Primary culture of Rhodnius prolixus (Hemiptera: Reduviidae) salivary gland cells

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In the present paper, we developed a primary culture of Rhodnius prolixus *salivary gland and main salivary canal cells. Cells remained viable in culture for 30 days. Three types of cells were indentified in the salivary gland cultures, with binuclear cells being the most abundant. The supernatants of salivary cultures contained mainly 16-24 kDa proteins and presented anticoagulant and apyrase activities. Secretion vesicles were observed budding from the cellular monolayer of the main salivary canal cells. These results indicate that* R. prolixus *salivary proteins may be produced in vitro and suggest that the main salivary canal may have a possible secretory role.*

Key words: *Rhodnius prolixus* - salivary gland - primary culture

Triatomines are insects that are haematophagous throughout the nymphal stages and as adults. They are vectors of the trypanosomatids *Trypanosoma cruzi* and *Trypanosoma rangeli*. To obtain a successful blood meal, triatomines produce a diverse set of molecules in their salivary glands that are released at the bite site during the feeding process (Soares et al. 2006) in order to counteract homeostasis, inflammation and immune responses from their vertebrate hosts (Ribeiro 1995).

The salivary apparatus of *Rhodnius prolixus* is composed of the salivary pump, the salivary canals and the salivary glands. The main salivary glands are two independent, elongated compartments found parallel to the oesophagus. Each main gland has a small transparent vesicle called the accessory gland (Perez 1969).

In triatomines, the main salivary glands are composed of a single layer of epithelial cells that surrounds the large central lumen and a thin basal lamina containing tracheolae, muscle fibres/myofibrils (Barth 1954, Lacombe 1999, Reis et al. 2003) and neuronal terminations (Meirelles et al. 2003). The morphology of the cells is still not completely known. In *Rhodnius domesticus*, Meirelles et al. (2003) showed that the main salivary glands have mononuclear cells, whereas Anhê et al. (2007) described binucleated cells in both the main salivary gland and the accessory gland from *R. prolixus* and *Rhodnius neglectus.*

The salivary canal originates from the main salivary gland and transports the saliva to the salivary pump. In *R. domesticus*, the canal is composed of epithelial cells covered by a basal lamina with tracheolae (Meirelles et al. 2003). The salivary pump forces the saliva through the canal and delivers it to the bite site (Perez 1969).

In *R. prolixus*, proteins belonging to the lipocalin family are the most abundant and diverse molecules in the saliva (Andersen et al. 2005). They include the notriphorins 1-7 (Champagne et al. 1995, Moreira et al. 2003, Knipp et al. 2007), biogenic amine binding proteins (Andersen et al. 2003) and ADP-binding proteins known as *Rhodnius* platelet aggregation inhibitor (Francischetti et al. 2000) as well as uncharacterised molecules related to *Triatoma pallidipennis* thrombin inhibitor triabin (Fuentes-Prior et al. 1997). In addition to the lipocalins, salivary components include apyrase (Ribeiro & Garcia 1981) and anti-haemostatic phospholipids, such as lysophosphatidylcholine (Golodne et al. 2003), as well as inhibitors of the complement system (Barros et al. 2009). In addition, novel molecules were identified in the study of the *R. prolixus* sialome (Ribeiro et al. 2004).

The molecular diversity of haematophagous insect saliva could lead to the identification of potential pharmacologically active compounds and could help to elucidate the evolutionary mechanisms of insect adaptation to haematophagy (Ribeiro et al. 2004). Thus, the present work aims to obtain a primary culture of the main salivary gland and salivary canal cells of *R. prolixus* in order to produce pure in vitro salivary compounds to be used in studies of identification and characterisation of novel bioactive molecules. The cultivation of *R. prolixus* salivary glands could also be useful in studying its interaction with *T. rangeli*.

The salivary glands (main and accessory glands) and the salivary canal were isolated from both male and female adult *R. prolixus* at 24 h or 5 days after feeding. Insects were anaesthetised on ice, disinfected by immersion in detergent (diluted to 5% in distilled water) for 2 min and immersed in 1% NaClO for 2 min and in 70% ethanol for 5 min. The organs were isolated under aseptic conditions using a laminar flow cabinet. The cul-

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tivation was carried out using the media IPL-41 (components weighed individually), TNM-FH (Sigma), Grace's (Sigma) and Schneider's (Sigma) supplemented with 30 mg/mL tryptose phosphate (Sigma), 20% inactivated fetal calf serum (FCS), 10 µg/mL mice epidermal growth factor (Sigma), 45 mg/mL glucose (Sigma), 30 mg/mL glutamine (Sigma), 2 mg/mL insulin (Sigma), 10X insect medium supplement (low protein < 150 µg/mL) (Sigma), 10 mg/mL tetracycline (Sigma), 2.5 mg/mL anfotericin B (Sigma) and 50 mg/mL gentamicin (Sigma) at pH 7.0.

The salivary glands were shred with entomological scissors and treated with type XI collagenase at a concentration of 2 mg/mL for 20 min and at 4 mg/mL for 40 min at RT. The salivary canal was not treated due to high cell sensitivity to enzymatic treatment. The tissues (15 salivary glands/well or 15 salivary canals/well) were cultivated in 6-well plates containing 25 µL of culture medium at 28°C in a humid chamber. The use of higher volumes of medium and wells with smaller diameters induced less cell adhesion and permitted the adhesion of the cells to the walls rather than to the bottom of the plate. The medium was replaced every five days. Culture supernatants were collected and maintained at -20°C until use.

Among all culture media tested, the best cellular adhesion was obtained with Schneider's insect medium (Sigma). The collagenase treatment enabled a clearer visualisation of the cell types of the salivary glands with less cellular damage. Glands and canals isolated 24 h after feeding had higher number of adherent cells and were used for the cultures.

Salivary gland cultures were analysed directly on the plates (Fig. 1) or after staining with Giemsa (data not shown) under an optic microscope. Three types of cells were observed in the salivary gland culture. The type I cells were elongated and fusiform and migrated from the tissue fragments to the edge of the plate wells (Fig. 1A). Cell migration started four days after the beginning of the culture and was observed until approximately 15 days of cultivation. These cells were abundant in the gland cultures. Type II cells were the most common in culture. They presented a globular shape with two evident nuclei (Fig. 1B) and could be visualised in cell clumps (Fig. 1D). Type III cells were rare and presented a globular shape with a unique nucleus (Fig 1C). Previous studies suggested that binucleated salivary gland cells may be responsible for saliva production and secretion. These cells have the largest nuclear mass, which

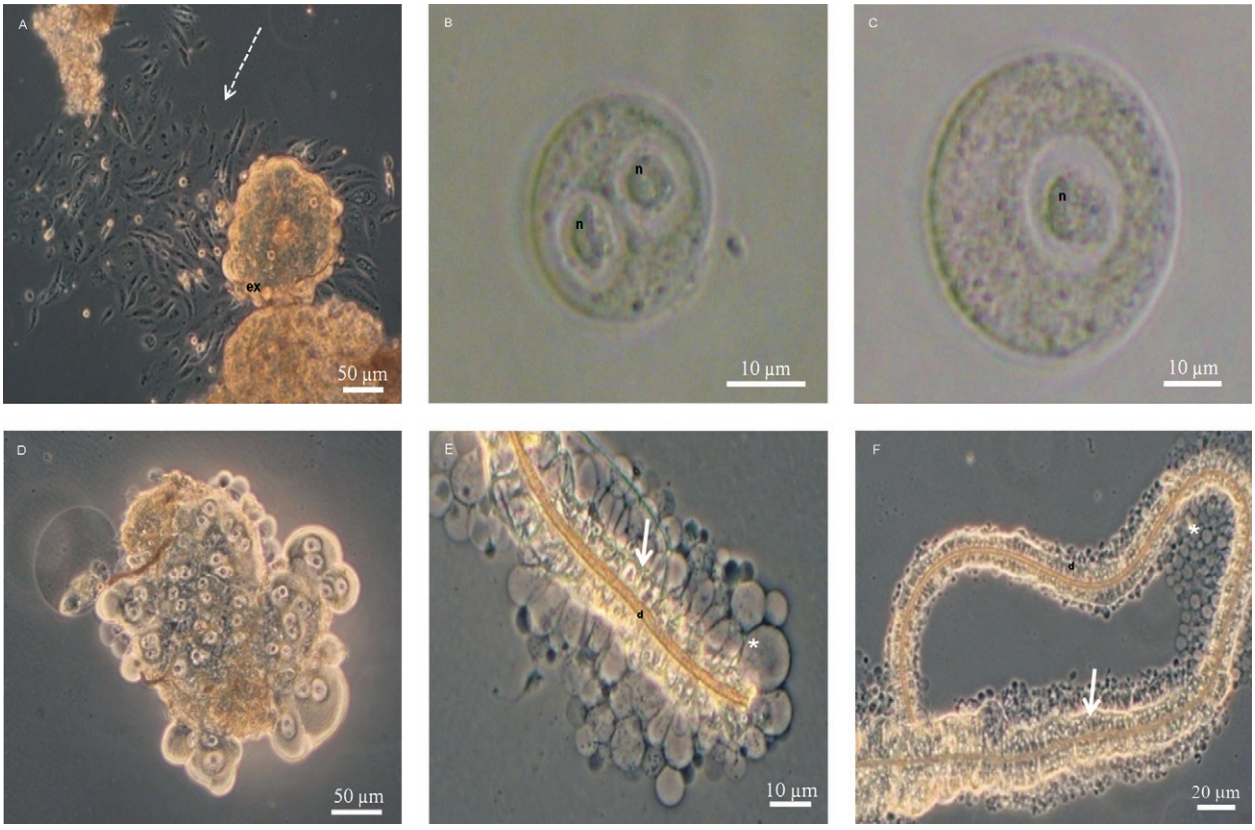

Fig. 1: primary culture of *Rhodnius prolixus* salivary gland and salivary canal cells. Photos were taken directly from the culture plates with no treatment. A: explants of salivary gland presenting type I cell migration with fusiform shape cells at day 7 of cultivation; B: close view of type II cells; C: close view of type III cells; D: overview of explants; E: close view of salivary canal; F: general view of salivary canal. Photos B-F were taken at day 12 of cultivation; d: salivary duct; ex: explants; n: nucleus; ---►: type I cell; —►: monolayer of juxtaposed cells; *: secretion granules.

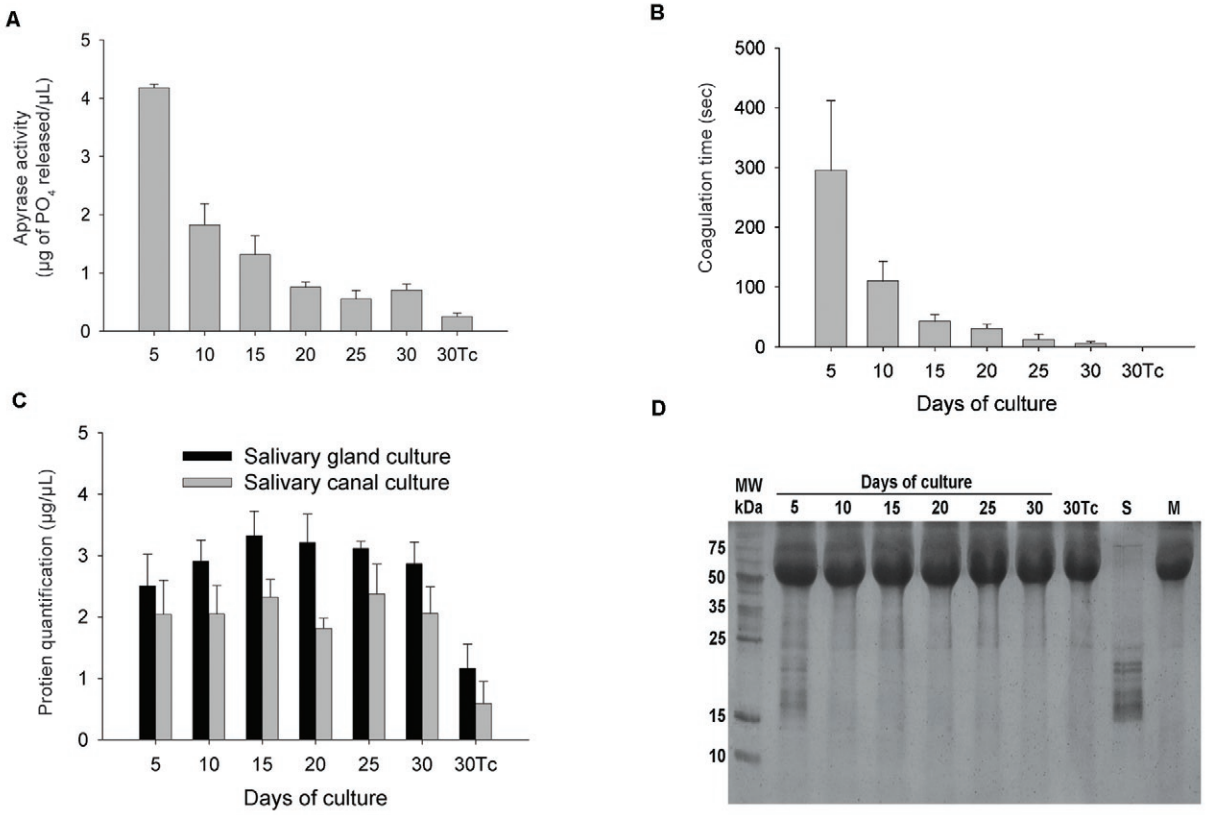

Fig. 2: biological activities and protein content from the supernatant of *Rhodnius prolixus* salivary gland cultures. Measurements were performed according to Araujo et al. (2006). Bars = mean ± standart error of 5 pools. Each pool is the supernatant of two cultures. 5 µL of the supernatant were used for the apyrase and anticoagulant assays. Values on graphs were subtracted from values of the medium alone. A: apyrase activity; B: plasma recalcification time assay; C: protein quantification; D: 15% SDS-PAGE; M: culture medium; MW: molecular weight standard; S: salivary content; 30Tc: extract of the salivary gland or canal cell culture at day 30. SDS-PAGE columns contain 2 µL from a pool of 5 samples of each culture supernatant, ~6 µg of salivary gland extract, ~6 µg of saliva and 2 µL of medium.

may indicate the higher metabolic activity necessary for rapid and regular cell regeneration (Barth 1954, Anhe & Azeredo-Oliveira 2008).

The salivary canal cultures presented groups of mononuclear cells arranged in a juxtaposed unique layer that formed the salivary duct (Fig. 1E, F). The canals remained in culture for 30 days. Interestingly, secretion granules could be observed around the cells from the 3rd day of cultivation onwards, reaching higher densities from day 3-16 and remaining present until day 30 of cultivation.

The supernatant of the salivary gland cultures presented both apyrase (Fig. 2A) and anticoagulant (Fig. 2B) activities that were highest at day 5 and decreased over time (Fig. 2A, B). The total amount of soluble proteins remained constant throughout the cultivation (Fig. 2C). SDS-PAGE analysis confirmed the presence of *R. prolixus* salivary proteins, which were best seen at the first days of cultivation (days 5-10) (Fig. 2D). Stronger bands were observed from 16-24 kDa, the approximate molecular weight expected for lipocalins (Ribeiro et al. 2004, Andersen et al. 2005). The presence of FCS in the medium (containing bovine serum albumin and other proteins) impaired the visualisation of proteins higher than ~66 kDa.

The total amount of proteins in the supernatant may indicate that the cells are producing proteins with molecular weights not visible in the SDS-PAGE and with activities other than anticoagulant or apyrase. These results are in line with the viability test, which confirmed low levels of cell death from day 5-25 of cultivation (Fig. 3), suggesting the supernatant has low levels of intracellular proteins derived from cell disruption.

The detection of salivary proteins in the supernatant after the medium was replaced indicates that the glands are actively secreting proteins. However, the presence of small amounts of proteins derived from cell disruption cannot be disregarded. The viability of the cells reduced with time, probably due to a lack of stimulus (e.g., hormones) in the medium or simply because of the biological cycle of the cells. Previous physiological studies of the salivary gland from Triatoma infestans described that cells entered an inactive state at 10-14 days after feeding. Five minutes after feeding, salivary gland cells were highly active, as characterised by an increased size, resumption of secretion and protoplasmatic regeneration (Barth 1954). These findings are in agreement with our results, in which more cells were adherent to plates in

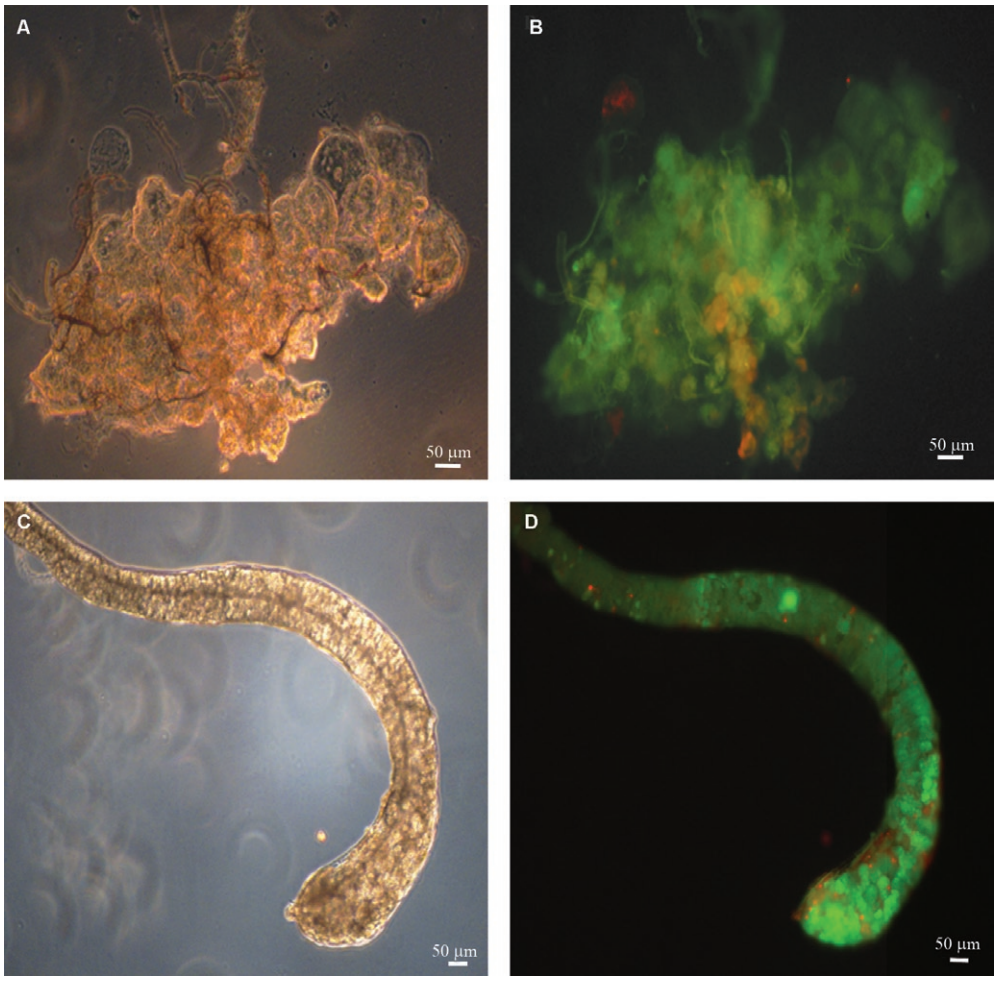

Fig 3: viability test of the cells from the primary cultures of *Rhodnius prolixus* at day 25 of cultivation. Cells were treated using the Live/Dead Viability/Citotoxicity kit (Molecular Probes®) according to manufacture instructions. The treatment was performed on the culture plates followed by photo documentation. A and C are phase contrast and B and D fluorescent view under optical microscope. Alive cells are marked green (excitation/emission 495/515 nm) and dead cells red (495/635 nm). A, B: salivary gland; C, D: salivary canal.

culture when isolated from salivary glands 24 h after feeding, with their highest protein activity observed in the supernatant within the first days of culture. Marshall (1982) maintained *Triatoma protracta* salivary glands in tubes containing culture medium for 35 days; the results of that study suggested that a constant level of protein is produced during the cultivation period. The supernatant collected on different days was injected intradermically and produced a similar area of wheal and erythema.

Protein quantitation indicated that the salivary canal produced and secreted molecules in the supernatant until the end of cultivation (Fig. 2C). Such values correspond with the identification of granules in cells of the salivary canal culture. These molecules were not visible in the SDS-PAGE (probably because they have molecular weights higher than 66 kDa) and had no anticoagulant or apyrase activities. Therefore, they may exert activities other than counteracting homeostasis, such as activation of salivary proteins (Amino et al. 2001, 2002) or supplementation of saliva with other proteins.

In conclusion, the results of the present paper showed that the salivary gland and the main salivary canal of *R. prolixus* can be cultivated in vitro. The primary cultures can be maintained for more than 30 days, but higher levels of activity in the supernatants were observed mainly in the first days. The presence of secretion vesicles in cells from the salivary canal is a novel finding and together with the possible role of hormones in the production of saliva, should be further investigated.

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