

## IDENTIFICATION AND ANTIMICROBIAL RESISTANCE OF MICROFLORA COLONIZING FERAL PIG (*SUS SCROFA*) OF BRAZILIAN PANTANAL.

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### ABSTRACT

Antimicrobial resistance of bacteria is a worldwide problem affecting wild life by living with resistant bacteria in the environment. This study presents a discussion of outside factors environment on microflora of feral pigs (*Sus scrofa*) from Brazilian Pantanal. Animals had samples collected from six different body sites coming from two separated geographic areas, Nhecolandia and Rio Negro regions. With routine biochemical tests and commercial kits 516 bacteria were identified, with 240 Gram-positive, predominantly staphylococci (36) and enterococci (186) strains. Among Gram-negative (GN) bacteria the predominant specimens of Enterobacteriaceae (247) mainly represented by *Serratia* spp. (105), *Escherichia coli* (50), and *Enterobacter* spp. (40) and specimens not identified (7). Antimicrobial susceptibility was tested against 17 drugs by agar diffusion method. Staphylococci were negative to production of enterotoxins and TSST-1, with all strains sensitive towards four drugs and highest resistance toward ampicillin (17%). Enterococci presented the highest sensitivity against vancomycin (98%), ampicillin (94%) and tetracycline (90%), and highest resistance pattern toward oxacillin (99%), clindamycin (83%), and cotrimoxazole (54%). In GN the highest resistance was observed with *Serratia marcescens* against CFL (98%), AMC (66%) and AMP (60%) and all drugs was most effective against *E. coli* SUT, TET (100%), AMP, TOB (98%), GEN, CLO (95%), CFO, CIP (93%). The results show a new profile of oxacillin-resistant enterococci from Brazilian feral pigs and suggest a limited residue and spreading of antimicrobials in the environment, possibly because of low anthropogenic impact reflected by the drug susceptibility profile of bacteria isolated.

**Key words:** Brazilian Pantanal, feral pig, antibiotic resistance pattern.

### INTRODUCTION

Pantanal is one of the most important wetlands ecosystems in the world comprehending a geographical region in the central South America continent, which border limit includes

Brazil, Paraguay and Bolivia. Cyclical flooding characterizes the region and Brazilian Pantanal embraces the biggest part of the area with 140.000 km<sup>2</sup> (15). Water environment has been shown to be the most efficient niche for exchange of genes of antimicrobial resistance among microorganisms and selection

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for resistance is proportional to time of exposure of bacteria to antimicrobial in the environment (2). Antimicrobial resistant bacteria have emerged around the world, and together with this phenomena the increasing of human mortality (17). The way bacteria acquire resistance may vary and for enterococci most of the cases of resistance is acquired throughout chromosomal mutation or gene acquisition (5). Fecal bacteria may survive in soil and one can speculate that the contact of feral pigs with environment could result in the exchange of resistant microorganisms after contact with other animals, since these agents may be present in all sort of environment, such as in contaminated soil (3, 43). In domestic animals, such as in pigs farms several studies showed the prevalence of resistant bacteria around world (1, 11, 43) and in this context the wild life may represent a risk for human and domestic animals (33). It was also showed the association of use of antibiotics as a group medication in pig farms and colonization of methicillin resistant *Staphylococcus aureus* (MRSA) in pig and the transmission between different properties in The Netherlands (46). Gram-negative bacteria (GN) can also be found in a diverse myriad of samples, but water, soil and feces represent the main source of contamination, and although fecal coliforms such as *E. coli* may not survive for long period in extra-intestinal conditions their presence may indicate recent fecal contamination generated by warm-blooded animals, including humans (21). The use of drugs in animal also may influence in microorganisms antimicrobial resistance profile, including the environment contamination (38, 40, 49). Although Schierack and colleagues (41) declared that no data are available from *E. coli* O157:H7 and *Campylobacter* spp. were isolated from fecal samples of feral pigs in the central coast of California – USA, and contamination of environment was discussed involving these animals as a potential risk factor for the spread of food borne pathogens contamination and crop fields damages (23, 24), besides shedding zoonotic pathogens in surface water (6). It is also assumed that feral pigs may play a role in

transmission zoonotic agents in Australia (33). Some other enterobacteria, such as non-fecal coliforms, and other groups of GN bacteria, characterized by their psychrotrophic nature and simple nutritional requirements, such as *Pseudomonas*, *Acinetobacter*, *Serratia*, *Enterobacter*, *Proteus* and *Vibrio*, in addition to the enterococci, may be recovered from environmental samples and enable them to persist for prolonged periods in environments such as water collections and soil, representing important contamination pathways (47). These microbes are common in the intestinal microbiota but in special conditions they became opportunistic and because of this characteristic they are known as amphibionts (29). It has been proposed by several authors that antibiotic resistance patterns (ARPs) of *Escherichia coli* (27, 32) and fecal streptococci (19, 50, 51) can be used as phenotypic “fingerprints” to determine the source of fecal pollution in natural waters or food. This study aimed to identify microflora colonizing feral pigs (*Sus scrofa*) of Brazilian Pantanal, localized in the Nhecolândia and Rio Negro wetlands areas and to examine their ARPs against drugs tested and staphylococci pathogenicity.

## MATERIAL AND METHODS

### Sample collection

The samples were collected in the sub region of Nhecolândia, Mato Grosso do Sul State (MS), Brazil (18°59'20”S and 56°37'07”W, see figure bellow), from 34 feral pigs (20 females and 14 males) in January 2006, from 12 animals (9 females and 3 males) in october 2008, and 10 animals (3 females and 7 males) in august 2008 in the sub region of Rio Negro (19°30'18”S and 55°36'44”W) (Figure 1). Feral pigs were live-captured in traps and all animals were humanely contended and then released after sampling. Commercial swabs (Copan Diagnostics, Italy) were used to collect samples from oral cavity, nasal cavity, ear canals, anus, prepuce and vagina. All samples were ice conserved and

transported to the laboratory.



**Figure 1.** Brazilian wetlands showing with subregions according. Source: EMBRAPA <http://www.cpap.embrapa.br/agencia/fazendas/fazesub.htm>

### Strains Isolation and Identification

The material was inoculated on chocolate agar (Acumedia, USA) supplemented by 5% defibrinate sterile horse blood and supplement VX at 37°C/24hs. Colonies were identified by Gram staining, cultured in blood agar (Acumedia, USA) and incubated at 37°C/24hs. Colony morphology, size, pigmentation and hemolytic pattern were observed, and tested for catalase (Sigma, USA) and oxidase production. *Enterobacteriaceae* strains were inoculated on MacConkey agar (Acumedia, USA) and identified by IMVIC and complementary tests of urease, manitol, DNase, lisina, saccharose, xilose, H<sub>2</sub>S, arabinose, maltose, inositol, and EMB agar. Hemolytic ability of *E. coli* strains was tested in 5% sheep blood agar.

Differentiation among the species of genera *Streptococcus* was conducted by tolerance test to 6,5% NaCl, growth in bile, esculin hydrolysis, production of pyrrolidonyl arylamidase (PYR) enzyme (PROBAC, Brazil). As controls strains *Enterococcus faecalis* ATCC29212 from Fiocruz-RJ, Brazil, and *Streptococcus dysgalactiae*, isolated from cow milk in the Laboratory of Animal Sanitary/CCTA/UENF. *Micrococcaceae* genera was differentiated by oxidase test (Difco, USA), susceptibility to bacitracin and furazolidone, with *Staphylococcus aureus* ATCC25923 and *Micrococcus luteus* ATCC4698 used as controls. Staphylococci pathogenicity was evaluated by testing for DNase production (DNase agar, Merck, Germany), coagulase production in rabbit plasma coagulase tube test (Difco, USA), and hemolysis in blood agar (Acumedia, USA) with *Staphylococcus aureus* ATCC25923 and *S. epidermidis* ATCC12228, used as positive and negative controls, respectively.

Commercial kits mini Api ID32 Staph, Api ID32E and rapid ID32 Strep (bioMérieux, France) with support of automated software (MiniApi, bioMérieux, Italy) were used for strains identification.

### Toxin detection in staphylococci

For enterotoxin production by staphylococci strains SET-RPLA (Oxoid, Denka Seiken, Japan) was used to detect SEA-SEE, and immunodiffusion test to detect TSST-1 by using specific rabbit polyclonal anti-TSST-1 affinity purified antibodies and purified staphylococcal TSST-1 toxin (12) as antigen and positive control.

### Antimicrobial assays

Susceptibility antimicrobial was realized by the disk diffusion method according to NCCLS (31) in Mueller Hinton agar-MHA (Acumedia, USA). For enterococci, MHA was supplemented with 5% defibrinated sheep blood. Gram-positive strains were tested toward amoxicilin (AMO, 30µg), ampicilin (AMP, 10µg), cephalotin (CFL, 30µg), cephoxitin

(CFO, 30µg), clindamicin (CLI, 2µg), erythromycin (ERI, 15µg), gentamicin (GEN, 10µg), oxacyllin (OXA, 1µg), penicillin G (PEN, 10UI), cotrimoxazole (SUT, 25µg), tetracycline (TET, 30µg) and vancomycin (VAN, 30µg). For GN the antimicrobial tested included amoxicillin+clavulanic acid (AMC, 20/10µg), ampicillin (AMP, 10µg), cephalotin (CFL, 30µg), cephoxitin (CFO, 30µg), ciprofloxacin (CIP, 5µg), chloramphenicol (CLO, 30µg), enrofloxacin (ENO, 10µg), gentamicin (GEN, 10µg), clotrimoxazole (SUT, 25µg), tetracycline (TET, 30µg), tobramycin (TOB, 10µg). All tests were assayed in triplicate.

## RESULTS AND DISCUSSION

The feral pig (*Sus scrofa*), one of the world's worst invasive species, was introduced to the Brazilian Pantanal about 200 years ago and is thought to compete with the native species, such as white-lipped peccary (*Tayassu pecari*) and collared peccary (*Pecari tajacu*). However, the competitiveness among these three species seemed not to occur, but feral pigs (*Sus scrofa*) may, nevertheless, impact the wildlife community in other ways as predators of eggs, by destruction of vegetation through rooting, or by functioning as disease reservoirs (15). Contact, throughout encounters, between these animals was observed (15), but no information about possible transmission of microorganisms was described so far. Although feral pigs from this environment have the habit of mud bath and frequent contact with water collections in natural environment, the scope of genera of bacteria isolated was restrict in number with the approach used in this work. Others have investigated the microbiota of feral pigs from different countries, including pathogenic bacteria (33, 34, 45). After bacteriological routine processing of swabs, 516 specimens were isolated, with 240 Gram-positive bacteria, among them 36 *Staphylococcus* and 186 *Enterococcus* identified. The methodology used also identified one strain of *Aerococcus viridans*, two *Lactococcus*

*lactis* subsp. *Lactis*, three *Sporosarcina*, four *Kocuria* spp. and eight *Bacillus* spp.. Gram-negative bacteria classification resulted in 276 strains, with two *Aeromonas* spp., six *Acinetobacter*, 21 *Pseudomonas* spp. and 247 (Table 1). *Serratia* spp. (n=105) and *E. coli* (n=50) were the GN species most prevalent in the study which were isolated from all body sites investigated. Environment may interfere on microbiota and involves factors such as water content, and the practice of using poultry litter in agriculture for crops nutrient purposes may not impact soil community of fecal indicator bacteria of farms, as observed under drought conditions (25). Neither fecal or water samples were examined in the present work, but studies showed that only 10 bacterial isolates are required to determine the most common clones in fecal samples (42), one can assume that the results showed may reflect the microbiota of feral pigs studied. *E. coli* may colonize specific intestinal sections (16). In Germany, the study of with 21 hunted feral pigs described clones of *E. coli* isolated from intestinal sections, all with different antimicrobial susceptibility profile when compared with susceptible strains isolated from domestic pigs (41). Strains of *E. coli* isolated in the present study had no hemolytic ability as observed in sheep blood agar, and contrary to other observations that found only one *E. coli* from jejunum portion of wild boar in Germany (41), and in accordance to others, commensal *E. coli* strains rarely contain virulence genes (10).

All *Staphylococcus* strains were submitted to classification by Api system, resulting in *S. simulans* (1), *S. saprophyticus* (1), *S. xylosus* (1), *S. warneri* (1), *S. epidermidis* (1), *S. haemolyticus* (3), *S. chromogenes* (5), *S. hyicus* (7), *S. sciuri* (11), and five coagulase-negative *Staphylococcus*. Studies from van Dijck and van de Voorde (45) found *S. aureus* and Poeta *et al.* (34) did not isolate staphylococci from wild life boars from forests of Belgium and Portugal, respectively. However, the identification of *Staphylococcus aureus* from domestic pigs is wide studied, including MRSA (4, 8, 14, 30, 46).

**Table 1.** Bacteria isolated from feral pigs (*Sus scrofa*) from Brazilian Pantanal, frequency of body colonization and number of animals, in the period of 2007 and 2008.

Bacteria (n)	Nasal cavity	Oral cavity	Ear canals	Anus	Vagina	Prepuce	Nº of pigs colonized
<i>Enterococcus</i> (n=186)	9	35	52	41	27	22	52/56
<i>Staphylococcus</i> (n=36)	7	4	14	1	7	3	23/56
<i>Lactococcus lactis lactis</i> (n=2)	0	0	0	1	0	1	2/56
<i>Aerococcus viridans</i> (n=1)	0	0	0	0	0	1	1/56
<i>Kocuria</i> spp. (n=4)	1	2	0	0	1	0	4/56
<i>Bacillus</i> (n=8)	0	2	3	1	0	2	4/56
<i>Sporosarcina</i> (n=3)	2	0	0	1	0	0	3/56
<i>Pseudomonas</i> spp (n=21)	1	0	18	1	0	1	17/56
<i>Aeromonas</i> (n=2)	1	0	0	0	0	1	2/56
<i>Acinetobacter</i> (n=6)	0	3	1	1	1	0	4/56
<b>Enterobactérias (n=247)</b>							<b>53/56</b>
<i>Kluyvera</i> (n=1)	0	0	0	0	1	0	1
<i>Ewingella</i> spp (n=1)	0	1	0	0	0	0	1
<i>Rahnella aquatis</i> (n=1)	0	0	1	0	0	0	1
<i>Buttiauxella agrestis</i> (n=2)	0	0	2	0	0	0	1
<i>Klebsiela</i> spp (n=2)	0	0	1	0	0	1	2
<i>Proteus</i> spp (n=2)	0	2	0	0	0	0	2
<i>Pantoea</i> spp (n=3)	0	0	2	0	0	1	3
<i>Erwinia</i> spp (n=3)	0	2	0	0	1	0	3
<i>Yersinia</i> spp (n=3)	0	1	1	0	1	0	3
<i>Edwardsiella tarda</i> (n=4)	0	2	0	2	0	0	3
<i>Citrobacter</i> (n=6)	0	1	5	0	0	0	4
<i>Hafnia alvei</i> (n=6)	0	1	4	0	1	0	4
<i>Cedecea</i> spp (n=11)	3	1	4	1	1	1	7
<i>Enterobacter</i> spp (n=40)	7	9	16	4	1	3	24
<i>Escherichia coli</i> (n=50)	0	6	8	29	6	1	28
<i>Serratia</i> spp (n=105)	15	25	41	10	5	9	33
Not identified (n=7)	0	0	4	2	1	0	6

### Antimicrobial susceptibility

Thirteen strains (36%) of *Staphylococcus* spp. were sensitive toward all drugs tested. The *S. xylosus* strain colonizing the prepuce of one animal showed multiple resistance toward amoxicillin, penicillin, ampicillin and erythromycin (Table 3). Ampicillin was the most ineffective drug against staphylococci with resistance observed in 17% of strains followed of erythromycin (14%). Bagcigil *et al.* (8) showed that 38% *S. aureus* isolated from nasal cavity of pigs, dogs, horses and cattle were erythromycin resistant in Denmark, mostly animals living in farms and in frequent contact with macrolid drugs, and all strains belonging to a clonal group expressing the gene *ermC*. Armand-Lefevre *et al.*

(4) studying *S. aureus* in pig farmers found high resistance to erythromycin among the isolates from farmers (66%), compared to controls (10% resistant), while 38% of the isolates from pigs were intermediate resistant toward the drug. The cause of staphylococci ampicillin and erythromycin resistance found the present study is to be investigated, since domestic pigs were not investigated yet in the area investigated.

Data from 186 isolates of *Enterococcus* in the present study showed high sensibility to vancomycin (98%), ampicillin (94%), tetracyclin (90%), penicillin G (83%), amoxicilin (70%) and cephalotin (69%), and with high resistance toward oxacillin (99%), clindamycin (83%) and cotrimoxazole (54%) (Table 3). Poeta *et al.* (34), evaluating the resistance of

*Enterococcus* strains from feral pigs toward 11 antimicrobial drugs, observed higher resistance against erythromycin (48,5%), tetracycline (44,8%) and ciprofloxacin (17,9%) and lower resistance against ampicillin (3,7%), cloranphenicol (4,5%), estreptomycin (6,7%) and kanamycin (9%). The results in the present work with enterococci resistance toward erythromycin was 13%, and lower than that observed against

the same drug in animals from Portugal (48,5%). Poeta *et al.* (34), observed 44,8% of tetracycline resistance among the isolates, while in the present work the level of resistance was practically insignificant (6%), while resistance against ampicillin presented results compatible, with 6% resistance in the present work against 3,7% in the Portuguese enterococci isolates.

**Table 3.** Antimicrobial susceptibility, in percentage, of Gram-positive bacteria isolated from feral pig (*Sus scrofa*) of Brazilian pantanal, in the period of 2007 and 2008.

	<i>Enterococcus</i> (n=186)			<i>Staphylococcus</i> (n=36)			<i>Kocuria</i> (n=4)		
	R	I	S	R	I	S	R	I	S
AMO	30	0	70	8	0	92	25	0	75
AMP	6	0	94	17	0	83	0	0	100
CFL	12	19	69	0	0	100	0	0	100
CFO	46	22	33	6	0	94	25	0	75
CLI	83	5	11	8	11	81	0	0	100
ERI	13	60	27	14	31	56	25	0	75
GEN	24	16	60	0	0	100	0	0	100
OXA	99	0	1	6	0	94	0	0	100
PEN	17	0	83	8	0	92	25	0	75
SUT	54	5	41	3	3	94	50	0	50
TET	6	4	90	0	0	100	0	0	100
VAN	0	2	98	0	0	100	0	0	100

The species *E. faecalis* is known as one of the main resistant against drugs from strains isolated from domestic pigs in different countries (1, 20, 49). *Enterococcus faecalis* and *E. faecium* present natural resistance to several antimicrobial drugs, including aztreonam, cotrimoxazole, clindamicin and cephalosporins, and habitually, lower sensibility toward aminoglycosides and penicillin G, moderate sensibility toward ampicillin and cloranphenicol, but high sensibility toward glycopeptides (22). Otherwise, when resistant to the last drugs the *Enterococcus* represent an epidemiological risk, since the genes may be transferable to other bacteria (5). There is no reference to clindamycin resistance in enterococci isolated from pigs.

The level of resistance toward cotrimoxazole in enterococci was also discussed by others studying domestic

pigs. Aubry-Damon *et al.* (7) associated a predominance of enteric bacteria resistant to drugs, among them cotrimoxazole, from pig farmer workers in France, and compared with isolates from pigs. The strains isolated from controls (no pig farmers) were sensitive to cotrimoxazole, suggesting the transmission of resistant bacteria for pig farmers.

Among 186 isolates from enterococci from feral pig of Brazilian Pantanal, three strains presented intermediate profile toward vancomycin. The plasmid gene *vanA*, responsible for the high resistance to this drug may be transferable to humans and animals (36, 37). A study with *E. faecalis* and *E. faecium* isolated from humans and pigs in Denmark showed that 17% of the pigs isolates and only 1,5% from humans isolates were vancomycin-resistant, and all possessed gene *vanA* (1). It has been assumed that vancomycin resistance is an intrinsic

characteristic of fecal coliforms (9). Enterococci may also change their antimicrobial profile according to environmental water contamination with antibiotic residue detection in surface water and groundwater from swine plant operations (38).

Both *Lactococcus lactis lactis* strains presented sensitivity to most antibiotics tested, and one strain was resistant to clindamycin and other intermediate toward cephoxitin. *Aerococcus viridans* strains were sensitive against all drugs, except toward oxacillin, which presented resistance profile.

Natural or intrinsic and acquired antibiotic resistance in enterococci was described as inherent characteristics of species of the genus or a consequence of insusceptibilities to physicochemical and environmental factors, but no mention about resistance to penicillin or their derivative is credited to enterococci unless overproduction of penicillin-binding protein (PBP) occurs (26). According to CASFM (Comité de l'Antibiogramme de la Société Française de Microbiologie) (13), enterococci may be a naturally oxacillin resistant bacteria. This is accordance with the results observed in this work, since virtually all enterococci strains presented resistance toward oxacillin. All together, these data indicate that the enterococci oxacillin resistance phenotype may be considered a stable genetic trait in this species isolated from feral pigs in Brazilian Pantanal, and never observed by others before. This alleged enterococci oxacillin resistance genetic trait deserves more investigations.

According to Table 2, for GN bacteria the susceptibility towards drugs tested showed that the bacteria with highest resistance was *Serratia marcescens*, with 98% resistance toward Cephalotin, 66% toward amoxicillin+clavulanic acid and 60% toward ampicillin. *E. coli* was the most sensitive with 10% resistance profile toward AMC and 7% toward CFL. Schierack and colleagues (41) found no resistance among *E. coli* strains from feral pigs, while strains from domestic pigs were more resistant. GN bacteria in the gut can present different profile toward drugs, resistance against tetracycline was higher than other drugs in *E. coli* (18). Taking the data from resistance profile of GN bacteria in the study and with other published data in domestic pigs, one can infer that anthropomorphic pressure in Brazilian Pantanal environment is low. Others have observed that cattle-ranching activities may favor feral pigs and the current anthropogenic changes in the landscape could lead to changes in competitive dynamics between these animals and native species (15), but exchange of bacteria and influence of such activity on resistance profile of microorganisms is yet to be studied. Cattle are considered the primary reservoir of *E. coli* O157 (28), but fecal shedding by other domestic livestock and wildlife has been described (35, 39) and cattle-ranching and agriculture practice for food purposes activities in California could be affected by surface water visited by feral pigs and, consequently, containing pathogenic bacteria (23, 24).

**Table 2.** Antimicrobial susceptibility, in percentage, of 233 *Enterobacteriaceae* isolated from feral pig (*Sus scrofa*) of Brazilian Pantanal, in the period of 2007 and 2008.

	<i>Serratia marcescens</i> (n=97)			<i>Enterobacter</i> spp. (n=35)			<i>Cedecea</i> (n=11)			outras (n=48)			<i>E. coli</i> (n=42)		
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
AMC	66	19	15	20	9	71	27	0	73	10	23	67	10	7	83
AMP	60	15	25	20	20	60	27	0	73	31	13	56	2	0	98
CFL	98	0	2	29	14	57	36	9	55	27	17	56	7	17	76
CFO	8	12	79	17	11	71	9	9	82	29	6	65	2	5	93
CIP	1	4	95	3	11	86	0	9	91	4	13	83	0	7	93
CLO	2	11	87	3	17	80	0	9	91	2	15	83	0	5	95
ENO	2	18	80	3	26	71	0	9	91	2	29	69	5	10	86
GEN	1	0	99	6	0	94	0	0	100	8	10	81	0	5	95
SUT	2	0	98	0	14	86	0	0	100	21	2	77	0	0	100
TET	51	30	20	3	3	94	9	0	91	8	13	79	0	0	100
TOB	4	5	91	6	3	91	0	9	91	6	10	83	0	2	98

In the literature no information is available on microbiota of feral pigs from Brazilian Pantanal. The environmental aspect emphasized in this work is based on the necessity to know the drug resistance of this microbiota to propose a possible interference of human activities in that environment. The study presented may reveal that controversial aspects on bacterial resistance towards drugs may occur specially in areas with association of heavy pressure of livestock and agricultural activities, or natural resistance is inherent to wild microorganisms associated to wild animals. However, most of the isolates were sensitive to drugs tested in this study and the results may reflect a regional characteristic of Brazilian wetlands like Pantanal, with cyclic water seasons reflecting on drug profile of microorganisms living in that environment, suggesting dispersion of residues of any kind of contamination, including antimicrobial drugs.

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