Antibiotic Resistant Microbial Contamination (*Enterobacter cloacae*) Derived from Egg Yolk and Frozen Semen Extender in Porcine *In Vitro* Fertilized Embryos

Seong-Sung Kwak[1,a], Se-Heon Jeong[1,a], Seung-Hoon Jang[1], Yubyeol Jeon[1], Young-Hee Nam[1,3], Dibyendu Biswas[1], Wan-Kyu Lee[2] and Sang-Hwan Hyun[1,*]

[1]*Laboratory of Veterinary Embryology and Biotechnology (VETEMBIO), College of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Republic of Korea*

[2]*Laboratory of Veterinary Bacteriology, College of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Republic of Korea*

[3]*Veterinary Service Laboratory, Department of Livestock Research, Gyeonggido Veterinary Service, Yongin 449-882, Korea*

ABSTRACT

The present study was to investigate the source of contamination during semen processing for *in vitro* uses. In the present study, frozen semen was prepared from liquid semen in our laboratory for *in vitro* fertilization (IVF) experiments due to lack of fresh semen. Antibiotics were added in the frozen semen extender (kanamycin and gentamicin) and *in vitro* culture (IVC) medium (gentamicin) for further inhibiting growth of microorganisms. Nevertheless, proliferations of microorganisms were observed in IVC culture drop during culturing of IVF embryos using frozen semen. Randomly 3 samples were taken from the liquid semen, frozen semen and egg yolk. Contaminated IVC medium, frozen-thawed semen, liquid semen and egg yolk were cultured in de Man, Rogosa and Sharpe (MRS) agar medium. Whitish colonies were detected in contaminated IVC drop, frozen-thawed semen samples and egg yolk but no colonies were formed in liquid semen samples. Gram-negative and rod-shaped identical bacteria were found in both frozen-thawed semen sample and contaminated IVC drop and egg yolk samples. *Enterobacter cloacae* were confirmed by API 20E kit according to manufacturer's instruction with identification value (% ID) 94.3% and T index 0.88. Antibiotic susceptibility tests were done according to Clinical and Laboratory Standards Institute (CLSI) by using ampicillin, amikacin, cephalothin, gentamicin, kanamycin, tetracycline, oxytetracycline, sulfamethoxazole trimethoprim, norfloxacin and ciprofloxacin test. Among them *Enterobacter cloacae* were resistant to ampicillin, amikacin, cephalothin, gentamicin, kanamycin but susceptible to tetracycline, oxytetracycline, sulfamethoxazole trimethoprim, norfloxacin and ciprofloxacin. From these findings it could be suggested that this contamination sources might be from egg yolk.

(Key words : egg yolk, frozen semen, IVF, *Enterobacter cloacae*, contamination)

INTRODUCTION

In vitro fertilization (IVF), one of the assisted reproductive technologies (ARTs), has been developed and used in human and veterinary medicine for various purposes. In human, it is available in aim of infertility treatment or investigation of human embryo development *in vitro*. In pigs, as an animal model for human diseases and xenotransplantation, it is a method of producing porcine preimplantation embryos to study *in vitro* porcine embryo development and gain transferable *in vitro* produced (IVP) porcine embryos. Media and condition for *in vitro* fertilization (IVF) and culture (IVC) are optimized to promote embryonic development, and this environment and media provide an ideal habitat for a variety of microorganisms. So, it is easy to be contaminated. Contamination during IVF practice may be harmful on IVF/IVC system and lead to disease transmission. Therefore, an IVF/IVC system should be a sterile system (Cottell *et al.*, 1996), and accurate procedures are needed for preparing and storing culture media.

Viral, bacterial, fungal and parasitic organisms have been identified in association with semen and embryos in human and animals (Sellers, 1983; Afshar and Eaglesome, 1990; Eag-

[a] These authors contributed equally to this work.

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* Correspondence : E-mail : shhyun@cbu.ac.kr

lesome and Garcia, 1992; Eaglesome *et al.*, 1992; Dejucq-Rainsford and Jegou, 2004; Bielanski, 2007; Hare, 2007). Especially, in human, identification of the microorganisms contamination showed that infections were mainly caused by *Escherichia coli* and *Candida* species (Kastrop *et al.*, 2007). Microorganisms contamination during IVF was reviewed by Bielanski (2007) and risk of contamination of germplasm during cryopreservation and cryobanking in IVF also reviewed by Bielanski and Vajta (2009). There are few contamination cases, however, reported in porcine embryo IVF/IVC system.

In this study, we made frozen semen for IVF/IVC experiments. Antibiotics were added in the frozen-thawed semen and IVC media (PZM-3) for inhibiting growth of microorganisms. Nevertheless, proliferation of microorganisms was observed in IVC drops. In this report, we identified the micro-organism and took an antibiotic resistance test. Also we investigated the route of contamination.

MATERIALS AND METHODS

1. Media and Reagent

Unless otherwise stated, all reagents and chemicals used in this case report were purchased from Sigma Aldrich Co. (St. Louis, MO. USA). Tissue culture medium-199 (TCM-199) was purchased from Invitrogen (Grand Island, NY, USA). The porcine zygote medium-3 (PZM-3) supplemented with 0.3% (w/v) bovine serum albumin (BSA) was prepared according to the method of Yoshioka *et al.* (2002). The four well culture dishes used in *in vitro* maturation (IVM) were purchased from Nunc Inc. (Copenhagen, Denmark).

2. Oocytes Collection and *In Vitro* Maturation

Ovaries were collected from a local slaughterhouse (Hannaeng Inc.) and washed with physiological saline supplemented with 100 IU/ml penicillin G and 100 μg/ml streptomycin sulphate. The ovaries were maintained at 32 to 35℃ in physiological saline during transport to the laboratory (2 hours after collection). The cumulus oocyte complexes (COCs) were aspirated using an 18-gauge needle attached to a 10 ml disposable syringe (Kim *et al.*, 2004) from superficial follicles 3 to 6 mm in diameter and pooled in to 15 ml conical tubes and allowed at 37℃ during 5 minute (Suzuki *et al.*, 2006) to settle down as sediment. The supernatant was discarded and the precipitate was resuspended with HEPES-buffered Tyrode's medium (TLH) containing 0.05% (w/v) polyvinyl alcohol (TLH- PVA) (Bavister *et al.*, 1983) and observed under a stereomicroscope. Only compact COCs with ≥3 uniform layers of compact cumulus cells (Bagg *et al.*, 2004; Tong *et al.*, 2004) and uniform ooplasm were recovered from the collected fluid and washed three times in the oocyte maturation medium and transferred 50 COCs to pre-warmed 500 μl of culture medium (TCM- 199; Invitrogen Corporation, Grand Island, NY, USA) which was supplemented with 0.6 mM cysteine, 0.91 mM pyruvate, 10 ng/ml epidermal growth factor, 75 μg/ml kanamycin, 1 μg/ml insulin, 10% (v/v) porcine follicular fluid (pFF), fresh 4 IU/ml pregnant mare serum gonadotropin (PMSG) and 4 IU/ ml hCG (Intervet, Boxmeer, Netherland) and was incubated at 39℃ in a humidified atmosphere of 5% CO_2 in 95% air. After 22 hours, the COCs were washed with PMSG- and hCG- free TCM-199 medium and were cultured with the same medium for another 20 hours. The pFF was aspirated from 3~7 mm follicles of prepubertal gilt ovaries. After centrifugation at 1,600× g for 30 min, the supernatants were collected and filtered sequentially through 1.2- and 0.45-μm syringe filters (Gelman Sciences, Ann Arbor, MI, USA). The prepared pFF was then stored at −20℃ until use. For maturation, the selected COCs were incubated at 39℃ in a humidified atmosphere of 5% CO_2 in 95% air. After 20~21 h of maturation with hormones, the COCs were washed two times in a maturation medium without hormone supplements and then cultured for 20~21 h without hormone supplements at 39℃ under 5% CO_2 in air. At the end of IVM, COCs were denuded with 0.5 mg/ml hyaluronidase for 1 min by gentle pipetting.

3. *In Vitro* Fertilization and Embryo Culture

A straw of frozen boar semen was thawed at 39℃ for 1 minute in a water bath and washed in 10 ml phosphate buffered saline (PBS) by centrifuging twice at 2,000 rpm for 2 min (Johnson *et al.*, 2000). After washing, the sperm pellet was resuspended in modified Tris-buffered medium (mTBM) which was pre-equilibrated for 18 hours at 39℃ under 5% CO_2 (Abeydeera *et al.*, 1997). After 42 hours of IVM, denuded oocytes were washed three times in TLH-PVA, and then evaluated under a stereomicroscope for oocytes selection that oocytes with visible polar body were used. Groups of 15 oocytes were randomly placed into 45 μl droplets of mTBM medium covered with pre-warmed mineral oil. After appropriate dilution, 5 μl of the sperm suspension was added to a 45 μl drop of fertilization medium (mTBM) to yield a final sperm concentration of 1×10^6 sperm/ml. The sperm concentration was determined with

a hemacytometer (MARIENFELD, Germany). The oocytes were co-cultured with sperm for 6 hours at 39℃ in a humidified atmosphere of 5% CO_2 and 95% air. After 6 hours, the attached sperms were completely removed from the surface of the zygotes by gentle pipetting. The zygotes were then washed three times with TLH-PVA before being cultured in 25 μl micro-drops (10 zygotes per one drop) of porcine zygote medium-3 (PZM-3) (Yoshioka *et al.*, 2002) covered with mineral oil pre-incubated under 5% O_2, CO_2 and 90% N_2 at 39℃ for 168 hours. Cleavage and blastocyst formation were evaluated under a stereomicroscope (Olympus, Tokyo, Japan) at 48 and 168 hours after insemination, respectively.

4. Sperm Preparation

The method of semen cryopreservation is essentially the same as that described by Pursel and Johnson (1975) and Wang *et al.* (1991) with a few modifications. Briefly, fresh semen was provided from the Veterinary Service Laboratory (Department of Livestock Research, Yong-in city, Gyeonggi-do, Republic of Korea) and delivered to our laboratory at 17℃ in portable incubator. Liquid semen was diluted 1:2 with Hulsenberger VIII diluents (Richter *et al.*, 1975) at 15℃ and centrifuged for 10 min at 3,680 g. The supernatant was discarded and spermatozoa were resuspended in Beltsville F5 (BF5) extender (Pursel *et al.*, 1978) to give 10×10^8 sperm/ml. After cooling to 5℃, the sperm suspensions were diluted with the same volume of BF5 solution containing 2 % (v/v) glycerol. The sperm suspensions were frozen in straws and stored in liquid nitrogen until required for IVF.

5. Microbiologic Examination

Contaminated IVC drops, frozen-thawed semen, egg yolk and liquid semen were sampled and sent to the microbiology laboratory for further investigation. These samples were cultured according to standard procedures. Samples were inoculated on de Man, Rogosa and Sharpe (MRS) agar (De Man *et al.*, 1960) plates for detection of Enterobacteriaceae. Plates were incubated aerobically at 37℃ and read within 24 h. Microbial identification was performed using API 20E kit (BioMérieux, Chemin de l'Orme, France). The API 20E kit was used according to the manufacturer's instructions. The API 20E biochemical profiles were calculated by API 20E V4.1 (BioMérieux, https://apiweb.biomerieux.com). A detailed description of the procedure for inoculating and reading the API 20E has been published (Aldridge *et al.*, 1978).

To test the antibiotic resistance of the isolated strains, the disk diffusion assay was carried out following the instructions of the Clinical and Laboratory Standards Institute (CLSI, Performance standards for antimicrobial disk susceptibility tests; 10th edition, 2009). The antimicrobial susceptibility test disks included ciprofloxacin (5 μg), ampicillin (10 μg), tetracycline (30 μg), gentamicin (10 μg), kanamycin (30 μg), cephalothin (30 μg), amikacin (30 μg), norfloxacin (10 μg), sulfamethoxazole and trimethoprim, and oxytetracycline (30 μg).

RESULTS

After IVF using frozen-thawed semen and IVC, multiplication of microorganisms were slightly observed on Day 1 (24 hours after IVC) and distinctly watched on Day 2 (42 hours after IVC) in all IVC drops (Fig. 1). As shown in Table 1, the cleavage rate was 32.2 % which was significantly lower than normal IVF/IVC system cleavage rate in our laboratory. On Day 7 (168 hours after IVC), no embryos reached to blastocyst.

Identical whitish colonies were detected by incubation of microorganisms in MRS media from the contaminated IVC drops, frozen-thawed semen and egg yolk (on Day 2), respectively. However, nothing was observed in incubation of liquid semen (Data not shown). After gram staining of respective colonies, gram-negative and rod-shaped identical bacteria were observed (Fig. 2). *Enterobacter cloacae* (*E. cloacae*) was identified by API 20E kit with identification value (% ID) 94.3% and T index 0.88. *E. cloacae* was resistant to ampicillin, amikacin, cephalothin, gentamicin, and kanamycin, but susceptible to tetracycline, oxytetracycline, sulfamethoxazole and trimethoprim, norfloxacin, and ciprofloxacin (Table 2).

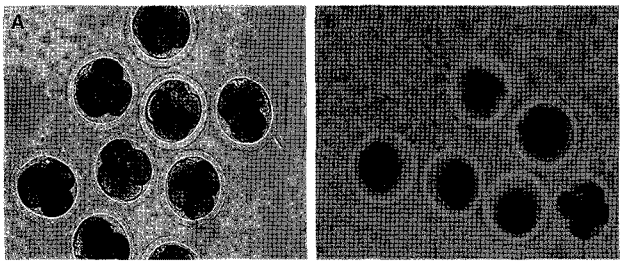

Fig. 1. Embryos in normal and contaminated IVC drops on Day 2 (48 h after IVC). Clear sight and cleaved embryos are seen in A (embryos in normal IVF/IVC drop). Multiplication of micro-organisms are seen in B (embryos in microbial contaminated drop), and embryos are almost degenerated or stayed in one-cell stage.

Table 1. Effect of microbial contamination on porcine embryo cleavage rate and blastocysts

	No. of oocytes examined(r*)	1-cell	2-cells	4-cells	6- to 8-cells	Degenerated oocytes	Cleaved oocytes (%)	Blastocysts (%)
Normal	197(3)	69	30	52	37	9	119(60.4)	42(21.3)
Contaminated	255(3)	137	34	43	5	36	82(32.2)	0(0)

r* : Replication.

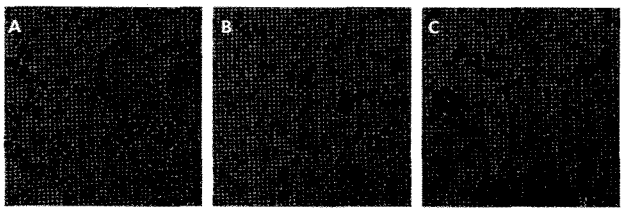

Fig. 2. Gram staining of microorganisms. Gram negative and rod-shaped bacteria are seen in A (from contaminated drops), B (from frozen-thawed semen), and C (from the egg yolk).

DISCUSSION

In this report, contamination of microorganisms in porcine embryo IVC drops was caused by *Enterobacter cloacae*. *E. cloacae* was resistant to ampicillin, amikacin, cephalothin, gentamicin, and kanamycin. This antibiotic resistant made possible to survive in antibiotics supplemented frozen-thawed semen (kanamycin and gentamicin) and IVC media (gentamicin). Contamination of antibiotic resistant *E. cloacae* leaded to reduce embryonic development and finally all embryos were dead during IVC.

Possible origins or routes of microorganisms contamination during IVF/IVC procedures were already infected boar semen, contamination during semen collection, processing or storage, animal-derived supplements used in diluents and extenders (egg yolk and milk), and poor laboratory hygiene (Bielanski, 2007). As the identical colonies were observed in incubation of contaminated IVC drops derived and frozen-thawed semen respectively, we could know that the frozen-thawed semen caused microbial contamination. The reason why more colonies were appeared at incubation of contaminated IVC drops derived than frozen-thawed semen was that *E. cloacae* could multiply highly during 48 h IVC. Incubation of fresh semen which used in making the frozen-thawed semen was not detected any colonies. Thus the extender added in frozen-thawed semen or coincidental contamination during preparation of frozen-thawed semen might cause the *E. cloacae* contamination. From among the materials added in frozen-thawed semen, egg yolk might be contaminated by microorganisms. Because all materials added in frozen-thawed semen were purchased from Sigma Aldrich Co. (St. Louis, MO. USA) and filtered by 0.45 μm filter before use except the egg yolk which was commercial product. Egg yolk had variety of antibiotic resistant bacteria such as *Staphylococcus aureus*, *Enterococcus faecalis*, *E. cloacae*, *Es-*

Table 2. Antibiotic resistance test of *Enterobacter cloacae*

No.	Symbol	Full name	R	I	S	mm	Result
1	AM10	Ampicillin	<13	14~16	>17	7	R
2	AN 30	Amikacin	<14	15~16	>17	7	R
3	CF 30	Cephalothin	<14	15~17	>18	7	R
4	CIP 5	Ciprofloxacin	<15	16~20	>21	24	S
5	GM 10	Gentamicin	<12	13~15	>15	7	R
6	K 30	Kanamycin	<13	14~17	>18	7	R
7	NOR 10	Norfloxacin	<12	13~16	>17	22	S
8	SXT	Sulfamethoxazole & Trimethoprim	<10	11~15	>16	18	S
9	T 30	Oxytetracycline	<14	15~18	>19	20	S
10	Te 30	Tetracycline	<14	15~18	>19	20	S

cherichia coli, *Pantoea agglomerans*, *Serratia liquefaciens*, *Citrobacter freundii*, and *Pseudomonas stutzeri*, and *E. cloacae* was resistant to ampicillin, amoxicillin + clavulanic acid, erythromycin, and tetracycline (Papadopoulou *et al.*, 1999). It supports that *E. cloacae* may be derived from the egg yolk.

Egg yolk is a major additive to protect against cold shock (Benson *et al.*, 1967). Its protective effect can be improved by adding Orvus Es Paste (OEP) to the extender (Graham E.F. *et al.*, 1971; Pursel *et al.*, 1978). Egg yolk is widely used but easy to be contaminated by antibiotic resistant bacteria. Therefore, the exact procedure for using egg yolk on producing cryopreserved semen should be needed.

Ruthless use of antibiotics on layers is regarded to cause antibiotic resistant bacteria living in layers and coming out in egg yolk. These bacteria can make harmful influences on IVF/IVC system and transfer disease during artificial insemination (AI) or embryo transfer (ET) procedure. Also it can exert bad influences on experimenter to get disease during investigations. In humans, *E. cloacae* is sometimes associated with urinary tract and respiratory tract infections (Sanders and Sanders., 1997). The emergence of antibiotic resistant bacteria has made it difficult to control their spread and contamination despite the supplement of antibiotics in extenders. Therefore, we should make simple, correct and more efficient methods of controlling the bacterial contamination, especially in preparing frozen-thawed semen for IVF. Commercially developed semen extenders (e.g. Biociphos-plus, from IMV) free of egg yolk and milk, can be recommended that it removed the potential risk of contamination of semen with bacteria and mycoplasmas from substances of animal origin (Bousseau *et al.*, 1998). Also we have to combine with various procedures for the disinfection of frozen-thawed semen such as washing procedures (swim-up and Percoll gradient centrifugation, etc), immunoextenders, ozone treatment, and so on (rewiewed by Bielanski A. *et al.*, 2007).

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