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RESEARCH

# Preclinical toxicity of Cuban pneumococcal conjugate vaccine candidate PCV7-TT

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**ABSTRACT**

*Streptococcus pneumoniae* is the principal causative agent of bacterial pneumonia, otitis media, meningitis as well as septicemia in children and elderly people. The World Health Organization considers this bacterium a significant health problem in many countries. *Pneumococci* comprise highly adapted commensals, their main reservoir on the mucosal surface of upper airways of carriers which enables transmission. Therefore, vaccines confer important protection. In this line, a new heptavalent conjugate vaccine against pneumococcal disease (PCV7-TT) has been developed. In this work, a repeated dose and local tolerance tests were performed in Sprague Dawley rats, as part of preclinical toxicity studies. Animals received four vaccine doses by intramuscular route at three concentration levels. They were tested for 43 days with dose intervals of 14 days each. Clinical observation, body weight, temperature, hematology, serum chemistry and pathological studies were designed. No clinical symptoms or deaths were recorded. Only slight swelling and hardening were observed at the immunization site. Body weight, temperature, hematology, serum chemistry were not changed. Similarly, there were no pathological alterations detected in organs, only a local response observed as a chronic inflammatory reaction similar to others vaccines having aluminum as adjuvant on its composition. Consequently, the PCV7-TT candidate vaccine is potentially safe and tolerable for human.

*Keywords:* Pneumococcal vaccine, combination vaccines, repeated dose, local tolerance test, Sprague Dawley rats

**RESUMEN**

**Toxicidad preclínica del candidato vacunal conjugado anti-pneumocóccico PCV7-TT cubano.** *Streptococcus pneumoniae* es el agente causal principal de neumonía bacteriana, otitis media, meningitis y septicemia en niños y ancianos. La Organización Mundial de la Salud considera a esta bacteria como un problema de salud significativo para varios países. Los pneumococos incluyen a una serie de bacterias comensales altamente adaptadas, cuyo reservorio principal es la superficie mucosal de las vías respiratorias superiores de los portadores, lo cual facilita su transmisión. Por lo tanto, las vacunas confieren una importante protección. Por tales razones, se ha desarrollado el nuevo candidato vacunal conjugado heptavalente PCV7-TT contra la enfermedad pneumocóccica. En este trabajo, se realizó una prueba de dosis repetida y tolerancia local al PCV7-TT en ratas Sprague Dawley, como parte de los estudios de toxicidad preclínica. Los animales recibieron cuatro dosis de la vacuna por la vía intramuscular a tres niveles de concentración. Se les evaluó durante 43 días a intervalos de 14 días, y se les realizó la observación clínica, y se les determinó el peso corporal, la temperatura, los parámetros hematológicos, la química sanguínea y los estudios patológicos. No se observaron síntomas clínicos  ni ocurrió la muerte de los animales ensayados. Solo hubo una ligera inflamación y endurecimiento en el sitio de la inmunización. Tampoco hubo cambios significativos en el peso corporal, la temperatura, la hematología ni en la química sanguínea. No se observó cambios patológicos en los órganos; solo hubo una respuesta local en forma de reacción inflamatoria crónica similar a la de otras vacunas que incluyen en su composición a sales de aluminio como adyuvante. En consecuencia, el candidato vacunal PCV-TT fue potencialmente seguro y tolerable para su posible administración en humanos.

*Palabras clave:* Vacuna anti-pneumocóccica, vacunas combinadas, dosis repetida, prueba de tolerancia local, ratas Sprague Dawley.

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## Introduction

Pneumococcal diseases remain as a serious health problem despite the effective vaccination used for more than a decade. Worldwide, an estimated 14.5 million episodes of serious pneumococcal diseases occur each year in children under five years old, resulting in more than 500 000 deaths, most of them in

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lower and middle-income countries [1, 2]. Two novel pneumococcal conjugate vaccines (PCV) are currently available for immunization against pneumococci in infants: Synflorix from GlaxoSmithKline (10-valent vaccine) and Prevnar-13 from Pfizer (13-valent vaccine). Several developing countries have introduced PCV thanks to big efforts done by World Health Organization (WHO) and GAVI alliance, but at the end of 2012 only 44 % of WHO member states had introduced PCV into national immunization programs [2, 3].

In this setting, pneumococcal vaccination was first introduced in Cuba in 2014. At the same time, a heptavalent conjugate vaccine (PCV7-TT, QuimiVio®) has been developed during the last 8 years, aimed for its future introduction as part of the National Immunization Program. PCV7-TT is a conjugate vaccine and contains the seven most prevalent pneumococcal serotypes in children below six years, which account for nearly 70 % of all isolated serotypes in the Americas and others regions [4, 5]: 1, 5, 6B, 14, 18C, 19F and 23F. PCV7-TT contains 2 µg of each serotype, except for serotype 6B with 4 µg, all of them conjugated to tetanus toxoid (TT) and containing aluminum phosphate as adjuvant. However, as part of the preclinical research of this vaccine candidate, it is mandatory to demonstrate the safety of the vaccine through toxicological studies. Therefore, we designed an assay of repeated dose toxicity in Sprague Dawley (SD) rats.

## Materials and methods

### Animal ethics

The study was conducted at the Center of Experimental Toxicology of the National Center of Laboratory Animals Breeding Production (CETEX/CENPALAB; Habana, Cuba). Protocols were approved by CENPALAB's Institutional Animal Care and Use Committee (CICUAL). They were conducted under Good Laboratory Practice (GLP) standards and complying with animal vaccination guidelines as recommended by WHO, the European Medicine Agency (EMA) and the Food and Drug Administration (FDA) [6, 8]. Animals were euthanized by Thiopental sodium overdose administrations with exsanguinations, and animal endpoint was performed according to the recommendations by Morton et al. and the Canadian Council on Animal Care [9-11].

### Vaccine composition and placebo

PCV7-TT (QuimiVio®) vaccine formulation (Batch Neu-12.02, Manufacturer: Finlay Institute of Vaccines, Havana, Cuba) contains 2 µg of capsular polysaccharide from *S. pneumoniae* serotypes 1, 5, 14, 18C, 19F, 23F and 4 µg of serotype 6B, each conjugated to TT, plus 125 µg aluminum phosphate and 0.058 mg Thiomersal. The vaccine is presented as a sterile suspension for injection in a single-dose vial; one human dose is contained in 0.5 mL. Placebo formulation (Batch NEU.PLA.02, Manufacturer: Finlay Institute of Vaccines, Havana, Cuba) was obtained containing the same composition of Thiomersal, Aluminum Phosphate and, additionally, Sodium Chloride 0.9 %. Vaccine and placebo compositions were stored stable between 2 and 8 °C until use.

### Animal housing and handling

Seven- to eight-week-old male and female, specific pathogen-free (SPF) Sprague-Dawley (Cenp: SPRD) rats were used from CENPALAB. Animals were in acclimation for seven days and further examined for suitability before starting the assay. The rats were housed in groups of five animals per plastic cage (TECNIPLAST, Type IV, 595×380×200 mm floor area 1820 cm2) in a room under controlled temperature 22 ± 3 °C, relative humidity 50 ± 20 %, and a 12 h light/dark cycle. Food and water were provided ad libitum, except the days preceding blood samples for clinical laboratory, in which the animals were subjected to an overnight fast on four hours previously. Animals were fed with sterile AlyCo® as provided by Cenpalab (Havana, Cuba).

### Immunization schedule

Animals were randomized using random numbers generated with the LABTOOLS software [12] and assigned to either four experimental groups (40 rats each, 20 male and 20 females): Group l: Placebo (control group); Group 2: PCV7-TT Low Dose (20 % human dose; 3.2 µg), Group 3: PCV7-TT Median dose (40 % human dose; 6.4 µg), Group 4: PCV7-TT High dose (human dose; 16 µg). The test substance was administered in the caudal thigh distributed in both hind limbs using a 26 G × ½ needle in the volume recommended to be used for the species tested [13]. Each animal received four intramuscular immunizations every 14 days in the morning, and they were sacrificed on day 43 at the end of the study.

### Clinical observations, mortality and body weight

All animals were observed for clinical signs by toxicity effects and mortality every day. Behavior of breathing patterns breath, alopecia, urination, defecation, lacrimation, eyelids ptosis, ataxia, piloerection, prostration, involuntary movements or any other signs after vaccination. The inoculation site was closely inspected searching for any change. Body weight was determined when the rats were received, grouped, before injection and every week, with the aid of digital balance (Sartorius®, Germany)..

### Haematological analysis and serum chemistry

At the beginning and at the end of in-life phase, blood samples were taken from the orbital sinus following anesthesia with Halothane (Piramal Health care, India). Hematology analyses were done by mixing bllod samples with ethylenediamine-tetraacetic acid (EDTA) and subsequently assayed using a Hematological Automatic Analyzer (Micros ABX, Roche Diagnostic Systems) for the following parameters: hemoglobin concentration (HGB), erythrocyte (ERI), hematocrit (HCT), medium corpuscular nombre incompleto (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), leucocytes (LEU), neutrophils (N), lymphocytes (L), monocytes (M) and eosinophils (E).

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Serum chemistry parameters (concentration) were analyzed using using a Hitachi 704 Clinical Chemistry Analyzer (Boehringer Mannheim Gmbh) and without EDTA, including: alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin (ALB), total protein (TP), glucose (GLUC), nitrogen urea (BUN), uric acid (UA), cholesterol (CHOLt-T), triglycerides (TG), creatinine (CREA), direct bilirubin (Bil/T), gamma glutamic transpeptidasa (GGT), Calcium (Ca), phosphor (Phos) and albumin/globulin ratio (A/G).

### Gross necropsy, organs weight and histopathology

A complete necropsy and histopathology analysis was conducted in all animals from each groups and sex. The brain, thymus, heart, lungs, liver, spleen, kidneys, adrenals, testes, and ovaries were taken and weighed following euthanasia to evaluated relative organs weight [6, 14]. Representative samples of the tissues were collected and preserved in neutral, phosphate-buffered 4 % formalin, then paraffin embedded tissues were sectioned and stained with hematoxylin and eosin (HE), in accordance with the WHO guidelines [6] and examined by a pathologist. A especial observation was performed at the inoculation site to evaluate the local tolerance of vaccine. Organ weight was determined by comparing experimental group data against data of the control group for each organ in the working range: control group mean ± 2 standard deviations for each organ [15, 16]. Historical records for the species were also considered.

### Statistical analysis

All data were entered into a database using the SPSS 11.5.1[17]. The animals' variables subjected to statistical interpretation were: body weight, hematological, serum chemistry and histopathological findings. Data was expressed as central tendency values with dispersion (means plus/ less standard deviation, lower and upper values). Statistical differences were set for $p \leq 0.05$. Normality assumptions (Kolmogorov-Smirnov) and homogeneity of variances (Levene test) were verified for each sex. When satisfied, a parametric analysis of variance (ANOVA) was applied. If they did not meet these criteria, the nonparametric alternative was used (Kruskal Wallis test). When necessary, paired comparisons were made in consecutive time intervals, using the paired t-test or the Wilcoxon test depending on the fulfillment of the approximation assumption for a normal distribution. For those cases showing global differences between groups, the LSD multiple comparison test or the Dunn's test were applied, according to compliance with distributional assumptions. Data resulting from the histopathological study were analyzed through the construction of the cross-classification tables, with the associated independence test (Fisher's exact test), indicating the statistical significance on each case.

## Results and discussion

The rat species is the animal model of choice for nonclinical vaccine toxicity evaluations, and the Sprague Dawley strain has been widely used due to its high sensitivity [14, 18-21]. That is why it was selected to test the toxicity of the PCV7-TT vaccine. All groups with the vaccine formulation get antibody titers higher to 3200 as statistical average and having into account the previously immune response saw in other animals models (manuscript in preparation). Moreover, repeat toxicity studies is one of the most complete evaluations although not the only one, while providing a wide spectrum of parameters to evaluate.

### Clinical observation, mortality and body weight

During the study, no clinical signs were shown and not any animal died. All the animals gained weight during the 43 days post-inoculation (Figure 1), with no significant differences detected between treatments as compared to the control group ($p \geq 0.05$).

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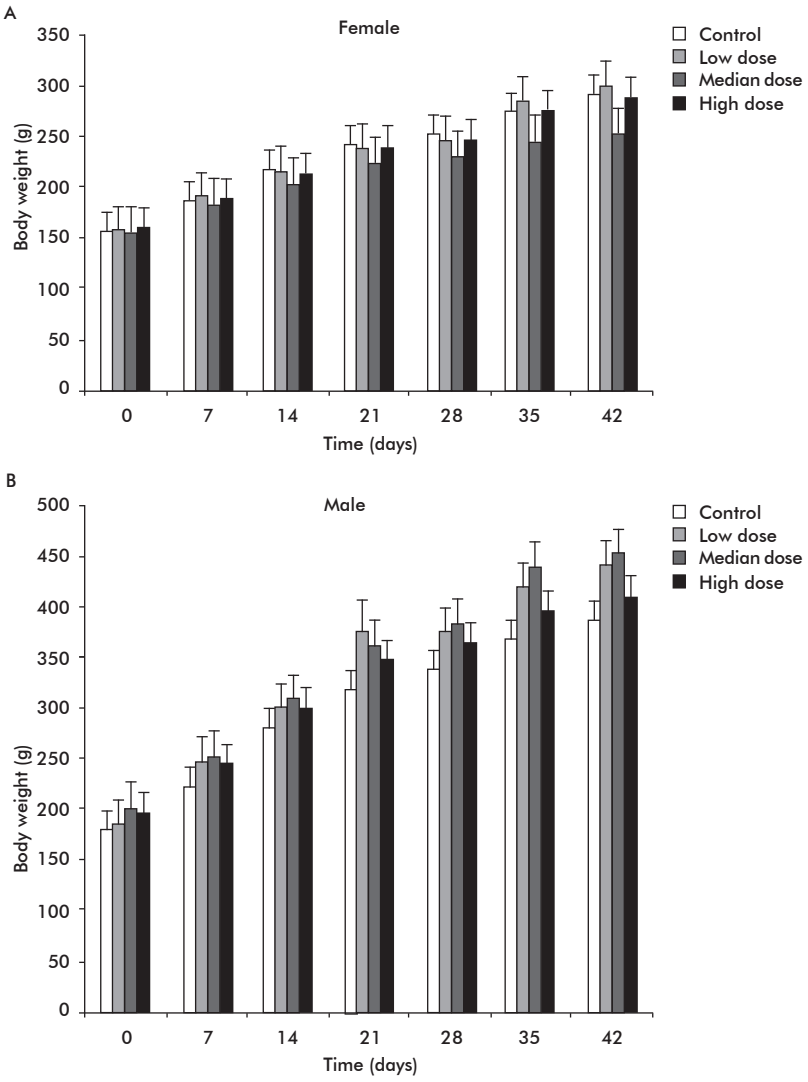


Figure 1. Performance of body weight in Sprague Dawley rats immunized either with PBS (control group) or Low dose (0.1 mL), Median dose (0.2 mL) or High dose of PCV7-TT vaccine. A) Female rats. B) Male rats. Each value stands for the mean ± SEM of the animals. Statistically significant differences were analyzed by the Kruskal-Wallis test ($p \leq 0.05$).

Table 1. Hematological studies of rats immunized either with control group, Low Dose, Media Dose and High Dose of PCV7-TT vaccine

| Female | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Group | HGB (g/dL) | ERI ($10^6$/mm$^3$) | HTC (%) | MCV (fL) | MCH (pg) | MCHC (g/dL) | PLT ($10^3$/mm$^3$) | LEU ($10^6$/mm$^3$) | N (%) | L (%) | M (%) | E (%) |
| Control | 15.52±1.2 | 7.99±0.7 | 47.2±4.0 | 59.0±1.2 | 19.4±0.4 | 32.9±0.4 | 684.4±149.8 | 9.64±1.98 | 17.4±5.2 | 82.2±5.0 | 0.20±0.4a | 0.20±0.4 |
| Low dose | 16.00±0.8 | 8.10±0.5 | 48.4±3.2 | 59.8±2.2 | 19.8±0.9 | 33.0±0.6 | 660.0±173.2 | 8.26±1.7 | 12.4±3.7 | 87.4±3.5 | 0.20±0.4a | 0±0 |
| Median dose | 15.80±0.4 | 7.92±0.3 | 47.3±1.4 | 59.7±1.2 | 19.9±0.4 | 33.3±0.5 | 703.0±64.0 | 10.4±1.1 | 12.0±5.3 | 85.5±7.2 | 2.00±1.4b | 0.50±0.5 |
| High dose | 15.10±0.5 | 7.88±0.3 | 46.4±1.1 | 59.0±1.5 | 19.1±0.4 | 32.4±0.4 | 742.8±138.5 | 8.12±1.0 | 17.6±8.1 | 82.0±7.4 | 0.20±0.4a | 0.20±0.4 |
| p | 0.2599 | 0.7530 | 0.4955 | 0.7996 | 0.2820 | 0.1069 | 0.8889 | 0.0516 | 0.4045 | 0.3774 | 0.0189 | 0.3647 |
| Male | | | | | | | | | | | | |
| Group | HGB (g/dL) | ERI ($10^6$/mm$^3$) | HTC (%) | MCV (fL) | MCH (pg) | MCHC (g/dL) | PLT ($10^3$/mm$^3$) | LEU ($10^6$/mm$^3$) | N (%) | L (%) | M (%) | E (%) |
| Control | 16.7±1.1 | 8.92±0.6 | 52.1±3.4 | 58.4±2.0 | 18.6±0.5 | 31.9±0.2 | 774.2±63.5a | 11.6±3.6 | 16.6±6.2 | 82.0±7.1 | 1.00±1.2 | 0±0 |
| Low dose | 16.3±0.4 | 8.92±0.4 | 51.2±1.8 | 57.4±1.1 | 18.3±0.5 | 31.8±0.4 | 784.0±65.5a | 10.4±1.4 | 13.2±2.6 | 86.0±2.2 | 0.40±0.5 | 0.40±0.5 |
| Median dose | 16.2±0.9 | 8.76±0.4 | 50.0±3.6 | 57.0±1.0 | 18.5±0.2 | 32.4±0.5 | 619.4±69.0b | 16.8±7.2 | 32.6±26.9 | 65.8±28.6 | 1.60±1.8 | 0±0 |
| High dose | 16.2±0.5 | 8.82±0.2 | 50.7±2.1 | 57.4±0.8 | 18.4±0.2 | 32.1±0.5 | 809.2±42.4a | 10.5±2.9 | 14.0±6.6 | 85.0±7.0 | 0.40±0.8 | 0.60±0.5 |
| p | 0.8088 | 0.9356 | 0.7063 | 0.6802 | 0.6298 | 0.3701 | 0.0006 | 0.1020 | 0.1497 | 0.1676 | 0.4800 | 0.0772 |

* Values represent the mean ± SEM of five animals in each group of treatment. Statistically significant differences were analyzed by the Kruskal-Wallis test ($p \leq 0.05$; highlighted). Different letters stand for statistical differences between groups. HGB - hemoglobin concentration, ERI- erythrocyte, HCT- hematocrit MCV - medium corpuscular, MCH- mean corpuscular hemoglobin, MCHC- mean corpuscular hemoglobin concentration, PLT- platelet count, LEU- leucocytes, N- neutrophils, L- lymphocytes, M- monocytes and E- eosinophils.

Table 2. Blood chemistry analysis of rats immunized either with control group, Low Dose, Media Dose and High Dose of PCV7-TT vaccine

| Female | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Group | ALP (U/L) | AST (U/L) | ALT (U/L) | ALB (g/L) | TP (g/L) | GLUC (mmol/L) | BUN (mmol/L) | UA (mg/dL) |
| Control | 84.6 ±18.9 | 133.0 ± 35.6a | 44.2 ± 3.11 | 51.6 ± 4.83 | 74.7 ± 5.08 | 5.69 ± 1.14 | 6.26 ± 0.71 | 104.6 ± 23.6 |
| Low dose | 82.0 ± 13.3 | 115.2 ± 4.32a | 44.2 ± 5.58 | 50.4 ± 2.22 | 75.6 ± 2.42 | 5.69 ± 1.08 | 6.54 ± 0.74 | 176.4 ± 47.1 |
| Median dose | 80.7 ± 18.2 | 216.7 ± 53.7b | 54.5 ± 6.55 | 47.1 ± 5.44 | 72.6 ± 5.88 | 5.69 ± 2.05 | 6.42 ± 0.67 | 137.2 ± 39.5 |
| High dose | 69.2 ± 11.2 | 128.2 ± 14.0 a | 45.8 ± 5.40 | 48.2 ± 2.64 | 73.0 ± 2.40 | 5.28 ± 0.72 | 7.10 ± 0.56 | 118.0 ± 39.1 |
| p | 0.4031 | 0.0187 | 0.0779 | 0.4591 | 0.6211 | 0.9127 | 0.3859 | 0.0553 |
| Male | | | | | | | | |
| Group | ALP (U/L) | AST (U/L) | ALT (U/L) | ALB (g/L) | TP (g/L) | GLUC (mmol/L) | BUN (mmol/L) | UA (mg/dL) |
| Control | 145.8 ± 27.6 | 161.2 ± 4.55 | 64.0 ± 9.61 | 45.7 ± 1.64 | 71.7 ± 3.66 | 4.63 ± 0.96 | 6.02 ± 0.61 | 158.2 ± 32.1a |
| Low dose | 124.0 ± 34.7 | 144.4 ± 26.3 | 60.0 ± 9.13 | 43.7 ± 2.31 | 71.0 ± 1.84 | 5.94 ± 0.35 | 6.00 ± 0.73 | 106.6 ± 35.2b |
| Median dose | 139.8 ± 21.2 | 146.2 ± 16.2 | 50.4 ± 12.1 | 43.4 ± 6.91 | 70.3 ± 5.04 | 5.95 ± 1.39 | 5.58 ± 0.26 | 136.4 ± 28.7b |
| High dose | 122.8 ± 30.7 | 134.6 ± 19.6 | 52.0 ± 10.3 | 44.0 ± 1.57 | 70.2 ± 3.51 | 6.51 ± 1.62 | 5.91 ± 0.65 | 104.6 ± 22.4b |
| p | 0.5202 | 0.1925 | 0.1614 | 0.1679 | 0.9204 | 0.1234 | 0.6231 | 0.0348 |
| Female | | | | | | | | |
| Group | CHOL-T (mmol/L) | TG (mmol/L) | CREA (µmol/L) | Bil/T (µmol/L) | GGT (U/L) | Ca (mmol/L) | Phos (mmol/L) | A/G |
| Control | 1.94 ± 0.31 | 0.53 ± 0.21 | 40.6 ± 2.70 | 2.00 ± 0 | 0.20 ± 0.44 | 3.00 ± 0.11 | 2.69 ± 0.09 | 2.24 ± 0.32 |
| Low dose | 2.39 ± 0.51 | 0.57 ± 0.12 | 45.8 ± 3.56 | 1.80 ± 0.44 | 0.60 ± 0.89 | 3.06 ± 0.22 | 3.29 ± 0.68 | 2.02 ± 0.14 |
| Median dose | 2.26 ± 0.27 | 0.44 ± 0.21 | 41.7 ± 8.65 | 1.75 ± 0.50 | 1.25 ± 0.95 | 2.90 ± 0.16 | 2.93 ± 0.46 | 1.87 ± 0.35 |
| High dose | 1.85 ± 0.37 | 0.42 ± 0.04 | 48.0 ± 6.42 | 1.60 ± 0.54 | 0.40 ± 0.54 | 2.88 ± 0.12 | 2.74 ± 0.47 | 1.96 ± 0.39 |
| p | 0.0570 | 0.2994 | 0.1475 | 0.5077 | 0.2773 | 0.3558 | 0.3094 | 0.2965 |
| Male | | | | | | | | |
| Group | CHOL-T (mmol/L) | TG (mmol/L) | CREA (µmol/L) | Bil/T (µmol/L) | GGT (U/L) | Ca (mmol/L) | Phos (mmol/L) | A/G |
| Control | 1.36±0.10 | 0.53±0.16 | 42.2±3.11 | 1.20±0.44 | 0.20±0.45 | 2.93 ± 0.09 | 3.39 ± 0.26ac | 1.76 ± 0.19 |
| Low dose | 1.45±0.09 | 0.59±0.17 | 38.8±5.71 | 1.40±0.54 | 0.40±0.89 | 2.76 ± 0.10 | 2.78 ± 0.18b | 1.62 ± 0.13 |
| Median dose | 1.56±0.26 | 0.72±0.31 | 42.2±2.77 | 1.60±0.54 | 0.20±0.45 | 2.90 ± 0.15 | 3.29 ± 0.13ac | 1.66 ± 0.39 |
| High dose | 1.40±0.25 | 0.52±0.18 | 41.2±3.70 | 1.20±0.45 | 0 ±0 | 2.78 ± 0.21 | 3.00 ± 0.26abc | 1.68 ± 0.19 |
| p | 0.4341 | 0.6405 | 0.3446 | 0.5132 | 0.7690 | 0.1875 | 0.0016 | 0.5928 |

* Values represent the mean ± SEM of the 5 animals in each group of treatments. Statistically significant differences were analyzed by the Kruskal-Wallis test ($p \leq 0.05$). Different letters mean statistical differences between groups. ALP- alkaline phosphatase, AST- aspartate aminotransferase, ALT- alanine aminotransferase, ALB- albumin, TP- total protein, GLUC- glucose, BUN- nitrogen urea, UA- uric acid, CHOLt-T- cholesterol, TG- triglycerides, CREA- creatinine, Bil/T- direct bilirubin, GGT- gamma glutamic transpeptidasa, Ca- Calcium, Phos- phosphor, A/G- albumin/globulin ratio (calculated).

Male rats gained weight faster than females ($p \leq 0.05$), and weight gain curves were in agreement with those previously observed in other experiments and reported in the scientific literature [14, 19-22].

## Haematological analysis and serum chemistry

Haematological analysis and serum chemistry parameteres were assessed (Tables 1 and 2). In gen-

eral, the red blood cells parameters assessed were within the physiological ranges described to the species and literature [23-25]. They were similar between sexes and treatment groups, didn't differ between them, while different in white blood cells. In these last series, differences were observed in only two parameters, monocytes in female rats where the difference was observed in the median dose with respect with the rest of the experimental groups, and platelet count parameter in male rats in the median dose group with respect to the other groups (Table 2).

Importantly, the difference observed did not indicate a toxic value, without physiological signification, an isolated case, with no dose-effect relationship and within the physiological range of this parameter. On the other hand, no histological changes or damage were found, in relation with animal age and sex [23, 24]. Similar differences were observed with the serum chemistry parameters assessed, where aspartate aminotransferase, uric acid and phosphorus differed between groups in the median dose too and unrelated to sex (Table 2). However, all the serum chemistry parameters were in the physiological range as reported for the animals' age, sex and species [23].

### Gross necropsy, organs weight and histopathology

Macroscopic studies performed in all organs and systems for each of the animals studied did not show any lesions that would suggest acute or chronic toxicity. However, the diagnosed macroscopic lesions identified as possible granulomatous processes at the inoculation site were further verified microscopically. It was evidenced in all vaccinated groups as nodular formation of abundance polymorphonuclear, neutrophils and macrophages (Figure 2A and 2B), the polymorphous were infiltrated in the muscular endomysium for the peripheral abscess. Very high significant differences were found as compared to the inoculation site in the control group vs. vaccines in the three dose levels ($p \leq 0.05$, Table 3). Moreover, the findings in the regional lymph nodes corresponding to the vaccine inoculation site were seen at the popliteal ganglion, such as, the presence of subcapsular and paracortical secondary follicles (Figure 2C).

These histological findings were consistent with observations in similar previous studies carried out in this species and when administering aluminum hydroxide or phosphate aluminum-containing vaccines as PCV7-TT. An inflammatory reaction at the inoculation site is characteristic of the vaccines with aluminum on its formulation, shown to efficiently potentiate the effector inflammatory responses, characterized mainly by the presence of macrophages, neutrophils and plasma cells [21, 25, 26].

Regarding the relative weight of organs (Table 4), only a significant difference was observed between heart weight of male rats, these was between the high dose with the rest of the groups. Anyway, reproducibility of the adverse event observed in one of the animals in the group with the highest dose should be studied in subsequent studies. It could be related to a supraphysiological concentration of the antigen, which is a condition quite infrequent in available vaccine formulations that could lead to a distorted biodistribution of the antigen beyond the inoculation site through the blood stream. A joint analysis of the relative weights of the organs, as well as the body weights of the animals under study, led us to conclude that this variable was not affected by the vaccine's groups, because the average is similar to other studies report [14] and as was mentioned before, organs not show any histological change or damage.

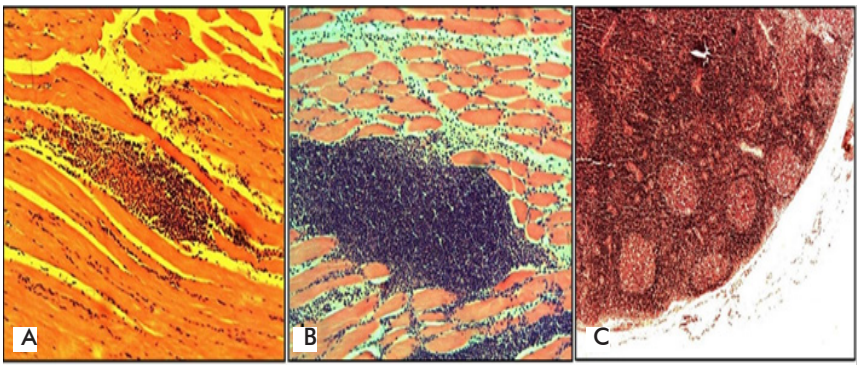


Figure 2. Histological findings related with inoculation site and immune organ from animals vaccined. a- Small nodular formation of macrophages y lymphocytes in the muscular endomysium, well delimited by fibre-muscular. b- Nodular formation of abundance polymorphonuclear, neutrophils and macrophages, infiltration of the polymorphous in the muscular endomysium at the abscess aseptic periphery. c- Popliteal ganglion showing hyperplasia with the presence of subcapsular and paracortical secondary follicles. HE stain, a 100× magnification was used.

Table 3. Frequency of histological changes associated to the immune response in SD rats of both sexes inoculated with either PCV7-TT or control group during the evaluation of repeated dose vaccine toxicity*

| | Female | |
|---|---|---|
| | Histological change/Group | |
| Group | Hyperplasia of subcapsular secondary follicles in lymph nodes | Hyperplasia of parcortical secondary follicles in lymph nodes |
| Control | 2/5 a (40 %) | 1/5 a (20 %) |
| Low dose | 4/5 b (80 %) | 5/5 b (100 %) |
| Median dose | 3/5 b (60 %) | 4/5 b (80 %) |
| High dose | 5/5 b (100 %) | 4/5 b (80 %) |
| n Total | 14/20 (70 %) | 19/20 (95 %) |
| | Male | |
| Group | Hyperplasia of subcapsular secondary follicles in lymph nodes | Hyperplasia of parcortical secondary follicles in lymph nodes |
| Control | 2/5 a (40 %) | 2/5 a (40 %) |
| Low dose | 5/5 b (100 %) | 3/5 b (60 %) |
| Median dose | 4/5 b (80 %) | 4/5 b (80 %) |
| High dose | 4/5 b (80 %) | 3/5 b (60 %) |
| n Total | 15/20 (75 %) | 12/20 (60 %) |

* n- Number of animals, % - percentage of the total of animals observed with the described alteration. Fisher's exact test ($p \leq 0.05$), different letters means differences between groups.

## Conclusions

In summary, the three levels dose of PCV7-TT vaccine showed a safety profile in SD rats. Neither local nor systemic adverse toxic effects were detected when a repeat dose of the vaccine was intramuscular administered. This indicates that PCV7-TT can be considered as potentially non-toxic and supports the clinical trials.

## Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Table 4. Relative organ weights (%) of SD rats of both sexes inoculated with either PCV7-TT or control group during the evaluation of repeated dose vaccine toxicity

| Female | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Group | Brain | Heart | Thymus | Lungs | Liver | Spleen | Kidneys | Adrenals | Ovary |
| Control | 0.731±0.07 | 0.382±0.02 | 0.152±0.03 | 0.497±0.04 | 2.933±0.24 | 0.216±0.02 | 0.723±0.06 | 0.070±0 | 0.053±0.01 |
| Low dose | 0.700±0.03 | 0.368±0.04 | 0.179±0.03 | 0.476±0.04 | 3.244±0.22 | 0.195±0.02 | 0.733±0.07 | 0.026±0 | 0.049±0.01 |
| Median dose | 0.616±0.31 | 0.384±0.01 | 0.134±0.08 | 0.425±0.21 | 2.340±1.17 | 0.180±0.09 | 0.773±0.08 | 0.027±0 | 0.049±0.01 |
| High dose | 0.711±0.05 | 0.445±0.14 | 0.154±0.03 | 0.515±0.07 | 3.067±0.20 | 0.223±0.03 | 0.722±0.05 | 0.027±0.01 | 0.055±0.01 |
| p | 0.2087 | 0.4842 | 0.4863 | 0.3604 | 0.0707 | 0.3642 | 0.7423 | 0.5332 | 0.7470 |
| Male | | | | | | | | | |
| Group | Brain | Heart | Thymus | Lungs | Liver | Spleen | Kidneys | Adrenals | Testicles |
| Control | 0.531±0.05 | 0.352±0.04a | 0.114±0.01 | 0.419±0.03 | 2.729±0.20 | 0191±0.01 | 0.710±0.06 | 0.015±0 | 0.407±0.04 |
| Low dose | 0.493±0.03 | 0.344±0.02a | 0.129±0.03 | 0.420±0.03 | 2.832±0.14 | 0.194±0.01 | 0.718±0.04 | 0.013±0 | 0.407±0.05 |
| Median dose | 0492±0.06 | 0.329±0.02a | 0.103±0.01 | 0.398±0.03 | 2.725±0.24 | 0.212±0.02 | 0.661±0.04 | 0.012±0 | 0.377±0.06 |
| High dose | 0.548±0.04 | 0.409±0.06b | 0.115±0.01 | 0.393±0.04 | 2.840±0.30 | 0.183±0.03 | 0.731±0.02 | 0.014±0 | 0.445±0.04 |
| p | 0.2088 | 0.0312 | 0.1212 | 0.5152 | 0.7642 | 0.1564 | 0.1239 | 0.1597 | 0.1963 |

* Values stand for the mean ± SEM of the 5 animals in each group. Statistically significant differences were analyzed by the Kruskal-Wallis test ($p \leq 0.05$; highlighted). Different letters mean statistical differences between groups

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