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Heterologus Gene Expression in a Cattle Tick *Rhipicephalus microplus* Embryonic Cell Culture

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Abstract: *Rhipicephalus microplus in vitro* cell culture have been useful in the study of acaricide resistance in ticks, a methodology for heterologous gene transference into these cultures may help to study several aspect of the cell biology and gene expression associated with acaricide resistance. In this study we assessed a pantropic retroviral gene transfer system in *R. microplus* embryonic cell cultures, for this purpose a Green Fluorescent Protein (GFP) was cloned downstream of the Drosophila heat shock protein 70 promoter within the pLNHX vector, pLNHX-GFP was cotransfected with pVSV-G vector coding for retroviral envelope protein into the GP-293 retroviral packaging cell line. An embryonic *R. microplus* cell line was transfected with the pantropic virus and evaluated for expression of the protein by fluorescence microscopy to detect the GFP. Infected embryonic tick cells expressed a green fluorescent signal that was not present in uninfected cultured cells. Relative Fluorescent Units (R.F.U) were determined as GFP expression values from infected and uninfected cells with mean values ranking at 1774 and 143 R.F.U. respectively, which demonstrates a successful heterologous gene expression in *R. microplus* cell culture. Our results showed that recombinant retroviral system can be used to express exogenous genes in *R microplus* cell cultures.

Key words: Retrovirus, transfection, *Boophilus microplus*, arthropod gene expression

INTRODUCTION

There is an increased scientific interest on the genetic manipulation of arthropods specially those pests inflicting heavy losses in agriculture and livestock industry. The southern cattle tick *Rhipicephalus (Boophilus) microplus* and related tick borne diseases plague the cattle industry in tropical and subtropical areas around the world with a tremendous economic impact. The chemical treatment with several acaricides formulas of tick infested cattle is the basic strategy to prevent and control tick transmitted diseases such as babesiosis and anaplasmosis in the cattle industry (De Castro, 1997). In Mexico numerous reports identify a constant increase of acaricide resistant ticks towards pyrethroids, organophosphates and amidines acaricides (Li *et al*., 2003; Rodriguez-Vivas *et al*., 2007; Soberanes-Céspedes *et al*., 2002). A tick resistance model based in tick cell culture in combination with the heterologous gene transference method by recombinant retrovirus will provide a useful tool for assessment of genetic regulation and biochemical pathways of individual genes related to acaricide resistance in *R. microplus*. Most pesticide resistance mechanisms in arthropods consist of either a change in sensitivity of the site of action which in case of pyrethroids and organophosphates are the sodium channel and Acetylcolinesterase (AchE) respectively (French-Constant *et al*., 1998), or enhanced activity of metabolic enzymes, such as esterases, mixed function oxidases and glutathion-S-transferases (Hemingway and Karunaratne, 1998). All these genes are important candidates for analysis on genetically manipulated tick cell cultures. These cells have been useful in our previous work in the study of *R. microplus* acaricide resistance at the cellular level (Cossio-Bayugar *et al*., 2002 a, b). The accurate identification of the genes involved in the *R. microplus* acaricide resistant phenomena will aid in a new approach to the development of better acaricides.

Heterologous gene transference by using retroviruses is a recent technology designed to introduce extraneous genetic material to a dividing cell culture (Miller, 1997), these vectors have long been used in

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mammalian systems for successful introduction and expression of heterologous genes of interest into different living models (Anderson, 1998; Eglitis and Anderson, 1988; Handler, 2001). In general retroviral vectors are made replication-incompetent for safety reasons, by deletion of essential genes that may be replaced by genes of interest. As a result Infectious particle production is only possible in packaging cell lines that supply the needed viral proteins (Handler, 2001). Retroviral infection specificity therefore is determined by the envelope proteins of the viral particle that recognizes specific receptors on the cell surface. The expansion of the host range beyond mammals has been achieved by pseudo-typing virions with the envelope glycoprotein from the Vesicular Stomatitis Virus (VSV-G). VSV-G mediates viral entry through lipid binding and plasma membrane fusion, thus it does not require specific protein receptor molecule for infection. Pseudo-typed VSV-G virus can mediate gene transfer into a wide range of organisms including cells from fish (Burns *et al*., 1993; Lin *et al*., 1994) newts (Burns *et al*., 1994), mosquitoes (Matsubara *et al*., 1996), moths (Franco *et al*., 1998), frogs (Burns *et al*., 1996) and birds (Kamihira *et al*., 2005). As with other replication-incompetent viruses, it can be safely produced and used with minimal biological containment concerns.

In this study we report the successful heterologus gene expression in *R. microplus* embryonic cell culture based on retroviral transfection.

MATERIALS AND METHODS

Reporter gene plasmid construction: The Monster Green® Fluorescent protein plasmid (phMGFP, Promega) was digested with XbaI and EcoRV (Eco321, Fermentas life Science) restriction enzymes to obtain the complete coding region for the green fluorescent gene. The resulting fragments were separated by electrophoresis in an agarose gel 1% TBE. The band corresponding to the Green Fluorescent Protein (GFP) gene was cut and purified form the agarose with Wizard ® SV Gel and PCR Clean up system (Promega). Blunt ends were generated on the fragment of interest using the blunting enzyme from the GeneJET™ PCR Cloning Kit (Fermentas) and ligated with T4 DNA ligase (Fermentas) in the pLNHX vector (Clontech Laboratories, Inc) previously digested with HpaI (Fermentas) restriction enzyme and treated with calf intestine alkaline phosphatase to avoid religation of the plasmid. This construction was denominated pLHNHX-GFP. The GFP gene was cloned downstream of the Drosophila heat shock protein 70 (hsp70) promoter (Phsp70). The pLHNHX-GPF was used to transform chemically competent *E. coli* cells (One Shot® TOP 10, Invitrogen™ CA, USA). The transformed bacteria were grown overnight in Luria medium and the plasmid purified by Wizard® Plus SV minipreps (Promega). The bacteria colonies containing the pLHNHX-GPF with the correct GFP orientation were selected by means of a double enzyme restriction digestion with BglII and Bcl1 (Fermentas).

Recombiant retrovirus generation: 10^6 GP2-293 cells were seeded in 60 mm Petri dishes 24 h before transfection in Dulbecco´s Modified Eagle´s Medium (DMEM, Gibco®, Invitrogen™ NY, USA). The cells were maintained at 37°C under 5% CO_2 atmosphere. A fresh preparation of liposome solution (Clonfectin™, Clontech Laboratories, Inc.) was prepared in HEPES-NaCl buffer at 55°C with a final concentration of 1 μg μL^{-1}. A solution containing 200 μL of serum free DMEM medium with 5 ug of liposome solution, 5ug of vector pLNHX-GFP and 5 ug of the retroviral capsid codifying vector pVSV-G was prepared and incubated at 37°C for 30 min according to the Clonfectin™ user manual. DMEM (1.8 mL) medium supplemented with 2.5% fetal bovine serum (FBS) was added to the mixture with subsequent 2 h incubation period at 37°C in 5% CO_2 atmosphere, afterwards the medium was removed and the cells were washed once with PBS and incubated at 37C in 5% CO_2 atmosphere for 72 h with 5 mL of fresh DMEM medium supplemented with 10% FBS according to protocol at www.clontech.com. After 72 h the supernatant was collected and centrifuged at 200 g for 5 min. The viral solution was concentrated by filtration in a 150 kDa cutoff membrane (Apollo™, Orbital Bioscience®) to reduce the volume to 50 μL. This viral solution (25 μL) was used to transfect a *R. microplus* embryonic cell culture.

Rhipicephalus (*Boophilus*) microplus embryonic cell culture: The tick strain used in this study was maintained at the Centro Nacional de Investigación Disciplinaria en Parasitología Veterinaria (CENID-PAVET) at Jiutepec, Morelos, México. A bovine was infested with 1×10^4 larval ticks. Fully engorged females were collected and maintained in a moistered chamber, at 28°C. After oviposition 10-day-old eggs were collected for the isolation of an embryonic cell cultures as described previously (Pudney *et al*., 1973; Holman and Ronald, 1980). Briefly: The eggs were washed with 10% benzalkonium chloride solution for 15 min and sterile water for 5 min, with Penicillin 100 U mL^{-1}, Streptomycin 100 μg mL^{-1}, amphotericin B 0.25 μg mL^{-1} for 5 min. Ten mililiter of 0.5% lactalbumin hydrolysate in Hanks´ balanced salt solution, PH 6.9 was added, the eggs were transferred to a sterile porcelain mortar and crushed under a porcelain rod with just enough pressure to crack the eggshells. The resulting tissue and eggshells suspension was filtered through a 70 μm cell strainer (BD Falcon™,

BD bioscience, CA USA) to remove the eggshells. The suspension was centrifuged at 100 g for 8 min. The supernatant was removed and 5 mL of fresh washing solution was added to the cell pellet a second centrifugation for 8 min at 200 g afterwards an additional cycle of washing-centrifugation was performed. The cell pellet was resuspended in 2 mL of Leibovits-15 and minimum essential medium with Hank´s salts supplemented with 20% fetal bovine serum heat inactivated at 56°C for 45 min, 10% tryptose phosphate broth and 0.1% bovine albumin fraction V with antibiotic-antimycotic. The resuspended cells were transferred to a 25 cm^2 cell culture flask containing 25 mL of growth medium. The cells were incubated at 28°C for 3 weeks. After 3 weeks the growth medium was removed and 10 mL of fresh medium was added. A week later the medium was removed and 5 mL of fresh medium was added. Growing embryonic cells were subcultured for viral transfection experiments.

Pantropic virus infection of embryonic cells: Embryonic *R. microplus* cells (1×10^5) were seeded in 2 well Nunc® LabTeck® chambers slide™ system 24 h before transfection. For each experimental chamber the cellular medium was removed and replaced with 2 mL of fresh medium with polybrene at 4 μg μL^{-1}. Concentrated viral solution (25 μL) was added to the cells to be transfected and free virus supernatant was added to the control cells. The cells with the virus were incubated for 24 h at 32°C under 5% CO_2 atmosphere. A medium change was done at 24 h and further 24 h incubating period under the same conditions were done. Results were observed under U.V. Zeiss Axioscope 40 epiflourescent microscope with a 450-490 nm excitation filter and an emition filter of 515 nm.

Relative fluorescence determination: Fluorescence determination of infected and uninfected cells were identified from digital images obtained at the U.V. microscope and their fluorescence values were determined as Relative Fluorescent Units (R.F.U) by using the Image Processor Epi Chemi^3 (UVP Life Sciences) feeding the image analysis software LabWorks 4.0 in its fluorimeter mode.

RESULTS

After 3 weeks of incubation period at 28°C of the egg macerate different cell types were observed on the culture flask: Large globular cells, few epithelial-like cells and some round cells similar as those described in previous works (Pudney *et al*., 1973; Holman and Ronald, 1980). After the 3 weeks incubation period subcultures were made for viral transfection. Cells from the 3rd subculture were used for the pantropic infection experiments (Fig. 1).

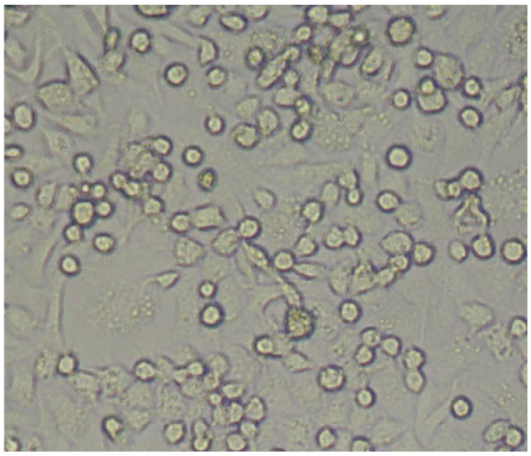

Fig. 1: *Ripicephalus microplus* embryonic cell culture. Inverted microscope image. Bright illumination; objective 40X. Two main cell forms are apparent: epithelial-like cells firmly attached to the polystyrene and globular cells which are easily detached and frequently floating in the medium

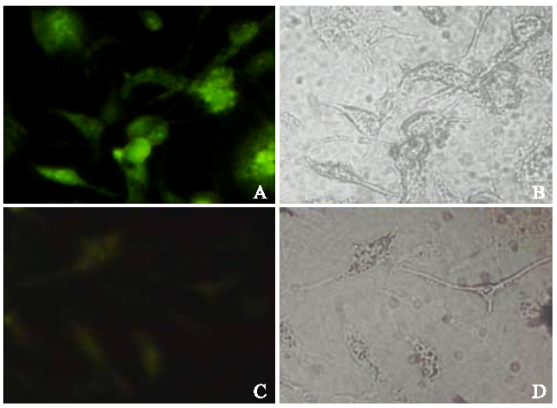

Fig. 2: Green fluorescent protein detection in *Ripicephalus microplus* embryonic cells. A. Epiflourescent microscope image of *R. microplus* cells expressing a transfected green fluorescent protein with a 450-490nm excitation filter and an emition filter of 515nm B. Bright field of the same field of infected cells in panel A. C. Epiflourescent microscope image of *R. microplus* uninfected cells. D. Bright field of the same area of uninfected cells in panel C. Objective 40X

Infected tick cells showed green fluorescence at the epiflourescent microscope when exposed to a 450-490 nm wavelength UV light. This fluorescence was distinctive to the transfected cells and seemed to be absent from uninfected control cells (Fig. 2), such a fluorescence was excluded from endogenous signal by using several

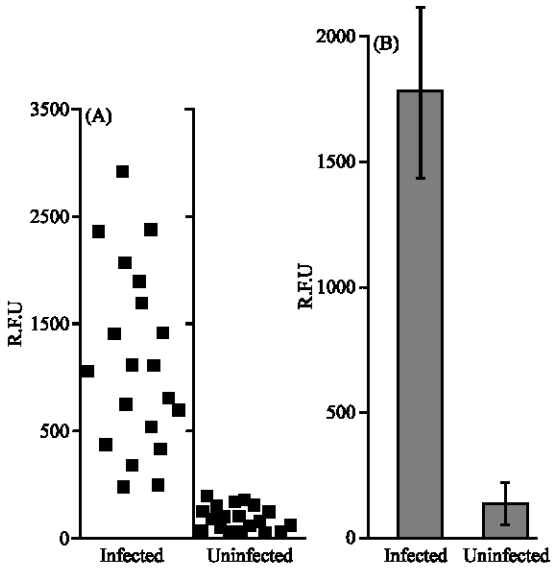

Fig. 3: Fluorescence determination of infected and uninfected cells. Individual cells from both groups were identified from digital images at the U.V. microscope and their fluorescence values were determined as Relative Fluorescent Units (R.F.U.) and plotted in separate groups A. The R.F.U. of both groups were processed for its mean and standard deviation values and graphically compared in B

wavelength interferometry filters and was attributed to the expression of GFP heterologous gene within the recombinant retrovirus, the fluorescence seems to by confined to the cytoplasm and some organelles like structures are visible. The fluorescent signal remained stable for several days after mounting the microscopic slide and showed similar fluorescent signal after 2 weeks with no apparent fading. Fluorescence determination of Individual cells from infected and uninfected cells were identified from digital images obtained at the U.V. microscope and their fluorescence values were determined as R.F.U and plotted in separate groups (Fig. 3). The R.F.U. of both groups were processed for its mean and standard deviation values showed a value of 1774±335 and 143±41 R.F.U. for infected and uninfected cells, respectively (Fig 3).

DISCUSSION

This is the first report on the use of a retroviral gene transference method in acarines arthropods and may be used for scientific research on the physiological pathway related to acaricide resistance and metabolic detoxification. The observed results were consistent with the expression of GFP in a *R. microplus* embryonic cell culture, this means that the tick cells were able to recognize the Drosophila promoter an induce the transcription and expression of GFP producing a distinctive fluorescence not seen in the uninfected control cells (Fig. 2). We attributed this fluorescence to the viral infection, because of the excitation and emition wavelength of the fluorescent detected, as well as to the distribution of the signal consistent with a viral protein at the cytoplasm and surrounding organelles probably transport vesicles and/or Golgi apparatus. Fluorescence determination showed a clear differentiation of at least three standard deviations or an order of magnitude between infected and uninfected cells, a difference attributed to the expression of the viral transfected GFP in the first group of cells which showed a mean R.F.U. level of 1774 compared to a mean level of 143 in the control group attributed to endogenous fluorescence or background noise (Fig. 3). Heterologous gene expression, in this case a coral fluorescent protein in *R. microplus* cell line demonstrates the possibility to work with other genes related to pesticide resistance ad/or host parasite relationship between *R. microplus* and *Babesia* sp. This are prioritary research subjects awaiting for an heterologous gene expression on ticks like the one described in this work since cellular and molecular aspects of *R. microplus* are considered as essential for discovering of new approaches for the control of acaricide resistant tick and tick borne diseases.

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