for the smaller laboratory.

Improvements in Sensitivity and Specificity

No test is both 100% sensitive and specific, and these two attributes are linked so that for example whilst it is possible to adjust test conditions to improve sensitivity there will be an associated loss in specificity. For instance, the following graph demonstrates this relationship as the positive/negative threshold is altered in the ELISA. When there is a high incidence of brucellosis it is desirable to maximise test sensitivity, whilst when the level of infected animals is reduced, it is important to maximise specificity. It has been repeatedly shown that both the indirect and competitive ELISA have higher sensitivity and detect infected animals earlier in the course of infection than the classical tests and this was most marked amongst animals in the most lightly infected herds.

Use in Vaccinated Animals

The use of vaccination is essential to quickly reduce the incidence of disease, but it compromises serological diagnosis and therefore this inhibits it's use. There are reports in the literature of variants of the indirect and competitive ELISA said to be able to discriminate antibody titres arising from infection or vaccination. If such tests are validated in the field, their use will enable the widespread use of vaccination whilst allowing the detection of infected animals and the assessment of vaccine coverage.

Quality

The quality assurance (QA) of serological tests is an essential requirement as inaccurate or incorrect test results will seriously affect the progress of eradication. The QA protocol should be administered independantly of the testing laboratories and assess test accuracy and precision as well as laboratories procedures and the training of staff.

Standardisation

The use of standardised methods and reagents is essential for the success of such an international programme. All methods used should be as described in the OIE Manual of Standards, and be standardised against International Standard Sera where available.

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New vaccines, new vaccinal strategy. Review of current research

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New generation vaccines can be classified depending on the obtention method, by classical methods or by mutagenesis or genetic engineering.
Among the classically obtained *Brucella* strains with smooth LPS, there is *B. suis* S2 which was apparently successful in field experiments in China and Lybia but that showed no protection in controlled experiments against *B. ovis* and *B. melitensis*.

Among the *Brucella* strains with rough LPS (lack of O-chain) there is *B. abortus* RB51, which was protecting against all *Brucella* species in a mouse model and apparently against *B. abortus* in cattle in USA but was not protective against *B. ovis* in controlled experiments in sheep.

Concerning vaccines attenuated by transposition mutagenesis we can distinguish also between smooth and rough strains. Up to now, the smooth mutant strains studied are those with lacking virulence factors and galactosidase, erythritol sensitivity, catalase and urease negative, superoxide-dismutase, cationic peptides sensitivity.... There is no available protection data.

Rough strains developed by transposition mutagenesis by some teams are the VRTM1 derived from virulent *B. melitensis* 16M and VRTS1 derived from *B. suis* 2570. Both strains showed growth curves similar to Rev.1 vaccine and were protective in the Balb/c model against *B. melitensis* 16M and *B suis* 2579 and 750. More studies are carrying out with these strains in order to complete the characteristics of that immunity.

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Conclusions and Recommendations

The experts agree with the general guidelines contained within the report of the Worshop held in Amman, Jordan, 14-17 february 1993 "FAO, WHO, OIE Guidelines for a Regional Brucellosis Control Programme for the Middle-East".

The expert agree with the recommendations of the abovementioned Amman Workshop that the use of Rev.1 vaccine at the dose of 10^8-10^9 by the conjunctival route, since no more information is available proving scientifically the efficacy and the innocuousness of the sub-cutaneous adult dose in sheep and goats, whilst this information is available for the conjunctival route. The dose of 10^8 reduces the chance of abortion.

There is now a Rev.1 reference strain available at the headquarters of the European Pharmacopoeia (BP 907, 67029 Strasbourg Cedex 1, France).

The quality control of the Rev.1 vaccine should conform to the methods described in the OIE Manual of Standards for Diagnostics Tests and Vaccines (1996).

Rev.1 should be used for the protection of cattle as soon as sufficient evidence is gained from controlled experiments. The 2 year initial phase will enable this research to be carried out.

The suggested research topics are an essential component of the programme and must be supported to ensure the success of the project.

For the measurement of progress, the use of bacteriological surveillance is an effective method when serological tests are compromised in a vaccinated population.

It is essential that serological methods would be available which can distinguish infected from vaccinated animals, when the vaccination phase will be completed. The development and evaluation of these methods must take place at the beginning of the project so that they are available for use to monitor the progress and success of the project at its mid-point and its end.

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