# Phagocytosis and Killing of Epidemic Methicillin-Resistant *Staphylococcus aureus* by Human Neutrophils and Monocytes

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***Staphylococcus aureus*** **is a pathogen that has been associated with nosocomial infections since the preantibiotic era. Since the introduction of antibiotics in medical practice in the 1940's, methicillin-resistant** *S. aureus* **(MRSA) strains have been emerging in various parts of the world. In view of the important role of the phagocytic system in the defense against this bacteria, we decided to study phagocytosis by neutrophils and monocytes of an epidemic MRSA strain in São Paulo, Brazil, in comparison with methicillin-sensitive strains. Complement system opsonins are fundamental for efficient ingestion of the resistant and sensitive strains by both types of phagocytes. We found no association of the opsonic requirement of the MRSA strain with the multiresistance phenotype. On the other hand, the MRSA strain was found to be more resistant to the effector mechanisms of neutrophils than both sensitive strains when opsonized with fresh serum, despite the phagocytosis results. This fact suggests that the intracellular killing of** *S. aureus* **is an additional parameter of bacterial virulence, but new approaches must be implemented to study the interactions of this MRSA strain with phagocytes in order to investigate the possible factors involved in its behavior in response to neutrophil effector mechanisms.**
**Key Words:** *Staphylococcus aureus*, **human phagocytes, phagocytosis, epidemic methicillin-resistant.**

*Staphylococcus aureus* is a pathogen that contains biochemical machinery that enables it to quickly colonize and invade its host through minor breaks in the skin and mucous membranes, as well as to protect itself against many of the host's defenses, thereby frequently reaching the bloodstream [1]. This bacterium has persisted as an important public health problem since the preantibiotic era, when staphylococcal bacteremia was frequently mortal for the affected patient [2]. Antibiotic use increased through the 1950's and

resistance rapidly developed [1], producing strains of *S. aureus* that were resistant to methicillin and oxacillin (MRSA) [3,4].

Peripheral blood phagocytes participate in the nonspecific defense against extracelullar bacteria. This interaction between cell and bacterium is important to eliminate the pathogen from blood or tissues. Neutrophils and monocytes are cells that actively participate in this process; they are able to kill these bacteria by phagocytosis, with consequent lysosomal degranulation and activation of oxidative metabolism.

There is much data concerning MRSA epidemiology, but studies on host defense mechanisms against these strains are rare. Some investigators have studied the possible association of multiresistance phenotype and resistance in the phagocytic system, but with contradictory results [5-8].

In view of this situation, we decided to study the phagocytosis and killing of an epidemic MRSA strain

in Brazilian hospitals by normal human neutrophils and monocytes.

## Material and Methods

### Human blood

Peripheral venous blood was collected from 57 healthy adult male blood donors from "Fundação Pró-Sangue do Hemocentro de São Paulo" (aged 18-49 years).

### Bacterial strains and culture conditions

The methicillin-resistant strain (MRSA) and a methicillin-sensitive strain of *S. aureus* (MSSA) were obtained from the Special Microbiology Laboratory of the Division of Infectious Diseases (Federal University of Sao Paulo). The MRSA strain had been isolated from a patient at a Hospital in São Paulo city, and it presented an epidemic pattern of chromosomal DNA when analyzed by pulsed-field gel electrophoresis, based on Sader et al. [9]. The MSSA strain was collected from a nasal carrier at Sao Paulo Hospital. Another methicillin-sensitive strain (CO - Cowan I - ATCC 12598), previously used to standardize the phagocytosis assay, was obtained from the Culture Collection Department of the Adolfo Lutz Institute. The strains were maintained lyophilized, and were cultured in Mueller Hinton broth (Difco Laboratories, Detroit, USA) at 37°C with shaking for 16 h for the phagocytosis assay. The MRSA strain was resistant to oxacillin, penicillin, ampicillin, cefoperazone, cefoxitin, ceftazidime, ceftriaxone, gentamicin, tobramycin, ciprofloxacin, norfloxacin, and lincomycin, and was sensitive to vancomycin, netilmycin, and nitrofurantoin, presenting intermediate sensitivity to rifampicin, according to the antibiogram method of Bauer et al. [10]. The MSSA and CO strains were sensitive to all antibiotics with the exception of intermediate sensitivity to cefoperazone and ceftazidime, and resistance to penicillin only in the MSSA strain.

All three *S. aureus* strains were producers of DNase, coagulase, fibrinogen binding protein, and protein A, and none of them presented a capsule by the China ink technique [11-15]. β-Lactamase production was detected only in the MRSA and MSSA strains [16].

### Bacterial opsonization

Bacteria of three strains were opsonized with a pool of normal human sera (fresh sera - FS or inactivated sera at 56°C for 30 minutes - IS) or unopsonized (without serum - UN). In separate experiments, opsonization with fresh serum from an agammaglobulinemic patient (AG) was compared with FS. Fresh sera were kept at -70°C. The levels of immunoglobulins and C3 complement protein in FS and AG were determined (Table 1). Bacteria were then incubated with 10% serum at 37°C for 30 minutes, to a final concentration of $2 \times 10^7$ cfu/mL in TC-199 medium buffered with 20 mM HEPES (TC199/HEPES).

### Leukocyte preparation

Leukocytes were separated from heparinized whole blood by sedimentation with 4.5% Dextran T-500 at 37°C for 30 minutes. The leukocyte-rich plasma was removed, washed twice with Hanks' balanced salt solution (HBSS) by centrifugation at 160 g for 10 minutes, and resuspended in TC199/HEPES at $2 \times 10^6$ phagocytes/mL.

### Phagocytosis and bacterial killing

Phagocytosis and bacterial killing by neutrophils and monocytes were assessed by a fluorochrome technique [17, 18], using acridine orange staining to determine living (green) and non-living (red/yellow) bacteria. Phagocytosis index (PI), and percent killed bacteria (%KB) were calculated as in Bellinati-Pires et al. [18].

### Statistical analysis

Each parameter was analyzed by Multifactor ANOVA (BMDP statistical software, Westwood, CA) and by the Multiple Comparisons Test (Tukey's Test).

**Table 1.** Levels of immunoglobulins and C3 protein of the complement system in pools of fresh normal human sera (FS) and in fresh serum from an agammaglobulinemic patient (AG)

| Serum | IgG (g/L) | IgM (g/L) | IgA (g/L) | C3 (g/L) |
|-------|-----------|-----------|-----------|----------|
| FS | 16.61 | 1.44 | 2.79 | 0.96 |
| AG | 4.25 | ND | ND | 0.87 |

ND = not detected. Normal range for adult individuals: IgG = 7.10 - 15.20 g/L (Tmax kit); IgM = 0.40 - 2.40 g/L (IAC kit); IgA = 0.90 - 3.10 g/L (IAC kit); C3 = 0.84 - 1.93 g/L (Tmax kit).

**Table 2.** Comparison of normal serum and agammaglobulinemic serum in the phagocytosis of *Staphylococcus aureus* strains

| Phagocytes | Parameters | | CO | MSSA | MRSA |
|------------|-----------|------|----|------|------|
| Neutrophils | PI | FS | 8.69 ± 0.92 | 8.67 ± 0.55 | 7.30 ± 0.52 |
| | | AG | 7.93 ± 0.66 | 8.15 ± 0.87 | 7.15 ± 0.59 |
| | %KB | FS | 50.11 ± 4.27 | 39.05 ± 3.79 | 34.50 ± 4.14 |
| | | AG | 45.95 ± 4.69 | 37.27 ± 5.57 | 35.94 ± 5.14 |
| Monocytes | PI | FS | 6.78 ± 0.74 | 6.29 ± 0.46 | 5.87 ± 0.34 |
| | | AG | 6.94 ± 0.84 | 6.23 ± 0.49 | 6.00 ± 0.39 |
| | %KB | FS | 58.49 ± 6.08 | 46.35 ± 3.33 | 46.66 ± 6.43 |
| | | AG | 55.43 ± 4.75 | 52.01 ± 7.73 | 45.59 ± 6.35 |

Phagocytosis of multidrug-resistant *Staphylococcus aureus* strain (MRSA), and two sensitive strains (MSSA and CO), by neutrophils and monocytes after opsonization with a pool of fresh normal human serum (FS) or serum from an agammaglobulinemic patient (AG). Results are reported as means ± SEM. PI = phagocytosis index; %KB = percent killed bacteria.

## Results

*Levels of immunoglobulins and C3 protein of the complement system in fresh sera used for opsonization (Table 1)*

Despite the low level of immunoglobulins in the AG serum, the complement C3 was within the normal range.

*Effect of bacterial opsonization, with fresh normal human serum (FS) or aggamaglobulinemic serum (AG), on the phagocytosis by neutrophils and monocytes*

No differences were observed in the effects of opsonization with FS versus AG on the ingestion and killing of bacterial strains by phagocytes (Table 2).

*Comparison of the MRSA, MSSA and CO strains after opsonization with fresh serum, inactivated serum or unopsonized*

It was evident that the CO strain was more phagocytized by neutrophils or monocytes than other strains. When fresh serum was used, the difference among PI values was significant between CO and MRSA for monocytes. However, when inactivated serum (IS) was used, increased differences between CO and MRSA or MSSA strains were observed in the PI values obtained for both types of phagocytes (Figure 1).

**Figure 1.** Comparisons of *Staphylococcus aureus* Cowan I (CO), sensitive (MSSA) and multi-drug resistant (MRSA) strains opsonized with a fresh or inactivated pool of human serum or unopsonized, with respect to phagocytosis by normal human neutrophils (A) and monocytes (B). Results are reported as mean ± SEM phagocytosis index (PI) (Multifactor ANOVA with repeated measures and Multiple Comparisons Test ✦ ,• = p<0.05).
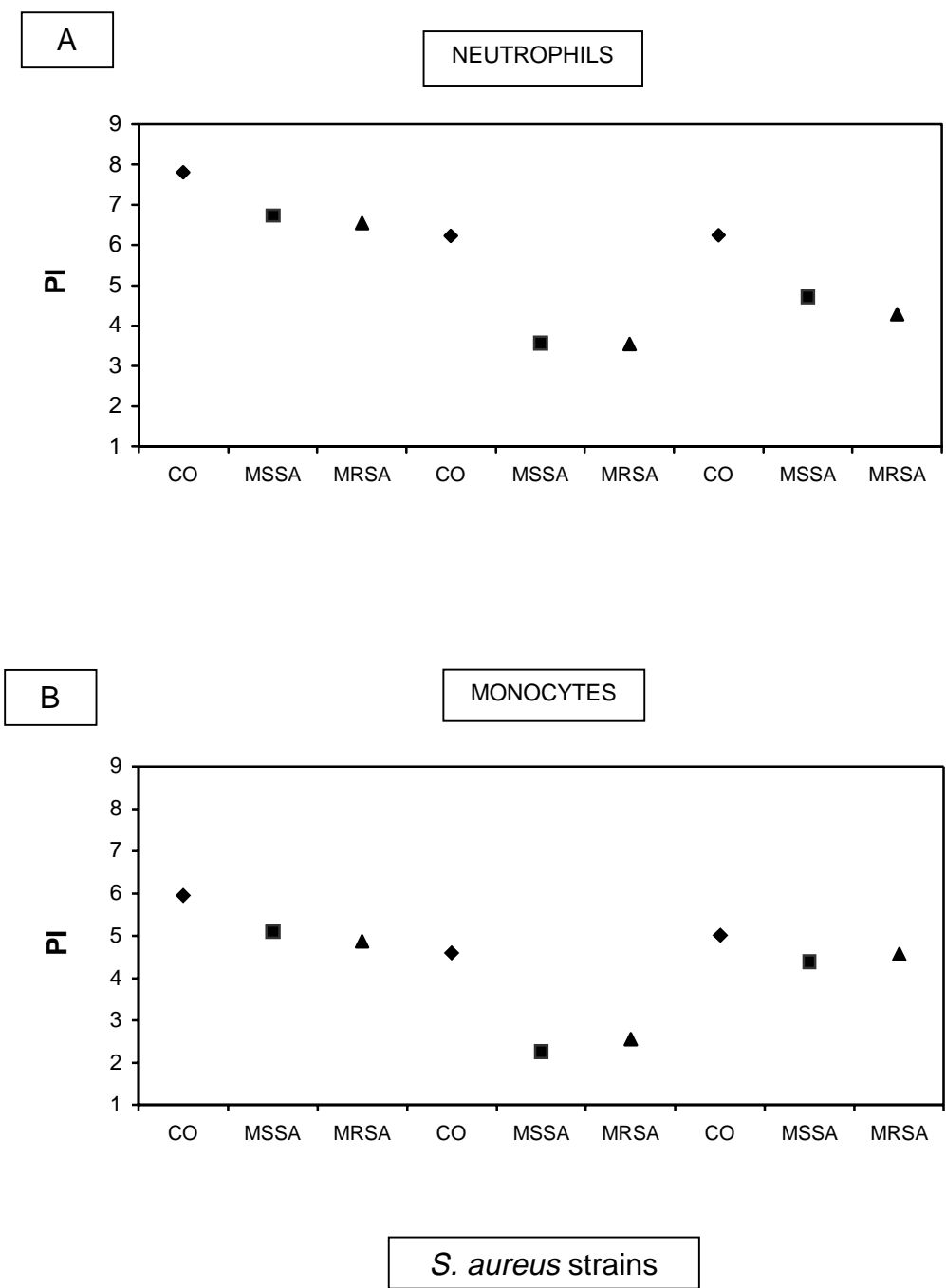
**Figure 2.** Comparisons of *Staphylococcus aureus* Cowan I (CO), sensitive (MSSA) and multi-drug resistant (MRSA) strains opsonized with a fresh or inactivated pool of human serum or unopsonized, with respect to intracellular killing by normal human neutrophils (A) and monocytes (B). Results are reported as mean ± SEM percent killed bacteria (%KB) (Multifactor ANOVA with repeated measures and Multiple Comparisons Test – ★ , • , ⊂ = p<0.05).
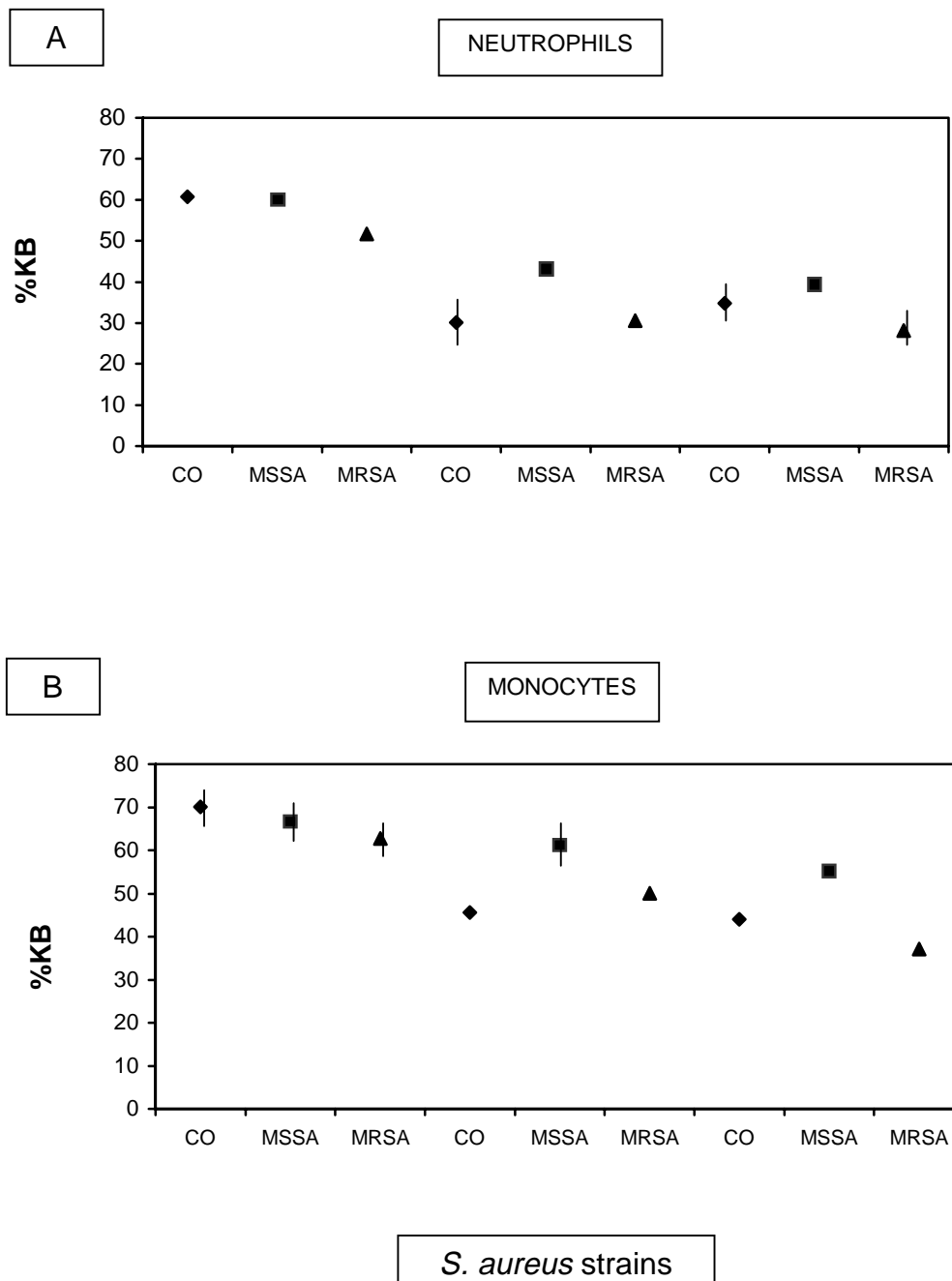
The MRSA strain was more resistant to killing by neutrophils than the other two sensitive strains (CO and MSSA), when opsonized with FS, and more resistant to killing by neutrophils than MSSA when non-opsonized (Figure 2).

## Discussion

Since virulent staphylococci have been found to survive the bactericidal action of neutrophils better than avirulent strains [19,20], we explored the possible relationship between expression of resistance to multiple drugs by strains of MRSA and survival of these organisms in neutrophils and monocytes. To answer this question, we selected a multidrug-resistant MRSA strain isolated from the intravenous catheter of a hospitalized Brazilian patient and two strains sensitive to multiple antibiotics (CO and MSSA strains).

In view of the high mortality caused by nosocomial infections with MRSA, we thought it important to verify whether, in addition to antibiotic resistance, other intrinsic bacterial factors could influence their virulence to the host. We examined the presence of some bacterial products, such as DNase, coagulase, fibrinogen-binding protein (FgBP), protein A (PA) and a capsule, which are among *S. aureus* virulence factors used in phagocytic system escape mechanisms [21-23]. The strains produced all the virulence factors that were tested, and we did not detect expression of a capsule by the China ink technique. These results may facilitate comparisons among strains in phagocytosis assays because they can usually be compared only on the basis of their antibiotic resistance polarity. Other authors also observed that MRSA strains were producers of coagulase [5,24] and FgBP [24] on independent multiresistance phenotype. We noted that the China ink technique was not sensitive enough to detect microcapsule expression or the absence of a capsule by the strains. Odierno et al. [15] also noted a lower sensitivity of this technique when they compared various techniques, although the China ink technique continues to be used because it permits direct results. As reported in the literature, microencapsulated strains

are the most commonly encountered types of strains among *S. aureus* clinical isolates in humans and animals [25]. PA, detected by immunoblotting and radial double immunodiffusion, was present in all three strains.

Although a possible loss of resistance phenotype to methicillin/oxacillin antibiotics by *S. aureus* strains cultured at 37°C *in vitro* has been reported [26], our MRSA strain maintained its resistance after repeated cultivation at 37°C, as evaluated by the antibiogram or E-Test (AB Biodisk, Sweden), even after 10 repeated cultures (data not shown). Our findings are in agreement with Peacok et al. [6] who also observed maintenance of resistance phenotype in a MRSA strain after incubation at 30, 35, and 37°C. Culture conditions such as broth or solid medium, medium composition, and growth period, are also thought to influence quantitative or qualitative expression of bacterial products [27-29]. This investigation was necessary because our strains were maintained in simple agar at 37°C for one month for the phagocytosis assay.

We applied the fluorochromic technique to evaluate the phagocytosis of each strain by normal human neutrophils and monocytes in order to determine the influence of serum opsonins on the strains. We observed that both types of phagocytes gave a diminished phagocytosis index (PI) for the three strains after treatment with inactivated serum (IS) or when serum was absent (UN), compared to treatment with a pool of fresh human serum (FS) (data not shown – n=18). These results confirm that the staphylococcal strains studied in this work were dependent on opsonization by heat-labile serum opsonins in order to be efficiently internalized by phagocytes. In the same set of experiments, we noted that only the killing by neutrophils of the CO strain, opsonized with IS or unopsonized, was affected. Actually, Gordon et al. [30,31], studying the phagocytosis of the *S. aureus* Cowan I strain and *Escherichia coli,* observed that ingestion via iC3b receptors augmented the neutrophil-mediated intracellular mortality of these bacteria.

When comparing bacterial opsonization with FS and fresh serum from an agammaglobulinemic patient (AG), we found no differences between treatments in the ingestion or killing of the three strains by the two types

of phagocytes. Considering that IgG levels in AG serum were 25% of FS IgG levels, with undetectable IgM and IgA levels and C3 protein within the normal range (Table 1), we conclude that specific immunoglobulins (Ig) had no effect on *S. aureus* ingestion or killing by phagocytes (Table 2). These data also confirmed the positive influence of heat-labile serum opsonins. In examination of the anti-phagocytic role of PA in the *S. aureus* evasion mechanisms, the results did not reveal any alterations in the phagocytic function of neutrophils and monocytes against any of the three bacterial strains, all of them PA producers, despite bacterial opsonization with normal or agammaglobulinemic serum. However, we can not rule out a possible anti-phagocytic activity of this protein in a systemic infection with *S. aureus* (bacteremia/septicemia) when a considerable increase in Ig levels occurs. In deep infections, in addition to Ig hyper-production, increased bacterial propagation and PA discharge occur, which may block the Fc IgG portions, inhibiting opsonization and phagocytosis via Fc receptors. Furthermore, free PA may binds to specific Igs produced during infection, inactivating the complement system by the classical pathway [23].

After evaluating the influence of complement system opsonins on the ingestion and killing of each strain separately, we performed a comparative study of three *S. aureus* strains in the same phagocytosis experiment. We found that the phagocytosis index (PI) obtained with the MRSA strain was lower than that obtained with the CO strain but similar to that obtained for the MSSA strain, both for neutrophils and monocytes, independent on the kind of opsonization (Figure 1). Thus, it does not seem that reduced ingestion of MRSA is associated with the multiresistance phenotype, at least under these experimental conditions.

In agreement with our data, Peacock et al. [6] did not observe any difference in the ingestion of a multi-drug resistant MRSA strain and 3 strains sensitive to various antibiotics by human neutrophils, after 15, 30, 60 and 120 minutes of phagocytosis in the presence of FS. They found that more than 99% of the bacteria were ingested after 60 minutes of incubation. Jordens et al. [5] also did not detect any difference in ingestion by human neutrophils of 7 multidrug-resistant MRSA

strains, 5 strains resistant only to penicillin and 2 sensitive strains opsonized with FS, after 30 minutes of phagocytosis.

Concerning killing by phagocytes, we observed that the MRSA strain had reduced values of %KB in neutrophils when compared to both sensitive strains after FS opsonization (Figure 2). This was a unique situation, in which the MRSA strain differed from sensitive strains for the same parameter. With respect to the CO strain, which is dependent on heat-labile serum opsonin activity for efficient killing by neutrophils, we think that serum inactivation or its absence in the phagocytic assay abolished the factors that cause differences between the CO and MRSA strains.

Based on *in vitro* phagocytosis data for the MRSA strain opsonized with FS, Vaudaux & Waldvogel [7] reported that 12 multi-drug resistant MRSA strains survived better within neutrophils than a sensitive strain after 30 and 60 minutes of incubation. However, they did not observe any correlation between level of methicillin resistance and strain survival in these cells. On the other hand, in 1979, when these same investigators decided to study the mortality of 8 MRSA strains in comparison with 2 sensitive strains after 1, 3, 5 and 24 hours of incubation with neutrophils [8], they did not confirm the differences observed in their previous study. Thus, they concluded that expression of methicillin resistance did not influence bacterial survival within neutrophils in their second study. Similarly, Peacock et al. [6] did not observe any difference in intracellular mortality of a multi-drug resistant MRSA strain in relation to 3 sensitive strains after 15, 30, 60 and 120 minutes of incubation with neutrophils, and observed that more than 93% of bacteria were ingested after 60 minutes of incubation. All of the above data were obtained by counting the number of *S. aureus* colony forming units by the pour plate technique, which differs from our microscopic methodology. Considering methodological variances and lack of data about interactions of multidrug-resistant staphylococci with the phagocytic system, it would be premature to attempt to compare our data with those obtained in these cited studies.

Peacock at al. [6] performed studies on mice inoculated intraperitoneally with a MRSA or 3 sensitive strains (MSSA1, MSSA2 and Wood 46) to establish the $LD_{50}$. They did not observe any difference between MRSA and MSSA1 and MSSA2, but the MRSA strain was considered to be less virulent than Wood 46. However, when they compared strains by means of intravenous injection of staphylococci in mice they showed that MRSA was more virulent than MSSA1 and Wood 46 and less virulent than the MSSA2 strain. Cutler [24], in a study of a MRSA strain and 3 variants of this strain whose resistance to some antibiotics was suppressed by culture at 42°C for 7 days, observed greater virulence for MRSA than for the other strains in mice and guinea-pigs subcutaneously injected with these bacteria. These contradictory data demonstrate the great difficulty in establishing parameters for evaluating staphylococcal virulence.

Some researchers have evaluated the association between resistance to antibiotics and *S. aureus* cell wall features. Cutler [24] suggested that alterations in staphylococcal cell walls related to the multiresistance phenotype could affect *S. aureus* virulence. Penicillin-resistant staphylococci have a greater cell wall lipid content and different immunoelectrophoretic mobilities than penicillin-sensitive variants of the same strains [32]; penicillin resistance and cell wall thickness could be associated [33] and gentamicin resistance may produce morphological changes in the cell wall [34]. It should be emphasized that strains resistant to methicillin/oxacillin have penicillin-binding proteins (PBPs) in their cell walls (named PBP2a), different from the PBPs of methicillin/oxacillin sensitive strains. These proteins, encoded by the chromosomal mecA gene, are responsible for the formation of *S. aureus* cell wall peptidoglycan chains, and are also targets for antibiotics such as penicillin [9,35,36]. Considering that these altered PBP2a continue to be accountable for cell wall structure and taking into account the above data about the cell wall, we can hypothesize that resistant strains have a morphology that confers greater protection against destruction by lysosomal

enzymes or by oxidative metabolism products of phagocytes. It would be interesting to investigate the cell wall structure of our MRSA strain more accurately, since it is also resistant to penicillin and gentamicin, among other antibiotics, facts that might involve cell wall alterations.

We would like to emphasize some behavioral differences between neutrophils and monocytes with respect to their interactions with the *S. aureus* strains evaluated in this work. Although neutrophils phagocytized many more bacteria than monocytes, the latter cells had a staphylococcidal capacity similar to or higher than that of the neutrophils ($p<0.05$). These observations raise questions about statements that monocytes are less efficient than neutrophils to eradicate extracellular pathogens [37], such as staphylococci. We also emphasize that, due to lack of data on phagocytosis and killing of MRSA by monocytes, our results could be useful as a starting point for future investigations about phagocytic functions against strains with different resistance phenotypes.

We conclude that, under the present experimental conditions, complement system opsonins are fundamental for the efficient ingestion of these three bacterial strains by human neutrophils and monocytes. Although the mortality of the MRSA strain was only lower than that of the sensitive strains in the presence of neutrophils after opsonization with FS, we could not demonstrate any correlation of the multiresistance phenotype with MRSA survival in response to phagocyte effector mechanisms.

On this basis, our experimental protocol can be utilized as a model for evaluating these bacteria with primed leukocytes, mimicking a situation of staphylococcal infection. We believe that this study provides new approaches to the evaluation of MRSA pathogenicity and its interaction with the human phagocytic system.

## Acknowledgements

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