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Original article

Apis mellifera semen: bacterial contamination and susceptibility to antibiotics

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Abstract – The aims of this study were to identify the bacterial genera and species present in semen of *Apis mellifera* and to evaluate the sensitivity of those bacteria to different antibiotics. Mesophilic aerobic and microaerophilic bacteria were characterized in semen samples from different hives, and the number of total viable bacteria per milliliter of semen was determined. Twenty-nine isolates were obtained, and 14 different genera were identified, some of them being monomicrobians and others polymicrobians. Colony-forming units (CFU) were variable from no bacterial development up to 4×10^5 CFU/mL. The most frequent genera were *Bacillus* and *Staphylococcus*. All the studied isolates were sensitive to gentamicin and amikacin. Spermatozoa viability in sperm samples diluted in buffer with antibiotic was similar to that of pure semen.

Apis mellifera **/ semen / bacterial content / antibiotics susceptibility**

1. INTRODUCTION

The ability to control mating has been one of the most challenging aspects of honeybee breeding programs (Cobey 1983; Palacio et al. 2003) as queens and drones meet and mate in free flights high in the air. Therefore, artificial insemination (AI) is a valuable tool for achieving controlled mating for research and stock improvement (Cobey 2007). In conjunction with AI, the development of methods for storing and preserving semen without compromising its quality and minimizing the risk of spreading pests and diseases is necessary to improve our existing technology.

Some attempts to assess semen quality have been conducted in *Apis mellifera* (e.g., Locke et al. 1990; Andere et al. 2008), and the structure and ultrastructure of honeybee spermatozoa is well characterized (Cruz-Landim and Cruz Höfling 1969; Cruz Höfling et al. 1970; Lensky and Schindler 1979; Lino-Neto et al. 2000). Other studies have addressed changes in drone and sperm during reproductive development to sexual maturity (Page 1986; Moors et al. 2005). A variety of parameters have been used to evaluate seminal quality: spermatozoal concentration, motility, viability, and morphology (Locke et al. 1990; Peng et al. 1990; Collins and Donoghue 1999; Schlüns et al. 2003). Additionally, Locke and Peng (1993) have

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studied the effects of drone age, semen storage, and contamination on semen quality.

Even under careful conditions, semen may get contaminated at the time of collection or subsequent processing (Russell et al. 1997; Kapoor 2003) or even during storage (Bielanski et al. 2003; Althouse and Lu 2005; Mazzilli et al. 2006). Bacteriological contamination is well documented in mammals (Eaglesome et al. 1992; Althouse 1999), and its possible role in disease transmission has been demonstrated as an important consideration in performing seminal assessment (Catena et al. 1998; Catena and Cabodevila 1999). One of us (Collins) has observed that bacterial contamination in honeybee semen stored without antibiotics caused significant deterioration in some samples.

Hives of *A. mellifera* support a diverse microbial biome (Gilliam 1997) in larvae, adults, pollen, and nectar (Gilliam 1978, 1979; Allipi 1995; Jeyaprakash et al. 2003; Kacániová et al. 2004; Piccini et al. 2004; García García 2006). Early work on the development of artificial insemination for honeybees reported high levels of septicemia in the queens after AI (Mackensen 1950). The addition of the antibiotic streptomycin to the saline solution used for semen collection is commonplace (Harbo 1985; Moritz 1984).

Both *Bacillus cereus* and *Bacillus brevis* were isolated from selected organs of virgin and mated queens including the spermatheca (Gilliam 1978). Nevertheless, the introduction of bacterial contamination to the queen during AI is still of concern.

Poole and Taber (1970) found that the use of antibiotics in honeybee semen preserved at low temperatures increased spermatozoa survival, as assessed through the production of fertilized eggs. More recently, Locke and Peng (1993) emphasized the need for research on antibiotics for use with honeybee semen as they are common components of semen diluents of others species. However, the merits of their use in semen storage have not been documented (Johnson et al. 1982; Williams and Harbo 1982; Moritz 1984). This study was carried out to identify the bacterial species present in the semen of *A. mellifera* and to evaluate the sensitivity of those bacteria to different antibiotics for possible use during semen storage.

2. MATERIALS AND METHODS

2.1. Semen collection

Semen samples were collected from drones of 26 hives in an experimental apiary (the Experimental Agriculture Experimental Station INTA–Balcarce, Buenos Aires Argentina) across four mating seasons (2005–2008). Colonies were headed by selected queens from the Honey Bee Genetic Program (MeGA) developed by the PROAPI (Integrated Project for Beekeeping Development). Colonies were fed with sugar syrup (sucrose/water, 2:1) and pollen, and two combs with drone cells were introduced to each colony in order to ensure drone availability. Mature drones were collected in traps at the entrance of each colony and placed in individual boxes labeled by source colony.

Two microliters of semen was collected from each colony using a Harbo syringe (Harbo 1985) and was diluted in 40 μL phosphate buffer solution, pH adjusted to 8.4 using 1 N NaOH. Samples were refrigerated at 4°C for transport to the laboratory for further processing.

2.2. Microbial analysis and isolate identification

Fresh diluted semen was cultured for mesophilic aerobic and microaerophilic bacteria to determine the number of total viable bacteria per milliliter of semen or colony-forming units (CFU). Ten microliters of semen dilution 1:20 and 1:100 was inoculated on trypticase soy agar Petri dishes employed as routine medium for growth of bacteria, in duplicate, and incubated at 37°C under aerobic conditions for 48 h. The CFU was calculated, and samples that showed no growth in 48 h of incubation were deemed negative.

Ten microliters of the initial dilution (1:20) was inoculated in pre-enrichment media Brain Heart Infusion broth (BHI; Merck, cod 10493), incubated aerobically for 24–48 h, and the bacterial development turbidity was observed visually. Another 10 μL was inoculated in Brucella broth and incubated under

microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) for 48 h. The bacterial growths found in both broths (BHI and Brucella broth) were inoculated on blood agar at 37°C for 24–48 h, aerobically and anaerobically. Colonies of different morphology were transferred to tryticase soy agar, stained with Gram's, and examined under a light microscope at ×400 magnification. Genus and species were determined according to standard biochemical tests and identification schemes (Cowan Steel 1999; Mac Faddin 2000).

2.3. Antimicrobial susceptibility test

Antimicrobial drug susceptibility testing was carried out using the standard disk diffusion method (Kirby et al. 1966) using Mueller-Hinton agar and interpreted based on the Clinical and Laboratory Standards Institute guidelines (CLSI 2008). The antibiotics were chosen according to studies carried out on semen diluents of honeybee semen and from their use with other species (Johnson et al. 1982; Williams and Harbo 1982; Moritz 1984; Gadea 2003; Aurich and Spergser 2007; Otti et al 2009). Disks containing gentamicin (10 μg, Oxoid CT00248), streptomycin (300 μg, Britannia CT1887B), nalidixic acid (30 μg, Oxoid CTB20197), penicillin (10 IU, Oxoid CT00438), or amikacin (30 μg, Oxoid CT107B) were tested.

A subculture from each isolate was done in BHI broth and incubated at 37°C during 24 h. Then, an inoculum with 0.5 turbidity on the Mc Farland scale (1.5×10^8 bacteria/mL) was prepared in the Microbiology laboratory of the College of Veterinary Sciences, Tandil, Buenos Aires, Argentina. One hundred microliters was seeded and distributed using a Drygalsky spatula. Plates were incubated aerobically at 35°C for 24 h. Inhibition zones were classified as sensitive, intermediate (moderately sensitive), or resistant according to the procedures of the CLSI (2008).

2.4. Minimum inhibitory concentration and minimum bactericidal concentration

According to the sensitivity shown against different antibiotics, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were estimated (NCCLS 2000). Solutions with the antibiotic selected at different concentrations from 0.78 to 800 μg/mL were prepared.

One milliliter of each isolate (equivalent to 0.5 McFarland) was added to each serial dilution tube (1.5×10^8 bacteria/mL inoculum). A positive control (a tube of broth without antibiotic and inoculum) and a negative control (a tube with antibiotic and without inoculum) were prepared for each isolate. All sample tubes including positive and negative controls were incubated at 35±0.5°C for 48 h. The lowest concentration of antibiotics that prevented bacterial growth, determined by the absence of turbidity, was defined as the MIC.

After incubation, aliquots from broths that showed no turbidity were inoculated in Mueller Hinton (Britania S.A. cod B0213705) agar plates. The lowest concentration that inhibited the bacterial growth was considered the MBC. Median, minimum, and maximum for MIC and MBC were reported.

2.5. Antibiotic effect on spermatozoa viability

To evaluate the antibiotic effect on the viability of the spermatozoa, 10 semen samples without diluent and antibiotic and 10 semen samples diluted with Kiev buffer pH 8.8 (3 vol semen/1 vol Kiev buffer; Moritz 1984) plus the selected antibiotic in the concentration determined before (MBC) were employed.

The Kiev buffer used in this experiment contained: 0.3 g glucose (Sigma, Cat# G-8270), 0.41 g potassium chloride (Sigma, Cat# P-3911), 0.21 g sodium bicarbonate (Sigma, Cat# P-3911), and 2.43 g sodium citrate dihydrate (Sigma, Cat# C-8532).

Three replicates for each semen sample were done. The spermatozoa viability (percentage live/dead) was estimated by direct count of 100 cells for each replicate, mixed with an acridine orange–ethidium bromide solution (McGahon et al. 1995) under epifluoresence microscope at ×400. Cells staining red were counted dead, while green cells were counted as live. To evaluate the antibiotic effect on the percentage of viability, an analysis of variance with repeated measures was performed using the MIXED procedures of Statistical Analysis Systems, version 9.1.3 (Little et al. 1996). Means and standard error are reported. When $p<0.05$, the differences were considered to be significant.

3. RESULTS

Microbial results indicated that, from the 26 semen samples, 24 were positive (92.3%). Many bacteria species were identified in semen samples of different hives, 87.5% were monomicrobians and the others were polymicrobians. The CFU was variable among hives, from no bacterial development up to 4×10^5 CFU/mL (Table I).

Twenty-nine isolates were obtained and 14 different genera were identified with standard tests. Some of them were identified to species level (12/14). A preponderance of Gram-positive bacteria (65.52%) was observed among the isolates.

Most frequent genera were *Bacillus* and *Staphylococcus* (Table I). *Staphylococcus epidermidis* was detected in seven hives, *Bacillus* in six. In addition, *Flavimonas oryzihabitans* was found in three. No bacterial development was registered in two of the studied hives.

The sensitivity to amikacin, penicillin, nalidixic acid, gentamicin, and streptomycin was studied in 11/14 of the isolated bacteria. All the isolates were sensitive to gentamicin and amikacin (Table II). Because in a preliminary study (data not shown) some bacteria isolates were resistant to amikacin, gentamicin was the chosen antibiotic to estimate the MIC and MBC (Table III). The MIC and the MBC had the same value in most of the studied hives, with a median 75 μg/mL (0.78 to 450) in both cases, being different only in four of 18 hives. The maximum gentamicin concentrations reached were 450 μg/mL. Bacteria of a specific genus and

Table I. Bacterial isolates and colony-forming units (CFU) per milliliter for each hive.

	Bacteria isolates	Frequency/hive	CFU/mL
n/d		2	
Monomicrobians	*A. delafieldii*	1	34,980
	Aerococcus viridans	1	31,680
	A. faecalis	1	17,820
	A. haemolyticum	1	6,270
	B. coagulans	1	1,980
	B. mycoides	1	7,260
	Bacillus spp.[a]	3	4,950–46,860
	Bacilo Gram-negative unidentified	3	11,550–117,480
	F. oryzihabitans	2	1,900–2,300
	S. cohnii	1	19,140
	S. epidermidis	4	1,000–270,600
	S. hominis	1	13,200
	Streptococcus mutans	1	28,380
Polymicrobians	*S. epidermidis*	1	399,300
	Corynebacterium spp.[a]		
	B. coagulans		
	S. epidermidis	1	20,460
	F. oryzihabitans		
	A. viridans	1	23,430
	S. epidermidis		
	B. pertussis		

n/d no development

[a] Identified at genus level

Table II. Antibiotic susceptibility of isolated bacterial species using the agar disk diffusion method.

Bacteria	Hive	Amikacin	Penicilin	Nalidíxic acid	Gentamicin	Streptomycin
A. delafieldii	18	S	R	I	S	R
A. viridans	26	S	R	R	S	S
A. faecalis	17	S	R	R	S	R
A. haemolyticum	4	S	R	S	S	S
B. coagulans	3	S	R	I	S	S
B. coagulans	6	S	S	S	S	S
B. mycoides	24	S	S	R	S	S
Bacillus spp.	7	S	S	S	S	S
Bacillus spp.	22	S	S	R	S	S
Bacillus spp.	19	S	R	R	S	S
Bacilo Gram (−) *n/d*	21	S	R	R	S	S
Corynebacterium spp.	3	S	S	R	S	S
S. cohnii	14	S	S	R	S	S
S. epidermidis	8	S	R	R	S	S

S sensitive, *R* resistant, *I* intermediate

species presented different sensitivity when it was isolated from different hives (e.g., *S. epidermidis* in hive 3, 5, 8, and 26). The mean percentage of spermatozoa viability (live/dead) in sperm samples diluted in Kiev buffer with the addition of gentamicin (450 μg/mL) was 70.67±1.23%, while it was 78.03±0.75% in the pure semen (without diluents or antibiotic), being not statistically different (F=2.94, P=0.1203).

4. DISCUSSION

This is the first report which describes the bacteria isolated from semen of *A. mellifera* drones. Most of the bacterial species have been mentioned in other research related to other organs of bees and to hives. Genera *Bacillus*, *Staphylococcus*, and *Micrococcus* have been the most frequently isolated by other authors (Gilliam 1971; 1978; Gilliam and Valentine 1974, 1976; Gilliam and Lorenz 1983; Gilliam and Taber 1991; Gilliam and Prest 1987). Soil surrounding the hive could be an important source of bacterial contamination as well (Snowdon and Cliver 1996).

The predominance of *Bacillus* species among the identified isolates in this work is in agreement with previous reports where bacteria were isolated from different organs of the mated queen honeybee (Gilliam 1978), suggesting that the microorganism could have entered the queen during copulation. Some of the isolates in semen of *A. mellifera* such as *Bacillus*, *Alcaligenes faecalis*, and *Acidovorax delafieldii* are widely distributed in natural sources such as water, soil, mud, and vegetation (Brenner 2005). *Bacillus coagulans* is usually isolated from a wide variety of animal species and in the guts of virgin and mated queens (Gilliam 1978). *Bacillus mycoides* and *F. oryzihabitans* have been isolated from soil and common environments (Giraldi 1991; Brenner 2005).

The finding of microorganisms of the genus *Staphylococcus* (such as *S. epidermidis*, *Staphylococcus hominis*, and *Staphylococcus cohnii*), considered as a constituent of the normal flora of the skin, mucous, and nasopharynx in humans (Brenner 1984), suggests that semen could be contaminated during sampling, processing, or both. *S. epidermidis* was also isolated in pig (Pineda and Santander 2007) and frozen cattle semen (Abro et al. 2009).

Collection of semen from drones using the standard method of eversion often results in

Table III. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of gentamicin for the different bacterial isolates.

Bacteria	Hive	MIC (μg/mL)	MBC (μg/mL)
A. viridans	25	100	200
A. viridans	26	12.5	25
A. haemolyticum	4	25	25
Bacillus spp.	22	25	25
B. coagulans	3	25	25
B. coagulans	6	0.78	0.78
B. mycoides	24	50	50
Bacillus spp.	19	1.56	1.56
Bacillus spp.	7	0.78	1.56
B. pertussis	26	0.78	0.78
Corynebacterium spp.	3	100	100
S. epidermidis	3	100	200
S. epidermidis	5	100	100
S. epidermidis	8	450	450
S. epidermidis	9	450	450
S. epidermidis	10	450	450
S. epidermidis	13	450	450
S. epidermidis	26	100	100

release of gut contents as well (e.g., feces, stomach contents; Cobey 1983).As *Bordetella pertussis* and *Arcanobacterium haemolyticum* are human respiratory pathogens, aerosol generation during the manipulation of the drones could be the origin of some semen contamination (Román and García 2005; Biosafety in Microbiological and Biomedical Laboratories 2007).

Although samples were obtained under controlled conditions, it is clear that these were not sufficient to avoid semen bacterial contamination. Additional hygienic conditions recommended for semen collection of other species (Martínez 1998; Althouse et al. 2000; Toniolli et al. 2002) should be considered for testing.

To our knowledge, this is the first study isolating bacteria from honeybee semen samples; thus, it was not possible to compare the CFU obtained in this work with other results in the same species. However, many authors have added antibiotics to drone semen before queen insemination (Johnson et al. 1982; Williams and Harbo 1982; Moritz 1984). Though in preliminary results (data not shown) some bacteria were resistant to amikacin, in the present study, all of the bacteria isolated were gentamicin- and amikacin-sensitive. Gentamicin employed at a concentration of 450 μg/mL was effective to control bacterial contamination without affecting sperm viability.

Aurich and Spergser (2007), working with stallions, reported that gentamicin reduced sperm motility during storage using 1 g/L. Other authors (Qureshi et al 1993; Akhter et al 2008) reported that 500-ug/mL concentrations were satisfactorily used for bovine and buffalo semen, respectively.

Similarly, aminoglucosides—including gentamicin—are the antimicrobials more frequently added to semen diluents in other species (Sone 1982; Mazurova and Vinter 1991; Althouse and Lu 2005), although some bacteria are resistant to this antibiotic (Althouse 1999; Althouse et al.

2000; Althouse and Lu 2005). Streptomycin was used to eliminate Gram-negative bacteria found in intestines of honeybees (Gilliam et al. 1988), but in this study, *A. delafieldii* and *A. faecalis* were resistant to this antibiotic.

This study assessed the bacteriological quality of *A. mellifera* semen following the methods used to evaluate semen bacteriological quality in other species. It will be relevant to evaluate bacteriological contamination in semen routinely to decide the best antimicrobial combination for use in honeybee semen. Moreover, regular bacteriological examination of semen will allow monitoring of health condition of the reproductive system of the drones destined to selection programs for breeding, which should decrease the probability of spreading diseases and to protect the quality of the genetic material. All these considerations will contribute to improved productivity in a genetic program.

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Sperme *d'Apis mellifera*: contamination bactérienne et sensibilité aux antibiotiques.

***Apis mellifera* / sperme / contenu bactérien / sensibilité aux antibiotiques**

***Apis mellifera* Sperma: Kontamination mit Bakterien und Empfindlichkeit gegenüber Antibiotika**

***Apis mellifera* / Sperma / Bakteriengehalt / Antibiotikaempfindlichkeit**

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