

**Microbiological evaluation of fresh semen from captivity kept collared peccary
(*Pecari tajacu*) at Brazilian Amazon****Avaliação microbiológica do sêmen fresco de caititus (*Pecari tajacu*) mantidos
em cativeiro na Amazônia Brasileira**

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ABSTRACT

This study aimed to identify the existent of microbiota in the fresh semen of collared peccary (*Pecari tajacu*), kept in captivity, and their susceptibility patterns to antimicrobial drugs. Ten males of this species were submitted into experimental induced ejaculation procedures throughout 14 months. The microorganisms found in the semen were evaluated within the following criteria: morphology, biochemistry, colony forming unit (CFU mL⁻¹), and antibiotic sensitivity. 225 Gram-positive strains were isolated, out of 88 samples. They were identified as following: *Streptococcus* (30.2%), *Staphylococcus* (30.2%), *Micrococcus* (33.7%), *Corynebacterium* (2.2%), *Enterococcus* (1.7%), and *Bacillus* (1.7%). The cell counting of contaminated semen material was under 300 CFU mL⁻¹. The evaluation of sensitivity against eleven antibiotics revealed that most of the bacteria found were sensitive to Gentamicin, Cephalothin, Amikacin, Ampicillin, Ceftriaxone, Cephotoxin and Penicillin. During the evaluation period, the presence of microorganisms in the semen here analyzed did not influence the quality of the semen production of the donor animals. Semen contamination is probably coming from the surrounding environment in which the animals were kept, during collection procedures. This study suggests that Gentamicin and Amikacin are better choices over Tetracycline as diluters of collared peccary's semen.

Keywords: Tayassuidae, Ejaculated, Microorganisms, antibiotics.

RESUMO

O objetivo deste estudo foi identificar a microbiota existente no sêmen fresco de queixada (*Pecari tajacu*), mantido em cativeiro, e seus padrões de suscetibilidade a medicamentos antimicrobianos. Dez machos desta espécie foram submetidos a procedimentos experimentais de ejaculação induzida ao longo de 14 meses. Os microrganismos encontrados no sêmen foram avaliados dentro dos seguintes critérios: morfologia, bioquímica, unidade formadora de colônias (UFC mL⁻¹) e sensibilidade a antibióticos. Foram isoladas 225 cepas Gram-positivas, de 88 amostras. Eles foram identificados da seguinte forma: *Streptococcus* (30,2%), *Staphylococcus* (30,2%), *Micrococcus* (33,7%), *Corynebacterium* (2,2%), *Enterococcus* (1,7%) e *Bacillus* (1,7%). A contagem de células do sêmen contaminado estava abaixo de 300 UFC mL⁻¹. A avaliação da sensibilidade contra onze antibióticos revelou que a maioria das bactérias encontradas eram sensíveis à gentamicina, cefalotina, amicacina, ampicilina, ceftriaxona, cefotaxina e penicilina. Durante o período de avaliação, a presença de microrganismos no sêmen aqui analisado não influenciou a qualidade da produção de sêmen dos animais doadores. Provavelmente, a contaminação do sêmen é proveniente do ambiente em que os animais foram mantidos, durante os procedimentos de coleta. Este estudo sugere que a gentamicina e a amicacina são melhores escolhas sobre a tetraciclina como diluidoras do sêmen de queixada.

Palavras-chave: Tayassuidae, ejaculado, microrganismos, antibióticos.

1 INTRODUCTION

Collared peccary (*Pecari tajacu*) is a species of great environmental diversity and adaptation. In captivity, it represents a highly potential in terms of productivity, when compared to other Amazon mammals (MAYOR et al., 2007; SILVA et al., 2016).

A wide range of bacteria commonly leads to reproductive diseases. Kuster and Althouse (2016) report that the reproductive performance of boar through artificial insemination is threatened by bacteriospermia. They describe a significant list of bacteria registered in semen that come from fecal material, preputial, skin and hair. During the semen collection to be used at *in vitro* fertilization procedures, it is quite common the occurrence of bacterial contamination coming from either the environment or from the donor animal's body (RIESENBECK et al., 2015). Such contamination is extremely hard to avoid. Therefore, it is crucial the addition of wide range of antibiotics to the semen's diluent (YÁNIZ et al., 2010).

A great number of authors have reported the presence of bacteria in the semen of different mammal species, such as: leopards (MORATO et al., 1998), sheep (MENDEZ NÁREZ et al., 1999), swallows (LOMBARDO; THORPE, 2000), pigs (KUSTER; ALTHOUSE, 2016), camel (GHONEIM et al., 2014), bovine and buffalo (SANNAT et al., 2015), goats (SOUZA et al., 2006), fishes (OLIVEIRA et al., 2007) and humans (WENG et al., 2014). However, there is no report found in the literature regarding the presence of such microorganisms in the semen of collared peccary.

It is suggested that the presence of microorganisms in the semen of collared peccary may interfere on its quality when used on *in vitro* fertilization procedures. As data regarding this topic is absent, studies focused in this topic are in high demand. The goals of this study were to identify the existent micro flora in fresh semen of collared peccary kept in captivity, as well as to evaluate their sensitivity towards the action of antibiotics.

2 MATERIAL AND METHODS

This study has followed all the rules established by the National Council for Animal Experiments of Brazil (CONCEA) and approved by Animal Ethics Committee (CEUA) of the Universidade Federal do Pará, registration nº 184/13 CEPAE-UFPA.

10 individuals, males, with average age of 76.8 (± 37.8) months and weighing on average 19.8 (± 2.7) kg, kept in the scientific captivity area of Embrapa Amazônia Oriental (Belém-PA, Brazil; 01°24'S; 48°20'W) were used. The animals were kept in 36 m² collective cubes, grouped by

family related individuals of up to 10 animals per cube. The male:female ratios in the groups were 1:2 or 1:3, under natural conditions of temperature, humidity and full darkness-lightness cycle. All the animals received food supplementation composed by pig food based on corn and soy (500 g animal⁻¹), diverse species of grass, fruit and water *ad libitum*. At 24 hours before collecting samples, the animals were isolated and submitted into fast from both food and liquids.

The animal's sedation was done in accordance to the protocol established by Souza et al. (2009) and Kahwage et al. (2010). After sedation, cleaning procedures such as hair removal, prepuce washing, feces removal, and rectus cleaning took place. The external and internal prepuce areas were cleaned up with pre-warmed NaCl 0.9 % solution (35 °C).

The semen collection was done in intervals of 15 days, by rectal electro stimulation, as described by Souza et al. (2009). Aliquots of the collected semen were used for seminal analysis, as well as for microbiological tests.

Samples were first evaluated for the following sperm analysis parameters: general appearance, color, total volume, concentration, pH, sperm's motility and vitality; morphology, viability and defected sperm cells.

For the microbiological assays, sample aliquots were inoculated in duplicates, in Petri dishes containing enriched bacteriological medium agar with 5% sheep blood defibrinated and MacConkey Agar. Petri dishes were then incubated at 37 °C, during 24 to 48 h. After incubation, the colonies were evaluated through their morphology per stain characteristics and biochemical tests were performed for their identification (QUINN et al., 1994).

The CFU counting was performed in accordance to the protocol preconized by World Organization for Animal Health (OIE, 2008) for cattle, with some few modifications.

The antibiotic susceptibility tests were done by the method of disc diffusion, proposed by Bauer et al. (1966) and reviewed by the Committee for Clinical Laboratory Standards Institute (NCCLS) (CLSI, 2019). The discs were inserted on Müeller-Hinton Agar, throughout 150 mm diameter Petri dishes. The antimicrobial discs used were: Penicillin (10 µg), Gentamicin (10 µg), Ampicillin (10 µg), Amikacin (30 µg), Cephalothin (10 µg), Cephotaxin (30 µg), Ceftazidime (30 µg), Ceftriaxone (30 µg), Chloramphenicol (30 µg), Erythromycin (15 µg), and Tetracycline (30 µg). The Petri dishes were incubated at 37 °C for 24 h. After the incubation, the measurements of growth inhibition halos were taken.

Odds Ratio tests were used to achieve the frequency based on the number of bacteria in the samples. The established significance level was 0.05.

3 RESULTS

The donor animals had symmetrical testicular with intermediate consistence. Out of 117 attempts to collect semen from the animals used in this study, only 88 of the ejaculated material contained sperms. The analyzed semen was characterized as the following averages: Volume: 0,81(\pm 0.86) mL; Concentration: 137.44(\pm 153) $\times 10^6$ spz mL⁻¹; Mobility: 52.66(\pm 28.79)%; Vigor: 2.2(\pm 0.8); Viability: 55.84(\pm 28.55)%; Major defects: 22.87(\pm 12.93)%; Minor defects: 9.11(\pm 5.88)% and total defects: 31.52(\pm 13.81)%.

Out of the total of 88 ejaculated obtained, 78 (88.6%) showed bacterial growth, and 225 strains were isolated. From the isolates, 135 (60%) were pure culture and 90 (40%) were mixed culture containing two or more genera. A total of six bacterial genera were identified: *Streptococcus* (30.2%), *Staphylococcus* (30.2%), *Micrococcus* (33.7%), *Corynebacterium* (2.2%), *Enterococcus* (1.7%) and *Bacillus* (1.7%). The estimated frequency and bacterial prevalence are presented on Table 1, in which it can be seen the two most prevalent genera: *Streptococcus* and *Micrococcus*. However, *Streptococcus* sp., *Staphylococcus* sp. and *Micrococcus* sp. were the most frequent strains found among the microorganisms isolated in this work.

Table 1. Frequency of isolated microorganisms from a total of 225 colonies isolated from the semen of *Collared peccarys*. N= individuals number, ODD= Odds ratio, P= Probability and RI (0.95)= Reference interval at 95%.

Microorganism	N	%	ODD	P	IR(0.95)
<i>Streptococcus</i> sp.	68	80.95	13.64	0.0001	7.69 - 24.21
Other genera	157	26.70			
<i>Staphylococcus</i> sp.	49	58.33	4.01	0.0001	2.51 - 6.39
Other genera	176	0.30			
<i>Staphylococcus</i> negative coagulase	19	22.62	0.72	0.27	0.42 - 1.22
Other genera	206	35.03			
<i>Micrococcus</i> sp.	76	90.48	32.13	0.0001	15.16 - 68.10
Other genera	149	25.34			
<i>Corynebacterium</i> sp.	5	5.95	0.44	0.005	0.24 - 0.77
Other genera	220	0.37			
<i>Enterococcus</i> sp.	4	4.76	0.11	0.0001	0.04 - 0.30
Other genera	221	0.38			
<i>Bacillus</i> sp.	4	4.76	0.11	0.0001	0.04 - 0.30
Other genera	221	0.38			

CFU counting, performed in serial dilutions is presented on Table 2. The following dilutions were: 1:10, 1:100 and 1:1,000 exceeded the limit of 300 CFU mL⁻¹. The dilutions 1:10,000 and 1:100,000 revealed a decline on the number of bacteria. In regard of CFU mL⁻¹ counting, it was observed a growth above 300 CFU mL⁻¹ on the less diluted conditions (Table 2).

Table 2. Total counting in CFU mL⁻¹ from collared peccary semen in its five serial dilution categories. CFU= Colony-forming unit.

Microorganism	CFU mL ⁻¹				
	10 ¹	10 ²	10 ³	10 ⁴	10 ⁵
<i>Streptococcus</i> sp.	>300	>300	>300	139.5	80.2
<i>Staphylococcus</i> sp.	>300	>300	>300	175.5	57.2
<i>Micrococcus</i> sp.	>300	>300	>300	88.5	58.2
<i>Corynebacterium</i> sp.	>300	>300	>300	125.5	66.0
<i>Enterococcus</i> sp.	>300	>300	>300	102.5	83.7

Table 3 presents results of the antibiotic susceptibility assays. Considering a sensitivity of $\geq 90\%$, *Streptococcus* sp. presented sensitivity to Amikacin and Gentamicin; *Micrococcus* sp. was only sensitive to Gentamicin; *Corynebacterium* sp. presented sensitivity to: Amikacin, Ampicillin, Cephotoxin, Gentamicin and Penicillin. Additionally, *Enterococcus* sp. presented sensitivity towards Cephotoxin, Chloramphenicol, Gentamicin and Penicillin.

Table 3. Antimicrobial susceptibility result of microorganisms found in the semen of collared peccary, raised in the metropolitan region of Belém (PA, Brazil).

Antimicrobial agents	Microorganisms									
	<i>Streptococcus</i>		<i>Staphylococcus</i>		<i>Micrococcus</i>		<i>Corynebacterium</i>		<i>Enterococcus</i>	
	N	%	n	%	N	%	N	%	n	%
Amikacin	72	90	64	88	48	75	5	100	3	75
Ampicillin	55	68	41	56	48	75	5	100	3	75
Cephalothin	62	77	51	70	49	76	1	20	1	25
Ceftriaxone	57	71	52	72	47	73	3	60	3	75
Cephotoxin	64	80	54	75	52	81	5	100	4	100
Ceftazidime	10	8	9	12	7	10	0	0	0	0
Chloramphenicol	55	68	48	66	41	64	4	80	4	100
Erythromycin	52	65	43	59	36	56	4	80	3	75
Gentamicin	76	95	64	88	60	93	5	100	4	100
Penicillin	54	67	44	61	39	60	5	100	4	100
Tetracycline	38	47	30	41	29	45	1	20	1	25

4 DISCUSSION

The semen bacterial biodiversity in all animals were similar to found in other studies analyzing semen of several animal groups, such as cattle (HERNÁNDEZ et al., 2002; PRADO; PEREZ, 2005), sheep (SOUZA et al., 2006) pigs (SONE, 1990; CORRÊA et al., 2001), fishes (OLIVEIRA et al., 2007), boar (PRIETO-MARTINEZ et al. 2014), and human (VILVANATHAN et al., 2016). On the other hand, *Enterococcus* sp., *Corynebacterium* sp. and *Staphylococcus* negative coagulase were less frequent. Similar found were obtained in studies of sheep semen (SOUZA et al., 2006), Pig's semen (ALTHOUSE; LU, 2005), *Piracanjuba* fish (OLIVEIRA et al., 2007) and cattle (PRADO; PÉREZ, 2005). However, Fernandes et al., (2013) founded frequency of 23.6% for *Bacillus* sp. in semen of sheep, which is 14 times higher than what was found in collared peccary semen.

About CFU counting, performed in serial dilutions the result diverges from the ones obtained by Prado and Perez (2005), in cattle's semen, in which less diluted semen leads to lower CFU mL⁻¹ concentration. However, it corroborates with the results found by Hernández et al. (2002) in bovine's thawed semen. The 1 in 100,000 dilution did not alter the sperms motility. This observation was also reported by Bennermann et al. (2000), as well as in Prado and Pérez (2005) study. However, Pineda and Santander (2007) stated that at 1 in 10,000 dilution, changes in the quality of the semen of pig could happen.

In regard of antimicrobial agents tested in this work, the sensitivity against Gentamicin, Amikacin and Cephotaxin confirmed the results found by Souza et al. (2006) in semen of sheep, and by Hernández et al. (2002) in cattle's semen. Nevertheless, in regard of the microorganisms isolated from semen of pig, the study of Gączarzewicz et al. (2016) presented high inhibitory activity against some antimicrobial agents as Gentamicin. In addition, Tetracycline, a very commonly antibiotic used as semen's dilutor, presented the lowest numbers of sensitivity on antibiotic susceptibility results.

The presence of such microorganisms in the semen has possibly no association or no relation to the efficiency of the male's reproductive system of the donor animal, once these microorganisms have been found in semen of healthy animals (KUSTER; ALTHOUSE, 2016). Therefore, it was observed that the presence of the six microorganisms did not interfere on the quality of semen analyzed. This fact also corroborates with studies of Sannat et al. (2015), and Kuster and Althouse (2016).

According to Riesenbeck et al. (2015), the semen contamination can be related to the fact the ejaculated material gets direct contact with the animal's prepuce secretions and hair. In addition, accidental handling by the technician, indirect contact to aerosol, sampling instruments, as well as the process of semen dilution and storage contribute to semen contamination. So, the presence of saprophytic bacteria in collared peccary semen might be occurring due to contamination during sampling and/or from the external environment in which the animals are raised. Yániz et al. (2010) also found microbiological contamination in studies with boar semen that are from other environment.

5 CONCLUSION

The presence of microorganisms in the semen of collared peccary during the entire time of this evaluation does not influence on the seminal quality of the animals. Moreover, it is very likely that the ejaculated material have been contaminated by external microbiota from the surrounding

environment. Finally, it is recommended to be reevaluated the use of Tetracycline as a common dilutor in the semen. Additionally, it would be very valuable the see more studies to confirm the use of Gentamicin and/or Amikacin as ideal antibiotics to be used as dilutors in the semen of collared peccaries.

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