ORIGINAL ARTICLE

STAPHYLOCOCCUS SPP. AND *mec*A GENE IN PREGNANT WOMEN: A NEGLECTED HEALTH RISK TO MOTHER AND CHILD

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Highlights: 1. Presence of the mecA gene in coagulase-negative Staphylococcus isolates. 2. Bacterial profile than what is already presented in the scientific literature. 3. Antimicrobial resistance can cause problems in women, fetuses and even newborns.

PRE-PROOF

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ABSTRACT

This study aimed to determine the phenotypic, molecular and epidemiological profile of methicillin resistant *Staphylococcus* spp. in pregnant women. Were included 100 asymptomatic pregnant women between 16 and 38 years old, who underwent microbiological examination by collecting a vaginal swab at the first trimester of pregnancy. The isolates were subjected to isolation, characterization, phenotypic and molecular tests were performed. Among the samples analyzed, were detected coagulase-negative *Staphylococcus* in 83%, coagulase-positive *Staphylococcus*, 6% and *Streptococcus* spp. in 5%, and there was no bacterial growth in 6%. The antibiotics that showed the highest resistance were amoxicillin + clavulanic acid and sulfamethoxazole + trimethoprim (92.77%) in coagulase-negative *Staphylococcus* and penicillin and sulfamethoxazole + trimethoprim in coagulase-positive *Staphylococcus* (100%), where in the latter *S. aureus* was the species identified in 66.67% of the samples. As for the identification of the *mec*A gene in *Staphylococcus* spp. samples, this gene was detected in 40.5% of the samples of coagulase-negative *Staphylococcus*, and it was not detected in the samples of coagulase-positive *Staphylococcus*. The epidemiological study showed that prior treatment with antibiotics was significantly ($p \leq 0.016$) associated with oxacillin resistance in vaginal swab samples. The presence of the *mec*A gene in coagulase-negative *Staphylococcus* isolates demonstrated a bacterial profile in this type of biological sample, different from what is already presented in the scientific literature. New studies are warranted to understand the epidemiology of the bacterial species involved and later to implement health education actions both in the target population and in health care professionals.

Keywords: Antibiotics; Epidemiology; Methicillin; Pregnancy; Resistance genes; *Staphylococcal carriage*.

STAPHYLOCOCCUS SPP. E O GENE *mec*A em gestantes: Um risco à saúde materna e infantil negligenciada

RESUMO

Este estudo teve como objetivo determinar o perfil fenotípico, molecular e epidemiológico de Staphylococcus spp resistentes à meticilina em mulheres grávidas. Foram incluídas 100 gestantes assintomáticas entre 16 e 38 anos, que realizaram exame microbiológico por meio de coleta de swab vaginal no primeiro trimestre de gestação. Os isolados foram submetidos a isolamento, caracterização, foram realizados testes fenotípicos e moleculares. Dentre as amostras analisadas, foram detectados Staphylococcus coagulase negativa em 83%, Staphylococcus coagulase positiva, 6% e Streptococcus spp. em 5%, e não houve crescimento bacteriano em 6%. Os antibióticos que apresentaram maior resistência foram amoxicilina + ácido clavulânico e sulfametoxazol + trimetoprima (92,77%) em Staphylococcus coagulase negativa e penicilina e sulfametoxazol + trimetoprim em Staphylococcus coagulase positiva (100%), sendo que neste último o S. aureus foi a espécies identificadas em 66,67% das amostras. Quanto à identificação do gene mecA em amostras resistentes à oxacilina, esse gene foi detectado em 40,5% das amostras de Staphylococcus coagulase negativa, e não foi detectado nas amostras de Staphylococcus coagulase positiva. O estudo epidemiológico mostrou que o tratamento prévio com antibióticos foi significativamente ($p \leq 0,016$) associado à resistência à oxacilina em amostras de swab vaginal. A presença do gene mecA em isolados de Staphylococcus coagulase-negativos demonstrou um perfil bacteriano neste tipo de amostra biológica diferente do que já é apresentado na literatura científica. Novos estudos são necessários para entender a epidemiologia das espécies bacterianas envolvidas e posteriormente implementar ações de educação em saúde tanto na população-alvo quanto nos profissionais de saúde.

Palavras-chave: Antibióticos; Epidemiologia; Metacilina; Gravidez; Genes de resistência.

INTRODUCTION

The World Health Organization[1] reports that bacterial drug resistance is a serious public health problem that has been growing in recent years, with high rates of infections caused by bacteria, including those caused by *Staphylococcus* spp.

Staphylococcus spp. have been described as routine members of the human microbiota on the surface of the moistest parts of the body, such as anterior nostrils, armpits and inguinal and perineal areas, while some species and subspecies have a predilection for the urogenital region, and these bacteria may cause skin infections, pneumonia, meningitis, endocarditis, and in some cases, osteomyelitis and toxic shock syndrome[2,3].

Currently, *Staphylococcus* spp. resistant to methicillin (MRS) have been detected, which are multidrug-resistant bacterial isolates and difficult to treat[4]. Previously, infections caused by these microorganisms were treated with simple antibiotics such as penicillin, but with selective pressure, bacteria began to produce penicillinases, a specific type of lactamase, capable of hydrolyzing the lactam ring and inhibiting antimicrobial activity[5].

In 1960, there was the introduction of methicillin, a synthetic antibiotic belonging to the group of β--lactams (penicillins) of small spectrum, as an alternative for bacteria that produce this enzyme, since it acts on the bacterial cell wall and is not influenced by penicillinase[6-8].

One year after the synthesis of methicillin, cases of resistance to this antibiotic were reported in hospitalized patients, and according to Gelatti et al.[9], this resistance can be associated to the presence of the *Panton Valentine* toxin, which is described in isolates belonging to the *Oceania Southwest Pacific Clone* (OSPC) and alternatively, by the presence of the *mec*A gene, part of a genomic element called the "*mec* staphylococcal chromosomal cassette" (SCCmec).

Staphylococcus spp. can be considered opportunistic bacteria since they have frequently caused infections in immunocompromised patients or those using invasive devices, and in this context, pregnant women are part of the risk group due to suppression of the immune system[10-12]. During pregnancy there is a hormonal modification, changing

the natural state of the vagina, with a significant reduction in lactobacilli and an increase in pH, which makes pregnant women susceptible to different forms of vaginal infections, especially bacterial infections[12, 13].

In pregnant women, MRS can cause endocarditis, endometritis, complicated pyelonephritis and even sepsis, in addition to compromising the course of pregnancy, leading to abortion, intrauterine fetal death, chorioamnionitis, premature birth and transplacental infection[14-17]. Due to the importance of bacterial drug resistance and to the possible consequences that can be harmful to maternal and child health, the objective of this study was to determine the phenotypic, molecular and epidemiological profile of MRS in pregnant women.

MATERIAL AND METHODS

Target population and sampling

This study included pregnant women, between 16 and 38 years old, who in the period from February to December 2018 during the first trimester of pregnancy, at a reference Clinical Analysis Laboratory in the respective municipality, responsible for carrying out a microbiological diagnosis in pregnant women registered and cared for in Basic Health Units (BHUs). These pregnant women at the time of the clinic visit did not show clinical signs for any disease.

The isolates were collected using a sterile swab, taking into account the number of pregnant women (n = 559) who had a bacterial culture in the respective municipality in 2017 and considering the formula for determining the number of isolates for discrete data[18]:

$n_0 = \frac{z^2.p.q}{(P-p)2}$ and $n = \frac{n0}{1+\frac{n0}{N}}$

n_0 = initial number

z –confidence level

p – value obtained from work previously done by other authors, or when it is not known, 50% is considered

N – population size

q = 100 – p

P – p = precision arbitrated by researcher (9%)

Resulting in:

$n_0 = \frac{1.96^2.50.50}{(9)2}$

$n_0 = 118.57$

$n = \frac{118.57}{1+\frac{118.57}{559}}$ n= 97.82

In this way, 100 swab isolates were collected

Culture, isolation and phenotypic characterization of isolates

The collection of vaginal secretion was performed by professional nurses using a swab via the vaginal introitus, only from the bottom of the vaginal sac region and ectocervical region, which does not cause stimulation of the pregnant woman's endocervical region, as directed by the Ministry of Health[19].

Subsequently, the isolates collected were sent immediately under refrigeration to the Laboratory of Preventive Veterinary Medicine and Public Health of the Postgraduation Program in Animal Science with emphasis on bioactive products at UNIPAR, where they were place in brain heart infusion (BHI) broth, and incubated at 37°C for 24 hours. The isolates were then cultured on Mannitol Salt Agar and incubated at 37ºC for up to 48 hours to isolate *Staphylococcus* spp.

Each colony was subjected to analysis of macroscopic and microscopic characteristics and tested for catalase and coagulase, allowing their classification into coagulase-positive *Staphylococcus* (STACP) and coagulase-negative *Staphylococcus* (STACN)[20].

Phenotypic antibiotic sensitivity tests

Antibiotic susceptibility tests were performed according to the Clinical and Laboratory Standards Institute[21]. Each *Staphylococcus* spp. isolated was subjected to the disk-diffusion assay and tested against amoxicillin + clavulanic acid (30 µg), ampicillin (10 µg), cephalothin (30 µg), cefotaxime (30 µg), clindamycin (2 µg), enrofloxacin (5 µg), gentamicin (10 µg), norfloxacin (10 µg), oxacillin (1 µg), penicillin (10 U), sulfamethoxazole + trimethoprim (25 µg) and tetracycline (30 µg).

Bacterial resistance index

To calculate the multidrug resistance index, we used the formula described by Krumperman[22], *a*/(*bc*), where *a* is the number of antibiotics to which the isolates showed resistance, *b* is the number of antibiotics tested, and *c* is the number of isolates. The index to define whether the isolates are high or low risk was 0.200, where below 0.199 is considered low risk and 0.200 and higher is considered high risk.

Another formula was used to evaluate the multidrug resistance index of each sample, also described by Krumperman[22]: *a*/*b*, where *a* is the number of antibiotics against which the isolate was resistant, and *b* the number of antibiotics tested.

Molecular diagnosis

Staphylococcus aureus identification

PCR was performed on the isolates with coagulase-positive *Staphylococcus* (STACP) to determine which of these isolates were *Staphylococcus aureus*. DNA was extracted with the Purelink Genomic DNA Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and the reactions were performed using the Sa442-1 primer (5'- AAT CTT TGT CGG TAC ACGATA TTC TTC ACG -3 'and the Sa442-2 primer (5'-CGT AAT GAGATT TCA GTA GAT AAT ACA ACA-3') as described by Martineau et al.[23]. The Applied Biosystems model Veriti™ 96-Well Thermal Cycler was used for DNA amplification.

The amplification products were electrophoresed on a 2% agarose gel stained with GelRed (Uniscience, Osasco, São Paulo, BR) using a 100-bp molecular marker, and the products were visualized as a single band of 241 bp.

mecA gene detection

DNA of all the *Staphylococcus* spp. was extracted using the Purelink Genomic DNA Kit (Invitrogen) according to the manufacturer's instructions, and PCR was performed using the mecA1 primer (AAAATCGATGGTAAAGGTTGG) and mecA2

primer (AGTTCTGCAGTACCGGTTG) according to Murakami et al.[24]. DNA was amplified using the Veriti™ 96-Well Thermal Cycler.

The amplification products were visualized by electrophoresis on a 2% agarose gel stained with GelRed (Uniscience) using a 100-bp molecular marker, and a single band of 533 bp was obtained.

Survey instrument

After reviewing and signing an informed consent form, each pregnant woman answered an epidemiological questionnaire containing information on treatment history about infections and/or previous use of antibiotics, and also social, cultural and behavioral information.

Statistical analysis

The results obtained after studying the variables associated with the presence of *Staphylococcus* spp. resistant to methicillin and also for the presence of the *mec*A gene were subjected to statistical analysis using the chi-square test (x^2) with Yates correction or Fisher's exact test, using the statistical program SPSS v. 21.0 at the 5% significance level.

RESULTS

Among the 100 isolates analyzed, coagulase-negative *Staphylococcus* was detected in 83 isolates (83%), coagulase-positive *Staphylococcus* in six (6%) and *Streptococcus* in five (5%), and there was no bacterial growth in six isolates (6%).

Among the coagulase-negative *Staphylococcus* isolates, the most notable results were that 77 (92.77%) of the isolates were resistant to amoxicillin + clavulanic acid and sulfamethoxazole + trimethoprim, followed by 74 (89.15%) resistant to ampicillin, 76 (91.56%) resistant to penicillin and 37 (44.57%) to oxacillin (Tables 1 and 2).

Table 1. Antibiotic resistance profile of coagulase-negative *Staphylococcus* samples from vaginal swabs of pregnant women seen at Basic Health Units (BHUs) in a city in the northwest region of Paraná State, Brazil, 2018.

Antibiotic-resistant samples		
Antibiotics	No.	%
Amoxicillin + Clavulanic acid	77	92.77
Sulfamethoxazole + Trimethoprim	77	92.77
Penicillin	76	91.56
Ampicillin	74	89.15
Tetracycline	41	49.39
Oxacillin	37	44.57
Clindamycin	30	36.14
Norfloxacin	14	16.86
Enrofloxacin	10	12.04
Gentamicin	9	10.84
Cefotaxime	9	10.84
Cephalothin	8	9.63

Table 2. Antimicrobial resistance profile of coagulase-positive *Staphylococcus* samples from vaginal swabs of pregnant women seen at Basic Health Units (BHUs) in a city in the northwest region of Paraná State, Brazil, 2018.

Antibiotic resistant		
Antibiotics	No.	%
Sulfamethoxazole + Trimethoprim	6	100
Penicillin	6	100
Ampicillin	5	83.33
Clindamycin	5	83.33
Tetracycline	4	66.67
Oxacillin	2	33.33

Cefotaxime	2	33.33
Cephalothin	1	16.67
Gentamicin	1	16.67
Enrofloxacin	0	0.0
Norfloxacin	0	0.0
Amoxicillin + Clavulanic acid	0	0.0

The multidrug resistance index of coagulase-negative *Staphylococcus* isolates was 0.424. This was higher than the cutoff according to Krumperman[22] indicating high risk to public health. The same Krumperman[22] formula was adapted and used for each antibiotic tested, showing that only gentamicin did not reach the established cutoff value (Table 3).

Table 3. Bacterial multidrug resistance index of coagulase-negative *Staphylococcus* isolates from vaginal swabs of 83 pregnant women seen at Basic Health Units (BHU) in a city in the northwest region of Paraná State, Brazil, 2018.

Bacterial resistance index			
Sample	Vaginal swab	Sample	Vaginal swab
3	0.462	51	0.769
4	0.462	53	0.308
5	0.462	54	0.385
6	0.154	55	0.385
7	0.846	58	0.385
8	0.538	59	0.308
10	0.385	60	0.692
11	0.308	62	0.308
12	0.154	63	0.615
13	0.538	64	0.308
14	0.462	65	0.615
16	0.462	66	0.154
17	0.308	68	0.462

18	0.615	69	0.385
19	0.769	70	0.615
20	0.385	71	0.462
21	0.462	72	0.308
22	0.538	73	0.308
23	0.462	74	0.385
24	0.231	75	0.308
28	0.308	76	0.615
29	0.615	77	0.462
30	0.692	79	0.385
31	0.385	80	0.538
32	0.385	81	0.615
33	0.615	82	0.385
34	0.154	83	0.385
35	0.538	84	0.231
36	0.231	85	0.385
37	0.385	86	0.385
38	0.308	87	0.538
39	0.462	89	0.538
40	0.308	90	0.154
41	0.154	91	0.615
42	0.462	92	0.385
43	0.308	93	0.462
44	0.385	94	0.231
45	0.538	95	0.385
46	0.769	98	0.462
47	0.462	99	0.385
48	0.538	100	0.000
50	0.538		

Legend: Krumperman (1983) suggests that samples with values over 0.200 represent cases that pose a high risk to public health.

Of the coagulase-positive *Staphylococcus* isolates, all six (100%) isolates were resistant to penicillin and sulfamethoxazole + trimethoprim, five (83.33%) resistant to ampicillin and clindamycin, four (66.67%) resistant to tetracycline, and two (33.33%) to oxacillin.

Of the six isolates of coagulase-positive *Staphylococcus*, four (66.66%) were identified as *Staphylococcus aureus*.

The multidrug resistance index of the coagulase-positive *Staphylococcus* isolates was 0.410, which was higher than the cutoff value, indicating a high risk to public health. The same formula by Krumperman[22] was used for each antibiotic tested, showing that amoxicillin + clavulanic acid, cephalothin, enrofloxacin, gentamicin and norfloxacin did not reach the cutoff value (Table 4).

Table 4. Bacterial multiresistance index of coagulase-positive *Staphylococcus* isolates from vaginal swabs of six pregnant women seen at Basic Health Units (BHUs) in a city in the northwest region of Paraná State, Brazil, 2018.

Bacterial resistance index			
Sample	Vaginal swab	Sample	Vaginal swab
49	0.308	88	0.308
61	0.462	96	0.462
78	0.462	97	0.462

Legend: Krumperman (22) suggests that samples with values over 0.200 represent cases that pose a high risk to public health.

Among the 89 isolates, the presence of the *mec*A gene in was detected in 15 (18.07%) of these coagulase-negative *Staphylococcus* isolates.

The variable “prior treatment with antibiotics” when analyzed with the vaginal swabs from pregnant women resistant to oxacillin showed statistical significance ($p \leq 0.016$).

DISCUSSION

Infections caused by MRS have caused serious problems inside and outside the hospital environment. Outside the hospital environment, it can promote colonization of people, increasing the chance of causing disease and even death in both sexes and all age groups, especially in sexually active women and pregnant women who are more susceptible to infections[9, 25].

Considering the possible pathogenicity and drug resistance of the bacteria, we evaluated the phenotypic, molecular and epidemiological profile of MRS in pregnant women in a city located in the northwest region of Paraná State in Brazil, which consists of an unprecedented work of great importance for public health, since infections by drug-resistant isolates may increase the risk of colonization of pregnant women, possibly reaching the fetus due to congenital infection[26]. These microorganisms have in many cases been neglected with regard to their clinical importance, and there is still a gap in studies of MRS involved in vaginal infections in pregnant women.

In this work, 83% of the isolates were identified as coagulase-negative *Staphylococcus* and 40.5% were positive for the presence of the *mec*A gene; however, there is a lack of research on the resistance profile of isolates of *Staphylococcus* spp. of cervicovaginal isolates. There is no reported association of coagulase-negative *Staphylococcus* and the presence of the *mec*A gene with vaginal infections, and for this reason, new studies are essential for the identification of possible coagulase-negative *Staphylococcus* species in this type of biological isolate that will provide greater knowledge related to the epidemiology of these bacterial species, which will assist in the implementation of preventive measures and later more assertive treatment regarding the choice of the most appropriate antibiotic to be used by the pregnant woman.

It is already known the frequency of use of amoxicillin + potassium clavulanic acid 500 mg + 125 mg tablets and sulfamethoxazole + trimethoprim 400 mg + 80 mg tablets in different specialties in the health area. Thus, the resistance profile detected in this study indicates the possibility of clinical use of these drugs by pregnant women in this municipality, once, they are drugs of routine use in the clinical practice of different diseases.

We found that 6% of the isolates were coagulase-positive *Staphylococcus* and the molecular diagnosis indicated that 66% were *Staphylococcus aureus*. In these isolates, *mec*A was not detected. These results do not corroborate those of Andrews et al.[15] in Birmingham, AL (United States); these authors detected *Staphylococcus. aureus* in 14.5% of pregnant women seen at an obstetric service, and in these cases, 24.3% of the isolates were resistant to methicillin and the general rate of colonization by methicillin-resistant *Staphylococcus. aureus* (MRSA) was 3.5%. This would possibly increase the risk of miscarriage, chorioamnionitis, complicated pyelonephritis and even sepsis, in addition to a possible transplacental infection, leading the authors to recommend follow-up of newborns[14,15,17].

It is known that *Staphylococcus aureus* resistant or not to methicillin is frequently associated with vaginal, vulvar or subcutaneous tissue infections, representing the main pathogen of this type of bacteria that cause infection in humans[27].

Riboli et al.[28] notes the importance of *Staphylococcus aureus* in newborns, which should be extrapolated to the present study, considering the resistance levels of coagulase-negative *Staphylococcus* determined not only in the phenotypic profile but also in the genotype in the present study.

According to Thurman et al.[29], the increase in the prevalence of MRS can be due to the inappropriate use of β-lactam antibiotics, where with selective pressure, bacteria begin to produce penicillinase, a specific type of lactamase, capable of hydrolyzing the β-lactam ring, thereby inhibiting the action of antibiotics in this group[4]. In the present study, it is observed that most isolates were resistant to some type of β-lactam, especially penicillin (91.57%) and the aminopenicillins amikacin (92.77%) and ampicillin (89.16%).

In the present study, 100% of the coagulase-positive *Staphylococcus* isolates showed resistance to sulfamethoxazole + trimethoprim. Thurman et al.[29] in Texas (USA) found that the incidence of MRSA in women with vulvar abscesses 64% and that the MRSA isolates were sensitive to clindamycin (72%), sulfamethoxazole + trimethoprim (96%) and doxycycline (96%), results are not similar to our findings. this study; it is possible that this resistance to these antibiotics developed due to their routine use in the local clinical practice.

The multi-resistance index of coagulase-negative *Staphylococcus* and coagulase-positive *Staphylococcus* was 0.424 and 0.410, respectively, which, according to Krumperman[22], indicates that they are of high risk to public health, since they are potentially dangerous to people. These bacteria increase the risk of contamination of environments and the possibility of spreading, becoming a major problem for hospitalized patients as well as community groups, especially immunocompromised adults, children, the elderly and pregnant women.

Resistant bacteria form biofilms and produce extracellular polysaccharide, which increases their pathogenicity, in which the adherence and persistence of microorganisms is favored, promoting a barrier to the action of antimicrobials. The concentration of antibiotics required to kill bacteria in biofilms is much higher than that needed to kill the same species in suspension, resulting in reduced drug susceptibility making treatment difficult[5].

The variable "prior treatment with antibiotics" showed a significant ($p \leq 0.016$) relation to oxacillin resistance. This finding indicates the importance of periodic assessment of the microbiological profile and sensitivity to different antibiotics in infections of the genitourinary tract in pregnant women, thereby allowing the selection of a more appropriate treatment, preventing in some way the emergence of potentially resistant isolates that could foster future complications both in the mother and in the fetus or newborn.

It is already known that pregnant women can transmit MRS to newborns by vertical transmission such as breastfeeding, maternal chorioamnionitis, and the birth canal itself. These microorganisms are one of the greatest problems in neonatal intensive care units, where their colonization of neonates can lead to serious morbidity or death[28].

This study highlights the importance of new research to determine not only other antibiotic resistance genes but also other pathogenicity factors, such as the production of enterotoxins, toxic shock toxin and biofilms, which can be related to coagulase-negative *Staphylococcus* species.

The antibiotic resistance shown in this study can lead to the emergence of highly pathogenic microorganisms, since the isolates obtained from vaginal isolates can pose a

high risk to public health and as a consequence can cause serious problems in pregnant women, fetuses and even newborns.

CONCLUSIONS

The presence of the *mecA* gene in coagulase-negative *Staphylococcus* isolates demonstrates a bacterial profile in this type of biological sample, different from what is already presented in the scientific literature. Therefore, new studies are warranted to understand the epidemiology of the possible bacterial species involved and later to implement health education actions both in the target population (pregnant women) and in health care providers (doctors, nurses and community agents connected to local health.

ETHICAL ASPECTS

This project was approved by Plataforma Brasil under protocol CAAE No. 88426418.0.0000.0109 and the Ethics Committee for Research Involving Humans at Universidade Paranaense under protocol CAAE: 88426418.0.0000.0109.

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