Blood dendritic cells in cattle infected with bovine leukemia virus (BLV): isolation and phenotyping

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Abstract

Dendritic cells (DCs) are most potent antigen presenting cells (APCs) with unique ability to prime effective immune responses. They express higher levels of MHC class II and accessory molecules on their surface, than other professional APCs. The investigations were performed on DCs generated from blood with the use of microbeads magnetically labeled with mouse anti human CD14. Flow cytometry was applied for determination of DCs immunophenotype in healthy and naturally infected with BLV cattle. For immunophenotyping mouse monoclonal antibodies anti bovine: CD11a, CD11b, CD11c, MHC-I and MHC-II were used. Our results demonstrated that dendritic cells infected with BLV expressed very high percentage of determinants: CD11a, CD11b, CD11c, MHC-I and MHC-II class. Leukaemic DCs exhibited DCs morphology and had a phenotype of mature DCs. The expression of gp51 glycoprotein of BLV on leukaemic DCs was detected in flow cytometry investigations.

Key words: cattle, bovine leukemia, dendritic cells, immunophenotype, flow cytometry

Introduction

Bovine leukaemia virus (BLV) belongs to the genus Deltaretroviridae, family Retroviridae and is closely related by genomic organisation and disease progression to human T-cell leukaemia viruses (HTLV-1 and HTLV-2) and simian T-cell leukaemia virus. BLV infection remains subclinical in the majority of cattle, but about one third of infected animals develop persistent lymphocytosis (PL). About 1-5% of infected animals develop lymphosarcoma with or without prior PL (Ferrer et al. 1980). The primary cellular target of BLV is the B-lymphocyte (Paul et al. 1977). The susceptibility of cells other than B lymphocytes to BLV infection is less clear.

Monocytes were first implicated as potential carriers of BLV in sheep on the basis of cell morphology and in situ hybridisation. Some authors reported that
BLV was present in 5% to 40% of adherence purified monocytes, but not in T cells or granulocytes from BLV infected cattle with or without PL. T cell susceptibility for BLV infection was evidenced, when immunoaffinity depletion of B cells and monocytes from peripheral blood or positive selection of T cells with immunomagnetic beads were performed (Schwartz et al. 1994).

Dendritic cells (DCs) are professional antigen-presenting cells, responsible for eliciting an efficient response against pathogens. DCs were first detected in the spleen of mice and described by Steinman and Cohn in 1973. They sample foreign pathogens in the periphery and migrate to lymphoid tissues, where they present processed antigens to naive T cells, initiating an immune response (Banchereau et al. 1998). These cells are distributed especially in tissues that interface with an external environment, such as the skin, gut and lungs (Sertl et al. 1986, Nestle et al. 1993, Nelson et al. 1994). Due to these locations, they can perform a sentinel function for incoming pathogens, and have the capacity of recruiting and activating cells of the innate immune system upon inflammation (Sallusto et al. 1998, Fernandez et al. 1999, Rescigno et al. 1999).

Uptake of pathogens by DCs induces a state of activation, which leads to the migration of an antigen-loaded DCs to lymphoid organs, where the cells of an adaptive immune response can be excited (Moll et al. 1993). DCs show a high degree of phenotypical and functional heterogeneity. Different DC lineages have been described, found at various anatomical locations and showing a distinct set of surface molecular markers. For example, CD8α-negative myeloid DCs were found in peripheral, nonlymphoid tissues and in the secondary lymphoid organs including Langerhans cells of the skin, interstitial dendritic cells in various organs, and marginal zone DCs in the spleen. DCs with CD8α molecule, called lymphoid DCs, are exclusively found in the T cell areas of the secondary lymphoid organs and in the thymus (Vremec et al. 1997).

Whereas the origin, development and function of myeloid DCs are well recognized, both developmental pathways and functions of lymphoid dendritic cells are still not exactly defined. DCs that circulate in blood or reside in solid tissues are immature (O’Doherty et al. 1994, Weissman et al. 1995). They can effectively endocytose antigens, although they express the accessory molecules needed for T cell activation at low levels. However, after antigen uptake and exposure to stimulatory signals, DCs mature. As a consequence of maturation, DCs express molecules that enable them to effectively bind and activate T cells in a manner that promotes immune responses (Banchereau et al. 1998), including those that are necessary for effective immunotherapy. The application of DCs ex-vivo transduced with a virus coding for one or multiple TAA (Tumour Associated Antigens) might have several potential advantages over the technically easier direct vaccination (Toes et al. 1999).

Two methods have been used to investigate dendritic cells in cattle: the cannulation of pseudoafferent lymphatic ducts and generation from monocytes cultured in the presence of GM-CSF and IL-4 (Sallusto et al. 1998).

The aim of the present study was generation of dendritic cells from bovine blood monocytes and determination of subpopulations and morphology of these cells in healthy and leukaemic cattle.

**Materials and Methods**

**Animals**

Investigations were performed on 19 cows naturally infected with BLV and 12 healthy cows, at the age of 4-7 years, polish black and white lowland breed.

**Generation of dendritic cells from monocytes**

Investigations were performed on two groups of cattle: healthy and naturally infected with BLV, positive in ELISA and PCR tests. For dendritic cells preparation, peripheral blood mononuclear cells were isolated from 100 ml of whole blood treated with EDTA-K2, by standard density centrifugation in Histopaque (Sigma). After centrifugation cells were collected and CD14 positive cells were separated by magnetic sorting using VarioMACS technique (Miltenyi Biotec GmbH) according to manufacturer’s protocol. Briefly, blood samples were centrifuged for 30 min, at 4°C, 2300 rpm. After thatuffy-coat was harvested and AutoMACS Rinsing Solution was added to the volume of 35 ml. This cells suspension was divided in two parts, loaded on the Histopaque gradient (1.077) and centrifuged for1h, 4°C, 2000 rpm. Then, cells from interphase were transferred to the new tubes, the AutoMACS Rinsing Solution was added and cells were centrifuged 3 times, 10 min each, 2000 rpm. The supernatant was discarded, cells were counted and concentration of 10^7/ml was prepared. To the suspension of 10^7 cells, the amount 80 μl of AutoMACS Rinsing Solution supplemented with 0.5% FCS was added, gently mixed and then 20 μl of immunomagnetic microbeads conjugated with monoclonal mouse anti human CD14 antibody was added and cells were incubated 15 min at temperature 4-8°C. Then, cells
were washed, centrifuged at 1500 rpm for 10 min. Supernatant was discarded and $10^8$ of the cells pellet was resuspended in 500 μl of AutoMACS Rinsing Solution with 1% of FCS. The cells separation was performed on the MACS LS Column (Miltenyi Biotec). The column was placed in the magnetic field of MACS Separator and column was prepared by filling and rinsing with 60 ml of buffer AutoMACS. Then 3 ml of AutoMACS Rinsing Solution with 1% of FCS was added on a magnetic column and cells suspension was placed into the column. After that, column was washed 3 times with 3 ml each of AutoMACS Rinsing Solution with 1% FCS. The eluted fluid containing unlabeled cells was discarded. The labeled cells were eluted from the column by removal the magnetic column from the magnetic source, then 5 ml of the AutoMACS Rinsing Solution with 1% of FCS was placed on the column and cells were flushed out by firmly applying the plunger supplied with the column.

**Dendritic cells culture**

Cells were cultivated, according to the procedure modified and described by Szczotka (Szczotka et al. 2009). Briefly, CD14+ isolated cells were cultured at a concentration of $1 \times 10^6$ cells/ml in standard culture flasks, in RPMI 1640 medium containing 10% FCS, glutamax and supplemented with antibiotics. The cells were incubated at 37°C, in a humidified atmosphere with 5% CO₂, in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4). On day 3, about half of the culture medium was removed from flasks and replaced with a fresh complete medium containing GM-CSF and IL-4. The cells were fed with fresh medium every 3 days, for 4 weeks. Every week one part of cells was analysed in flow cytometer and under a microscope: cells from cultures were collected, washed and submitted for light and scanning microscopy examination, as well as flow cytometry.

**Flow cytometry of dendritic cells**

Dendritic cells were counted and a concentration of cells 600 000/ml was prepared. Then, cells were coupled with 1 μl of mouse monoclonal antibodies directed against human CD14 (Miltenyi Biotec), and mouse anti bovine CD11a, CD11b, CD11c, MHC-I, MHC-II and anti-BLV-gp51 (VMRD Pullman) and incubated for 15 min at room temperature. Next, cells were 3 times washed in phosphate-buffered saline (PBS) containing γ globulin- free horse serum, centrifuged and the cell pellet was incubated with 1 μl of goat-anti- mouse (IgG+IgM) H+L-FITC conjugate (Biosource Camarillo, CA) for 15 min, at room temperature, in darkness. After 3 washings in PBS buffer with horse serum and another 3 washings in PBS buffer without horse serum, the cell pellet was resuspended in PBS buffer containing formaldehyde and flow cytometry analysis was performed.

**Determination of dendritic cells morphology**

Morphology of dendritic cells was determined with a light and scanning electron microscopes. For the analysis with the light microscope smears of cells were fixed and stained according to the May-Grünwald method. For scanning microscopy, the cells were attached to the plastic coverslip that had been coated with 15 poly-L-lysin in water for 15 min. The cells were fixed with 1.2% glutaraldehyde in 0.1 M PBS, pH 7.4, passed through an alcohol gradient, dried and prepared for examination with scanning electron microscope (SEM).

**Results**

After immunomagnetic separation procedure we obtained positively enriched (95%-97%) labeled CD14 cells and 98% of viability (estimated by trypan blue exclusion). The CD14 positive cells had typical monocytes morphology, with large nucleus and small cytoplasm. These cells cultured in the presence of GM-CSF and IL-4 in growth medium generated to the dendritic cells. These cells had characteristic for blood and lymph dendritic cells dendrites and veiled cytoplasm. In the cell culture we observed dendritic cells on different levels of maturity. There were immature cells with characteristic Birbeck granules, as well as mature cells, in which granules were small or absent, but they had dendrites-like processes and lamellipodia (Fig. 1, 2). DCs observed in SEM had delicate veiles and edges divided on the end (Fig. 3, 5). These structures enable to take up and process many particles of antigens in the same time and to present them to the lymphocytes (Fig. 6). In the 72 h culture of monocytes almost all cells had typical for DCs shape, veiles and edges (Fig. 3). Fig. 4 presents dendritic cells harvested from the cell culture and prepared for investigations in flow cytometry.

The results of dendritic cells immunophenotyping and the fluorescence intensity of cells investigated with flow cytometer are presented in Fig. 7 and Fig. 8.
Fig. 1. Formation of dendritic cells in monocytes cell culture (24 h cell culture, x20).

Fig. 2. The 24 h monocytes cell culture (SEM x1000).

Fig. 3. The 72 h cell culture of DCs (SEM x2000).

Fig. 4. Dendritic cells harvested from cell culture for flow cytometry (x100).

Fig. 5. Mature blood dendritic cell (SEM x10000).

Fig. 6. Blood dendritic cell with typical morphology: long dendrite and veil (SEM x10000).
Fig. 7. Expression of surface molecules related to dendritic cells generated from monocytes of a healthy cow.
CD14 (4.8%)

CD14 (8.8%)

CD11a (11.8%)

CD11a (7.7%)

CD11b (69.4%)

CD11b (76.4%)

CD11c (93.7%)

CD11c (91.4%)

Fig. 8
Fig. 8. Expression of BLV-gp51glycoprotein and surface molecules related to dendritic cells generated from monocytes of two cows naturally infected with BLV.

Table 1. The immunophenotype of dendritic cells in 19 leukaemic and 12 control cows (% cells – mean values).

<table>
<thead>
<tr>
<th></th>
<th>BLV(+)</th>
<th>CD 14</th>
<th>CD 11a</th>
<th>CD 11b</th>
<th>CD 11c</th>
<th>MHC I</th>
<th>MHC II</th>
<th>gp51</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 week</td>
<td>83.6</td>
<td>42.5</td>
<td>89.5</td>
<td>58.4</td>
<td>84.6</td>
<td>17.0</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>2 weeks</td>
<td>87.9</td>
<td>53.0</td>
<td>84.9</td>
<td>30.0</td>
<td>79.5</td>
<td>16.4</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>3 weeks</td>
<td>92.7</td>
<td>63.2</td>
<td>95.7</td>
<td>31.6</td>
<td>77.5</td>
<td>20.5</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>4 weeks</td>
<td>31.0</td>
<td>13.8</td>
<td>42.1</td>
<td>10.1</td>
<td>4.7</td>
<td>4.0</td>
<td>1.2</td>
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<table>
<thead>
<tr>
<th></th>
<th>BLV(-)</th>
<th>CD 14</th>
<th>CD 11a</th>
<th>CD 11b</th>
<th>CD 11c</th>
<th>MHC I</th>
<th>MHC II</th>
<th>gp51</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 week</td>
<td>49.4</td>
<td>38.8</td>
<td>67.3</td>
<td>26.5</td>
<td>80.4</td>
<td>20.5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2 weeks</td>
<td>41.6</td>
<td>27.3</td>
<td>75.7</td>
<td>26.0</td>
<td>51.3</td>
<td>9.2</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>3 weeks</td>
<td>88.4</td>
<td>23.4</td>
<td>72.2</td>
<td>11.2</td>
<td>28.9</td>
<td>10.0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4 weeks</td>
<td>12.7</td>
<td>10.4</td>
<td>41.3</td>
<td>2.1</td>
<td>1.4</td>
<td>30.7</td>
<td>–</td>
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Mann-Whitney U Test was used for statistical analysis, significant at (p<0.05).
Dendritic cells isolated from leukaemic cattle had high expression of CD14. In the first week of incubation the cells culture was composed of 83.6% of these cells. We observed that these values raised in the next two weeks up to 92.7% with decrease to 31% one week later. In the control group this percentage was significantly lower (49.4%), but in the third week of culture it increased almost twice (up to 88.4%), then rapidly decreased to 12.7%. The level of cells with CD11a marker expression elevated from 42.5% in the first week of culture to 63.2% in the third week, then was only 13.8%. The percentage of CD11a cells in the third week was much higher in infected cows and was statistical significant (p<0.05), but in the fourth week the percentages of these cell decreased. In the control animals the percentage of these cells was lower (38.8%) and had tendency to constantly diminish, up to 10.4%. The percentage of cells with CD11b marker was high in BLV-positive animals in the first week of culture, reaching (89.5%) and increased in the third week to the value of 95.7% (statistical significant). In the control animals the percentage of these cells was lower (38.8%) and had tendency to constantly diminish, up to 10.4%. The percentage of cells with CD11b marker was high in BLV-positive animals in the first week of culture, reaching (89.5%) and increased in the third week to the value of 95.7% (statistical significant). In the control animals the percentage of these cells was lower (38.8%) and had tendency to constantly diminish, up to 10.4%. The percentage of cells with CD11b marker was high in BLV-positive animals in the first week of culture, reaching (89.5%) and increased in the third week to the value of 95.7% (statistical significant). In the control animals the percentage of these cells was lower (38.8%) and had tendency to constantly diminish, up to 10.4%. The percentage of cells with CD11b marker was high in BLV-positive animals in the first week of culture, reaching (89.5%) and increased in the third week to the value of 95.7% (statistical significant). In the control animals the percentage of these cells was lower (38.8%) and had tendency to constantly diminish, up to 10.4%. The percentage of cells with CD11b marker was high in BLV-positive animals in the first week of culture, reaching (89.5%) and increased in the third week to the value of 95.7% (statistical significant). In the control animals the percentage of these cells was lower (38.8%) and had tendency to constantly diminish, up to 10.4%. The percentage of cells with CD11b marker was high in BLV-positive animals in the first week of culture, reaching (89.5%) and increased in the third week to the value of 95.7% (statistical significant). In the control animals the percentage of these cells was lower (38.8%) and had tendency to constantly diminish, up to 10.4%. The percentage of cells with CD11b marker was high in BLV-positive animals in the first week of culture, reaching (89.5%) and increased in the third week to the value of 95.7% (statistical significant). In the control animals the percentage of these cells was lower (38.8%) and had tendency to constantly diminish, up to 10.4%. The percentage of cells with CD11b marker was high in BLV-positive animals in the first week of culture, reaching (89.5%) and increased in the third week to the value of 95.7% (statistical significant). In the control animals the percentage of these cells was lower (38.8%) and had tendency to constantly diminish, up to 10.4%. The percentage of cells with CD11b marker was high in BLV-positive animals in the first week of culture, reaching (89.5%) and increased in the third week to the value of 95.7% (statistical significant). In the control animals the percentage of these cells was lower (38.8%) and had tendency to constantly diminish, up to 10.4%. The percentage of cells with CD11b marker was high in BLV-positive animals in the first week of culture, reaching (89.5%) and increased in the third week to the value of 95.7% (statistical significant). In the control animals the percentage of these cells was lower (38.8%) and had tendency to constantly diminish, up to 10.4%. The percentage of cells with CD11b marker was high in BLV-positive animals in the first week of culture, reaching (89.5%) and increased in the third week to the value of 95.7% (statistical significant). In the control animals the percentage of these cells was lower (38.8%) and had tendency to constantly diminish, up to 10.4%.
Discussion

For many years it has been very difficult to obtain large numbers of DCs. With the development of recombinant cytokines and culture techniques it is now possible to generate large numbers of DCs in vitro (Ye et al. 1996). These cells can be adapted as antigen carriers for tumour vaccination (Weissman et al. 1995, Wischatta et al. 2000, Yi et al. 2002). Due to their high expression levels of costimulatory and adhesion molecules and their exquisite ability to produce cytokines, such as IL-12 and IL-18, they are the most effective T-cell activators (Xiao et al. 2003, Xiao et al. 2004). These characteristics make them of particular interest as adjuvant agents in cancer vaccine preparations. Promising clinical results have even been obtained by using DCs fused with whole tumour cells, by ex vivo transduction of DCs using either RNA or replication-defective recombinant viral vectors to introduce genes encoding antigen. Generation of dendritic cells from the blood monocytes was very useful for DCs immunophenotype determination in flow cytometry and microscopical analysis.

Our observations performed during 4 weeks showed that CD surface antigens presented on the DCs were different and dependent on the maturity of these cells. We observed statistical essential differences in the expression of CD11a, CD11b and CD11c on dendritic cells in third week of culture in vitro and rapid decrease of the expression of almost all CD markers in the fourth week in both group of cows. Mature DCs are more immunogenic than immature DCs in mice (Inaba et al. 2000), and there is good evidence that this also applies to humans. Mature DCs express a high number of costimulatory molecules and more MHC-peptide complexes with a longer half-life (Kukutsch et al. 2000). In direct comparison in melanoma patients, intranodally injected peptide-pulsed mature DCs led to a potent T cell response whereas immature DCs failed to do so (Jonuleit et al. 2001). Recent studies have shown that immature DCs can even silence the immune system. Repetitive stimulation of naïve CD4+ T cell with immature DCs results in IL-10 producing regulatory T cells (Jonuleit et al. 2000). The experiments performed by Dhodapkar (Dhodapkar et al. 1999) in vivo on healthy volunteers and advanced melanoma patients with fully mature DCs have demonstrated that both antigen-specific CD8+T cells and IFN-γ Th1 T cells can be rapidly induced (Schuler-Thurner et al. 2002). They recommended the use of mature DCs for cancer therapy. Mature DCs exhibit a stable phenotype and are more immunogenic, easier to cryopreserve and even resistant to CTL-mediated lysis (Moll et al. 1993). The results presented in this report demonstrate that bovine in vitro-derived DCs can be generated using methods available for human DC generation. These DCs will aid further characterization of bovine dendritic cells biology and their relations with infectious agents.

DCs possess a unique ability to environmental stimuli, leading to different functional phenotypes based on cell surface markers and production of cytokines such was observed in Langerhans cells, plasmacytoid and myeloid DCs. In a clinical perspective this is of interest since immunomodulation of specific DCs subset leads to activation of different DCs involved in the generation of T cell mediated anti-tumour immunity. In patients with a high risk of metastatic disease or in a state of minimal residual disease, potentiation of systemic or local T cell specific immunity by modulation of DCs in tumour draining lymph nodes may establish effective immunosurveillance (Zhou et al. 2002).

DCs play a very promising role in therapy of leukemias and tumors or in immunomodulation, but still questions remain over what is the best type of subtype of DCs to be used, route of administration, dose of antigen required to induce a response, and maintenance of therapy and control.

References


Distribution of the ymoA and ystA genes and enterotoxins Yst production by Yersinia enterocolitica strains isolated from humans and pigs

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Abstract

Yersinia (Y.) enterocolitica is the third etiological agent of human diarrhea in terms of the number of confirmed clinical cases. One of the important virulence markers is the yst gene which encodes the production of enterotoxins Yst (Yersinia stable toxins). However, not all strains with yst genes produce enterotoxins, what seems to be caused by the ymoA gene encoding the production of the YmoA protein inhibiting the expression of various genes. The purpose of our study was to evaluate the distribution of the ymoA and ystA genes and Yst production by Y. enterocolitica isolated from humans and pigs. All the studied strains obtained from pigs had the ystA gene which indicates that they belong to the group of strains commonly regarded as pathogenic, but the ability to produce YstA was detected in only 14 out of 96 examined strains. The fragments of ystA gene were also detected in all Y. enterocolitica strains isolated from human cases of diarrhea. Amplification of a fragment of the ymoA gene was detected in all the studied strains, both from humans and pigs, based on the presence of a 330 bp band. Thus no correlation was identified between the occurrence of the ymoA and ystA genes and the production of a specific type of enterotoxin.

Key words: Y. enterocolitica, enterotoxins Yst, YmoA protein, multiplex PCR, suckling mouse bioassay

Introduction

Yersinia (Y.) enterocolitica is the third aetiological agent of human diarrhea in terms of the number of confirmed clinical cases, after Campylobacter spp. and Salmonella spp. (Osek et al. 2011). The correlation between strains isolated from pigs and those which evoke clinical signs in humans has been sufficiently proven, justifying selection of this animal species as the study object (Boghenbor et al. 2006, Kelesidis et al. 2008, Farzan et al. 2009, Laukkanen et al. 2009). Due to the symptomatic diversity of the disease, it is extremely difficult to recognise it by the clinical picture alone, both in humans and in animals. Laboratory tests necessary for proper diagnosis are time- and labour-consuming. Therefore, molecular methods involving detection of virulence markers are more frequently applied. One of the most important virulence markers is the yst gene which encodes enterotoxins Yst (Yersinia stable toxins) production.
Yst is a 30-amino-acid peptide whose properties are very similar to those of STI (a heat-stable toxin type I) produced by *Escherichia* (*E.*) *coli*. They have the same mechanism of action, based on guanylate cyclase activation resulting in the increased cGMP levels in enterocytes and extracellular accumulation of liquid in the intestine (Delor et al. 1990, Revell et al. 2001). Opponents of the theory indicating Yst as the key factor of diarrhea induction in the course of yersiniosis have pointed the lack of Yst production in a temperature over 30°C and the biological activity of those environmental strains commonly regarded as non-pathogenic. Taking into account that the temperature in the intestines is around 37°C, the role of the enterotoxin Yst in diarrhea induction has seemed doubtful. However, the studies of Singh et al. (2004) showed that the ability to produce Yst at a temperature of 37°C in a slightly alkaline environment (pH 7.5) is the same as the ability to produce Yst at temperatures below 30°C. That kind of alkaline environment is present in the end sections of the gastrointestinal tract where *Y. enterocolitica* is usually found. Environmental strains, on the other hand, show the ability to produce an alternative enterotoxin whose mechanism of action is similar to the classical Yst and very comparable to STI. Ramamurthy et al. (1997) called this variant YstB to distinguish it from the original YstA. The *ystB* gene encoding the production of the new variant of Yst was found in 36 out of 304 strains of *Y. enterocolitica* originating from 18 different countries and was always correlated with 1A biotype strains. Additionally, out of 36 strains with the *ystB* gene tested for biological activity using the suckling mouse bioassay, as many as 18 produced the enterotoxin. The role of 1A biotype strains, so far considered non-pathogenic, in the etiology of yersiniosis is also confirmed by the increasing number of clinical cases (Bottone 1999, Grant et al. 1999). One should therefore revise the existing classification into pathogenic and non-pathogenic strains based only on their biotype. Huang et al. (1997) determined the nucleotide sequence of the *ystC* gene, another variant of the classic *yst* gene encoding the enterotoxin production. While *ystA* and *ystB* consist of 30 amino-acids, *ystC* consists of 53 amino-acids and has a larger molecular mass. On the amino-acid level, there is approximately a 50% similarity between C- and N-ending *YstC* and *YstA* chains. C-ending 13-amino-acid region of *ystA* and *ystB* correspond to a strongly conservative sequence characteristic for all thermostable toxins (so-called “toxic domain”).

The ability of Yst production has been demonstrated in pathogenic strains isolated from clinical cases of yersiniosis. It indicates that this plays an important role in the etiology of diarrhoea occurring in the course of the disease. However, not all the strains possessing *yst* genes produce the enterotoxins. It seems to be caused by the *ymoA* gene encoding production of the *YmoA* protein inhibiting the expression of various genes (Platt-Samoraj et al. 2006). This protein, considered to belong to the growing family of conservative Hha proteins with a small molecular mass, shows similarities to proteins of the H-NS group (Ellison et al. 2006). In intestinal bacteria H-NS proteins play an important role, both as structural proteins and as gene expression modulator, including virulence markers (Dorman 2004). The expression of genes directly responsible for the pathogenicity of *Y. enterocolitica* has been the subject of many studies. It was proved that this process is significantly influenced by temperature, but the mechanism is not entirely clear. It is supposed that *YmoA* is the key regulator of gene expression by environmental factors, including temperature (Madrid et al. 2007, Banos et al. 2008). It was also proved that the *YmoA* protein inhibits the expression of the *inv* gene encoding invasion – an important virulence factor responsible for the transport of bacteria cells through M cells (Ellison et al. 2003) and participating in the temperature-dependent production of *Yersinia* outer proteins (Yops) and *Yersinia* adhesin (YadA) – plasmid virulence markers (Cornelis et al. 1991). World literature points to the role of *YmoA* and related proteins (H-NS) in the regulation of gene expression in various microorganisms, e.g. *E. coli*, whereas there are only few reports on *Y. enterocolitica* (Cornelis et al. 1991, Ellison et al. 2003, 2006, Brzostek et al. 2007, Banos et al. 2008, Fang et al. 2008).

The purpose of our study was to evaluate the distribution of the *ymoA* and *ystA* genes and Yst production by *Y. enterocolitica* isolated from humans and pigs.

**Materials and Methods**

**Bacterial strains**

The material for the study consisted of 86 *Y. enterocolitica* strains isolated from humans and 96 *Y. enterocolitica* strains isolated from clinically healthy fattening pigs. The *Y. enterocolitica* strains from humans were isolated in the 2010 and 2011 years from clinical cases of yersiniosis and confirmed by the National Institute of Public Health – National Institute of Higiene in Poland. Both human and pig strains of *Y. enterocolitica* were then bio- and serotyped according to the PN-EN ISO 10273 standard. *Y. enterocolitica* strains isolated from the fattening pigs mostly belonged to biotype 4 – only 1 strain belonged...
The purified DNA was stored in a test tubes at -20°C following the manufacturer’s instructions. Most Y. enterocolitica strains isolated from the humans belonged to biotype 4, serotype O:3; only 6 strains belonged to biotype 1B, serotype O:8 and 3 belonged to biotype 2, serotype O:9.

DNA isolation

Genomic DNA isolation was performed with the “Genomic Mini” kit (A&A Biotechnology, Gdynia, Poland) designed for the isolation of DNA from bacteria, cell cultures, and solid tissues. The isolation was carried out following the manufacturer’s instructions. The purified DNA was stored in a test tubes at -20°C for further analyses.

Primers and multiplex PCR conditions

The multiplex PCR method involved the amplification of four gene fragments: ystA, ystB, ystC and ymoA. The primer sequences (synthesised at Oligo, Warsaw, Poland) were obtained from the papers by Platt-Samoraj et al. (ystA, ystB) (2007), Grant et al. (ymoA) (1998) and Bancerz-Kisiel et al. (ystC) (2011b).

A multiplex PCR was carried out using HotStarTaq Plus DNA Polymerase (Qiagen, Hilden, Germany) and the HotStarTaq Plus Master Mix Kit (Qiagen, Hilden, Germany). The reaction mixture of 20 μl volume contained: about 120 ng of isolated DNA (from 1 to 3 μl), 10 μl HotStarTaq Plus Master Mix 2x, 2 μl CoralLoad Concentrate 10x, 0.1 μl of each of the primers (final concentration 0.5 μM), and the total was supplemented up to 20 μl with RNase-Free Water. The applied reaction conditions were published previously (Bancerz-Kisiel et al. 2011b). Electrophoresis separation was performed in a 2% agarose gel containing 0.5 μg/ml of ethidium bromide, and the size of the obtained products was evaluated by means of a comparison with the standard GeneRuler™ 100 bp Ladder Plus (Fermentas, Lithuania).

Suckling mouse bioassay

The ability to produce enterotoxin by Y. enterocolitica isolated from pigs was demonstrated using suckling mouse bioassay (infant BALB/c mice) according to Giannella (1976) with two small modifications. The first modification was the way by which the supernatant for the inoculation was prepared. In our study each strain was cultured into a tryptic soy broth containing 0.6% yeast extract (TSB-YE) and incubated at 25°C for 48 hours. The second modification was the 2 hours interval between supernatant inoculation and euthanasia, according to Nunes et al. (1981), who showed that the peak of the Yst production takes place between 1 and 2 hours after inoculation. The particular modifications and the reasons for them were precisely described by the authors previously (Bancerz-Kisiel et al. 2011a). Ratio of intestinal mass to the rest of the body mass in the group of three examined sucklings was the basis for the bioassay interpretation. Ratio ≤ 0.074 was regarded as a negative result, 0.075 – 0.082 as a doubtful result and ratio ≥ 0.083 was regarded as a positive result.

The ability to produce Yst by Y. enterocolitica isolated from humans was confirmed by the fact that all the strains were isolated from clinical cases of yersiniosis with diarrhea.

Results

Molecular examinations of human and animal Y. enterocolitica strains showed that all the studied strains had the ystA gene. An amplification of a fragment of the ymoA gene was also detected in all the studied strains. No 180 and 134 bp bands indicating, respectively, the presence of the ystB and ystC gene amplicons were also obtained. The results of the multiplex PCR were documented with photographs of the agarose gels (see example in Fig. 1).

The study of the ability to produce enterotoxin Yst by Y. enterocolitica strains isolated from pigs using suckling mouse bioassay showed positive results in 14 cases, constituting 14.58% of all the examined strains. Doubtful results were obtained in case of 33 strains (34.37%), whereas the remaining 49 strains (51.04%) did not show Yst production. The ability to produce Yst by Y. enterocolitica isolated from humans were confirmed by the fact that all the strains were isolated from clinical cases of yersiniosis with diarrhea.

The results of multiplex PCR, detecting virulence markers and enterotoxic properties taking into consideration the biotype, serotype and the number of examined Y. enterocolitica strain are presented in Table 1.

Discussion

The results obtained in our study reveal several new and interesting data on epidemiology and the pathogenesis of Y. enterocolitica infections and corre-
Fig. 1. Electrophoretic separation of products of ystA, ystB, ystC and ymoA gene amplification from Y. enterocolitica strains in multiplex PCR.

M – size marker GeneRuler™ 100bp DNA Ladder Plus (Fermentas).
Lane 1 – positive control, contains DNA isolated from a reference strain of Y. enterocolitica O:8 (ACTT 23715).
Lane 2 – positive control, contains DNA isolated from a reference strain of Y. enterocolitica O:5.
Lane 3 – negative control, does not contain bacterial DNA, contains ystA, ystB, ystC and ymoA gene primers.
Lanes 4 – 6 – products of amplification of ystA and ymoA gene fragments Y. enterocolitica strains isolated from pigs.
Lanes 7 – 9 – products of amplification of ystA and ymoA gene fragments Y. enterocolitica strains isolated from humans.

Table 1. Comparison of the biotypes, serotypes, genotypes and enterotoxic properties of Y. enterocolitica strains isolated from humans and pigs.

<table>
<thead>
<tr>
<th>Source</th>
<th>Biotype</th>
<th>Serotype</th>
<th>Number</th>
<th>Virulence markers</th>
<th>Enterotoxins production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ystA</td>
<td>ystB</td>
</tr>
<tr>
<td>Humans</td>
<td>1B</td>
<td>O:8</td>
<td>6</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>O:9</td>
<td>3</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>O:3</td>
<td>77</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Pigs</td>
<td>2</td>
<td>NI</td>
<td>1</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>O:3</td>
<td>77</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>O:9</td>
<td>8</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>NI</td>
<td>10</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NI – non-identified</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

According to the reports of Ramamurthy et al. (1997) based on a study of 304 Y. enterocolitica strains, as much as 78.5% of strains categorized as pathogenic had the ystA gene, while ystB and ystC genes in this group were very rare. In the group of non-pathogenic strains, the ystA gene was considerably less common (26.1%) than the ystB gene (67.4%). The ystC gene was detected in only 3 strains of Y. enterocolitica: 2 from Japan and 1 from Belgium. In our study all the examined Y. enterocolitica strains isolated from pigs showed the presence of ystA gene and should be categorized as pathogenic what, in connection with their pathogenic properties, indicates a risk for the health of humans. Classically pathogenic strains isolated from human cases of diarrhea produce thermostable enterotoxin YstA (Delor et al. 1990, Mikulskis et al. 1994, Ramamurthy et al. 1997, Singh and Virdi 2004) and the fragments of the ystA gene were detected in all human Y. enterocolitica strains examined in our study.

We also proved that the target ymoA gene was present in all the studied Y. enterocolitica strains, both human and animal. A similar result was obtained in...

the study of Grant et al. (1998) concerning 20 biotype 1A strains, where ymoA gene amplification products were found in all the studied strains. Also Bhagat et al. (2007) in their molecular tests of biotype 1A strains of *Y. enterocolitica* found sequences characteristic for the ystB and ymoA genes to be present in all examined strains. There were no reports regarding occurrence of *ymoA* gene together with *ystA* gene. In the context of the above mentioned reports and the authors’ own studies we should notice that the presence of *ymoA* or *ystA* genes is insufficient criterion to determine enterotoxigenic activity, what was observed in the authors’ investigation.

An important finding of the study was also showing that the majority of *Y. enterocolitica* strains, especially isolated from pigs, belonged to biotype 4 which is common in Poland but mainly in humans. Similarly, bioserotypes 4/O:3 and 2/O:9 proved to be predominant, both in humans and pigs. It seems particularly interesting in the context of studies conducted recently by Boghenbor et al. (2006) intended to determine the genotype of *Y. enterocolitica* strains originating from pigs and humans. The study proved that 83-87% of *Y. enterocolitica* strains belonging to serotypes 2/O:9, 3/O:3 and 4/O:3 showed great phylogenetic similarity so not only the typically pathogenic strains included in bioserotype 4/O:3 may cause infection in humans (Boghenbor et al. 2006). The other important finding of our study was showing the absence of biotype 1A strains of *Y. enterocolitica* obtained from pigs. The reliability of this result was confirmed both by serotyping (serotype O:5 was not found) and multiplex PCR (no amplicons of *ystB* and *ystC* genes). This finding is in line with reports of Woźniak-Kosek et al. (2001) but in opposite with reports of other authors showing the predominance of biotype 1A strains in isolates obtained from pigs (Thoerner et al. 2003, Kot et al. 2007).

Bio- and serotyping of *Y. enterocolitica* strains isolated from human cases of yersiniosis showed predominant causative role of bioserotype 4/O:3. Only a few 2/O:9 strains were isolated but significantly more interesting is the fact that 6 most pathogenic 1B/O:8 strains were isolated in Poland. This *Y. enterocolitica* strains considered as prevalent for USA were as yet uncommon in Poland.

Trying to expand the knowledge on mechanisms inducing the production of enterotoxins by demonstrating the correlation between the occurrence of the *ymoA* and *ystA* genes and the production of Yst by *Y. enterocolitica*, we showed that studies should be continued. Using more sophisticated techniques we should define phylogenetic relationship between human and animal *Y. enterocolitica* strains, the possible influence of mutation in *ymoA* and *ystA* genes on the enterotoxin YstA production and the expression level of this genes.

**Acknowledgements**

The study was supported by the Committee of Scientific Research (KBN, grant No. N N308 320235) and the EU under the European Social Fund. The human *Yersinia enterocolitica* strains were obtained by courtesy of the National Institute of Public Health – National Institute of Higiene in Poland and dr hab. n. med. Eugenia Gospodarek, prof. UMK.

**References**


Delor I, Kaeckenbeeck A, Wauters G, Cornelis GR (1999) Nucleotide sequence of *yst*, the *Yersinia enterocolitica* gene encoding the heat-stable enterotoxin, and preva-


Comparison of PCR methods for detection of classical swine fever virus and other pestiviruses

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Abstract

Classical swine fever (CSF) is a notifiable, highly contagious disease of swine controlled mainly with costly administrative methods. Swine may be infected not only with classical swine fever virus (CSFV), but also with other, non porcine, genetically and antigenically related pestiviruses. Differentiation of infections with CSFV and other pestiviruses is a crucial element of diagnostics.

In the present study two real-time PCR methods and conventional one-tube nested PCR for specific detection of CSFV were compared. Additionally, two methods designed for detection of all pestivirus species real-time SYBR Green I and one-tube nested PCR were included into the study. Analyzed methods varied considerably regarding their sensitivity and specificity, what suggests that careful selection of diagnostic methods and their evaluation on a regular basis is necessary.

Key words: pestiviruses, CSFV, real-time PCR, nested PCR, diagnostics

Introduction

Classical swine fever (CSF) is an economically important disease notifiable within EU and listed by OIE. Most of European countries are free from CSF, however, during the last five years several episodes of CSF were identified in pigs (Lithuania, Serbia, Slovakia, Bulgaria, Romania) as well as in wild boars (Lithuania, Slovakia, Bulgaria, Germany, Hungary). The stamping out policy of CSF control adopted within EU resulted in fully susceptible population. Several examples have shown that introduction of the virus into naïve population may cause tremendous economical losses (Ribbens et al. 2012). During the CSF epidemic in the Netherlands in 1997-1998 approximately 12 million pigs were slaughtered and the cost of disease eradication reached 2.3 billion USD (Stegeman et al. 2000).

The etiological agent of the disease is CSF virus (CSFV), classified in the genus Pestivirus, family Flaviviridae together with bovine viral diarrhoea virus 1 (BVDV-1), BVDV-2 and border disease virus (BDV) (Becher et al. 2003). BVDV and BDV cause non-notifiable infections in ruminants but they are also able to infect pigs, sometimes causing misinterpretations in diagnostic tests (de Smit et al. 1999, Oguzoglu et al. 2001, Loeffen et al. 2009). Therefore, rapid and effective detection of infections with pestiviruses and their differentiation is a crucial aspect in diagnosis of CSF.

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Currently, tests prescribed by OIE for international trade are neutralizing peroxidase-linked assay (NPLA), fluorescent antibody virus neutralization test and ELISA. For identification of the virus fluorescent antibody test on cryostat sections and virus isolation are recommended. However, ELISA methods widely used in monitoring of population for CSFV-specific antibodies are prone to non-specific results due to cross-reactions with antibodies specific to ruminant pestiviruses (de Smit et al. 1999, Bingham et al. 2010). During the CSF outbreak in the Netherlands in 1997/98 26.5% of samples initially diagnosed as CSFV-positive by ELISA were eventually recognized as infections with ruminant pestiviruses (de Smit et al. 1999). Also for NPLA, considered as a gold standard in detection of CSFV antibodies, incorrect results in double BVDV and CSFV infections were reported (Wieringa-Jelsma et al. 2006). Traditional virus isolation method is time-consuming and laborious. Moreover, it may be influenced by the presence of neutralizing antibodies. OIE manual also refers to RT-PCR as a method of increasing significance in preclinical diagnosis of CSF and screening of infected herds.

The aim of the study was to compare the usefulness of different PCR methods applied in differential diagnostics of pestiviruses. The sensitivity of two PCR methods detecting RNA of all pestiviruses species (pan-pestivirus) and three methods designed to detect CSFV RNA were compared. Methods evaluated in the study included real-time PCR based on SYBR Green I intercalating dye and specific TaqMan probes as well as two conventional PCR methods.

Materials and Methods

Viruses

Strains representing all species within Pestivirus genus were used in the study. Two CSFV strains, Alfort/187 (genotype 1) and field strain 1795/94 (genotype 2), BVDV-1 strain NADL, BVDV-2 strain Short and BDV strain Moredun were propagated and titrated using PK15 (CSFV), MBDK (BVDV-1 and BVDV-2) and SFTR cell line (BDV).

RNA extraction and reverse transcription

Total RNA was extracted using Total RNA Prep Plus kit (A&A Biotechnology) according to the manufacturer’s recommendations and eluted in 100 μl of RNase-free water. cDNA was synthesized using MMLV reverse transcriptase (Invitrogen) and 2.5 μM of random nonamers (Sigma) based on previously optimized method (Podgorska and Stadejek 2010), aliquotted and stored at -70°C until further analysis.

Polymerase chain reaction

Real-time PCR reactions were carried out in MicroAmp Optical Tubes (Applied Biosystems) closed with Optical Caps (Applied Biosystems). Amplification was performed using Stratagene MxPro 3005P (real-time PCR) or Biometra (conventional PCR) equipment. All PCR reactions were run in triplicates. Thermal conditions of each test and sequences of oligonucleotides used for amplification are presented in Tables 1 and 2.

Two methods designed to detect all pestivirus species were applied (Table 1, 2). Real-time PCR based on SYBR Green I (Stadejek et al. 2006) was carried out in a volume of 25 μl containing 1x QuantiTect SYBR Green PCR Master Mix, 0.8 μM of csfv 6 and csfv 7 primers, 1 μl of cDNA and nuclease-free water. The PCR reaction was followed by analysis of the melting temperature of amplification product to control the specificity of reaction. The other pan-pestivirus method was a conventional two-step nested PCR (PP-nPCR) based on Vilcek et al. (1994) with further modifications (Stadejek and Pejsak 2000). First step was performed based on 50 μl reaction mix containing 0.4 μM of external primers (V324/V326), 2.5 mM MgCl₂, 0.4 mM of dNTPs, 1 μl of 10% Triton X-100, 2.5 U Taq polymerase (Fermentas), 1x Taq Buffer with KCl, RNAse-free water and 5 μl of cDNA. The reaction mix was covered with a layer of mineral oil and subjected to amplification. Next, the amplification tubes were thoroughly mixed to dissolve reagents previously dried in the lids in 22% trehalose (20 pmols of internal primers, 1 μl of a mixture of 10 mM dNTP and 1.25 U Taq polymerase) then briefly centrifuged and subjected to nested PCR reaction.

Two different TaqMan-based real-time PCR methods, TaqMan I (Stadejek et al., unpublished) and TaqMan II (Hoffmann et al. 2005), as well as conventional two-step nested PCR (CSFV-nPCR) (Katz et al. 1993) were used for specific detection of CSFV (Table 1, 2). Reactions were carried out in 25 μl volume containing 5 μl of cDNA, 0.4 μM (TaqMan I) or 0.8 μM (TaqMan II) of primers, 1x Quantitect – Probe RT-PCR Master Mix (Qiagen), 0.8 μM (TaqMan I method) or 0.1 μM (TaqMan II) of dually-labeled probe and RNase-free water. CSFV-nPCR was based on external (A/D) and internal (B/C) pairs of primers. The concentration of reagents was the same as described above for PP-nPCR.
Table 1. Characteristics of the oligonucleotides used in the study.

<table>
<thead>
<tr>
<th>Method</th>
<th>Oligonucleotides</th>
<th>Sequence (5’→3’)</th>
<th>Amplified region of the genome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan I</td>
<td>→ csfv 6</td>
<td>CTA GCC ATG CCC IYA GTA GGA</td>
<td>5’ UTR</td>
<td>Uttenthal et al. 2003</td>
</tr>
<tr>
<td></td>
<td>← csfv 7</td>
<td>CTC CAT GTG CCA TGT ACA GCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>→ TaqMan probe</td>
<td>6FAM- CCC TGG GTG GTG TCA GTG AGT ACA G-TAMRA</td>
<td>5’ UTR</td>
<td>Stadejek et al. 2006 (unpublished)</td>
</tr>
<tr>
<td>TaqMan II</td>
<td>→ CSF100-F</td>
<td>ATG CCC AYA GTA GGA CTA GCA</td>
<td>5’ UTR</td>
<td>Hoffmann et al. 2005</td>
</tr>
<tr>
<td></td>
<td>← CSF192-R</td>
<td>CTA CTG ACG ACT GTG CTG TAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>→ TaqMan probe</td>
<td>6FAM-TGG CGA GCT CCC TGG GTG GTC TAA GT -TAMRA</td>
<td>5’ UTR</td>
<td></td>
</tr>
<tr>
<td>CSFV nPCR</td>
<td>→ A</td>
<td>ATA TAT GCT CAA GGG CGA GT</td>
<td></td>
<td>E2</td>
</tr>
<tr>
<td></td>
<td>← B</td>
<td>CTG TGG CTA ATA GTG ACC TAC</td>
<td></td>
<td>E2/NS2 1993</td>
</tr>
<tr>
<td></td>
<td>← C</td>
<td>CAT TTC TTT ATG GGC TCA TAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>← D</td>
<td>ACA GCA GTA GTA TCC ATT TCT TTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SYBR Green I</td>
<td>→ csfv 6</td>
<td>CTA GCC ATG CCC IYA GTA GGA</td>
<td>5’ UTR</td>
<td>Uttenthal et al. 2003</td>
</tr>
<tr>
<td></td>
<td>← csfv 7</td>
<td>CTC CAT GTG CCA TGT ACA GCA</td>
<td></td>
<td>Stadejek et al. 2006</td>
</tr>
<tr>
<td></td>
<td>→ V324 (324)</td>
<td>ATG CCC WTA GTA GGA CTA GCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>← V326 (Pest2)</td>
<td>TCA ACT CCA TGT GCC ATG TAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>→ A11</td>
<td>AGT ACA GGA TAG TCG TCA GTG GTT CG</td>
<td>5’ UTR</td>
<td>Vilecek et al. 1994</td>
</tr>
<tr>
<td></td>
<td>← A14</td>
<td>CAA CTC CAT GTG CCA TGT ACA GCA G</td>
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</table>

Table 2. PCR reaction conditions.

<table>
<thead>
<tr>
<th>Method</th>
<th>PCR reaction conditions</th>
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<tbody>
<tr>
<td></td>
<td>stage</td>
</tr>
<tr>
<td>TaqMan I</td>
<td></td>
</tr>
<tr>
<td>II (40 cycles)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>TaqMan II</td>
<td></td>
</tr>
<tr>
<td>II (42 cycles)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>SYBR Green I</td>
<td></td>
</tr>
<tr>
<td>II (42 cycles)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td></td>
</tr>
<tr>
<td>II (35 cycles)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>PP-nPCR,</td>
<td></td>
</tr>
<tr>
<td>CSFV-nPCR</td>
<td></td>
</tr>
<tr>
<td>III (30 cycles)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* collection of the fluorescence data
Table 3. Summary results of amplification. Number of positive results per three repeats of every reaction is indicated.

<table>
<thead>
<tr>
<th>Strain &amp; Genotype</th>
<th>Dilution</th>
<th>CSFV-specific</th>
<th>Pan-pestiviruses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Titer (TCID&lt;sub&gt;50&lt;/sub&gt;/ml)</td>
<td>TaqMan I</td>
</tr>
<tr>
<td></td>
<td>10–1</td>
<td>E+6</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>10–2</td>
<td>E+5</td>
<td>1/3</td>
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<td>E+3</td>
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<td></td>
<td>10–6</td>
<td>E+1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10–7</td>
<td>E+0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10–1</td>
<td>E+5.55</td>
<td>–</td>
</tr>
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<td>E+4.55</td>
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<td>10–5</td>
<td>E+1.55</td>
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<td></td>
<td>10–6</td>
<td>E+0.55</td>
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<td></td>
<td>10–7</td>
<td>E–1.55</td>
<td>–</td>
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<tr>
<td>BVDV-1 NADL</td>
<td>N</td>
<td>E+6.05</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10–1</td>
<td>E+5.05</td>
<td>–</td>
</tr>
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<td></td>
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<td>–</td>
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<tr>
<td></td>
<td>10–7</td>
<td>E–1.05</td>
<td>–</td>
</tr>
<tr>
<td>BVDV–2 Short</td>
<td>N</td>
<td>E+5.8</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10–1</td>
<td>E+4.8</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10–2</td>
<td>E+3.8</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10–3</td>
<td>E+2.8</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10–4</td>
<td>E+1.8</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10–5</td>
<td>E+0.8</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10–6</td>
<td>E–1.8</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10–7</td>
<td>E–2.8</td>
<td>–</td>
</tr>
<tr>
<td>BDV Moredun</td>
<td>N</td>
<td>n.d.**</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10–1</td>
<td>n.d.</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10–2</td>
<td>n.d.</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10–3</td>
<td>n.d.</td>
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<td></td>
<td>10–4</td>
<td>n.d.</td>
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<td>10–5</td>
<td>n.d.</td>
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<td></td>
<td>10–6</td>
<td>n.d.</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10–7</td>
<td>n.d.</td>
<td>–</td>
</tr>
</tbody>
</table>

*N – not diluted
**n.d. – not determined

Sensitivity of all PCR methods was assessed by amplification of dilution series of individual strains of known (except BDV) concentration (Table 3).

Statistical analysis

The results of particular methods regarding individual strain detection was evaluated with McNemar’s test. The level of significance was set at p=0.05. Also, the agreement between tests was assessed based on Cohen’s kappa coefficient. Both statistical tests were performed using GraphPad statistical software (http://graphpad.com/).

Results

The results of this study have shown differences in sensitivity of the evaluated methods (Table 3). None
of the methods specific for CSFV detected other pestiviruses species. The highest sensitivity in detection of CSFV was obtained using the TaqMan II method which allowed to detect Alfort/187 strain in a concentration of 10^4 TCID_{50}/ml in 2 of 3 repeats and 10^{1.55} TCID_{50}/ml of the field strain 1795/94 in all 3 repeats. CSFV-nPCR gave comparable results detecting the same concentration of CSFV strains but in 1 of 3 and 2 of 3 repeats, respectively. There was no statistically significant difference between these two methods (p>0.05) and the kappa coefficient indicated substantial agreement regarding Alfort (κ = 0.65) and good agreement regarding 1795/94 (κ = 0.8) strain. Significantly lower performance was observed for TaqMan I method (p<0.05). The test detected 10^4 TCID_{50}/ml of genotype 1 strain Alfort/187 but failed to detect field CSFV strain 1795/94 of genotype 2. The agreement with the best working TaqMan II method regarding Alfort strain was poor (κ = 0.18). What was interesting, PP-nPCR method designed to detect all pestiviruses detected CSFV at a similar level as Taq-Man II regarding both Alfort/187 and 1795/94 (p<0.05) with a moderate (κ = 0.75) and substantial (κ = 0.71) agreement, respectively. PP-nPCR detected Alfort strain in 1 log higher concentration compared to TaqMan II and CSFV-nPCR methods. Only slightly lower sensitivity was recorded for 1795/94 strain. In that case, PP-nPCR detected 10^{1.55} TCID_{50}/ml of 1795/94 CSFV in 1 per 3 reactions while TaqMan II was able to detect the virus in all 3 reactions. On the other hand, SYBR Green I detected only 10^3 TCID_{50}/ml of Alfort/187 and 10^{1.55} TCID_{50}/ml of 1795/94 strain. PP-nPCR gave significantly better results (p<0.05) in detection of BVDV-1 and BVDV-2 compared to SYBR Green I assay. The difference was as high as 4 and 2 logs for BVDV-1 and BVDV-2, respectively. BDV strain Moredun was detected only using PP-nPCR test.

**Discussion**

Despite strict and costly methods of CSF control within EU events of re-introduction of the disease still occur causing serious economic damage (Ribbens et al. 2012). Although most of the EU countries are free from CSFV, infections with other pestiviruses (BVDV-1, BVDV-2 and BDV) are present in ruminants (Loeffen et al. 2009). Poland is free from CSFV but BVDV infections in cattle are common (Polak and Zmudzinski 1999). Also, seroconversion to pestiviruses has been detected in free living ruminants in Poland (Fabisiak et al. in press). The status of BDV in Poland has not been determined. It was experimentally proved that ruminant pestiviruses may also infect swine, sometimes causing symptoms suggesting infection with low virulent CSFV (Paton and Done 1994). Such infections may also interfere with diagnostic and intervention programs, especially based on marker DIVA vaccines (Loeffen et al. 2009, Passler and Walz 2010). Influence on CSFV transmission in pig population and possibly delayed identification of CSF outbreak were also discussed (Wieringa-Jelsma et al. 2006, Loeffen et al. 2009). Therefore, constant monitoring of swine population not only for CSFV but also for the presence of ruminants pestiviruses is necessary.

PCR methods offer a promising alternative in the diagnosis of CSF and differentiation of the virus from other pestiviruses (Hoffmann et al. 2005). Amplification and sequencing of the specific regions in pestiviruses genome (5’ untranslated region, E2, NS5B genes) gives an additional advantage of further phylogenetic analysis which is of great importance in epidemiology and tracing of the origin of CSF outbreaks (Paton et al. 2000).

In the present study 3 PCR methods specifically directed for detection of CSFV and 2 pan-pestivirus specific PCR methods were evaluated regarding a set of strains representing all pestiviruses species. Compared methods significantly differed in sensitivity to detect CSFV. Real-time PCR TaqMan II proved to be the most sensitive. It detected 1000 times less concentrated Alfort/187 compared to other CSFV-specific TaqMan I method (Table 3). Moreover, the latter assay failed to detect genotype 2 CSFV field strain 1795/94. However, 1795/94 was detected by SYBR Green I method based on the same primers (Table 1). Most probably the problem was associated with the specificity of a TaqMan probe which was not able to recognize genotype 2 strain. That false negative result underlines the necessity of an evaluation of applied RT-PCR methods regarding currently circulating CSFV strains on a regular basis.

Out of the two pan-pestivirus specific tests PP-nPCR gave significantly better results (p<0.05) regarding BVDV-1 and BVDV-2 compared to real-time SYBR Green I assay. Moreover, it was the only method that detected BDV strain. Although SYBR Green I allowed for elimination of electrophoresis stage and minimization of the risk of contamination, its low sensitivity suggest that conventional gel-based PP-nPCR should be a method of choice in detection of BVDV and BDV. This method could be used for monitoring of the swine population for the presence of infections with pestiviruses and collect data important for epidemiology and diagnosis of CSF. Subsequent use of CSFV-specific systems would allow further differentiation of detected pestiviruses.
What is important, both conventional PCR methods used in the study, PP-nPCR and CSVF-nPCR, have shown relatively high sensitivity in detection of CSFV. The results were comparable to the best-working TaqMan II method and much better than two other real-time tests used in the study. Although classical nested-PCR is a multistage method which increases the risk of laboratory contamination, not all laboratories can afford expensive real-time PCR equipment. The abovementioned results indicate that conventional PCR methods may be used as an alternative for CSFV detection without major impairment of sensitivity.

Our previous study reported that optimization of reverse transcription improved the sensitivity of RT-PCR for detection of CSFV about 1000 times (Podgorska and Stadejek 2010). In the present study we indicated that further improvement of similar magnitude may be obtained by a proper selection of diagnostic method. In the worst case scenario (TaqMan I and CSFV strain 1795/94) the infection with CSFV may not be detected what can result in serious economic losses. Additionally, method for monitoring of pig population for the presence of ruminant pestiviruses infections was proposed.

Acknowledgements

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References


Effect of a two-week treatment with low dose of ortho-substituted polychlorinated biphenyls (PCB104 and PCB153) on VEGF-receptor system expression in the choroid plexus in adult ewes

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Abstract

Ortho-substituted polychlorinated biphenyl (PCB) congeners, which constitute a large part of PCB residues found in the environment and in animal tissues, are known to exert potent vascular effects and can activate endothelial cells in the periphery and in the brain. The choroid plexus (CP) is responsible for cerebrospinal fluid (CSF) production and its epithelial cell layer is responsible for structure and functions of the blood-CSF barrier. The aims of this study were: 1) to investigate if environmentally relevant doses of PCB153 and similar doses of PCB104 caused changes in the expression of vascular endothelial growth factor (VEGF) – receptor system, which maintains CP function, and 2) to determine the level of both congeners in blood plasma after their oral administration. Studies of both congeners were performed on ovariectomized ewes treated per os with low doses (0.1 mg/kg, three times a week for two weeks) of PCB153 (n=4) or PCB104 (n=4) and vehicle (control, n=4). The effects of PCB153 and PCB104 treatment on mRNA expression of two isoforms of VEGF (VEGF₁₂₀ and VEGF₁₆₄) and their receptors Flt-1 and KDR were determined using real-time PCR. Plasma concentration of PCBs was measured using high resolution chromatography/tandem mass spectrometry (HRGC/MS-MS). We observed that neither PCB153 nor PCB104 significantly altered the mRNA of the VEGF-receptor system in the CP. In PCB treated animals plasma concentration of PCB153 (1.425 ± 0.16 ng/g of dry mass, DM) was about 150 times higher than PCB104 (0.009 ± 0.007 ng/g DM). In control animals the PCB153 level was 0.14 ± 0.031 ng/g DM, while the PCB104 level was below detection level. This indicates that increase in plasma PCB153 concentration to levels similar to those reported in humans and of PCB104 concentration to levels 100 times higher than those found in human plasma did not affect the VEGF-receptor system in the CP in adult ewes. The significantly lower increase of PCB104 than PCB153 concentration in blood after oral administration suggests different absorption of both congeners from the digestive tract.

Key words: polychlorinated biphenyls: PCB104, PCB153, vascular endothelial growth factor, choroid plexus, ewes
Introduction

Polychlorinated biphenyls (PCBs) are chemicals that comprise a group of 209 congeners with varying degrees of chlorination, which determines their physical, chemical, and biological properties (Safe 1994). From a structural point of view, there are at least two distinct classes of PCBs: the non-ortho (coplanar) and ortho-substituted (planar) congeners. Ortho-substituted PCBs constitute a large part of the PCB residue found in the environment and animal tissues (Kodavanti et al. 1998, Faroon et al. 2001, Costera et al. 2006). The most environmentally relevant ortho-substituted PCB – 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB153) has been demonstrated to affect the reproductive function and puberty onset in young goats which were nataally exposed to a low dose of PCB153 (Lyche et al. 2004, Oskam et al. 2005). We recently demonstrated that a low dose of PCB153 affects gonadotropin secretion in adult female ewes through an action on both pituitary Luteinizing Hormone (LH) release and the hypothalamic Gonadotropin Releasing Hormone (GnRH) pulse generator (Skipor et al. 2012). Although the brain appears to be better protected against PCBs than other tissues because of the blood-brain and blood-cerebrospinal fluid (CSF) barriers (Skipor and Thiery 2008), ortho-substituted PCBs have been reported to accumulate preferentially in the brain and CSF compared to other PCBs (Kodavanti et al. 1998, Takasuga et al. 2004, Montie et al. 2009). Recently, measurement of PCB concentration in the ovine CSF showed that PCB153 was the most abundant congener in the CSF (Skipor et al. 2012).

There is evidence that PCB153 disrupts expression of tight junction proteins in the blood-brain barrier which restricts most paracellular movement of ions and solutes across the brain barriers (Eum et al. 2008, Seelbach et al. 2010). Among ortho-substituted PCBs, 2,2′,4,5,5′-pentachlorobiphenyl (PCB104) also affects tight junction proteins expression in brain endothelial cells, stimulates inflammatory mediators in vascular endothelial cells and induces prometastatic responses (Choi et al. 2003, Sipka et al. 2008). It has been demonstrated that vascular endothelial growth factor (VEGF) is involved in PCB104 induced endothelial hyperpermeability (Eum et al. 2004). VEGF is continuously and highly expressed in the choroid plexus (CP), in which the blood-CSF barrier is located (Skipor and Thiery 2008). In the ovine CP two isoforms, VEGF₁₂₀ and VEGF₁₆₄, are expressed (Szczepkowska et al. 2012). VEGF plays an important role in regulation of the stability of the endothelial cells in the CP (Maharaj et al. 2008) and is involved in maintaining endothelial cells’ fenestrated phenotype in the CP capillaries (Roberts and Palade 1995, Esser et al. 1998). The effects of VEGF are transduced mainly by two high-affinity receptors belonging to the tyrosine kinase-family: the fms-like tyrosine kinase (Flt-1) and the fetal liver kinase-1/kinase insert domain-containing receptor (Flk-1/KDR). The CP has been shown to contain mRNA (Nico et al. 2004) and protein (Witmer et al. 2002, Maharaj et al. 2008, Yang et al. 2010) of VEGF receptors.

Studies on PCB toxicity are mainly based on in vitro models or in vitro experiments in which mice or rats are intraperitoneally injected with PCBs (Sipka et al. 2008). The in vivo effects of PCB153 and PCB104 on expression of the VEGF system in the CP in domestic animals exposed to PCBs through the food chain, the most common route of PCB exposure, has not yet been reported. Therefore, the aim of this study was to evaluate the effect of chronic oral administration of low doses (0.1 mg/kg of body weight, bw) of PCB104 and PCB153 on expression of the VEGF system in the CP in ewes. Additionally, the concentration of both PCB congeners was measured in blood plasma.

Materials and Methods

Animal management and experimental design

The experiment was conducted on adult Polish Lowland ewes (n=16, 60-70 kg bw). To prevent variability linked with the estrous cycle or interaction with steroids the animals were ovarioctomized (under xylazine anaesthesia (0.25 ml/kg, im; Rometar, Spofa, Prague, Czech Republic) about 20 days before PCB administration. All animals were maintained indoors under natural lighting conditions (October-November), each group being in separated pens in order to avoid contamination through the ingestion of PCB-contaminated faeces. The ewes were fed a diet of hay, straw, and commercial concentrates with water and mineral licks available ad libitum. Experimental procedures were conducted in accordance with the Polish Guide for the Care and Use of Animals (1997) and approved by the Local Ethics Committee (agreement no 4/2008).

After recovery, the animals were randomly allocated to three groups: group 1 – control (control), group 2 – PCB104 treated (PCB104) and group 3 – PCB153 treated (PCB153). Animals in the PCB104 and PCB153 groups were treated with appropriate PCB congeners by oral gavage three times weekly for 14 days, at a dose of 0.1 mg/kg of bw.
Table 1. Sequences of oligonucleotide primers used for Real Time – PCR analyses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5’→3’)</th>
<th>Product size</th>
<th>Literature source</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A120</td>
<td>Forward: AAGGCCAGCACATAGGAGAG Reverse: CCTCGGCTTIGTCAATTITG</td>
<td>101bp</td>
<td>Kaczmarek et al. (2008)</td>
</tr>
<tr>
<td>VEGF-A164</td>
<td>Forward: GAGGCAAGAAATCCTCGTG Reverse: TCACATCTGCAAGTACGTTCG</td>
<td>150bp</td>
<td>Kaczmarek et al. (2008)</td>
</tr>
<tr>
<td>Flt-1</td>
<td>Forward: TGGATTTCAGGTGAGCTTGGA Reverse: TCACCGTGCAAGACAGCTTC</td>
<td>68bp</td>
<td>Redmer et al. (2005)</td>
</tr>
<tr>
<td>KDR</td>
<td>Forward: CTTCCAGTGGGCTGATGACC Reverse: GCAACAAACGGCTTTTCATGT</td>
<td>67bp</td>
<td>Redmer et al. (2005)</td>
</tr>
<tr>
<td>NRP-1</td>
<td>Forward: GATTGCGGTGGACGATATTAGC Reverse: GGTTTTGCGCAGTCCTCTTG</td>
<td>60bp</td>
<td>Vonnahme et al. (2006)</td>
</tr>
<tr>
<td>RPL19</td>
<td>Forward: AATCGCCAATGCCAACTC Reverse: GGGACTGGTGTTGCTTCAAGAC</td>
<td>156bp</td>
<td>Herman et al. (2010)</td>
</tr>
<tr>
<td>PPIC</td>
<td>Forward: TGGCACTGGTGGTATAAGCA Reverse: GGCTTGGTCAAGGTGATAA</td>
<td>145bp</td>
<td>Herman et al. (2010)</td>
</tr>
</tbody>
</table>

Animals from the control group were treated with sunflower oil used as a vehicle for PCBs. Blood samples (20 ml) were collected from the jugular vein 15 days after beginning the PCB treatment (1 day after the end of PCB treatment) and then centrifuged and stored at -20°C for further analysis of PCBs. The animals were then sacrificed and, immediately after decapitation, the brains were dissected out and the CP from the lateral ventricles were collected and stored at -80°C until further analysis.

**Calculation of doses and PCB administration**

PCB104 (2,2′,4,5,6′-pentachlorobiphenyl) and PCB153 (2,2′,4,4′,5,5′-hexachlorobiphenyl) from AccuStandard (New Haven, CT, USA) were purchased locally at Tusnovics Instruments Polska and were 99.9% pure. Each PCB was dissolved in sunflower oil to a concentration of 1 mg/ml. Literature data (Lyche et al. 2004b) indicated that similar doses of PCB153 given orally in sheep for 30 days resulted in a plasma concentration of PCB153 of 30 ng/g (wet-weight). Costera et al. (2006) demonstrated that, in goats exposed to contaminated hay (concentration of PCB153 of about 460 ng/kg of dry mass (DM)), the time to reach a steady state concentration in milk was 15 days for most PCB congeners.

**Relative gene expression assays**

CP tissue, frozen and cut into small pieces (20 mg), was homogenized in Fenozol in Lysing Matrix D (MP Biomedicals, Illkirch, France) with a FastPrep-24 instrument (MP Biomedicals). RNA was extracted using a Total RNA Kit (A&A Biotechnologies, Poland), and Amplification Grade DNase 1 (Sigma Aldrich, Germany) was used to eliminate possible genomic DNA contamination, according to the manufacturer’s instructions. The concentration and quality of RNA isolated from the CP tissue were determined using a NanoDrop (Thermo Scientific, USA) and 2% agarose gel electrophoresis. One microgram of total RNA was retained for further use in RT (reverse transcription) reaction. The RT reaction was performed with Quanti Tect Reverse Transcription Kit according to the protocol supplied by the manufacturer (Qiagen, USA). The resulting cDNA was stored at -20°C until further analysis.

To evaluate the effects of PCB treatment on mRNA expression of components of the VEGF-receptor system, real-time PCR was performed with the use of an ABI Prism 7900 sequence detection system and a Power SYBR green PCR master mix (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). Specific primer pairs for the different genes were used according to the literature (Table 1). All primers were synthesized by IBB PAN (Poland). PCR-derived DNA fragments (VEGF-A120, VEGF-A164, Flt-1, KDR, NRP-1, reference genes- RPL19 and PPIC) were separated by electrophoresis on 2% agarose gels supplemented with 0.01% ethidium bromide and examined under UV light (Gel Logic100, KODAK). Each real-time PCR reaction well (20 µl) contained 2 µl of diluted RT product, 0.2 µM forward and reverse primers each and 10 µl of Power SYBR green PCR master mix. The following protocol was used: 95°C for 15 min for Hot Start AmpliTaq Gold DNA polymerase and 40 cycles of 95°C for 10 s (denaturation), 55°C for 20 s (annealing), and 72°C for 20 s (extension).
Fig. 1. Mean levels (±SD) of PCB104 (A) and PCB153 (B) in blood plasma of ovariectomized ewes receiving vehicle (C-PCB104 and C-PCB153), PCB104 (T-PCB104 – A) or PCB153 (T-PCB153 – B) per os (0.1 mg/kg of body weight; 6 doses over 14 days). Note that PCB104 in control group was below detection limit.

After the cycles, a final melting curve analysis under continuous fluorescence measurement was performed to evaluate the specific amplification. The results were analyzed using Real-time PCR Miner (available on-line: http://www.miner.ewindup.info/version2), based on the algorithm developed by Zhao and Fernald (2005).

**PCB determination in blood plasma**

Blood plasma samples (20 ml) were freeze dried and spiked with clean-up standards using 1 ml of $^{13}$C-PCBs/g of the sample with standard mixture containing 10 ng/ml of $^{13}$C-PCB104 and 20 ng/ml of $^{13}$C-PCB153, and extracted with toluene for 16 h in 20 ml Soxhlet apparatus. The extract was cleaned up according to the previously published procedure (Surma-Zadora and Grochowalski 2008). The cleaned-up extract was evaporated to approximately 1 ml, transferred to 5 ml of dichloromethane and placed in a polyethylene semipermeable membrane. Dialysis was performed within 24 h with hexane as a recovery solvent. The hexane dialysate was then cleaned-up on a column which contained 10 g of 50% H$_2$SO$_4$ on silicagel. The column was eluted with 100 ml of hexane, evaporated to approximately 1 ml and transferred to another column containing 5 g of acidic Alumina (Merck 101078, 0.063 – 0.200 mm of activity I) and eluted firstly with 15 ml of hexane (fraction was discarded). The PCBs were then eluted in the second fraction of 50 ml of 2% dichloromethane in hexane. The eluate was evaporated just to dryness and reconstituted into 50 μl of nonane containing 100 pg/ml of $^{13}$C-1,2,3,4-tetrachlorodibenzo-p-dioxin ($^{13}$C-1,2,3,4-TCDD) as a precision and recovery standard.

The determination of PCBs was performed in an accredited laboratory (Laboratory for Trace Organic Analyses, Krakow University of Technology, Poland) using the isotope dilution method, high resolution chromatography/tandem mass spectrometry (HRGC/MS-MS) on a Thermo Scientific GCQ-1100/Trace2000 system adjusted to double fragmentation mode equipped with Xcalibur data acquisition and analysis software. Separation of PCB congeners was performed on a 30 m × 0.25 mm i.d. DB5MS capillary column of 25 μm film and DB17 30 m × 0.25 mm i.d. A sample of 2 μl volume was injected into a SSL injector at 260°C. The GC oven temperature was held at 100°C for 1 minute, and then raised to 180°C at 20°C/min, then 2°C/min to 260°C, and then 20°C/min to a final temperature of 300°C. The temperature was held at 300°C for 5 minutes.

The method is of high specificity and gives non-interfered signals in the determination of PCB104 and 1CB153 congeners. Recoveries of PCB104 and PCB153 were in the range of 65 – 90% and 85-120%, respectively.

**Data analysis**

PCB data are expressed as the mean ± SD. The real-time PCR results are presented as the relative gene expression (mean ± SEM) of the target gene vs. the reference gene (RPL19). The significance of differences were statistically analyzed using one-way ANOVA (PRISM 4, Graph Pad, USA).
Results

PCB determination in blood plasma

The mean level of PCB congeners in blood plasma of sheep after oral administration of a low dose (0.1 mg/kg/per day) of PCB153 and PCB104 is presented on Fig. 1. In control sheep concentration of PCB153 was 0.14 ± 0.031 ng/g of DM, while the PCB104 level was below the detection limit (0.0001 ng/g DM). After oral administration of PCB153 and PCB104, their concentrations in blood plasma were 1.425 ± 0.16 ng/g DM and 0.009 ± 0.007 ng/g DM, respectively.

Real Time PCR analysis

PCB104 and PCB153 treatment had no significant effect on the expression of all tested genes: VEGF-A120, VEGF-A164, Flt-1, and KDR (Fig. 2) in comparison with the control group.

Discussion

This is the first study investigating the effect of environmentally relevant PCB153 in vivo on the VEGF-receptor system in the CP. In this study PCB153 action was compared with that of PCB104, formerly documented as affecting the VEGF system in vitro (Eum et al. 2004). Neither PCB153 nor PCB104 exposure significantly altered the mRNA of the VEGF-receptor system expression in the CP in ovariectomized adult sheep. The high variability of PCB concentration (78.5% for PCB104 and 21.8% for PCB153) observed in our study may account for large individual differences in PCB action on VEGF-receptor system mRNA expression. An earlier study demonstrated that maternal exposure to low doses (98 μg/kg bw) of PCB153 during gestation and lactation suppressed prepubertal plasma LH concentrations, delayed the onset of puberty of the female offspring, altered bone composition, and suppressed neonatal immunity in goats (Lyche et al. 2004a, b, Lyche et al. 2006, Lundberg et al. 2006). Recently we observed symptoms of estrogenization (enlarged vulva and edematous uterus) in PCB153 treated ewes, in which the plasma concentration of PCB153 was 196 pg/ml, equivalent to 2.4 ng/g DM
as in our earlier study (0.196 ng/ml) is similar to the PCB153 concentration measured in the human population in the United States (http://www.cdc.gov/exposurereport). According to a national report on human exposure to environmental chemicals, the geometric mean of serum PCB153 concentration in humans was 0.167 ng/g (0.151-0.185 ng/g, 95% confidence interval, CI) in 2001-2002 and 0.121 ng/ml (0.114-0.128 ng/ml, 95% CI) in 2003-2004.

In contrast to PCB153, PCB104 is not environmentally and tissue relevant (http://www.ewg.org/reports/bodyburden2, Fielden et al. 1997), mainly due to the low distribution in commercial PCB mixtures (Frame 1997). In our studies, treatment of ewes with a low dose of PCB104 increased its concentration in blood plasma from an undetectable level to 0.009 ng/g DM (corresponding to 0.75 pg/ml). In humans, measurement of PCB104 in umbilical blood plasma gave 0.9 pg/g of lipid weight, corresponding approximately to 0.007 pg/ml (http://www.ewg.org/reports/bodyburden2). Therefore, we demonstrated here that a 100 times higher concentration of PCB104 than those found in humans did not affect the VEGF-receptor system in the CP. The dose of PCB104 used in in vitro studies promoting an increase in the microvascular permeability and the transmigration of breast cancer cells following PCB104 treatment ranged from 2-15 μM, which corresponds to 650-4860 ng/ml. Such high concentrations of total PCBs (3-6 μM) were detected in human blood plasma in industrialized areas of the Netherlands and in the Aland/Turku Archipelago (Koopman-Esseboom et al. 1994, Hagmar et al. 1998).

In our studies, treatment of ewes with the same low doses (0.1 mg/kg bw) of PCB104 and PCB153 resulted in about 150 times higher concentration of PCB153 in blood plasma compared to PCB104. This could result from feeding ewes with hay containing PCB153 since the concentrations of PCB153 congener in hay is among the highest compared to other congeners (Chauhan et al. 2000). Costera et al. (2006) showed that amounts of PCB153 in hay may reach 460 ng/kg/DM. It is also possible that our findings may result from the lower resistance to the biotransformation for PCB104 than for PCB153, which was demonstrated to be very resistant (Thomas et al. 1999). Indeed, about 22% of the total PCB residue in human tissue consists of PCB153 (Jensen and Sundstrom 1974). It has been suggested that binding of PCB with transthyretin in blood plasma protects against their rapid removal from circulation. PCB153 binds with transthyretin more effectively than PCB104 (IC50, 90 vs. >1000) (Chauhan et al. 2000). In our study, the mean concentration of PCB153 in blood plasma was about 1.4 ng/ml. This is below the levels measured in the plasma of pregnant goats (30 ng/g wet weight) long-term treated with a similar dose of PCB153 (Lyche et al. 2004b). Such discrepancy between two closely related animal families could be due to species difference, as differences in reactivity to PCB treatment has also been reported between rats and mice (Craft et al. 2002). Alternatively, it could stem from the use of pregnant goats by Lyche et al. (2004b) and ovariectomized sheep in our study. Finally, the endocrine status of the female rather than a difference in regimen of the treatment may be responsible for such a discrepancy, since we demonstrated that in sheep two-week oral administration of low doses of PCB153 (0.3 mg/kg bw) induced higher levels of PCB153 in short days than long days (Skipor et al. 2012).

In summary we demonstrated that increase of plasma PCB153 concentration to the level present in humans and PCB104 concentration to the level exceeding 100 fold that present in human plasma did not affect the VEGF-receptor system in the CP in adult ewes.

Acknowledgement

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Use of rapid human respiratory syncytial virus strip tests for detection of bovine respiratory syncytial virus in experimentally vaccinated calves

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Abstract

Three different rapid strip tests: TRU RSV, BinaxNOW RSV and RSV Respi-strip were compared with RT-PCR and ELISA BRSV Ag for the ability to detect bovine respiratory syncytial virus (BRSV) in nasal swabs collected from calves experimentally vaccinated with live vaccine Rispoval RS-PI3. The reference strains of BRSV (375 and A51908) were detected by ELISA BRSV Ag whereas the strains of human respiratory syncytial virus (HRSV) and bovine parainfluenza virus type 3 (BPIV-3) were not. All rapid strip tests as well as RT-PCR reacted positively both to HRSV and BRSV reference strains and negatively to BPIV-3. The detection limit for RT-PCR was 39.1 TCID50 (strain 375 of BRSV), whereas for each of the rapid tests it was approximately 156 TCID50 and 312 TCID50 for antigen ELISA. Diagnostic sensitivity in detecting BRSV in nasal swabs for TRU RSV and RSV Respi-strip tests was 33% and 50% for BinaxNOW RSV. Diagnostic specificity of TRU RSV was 100%, whereas for both BinaxNOW and Respi-strip it was 87%. We concluded that TRU RSV could be used as a supportive rapid test for BRSV screening in nasal swabs taken directly on a farm. However, due to the small group of animals used in the experiment, the results should be regarded as preliminary and the study should be repeated on a larger number of animals.

Key words: BRSV, diagnosis, strip tests

Introduction

Bovine respiratory syncytial virus (BRSV) is an enveloped, negative-stranded RNA virus belonging to the Pneumovirus genus of the Paramyxoviridae family. It is one of the major viral pathogens responsible for respiratory tract diseases in cattle worldwide. Infection with BRSV can affect cattle of all ages and breeds and is characterized by increased respiratory rate, nasal discharge, fever and cough. The morbidity is high (60 to 80%), whereas the mortality rate seldom exceeds 5-10%, although occasionally in some outbreaks it can reach up to 20% (Valarcher and Taylor 2007). Clinical signs observed during acute disease may raise the suspicion of BRSV infection but to make a definitive diagnosis laboratory confirmation is needed. The most widely used diagnostic methods for direct detection of BRSV in field specimens are the virus isolation test, antigen enzyme-linked immunosorbent assay (ELISA) and reverse transcriptase.
polymerase chain reaction (RT-PCR) (Larsen 2000). Although virus isolation in cell cultures is considered as the “gold standard” among virological methods, in the case of BRSV infection this test can be unsuccessful. Attempts at virus isolation often fail because of the lability of the virus even in optimally stored samples. Moreover, BRSV replicates slowly and several blind passages are often required before any cytopathic effect can be seen. This makes the virus isolation test laborious and time consuming and therefore not suitable for routine diagnostics.

Recently, various RT-PCR assays targeting fusion (F), glycoprotein (G) and nucleoprotein (N) genes of BRSV were developed for detection of the virus (Vilcek et al. 1994, Socha and Rola 2011). Although RT-PCR and especially real time RT-PCR has been shown to be more sensitive than other tests, each of these methods is relatively complicated and requires a well equipped laboratory and trained personnel. Therefore there is a need for a simple and rapid diagnostic test that would allow detection of BRSV in field conditions.

Rapid immunochromatographic strip tests detecting viral antigens have been developed for HRSV (Popow-Kraupp and Aberle 2011). HRSV is the prototype of the Pneumovirus genus and BRSV is closely related to it with a high degree of genetic, antigenic and epidemiological similarities. These features, could theoretically make it possible to adapt rapid HRSV strip tests for the diagnosis of BRSV.

The aim of this study was to evaluate the possibility of using rapid HRSV immunoassay tests for detection of BRSV.

Materials and Methods

Reference strains

Two BRSV strains: 375 (VR-1339) and A51908 (VR-794) and two other paramyxoviruses, HRSV strain A2 (VR-1540) and BPIV3 strain SB (VR-739), were used in the study as the positive and negative controls for RT-PCR, ELISA BRSV Ag, rapid TRU RSV, BinaxNOW RSV and RSV Respi-strip tests, respectively.

Experimental infection and collection of nasal swabs

Blood samples were taken before infection from all tested calves and examined using the indirect ELISA BRSV SVANOVIR test (Svanova) to detect BRSV-specific antibodies. Five clinically healthy and serologically negative calves approximately 6-8 weeks old were finally selected for experiment. Three calves were inoculated intranasally with 2 ml of live Rispoval RS-PI3 (Pfizer) vaccine containing no less than $10^{5.0}$ TCID$_{50}$ of attenuated strain 375 of BRSV. The vaccine was administrated intranasally using an applicator provided by the manufacturer of the vaccine. Control calves received 2 ml of sterile water. Both groups of calves were housed separately in isolation to prevent the spread of the vaccine virus.

Nasal swabs were taken from the inoculated and control calves at -1, 0, 1, 2, 6, 8, 9, 14, 21 and 28 days post inoculation (dpi). Swabs were collected from the caudal part of the nasal cavity with a sterile plastic applicator (UTM-RT Copan system) and placed in liquid transport medium. In the laboratory swabs were shaken, centrifuged (300 x g, 10 min) and used directly for testing.

Strip tests

Three different rapid strip tests: RSV Respi-strip (Coris), TRU RSV (Meridian Bioscience), BinaxNOW RSV (Inverness Medical) designed for diagnostics of HRSV were evaluated in the study. The BinaxNOW RSV and RSV Respi-strip tests detect F protein antigen, whereas the TRU RSV test detects F and N antigens. All the tests were run according to the manufacturer’s instructions. Interpretation of the final result was similar for each of the strip tests. A positive test result was obtained when both a sample line and a control line were visible. If only a control line was visible the test was negative. If control line was not visible the test was invalid.

ELISA BRSV Ag

Pulmoest BRSV Antigen Detection ELISA (Euroclone) designed for the detection of BRSV antigens was used in the study. The test was performed according to the manufacturer’s instructions. Briefly, the optical density of the sample was measured using a microplate spectrophotometer at 450 nm. For each sample the percent of positivity was calculated. The sample was positive if this value was higher than the value given in the Quality Control data sheet of the test batch.

Analytical sensitivity and specificity

The detection limit of all tests was determined by analysing a 2-fold dilution series of BT (bovine
Table 1. Specificity of RSV strip tests compared with ELISA BRSV Ag and RT-PCR.

<table>
<thead>
<tr>
<th>Controls</th>
<th>ELISA BRSV Ag</th>
<th>RSV Respi-strip</th>
<th>TRU RSV</th>
<th>BinaxNOW RSV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT-PCR</td>
<td>Cit – Coris</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRSV A51908</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BRSV 375</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HRSV A2</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BPIV 3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Negative Swab</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 2. Shedding of BRSV in calves experimentally vaccinated with live vaccine Rispoval RS-PI3 determined by RT-PCR.

<table>
<thead>
<tr>
<th>Calf no.</th>
<th>Days post vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>1.</td>
<td>–</td>
</tr>
<tr>
<td>2.</td>
<td>–</td>
</tr>
<tr>
<td>3.</td>
<td>–</td>
</tr>
<tr>
<td>Controls</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 3. Results of rapid strip tests for detection of BRSV in nasal swabs*.

<table>
<thead>
<tr>
<th>Test</th>
<th>Diagnostic sensitivity (%)</th>
<th>Diagnostic specificity (%)</th>
<th>Positive predictive value (%)</th>
<th>Negative predictive value (%)</th>
<th>True positives</th>
<th>False positives</th>
<th>True negatives</th>
<th>False negatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV Respi-strip</td>
<td>33</td>
<td>87</td>
<td>57</td>
<td>71</td>
<td>4</td>
<td>3</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>TRU RSV Test</td>
<td>33</td>
<td>100</td>
<td>100</td>
<td>74</td>
<td>4</td>
<td>0</td>
<td>23</td>
<td>8</td>
</tr>
<tr>
<td>BinaxNOW RSV</td>
<td>50</td>
<td>87</td>
<td>67</td>
<td>77</td>
<td>6</td>
<td>3</td>
<td>20</td>
<td>6</td>
</tr>
</tbody>
</table>

* Specimens were defined as true positive if BRSV was detected by RT-PCR.

Turbinate (ciliated) cell cultures infected with BRSV strain 375. These dilutions contained from 20 000 TCID_{50} to 19.5 TCID_{50} of the virus. They were directly used for examination with the rapid strip tests and ELISA BRSV Ag. For RT-PCR, RNA was isolated from them first. To determine diagnostic specificity of the tests we investigated reference strains for BRSV and other paramyxoviruses described above. A further negative control, consisting of transport medium from the UTM-RT Copan system, was included.

Diagnostic specificity, diagnostic sensitivity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for each of the strip tests. Diagnostic sensitivity was calculated by dividing the total number of true positive results (TP) by the sum of false negatives (FN) and TP, whereas diagnostic specificity was calculated by dividing the total number of true negatives (TN) by the sum of TN and false positives (FP). PPV was calculated by dividing TP by the sum of TP and FP, and NPV by dividing TN by the sum of FN and TN. The results were multiplied by 100 and expressed as a percentage.

**RNA extraction and RT-PCR**

Total RNA was extracted from the supernatant of nasal swabs using TRI reagent (Sigma) according to the producer’s instructions.

RT-PCR was performed using the Titan One-Tube RT-PCR System (Roche). Primers specific to gene encoding nucleoprotein N (N-F 5’-GTCAGCTTAACATCAGAGTTCAAG-3’ and N-R 5’-ACATAGCCTACATTACACCACAATCTA-3’) giving a 114 bp long product were used (Zulauf 2007). Reverse transcription was performed for 1 h at 48°C.
followed by 2 min denaturation at 94°C. The amplification was done in 40 cycles according to the following scheme: 45 s denaturation at 94°C; 45 s of hybridisation at 51°C and 1.5 min of elongation at 72°C. Reaction was finished by final elongation for 7 min at 72°C. The PCR products were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide and visualized under UV light. The RT-PCR assay was used as the reference test for this study. Samples positive by this method were considered true positives and samples negative were considered true negatives.

Results

All rapid strip tests and RT-PCR reacted positively both with HRSV strain A2 and BRSV reference strains, and negatively with BPIV-3. The ELISA BRSV antigenic test reacted positively only with strains of BRSV. A summary of the results concerning the specificity of evaluated tests is presented in Table 1.

Analytical sensitivity of the tests was determined for the same virus strain of BRSV. The detection limit for RT-PCR was 39.1 TCID$_{50}$. Analytical sensitivity of the strip tests was lower than RT-PCR and each of these rapid tests detected approximately 156 TCID$_{50}$ of the BRSV. The detection limit for ELISA BRSV Ag was 312 TCID$_{50}$.

All three calves vaccinated with the live vaccine Rispoval RS-PI3 were RT-PCR positive when tested using nasal swabs. In total, the vaccine virus was found in 12 swabs out of 30 swabs collected. In calf No. 1 vaccine virus was excreted with nasal discharges continuously for 8 dpi, in calf No. 2 virus shedding lasted from the 2nd to 6th dpi, and in calf No. 3 from the 1st to 8th dpi. Two control calves remained negative in RT-PCR throughout the study (Table 2).

The results concerning virus shedding in vaccinated calves obtained with rapid strip tests are summarized in Table 3. Using the RSV Respi-strip test the BRSV was detected in 7 swabs derived from calves No. 2 (1, 2, 8 and 9 dpi) and 3 (2, 8 and 9 dpi). Swabs collected from calf No. 1 were negative in this test. Four nasal swabs were positive in the TRU RSV test. The virus was detected in calves No. 1 (0, 1 dpi), No. 2 (2 dpi) and No. 3 (1 dpi), respectively. Diagnostic sensitivity of both these tests in detecting BRSV in nasal swabs was 33%.

BinaxNOW RSV test was positive for 9 swabs. The vaccine virus was detected in calves No. 1 (0, 1 dpi), in calf No. 2 (0, 1, 2 dpi) and calf No. 3 (1, 2, 6 and 9 dpi). Diagnostic sensitivity of the test was 50%.

Only one sample was positive in ELISA BRSV Ag. This was the sample from calf No. 2, collected at day 2 pi. However, a slight increase in the percent of positivity was visible in all vaccinated calves during the first days post infection. Results of this test are shown in Fig. 1.

Discussion

Most of the HRSV rapid strip tests currently available on the market are designed to detect either glycoprotein F, nucleoprotein N or both. They belong to the most conservative proteins both in HRSV and BRSV, showing 81% identity for glycoprotein F and 93% for nucleoprotein N at the amino acid sequence level (Valarcher and Taylor 2007). It has been shown that monoclonal antibodies to HRSV cross-react with most of the epitopes of F and N proteins of BRSV (Stine et al. 1997, Fogg et al. 2001).

Based on this knowledge and previous successful use of the HRSV enzyme immunoassay for detection of BRSV (Osorio et al. 1989), we assumed that HRSV strip tests could be used in the diagnostics of BRSV as well. In our studies three commercial immunochromatographic strip tests were evaluated for detection of BRSV: BinaxNOW RSV, TRU RSV and RSV Respi-strip. These were chosen based on previous evaluations of various immunochromatographic tests used in the diagnostics of HRSV (Gregson et al. 2005, Selvarangan et al. 2008, Miernyk et al. 2011, Sánchez-Yebra et al. 2012). Although all of these showed generally lower sensitivity compared to the recommended laboratory diagnostic methods, their high specificity, low cost, simplicity and short time required for testing (Gregson et al. 2005, Miernyk et al. 2011, Sánchez-Yebra et al. 2012) mean that these tests can be used as complementary tests in HRSV diagnostics. Moreover, all of these tests were specific to highly conservative proteins of pneumoviruses, which theoretically should also make detection of BRSV possible.

Results of our studies confirmed that rapid immunochromatographic HRSV tests would be able to
detect BRSV. All of the strip tests reacted positively with the reference strains, both HRSV and BRSV, but no reaction with negative controls was recorded. What was particularly important, no reaction with another paramyxovirus e.g. BPIV3 was seen. Positive reaction with HRSV, to which these tests were dedicated, should not be a problem because it is known that this virus has a highly restricted host range and does not have the ability to infect cattle (Bossert and Conzelmann 2002).

All of the strip tests used in this study were previously evaluated in HRSV diagnostics. The specificity of all of these assays was equally high and reached a level of 97-98%, whereas diagnostic sensitivity differed considerably for each of them and amounted to 58% for TRU RSV, 72% for BinaxNOW and 91% for RSV Respi-strip (Wybo et al. 2009, Miernyk et al. 2011, Sánchez-Yebra et al. 2012).

In our study we performed similar calculations for the same three strip tests, which allowed us to observe possible differences in their performance when used for diagnosis of BRSV infections. Compared to RT-PCR, the specificity of strip tests was relatively high, ranging from 87% for RSV Respi-strip and BinaxNOW to 100% for TRU RSV. However, their diagnostic sensitivity was lower, ranging from 33% for TRU RSV and RSV Respi-strip to 50% for BinaxNOW. Although the specificity of strip tests was similarly high for both HRSV and BRSV, their sensitivity was clearly lower when used in the detection of bovine pathogen. This could be explained by existing differences in amino acid sequence of F and N proteins between BRSV and HRSV.

Although it has been shown that some HRSV specific monoclonal antibodies are able to recognize F and N proteins of BRSV, they bind only to selected epitopes which can lead to an overall weaker reaction with the reference strains, both HRSV and BRSV, but no reaction with negative controls was recorded. What was particularly important, no reaction with another paramyxovirus e.g. BPIV3 was seen. Positive reaction with HRSV, to which these tests were dedicated, should not be a problem because it is known that this virus has a highly restricted host range and does not have the ability to infect cattle (Bossert and Conzelmann 2002).

Variability of the diagnostic sensitivity of the evaluated tests could be surprising when compared with their equal limit of detection. This discrepancy could be due to the fact that diagnostic sensitivity was evaluated on nasal swab samples acquired from vaccinated animals whereas for calculation of analytical sensitivity serial dilutions of infected cell cultures were used. Observed differences in diagnostic sensitivity could therefore represent the different ability of the evaluated assays to cope with contaminations or different titres of the virus in the nasal swabs, which could affect the performance of the tests.

Based on the calculated positive predictive value (PPV) and negative predictive value (NPV) of the strip tests, it can be concluded that the most reliable was TRU RSV. Although its sensitivity was lower compared to BinaxNOW RSV (50%), it was characterized by very high specificity (100%) as well as PPV (100%) and NPV (74%). This means that samples positive by TRU RSV were true positive. TRU RSV could therefore be used as a supportive rapid screen test for BRSV detection on a farm. However, due to the limited sensitivity, negative results would have to be confirmed by more sensitive tests such as RT-PCR. On the other hand, based on the calculated limit of detection and sensitivity, it was shown that TRU RSV outperforms the ELISA BRSV Ag test as a BRSV diagnostic method, even though it was designed for detection of HRSV. Immediate testing using TRU RSV could also reveal some positive samples as negative by cell cultures due to the possible inactivation of the virus during transport. However, due to the fact that the tested group of animals was very small, the results should be treated with caution and regarded as preliminary. Further research on a larger test group is needed before applying the results of the study in veterinary practice.

In the longer perspective, an ideal solution would be strip tests dedicated to BRSV, which would offer not only high specificity like TRU RSV, but also much higher sensitivity.

References


Gregson D, Lloyd T, Buchan S, Church D (2005) Comparison of the RSV respi-strip with direct fluorescent-antigen detection for diagnosis of respiratory syncytial virus infec-