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Evaluation of a canine transmissible venereal tumour cell line with tumour immunity capacity but without tumorigenic property

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Abstract

Introduction: Canine transmissible venereal tumour (CTVT) is a sexually transmitted tumour affecting dogs worldwide, imposing a financial burden on dog owners. A stable culture cell line in continuous passages for >18 months has only been achieved once. The present study investigated a stable CTVT cell line isolated from a bitch and its potential as a vaccine. **Material and Methods:** A biopsy from a 2-year-old mongrel bitch with CTVT was obtained for histopathological confirmation and isolation of tumour cells. The isolated cells were cultured to passage 55 and characterised by flow cytometry, with karyotyping by GTG-banding and by PCR detection of myc S-2 and LINE AS1. The isolated CTVT cell line was also used as a preventive vaccine in a canine model. **Results:** Histopathological analysis of the isolated tumour cells revealed typical CTVT characteristics. Constant proliferation and stable morphological characteristics were observed during culture. Phenotypic analysis determined the expression of HLA-DR$^+$, CD5.1$^+$, CD14$^+$, CD45$^+$, CD83$^+$, CD163$^+$, and Ly-6G-Ly-6C$^+$. GTG-banding revealed a mean of 57 chromosomes in the karyotype with several complex chromosomal rearrangements. LINE-c-myc insertion in the isolated CTVT cell line at 550 bp was not detected. However, a 340-bp band was amplified. Isolated CTVT cell line inoculation at a concentration of 1×10^8 did not induce tumour growth in bitches, nor did a challenge with primary CTVT cells. **Conclusion:** The present study successfully identified and isolated a stable CTVT cell line that may be useful in CTVT prevention.

Keywords: dog, transmissible venereal tumour, stable cell line isolation, vaccine, flow cytometry.

Introduction

Canine transmissible venereal tumour (CTVT) is one of four transmissible tumours known to date; the others include Tasmanian devil facial tumour, Syrian hamster sarcoma, and soft-shelled clam haemic neoplasia. In addition, CTVT is the only globally known transmissible tumour (12, 19, 23). The tumour occurs in sexually mature animals and is transmitted during coitus. It predominantly affects the external genitalia, but other organs (nose, ears, mouth, and eyes) may also be affected (6, 26, 28). In addition, CTVT may also be transmitted to foxes, wolves, and coyotes (20, 22). This tumour contains 57–59 chromosomes, instead of the 78 that naturally occur in dog somatic cells and is transmitted by neoplastic cell implantation (27). A characteristic of CTVT cells during the transmission phase is that they express major histocompatibility complex (MHC) antigens I and II at low levels (18, 19). They also inhibit certain types of antigen-presenting cells and tumour growth factor (TGF)-β1, a cytokine which suppresses the cytotoxicity of tumour-infiltrating lymphocytes, leading to immune system evasion and host implantation (17, 18, 22). CTVT cells and their biological characteristics were first described in 1876 by Nowinsky (21) and have become a popular model for cancer biologists (19). Although CTVT has been subjected to immunohistochemical analysis for several tumour markers, including keratin, vimentin, desmin, CD3, α-smooth muscle actin, immunoglobulins G and IgM, λ-light chains, κ-light chains, lysozyme, ACM1, and

A-1-antitrypsin, its origin and immunophenotype remain unclear. The expression of certain markers suggests its histiocytic origin, but differential diagnosis should be based on clinical and histopathological criteria (17). In the present study, cell surface markers were analysed by flow cytometry to detect any alterations between CTVT cell passages.

Despite 130 years of research on CTVT, several characteristics of this transmissible cancer remain poorly understood (2, 7) and no vaccine is available yet. The literature regarding the *in vitro* culture of CTVT cells is limited. In 1951, Bloom *et al.* (3) reported the successful short-term culture of CTVT cells. Later, in 1962, Prier (25) described certain nutritional requirements for monolayer cultures and reported the morphological characteristics and chromosome pattern of the venereal tumour cells in short-term culture. The most recent article was published in 1968 by Adams *et al.* (1) who described the establishment of a stable culture cell line in continuous passages for >18 months. They identified two morphologically distinct ubiquitous cell types that are spindle cells with reticular-like elements and round cells. To the best of our knowledge, there are currently no other reports of *in vitro* cell culture and isolation of a CTVT cancer cell line. In our previous studies, primary CTVT cell cultures were used to establish the tumour *in vivo* as a model of cancer to evaluate the efficacy of autologous immunotherapy with autologous dendritic cells and cytotoxic T cells. The results demonstrated that the long-term maintenance of primary CTVT cell cultures was difficult to achieve. The aim of the present study was to establish a stable CTVT cell line isolated from a bitch and explore its potential use as a vaccine in the prevention of CTVT.

Material and Methods

Animals. A total of 17 healthy mongrel bitches (aged ~3 years and weighing 15 ± 3 kg) were obtained through the Faculty of Veterinary and Zootechnics of the Autonomous University of Nuevo León (AUNL; San Nicolás de los Garza, Mexico). The dogs were housed in climate-controlled rooms (1.2 × 2.4 m) in the bioterium of the Biological Science Faculty of the AUNL.

Experimental groups. Three bitches, vaccinated with primary CTVT cells derived from a fresh biopsy, were used as a control for the tumour growth group. To corroborate if the isolated CTVT cell line induced tumoral growth, seven bitches were vaccinated. The capacity of the isolated CTVT cell line to prevent the tumoral growth was evaluated using another seven bitches that had previously been vaccinated with the isolated CTVT cell line and then challenged with an inoculation of a primary CTVT cell derived from a fresh biopsy. The primary CTVT cells derived from a fresh biopsy were donated by the Veterinary Hospital of the Faculty of Veterinary Medicine of the AUNL. The dogs used as control for the tumour growth group were treated with vincristine (0.025 mg/kg for 3–6 weeks). At the end of the experiment, all dogs were ovariohysterectomized and adopted.

Bitches were selected for their behaviour and easier management of the tumour, due to its location in the interior of the vulva. Males were excluded, as they exhibited aggressive behaviour and the tumour cells would have needed to be implanted at the basis of the penis, making tumour visualization and management difficult.

Isolated CTVT cell line. The tumour biopsy material was donated by the Veterinary Hospital of the Faculty of Veterinary Medicine of the AUNL and obtained from the vulva of a 2-year-old mongrel bitch. CTVT was diagnosed by clinical and histopathological examination at the Veterinary Hospital. Briefly, formalin-fixed tissue samples were washed and dehydrated in graded ethanol and embedded in paraffin wax for histopathological study. Fixed tissues were sectioned at 5 µm and stained with haematoxylin and eosin for microscopic examination (27). For the established procedure and maintenance of CTVT cells, the tumour was rinsed with phosphate-buffered saline (PBS) to remove the blood and mechanically disintegrated with a Medimachine System (BD Biosciences, USA) to obtain the CTVT cells. Approximately 2×10^6 viable cells were recovered, as determined by trypan blue exclusion staining. The cells were cultured in a 75 cm^2 culture flask (Corning, USA) containing Dulbecco's modified Eagle's medium (DMEM/F-12) and 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (Gibco; Thermo Fisher Scientific, USA) with 10% foetal bovine serum (FBS) (Gibco), as well as antibiotics and antimycotics (Antibiotic-Antimycotic 100X; Gibco), at 37°C with 5% CO_2. StemPro Accutase (Gibco) was used to detach the cells from the flask.

For the CTVT cell line cryopreservation, cells were adjusted to a concentration of 2×10^6 per vial with DMEM containing 10% FBS and 10% dimethyl sulphoxide, as described in the ATCC primary cell culture guide (29). To corroborate the viability of the frozen CTVT cell line, one vial was defrosted at room temperature and the pellet was washed twice for 10 min with DMEM containing 10% FBS at 500 g. Subsequently, cells were cultured in a 75 cm^2 culture flask (Corning) and maintained under standard conditions until they attained 100% confluence. The morphology of the CTVT cell line was evaluated by inverted microscopy.

Flow cytometry. The phenotypic analysis of CTVT cell line was performed by collecting the cells with Accutase and adjusting to a cellular concentration of 5×10^4. Subsequently, antibodies (1:100 against CD5.1 (fluorescein isothiocyanate (FITC) mouse anti-mouse; BD Biosciences), CD14 (APC-Cy7 mouse anti-human; BD Biosciences), CD34 (FITC mouse anti-

human; BD Biosciences), CD45 (peridinin chlorophyll (PerCP) rat anti-mouse; BD Biosciences), CD80 (FITC mouse anti-human; BD Biosciences), CD83 (phycoerythrin (PE) mouse anti-human; BD Biosciences), CD163 (FITC anti-human; Biolegend, USA), CD4/CD8/CD3 (FITC CD4, PE CD8 and PerCP CD3 anti-human; BD Biosciences), Ly6C-Ly6G (PE rat anti-mouse; BD Biosciences), HLA-DR (PE-Cy mouse anti-human; BD Biosciences) and vimentin (PE anti-mouse; Thermo Fisher Scientific) were added to each tube separately and incubated for 30 min on ice. The cells were then washed with PBS containing 0.5% albumin, the supernatant was discarded to remove the excess antibody, and the pellet was diluted in 100 µL PBS for flow cytometry analysis which was performed with BD Accuri C6 Plus software (BD Biosciences).

Myc S-2 and LINE AS1 PCR. Primers Myc S-2 and LINE AS1 were designed to cover a 553-bp segment extending from the 5'-end of the first c-Myc exon of the LINE insertion that was previously used to identify CTVT (15). The expression of this sequence was determined in the CTVT cell line, using primers previously reported by Liao *et al.* (15) and synthesised by the Institute of Biotechnology, Unit of Synthesis and Sequencing of The National Autonomous University of Mexico (UNAM): the primer for MycS-2 was ATTCCTACGAATGAATGATTGGCCAGA and for LINE AS-1 was CAGACACATAGATCAGTG GAACAGAAT.

DNA extraction was performed using the Wizard Genomic DNA Purification kit (Promega Corporation, USA) according to the manufacturer's instructions. A PCR was performed as described previously (29). In brief, samples were processed in a Mastercycler (Eppendorf, Germany) under the following conditions: 1 cycle at 94°C for 5 min; 35 cycles at 94°C for 1 min, at 64°C for 50 sec, and at 72°C for 1 min, and a final elongation step at 72°C for 5 min. PCR products (10 µL) were subjected to gel electrophoresis (1.5% agarose gel prepared with Tris-borate-ethylenediamine tetra-acetic acid (EDTA) buffer) at 100 V for 50 min. The size of the PCR products was evaluated with a HyperLadder 100 bp DNA ladder marker (Bioline, UK). The products were stained with ethidium bromide solution (0.5 g/mL) and visualised at 260 nm with a UV illuminator (Pharmatek Scientific Systems, India).

Isolated CTVT cell line karyotype. Karyotype and GTG-banding analysis were performed by the Genos Medical Center Specialised in Genetics (Mexico City, Mexico). Briefly, CTVT cells (5×10^6) were cultured at 37°C for 72 h in 10 mL of chromosome medium 1A (Gibco). Cell cycles were arrested with colchicine (10 µg/mL) for 30 min. Chromosome preparations were made by incubating the cell suspension in 0.075 M KCl at 37°C for 13 min, followed by fixation on glass slides with a freshly prepared mixture of methanol: acetic acid (3:1) at −20°C. GTG banding was performed by incubating the glass slides in a 0.05% trypsin solution (Gibco) at 37°C for 15 sec, followed by rinsing the slides in PBS and 5% Giemsa-Wright staining for 8 min (32). The slides were rinsed with water, air dried, and visualised by microscopy with a Media Cybernetics camera and Image Pro software (Media Cybernetics, USA).

Isolated CTVT cell line inoculation. The isolated CTVT cell line cells were adjusted to a concentration of 1×10^8 in PBS (1 mL) and inoculated into the walls of the vulva of seven mongrel bitches under general anaesthesia (1–2 mg/kg (im.) of xylazine, 15–25 mg/kg (im.) of ketamine) using a syringe with a 21G × 11/4″ needle, as approved by the Animal Ethics Committee (CEIBA, Autonomous University of Nuevo Léon). The tumour growth was evaluated weekly for 1 year.

Isolated CTVT cell line vaccination and challenge in a model of CTVT. The isolated CTVT cell line was adjusted to a concentration of 1×10^8 in PBS (1 mL) and subcutaneously inoculated in seven bitches. After 21 days, the primary CTVT cells (obtained from a fresh tumour biopsy of CTVT from the Faculty of Veterinary Medicine and Zootechnics of the AUNL) were adjusted to a concentration of 1×10^8 cells in 0.5 mL PBS and injected intravulvally to evaluate the capacity of the isolated CTVT cell line to prevent tumoral growth. Also, three bitches were vaccinated using a primary CTVT cell from a fresh biopsy and used as a control for the tumour growth group. All the procedures were performed under general anaesthesia with xylazine hydrochloride (1.1 mg/kg; PISA, Mexico) and tiletamine hydrochloride/zolazepam hydrochloride (7.5 mg/kg; Virbac, Mexico).

Tumour size. The tumour size was measured weekly with calipers. Tumour volume was calculated using the following formula: length (mm) × width (mm) × height (mm) × π/4.

Statistical analysis. The results were analysed using Student's *t*-test, to compare the tumour volumes between treated groups. Student's *t*-test was performed with SPSS (SPSS Statistic Modeler 15.0, IBM, USA). $P \leq 0.05$ indicated a statistically significant difference.

Results

Morphological and histopathological analysis. The results demonstrated that the primary dog tumour had the clinical characteristics of CTVT. The size was ~165 cm^3 and consisted of several cauliflower-like multinodular masses present in the vulva, which was turgid and deformed, and extending into the vestibule of the vagina (Fig. 1). The histopathological analysis of the tumour cells revealed tight clusters or cords, composed of large round homogeneous neoplastic cells, interspersed by sections of a delicate stroma. The cells had dense eosinophilic cytoplasm. A number of vacuolated, large nuclei with anisocytosis and anisokaryosis were also present, with condensed or granular chromatin and prominent nucleoli. Mitotic

figures (2–3 per field), with large areas of haemorrhage and pyknotic cells were observed. Marked lymphocytic infiltration was observed around blood vessels at the tumour margins. The neoplasm was delimited but not encapsulated (Fig. 2).

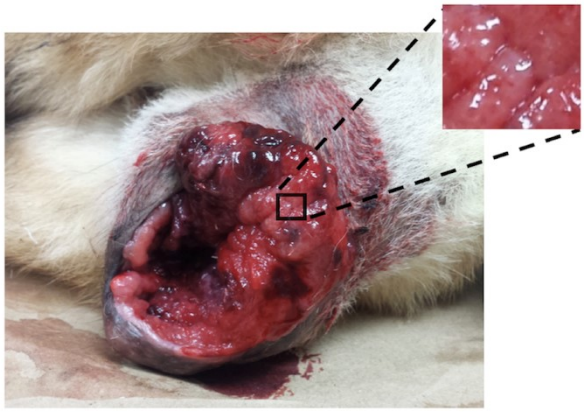

Fig. 1. Canine transmissible venereal tumour in a mongrel bitch. The tumour biopsy was collected surgically. The vagina showed multiple lesions, light roseate to deep red in colour, with areas of haemorrhage and necrosis. It was turgid at palpation with cauliflower multinodular masses. It was soft, very vascularised, and friable

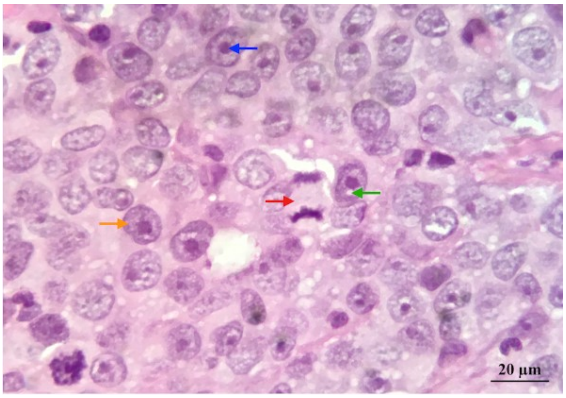

Fig. 2. Histopathology of CTVT cells shows homogeneous large round neoplastic cells that are distributed in clusters, prominent nuclei and nucleoli (blue arrow), dense eosinophilic cytoplasm (orange arrow), sometimes vacuolated (green arrow), and a mitotic figure in the centre (red arrow). Stained with haematoxylin and eosin. Visualised using a clear field microscope (100×)

Isolated CTVT cell line. Following the mechanical disintegration of CTVT cells, these were seeded into a culture flask. Round, ovoid, and clear cell morphology was observed, characteristics similar to those of the suspension cells (Fig. 3A). After 12 h of culture, ~50% of the cells began to attach to the culture flask, exhibiting fibroblast-like or spindle shapes (Fig. 3B). At 24 h, the cells were completely attached, with a fibroblast-like shape and cytoplasmic vacuoles (Figs 3C and D). Complete confluence was obtained after 96 h of culture and then the first passage was complete (Fig. 3E). The cells were maintained under standard culture conditions as mentioned previously, and 55 passages were performed. Constant proliferation and stable morphological characteristics were observed. For preservation, the cells were frozen in several vials in vapour phase liquid nitrogen at −130°C. One vial of cryopreserved cells was defrosted each month to corroborate the viability and proliferation, and similar growth behaviour to the first culture was detected.

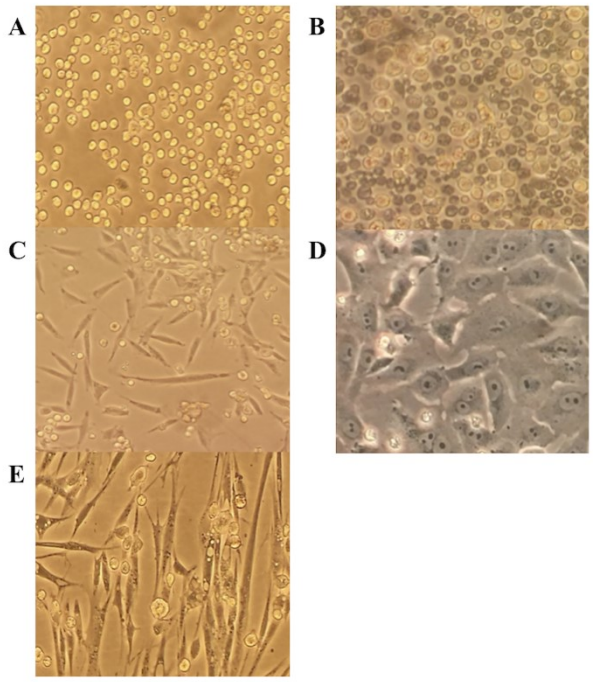

Fig. 3. Morphological characteristics of stable CTVT culture cell line. A – CTVT cells before attachment show a round form with refringent appearance. B – After 12 h the cells begin to attach to the flask and take on a grey dark colour. C – Round and spindle cells are observed (10×). D – 100% confluence is observed after 96 h (40×). E – The cells are completely elongated with spindle shape and vacuoles, characteristic of CTVT cells (40×)

Isolated CTVT cell line characterisation. Through flow cytometry, it was determined that the cell population size and granularity was uniform, with an overall viability of 94.5% (Fig. 4). The expression of the following surface markers of CTVT cells was observed: HLA-DR$^+$ (0.6%), CD5.1$^+$ (1.7%), CD14$^+$ (43.6%), CD45$^+$ (11.8%), CD83$^+$ (0.7%), CD163$^+$ (4.1%), and Ly6G-Ly6C$^+$ (60.2%). Expression of the markers CD80, CD34, CD4, CD8, and CD3 was not detected (Figs 4, 5). Furthermore, loss of vimentin (4.5%), a malignancy marker, was observed, compared with the overexpression of vimentin in the CTVT primary tumour (41%) (Fig. 6).

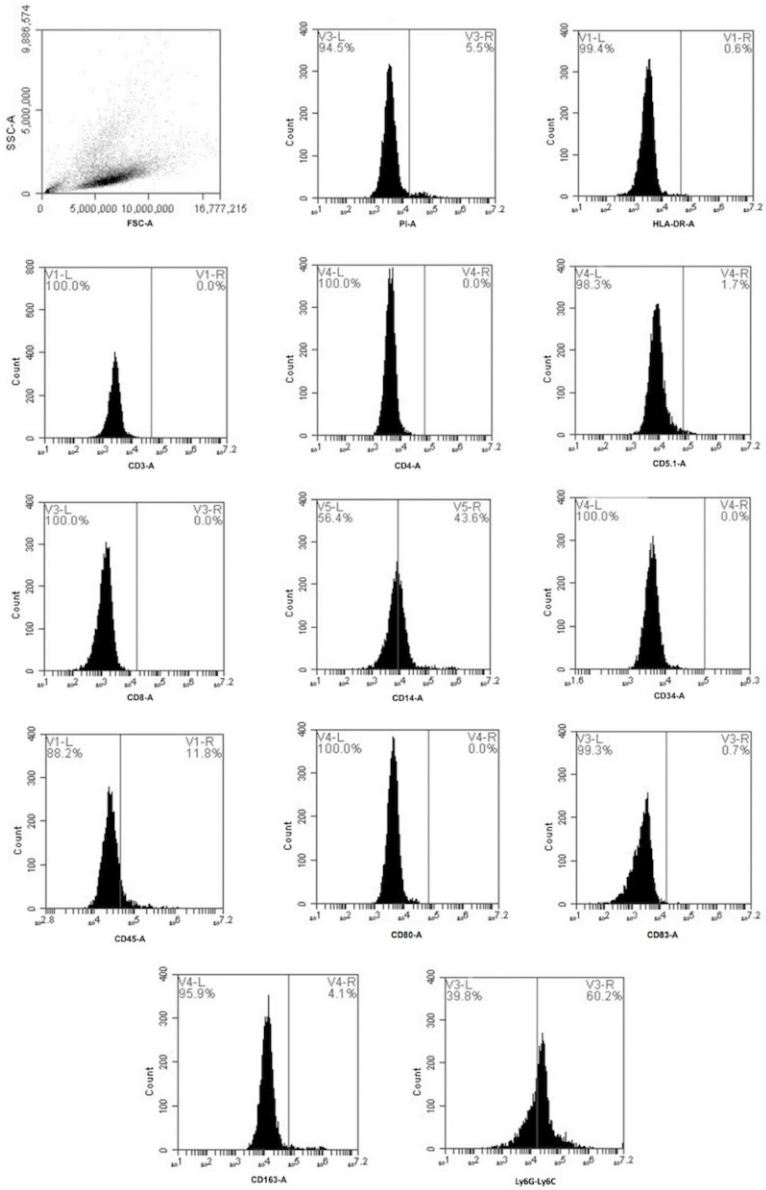

Fig. 4. The expression of the following surface markers of CTVT cell line: PI, HLA-DR, CD3, CD4, CD5.1, CD8, CD14, CD34, CD45, CD80, CD83, CD163 and Ly6G-Ly6C. Representative histogram of three repetitions

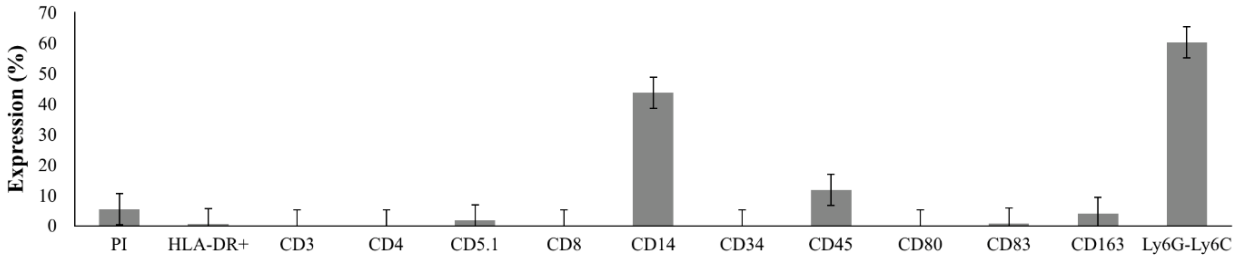

Fig. 5. Surface markers of CTVT cell line. The surface markers PI, HLA-DR, CD3, CD4, CD5.1, CD8, CD14, CD34, CD45, CD80, CD83, CD163, and Ly6G-Ly6C were analysed by flow cytometry. The results are the averages of three repetitions (± SD 3)

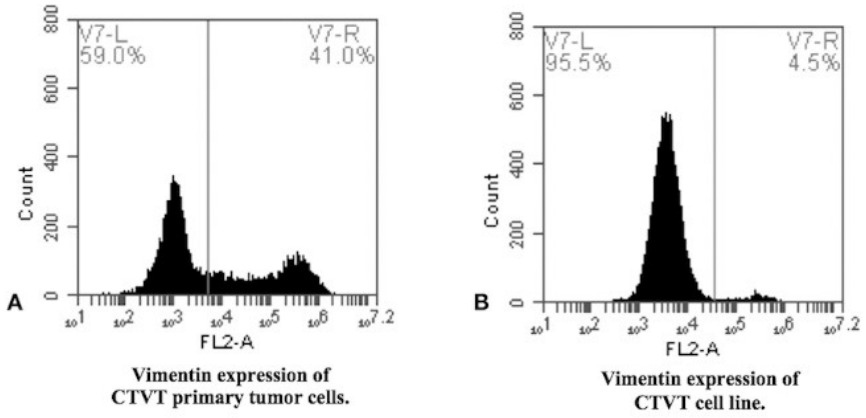

Fig. 6. Vimentin expression. A – CTVT primary tumour (41%). B – CTVT cell line (4.5%)

A CTVT cytogenetic study was performed in five metaphases demonstrating multiple complex arrangements with loss of several chromosomes leaving 56–59: add(1)(q21), del(1)(q11), t(1;12)(p12;q12), −2, del(2)(q13), −3, −3, −4, add(4)(p12), del(5)(q13), der(5), +der(5), der(6)×5, del(7)(p12), der(7)×2, t(8;2), −9, −9, inv(10)×2, der(11)×2, −12, −13, del(13)(q22q32), +14, +14, +14, t(14;2), −15, −15, add(16)(p13.3), −17, −17, −18, −18, i(19)(p10), −20, −20, −21, −21, −22, +19 mar(5) (Fig. 7).

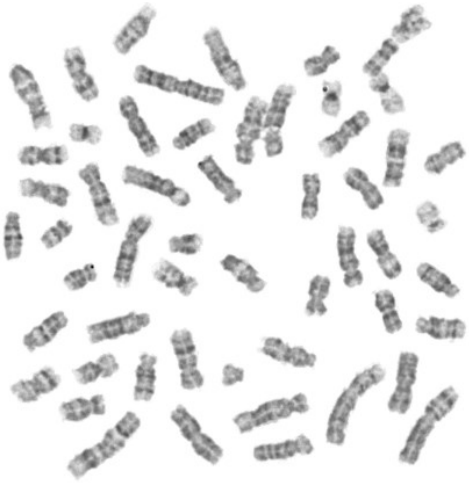

Fig. 7. Representative metaphases of CTVT cell line; 56 chromosomes

In the CTVT cell line, the LINE-c-myc insertion expressed at 550 bp was not detected. However, a 340 bp band was amplified (Fig. 8). The inoculation of the CTVT cell line at a concentration of 1×10^8 via submucosal vaginal delivery did not induce tumour growth in bitches during a one-year observation (data not shown).

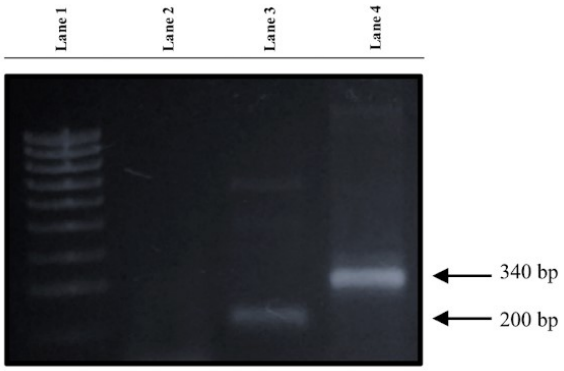

Fig. 8. Gel electrophoresis of MycS-2/LINE AS-1 PCR products from CTVT cell line. Lane 1 – 100 bp ladder molecular markers; Lane 2 – no template; Lane 3 – actin as internal positive control; Lane 4 – CTVT cell line

Inoculation with isolated CTVT cell line prevents CTVT development. CTVT tumour growth was visible and palpable at 7 weeks post-inoculation with primary CTVT cells and continued to develop over time (Fig. 9), reaching a volume of 35 cm^3 after 16 weeks. However, when bitches were previously inoculated with the isolated CTVT cell line and challenged with the primary CTVT cells on the 3rd week, tumorigenesis was not observed over 16 weeks, compared with the female group treated with primary CTVT cells alone (Figs 9 and 10).

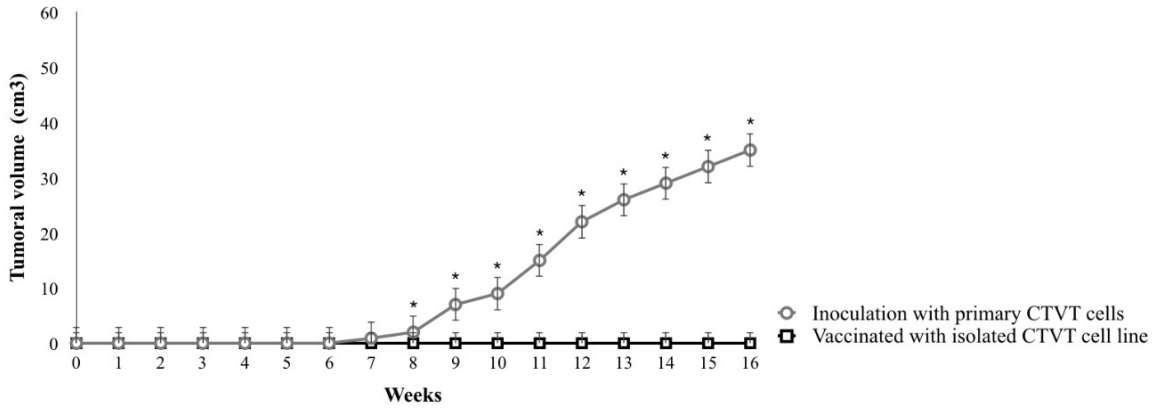

Fig. 9. Inoculation with the isolated CTVT cell line as an effect on CTVT development. The tumour is visible and palpable by the seventh week post inoculation with primary CTVT cells (control). However, when bitches were previously inoculated with the isolated CTVT cell line and then challenged with the primary CTVT cells tumorigenesis was not observed

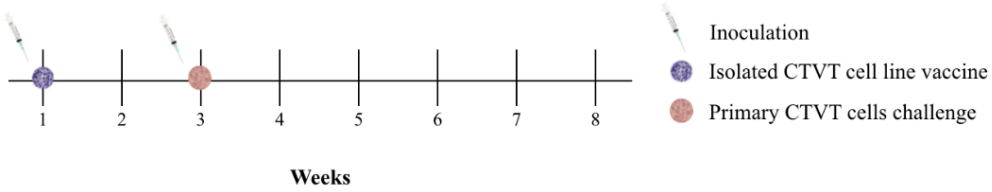

Fig. 10. Vaccination/challenge scheme. In the first week bitches were inoculated with the isolated CTVT cell line vaccine and in the third week were challenged with the primary CTVT cells

Discussion

The present study demonstrated the successful isolation and long-term culture of a stable CTVT cell line and its potential as a CTVT prevention strategy. CTVT is endemic in at least 90 countries worldwide (31) and appears to be transmitted through live cell transfer between animals, particularly during sexual contact. CTVT is established in the host by downregulating MHC expression on the tumour cell surface, thus "hiding" from the host immune system (12, 16). Currently, a vaccine preventing CTVT or reducing its prevalence and spread does not exist. The present study was performed in order to isolate CTVT cells, identify molecular and/or genetic mechanisms underlying the development of this tumour, define potential molecular markers, and provide an experimental platform to test and understand the mechanisms of action of transmissible tumours (11, 14). However, despite the successful isolation and proliferation of the CTVT cell line, tumour growth *in vivo* was not possible. The cancer cell line was characterised and used to prevent the implantation of CTVT, prompted by previous reports indicating that this tumour is immunogenic (5). In the present study, the CTVT cells also exhibited histomorphological characteristics previously reported by Stockman *et al.* (30) in 2011, such as a round shape, string like arrangement in strings, having a high nucleus: cytoplasm proportion, and round-shaped nuclei with chromatin ranging from delicate to coarse and prominent nucleoli. The karyotype in our CTVT cell line confirms the number of chromosomes to be 56–59, characteristic of CTVT, with a different number of somatic cells from that characteristic of dogs (78 chromosomes) (8). Furthermore, detection was possible in the present study of certain markers of this tumour, such as HLA-DR (28) (negative or low expression, 0.6%) and other markers not previously reported: $CD14^+$ (43.6%), $CD45^+$ (11.8%) and Ly6G-$Ly6C^+$ (60.2%). Although the surface markers CD5.1 (1.7%), CD83 (0.7%), CD163 (4.1%), CD34 (0%), CD80 (0%), and CD4, CD8, and CD3 (0%) were also evaluated, the sample material was not considered positive for them, due to the low (<5%) or undetectable expression levels.

Furthermore, the 550-bp LINE-c-myc insertion in our CTVT cell line was not detected by PCR. Similar results were obtained by Liao *et al.* (15), where spindle cells that were attached to the flask did not have the LINE-c-myc insertion. It was suggested that loss of the LINE gene insertion may be a result of terminal differentiation during the passages. However, in the same study, the LINE c-myc insertion was detected by PCR in CTVT cells obtained directly from the tumour (14). In the present study, the CTVT cell line only had an amplified band of 340 bp, similar to the findings of

Fonseca *et al.* (10), who reported a 340-bp band from 35 dog tumour biopsies.

In addition, the primary CTVT cell line was inoculated intravaginally to test its tumorigenic potential in the present study, which was methodologically unlike Chien *et al.* (9) who implanted the cells subcutaneously into the backs of the dogs. However, no tumour growth was observed over a one-year period of evaluation, which was a result consistent with observations made by Adams *et al.* (1) who failed to induce tumour growth when CTVT cells were inoculated intravenously, subcutaneously, or intraperitoneally, suggesting that established cell lines do not necessarily have tumorigenic potential. Flow cytometry analysis was performed in the aim of explaining this result, and it detected low vimentin expression in the CTVT cell line, indicating a possible association of this marker with the loss of malignant potential. Furthermore, it has been demonstrated that increased levels of vimentin mRNA after chemotherapy are correlated with a poor prognosis in breast cancer patients (4). When MCF7 breast cancer cells (vimentin-negative) were transfected with vimentin, its overexpression was associated with reorganisation capacity of the cytoskeleton, which enhances directional migration ability (4). The reasons why vimentin was considerably reduced in the present study and why tumorigenic potential was lacking remain unclear. However, it may be useful in the development of a preventive vaccine for CTVT and more experiments should be performed. The immunisation's prevention of tumour development when bitches were challenged with inoculation of primary CTVT cells, was likely due to the fact that live vaccines have a significantly higher immunogenicity, by eliciting a wider range of immunological responses, humoral (B cells) and cellular ($CD8^+$ and $CD4^+$ T cells). In addition, it has been reported that a single vaccine administration is usually sufficient to induce long-term, sometimes even lifelong, protection (14). Although this is considered a preliminary study due to the small number of dogs used (n = 7), it is a demonstration of the immunogenic capacity of the CTVT cancer cell line, as well as its potential to be used as preventive vaccination against this disease in immunocompetent dogs. Furthermore, studies in nude mice should be performed to corroborate the non-tumorigenic nature of the cell line. The dogs used as a control for the tumour growth group were treated with vincristine at the end of the experiment and, once healthy, all the bitches, including those vaccinated with the CTVT cell line, were ovariohysterectomised and adopted. The results of the present study provided evidence supporting the use of this vaccine in all dogs with a mature and functional immune system (3 months of age) or of reproductive age. This may be administered in two applications, with a 14-day interval, by subcutaneous injection, and it should be contraindicated in sick dogs or dogs with immunodeficiencies. However, these suggestions should be investigated in the next phase of experimentation.

In conclusion, we reported the identification and isolation of a stable CTVT cell line. This cell line had immunogenic and non-tumorigenic characteristics, which support its potential to be used as a preventive vaccine. However, further studies are required to confirm these results.

Conflict of Interests Statement: The authors have declared that they have no conflict of interests regarding the publication of this article.

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Animal Rights Statement: This study was performed in accordance with the recommendations of the Animal Ethics Committee of the Institution (CEIBA) and Mexican law (NOM-062-ZOO-1999). All experimental protocols were approved by the Animal Ethics Committee of the Biological Science Faculty of the AUNL (No. UANLFCB-0028) and performed accordingly. Samples were collected only from animals for laboratory analyses, avoiding unnecessary pain and suffering of the animals. The owners gave their written consent for sample collection, and the locations where we sampled are not privately owned or protected in any way. The studies did not involve endangered or protected species.

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