

## Determination of genotoxicity of classical swine fever vaccine *in vitro* by cytogenetic and comet tests

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**Chromosome damage in lymphocyte cultures induced by live virus vaccine against classical swine fever (CSF) has been observed in previous studies. *In vivo* cytogenetic tests were made with several doses of vaccines used in Argentina to control the disease. These studies have shown that genotoxic effects increased with dose. In the present study, two different *in vitro* assays were performed by recording the frequency of cells with chromosome alterations and by assessing the ability of the vaccine to damage DNA, using the single cell gel microelectrophoretic assay (comet test). Frequencies of cells with chromosomal alterations increased significantly when compared with controls and were dose ( $\mu\text{l/ml}$ ) dependent: 0 = 1.23, 5 = 2.29, 10 = 5.42 and 20 = 11.71%. In the comet assay the variables measured, tail length (TL) and tail moment (TM), also increased. For control cultures TL was 2.32  $\mu\text{m}$ , whereas with concentrations of 20 and 100  $\mu\text{l/ml}$  TL were 12.47 and 42.3  $\mu\text{m}$ , respectively. TM of control cultures was 0.18, whereas with vaccine concentrations of 20 and 100  $\mu\text{l/ml}$  TM were 5.52 and 24.52, respectively. Comet frequency distributions differed significantly among treatments. These results agree with previous *in vivo* observations. Regarding CSF pathogeny, our results support a direct effect of CSF vaccinal virus on lymphocyte DNA. Genotoxicity of CSF vaccine was corroborated *in vitro* at the cytogenetic and molecular levels.**

### Introduction

Classical swine fever (CSF) is the most important disease in swine production worldwide. CSF virus is very infectious and causes high morbidity and mortality rates.

CSF pathogeny is still a matter of discussion. One hypothesis supports a direct effect of the virus on lymphocytes (1), with viral RNA localized in follicles at very early stages of the disease, and the germinal centers are the micro anatomic sites of virus replication preceding lymphocyte depletion. The second hypothesis proposes an indirect mechanism (2) stating that depletion occurs before detection of the viral antigen and of cell apoptosis. Pathogenic studies on granulocytopenia and bone marrow atrophy during CSF revealed apoptosis induction in non-infected cells (3).

CSF virus can induce chromosome alterations in diseased and immunized pigs using live attenuated virus vaccines

(4–6). Gustavsson (7) considered the mutagenic capacity of attenuated virus as a useful line of mutation research in countries where vaccination is used to control CSF. Consequently, in 1996 we started a research program at the National University of Río Cuarto (UNRC) to evaluate the genotoxicity of the CSF vaccine currently used in Argentina, running *in vivo* tests (8–11). In this country CSF is an endemic disease that was controlled by compulsive immunization with live attenuated virus vaccines until May 2004, when this program was discontinued.

The objective of this study was to determine the genotoxic activity of CSF vaccine by running two *in vitro* assays using lymphocyte cultures, cytogenetics analysis and DNA damage (as evaluated using the single-cell microgel-electrophoresis or 'comet assay'). This latter assay of genotoxicity estimation has previously been applied to detect DNA damage induced by the influenza virus A2/HK/68 in *in vitro* infection experiments with human leukocytes (12).

### Materials and methods

Five piglets of the same litter not immunized against CSF, from the UNRC Experimental Farm were used for this study. All animals belonged to a F1 (Landrace  $\times$  Yorkshire)  $\times$  Patented Terminal Hybrid cross and were properly identified and labeled. Pigs were 40 days old, with a weight of 20 kg, and presented similar physiological conditions.

For the cytogenetic analysis three peripheral blood lymphocyte (PBL) cultures per piglet were made following our procedure (13), except that the incubation time was 44 h. After 24 h of incubation the cultures were exposed to  $10^{3.5}$  TCID<sub>50</sub>/ml of the vaccine, titration was made by the National Animal Health Service (SENASA) (14). The cultures were exposed at three different times: 0, 10 and 20 h of incubation.

To measure cytotoxicity, 15  $\mu\text{l}$  of each original cell culture was mixed with 15  $\mu\text{l}$  of a 0.005% solution of trypan blue dye (CAS 72-57-1). Cells were analyzed with a light microscope and the percentage of viable cells was determined. Statistical analysis was not performed.

The maximal tolerable dose (MTD) of vaccine in cultures was determined by testing four final vaccine concentrations in the culture: 200, 100, 60 and 20  $\mu\text{l/ml}$ , with two control cultures by pig. The concentration that reduced the mitotic index (MI) to 20–25% of the control was taken as the MTD. MI was calculated observing 1000 lymphocytes per replication. For the cytogenetic assay three-fourth of that concentration was added to the cultures (15). For the three treatments and controls a total of 10 cultures per pig were performed.

Assays were done with three best-known vaccine brands of Argentina named for the purpose of this study as A, B and C. Chromosome alterations were counted in  $\sim 100$  cells per culture following Savage classification (16).

For the comet assay, blood samples were taken from five pigs belonging to a pig farm in Chillán, Chile, with the same characteristics of those used for the cytogenetic analysis. The same technique of lymphocyte culture was used, without including the mitogen, following international protocols. The procedure (17) with minor modifications was applied to evaluate DNA damage. Briefly, conventional slides were covered with a first layer of 0.5% of normal agarose. Then 75  $\mu\text{l}$  of 0.5% low-melting agarose (CAS 9012-36-6) was mixed with 5  $\mu\text{l}$  of the cell suspension and covered with a layer of 0.5% of low-melting agarose. The slides were immediately covered. After agarose solidification at 4°C for 5 min, the coverslips were removed and the slides were immersed overnight at 4°C in a freshly prepared lysing solution [2.5 M NaCl, 100 mM Trizma base (CAS 77-86-1), 1% Triton X-100 (CAS 9002-93-1) and 10% DMSO]. Two slides were prepared from each control and treatment group under dimmed light. The slides were then placed in a horizontal gel

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electrophoresis unit filled with alkaline buffer (300 mM NaOH and 1 mM Na<sub>2</sub>EDTA, pH > 13) and left for DNA unwinding for 20 min. Afterwards, electrophoresis was performed for 30 min at 4°C under dim light, the same alkaline buffer at 0.75 V/cm (25 V, 300 mA) for 20 min was used. After electrophoresis, the slides were washed with neutralizing buffer (0.4 M Tris, pH 7.5) and the DNA was stained with 50 µl of ethidium bromide (CAS 1239-45-8; 2µg/ml).

Vaccine was added to the cultures at the final concentrations of 100 and 20 µl/ml (MTD), after 20 h of incubation and during 24 h.

To evaluate DNA damage 200 comets were analyzed in each treatment and controls. Cell images from each pig were randomly selected from two different slides (100 each) and analyzed using an epifluorescence microscope (Zeiss Axioskop II Mot) equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. A ×20 objective was used to project the image into a highly sensitive camera. Imaging was performed using the Comet Imager Software V 3.4.6 designed by Metasystem-Germany, which acquires, computes and integrates intensity profile for each cell, estimates the comet cell components, head and tail, and evaluates a range of derived parameters including tail moment (TM), an index of DNA damage that considers both the tail length (TL) and the fraction of DNA in the comet tail (TM = %DNA in tail × TL/100), TL and Head Size (HS) (18–21).

For the statistical analysis, each value of a cell with chromosome alteration was transformed to frequency, dividing by the total value in each case. The Shapiro-Wilk test indicated that the number of cells with chromosome alterations did not follow a normal distribution; therefore, the Kruskal–Wallis non-parametric range test was applied.

The χ<sup>2</sup>-test for distribution heterogeneity was used for the comet assay, since TL and TM were not represented by Gaussian distribution. Standard units of the sample were considered to obtain class intervals that were calculated by

deducing the minimal value from the maximal and dividing by the number of classes. TL and TM were the variables analyzed.

**Results**

The viability of untreated and treated PBL was always >85%. The vaccine effect on MI was evident particularly with 60, 100 and 200 µl/ml treatments (Figure 1). Results indicate an inverse correlation between vaccine dose and MI, since the latter was significantly lower as dose increased.

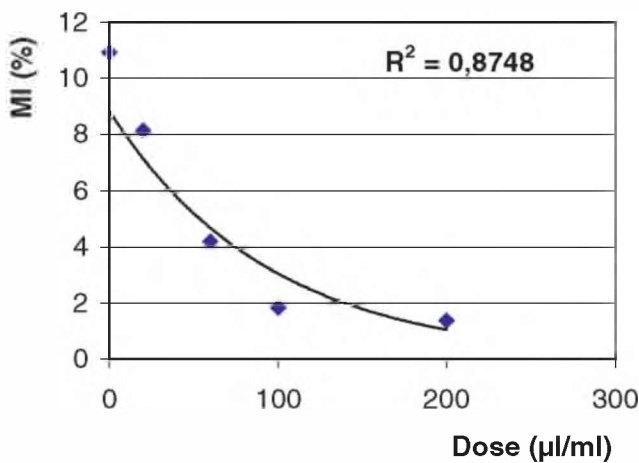
The MI of control cultures, 11.081 ± 0.1703, did not differ from the 20 µl/ml treatment (8.226 ± 0.0794), therefore this vaccine concentration was considered the MTD for the subsequent cytogenetic analysis, according to international standards for *in vitro* experiments on genetic toxicology.

The induction of cells with different types of chromosome alterations, monochromatid and isochromatid breaks (B' + B''), chromatid exchanges (RB), multiple alterations (Mu) and pulverizations (Pu), was observed only after 20 h of culture exposure to the vaccine.

No significant difference was found among PBL cultures from each animal. Consequently, values from the five piglets were aggregated for statistical analysis.

To analyze the effect of vaccine dose, results corresponding to each vaccine brand were aggregated. Frequency of cells with chromosome alterations increased with dose, being significantly different for all doses (P < 0.001). Taking each type of chromosome alteration separately (Table I), the statistical analysis discriminated between two groups: 5 and 10–20 µl/ml, except for pulverization that disagreed in the three doses.

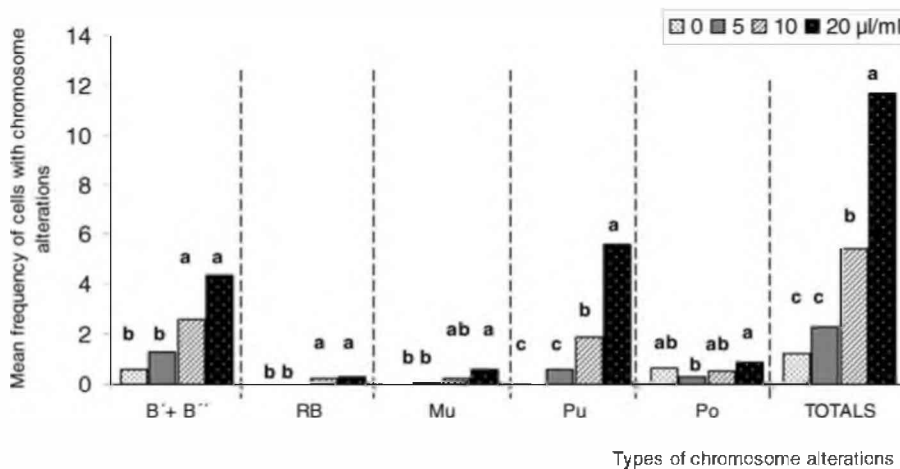
The kinetics of the frequency of each type of chromosome alteration (Figure 2), according to dose, showed an increase



**Fig. 1.** Exponential decrease of mitotic index (MI) with increased vaccine dose.

**Table I.** Frequency of cells with chromosome alteration types induced by the vaccine at different doses

Dose (µl/ml)	Frequency of chromosomal alterations					Total
	B' + B''	RB	Mu	Pu	Po	
0	0.5711	0	0	0	0.6635	1.2347
5	1.3139	0	0.0596	0.6025	0.3179	2.2938
10	2.5748	0.2274	0.2307	1.8865	0.5088	5.4282
20	4.3739	0.2809	0.5677	5.5993	0.8975	11.7190



**Fig. 2.** Mean frequency of cells with chromosome alterations for different vaccine doses. Same letters indicate no statistical significant difference. B' + B'', monochromatid and isochromatid breaks; RB, chromatid exchanges; Mu, multiple alterations; Pu, pulverization; Po, polyploid cells.

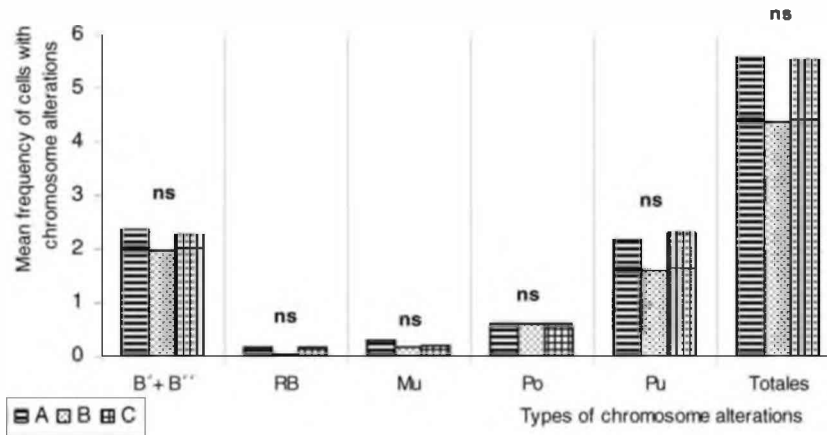


Fig. 3. Mean frequency of cells with chromosome alterations discriminated by brand (A, B, C). Same letters indicate no statistical significant difference.

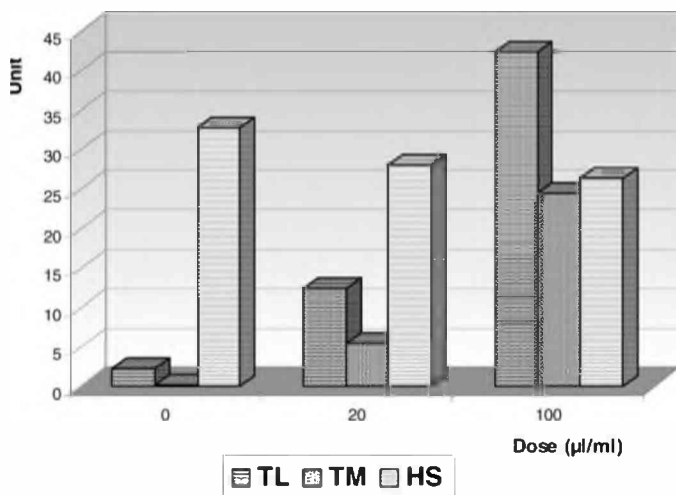


Fig. 4. Mean values of TM, TL and HS according vaccine dose.

mainly depending on cells with chromosome pulverization (Pu) and breaks ( $B' + B''$ ). Cells with multiple abnormalities (Mu) were observed in all tested doses, while chromatid exchanges (RB) were observed only in those cultures that received the two highest doses. There was no significant difference among all doses in the frequency of polyploid cells (Po).

No difference was observed among vaccine brands considering the mean frequency of cells with chromosome alterations (Figure 3). However, the cytogenetic effect induced by the vaccine B showed consistently slightly lower mean values.

Mean values of the quantification of TL and TM, and HS for each dose indicated that with the dose increment there was a sharp rise of TL and TM values, while HS decreased gradually (Figure 4; Table II). For concentrations 20 and 100  $\mu\text{l/ml}$  there were highly significant differences in the frequency distribution of TL and TM.

The general tendency of TL and TM frequency distributions can be summarized by combining treatment and control data into a single graph for each variable (Figure 5). It was clearly seen that, in controls, most DNA remained in the nucleus with very little migration (frequencies combined at lower interval values). In the cultures treated when vaccine concentration was higher, more DNA migrated across the gel and formed

Table II. Mean values of TL, TM and HS in control and pigs treated with 20 and 100  $\mu\text{l/ml}$  of vaccine

Dose ( $\mu\text{l/ml}$ )	TL ( $\mu$ )	TM (U)	HS ( $\mu$ )
0	2.326 $\pm$ 0.105	0.180 $\pm$ 0.012	32.758 $\pm$ 0.102
20	12.477 $\pm$ 0.516	5.522 $\pm$ 0.236	28.049 $\pm$ 0.131
100	42.313 $\pm$ 0.730	24.519 $\pm$ 0.559	26.337 $\pm$ 0.162

comets with long tails (frequencies combined at highest and intermediate interval values).

The comet assay allowed the separation of cells with different categories of DNA damage depending on TM value (22,23). For these experimental conditions, cells with  $\text{TM} < 2$  were regarded as undamaged (comet type 1); cells with  $\text{TM} \geq 2$ , were considered damaged (comet types 2, 3, 4 and 5). Within damaged cells, those having TM values  $> 30$  were classified as apoptotic (comet type 5b).

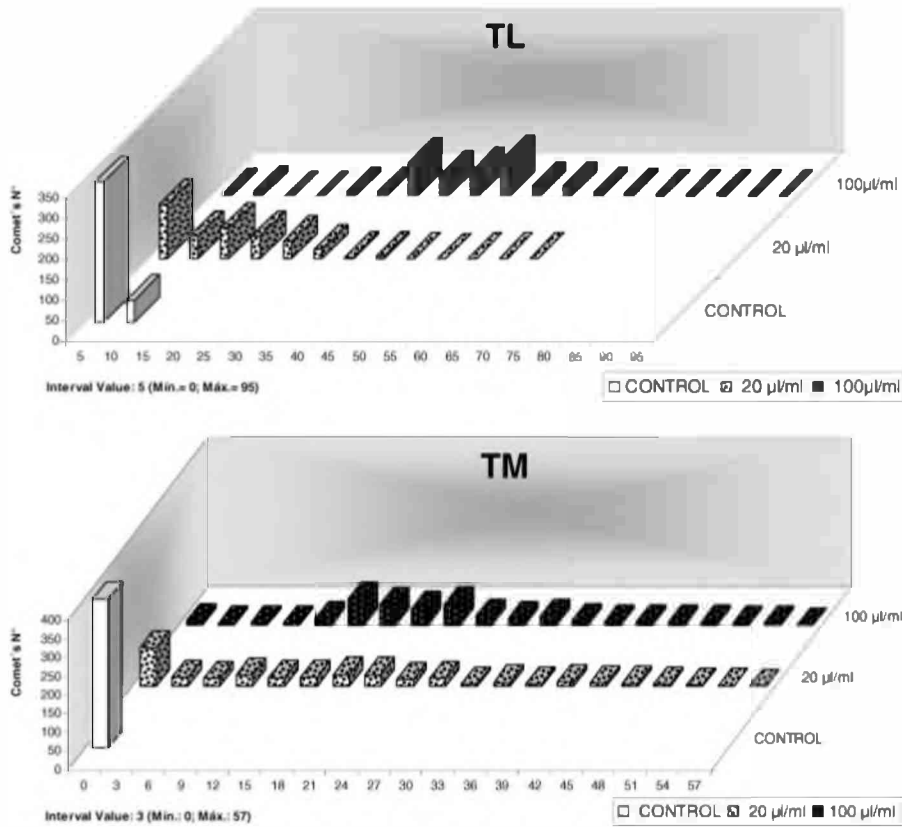
The aggregation of cells according to these criteria (Figure 6) revealed that in control cultures 100% of cells were undamaged ( $\text{TM} < 2$ ), while in those treated with 20  $\mu\text{l/ml}$  of vaccine,  $\sim 70\%$  of comets had TM values indicating damage, but not apoptosis. On the other hand, comets obtained from cultures treated with the highest vaccine dose showed 97.5% of values indicating damage, 25% of them being apoptotic.

## Discussion

The two *in vitro* genotoxicity assays carried out in this study ratify previous *in vivo* observations regarding the genotoxic effect of the live CSF virus vaccine (9–11). The drastic reduction of MI, observed by other authors (5) in CSF diseased pigs, is in agreement with 100 and 200  $\mu\text{l/ml}$  vaccine doses in our study.

At 10 h of incubation lymphocytes remained in the  $G_0$  phase, probably because of cytotoxicity since the CSF virus reduces proliferation of mitogenized cells (24–26). Only after 20 h of incubation was the cytogenetic analysis possible in the first metaphase after exposure since DNA synthesis begins  $\sim 18$  hours after mitogen stimulation. Consequently, alterations were of the chromatid type agreeing with previous studies on virus genotoxicity (27–30).

Damage in control cultures was 1.25%, being similar to the basal damage observed in *in vivo* assays (8,9), and the observed alterations were monochromatid gaps and polyploid cells that



**Fig. 5.** Comparative histograms of distribution of comets in cultures exposed to 20 and 100 µl/ml of vaccine.

are normally found in any lymphocyte culture. With the smallest concentration (5 µl/ml) the frequency of cells with alterations increased to 2.3%. This value was not statistically different from control average; however, it might have biological importance, because serious types of chromosomal alterations, such as ‘Mu’ and ‘Pu’, were induced at such a low concentration by the vaccinal virus.

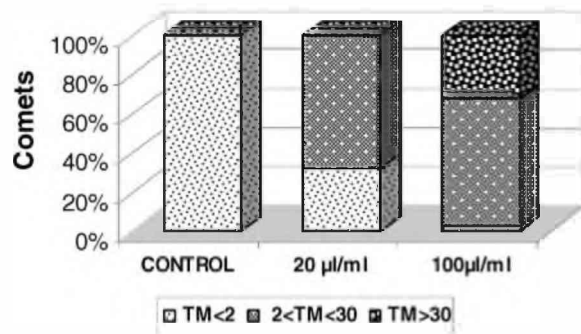
Polyploid cells were present in low and similar frequencies in all concentrations. This might have been due to elimination of polyploid cells as happens in cancerous tissue (31).

The lack of statistical differences among frequencies of cells with chromosome alterations induced by vaccines of three different brands was not surprising considering that all of them use the China virus, strain of known mutagenic effect (4–6).

The use of the comet assay in this study might be the first instance of the use of this test to evaluate the genotoxicity of a pig virus vaccine and was particularly useful, since individual cells are used as in the cytogenetics analysis, allowing both results to be compared.

Comet test results showed that CSF vaccine induced DNA migration, which increased with dose. With 20 µl/ml TL and TM values were ~6 and 26 times higher than that of controls and with 100 µl/ml were ~21 and 120 times higher than that of controls, respectively.

Average TM values for 0 µl/ml dose were lower than 1 unit, with 2 being the general upper limit for control cells (18,19). The presence of comet figures in control cultures is due to both the alkaline condition of the electrophoresis that can detect DNA alkali labile regions and open excision repair sites (32). With 20 and 100 µl/ml of vaccine, 76 and 97% of TM values, respectively, were >2. TM values >30 indicate apoptosis (18);



**Fig. 6.** Cluster of comets showing different degrees of DNA damage by treatment.

in our studies with 100 µl/ml vaccine, 35% of the comets had TM values >30, indicating occurrence of apoptosis. These results agree with the capacity of E<sup>ms</sup> glycoprotein of CSF virus in the induction of lymphocyte apoptosis (33). In apoptotic cells almost the entire volume of DNA migrated outside the comet head producing very protruding tails due to massive DNA migration, making them easily distinguishable (34).

Regarding CSF pathogeny, our *in vitro* assays strongly support a direct effect of CSF vaccinal virus over lymphocyte DNA, considering that the cultures were directly exposed to the vaccine and 24 h later not only chromosomal alterations induction but also direct DNA damage were observed. Considering that the complete attenuation of CSF virus requires the blockage of the glycoprotein E<sup>ms</sup> viral activity, we can conclude that the mentioned protein could stay active in the

CSF vaccines evaluated in this study and used in Argentina for many years resulting in genotoxicity. This could be revealed at the cytogenetic level regarding induction of chromosomal alterations and at a molecular level in DNA damage.

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