Porcine circovirus type 2 detection in in vitro produced porcine blastocysts after virus sperm exposure

Porcine circovirus type 2 detection in *in vitro* produced porcine blastocysts after virus sperm exposure

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Running title: PCV2 and porcine blastocysts

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ABSTRACT

This study was aimed at assessing the capability of semen experimentally infected with porcine circovirus type 2 (PCV2) to produce porcine blastocysts PCR positive for PCV2. Embryos were obtained from *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of porcine oocytes or by parthenogenesis. Sperm suspension was exposed to PCV2b and utilized for IVF. PCV2 spiked semen did not reveal any reduction in sperm viability and motility but its ability to produce infected blastocysts was irrelevant as only one out of 15 blastocysts obtained by IVF were PCV2b; however 2 blastocysts were PCV2a positive. Furthermore, the presence of PCV2 was demonstrated also in embryos obtained by parthenogenesis (1 out of 17 was PCV2b and one PCV2a positive). Even if PCV2 firmly attaches to the surface of spermatozoa, experimentally spiked sperm were not effective in infecting oocytes during IVF and in producing PCR positive embryos. The infected blastocysts we obtained derived most probably from infected oocytes recovered at abattoir.

Keywords

Blastocysts, IVM-IVF, PCV2, pig, spermatozoa.

INTRODUCTION

Porcine circovirus type 2 (PCV2) related conditions cause economic losses to the pig industry worldwide. Apart from post-weaning multisystemic wasting syndrome (PMWS), PCV2 is associated with a number of conditions collectively known as porcine circovirus diseases (PCVD) (Segales et al. 2004, 2005; Chae 2005). A recent line of studies has focused on PCV2-associated reproductive failure, including irregular return to oestrus, failure of pregnancy progressing to abortion, or reduced litter size with PCV2 being detected in aborted and stillborn foetuses (O'Connor et al. 2001; Ladekjaer-Mikkelsen et al. 2001; Sanchez et al. 2001). Despite the fact that PCV2 is now being regarded as a potentially important emerging pathogen in swine reproduction, its possible interaction with oocytes, spermatozoa and embryos has not been deeply investigated.

In general, oocytes may become infected before ovulation by contact with infectious agents present in either granulosa cells or follicular fluid and after fertilization during the passage through the oviduct and the uterus (Bielanski et al. 2004). These Authors showed that only a small proportion of cumulus–oocyte complexes (COCs) recovered from PCV2 seropositive gilts is positive for viral DNA. They suggested that COCs may have the potential for the production of PCV2-contaminated embryos when follicular fluid containing COCs from infected animals is pooled for *in vitro* fertilization (IVF) as already observed for bovine herpesvirus-1 (BHV-1) and bovine viral diarrhea virus (BVDV) during *in vitro* production of bovine embryos (Bielanski et al. 1993).

In contrast, all *in vivo* fertilized, uterine stage, embryos (developmental range: from four cells to hatched blastocysts) from infected sows tested were negative for PCV2 DNA (Bielanski et al. 2004). These authors suggested that this could be due to the fact that,

despite the excretion of some pathogenic agents into the reproductive tract, uterine stage embryos may not be associated with infectious agents and/or they may be rendered free from infectious agents by a simple sequential washing procedure (Stringfellow 1998). Recently PCV2 nucleic acid has been detected in semen samples from healthy and experimentally infected boars but information regarding the fraction in which PCV2 occurs in semen are conflicting. Pal et al. (2008) showed PCV2 DNA to be present mainly in the cell fraction as opposed to the seminal plasma fraction, whereas Kim et al. (2001) found that PCV2 DNA is mainly present in seminal fluid and nonsperm cell fractions and in only few percent of sperm head.

Due to a recent increase in the use of pig as a model in modern biotechnology applications such as *in vitro* fertilization, *in vitro* embryo production and sperm sexing technology, the potential for infectious disease transmission by pig gametes is a topic of considerable interest.

To the best of our knowledge, no studies have been performed to date on the possibility of inducing PCV2 infection through infected sperm cell.

Basing on above mentioned observations, this study was set up to evaluate the ability of PCV2 experimentally infected semen to produce blastocysts PCR positive for PCV2 from IVM (*in vitro maturation*) - IVF (*in vitro fertilization*) oocytes.

MATERIALS AND METHODS

Unless otherwise specified, all the reagents were purchased from Sigma–Aldrich (Milan, Italy).

Virus

The virus used was a PCV2b strain isolated from an outbreak of PMWS in Italy and genotyped as reported by Hesse et al. (2008). It was propagated in circovirus-free PK15 cells and its identity was confirmed by PCR analysis and reactivity with specific PCV2 monoclonal antibodies. The viral suspension titre was determined by growing serial dilutions in circovirus-free PK15 cell monolayers, followed by immunofluorescent labelling for viral antigen.

Semen processing and exposure of boar spermatozoa to PCV2b

The boar used to collect semen had a proven fertility, was housed since five years in our facility (without contact with other pigs) for experimental purposes and was serologically negative to PCV2. Sperm-rich fraction of ejaculate was collected by gloved hand technique and extended in equal volumes of Androhep^{TM} (Minitub, Tiefenbach, Germany). The semen resulted negative by PCR to PCV2 DNA (Sarli et al. 2012).

Sperm suspension containing $3x10^9$ spermatozoa/100 mL of Androhep was incubated for 4 h at 16°C with 10 mL of a PCV2b viral suspension containing $10^{3.9}TCID_{50}$/mL.

Semen evaluation

To evaluate a potential negative effect of viral suspension on sperm cells, viability of spermatozoa was analyzed before and after the above described incubation with viral suspension. Briefly, 25 μL of semen were incubated with 2 μL of a 300 μM Propidium Iodide (PI) stock solution and 2 μL of a 10 μM SYBR-14 stock solution (both obtained from the Live/dead sperm viability kit; Invitrogen, Eugene, OR, USA), for 5 min at 37°C in the dark. After incubation, 10 μL of sperm suspensions were analyzed with a

Nikon Eclipse epifluorescence microscope using a double-band-pass filter for green and red fluorescence. The spermatozoa with green or red fluorescence on the head were considered live or dead, respectively. At least 200 spermatozoa were scored for each sample.

In vitro maturation (IVM) of cumulus-oocyte-complexes

Ovaries were collected at a local abattoir and transported to the lab within 2 h in a thermos filled with physiological saline at 30-35°C. Cumulus oocyte complexes (COCs) from follicles 3-6 mm in diameter were aspirated using a 18 gauge needle attached to a 10 mL disposable syringe. Under a stereomicroscope, intact COCs were selected and transferred into a petri dish (35 mm, Nunclon, Roskilde, Denmark) prefilled with 2 mL of modified PBS supplemented with 0.4% BSA. Only COCs with complete and dense cumulus oophorus were used. After three washes in NCSU 37 (Petters & Wells 1993) supplemented with 5.0 mg/mL insulin, 0.57 mM cysteine, 10 ng/mL epidermal growth factor, 50 µM ß-mercaptoethanol and 10% PCV2-PCR-negative porcine follicular fluid (IVM medium), groups of 50 COCs were transferred to a Nunc 4-well multidish containing 500 µL of the same medium per well and cultured at 39°C in humidified atmosphere of 5% CO_2/7% O_2 in air. For the first 22 h of *in vitro* maturation, the IVM medium was supplemented with 1.0 mM dibutyryl cyclic adenosine monophosphate (db-cAMP), 10 IU/mL eCG (Folligon, Intervet, Boxmeer, The Netherlands) and 10 IU/mL hCG (Corulon, Intervet). For the last 20 h COCs were transferred to fresh maturation medium (Funahashi et al. 1997). Part of the oocytes was washed in PBS, treated with pronase, in order to remove zona pellucida, rinsed twice in nuclease free water, frozen in 12 pools of 50 and stored at -80°C until PCR analysis.

Collection of immature oocytes, cumulus cells and follicular fluid

COCs and follicular fluids from 80-90 follicles, 3-6 mm in diameter, were aspirated and placed in a petri dish. COCs were then transferred to another petri dish containing 500µL of modified PBS supplemented with 0.4% BSA; the remainig follicular fluids were centrifuged and the supernatants were stored at -80°C until PCR analysis.

Oocytes were mechanically denuded from cumulus cells, then treated with pronase and frozen at -80°C. Cumulus cells were counted using a Thoma's hemocytometer then centrifuged and the pellets stored at -80°C until PCR analysis.

***In vitro* fertilization (IVF) of cumulus-oocyte-complexes**

Matured oocytes were fertilized with infected (infected group; INF) and non-infected (control group; CTR) spermatozoa as follows. Sperm suspensions were washed twice in Brackett&Olifant medium (Bracket & Olifant 1975) supplemented with 12% fetal calf serum (FCS; Gibco, Invitrogen, Monza, Italy) and 0.7 mg/mL caffeine (IVF medium). Sperm concentrations were evaluated and 50 matured oocytes, freed from cumulus cells by gentle repeated pipetting, were transferred to 500 µl IVF medium containing $1x10^6$ sperm/mL. After 90 min of co-culture, oocytes were transferred to fresh IVF medium and cultured for an additional 18 h period. Oocytes were then washed twice in NCSU-23 and cultured in 500 µL of the same medium. On day 5 post fertilization, 250 µL of medium were replaced with fresh pre-equilibrated NCSU-23 containing 20% (v/v) FCS to reach a final FCS concentration of 10% (v/v). At day 7 post-fertilization, embryos were examined by stereomicroscope and blastocysts were treated with pronase, in order to remove zona pellucida, rinsed twice in nuclease free water, individually frozen in 5

154 µL water and stored at –80°C until PCR analysis. Aliquots of infected spermatozoa used
155 for the IVF trials were washed twice with PBS and stored at –80°C until PCR analysis.
156
157 **Parthenogenetic activation**
158 After IVM, oocytes were denuded as described above, washed three times in IVF
159 medium and then parthenogenetically activated according to the method described by
160 Boquest et al. (2002) slightly modified. Briefly, the oocytes were transferred to IVF
161 medium containing 5 mM ionomycin for 5 min, then washed twice and incubated in
162 NCSU-23 containing 2 mM 6-dimethylaminopurine (6-DMAP) for 3 h at 39°C.
163 Presumptive parthenotes were washed twice in NCSU-23 and cultured in groups of 50
164 in 500 µL of the same medium. On day 5 postactivation, 250 µL of medium were
165 replaced with fresh pre-equilibrated NCSU-23 containing 20% (v/v) FCS to reach a
166 final FCS concentration of 10% (v/v). At day 7 post-activation, percent of blastocysts
167 was determined and treated as described above.
168
169 **Effect of sperm washing on virus presence in experimentally infected spermatozoa**
170 Sperm suspension containing $3x10^9$ spermatozoa/100 mL of Androhep, was incubated
171 for 4 h at 16° C with 10 mL of a PCV2b viral suspension containing $10^{3.9}TCID_{50}$/mL.
172 Then 3 aliquots of 1 mL of sperm suspension ($30x10^6$ spermatozoa) were washed with
173 10 mL PBS 5 times. After that spermatozoa were stored at –80°C until PCR analysis.
174
175 **PCV2 detection in blastocysts, oocytes, cumulus cells and follicular fluid**
176 Blastocysts and pool of oocytes were lysed as previously described (Spinaci et al. 2007)
177 and 2 µL were used to perform the first round of PCR for PCV2. Cumulus cells (110000

178 to 880000 cells/pool) were submitted to DNA extraction with ZR Genomic DNA Tissue
179 Miniprep (Zymo Research, Corp. Irvine, CA, USA) according to manufacturer's
180 instruction. Follicular fluid pools were assayed as follows: 200 µL samples underwent
181 DNA isolation by using a commercially available kit "NucleoSpin Tissue" (Macherey-
182 Nagel, Düren, Germany) in accordance to manufacturer's instruction. Twenty ng of
183 DNA from cumulus cells and follicular fluid were used for first PCR round for PCV2.
184 The detection of PCV2a or PCV2b was conducted by nested-polymerase chain reaction
185 (n-PCR). In the first round of PCR, the outer primers used were specific for PCV2. A
186 forward primer 5'-CAGTTCGTCACC-3' (designed using the Beacon Designer 2.07
187 Software, premier Biosoft International, Palo Alto, CA, USA) and reverse primers 5'-
188 CCGCACCTTCGGATATACTGTC -3' primer (Ouradani et al. 2000) were used.
189 Amplification of DNA was achieved by 35 cycles of 95°C for 1 min, 55°C for 1 min
190 and 72°C for 1 min.
191 In the second PCR run (n-PCR), a common forward primer, 5'-
192 CACGGATATTGTAGTCCTGGT-3' (Ouradani et al. 2000) and different reverse
193 primers sequences, were used to amplify either PCV2a 5'-
194 GGGGGACCAACAAAATCTC-3' or PCV2b (5'-GGGGCTCAAACCCCCGCTC-3')
195 (Hesse et al. 2008). Two µL of first PCR were used to performed the n-PCR.
196 Amplification of DNA was achieved by 35 cycles of 95°C for 1 min, 62°C for 1 min
197 and 72°C for 1 min. The reaction mixtures contained 3 mM $MgCl_2$; 200mM of each
198 dNTP, 0.2 µM of each primer; 0.5U of Go Taq Polymerase (Promega Corp, Madison,
199 WI, USA). All reactions were performed in duplicate. PCR products were analyzed on
200 2% agarose gel stained with ethidium bromide under UV light. The expected PCR
201 products of the first and n-PCR were 627 bp or 391 bp respectively. The specificity of

the primers used was demonstrated by PCR amplification of PCV2a and PCV2b control viruses ($10^{3.3}$ $TCID_{50}$/mL and $10^{3.9}$ $TCID_{50}$/mL for PCV2a and PCV2b respectively). The sensitivity of the PCR was estimated through serial dilutions from $1x10^9$ to 10 DNA copies of reference positive control (PCV2a and PCV2b). The PCR (first round) had sensitivity of $1x10^5$ whereas the n-PCR was 10 times more sensitive ($1x10^4$). Amplification of ß-Actin gene was performed for all sample as internal control as previously described (Forni et al. 2003).

PCV2 detection in sperm samples

Aliquots of sperms ($6x10^6$ spermatozoa) were resuspended with denaturing buffer (20 mM TrisHCl; EDTA 20mM; DTT 80mM; SDS 4%; NaCl 200mM; proteinase K 20 mg/mL) and incubated for 3h at 55°C then submitted to genomic DNA extraction by using ZR Genomic DNA (Zymo Research, Corp. Irvine, CA, USA) according to manufacturer's instruction. The first round of PCR was performed with 50 ng of extracted DNA and PCR product was analyzed on 2% agarose gel stained with ethidium bromide under UV light.

Experimental design

Four experiments were performed:

Experiment 1: evaluation of PCV2 presence in blastocysts obtained by IVF with *in vitro* infected or control semen;

Experiment 2: evaluation of PCV2 presence in blastocysts obtained by parthenogenesis;

Experiment 3: evaluation of PCV2 presence in *in vitro* matured oocytes and immature oocytes, cumulus cells and follicular fluid;

Experiment 4: evaluation of PCV2 presence in *in vitro* infected spermatozoa after increasing washing steps.

Statistical analysis

Data on the number of blastocyst obtained were pooled from two replicates and analyzed using SPSS 11 (SPSS Inc., Chicago, IL, USA) with chi-squared test; the level of significance was set at $P<0.05$.

RESULTS

Sperm viability

Sperm viability was not affected by the treatment with viral suspension: it was 82.97± 3.24% before incubation and 81.44 ± 2.8% (mean±SE) after incubation.

Experiment 1: evaluation of PCV2 presence in blastocysts obtained by *in vitro* infected semen

Sperm cells exposed for 4 h to PCV2b were PCR positive. Exposure of spermatozoa to PCV2b did not exert any effect on the percentage of embryos that developed to blastocyst stage (Table 1). In CTR group, 4 out of 15 blastocysts tested were PCV2a positive. In INF group, 1 out of 15 blastocysts screened were PCV2b positive and 2 PCV2a positive (Table 2, Fig. 1).

Experiment 2: evaluation of PCV2 presence in blastocysts obtained by parthenogenesis

At day 7 the percent of blastocysts parthenogenetically activated was 23.92%.

One out of 17 blastocysts parthenogenetically activated was PCV2b positive and one PCV2a positive.

Experiment 3: evaluation of PCV2 presence in *in vitro* matured oocytes and immature oocytes, cumulus cells and follicular fluid

None of the twelve pools of fifty *in vitro* matured oocytes was positive to PCV2b or PCV2a. On the basis of these results PCV2b or PCV2a positivity was assayed in six more consistent pools of immature oocytes, cumulus cells and follicular fluid (from 80-90 follicles). The results obtained are presented in Table 3 and Figure 2.

Experiment 4: evaluation of PCV2 presence in *in vitro* infected spermatozoa after increasing washing steps

As shown in Figure 3 a sequential washing procedure was not effective in removing virus from spermatozoa.

DISCUSSION

The purpose of the present work was to evaluate the ability of PCV2 experimentally spiked semen to produce blastocysts PCR positive for PCV2. Embryos were obtained from *in vitro* maturation and fertilization of porcine oocytes from ovaries collected at a local abbattoir.

To our knowledge, the present study is the first one in which spermatozoa have been contaminated with PCV2 and utilized for IVF under experimental conditions.

Our results clearly demonstrate that PCV2 firmly attaches to the surface of spermatozoa (as demonstrated in experiment 4) but they don't seem to be effective in infecting the oocytes during IVF and in producing infected embryos.

The infected blastocysts we obtained derived most probably from infected oocytes obtained at abattoir; in fact, 6 out of 30 blastocysts tested were PCV2a positive. Moreover parthenogenetic blastocysts PCV2b or PCV2a positive were obtained. Similar contamination has been observed also by Bureau et al. (2005).

In order to confirm this hypothesis, we evaluated the presence of PCV2a or PCV2b in different pools of fifty *in vitro* matured oocytes but none of the pools was positive. When more consistent oocyte pools were assayed, few of them resulted positive for PCV2a or PCV2b. Moreover, PCV2 positivity was also recorded in follicular fluids and cumulus cells from pool of follicles.

These results confirm that oocyte from ovaries collected at the slaughterhouse can be infected and therefore could generate infected embryos. Why experimentally spiked semen was not effective in producing infected embryos in our study is not clear.

A hypothesis could be that the virus bound to sperm cells could be entrapped, during the zona pellucida crossing, in the ZP network of filaments with pores/holes and meshes or to the presence of carbohydrates and glycoproteins that can function as receptors for viruses. The PCV2 capsid is constituted by capsomeres, proteins with a high affinity for glycosilated proteins (Misinzo et al. 2006). Since porcine ZP is composed of glycoproteins (pZPA, pZPB, pZPC) (Harris et al. 1994), an interaction between the viral capsid and these glycoproteins might be responsible for the sticky behaviour of the porcine ZP towards PCV2.

297 It is also important to note that Bureau et al. (2005) reported that hyaluronidase is able
298 to remove PCV2 from contaminated embryos. In fact, those authors demonstrated that
299 when trypsin was used as proteolytic enzyme in washing buffers, more virions remained
300 bound to the zona pellucida than when hyaluronidase was used.
301 It's well known that sperm head surface is involved in acrosome reaction which results
302 in the release of digestive enzymes (including hyaluronidase) required for zona
303 penetration (Yanagimachi et al. 1994; Flesch & Gadella 2000). Therefore we can
304 hypothesize that, during the fertilization process, membrane capacitation modifications
305 first and acrosome reaction subsequently, with hyaluronidase and other enzymes release,
306 could have induced, at least in part, the detach of PCV2 from sperm head plasma
307 membrane. More recently Blomqvist et al. (2011) showed that Single Layer
308 Centrifugation (SLC) on Androcoll-P, followed by swim-up, is effective in removing
309 PCV2 virus from boar semen. Moreover, Bucci et al. (2013) demonstrated that SLC
310 using Androcoll-P induces some capacitation-related changes in boar sperm membrane,
311 possibly as a consequence of the complete removal of seminal plasma and the partial
312 removal of cholesterol from sperm membrane due to Androcoll centrifugation.
313 Basing on the above mentioned observations, we tested the grade of spermatozoa
314 infection after 2 h at 39°C in capacitation medium and acrosome reaction induction by
315 adding A23187 as previously described by Bucci et al. (2012). Spermatozoa subjected
316 to *in vitro* induction of capacitation and acrosome reaction showed a reduction of PCV2
317 amount (unpublished data). These results could explain, at least in part, the inability of
318 sperm cell to produce infected blastocysts.

In conclusion, the present study shows that PCV2 attaches to the surface of spermatozoa but it seems not to have the ability to carry the virus into the oocyte at fertilization and to produce infected embryos.

Conflict of interest statement

None of the authors of this manuscript have any conflict of interest to declare.

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439

440 **Table 1** Blastocysts obtained by IVF with infected (INF) or non-infected spermatozoa
441 (CTR).

442

443

Groups	No. oocytes	No. blastocysts (%)
CTR	250	36 (14.4)
INF	250	33 (13.2)

444

445 **Table 2** Evaluation of PCV2a or PCV2b presence in blastocysts obtained by IVF.

446

Groups	No. blastocysts examined	No. blastocysts positive	
		PCV2a	PCV2b
CTR	15	4	0
INF	15	2	1

447

448

449 **Table 3** PCV2a and PCV2b positivity in the six pools of immature oocytes, cumulus
450 cells and follicular fluid.
451

	PCV2a positivity			PCV2b positivity		
Pool n°	Oocytes	Cumulus cells	Follicular fluids	Oocytes	Cumulus cells	Follicular fluids
1	-	-	-	-	-	+
2	-	-	-	-	-	-
3	-	-	+	-	-	-
4	-	+	-	+	+	-
5	+	-	-	-	+	+
6	-	-	-	-	-	+

452

453

454

455 **Figures captions**

456

457 **Figure 1** Representative image of agarose gels of nested PCR for PCV2a and PCV2b
458 products (**A, B**) and ß-actin (**C**) of blastocysts. **A**: CTR blastocysts; **B:** blastocysts
459 obtained after IVF with PCV2b experimentally spiked semen. Lanes 1-5; 10-14:
460 blastocysts; Lane 7: PCV2a positive control; Lane 8: PCV2b positive control; Lane 6:
461 negative control of PCR; MWM: DNA molecular weight marker.

462

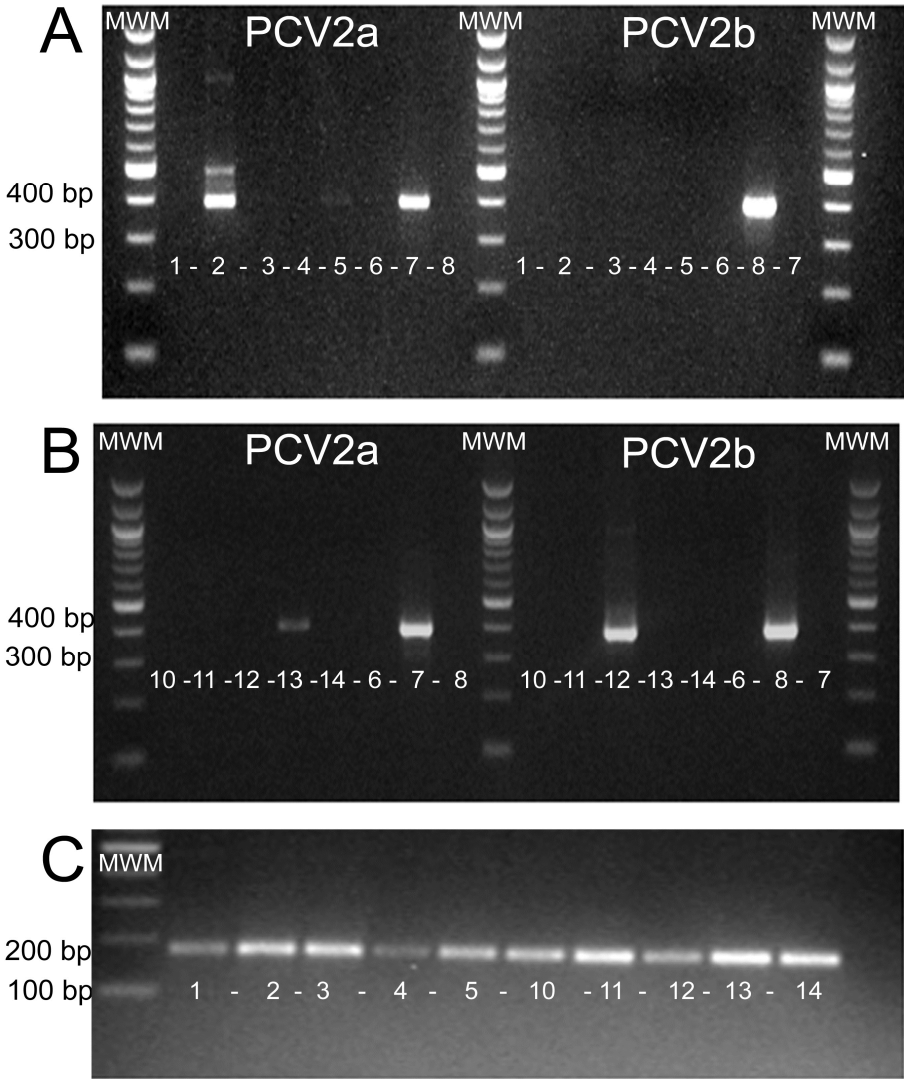
463 **Figure 2** Representative image of agarose gels of nested PCR for PCV2a and PCV2b
464 products of different pools (n=6; Lanes 1-6) of: ocytes (**A**); cumulus cells (**B**); follicular
465 fluid (**C**). Lane 7: DNA molecular weight marker. Lane 8: PCV2a and PCV2b positive
466 control.

467

468

469 **Figure 3** Representative image of agarose gel of PCV2 PCR of infected spermatozoa
470 (Lane 4) and washed infected spermatozoa (Lanes 1-3). Lane 5: negative control of
471 PCR; Lane 6: PCV2b positive control; MWM: molecular weight marker.

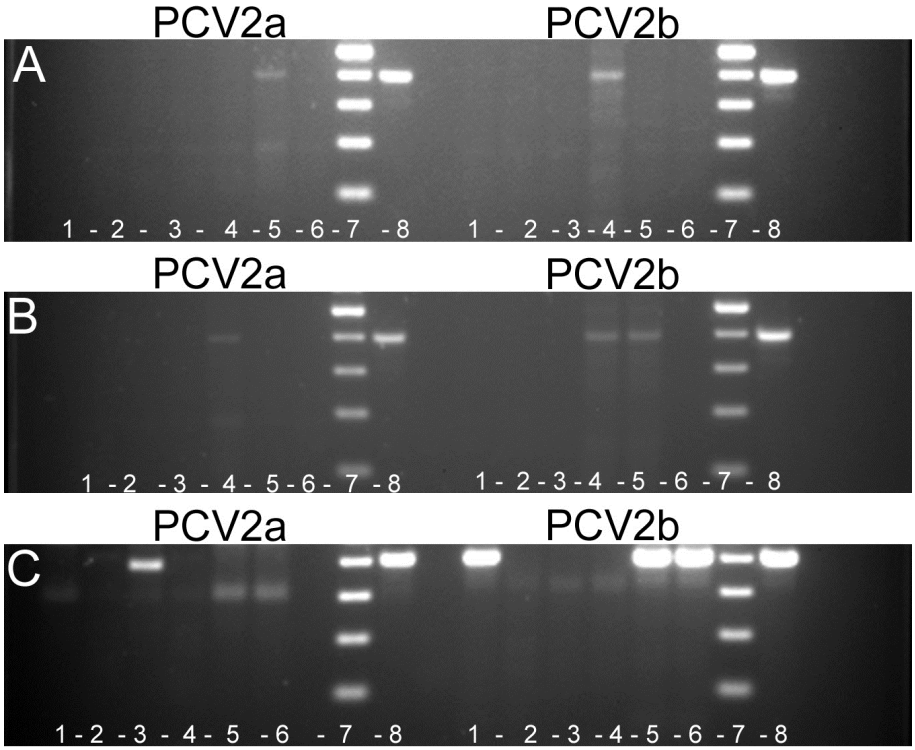
472



473

474 **Figure 1**

475



476

477 **Figure 2**

478

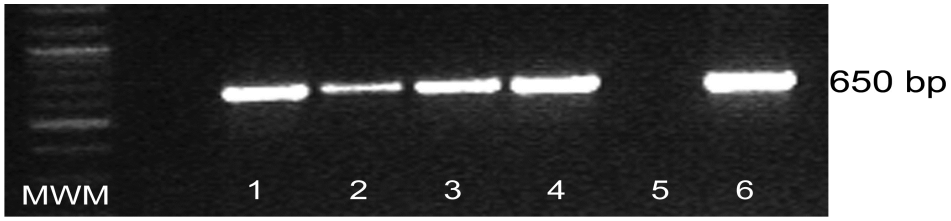


Figure 3