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Validation of Simple In vitro alkaline DNA Comet assay for detection and evaluation of Genotoxicity and Biosafety of inactivated veterinary vaccines on Peripheral blood cell lymphocytes

Abeer S. El-Maghraby [*]; Mohamed F. Azooz *;Abeer M. Anwer[**] ;Ahlam Mourad; Esraa Fouad; Alaa R.I. Morsy; Gehad A. Yousef and Nassif, S.A. *.

*Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), *Abbasia*, P.O. Box 131, Egypt, Agricultural Research Center (ARC)

**Immunopharmacology Unit, Animal Reproduction Research Institute, ARC, Egypt

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ABSTRACT

The comet assay or single-cell gel electrophoresis has developed into one of the standard techniques for determining DNA damage, with applications in genotoxicity testing, molecular epidemiology, as well as basic studies on DNA damage and repair. The Comet assay was used to evaluate the effect of veterinary vaccines on Peripheral blood cell lymphocytes under alkaline conditions, and it was discovered that the method's ability to separate cells with different classes of DNA damage based on tail moment (TM) value was possible. The aggregation and classification of cells in accordance to TM value revealed that in control cultures 100% of cells have been undamaged (TM<2), additionally on those dealt with 25 , 60 and 100ul/ml of inactivated vaccines No 1 – against Newcastle, infectious bronchitis , infectious bursal disease and turkey rhinotrachities viruses ; No 2 – against avian influenza H5N2 virus; No 3 – against avian influenza H9N2; No 4 – against Newcastle disease virus ; No 5 – against Newcastle, infectious bursal disease and Egg drop syndrome viruses ; No 6 – inactivated vaccine against Reo virus; 100% of comets had TM values <2 indicating undamaged DNA. The classification of comets, in accordance to tail DNA% revealed that, the DNA damage in the control group's cells was categorized as low (5–25%). Low levels of damage were seen in lymphocyte cell cultures (5–25%) exposed to 25, 60 and 100ul/ml concentrations of in activated vaccines No 1 – against Newcastle, infectious bronchitis, infectious bursal and turkey rhinotrachities viruses; No 2 – against avian influenza H5N2 virus; No 3 – against avian influenza H9N2; No 4 – against Newcastle disease virus; No 5 – against Newcastle, infectious bursal and Egg drop syndrome viruses; No 6 – inactivated vaccine against Reo virus. The experimental work carried out has allowed us to recommend comet assay for the evaluation of the biological safety of inactivated veterinary vaccine preparations.

Corresponding author: Abeer S. El-Maghraby, Department of Bacterial Large Animal Vaccines (BLAVD), Central Laboratory for Evaluation of Veterinary Biologics (CLEVB), (ARC).
E-mail address: Abeer.maghraby17774@gmail.com
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INTRODUCTION

Inactivated veterinary vaccines are produced by killing the pathogens with chemical agents, heat, or radiation **Elena et al. 2012**. The inactivation using various chemical substances eventually leaves residues in the final products, some of which are hazardous and induce allergic reactions. Genotoxicity is a relatively new specialization that lies at the intersection of toxicology and genetics, which is thus the name toxicological genetics.

Comet assay was created by Östling and Johanson in 1984 and refined by **Singh et al. (1988)**, who ascribed increased sensitivity to the procedure by using alkaline solution and was shown to be useful in cytogenetic studies (**Flores et al. 2008**).

Azqueta et al. (2019 and **2020)** reported that the alkaline comet assay is a technically simple, sensitive assay to detect DNA damage (strand breaks and other lesions that are converted into strand breaks under alkaline conditions) and DNA repair activity. In the Comet assay, a damaged cell takes on the appearance of a comet, with head and tail regions.

The main benefit of the comet assay is a test for the detection of DNA damage and repair activity, is that it can be used to measure the migration of DNA in agarose gels as a result of the relaxation caused by strand breaks in an alkaline environment **Møller, et al. (2020)**. Image analysis software offers several geometric and concentration measurement parameters to calculate the amounts of DNA in the head and tail regions and the extent of migration to the tail region. Given that the damage caused by the aforementioned comet can be seen clearly, it is recommended that DNA break frequencies be expressed as a percentage of tail DNA. However, according to Møller, *et al.,* 2020, many academics continue to support the use of tail moments. The two descriptors, according to **Azqueta et al. (2019a & b)** are similarly affected by assay conditions. As the length and density of the tail reflect the number of single-strand breaks in the DNA, **Srivastava** and **Singh (2020)** and **Sumiyantoro, et al. (2020)** claim that the proportion of DNA in the tail offers a quantitative measure of DNA damage.

Advantages of the Comet assay over other genotoxicity assays such as chromosomal aberrations, sister chromatid exchanges, alkaline elution, and micronucleus assays include its demonstrated sensitivity to detect low levels of DNA damage (one per 1010 Da DNA break) requiring a small cell area (~ 10,000) per sample, allows flexible use of proliferating and non-proliferating cells, is inexpensive, easy to apply, and requires a short time to complete studies (**Milic et al. 2021).**

Therefore, the main objective of this study was to determine the genotoxic activity of activated veterinary vaccines on peripheral blood cell lymphocytes by an in vitro assay using alkaline DNA comet (single cell gel electrophoresis).

MATERIALS And METHODS

Vaccine preparations of various producers, well-known in veterinary practice, were also used below there is a list of studies in activated veterinary vaccines:
No 1: against Newcastle, infectious bronchitis, infectious bursal and turkey rhinotrachities viruses ; No 2 : against avian influenza H5N2 virus; No 3 : against avian influenza H9N2; No 4 : against Newcastle disease virus ; No 5 : against Newcastle, infectious bursal and Egg drop syndrome viruses ; No 6 : inactivated vaccine against Reo virus;

The genotoxic effect of veterinary vaccines on lymphocytes was estimated using comet assay and DNA damage was determined according to procedures described by **Singh et al. (1988).**

20 mL of peripheral blood was collected from 10 chickens by venous puncture of the jugular vein, using a vacuum tube containing potassium heparin. Separation of lymphocyte by centrifugation through a ficoll-hypaque at 400 xg. Lymphocyte counts were concentrated as $1x10^6$cells/mL and cultured in 96 well tissue culture plates with 10% fetal calf serum at 37° C and for 72h at 5% CO2. Vaccines were added to the cultures at the final concentrations of

100, 60 and 25 µL /ml (MTD), after 20 h of incubation and 24 h. The cells were washed with 2.0 ml of sterile PBS and centrifuged at 1120G, and the supernatant was discarded. The pellet was homogenized with 200 µL of PBS.

10 µL of cell sediment was mixed with 90µL of low melting point agarose (0.7% in PBS) at 37ºC. The final volume of 100 µL of the suspension cell was aspirated using a pipette and transferred to a fully frosted microscope slide coated with 1.5% normal melting point agarose. The slides were covered with a coverslip and incubated at 4°C for 20 minutes to solidify the agarose. After this, the coverslip was carefully removed. the slides were placed into a flask containing cold lyses buffer (2.5 mol/L NaCl, 100 mol/L Na2EDTA, 10 mol/L Tris, (pH 10) with freshly added 1% Triton X-100 and 10% DMSO for 2 h at 4ºC. After draining the lysis solution, the slide was rinsed with distilled water for 15 min. After repeated washes with distilled water twice, the slide was placed in a horizontal electrophoresis tank containing freshly made buffer (300 mol/L NaOH, 1 mol/L Na2EDTA (pH>13) for 15 min at 4ºC. Electrophoresis was carried out in the same buffer for 30 min by applying an electric field of 25 V (0.8 V/cm) and adjusting the current to 300 mA by slowly changing the buffer level in the tray. The electrophoretic vat was coated with ice sheets to ensure that the material remained at 4ºC during the experiment. After electrophoresis, the slide was rinsed gently with (0.4 mol/L Tris (pH 7.5) for 5 min. This step was again repeated. Then the slide was dried at room temperature and kept in a refrigerator in a sealed container until analysis. Duplicate slides were prepared for all the samples. The slides were immersed in distilled water for 30 min, and stained with (50µL of ethidium bromide (2mg/mL). All of these steps were carried out under dimmed light to prevent additional DNA damage caused by visible light. A total of 50 randomly selected cells for each slide were analyzed. Imaging was performed with a fluorescence microscope (Zeiss Axiovert L410 Inc., Jena Germany), connected to a digital camera (Olympus Inc., Tokyo, Japan), and equipped with a 549 nm excitation filter, and a 100-W mercury lamp. The percentage of DNA in the comet tail (DNA damage) was automatically calculated using a 'Toolbox' from the IN cell Investigator analysis package (GE Healthcare Life Sciences), Tail moment was calculated as a product of tail length multiplied by tail % damage.

The comet assay allowed the separation of cells with different categories of DNA damage depending on tail moment (TM) value (**Piperakis et al. 1999, Abd-allah et al. 1999 and Genghini et al. 2006)**. TM appeared to be the most statistically significant measurement for analyzing comet results **(Kumaravel and Jha 2006).** Cells with TM<2 were regarded as undamaged (comet type 1); cells with TM >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 cells were classified into five categories based on tail DNA (%): undamaged (5%), low damage (5–25%), moderate damage (26–45%), high damage (46–80%) and extreme damage (>80%) (**Nan, et al. 2016, Srivastava and Singh 2020)**

Statistical Analysis

The results were expressed as mean ± standard error of means. The data were statistically analyzed using (ANOVA ONE WAY) the program statistical package for social sciences (SPSS) version 17. The data means were considered different at $p < 0.05$.

RESULTS

As shown in Table 1, for these experimental conditions, and according to the classification of comets, according to tail moment (TM) value. In control cultures, 100% of cells were undamaged category (TM<2) (fig 1) , and also in those treated with 25 , 60 and 100ul/ml concentrations of in activated vaccines No 1 : against Newcastle, infectious bronchitis , infectious bursal and turkey rhinotracheitis viruses; No 2 : against avian influenza H5N2 virus; No 3 : against avian influenza H9N2; No 4 – against Newcastle disease virus ; No 5 : against Newcastle, infectious bursal and Egg drop syndrome viruses: No 6 : inactivated vac-

cine against Reo virus; 100% of comets had TM values<2 indicating the undamaged category.

As shown in table 1 according to the classification of comets, according to tail DNA%. The DNA damage in cells of the control group was considered a low damaged category (5-25%). It was observed low damaged lymphocytes cell cultures (5-25%) (fig 2) exposed to 25, 60 and 100ul/ml concentrations of inactivated vaccines No 1 : against Newcastle, infectious bronchitis, infectious bursal and turkey rhinotrachities viruses; No 2: against avian influenza H5N2 virus; No 3: against avian influenza H9N2; No 4: against Newcastle disease virus; No 5: against Newcastle, infectious bursal and Egg drop syndrome viruses; No 6: inactivated vaccine against Reo virus.

DISCUSSION

We have obtained the characteristics of DNA-degrading activity of both veterinary vaccines and control samples using DNA Comet assay for determination of their genotoxic properties in lymphocytes cultures. The genotoxic effects of activated veterinary vaccines on lymphocytes blood cultures were performed using single cell gel electrophoresis test with three scales (tail length, tail DNA% and olive tail moment). According to the classification of comets and according to the tail moment (TM) value. In control cultures, 100% of cells were undamaged category (TM<2) (fig 1), and also in those treated with 25, 60 and 100ul/ml concentrations of in activated vaccines No 1 : against Newcastle, infectious bronchitis , infectious bursal and turkey rhinotrachities viruses; No 2 : against avian influenza H5N2 virus; No 3 : against avian influenza H9N2; No 4 – against Newcastle disease virus ; No 5 : against Newcastle, infectious bursal and Egg drop syndrome viruses : No 6 : inactivated vaccine against Reo virus; 100% of comets had TM values<2 indicating undamaged category.

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The present study resulted in new important scientific advances of knowledge and possible paradigm shift as it deals with the use of the Comet test to assess the effect of veterinary vaccines on the genetic material and its effect, in particular, on Peripheral blood cell lymphocytes .The study itself is considered a new addition in terms of shedding light clearly on the level of biosafety of veterinary vaccines. The study indicates the possibility of using the Comet test to neutralize the safety level of veterinary vaccines, which may lead to a new addition to ensuring the quality and evaluation of veterinary vaccines, as the study contributes to the issuance of veterinary vaccines, high safety, and does not affect the genetic material of the animal, which is reflected positively on the general health of human being.

CONCLUSION

The method of alkaline gel electrophoresis of single cells (Comet assay) is suitable for determining of Genotoxicity of inactivated veterinary vaccines on Peripheral blood cell lymphocyte

Table 1. Comet analysis of DNA breakage of Peripheral blood cell lymphocytes cells exposed to inactivated veterinary vaccines.

	% damage	**Tail length**	**% DNA in tail**	**Tail moment**
Control	3.66±0.176	6.19±0.40	11.86±1.15	0.654±0.09
		1st vacc		
1st conc	6.03±0.26 c	4.17±0.37 c	11.59±0.21 b	0.455±0.05 b
2nd con	6.76±0.145 b	7.96±0.23a	20.36±1.88a	1.32±0.24a
3rd conc	9.16±0.176a	7.47±0.14a	13.1±0.173 b	1.001±0.04ab
		2nd vacc		
1st conc	26.8±0.44a	8.77±0.81a	19.31±1.48a	1.64±0.38a
2nd con	11.4±0.30 b	5.93±0.485a	13.49±1.2 b	1.20±0.3a
3rd conc	7.36±0.31 c	7.57±0.94a	16.47±0.50ab	1.35±0.20a
		3rd vacc		
1st conc	6.6±0.23 b	8.93±0.45a	16.63±0.17a	1.52±0.04a
2nd con	7.76±0.14a	5.58±0.953 b	17.67±0.67a	1.10±0.11 b
3rd conc	6.13±0.18 b	5.54±0.446 b	16.2±0.27a	0.817±0.016 c
		4th vacc.		
1st conc	11.73±0.14a	6.39±0.745a	15.5±1.46a	1.15±0.29a
2nd con	11.56±0.42a	8.14±0.763a	13.51±1.35a	0.632±0.18a
3rd conc	11.34±0.33a	8.01±0.924a	12.42±0.562a	0.727±0.13a
		5th vacc		
1st conc	8.86±0.20 b	7.48±0.820a	16.7±1.32a	0.824±0.12a
2nd con	8.76±0.14 b	8.97±0.820a	11.05±1.53 b	1.01±0.30a
3rd conc	22.4±0.29a	8.05±0.446a	15.05±0.529ab	1.13±0.017a
		6th vacc		
1st conc	8.03±0.29 b	7.74±0.446a	17.7±0.419a	1.37±0.05a
2nd con	9.63±0.20a	7.95±1.73a	15.55±0.02a	1.33±0.109a
3rd conc	9.96±0.29a	8.87±0.387a	10.02±1.02 b	0.867±0.045 b

Results are represented as mean ±SE. a, significant to control ($p<0.05$).

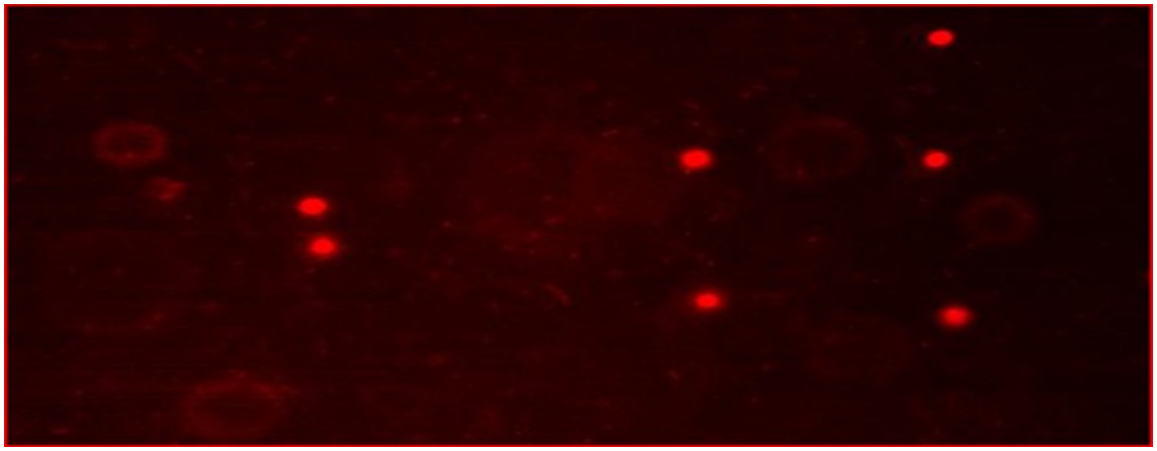

Fig 1. Electrophoreses images of DNA of negative control lymphocyte cells, not treated with genotoxicants Normal cell results: DNA is tightly compressed and maintained the circular disposition of normal nucleus

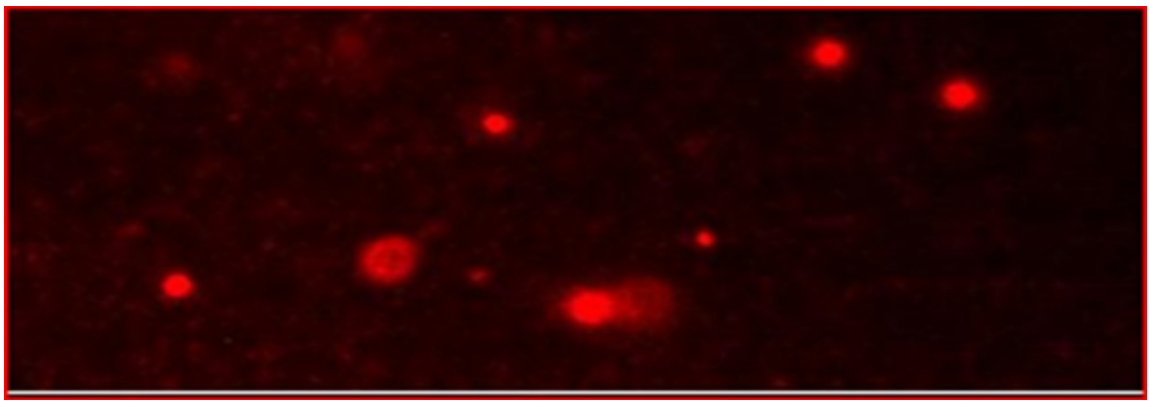

Fig 2. Electrophoreses images of DNA of the Positive Cell Results: The increase in DNA damage was mostly evidenced by an increase of comet tail

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