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Development of neutralization test against Bacillus anthracis toxins to evaluate the antibody in response to Sterne 34F2 vaccination

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Introduction
Anthrax is a zoonotic disease caused by *Bacillus anthracis* spore-forming bacterium. Since it is primarily a disease of animals, the control in animals, and humans depend on the prevention in livestock, principally cattle, sheep, and goats. Most veterinary vaccines utilize the toxigenic, uncapsulated (pXO1+/pXO2-) *B.anthracis* strain 34F2 which affords protection through the production of neutralizing antibodies directed to the toxin's components. The titration of specific antibodies in sera of vaccinated animals is crucial to evaluate the efficacy of the vaccination and to obtain epidemiological information for an effective anthrax surveillance (1).
The aim of this study was to set up and demonstrate the efficacy of a *B.anthracis* toxin neutralization test (TNA) for the in vitro evaluation of the neutralizing antibody response induced following immunization with Sterne 34F2 vaccine. This test would allow to evaluate the efficacy of the Sterne vaccine to be measured without the need of experimental infection with pathogenic strains of *B.anthracis.*

Methods
In this study we used 10 New Zealand White rabbit and 10 sheep of a southern Italy farm vaccinated with two doses of Sterne 34F2 vaccine (2). Five animals for species were used as a control. Sera were collected before the first vaccination (T0), before the booster dose (T1) and 15 days after the second dose (T2).
The TNA assay was conducted in 96-well plates using the mouse macrophage line RAW 264.7. The 1:160 diluition of serum samples were tested in triplicate wells for the presence of antibodies that neutralized the infectivity of *B.anthracis* toxins in RAW cell monolayers. At each dilution of serum samples, 200 ng/ml of Recombinant Anthrax Protective Antigen and 640 ng/ml of Anthrax Lethal Factor were added (3).
To assess cell viability, we used the Vybrant® MTT Cell Proliferation Assay Kit (Life Technologies) for our experiments, following the protocol indicated by the company.

Results
The concentration of recombinant toxins used resulted in 80% mortality of cells in the absence of neutralizing antibodies. In the present study, all serum samples analyzed from vaccinated animals showed production of neutralizing antibodies at both T1 and T2, while no neutralizing antibody response was found at T0 and in all control samples.
In vaccinated sheep, a significant booster effect of the vaccine dose was noted, rising from a cell viability of 32% at T1 to about 80% at T2.
In contrast in rabbits there was no booster effect, in fact at both T1 and T2 the cell viability was about 80% (Fig.1).

Conclusions
The test developed and standardized in the present project showed that animals vaccinated with the live attenuated Sterne 34F2 strain produced a good amount of antibodies neutralizing *B.anthracis* toxins, demonstrating how important this test is for determining post-vaccinal serology.
In conclusion, the toxin neutralization test can offer some advantages such as (i) doesn't require the use of the challenge test with live pathogen strains, without the need of special safety measures; (ii) shows the real neutralizing effect of the antibodies produced.