Evaluation of veterinary autogenous vaccines safety by MTT in-vitro *cytotoxicity assay*

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Veterinary autogenous vaccines,
ATT - MTT assays,
Alternative methods.

Summary
In Italy, veterinary autogenous vaccines manufacturing is regulated by the legislative decree of the Ministry of Health, March 17th, 1994, n. 287. The production is performed by the network of the 'Istituti Zooprofilattici Sperimentali' (IZSs), public health institutes scattered all over the Italian territory. The aim of this research was to evaluate the feasibility of an *in vitro* method to test the abnormal toxicity of autogenous bacterial vaccines as an alternative to animal models routinely employed. For this purpose, the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZSAM) in partnership with the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER), evaluated the toxicity of 49 batches of autogenous bacterial vaccines, previously shown to be safe in guinea pigs and mice, on animal model, by means of the methyl tetrazolium (MTT) assay. All vaccines showed cytotoxic effects when tested 1:2 diluted and undiluted; overall, all vaccines lost toxicity at 1:128 dilution. As expected, these findings suggest a different susceptibility of this assay compared to the laboratory animal model. On the other hand, these results do not clarify which components of the vaccines are responsible for the cytotoxic effect. Overall, more experiments are warranted in order to standardize the MTT assay which could be coupled with the trials in laboratory animals.

Valutazione della sicurezza di vaccini stabulogeni veterinari attraverso il methyl tetrazolium test *(MTT)*

Parole chiave
Vaccini stabulogeni veterinari,
Prova di "tossicità anormale" (ATT),
MTT test,
Metodi alternativi.

Riassunto
La produzione dei vaccini stabulogeni e degli autovaccini ad uso veterinario in Italia è disciplinata dal Decreto Ministeriale n. 287 del 17 marzo 1994, recante indicazioni relative alla "produzione, l'impiego ed il controllo dei medicinali veterinari immunologici inattivati, aventi caratteristiche di vaccini stabulogeni ed autovaccini", e rientra tra le attività principali svolte dagli Istituti Zooprofilattici Sperimentali. La normativa prevede che, prima del rilascio, ciascun lotto di vaccino sia sottoposto alla prova di "tossicità anormale" (ATT - *abnormal toxicity test*) su animali da laboratorio. Il Decreto Legislativo n. 26 del 4 marzo 2014, "sulla protezione degli animali utilizzati a fini scientifici", recepimento della Direttiva europea 2010/63/UE, ribadisce l'importanza di identificare e validare metodi alternativi in accordo con i principi delle 3R di riduzione, affinamento e sostituzione (*replacement, reduction, refinement*) delle prove biologiche sugli animali da esperimento. A tal fine, 49 lotti di vaccini stabulogeni di origine batterica, precedentemente sottoposti, con esito positivo, alla prova di tossicità anormale, sono stati testati con il test di citotossicità cellulare MTT (*methyl tetrazolium test*) utilizzando la linea cellulare continua L929. Tutti i vaccini saggiati alla diluizione 1:128 non hanno manifestato azione citotossica, alla diluizione 1:32 il 68% dei vaccini risultava idoneo,

mentre alle diluizioni 1:2 e tal quale c'è stata una riduzione di vitalità cellulare > 30% in tutti i vaccini. Dai dati ottenuti si evidenzia una diversa suscettibilità del test MTT rispetto al saggio di tossicità anormale su animali. Non è stato finora possibile comprendere quali costituenti siano effettivamente responsabili della citotossicità dei vaccini, inoltre è emersa la necessità di produrre controlli positivi affinché la prova possa essere standardizzata. Dai risultati ottenuti si evince che, se ulteriormente saggiato e standardizzato, il test MTT potrebbe costituire un metodo alternativo e complementare al tradizionale saggio *in vivo* attualmente in uso.

Introduction

Autogenous vaccines

Veterinary vaccines are an important tool for controlling animals infectious diseases with a tremendous impact on antimicrobial resistance. Their use has been indeed promoted in the last years (Thibault 2004, Monath 2013, OIE 2018) rather than treat animals with antibiotics, into a 'One Health' vision (Kaplan *et al.* 2009, Evans and Leighton 2014).

The current legislation regarding autogenous vaccines, Decree of the Italian Ministry of Health, 17 March 1994, n. 287[1], establishes that these products can be released after a safety control, named 'abnormal toxicity test' (ATT), that has to be carried out in laboratory mice and guinea pigs (EP 01/2008:20609) by injecting subcutaneously a specified volume of final product, and waiting the following 7 days for any adverse reaction to occur[2]. Anyway, side effects in these animals were never reported by the Istituti Zooprofilattici Sperimentali (IZSs), the legal Italian publich health authorities which are in charge for this task. For this and for other reasons, such as the low specificity of the test (e.g. strain-related differences, or the fact that stressful conditions can produce different results), the reliability of *in vivo* methods is questionable[3] (Kumar *et al.* 2018).

On September 2010, a new European Union Directive for the protection of vertebrate animals used for experimental purposes, Directive of 22 September 2010, n. 63 of the European Parliament and of the Council, increased the protection of experimental animals and posed specific rules regarding the correct design of a scientific project[4]. This task will be achieved by defining the most suitable animal model to be employed and by reducing their number with increased animal welfare (Combrisson 2014).

The three R's Principles expressed in 1959 by Russell and Burch, represent the main ethical guideline addressing the EU Directive 63/2010. It foresees a reduction in the number of animals (Reduction), their substitution with effective *in vitro* and *in silico* models or with animals with the lowest capacity to experience pain (Replacement) and an improvement of welfare conditions (Refinement)[4] (Fabre 2009).

While in many fields it hasn't been possible yet to replace animal models, in some other, as toxicology (mainly acute topic toxicity, such as skin irritation), several *in vitro* tests were validated[3] (Faller and Bracher 2002, OECD 439 2015, Ohtake *et al.* 2018).

Within these alternative tests, the 3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl-2-H-tetrazolium bromide-MTT assay is included. MTT assay has been widely employed in many fields, ranging from regenerative medicine, dermatology and orthodontics, to immunology and toxicology (Thoneman *et al.* 2002, Di Francesco *et al.* 2005, Malkoc *et al.* 2010, Patnaik and Padhy 2018, Qi *et al.* 2018). This test recognizes live cells for their capability to reduce tetrazolium MTT salt, a yellowish reagent, to an intracellular, purple product, named formazan; on the contrary, dead cells lack the ability to process any substrate, therefore they still not induce any color change and wells remain yellow. The amount of live/dead cells can be quantified by a spectrophotometer that measures the amount of light that passes through the purple/yellow solution.

[1] Ministry of Health. Ministerial Decree of 17 March 1994, n. 287. Regolamento recante norme sulla produzione, l'impiego ed il controllo dei medicinali veterinari immunologici inattivati, aventi caratteristiche di vaccini stabulogeni ed autovaccini. *GU*, **111**, 14.05.1994.

[2] Coordination group for mutual recognition and decentralized procedures Veterinary medicine (CMDv) & Heads of Medicines Agencies (HMA). 2017. Recommendations for the manufacture, control and use of inactivated autogenous veterinary vaccines within the EEA. EMA/CMDv/452656/2016. REC-002-01. London, 20 March 2017. http://www.hma.eu/fileadmin/dateien/Veterinary_medicines/CMDv_Website/Procedural_guidance/Miscellaneous/Recommendations_ manufacture_control_use_inact_autogenous_vaccines.pdf.

[3] Tellner P. & European Federation of Pharmaceutical Industries and associations (EFPIA). 2017. Deletion of test for abnormal toxicity from European pharmacopoeia. European Federation of Pharmaceutical Industries and associations (EFPIA). Version: FINAL, 30/06/2017 https://www.efpia.eu/media/219814/deletion-of-test-for-abnormal-toxicity- from-european-pharmacopoeia.pdf.

[4] European Parliament and Council Union (EU). 2010. Directive 2010/63/EU, 22 September 2010 on the protection of animals used for scientific purposes. *Off J*, **L 276/33**, 20.10.2010.

The present study aimed to evaluate the effectiveness of the MTT assay in assessing the toxicity of bacterial autogenous vaccines.

Materials and methods

Autogenous vaccines manufacturing

A total number of 49 autogenous vaccines, produced by IZSAM, during the years 2013-2018, were tested by MTT. Vaccines were produced following an official request using, as starting material, microbial agents isolated during outbreaks in domestic animals (Figure 1).

The isolates were propagated into specifics growth medium and subsequently, after purity and identity tests, pure liquid cultures were amplified in the proper amount of growth medium, depending on the number of requested doses. After growth, bacterial cultures were harvested and inactivated by dilution in 0.4% sterile saline solution containing formaldehyde (37%), followed by incubation at 37 °C for 24 hours. For *Clostridium* spp., the inactivation was achieved by adding 0,6% sterile saline solution containing formaldehyde (37%), followed by incubation for 21 days at 37 °C. Once bacterial inactivation was confirmed, the product was diluted in saline solution containing sodium ethylmercurithiosalicylate 0.005% as preservative, to reach a final concentration ranging between 2.2 x 10^9 to 3 x 10^9 CFU/ml. Finally, 10% of aluminum hydroxide was added as adjuvant.

Before the delivery, final products were tested to establish the residual concentration of free-formaldehyde, that should not exceed 20 ppm (Ph. Eur. 01/2008:20418), and were also submitted to evaluate the abnormal toxicity in laboratory animals, in accordance to MD n. 287/1994. Three mice were inoculated subcutaneously with 0.5 ml of each vaccine and observed for 7 days (21 days for *Clostridium* spp. vaccines) for any adverse reaction. For *Clostridium* spp. autogenous vaccines, 3 guinea pigs were also injected subcutaneously of vaccine, following the same protocol provided for mice.

Vaccines were rejected when a local or a systemic reaction was observed (e.g. increase in body temperature, variation in feed and water intake) within 7 days (Ph. Eur. 04/2013:50209).

MTT test

L929 cell line, provided by IZSLER (cod. BS CL 56), deriving from mouse fibroblast cells located in the adipose tissue (Nordin *et al.* 1991, Badole *et al.* 2013), were employed for the MTT assay.

L929 cells were grown in 75 cm^2 cell culture flasks with filtered cap (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and were cultured in MEM (modified Eagle's medium Sigma Aldrich, Saint Louis, Missouri, USA) without phenol red, supplemented with 10% fetal bovine serum (BFS) (Sigma Aldrich, Saint Louis, Missouri, USA), $NaHCO_3$ 2.2 g/L and glutammine 200 mM (Sigma Aldrich, Saint Louis, Missouri, USA). Cells were incubated at 37 °C at 5% CO_2 and were daily observed under inverted microscope (20X-40X); at 90-100% of confluence, cells were trypsinized, centrifuged (300 g x 10 min at 4 °C) and diluted in MEM medium with phenol red (Sigma Aldrich, Saint Louis, Missouri, USA) supplemented with 10% BFS, to obtain a final concentration of 3 x 10^5 cells/ml.

Cell suspension was transferred onto 96-well flat bottomed cell culture plates (BD Biosciences, Massachutes, USA) (100 µl of cell suspension/well) excluding the peripherals wells of the plate filled with 100 µl of MEM without phenol red. Plates were incubated for 24 hours at 37 °C at 5% CO_2. At the end of incubation, plates were observed under inverted microscope (magnification 20X-40X) to verify that a minimum of 70% of growth, necessary to perform the MTT assay, has been reached.

Thirty-five out of forty-nine vaccines were tested starting from the original and undiluted material up to dilution 1:32. A positive control, consisting of sterile saline solution containing 0.5% phenol, was also enrolled for the study. The remaining 14/49 vaccines were tested with the same procedure but up to 1:256 dilution. For these samples, a different positive control, consisting of a sterile saline solution containing sodium ethylmercurithiosalicylate 0.005%, was used (Table I).

To perform the MTT test, the supernatant of the cell culture plates, at 70% of confluence, was discharged. Samples included by columns 3,4,5 and rows B-G, contained 100 µl of undiluted and two-fold diluted vaccines, in triplicate. MEM without phenol red was employed as diluent. Wells included by columns

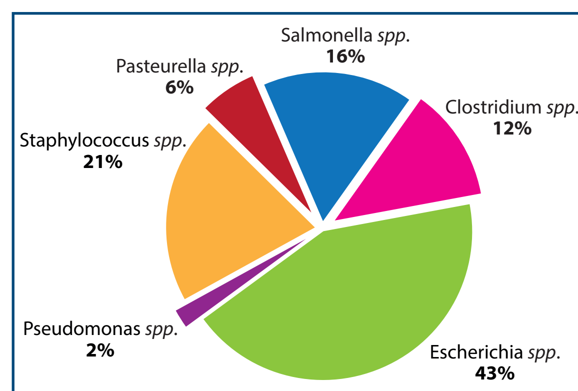

Figure 1. *Bacterial autogenous vaccines tested by MTT assay.*

2 and 11 and rows B-G were used as blank by adding 100 µl of MEM without phenol red. Wells belonging to columns 6 and 7 were employed as negative control (NC) by adding 100 µl of MEM without phenol red but containing 10% BFS. Finally, wells belonging to columns 8, 9 and 10, rows B-G, served as positive control (PC). Plates were then incubated for 24 hours at 37 °C, 5% CO_2.

After 24 hours, plates were evaluated to detect cytotoxic effect; then, the medium was discharged and plates washed once with 100 µl of PBS.

Subsequently, 50 µl of MTT 1X (50 mg/50ml) reagent (Merck Millipore, Italy) was added to each well. Plates were incubated at 37 °C for 2 hours. After incubation, 100 µl of isopropyl alcohol 100% (Sigma Aldrich, Italy) was added to each well. After a gentle shaking of the plates for 30 minutes at room temperature, the optical density (OD) was measured at a 550 nm wavelength on a spectrophotometric Benchmark Microplate reader and results were processed by Microplate Manager 5.2 (Biorad, Italy). For each dilution the mean cell viability (CV%) was calculated as follows:

Mean cell viability (CV%) =
[Mean OD (samples)/ Mean OD Blank] X100

where:

Mean OD samples = mean optical density for each sample, tested in triplicate, columns 3, 4, 5, rows B-G;

Mean OD Blank = mean optical density of the blanks, for each sample tested in duplicate in columns 2-11, row B-G.

The results for each one of the 49 MTT assays were considered valid if mean OD values for blank and PC were ≥ 0.2 and < 0.2, respectively (Table II).

A minimum of 70% cell viability was considered as cut-off (final dilution FD70).

Statistical analysis

The percentages of non-cytotoxic vaccines for each dilution were calculated. In order to associate an uncertainty with this probability, the 95% confidence intervals, according to a Bayesian approach, were calculated by means of the Beta distribution.

Results

Vaccines were not toxic in laboratory animals

The ATT test performed with the 49 autogenous vaccines did not elicit adverse reaction in laboratory animals.

Table I. *Organization of the 96 wells cells culture plates for each vaccine.* A total of 35 vaccines were tested up to the 1:32 dilution (**A**); the remaining 14 vaccines were tested up to the 1:256 dilution (**B**).

A												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM
B	MEM	Blank	Sample U	Sample U	Sample U	NC	NC	PC	PC	PC	Blank	MEM
C	MEM	Blank	Sample 1:2	Sample 1:2	Sample 1:2	NC	NC	PC	PC	PC	Blank	MEM
D	MEM	Blank	Sample 1:4	Sample 1:4	Sample 1:4	NC	NC	PC	PC	PC	Blank	MEM
E	MEM	Blank	Sample 1:8	Sample 1:8	Sample 1:8	NC	NC	PC	PC	PC	Blank	MEM
F	MEM	Blank	Sample 1:16	Sample 1:16	Sample 1:16	NC	NC	PC	PC	PC	Blank	MEM
G	MEM	Blank	Sample 1:32	Sample 1:32	Sample 1:32	NC	NC	PC	PC	PC	Blank	MEM
H	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM
B												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM
B	MEM	Blank	Sample 1:64	Sample 1:64	Sample 1:64	NC	NC	PC	PC	PC	Blank	MEM
C	MEM	Blank	Sample 1:128	Sample 1:128	Sample 1:128	NC	NC	PC	PC	PC	Blank	MEM
D	MEM	Blank	Sample 1:256	Sample 1:256	Sample 1:256	NC	NC	PC	PC	PC	Blank	MEM
E	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM
F	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM
G	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM
H	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM	MEM

U = undiluted; NC = negative control; PC = positive control; MEM = modified Eagle's medium.

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Most of vaccines lost toxicity at 1:32 dilution

All vaccines under investigation were cytotoxic when tested undiluted and 1:2 diluted, as they reduced more than 70% of L929 cell viability; cytotoxicity progressively decreased up to 1:128 dilution (Table III).

Interestingly, 32 out of 49 vaccines lost their citotoxicity at 1:32 dilution (p value: 87.76%; lower confidence limit: 75.6; higher confidence limit: 94.18) (Table IV).

Discussion

The MTT assay (Fotakis and Timbrell 2006), is a cell viability test able to evaluate the *in vitro* cytotoxic effect of several compounds after interaction with a cell monolayer (Mosmann 1983, Lin *et al.* 2015).

In our study the MTT assay was employed to detect vaccines cytotoxicity; low dilutions induce cytotoxicity which decreases in a concentration-dependent manner. Nevertheless, the assay lacks of the ability to recognize if the cytotoxic effect is due to one or more vaccines components. When employed as positive control the preservative itself damaged L929 cells in all PC wells, suggesting that vaccine's cytotoxic effect may be related mainly to it.

Our data show that MTT has a different susceptibility with respect to the *in vivo* ATT. In our settings, in order to find the dilution at which cytotoxicity is completely lost, 14 vaccines, that showed DF70 ≥ 1:32, were re-tested at higher dilutions, employing the protocol previously described. All 14 vaccines showed values ranging from 1:32 to 1:128 (Table III); therefore, with all due caution, 1:128 dilution could represent the dilution limit (DF70) from which, if cytotoxic effects are absent, the safety of autogenous vaccines can be ensured.

Moreover, in order to apply and validate the assay, vaccines which were previously rejected by ATT, so toxic for live animals, need to be tested by MTT. In this way, we could easily define the DF70 for safe and unsafe vaccines with relevant consequences in terms of reliability of the assay and reduction of the use of live animals. Reasonably we do not

Table II. *Spectrophotometric reading (wavelength: 550 nm) for autogenous vaccine n. 35.* The mean OD values for Blanks and PC were 0.58, and 0.04, respectively; therefore MTT assay for vaccine n. 35 was considered valid.

	Blank	35	35	35	NC	NC	PC	PC	PC	Blank
Undiluted	0.515	0.045	0.049	0.047	0.673	0.749	0.041	0.043	0.057	0.546
1:2	0.595	0.046	0.045	0.045	0.748	0.716	0.046	0.047	0.049	0.62
1:4	0.532	0.043	0.049	0.047	0.641	0.629	0.048	0.05	0.051	0.675
1:8	0.663	0.228	0.242	0.241	0.825	0.802	0.051	0.05	0.066	0.593
1:16	0.693	0.662	0.645	0.6	0.879	0.879	0.045	0.043	0.088	0.771
1:32	0.503	0.475	0.48	0.634	0.724	0.641	0.049	0.05	0.059	0.582
Mean value	**0.54**						**0.04**	**0.05**	**0.05**	**0.62**

NC = negative control; PC = positive control.

Table III. *Numbers in bold indicate how many vaccines showed (per pathogen) a reduction of cytotoxicity for at least the 70% of L929 cells; the corresponding dilution represents the dilution limit for each vaccine (final dilution-FD70).*

	CV Values ≥ 70%							
Dilutions	***S. abortus ovis***	***S. abortus equi***	***Clostridium* spp.**	***E. coli***	***Pseudomonas* spp.**	***Staphylococcus* spp.**	***Pasteurella* spp.**	**Total**
Undiluted								0
1:2								0
1:4	**1**							1
1:8		**1**						1
1:16	**1**	**3**	**2**	**1**		**2**		9
1:32	**1**		**4**	**18**	**1**	**8**		32
1:64				**2**			**1**	3
1:128	**1**						**2**	3
1:256								
Total	4	4	6	21	1	10	3	49

Table IV. *P values, corresponding to the cumulative number of vaccines that lost cytotoxicity at a specific dilution/ total of examined vaccines.* For each p value, confidence limits for beta distribution (95%) are also reported.

Dilution	Loss of cytotoxicity	Examined	p	l.c.l. (95%)	u.c.l. (95%)
1:4	1	49	2.04%	0.49%	10.65%
1:8	2	49	4.08%	1.25%	13.71%
1:16	11	49	22%	13.06%	35.96%
1:32	43	49	87.76%	75.69%	94.18%
1:64	46	49	93.88%	83.45%	97.78%
1:128	49	49	100.00%	94.18%	100.00%

support the single use of this assay. MTT can, indeed, efficiently couple ATT as a front-line screening tool for the mandatory *in vivo* trials of the most promising vaccines (Schwanig *et al.* 1997, Kumar *et al.* 2018).

In conclusion, further investigations are required to standardize ATT for autogenous vaccines in order to promote, if solid and robust data are obtained, amendments to the current legislation.

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