University of South Bohemia
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Interaction of *Borrelia burgdorferi* spirochetes with the salivary glands of *Ixodes ricinus* and with tick cells *in vitro* observed by immunofluorescence microscopy

Bachelor thesis

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Annotation: The first part of this work is aimed on the artificial infection of *Ixodes ricinus* tick cells with *Borrelia burgdorferi* spirochetes. I studied the effect of incubation of *B. burgdorferi* spirochetes with anti-OspA and anti-OspC antibodies on adherence to and penetration into the tick cells.The second part of the study is focused on the ability of *B. burgdorferi* to invade the tick *I. ricinus* salivary glands. A role of OspC in this process was studied. Antibodies directed against *B. burgdoferi* spirochetes were used to localise the bacteria by immunofluorescence microscopy.

Anotace: První část této práce je zaměřena na umělé infikování buněk klíštěte *Ixodes ricinus* boreliemi druhu *Borrelia burgdorferi.* Zkoumal jsem, zda-li má inkubace borelií s protilátkami anti-OspA a anti-OspC vliv na jejich schopnost přilnout a následně proniknout do klíštěcích buněk. Druhá část této studie se zabývá schopností borelií druhu *B. burgdorferi* proniknout do slinných žláz klíštěte *I. ricinus*. Byla zkoumána role proteinu OspC v tomto procesu. Protilátky namířené proti boreliím byly použity k lokalizaci bakterií pomocí imunofluorescenční mikroskopie.

I hereby declare that I worked out this bachelor thesis on my own, only using the cited literature.

I declare that in accordance with the Czech legal code § 47b law No. 111/1998 in its valid version, I consent to the publication of my bachelor thesis (in an edition made by removing marked parts archived by the Faculty of Science) in an electronic way in the public access to the STAG database run by the University of South Bohemia in České Budějovice on its web pages.

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1. Introduction

1.1. The ticks of the genus *Ixodida*

The *Ixodida* belong to obligate hematophagous organisms and one of the most common ectoparasites of domestic animals. The *Ixodida* include three families, the *Ixodidae* (hard ticks), *Argasidae* (soft ticks) and *Nuttalliellidae* that is comprised of only one rare African species (Hoogstraal, 1956).

In order to get a blood meal, ticks have to penetrate the host's skin and rupture the blood vessels. *Ixodid* ticks belong to slow feeders. They are able to feed for even longer than a week (Ribeiro, 1987). On the other hand, argasids feed up to an hour, their penetration is deeper and leakage of blood can continue long after the tick has stopped feeding (Binnington and Kemp, 1980).

A comparison of hemostatic inhibitors of hard and soft ticks indicates that the two main tick families have independently evolved their antihemostatic apparatuses. Independent evolution of these apparatuses suggests a quite brisk divergence between tick families. It is assumed that this fork came about 120 and 92 MYA. This goes hand in hand with present molecular phylogeny opinions on the early radiation of modern birds and placental mammals. It is nowadays believed that this event might have been the decisive factor which caused the evolution of hematophagy in ticks (Mans et al., 2002).

Ticks have not been always blood-feeding organisms. It has been implied that they predominantly fed on body fluids of animal corps before adaptation to a blood-feeding environment (Walter and Proctor, 1998). Moreover, a phenomenon of cannibalism was observed in some soft ticks (Balashov, 1972).

1.2. Tick species *Ixodes ricinus*

Ixodes ricinus abounds mainly in cool humid environments (forests) of Europe where its primary host is a deer. It is the only *Ixodes* species, which parasites on cattle in North Africa. *I. ricinus* transmits a broad range of pathogens to humans and animals. Birds, lizards, dogs and wild carnivores all belong to its most favourite hosts. In Europe, this tick is responsible for transmission of a number of pathogens

such as *Borrelia burgdorferi, Babesia bigemina* and *Anaplasma phagocitophylum.* Moreover, tick-borne encephalitis (TBE), a viral infection of the central nervous system, is spread by this tiny blood-sucking parasite. The period of activity of the adults starts in March and ends in October (Estrada-Pena et al., 2004).

The climate is considered to be the major restricting factor for the geographical distribution of *I. ricinus* (Jaenson et al., 1994). A vast majority of unfed nymphs of *I. ricinus* is not able to survive longer than 30 days at -10°C (Dautel and Knuelle, 1997). Nevertheless, the geographical border shifts constantly due to the climate changes. In Sweden in the late 1980s, the geographical distribution range of *I. ricinus* used to be located below 61°N (Jaenson et al., 1994), but nowadays the ticks are observed commonly up to 66°N (Gray et al., 2009).

In Central Europe, the height 700-800 meters above sea level was regarded as the upper limit of the occurrence of *I. ricinus* (Cerny et al., 1965). Field studies that were performed in the Šumava National Park (Czech Republic) in 2001 showed that *I. ricinus* is recently present as high as 1100-1200 meters above sea level (Daniel et al., 2003).

1.3. *Borrelia burgdorferi*

Borrelia burgdorferi is a pathogen that is capable to colonize many various organisms., i.e. arthropods, birds or mammals. *B. burgdorferi* is a typical representative of spirochete-like bacteria. It is usually 20-30 µm long, 0.2-0.5 µm wide and is spiral or coil-shaped. However, individual spirochetes can vary in length, width and in number of the coils. The cell consists of a protoplasmic cylinder containing the cytoplasm surrounded by plasma membrane and gram-negative type cell wall. Moreover, it contains flagellar apparatus which is covered by a periplasm and a lipoprotein-based outer surface membrane (Barbour and Hayes, 1986).

The genome of the bacterium is relatively small. Linear chromosome and plasmids (nine linear and twelve circular plasmids) consist of 950 and 620 kilobases, respectively (Casjens et al., 2000). Plasmids contain many of the genes directly related with borrelia pathogenicity. The vast majority of plasmid genes have no similarity to genes outside *Borrelia* genus. It is believed that these genes fulfill

specialized functions probably related to spirochetes adaptation in their hosts (Barbour, 1988).

More than 8% of *B. burgdorferi* coding genes are dedicated to producing lipoproteins. This is a very high number contrary to other pathogenic bacteria (Fraser et al., 1997). *B. burgdorferi* has an ability to change the composition of the surface lipoproteins structure at various stages of its life cycle. It considerably facilitates the spirochetes adaptation, immune evasion and survivability in diverse host environments (Fikrig et al., 2000). Despite the strong humoral responses which are exerted on the spirochetes, they are able to continuously infect mammalian hosts (Liang et al., 2002).

Gene expression in spirochetes is regulated by temperature (Schwan et al., 1995), pH (Carroll et al., 1999), cell density (Indest et al., 1997) but also host factors (Revel et al., 2002). In the tick vector, spirochetes produce different sets of lipoproteins during the feeding and starving periods of ticks (the pH of the tick gut is lower during the feeding process). Diverse lipoproteins are also expressed in the mammalian hosts. It is hypothesized that *B. burgdorferi* might be able to downregulate the expression of surface proteins that otherwise would be recognized by mammalian antibodies which are permanently produced during infection (Liang et al., 2002).

1.4. Adaptation in vertebrates

B. burgdorferi invades the vertebrate hosts during tick feeding. During the engorgement process, a strong multiplication of spirochetes appears in the gut of the tick (de Silva et al., 1995). The spirochetes increase their numbers several hundred times (Piesman et al., 2001). It is suggested, that the newly formed proteins ease the transmission from tick gut to its salivary glands and further to the dermis of the host (Gilmore and Piesman, 2000).

The spirochetes are transmitted via saliva into the host skin where they multiply for several days. Afterwards they invade various organs of mammalian host. The highest concentrations can be found in the spleen, urinary bladder, joints, heart and skin (Steere, 2001). *B. burgdorferi* has an ability to bind host-derived proteins on its surface, for instance a fibronectin. Binding this glycoprotein may help

the spirochete from being recognized by the host immune system (Burman et al., 1998).

In the tick, a diverse population of spirochetes with antigenic and genetic variability is present. It is caused by transcription and recombination events that constantly happen in spirochetes in the gut during the feeding and also later on during the transmission to mammals (Ohnishi et al., 2001). Antibodies produced by the infected mammal selectively destroy only the 'weak' phenotypes of spirochetes. The more adaptive ones are able to survive in the inhospitable conditions by downregulation of those proteins which are targeted by host antibodies. According to Liang et al.(2002), *B. burgdorferi* expresses about 116 genes during early mammalian infection and almost 3/4 of these genes are downregulated later on. It has been hypothesized that *B. burgdorferi* induces certain mechanisms which inhibit phagocytosis or ingestion by host cells (Georgilis et al., 1991).

Once being in the mammalian host, *B. burgdorferi* starts expressing genes, that facilitate the colonization of the new host, such as:

- *bbk32* - fibronectin-binding protein
- *dpb A* - decorin-binding protein
- *ospE/F* - complement inhibition
- *vls* - immune escape (summarized by Anguita et al., 2003)

1.5. Adaptation in ticks

B. burgdorferi has to accommodate to two totally dissimilar environments when transmitted from vertebrates to ticks and *vice versa*. Ticks acquire *B. burgdorferi* infection when larval or nymphal ticks feed on an infected host. According to Schwan and Piesman (2000), the average feeding time of *Ixodes* ticks is approximately 96 hours, but both larval and nymphal stadiums of ticks are infected by spirochetes in the first 24 hours of attachment.

The bacteria have evolved so perfectly, that they are able to survive cardinal temperature differences in poikilothermic organism. They have to sustain long periods of starving. When the blood meal finally comes, they have to sense the right stimuli and start travelling from the gut to the salivary glands (Pal and Fikrig, 2003).

Recent studies try to find out how individual tick species support their own colonization by spirochetes. It was shown that there are clear differences in the innate immune responses of different tick species. For instance, *Dermacentor variabilis* produces antimicrobial peptides in the hemolymph and therefore does not promote the bacterium life cycle. On the other hand, *Ixodes scapularis* is able to synthesize ligands which support the survival of *B. burgdorferi* within ticks (Johns et al., 2000 and 2001; Pal et al., 2004).

1.6. Outer surface proteins in the life cycle of borrelia

The genome of *B. burgdorferi* encodes high number of lipoproteins, several of which have been identified. Different lipoproteins are expressed at different stages of the spirochete life cycle (Ohnishi et al., 2001). A big attention has been focused especially on outer surface protein A (OspA) and even more to outer surface protein C (OspC).

OspC is a dimer, predominantly composed of α-helixes, with an alleged binding pocket for an unidentified ligand. It is totally dissimilar to OspA, the only other outer surface lipoprotein from *Borrelia* genus with known architecture. OspC shows the most resemblance to the extracellular domain of the *Salmonella typhimurium* aspartate receptor and the surface glycoprotein from *Trypanosoma brucei* (Eicken et al., 2001). The *ospC* gene is located on a plasmid cp26 (Marconi et al., 1993).

OspA is produced when *B. burgdorferi* spirochetes enter the tick vector and remains a major surface lipoprotein during the gut colonization (Schwan and Piesman, 2000). It serves as a gut adhesin. Until the next feeding, when the spirochetes are translocated, OspA is produced at fairly high levels (Pal et al., 2000).

TROSPA (Tick receptor for OspA) has been identified recently in the tick gut. It is a tick protein that is required for the successful spirochetal colonization of the arthropod. The expression of TROSPA is much higher within ticks that are colonized by spirochetes when compared to ticks in which no borrelia are present. Both the blockade of TROSPA antibodies and the inhibition of TROSPA expression lower the spirochetes adherence to the tick gut (Pal et al., 2004b).

OspB is another known surface lipoprotein which facilitates the binding to the tick gut. OspB is structurally very similar to OspA. It is encoded on the linear plasmid lp54, together with OspA (Fraser et al., 1997). In contrast to OspA, its receptor molecule has not been identified yet (Neelekanta et al., 2007). Moreover, OspD, has also been shown to have a certain influence in the adherence of spirochetes to the tick gut cells (Li et al. 2007).

When a tick starts feeding, it triggers the downregulation of OspA. On the other hand, spirochetes start to significantly increase the production of OspC (de Silva et al., 1996). Several lines of evidence suggest that OspA and OspC are in an inverse relationship. While OspA is needed to adhere to the gut wall, OspC may be involved in the migration of spirochetes from the gut to the salivary glands and furthermore, in establishing an infection within the vertebrate (Gilmore and Piesman, 2000). However, the induction of OspC that turns up during the blood meal does not require the displacement of OspA from the surface.

Pal et al. (2004a) focused in his study on the clarification of the role of OspC during the invasion of the salivary glands. While the spirochetes, that produced OspC, were capable to invade the salivary glands, the OspC-deficient bacteria were not. Once the OspC-deficient spirochetes were complemented with plasmid containing the OspC gene, the colonization of the salivary glands was completely restored. It points out, that OspC is a very important constituent enabling spirochetes binding to the tick salivary glands.

Signals that evocate the switch of expression from OspA to OspC are believed to be rather external than internal. During the tick feeding, the temperature in the tick gut raises from 23°C up to 37°C (Piesman et al., 2001), pH lowers from 7.4 to 6.8 (Yang et al., 2000) and when the spirochetes in the gut come into contact with the blood meal, spirochete cell density increases due to replication (Piesman et al., 2001).

A study carried out by Obonyo et al. (1999) indicates that temperature has totally opposite effect on expression of OspA and OspC by *B. burgdorferi.* Two different strains of this species were cultivated with cells isolated from the tick *Ixodes scapularis*. While the expression of OspC increased with increasing temperature, OspA production lowered with increasing temperature. The raise in

OspC production was more pronounced than the decline in OspA. OspA content was not dramatically influenced by temperature. However, the temperature alone does not appear to be a sufficient signal for OspC induction, because borrelia in the gut of unfed ticks exposed to higher temperatures do not induce OspC expression (Schwan et al., 1995)

1.7. Different opinions on the OspC function

As was already mentioned, during the blood meal, many spirochetes upregulate OspC and downregulate OspA. Based on this relationship it is generally believed (and previous studies pointed to it) that OspC is responsible for the invasion of the salivary glands and the subsequent infection of the host. However, Ohnishi et al. (2001) has demonstrated that OspC was not produced by the vast majority of spirochetes actually invading the salivary glands. His work showed that ticks had to be attached to mice for at least 53 h for stable transmission of spirochetes. The immature specimens which invaded the mice earlier produced predominantly OspA. Moreover, they were noninfectious. Bacteria that entered the host after the 53 h time period consisted mainly of infective specimens that produced neither OspA nor OspC and a minority that produced only OspC. It demonstrates, that the population of spirochetes entering the host is antigenically and genetically polymorphic. It is proposed that OspC facilitates the escape of spirochetes from the tick gut. However, as soon as the bacteria leave the gut, they individually may start downregulating the OspC synthesis, without any influence on the capability of the spirochetes to enter the salivary glands and later on the host (Ohnishi et al., 2001).

The problem of OspC versatility has been discussed even more by Tilly et al. (2006). She also proclaims that OspC is the primary surface protein synthesized by *B. burgdorferi* during the tick feeding but whose production is decreased after the bacteria are transmitted to the mammal. However, she adds that OspC is indeed "indispensable for establishing infection by *B. burgdorferi* in mammals, but is not required at any other point of the mouse-tick infection cycle." Grimm et al. (2004) came with the same findings. Moreover, Tilly argues with Ohnishi et al. (2001) about the importance of OspC in the feeding tick. According to her studies,

spirochetes lacking OspC are capable to replicate in and invade the tick salivary glands but they do not manage to infect the mice.

OspC performs its crucial protective function within 48 h after the borrelia transmission into the mammal (Tilly et al., 2007). However, once the spirochetes adapt to a mammalian host, they can infect and proliferate in the absence of OspC. It is hypothesized "that another protein uniquely present on host-adapted spirochetes performs the same essential function initially fulfilled by OspC". This, by now unknown protein, substitute for OspC and is produced during the persistent infection (Tilly et al., 2009).

The analogous surface-exposed proteins substitution happens also within other *Borrelia* species. *Borrelia hermsii* spirochetes encode the numerous Vsp/Vlp family of outer surface proteins that are homologous to OspC (Carter et al., 1994). Vsp33 (structurally similar to OspC) of *B.hermsii* is present on relapsing fever spirochetes during the migration of bacteria into the host (Schwan and Hinnenbusch, 1998). To promote persistent infection, *B.hermsii* undergoes antigenic variation by expressing different alleles of the *vsp/vlp* gene family in the same way as *B.burgdorferi* does in the case of OspC.

1.8. Tick salivary glands

The salivary glands in ticks are a multifunctional and morphological complex that is critical for the biological success of ticks both during the lengthy periods off their hosts and also during the feeding periods on the host. Hygroscopic fluid produced by the salivary glands allows ticks to absorb water and therefore remain hydrated during the long interval between bloodmeals. When feeding, the rate of salivary fluid secretion increases, allowing the ixodid tick to return about 70% of the fluid and ion content to the host and so to concentrate the bloodmeal. The produced saliva contains many bioactive components that enable the blood-feeding. Various enzymes, inhibitors, histamine agonists and antagonists, prostaglandins, antihaemostatic factors and immunomodulating factors were identified in the tick saliva (Bowman and Sauer, 2004; Sauer et al., 1995).

1.9. Role of the salivary glands

The site of the tick attachment to its host is significantly altered by bioactive molecules present in tick saliva. Exploitation of the pharmacological properties of tick saliva is reckoned to be a decisive factor in successful transmission of tick-borne pathogens. Such an enhancement of pathogen transmission via the action of tick saliva is referred to as saliva-activated transmission (SAT) (Nuttall and Labuda, 2004).

A feeding-inducible protein, Salp15, in saliva of ticks species *Ixodes scapularis* shows an immunosuppressive properties. It inhibits CD4+ T cell activation (Anguita et al., 2002). Salp15 Iric-1, a homologue to *I. scapularis* Salp15 salivary protein, was recently identified in tick *I. ricinus*. It was shown, that spirochetes which bind these proteins are protected from antibody-mediated killing. Further, the binding of Salp15 and Salp15 Iric-1 to OspC was confirmed (Ramamoorthi et al., 2005; Hovius et al., 2008).

The transmission of spirochetes through the tick saliva provides them with another advantages. Tick saliva reduces the rate of phagocytosis (Ribeiro et al., 1990) and inactivates the host complement system (Ribeiro, 1987). Moreover, it was observed that salivary glands extracts enhance chemotactic migration of *B. burgdorferi* spirochetes (Shih et al., 2002).

2. Objectives

The aim of the thesis is to describe some of the factors affecting the invasion ability of *B. burgdorferi* spirochetes within the context of bacteria-tick cell interaction and bacteria-salivary glands interaction.

3. Material and methods

3.1. Material

3.1.1. Animals

Ticks of the species *Ixodes ricinus* were obtained from the housing facility of the Institute of Parasitology, Biology Centre of Academy of Sciences of the Czech Republic in České Budějovice. Salivary glands from fed adult females were used for the experiment.

3.1.2. Chemicals

Tab 1: Prepared solutions and buffers

anti-OspC antibody BBM45		monoclonal, purified antibody, (Baxter, Austria)*
anti-OspA antibody H5332		monoclonal, medium from cells, (Baxter, Austria)
PBS		1x PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.47 mM KH_2PO_4 , pH 7.2)
PBS -Tween 20		0.05% Tween 20 in PBS
Immunostaining	Block buffer	4.5 ml PBS-Tween 20, 0.5 ml goat serum, 3% BSA
	Primary Abs	monoclonal 4C12/C2, anti-Borrelia medium, (Baxter, Austria)
	Secondary Abs	SwAM - conjugated with FITC
Permeabilization agents	Triton X-100	0.2% Triton X-100, 1% NGS in PBS
	Proteinase K	2% Proteinase K in PBS

* **by courtesy of Dr. Ian Livey (Baxter, Austria)**

3.2. Methods

3.2.1. Tick cells cultivation

Cell line derived from the tick *Ixodes ricinus* IRE19 (Leslie Bell-Sakyi; University of Edinburgh; UK) was grown in L15 culture media supplemented with 20% BOFES, 10% TPB and 1% mixture of antibiotics-antimycotics (Sigma; penicilin, streptomycin, amphotericin B) at 34°C.

3.2.2. Bacterial growth conditions

The B31 strain of *Borrelia burgdorferi* was grown at 35°C for 2 weeks in BSK-H medium (Sigma) with 6% rabbit serum (Sigma) and ATB [phosphomycin (0.02 mg/ml), rifampicin (0.05 mg/ml), amphotericin B (2.5 µg/ml)]. The number of spirochetes was determined by dark-field microscopy.

3.2.3. Samples preparation - salivary glands

Salivary glands from fed adult females of tick *I. ricinus* were prepared for confocal microscopy as follows:

Salivary glands were dissected from the live fed ticks and immediately washed in PBS. Subsequently, the glands were incubated with *B. burgdorferi* (one part of spirochetes was previously treated with anti-OspC antibodies).
The incubation lasted either 4 h or overnight at 34 °C. Various strategies were applied in next steps of immunofluorescent detection procedure.

1. The salivary glands were fixed by pre-cooled 100% acetone (15 min). Washing with PBS followed.
2. The salivary glands were fixed in paraformaldehyde/glutaraldehyde mixture (0.5 h at room temperature). Subsequently the glands were washed with pre-cooled 0.02 M glycine in PBS (3x 5 min), permeabilized with Triton X-100 for 20 min on ice and washed once again with 0.02 M glycine in PBS (2x 5 min).
3. The salivary glands (incubated with *B. burgdorferi* treated with anti-OspC) were fixed by incubation in paraformaldehyde/glutaraldehyde mixture (0.5 h at room temperature). Subsequently, the glands were washed with pre-cooled 0.02 M glycine in PBS (3x 5 min), permeabilized with proteinase K for 20 min on ice and washed once again with 0.02 M glycine in PBS (2x 5 min).

3.2.4. Samples preparation - tick cells

$2x10^6$ of spirochetes in about 40 µl of BSK-H cultivation medium were mixed and incubated with each:

1. 1 µl of anti-OspC antibodies
2. 1 µl of anti-OspA antibodies
3. 1 µl of anti-OspC and 1 µl of anti-OspA antibodies
4. no antibodies

at 28°C for 1 h. Tick cells were incubated at the same time under the same conditions. The spirochetes and the tick cells were mixed to a final volume of 1 ml so as it contained $2x10^6$ of spirochetes and $2x10^4$ of tick cells. All samples were incubated at 28°C for 4 h, washed in PBS (3x 2 min) and fixed by ice-cold acetone (20 min). The samples adhered to cover slips were fastened onto the glass slides with nail polish. Immunostaining was carried out the same way as with the salivary glands.

The glass slides were examined in the fluorescence microscope (Olympus BX-60) and shots were taken by camera Olympus C-3030 ZOOM. Pictures were modified and composed in Adobe Photoshop program.

3.2.5. Immunostaining

Salivary glands were incubated first in block buffer for 1,5 h at room temperature and then in primary antibody (1:50 in PBS). After 2 h the salivary glands were washed in PBS-Tween 20 (3x 2 min). After the addition of secondary antibody (1:60 in PBS), all successive steps were performed in the dark. Incubation with secondary antibody lasted for 2 h, followed by washing with PBS-Tween 20 (3x 2min). The cells nuclei were stained with DAPI (1:1000 in PBS) for 15 min at room temperature and washed with PBS-Tween 20 (3x 2min) afterwards. The salivary gland were transferred to glass slides and embedded with anti-fading reagent DABCO. The samples were sealed up with cover slips which were subsequently fastened onto the glass slides with nail polish. The glass slides were examined in a confocal microscope. Pictures were modified and composed in Adobe Photoshop program.

4. Results

4.1. Tick cells-spirochetes interaction

In this study, I examined the invasion ability of *B. burgdorferi* B31 spirochetes within the context of bacteria-tick cell interaction. Further, I investigated whether they are able to penetrate or only adhere to the tick cells within 4 h incubation period. Moreover, the effect of anti-OspA and anti-OspC was assessed.

The obtained data were divided into four categories according to the relative position of spirochetes and tick cells. The categories are as follows:

1. Spirochete is inside the cell
2. Spirochete is on the surface of the cell
3. Spirochete is partly inside and partly outside the cell
4. No spirochetes are present in or on the cell

The measurement was carried out on 200 tick cells. 50 cells for every category were examined. Results are summarized in Tab2.

Tab 2: Percentage of cells attacked by at least one *B. burgdorferi* spirochete. ($^{+}$ It means that 24% of cells were penetrated by at least one spirochete treated with no antibody)

	inside [%]	surface [%]	positive* [%]	no *borrelia* [%]
no antibody	24^{+}	8	8	70
anti-OspA	32	36	26	36
anti-OspC	34	10	14	54
anti-OspA/C	24	6	6	70

* Spirochetes that are partly inside and partly outside the cell

The sum value does not give together 100% because one cell can be invaded by more than just one spirochete.

Results obtained indicated that neither OspA nor OspC have an influence on the *B. burgdorferi* B31 spirochetes binding ability to the tick cells. Spirochetes that were not treated with any antibody and spirochetes treated with both anti-OspA and anti-OspC showed comparable results with regard to their invasion ability. Selected photos depicting tick cell-spirochete interactions are enclosed as Appendix.

4.2. Salivary glands-spirochetes interaction

In this experiment the ability of *B. burgdorferi* B31 spirochetes to bind the salivary glands and a role for OspC in spirochete adherence to this tissue were examined. Several opinions about the importance of OspC in the salivary glands invasion by spirochetes were discussed in the section “Introduction“. According to images of salivary glands obtained by confocal microscopy (enclosed as Appendix), we can see that anti-OspC did not protect *I. ricinus* salivary glands from *B. burgdorferi* B31 colonization. Spirochetes invaded the salivary glands regardless of whether they were treated with anti-OspC or not.

5. Discussion

The purpose of the study was to even more clarify the function of OspA and OspC, since there exists different opinions on their role in the borrelia life-cycle. My results from the first experiment indicate that both OspA and OspC have no influence on the ability of *B. burgdorferi* B31 spirochetes to invade the tick cells. However, a question arises whether these results were caused by the failure of anti-OspA and anti-OspC antibodies to bind to the target proteins. The effectiveness of anti-OspA was already proved by other members of the laboratory. On the contrary, the ability of the used anti-OspC antibody to bind *B. burgdorferi* B31 strain was doubted (information obtained by personal discussion with my supervisor J. Štěrba). The inability of anti-OspC antibody employed in this experiment to bind *B. burgdorferi* B31 strain was also confirmed by A. Walnerová (A. Walnerová, University of South Bohemia, České Budějovice, bachelor thesis, 2010). Therefore, we can only conclude that the OspA-TROSPA interaction is not the driving force (at least not the main one) responsible for the tick cell invasion. This outcome is in line with expectations, as there has not been yet proposed any study that indicates any other role of OspA in the borrelia life-cycle, than just in adherence to the tick gut (Pal et al., 2004a). The experiment was carried out three times but only once reasonably good photos were obtained. The other two attempts were not worth evaluating since the samples were debased by impurities. The tick cells used in the experiment were a mixture from different tissues and we do not know exactly what kinds of cells they were.

Spirochetes invade and colonize a variety of tissues within a given host or vector and salivary glands belong to one of these tissues. There are opinions that both support (Pal et al., 2004a) and oppose (Grimm et al., 2004; Tilly et al., 2007) the necessity for OspC during invasion of salivary glands. *Borrelia burgdorferi* B31 spirochetes were able to invade salivary glands regardless of whether they were treated with monoclonal anti-OspC or not. The results also indicate that anti-OspC used in the experiments were not functional.

6. Conclusion

Anti-OspC antibodies used in this work did not fulfill their function, that is, does not block outer surface protein C of *Borrelia burgdorferi* B31 strain, and therefore did not protect neither ixodid tick cell lines nor tick salivary salivary glands from spirochete invasion. OspA is not responsible for the colonization of tick *Ixodes ricinus* IRE19 cells.

7. List of abbreviations

BOFES	bovine foetal serum
BSA	bovine serum albumin
BSK-H	Barbour-Stoenner-Kelly-H
FITC	fluorescein isothiocyanate
NGS	natural goat serum
PBS	phosphate buffered saline
TPB	triptose phosphate broth
Vlp	variable large protein
Vsp	variable small protein

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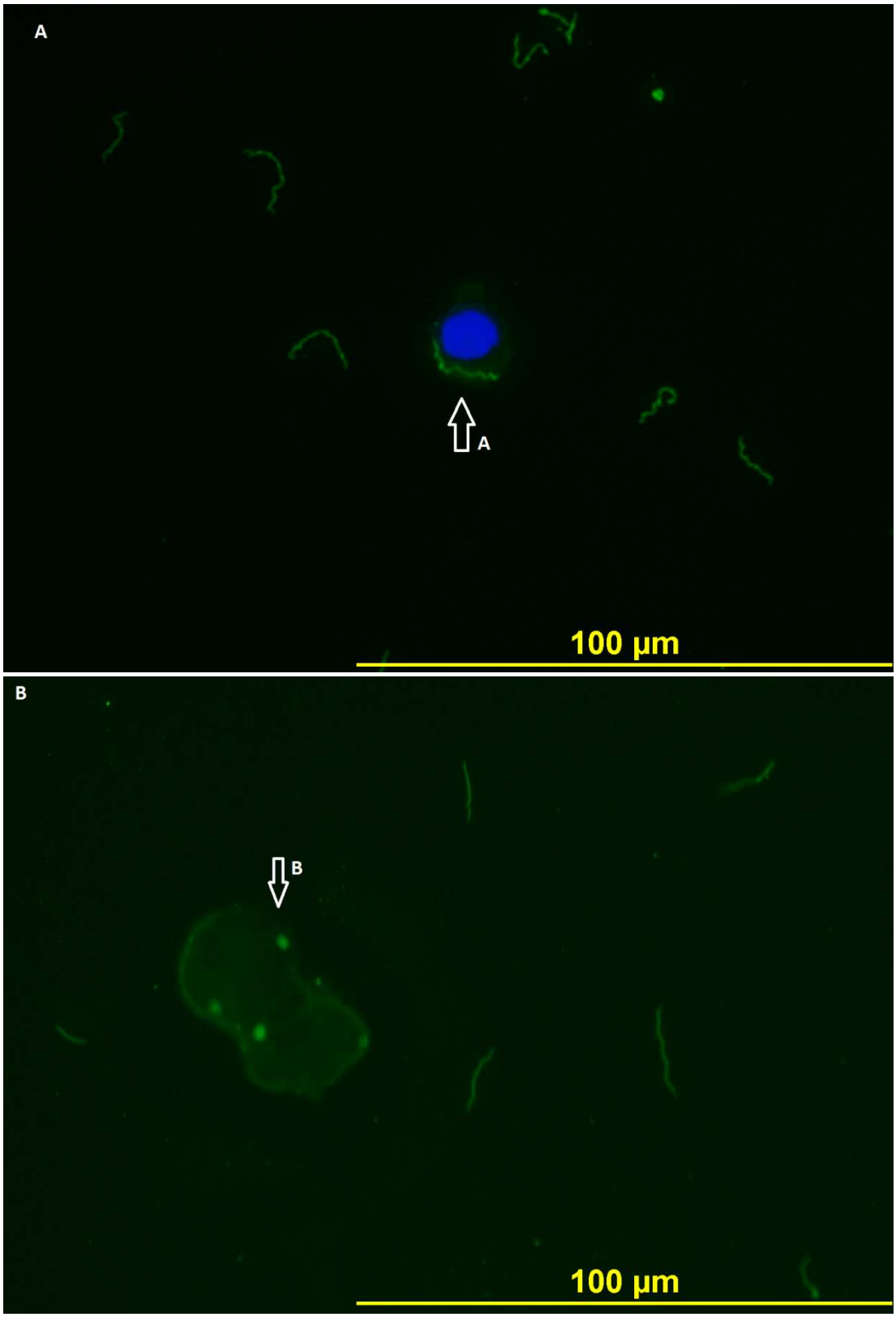
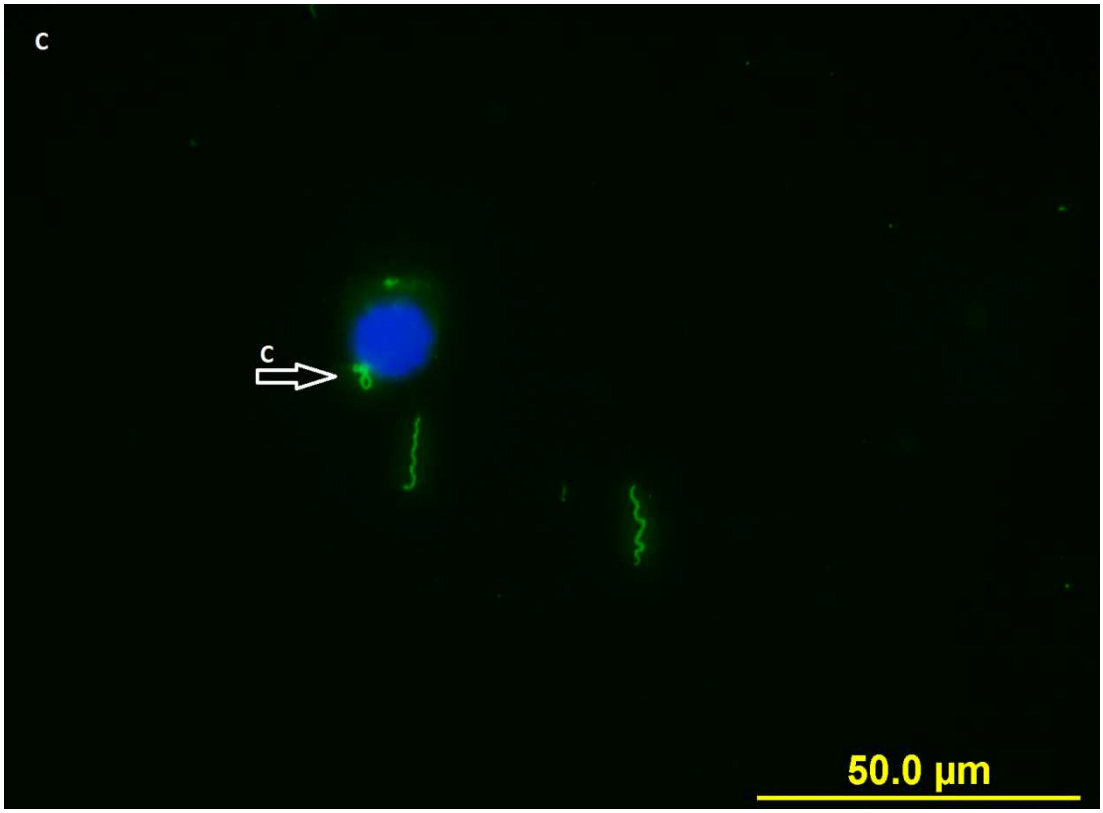
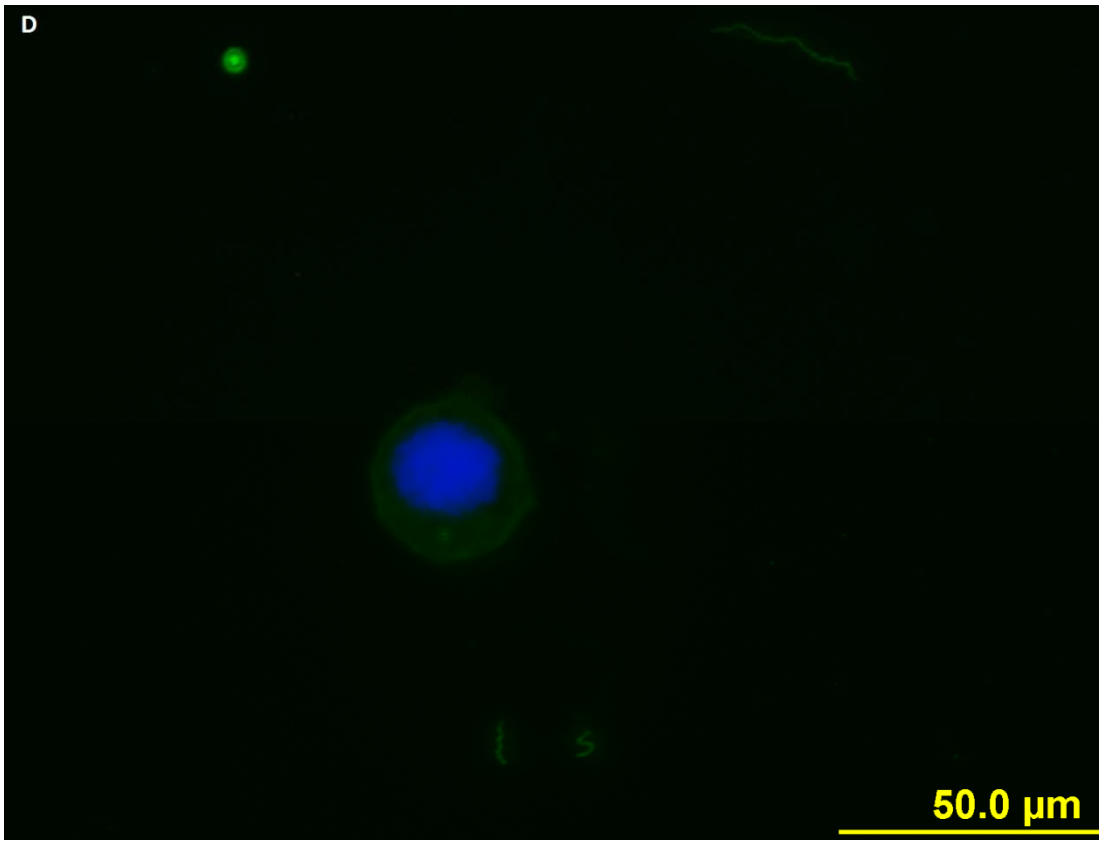
9. Appendix

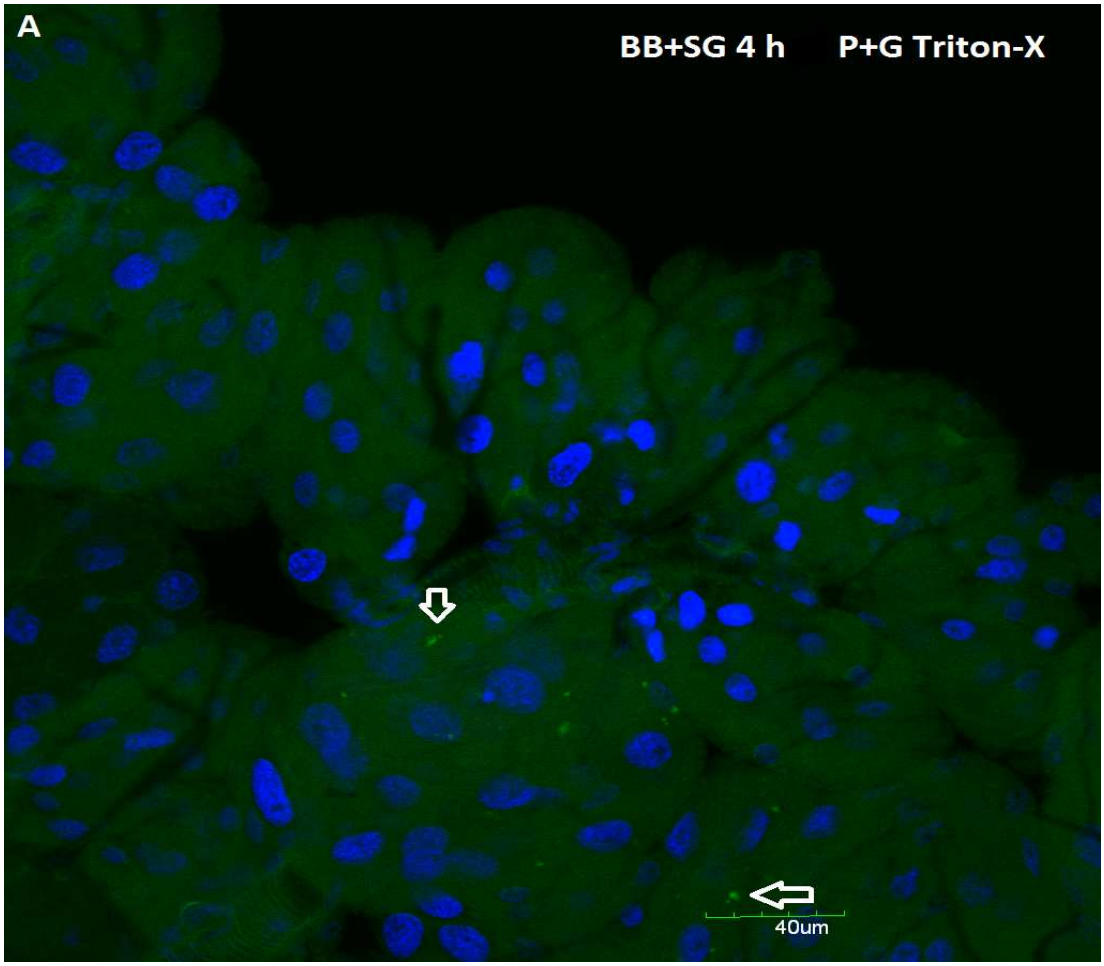
Pic 1: Localisation of spirochetes within the tick cells. (A) Spirochete on the surface of the cell. (B) Spirochete inside the tick cell. (C) Spirochete partly inside and partly outside the cell. (D) Not invaded cell. The cells nuclei were stained with DAPI.

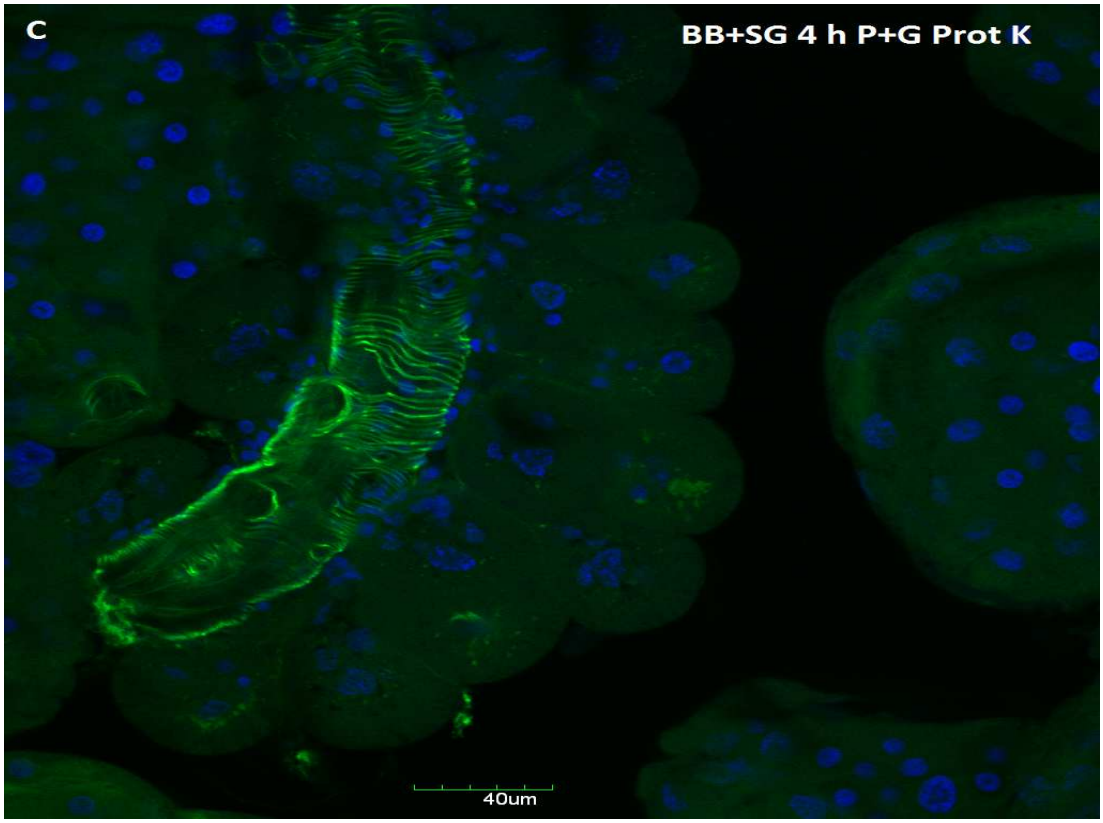


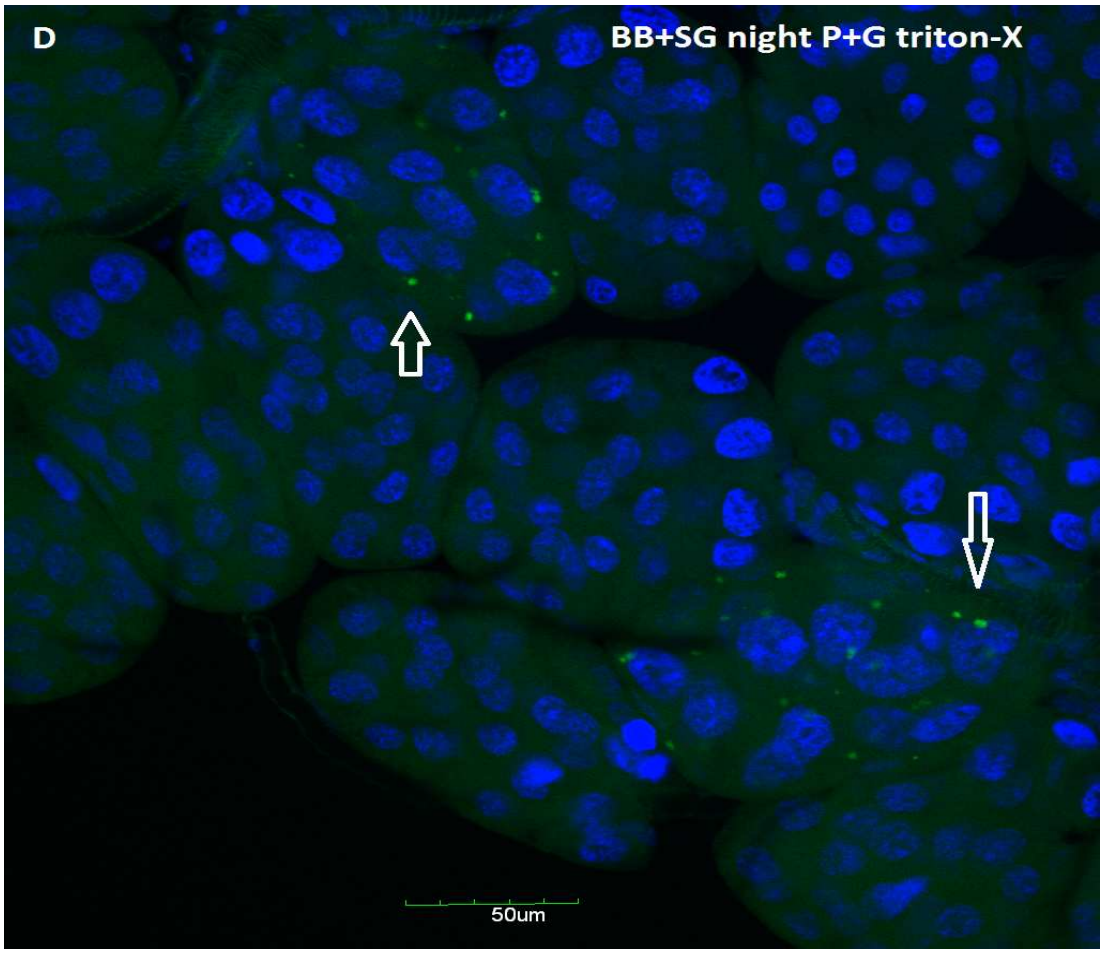

C
C
50.0 µm


D
50.0 µm

Pic2 : Localisation of *B. burgdorferi* B31 spirochetes within the salivary glands of tick *I. ricinus*. Arrows pointed to spirochetes. Salivary glands fixed by paraformaldehyde/glutaraldehyde, permeabilized by (A) triton-X and incubated for 4 h with non-immunized *borrelia* spirochetes; (B) proteinase K and incubated overnight with anti-OpsC immunized *borrelia* spirochetes; (C) proteinase K and incubated for 4 h with anti-OpsC immunized *borrelia* spirochetes; (D) triton-X and incubated overnight with non-immunized *borrelia* spirochetes. The cells nuclei were stained with DAPI.




C
BB+SG 4 h P+G Prot K
40um


D
BB+SG night P+G triton-X
50um