Antibiotic Resistance, Biofilm Formation, and Intracellular Survival As Possible Determinants of Persistent or Recurrent Infections by *Staphylococcus aureus* in a Vietnamese Tertiary Hospital: Focus on Bacterial Response to Moxifloxacin

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Resistance is notoriously high in Asia but may not entirely explain therapeutic failures. Specific modes of bacterial life, such as biofilm or intracellular survival, may also contribute to the persistent and/or recurrent character of infections. Most *Staphylococcus aureus* isolates form biofilm and many survive and even thrive intracellularly. We collected 36 nonduplicate *S. aureus* isolates (including 18 methicillin-resistant *S. aureus*) from patients with clinical evidence of persistent or recurrent infections in a large tertiary Vietnamese hospital. We examined their antibiotic resistance profile (minimal inhibitory concentration determination) and clonal relatedness (*spa* and *agr* typing, pulsed field gel electrophoresis profiles). We then assessed the activity of moxifloxacin in both biofilms and infected phagocytes (moxifloxacin previously proved to be one of the most active antibiotics against reference strains in these models). *spa*-types t189 and t437 and *agr* group I were the most frequent. Among the 36 isolates, 30 were multidrug resistant but 30 were recovered from patients having received an active drug. All tested isolates produced biofilm and survived inside phagocytes. At its human C_{max}, moxifloxacin was inactive on biofilms made by moxifloxacin-susceptible as well as moxifloxacin-resistant isolates. It caused only a modest intracellular colony-forming unit decrease against moxifloxacin-susceptible isolates and was inactive against those resistant to moxifloxacin. While our data confirm for this collection the high resistance levels and prevalence of endemic *spa*- or *agr*- types in Asia, they show that tolerance in both biofilm and phagocytes are correlated and markedly limit moxifloxacin activity, which goes in line with the suggested role of these modes of life in persistence or recurrence of infections.

Keywords: *Staphylococcus aureus*, resistance, biofilm, intracellular infection

Introduction

BACTERIAL RESISTANCE IS notoriously high in Asia, related to wide and largely inappropriate use of antibiotics,[1] as recently shown in Vietnam.[2] Resistance, which creates difficulties for health care professionals for selecting appropriate treatments, is therefore thought to be the main contributor to the persistent and/or recurrent character of many infections. Other mechanisms, however, may also explain this type of clinical and/or microbiological failure, such as biofilm formation and/or occurrence of intracellular

niches, two situations where antibiotic activity is markedly hampered toward isolates categorized as susceptible by conventional testing.[3]

In this study, we focused on *Staphylococcus aureus* isolates collected in one of the largest tertiary hospitals in Vietnam, with patients originating from all the Northern part of the country. We specifically selected isolates obtained from patients with clinical evidence of either persistent infections (*i.e.*, infections that did not resolve after 5 days of treatment by antibiotics to which the initial isolates were reported as susceptible) or recurrent infections (*i.e.*, infections that

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reactivated a few days after improvement of the patient's conditions and subsequent discontinuation of the initial antibiotic). Our aims were (i) to determine the capacity of these isolates to form biofilm *in vitro* and to survive in human monocytes and (ii) to measure their response to an active antibiotic in these two types of models compared with a reference strain. To this effect, we selected the antibiotic moxifloxacin, for its previously demonstrated high activity in these models.[4,5]

Materials and Methods

Bacterial strains and characterization

Nonduplicate *S. aureus* (*n* = 36) isolates were prospectively collected between July and December 2015 from patients hospitalized at the Bach Mai Hospital, Hanoi, Vietnam, from the two wards in which the majority of

staphylococcal infections diagnosed in this hospital are encountered (Table 1). These patients showed clinically and microbiologically documented persistent and/or recurrent infections (Table 1) (Ethics Committee approval from the Hanoi University of Pharmacy: 1102/QD-DHN). They were isolated on Mannitol Salt Phenol Red agar and identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Isolates were routinely grown on Tryptic Soy Agar (TSA) plates. Their growth rate was determined by following the increase in optical density at 620 nm (OD_{620nm}) over incubation time in cation-adjusted Mueller Hinton Broth (CA-MHB), using a starting inoculum at a $OD_{620nm} = 0.005$.

Minimal inhibitory concentrations (MICs) were measured by microdilution in CA-MHB following Clinical and Laboratory Standards Institute recommendations, with susceptibility assessed according to European Committee on

TABLE 1. DESCRIPTION AND TYPING OF CLINICAL ISOLATES

Strain number	Origin[a]	Unit	mecA[b]	agr[c]	spa-type[d]	Lineage ST[e]	Lineage CC[e]	PFGE	Profile[f]
69613	Pus (Abscess)	Urology	−	I	t034	ST398	CC398	nt	1a
30461	Catheter	Urology	−	I	t437	ST59, ST338	CC59	XJ	2a
19255	Blood	Urology	−	I	t437	ST59, ST338	CC59	XJ	2a
30462	Catheter	Urology	−	I	t2883	ST188	CC1	WX	3a
20975	Catheter	Urology	−	I	t189	ST188	CC1	WX	3b
69781	Pus	Urology	−	I	t189	ST188	CC1	WX	3b
30549	Catheter	Urology	−	I	t437	ST59, ST338	CC59	XJ	2a
30566	Catheter	Urology	−	I	t189	ST188	CC1	WX	3b
17883	Blood	Urology	−	I	t18690			XL	4
34427	Pus (tophi)	Rheumatology	−	I	t189	ST188	CC1	WX	3b
34467	Articulation	Rheumatology	−	I	t034	ST398	CC398	nt	1a
13890	Blood	Rheumatology	−	I	t189	ST188	CC1	WX	3b
14305	Blood	Rheumatology	−	IV	t159	ST121	CC121	N	5
69783	Articulation	Rheumatology	−	I	t304	ST6, ST8	CC8	XM	6
35994	Articulation	Rheumatology	−	I	t189	ST188	CC1	WX	3b
36606	Abscess	Rheumatology	−	IV	t159	ST121	CC121	N	5
30342	Catheter	Urology	−	I	t189	ST188	CC1	WX	3b
34791	Pus (tophi)	Rheumatology	−	I	t189	ST188	CC1	WX	3b
30337	Catheter	Urology	+	I	t437	ST59, ST338	CC59	XJ	2a
69474	Catheter	Urology	+	I	t437	ST59, ST338	CC59	XJ	2a
69493	Pus	Urology	+	I	t437	ST59, ST338	CC59	XJ	2a
69486	Pus	Urology	+	I	t437	ST59, ST338	CC59	XJ	2a
69544	Pus (catheter)	Urology	+	I	t437	ST59, ST338	CC59	XJ	2a
69687	Pus (catheter)	Urology	+	I	t437	ST59, ST338	CC59	XJ	2a
69700	Pus (catheter)	Urology	+	I	t437	ST59, ST338	CC59	XJ	2a
22714	Blood	Urology	+	I	t437	ST59, ST338	CC59	XK	2b
25619	Blood	Urology	+	I	t189	ST188	CC1	WX	3b
30609	Catheter	Urology	+	I	t189	ST188	CC1	WX	3b
69505	Pus (tophi)	Rheumatology	+	I	t437	ST59, ST338	CC59	XK	2b
34613	Abscess	Rheumatology	+	I	t437	ST59, ST338	CC59	XJ	2a
34724	Abscess	Rheumatology	+	I	t437	ST59, ST338	CC59	XJ	2a
69519	Pus (Abscess)	Rheumatology	+	I	t189	ST188	CC1	WX	3b
36271	Pus	Rheumatology	+	I	t437	ST59, ST338	CC59	XJ	2a
69867	Pus (tophi)	Rheumatology	+	I	t1250	ST398	CC398	nt	1b
36253	Abscess	Rheumatology	+	I	t189	ST188	CC1	WX	3b
69915	Pus tophi	Rheumatology	+	I	t189	ST188	CC1	WX	3b

[a]As reported from medical files.
[b]Determined by PCR; all MSSA strains were *mecA* and *mecC* negative.
[c]Accessory gene regulator (*agr*) operon variable region.
[d]*Staphylococcus* protein A gene typing.
[e]Based on Ridom *spa* server database and literature.
[f]To facilitate the comparison between isolates, those with an identical profile were annotated with the same number (a and b correspond to similar but not identical profiles).
PFGE, pulsed-field gel electrophoresis; nt, nontypable; MSSA, methicillin-susceptible *Staphylococcus aureus*.

Antimicrobial Susceptibility Testing (EUCAST) interpretive criteria (version 9.0; 2019). Their methicillin-resistant *S. aureus* (MRSA) character was determined by PCR of *mecA* and *mecC* genes.[6] Molecular typing included determination of the accessory gene regulator (*agr*) group par multiplex PCR, sequencing of the *Staphylococcus* protein A (*spa*) gene and Pulsed-Field Gel Electrophoresis (PFGE) and was performed exactly as previously published.[7,8] Multiple isolates from single patients were considered only if showing different *spa*-type/PFGE profile and/or antibiotic susceptibilities.

Biofilm formation, colony-forming units counting, and evaluation of moxifloxacin activity

Biofilms were grown in 96-well plates, using an initial inoculum at an OD_{620nm} of 0.005 in Tryptic Soy Broth supplemented with 1% glucose and 2% NaCl (TGN[5]) where T stands for Tryptic, G for glucose, and N for NaCl. After 24 hr incubation at 37°C, biofilms were gently washed twice with sterile phosphate-buffered saline (PBS), collected in sterile water, and sonicated 5 min using a sonication bath to insure the liberation of bacteria from the matrix. After appropriate dilution in PBS, aliquots were spread on TSA and colony-forming units (CFUs) were counted after overnight incubation at 37°C. To measure moxifloxacin activity, 24-hr biofilms were reincubated during 24 hr in the presence of moxifloxacin or in control medium and then treated as described above for CFUs enumeration.

Intracellular infection and evaluation of antibiotic activity

Infection of THP-1 human monocytes was performed following a previously established protocol.[4] In brief, bacteria opsonized with human serum were incubated at 37°C for 1 hr at an inoculum of four bacteria per cell to allow phagocytosis. After removal of the medium, infected cells were incubated at 37°C for 45 min with gentamicin at $100 \times MIC$ to eliminate extracellular bacteria, and thereafter washed twice with PBS at room temperature to eliminate gentamicin (this necessary step limited the study to isolates with gentamicin MIC ≤8 mg/L). They were then incubated at 37°C for 24 hr with moxifloxacin, washed with PBS, and collected in sterile water. CFUs were enumerated on TSA plates as described above, and proteins assayed by the Folin–Ciocalteu method. Results were expressed as the change in the number of CFUs per mg of cell protein from the post-phagocytosis inoculum.

Results

Characterization of the isolates

No major difference in the growth rate could be evidenced between the 36 isolates collected (Supplementary Fig. S1). Out of them, 18 were MRSA (*mecA* positive), whereas 2 were borderline oxacillin-resistant *S. aureus/* modified *S. aureus* (methicillin resistance but lack of *mecA* and *mecC* genes). The accessory gene regulator (*agr*) group I was largely predominant in this collection, with only two isolates showing a group IV profile. Most of the isolates belonged to *spa*-types t189 (*n* = 13; 5 MRSA) and t437 (*n* = 15; 12 MRSA), associated to clones ST188 and CC59,

respectively. The remaining isolates (*n* = 8) were spread over six other *spa*-types. Most of the isolates with a same *spa*-type also shared the same PFGE profile as well as the same *agr* group (Table 1).

The MICs of 26 antibiotics were determined against these isolates (see Table 2 for all MIC distributions and Table 3 for MIC values of representative antibiotics). No isolate was resistant to vancomycin, linezolid, or fusidic acid. Resistance rates to other antibiotic classes were 8% (tetracyclines), 11% (rifampicin; among rifampicin-resistant isolates, there was only one MRSA), 39% (fluoroquinolones), 44% (aminoglycosides), 72% (chloramphenicol), and 81% (macrolides and lincosamides).

According to the definition from Magiorakos *et al.*[9] and using EUCAST R (resistant) breakpoints, 30/36 (83%) isolates could be categorized as multidrug resistant. Among them, 12 belong to the *spa*-type t189 and 13 to the *spa*-type t437, which corresponds to 92% of t189 and 87% of t437 isolates, respectively. Among β-lactam-resistant isolates, 4 were coresistant to 2 other antibiotic classes, 12 to 3 other classes, 2 to 4 other classes, 4 to 5 other classes, 7 to 6 other classes, and 3 to 7 other classes. Coresistance to macrolides, lincosamides, fluoroquinolones, gentamicin, and chloramphenicol was observed in 8 strains, among which were 5 methicillin-susceptible *S. aureus* (MSSA). Isolates resistant to ≥5 classes belonged mainly to *spa*-type t189 (11/13) and less frequently to *spa*-type t437 (2/15). Of interest, all t189 isolates were moxifloxacin resistant, whereas all t437 isolates were moxifloxacin susceptible. Likewise, 12/13 t189 were gentamicin resistant, whereas 14/15 t437 were gentamicin susceptible.

Quite importantly, and in spite of these high levels of resistance, most of these isolates (30/36) had actually been recovered from patients who received at least one active antibiotic (MIC less than or equal than the EUCAST "R" breakpoint) during their initial treatment (Table 3). This strongly suggested that resistance was not the only culprit for microbiological failure. We therefore tested the capacity of all isolates to form biofilms as well as to survive intracellularly. To compare the impact of these two modes of life on antibiotic activity against these clinical isolates, we selected moxifloxacin as a study drug. This fluoroquinolone, indeed, proved to be one of the most active antibiotics in these two models, based on previous work from our laboratory,[4,5] a necessary condition to allow for accurate measurement of a potential decrease of activity against clinical isolates compared with reference strains.

Biofilm formation and intracellular survival

These features were evaluated against part of the collection, including both moxifloxacin-susceptible and -resistant isolates. When looking at intracellular survival, only isolates with gentamicin MIC ≤8 mg/L could be considered. Biofilms were quantified here by recovering bacteria after sonication and counting the number of CFUs, which was more appropriate than looking at biofilm biomass to compare bacterial proliferation and antibiotic activity in biofilm versus those measured intracellularly. All tested isolates were much stronger biofilm producers than the reference strain ATCC25923, with a mean value of 7.5 versus 5.9 $\log_{10}$ CFU recovered from biofilms after 24 hr of growth.

TABLE 2. MINIMAL INHIBITORY CONCENTRATION DISTRIBUTION AND PERCENTAGE
OF RESISTANCE IN CLINICAL ISOLATES

Antibiotic	MIC_{50} (mg/L)	MIC_{90} (mg/L)	EUCAST breakpoints (mg/L) S≤/R>	Nb (%) resistant strains
Penicillin G (PEN)	16	>16	0.125/0.125	33 (92%)[a]
Amoxicillin (AMX)	16	>32	na	na
Amoxicillin/clavulanic acid (AMC)	2	16	na	na
Oxacillin (OXA)	0.5	8	na	na
Cefoxitine (FOX)	8	32	na	50% MRSA[b]
Cefuroxime (CXM)	2	8	na	na
Cefoperazone/sulbactam (CFP/SUL)	4	8	na	na
Ceftriaxone (CRO)	4	16	na	na
Imipenem (IMI)	0.032	0.064	na	na
Meropenem (MEM)	0.25	2	na	na
Vancomycin (VAN)	1	1	2/2	0
Linezolid (LZD)	2	2	4/4	0
Tedizolid (TDZ)	0.25	0.25	0.5/0.5	0
Fusidic acid (FUS)	0.064	0.125	1/1	0
Erythromycin (ERY)	>256	>256	1/2	29 (81)
Azithromycin (AZM)	>256	>256	1/2	29 (81)
Solithromycin (SOL)	0.5	>256	na	
Clindamycin (CLI)	>256	>256	0.25/0.5	29 (81)
Chloramphenicol (CHL)	0.5	32	8/8	26 (72)
Doxycycline (DOX)	2	4	1/2	9 (25)
Gentamicin (GEN)	0.5	32	1/1	
Moxifloxacin (MXF)	0.064	2	0.25/0.25	14 (39)
Levofloxacin (LVX)	0.25	8	1/1	14 (39)
Ciprofloxacin (CIP)	0.5	32	1/1	14 (39)
Rifampicin (RIF)	0.008	>0.5	0.06/0.5	4 (11)
Cotrimoxazole (STX)	0.125	>16	2/4	10 (28)

[a]Susceptible strains are not β-lactamase producers.
[b]Strains with MICs >4 mg/L are MRSA due to expression of *mecA* (see Table 1).
MIC, minimal inhibitory concentration; na, not applicable; MRSA, methicillin-resistant *Staphylococcus aureus*.

Conversely, they could not be distinguished from the reference strain with respect to phagocytosis and intracellular growth in THP-1 monocytes (Fig. 1, upper panels). No difference could be evidenced among *spa*-types regarding these properties (Supplementary Fig. S2).

Activity of moxifloxacin against biofilms and intracellular bacteria

We then examined the activity of moxifloxacin at a concentration corresponding to its average human C_{max} (4 mg/L; total concentration) and after 24 hr of incubation in these two models (Fig. 1, lower panels). Isolates were divided in two groups according to the moxifloxacin MIC (namely [i] 0.03–0.06 mg/L and [ii] 1–2 mg/L), corresponding to isolates categorized as susceptible or resistant (EUCAST interpretive criteria). In biofilms, moxifloxacin caused an ∼1.5 log_{10} CFU decrease against the reference strain ATCC25923 but only a mean decrease of about 0.3 log_{10} CFU for the moxifloxacin-susceptible isolates, and no decrease for those resistant to moxifloxacin. Intracellularly, moxifloxacin caused a reduction of the intracellular inoculum of about 2 log_{10} CFU (from the initial postphagocytosis inoculum) for ATCC25923, but variable reductions (0.5–2.5 log_{10} CFU decreases) for the moxifloxacin-susceptible isolates, and no reduction of CFU (bacteriostatic effect only) for the moxifloxacin-resistant isolates. Figure 1F shows that there was a significant correlation between the changes in CFU induced by moxifloxacin in these two models.

Discussion

In this collection of isolates obtained from patients with persisting or recurrent infections in Vietnam, we noted high resistance levels (as anticipated and consistent with other studies[2,10,11]), but these were unlikely to be responsible for the microbiological failures we described since patients were initially treated with antibiotics to which the isolates were considered susceptible. Rather, we show here that biofilm formation and, to a lesser extent, intracellular hiding allow for protection against an active antibiotic and could be a reason for persistence and/or recurrence.

Although our study was not designed or powered as an epidemiological survey, this collection is probably representative of the current epidemiology in this part of the world, as it shows *spa*-types that are widely distributed in Asia,[12] and more specifically in China,[13] with *spa*-type t189 being more common among MSSA and *spa*-type t437, among MRSA, as previously described.[14] ST59-t437-*agr* group I, which was recently reported as the most frequent genotype in MRSA isolated from hospitalized children in China,[15] was also predominant in this study. More globally, *agr* group I is also highly prevalent in MSSA in China,[16] which is also what we observed here. We also found similar proportions of MRSA and of resistance as in other surveys.

Specifically, we noticed worrying resistance levels not only to β-lactams but also to macrolides and lincosamides, chloramphenicol, and to a lower extent, aminoglycosides and fluoroquinolones. Data for Vietnam remain limited.

TABLE 3. MINIMAL INHIBITORY CONCENTRATIONS FOR REPRESENTATIVE ANTIBIOTICS OF REFERENCE STRAINS AND CLINICAL ISOLATES WITH INDICATION OF THE ANTIBIOTICS RECEIVED BY THE CORRESPONDING PATIENT

Strain number	Antibiotics received by patients[a]	Phenotype of resistance to β-lactams	OXA	FOX	VAN	LZD	FUS	AZM	CLI	CHL	DOX	GEN	MXF	RIF	SXT 1/19
ATCC25923		MSSA	0.125	4	1	2	0.125	0.5	0.064	4	0.25	0.25	0.032	0.008	0.125
ATCC33591		MRSA	>16	>32	1	1	0.125	>256	>256	**32**	>16	0.5	0.064	0.004	1
69613	None	MSSA	0.25	2	1	2	0.064	≥256	>256	8	0.5	**32**	0.064	0.008	0.064
30461	CFP/SUL	MSSA	0.25	2	1	2	0.125	>256	>256	**64**	**2**	0.25	0.064	0.008	0.125
19255	CFP/SUL	MSSA	0.25	4	1	2	0.064	>256	>256	**64**	**2**	0.25	0.064	0.008	0.25
30462	None	MSSA	0.125	4	2	4	0.125	>256	>256	**64**	**8**	**16**	**2**	0.016	0.25
20975	CFP/SUL	MSSA	0.125	4	1	2	0.064	1	0.125	**16**	0.25	0.5	**2**	0.008	0.064
69781	CFP/SUL	MSSA	0.25	4	1	2	0.064	>256	>256	**32**	0.25	**32**	**2**	**>0.5**	**>16**
30549	CFP/SUL	MSSA	0.125	4	1	2	0.25	2	0.125	64	4	0.25	0.032	0.008	0.064
30566	CFP/SUL	MSSA	0.5	4	1	2	0.064	>256	**256**	**32**	0.5	**32**	**2**	**>0.5**	**>16**
17883	LVX	MSSA	0.125	2	1	2	0.125	0.5	0.125	**16**	0.125	0.5	0.032	0.008	0.125
34427	CRO, **CLI, CIP,** VAN	MSSA	0.25	4	1	2	0.064	>256	>256	8	**4**	**32**	**2**	0.008	2
34467	**CFP, CLI,** MXF, CRO, MEM, VAN	MSSA	0.5	4	1	2	0.064	≥256	>256	**16**	0.5	0.5	0.064	0.008	0.064
13890	VAN	MSSA	0.125	4	2	2	0.125	>256	**4**	**64**	0.25	**8**	**1**	0.008	0.125
14305	LVX, VAN	MSSA	0.125	4	1	2	0.125	1	0.125	4	0.25	0.5	0.032	**>0.5**	0.064
69783	MEM, MXF, VAN, CRO, LVX	MSSA	0.125	4	1	2	0.125	1	0.125	**64**	0.5	**8**	0.032	0.004	0.125
35994	VAN, IMI	MSSA	0.25	4	1	2	0.125	>256	>256	**16**	0.25	**32**	**2**	0.008	**>16**
36606	CLI, CFP	MSSA	0.25	4	1	2	0.125	1	0.125	8	0.25	0.5	0.032	0.008	0.125
30342	**CRO**	BORSA or MODSA[a]	2	**8**	1	2	0.125	>256	>256	**16**	0.5	**32**	**2**	**>0.5**	**>16**
34791	**CLI, CIP, IMI**	BORSA or MODSA	0.25	**8**	1	2	0.064	>256	>256	**64**	0.25	**32**	**2**	0.008	**16**
30337	**CFP,** MXF	MRSA	1	**16**	1	2	0.125	>256	>256	**64**	**2**	0.5	0.064	0.016	0.064
69474	**CLI,** MXF	MRSA	1	**8**	1	2	0.064	>256	>256	**64**	**16**	0.25	0.064	0.008	0.064
69493	MXF, VAN	MRSA	2	**8**	1	2	0.064	>256	>256	**64**	**2**	0.25	0.064	0.016	0.125
69486	**CLI, MER**	MRSA	1	**8**	1	2	0.25	2	0.125	8	**2**	0.25	0.064	0.008	0.125
69544	**CFP,** MXF	MRSA	4	**>32**	1	2	0.064	>256	>256	8	**2**	0.25	0.032	0.008	0.25
69687	**CFP/SUL**	MRSA	8	**32**	1	4	0.125	>256	>256	**64**	**2**	0.25	**0.25**	0.008	0.125
69700	MXF	MRSA	2	**16**	1	2	0.125	>256	>256	**64**	**2**	1	0.064	0.016	0.064
22714	LVX, VAN	MRSA	8	**32**	1	2	0.064	>256	>256	**64**	**4**	**>32**	0.064	0.008	0.064
25619	**IMI**	MRSA	8	**32**	1	2	0.064	>256	>256	8	**4**	**32**	**2**	0.004	**>16**
30609	**IMI**	MRSA	16	**>32**	1	2	0.064	>256	>256	8	**8**	**32**	**2**	0.004	**>16**
69505	**CFP,** CIP	MRSA	4	**16**	1	2	0.25	>256	>256	**64**	**2**	0.5	0.032	0.008	4
34613	CIP, **CFP,** MXF, VAN	MRSA	0.5	**8**	1	2	0.064	>256	>256	**>64**	**2**	0.5	0.032	0.008	0.064
34724	CIP, **CFP,** MXF, VAN	MRSA	1	**16**	1	2	0.064	>256	>256	**64**	0.5	0.25	0.064	0.008	0.064
69519	VAN, **MEM, IMI**	MRSA	4	**>32**	2	2	0.125	>256	>256	8	0.25	**32**	**1**	0.008	**>16**
36271	**CLI,** MXF	MRSA	1	**8**	1	4	0.125	>256	>256	**128**	**2**	0.25	0.032	0.004	0.064
69867	VAN, MXF	MRSA	4	**16**	1	1	0.064	**256**	>256	**32**	**4**	0.5	0.064	0.125	0.125
36253	**CFP, CIP,** VAN	MRSA	4	**16**	1	2	0.064	>256	>256	**16**	0.25	**32**	**2**	0.008	**>16**
69915	**CFP, CLI**	MRSA	4	**16**	1	2	0.064	>256	>256	8	**4**	**32**	**1**	0.008	**>16**

See Table 2 for antibiotic acronym definitions.
Values in bold are higher than EUCAST R breakpoints (see Table 2).
[a]As reported in medical files.
BORSA, borderline oxacillin-resistant *S. aureus*; MODSA, or modified *S. aureus*; EUCAST, European Committee on Antimicrobial Susceptibility Testing.
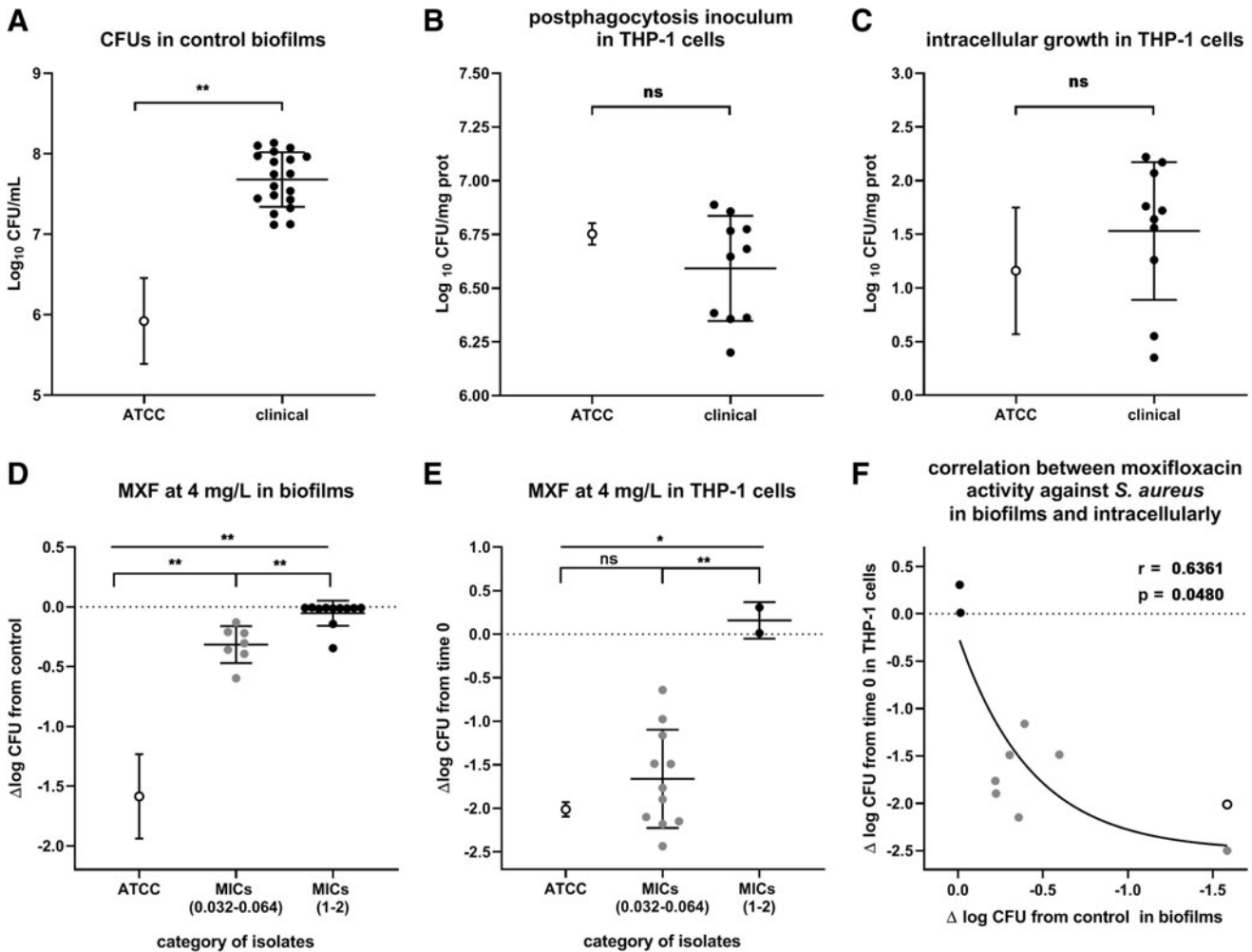
FIG. 1. *Upper panel*: biofilm formation and intracellular infection of THP-1 monocytes by the reference strain ATCC 25923 and selected clinical isolates. **(A)** CFU counts in 24-hr biofilms from ATCC25923 and 19 randomly selected clinical isolates. **(B)** Postphagocytosis inoculum in THP-1 cells exposed during 1 hr to serum opsonized *Staphylococcus aureus* (ATCC 25923 and eight isolates), after elimination of extracellular bacteria using $100 \times$ MIC gentamicin (note that the number of isolates was limited to those with low [≤ 8 mg/L] gentamicin MICs). **(C)** Intracellular growth of the same isolates after 24 hr of incubation in the presence of $1 \times$ MIC gentamicin (to avoid extracellular contamination). *Lower panel*: Activity of moxifloxacin against biofilms or intracellular bacteria. **(D)** Reduction in CFU after 24 hr of incubation of 24 hr biofilms with moxifloxacin at its human C_{max} (4 mg/L). **(E)** Reduction in CFU from the initial postphagocytosis after 24 hr of incubation of infected THP-1 cells with moxifloxacin at its human C_{max} (4 mg/L). In both panels, susceptible (*gray dots*) and resistant (*black dots*) isolates were analyzed apart from one another. **(F)** Correlation between the activity of moxifloxacin against biofilm and intracellular bacteria. Statistical analysis: analysis of variance **(D–E)** or *t*-test **(A–C)** with Welsh's correction. $*p < 0.05$; $**p < 0.01$. ns, not significant; CFU, colony-forming unit; MIC, minimal inhibitory concentration.

Thirty-seven percent of staphylococci isolated from bloodstream infections in a tertiary infectious diseases hospital in North Vietnam have been reported as MRSA.[10] A similar proportion (40%) was found among isolates causing infective endocarditis in tertiary care hospitals of South Vietnam.[11] A recently published large-scale study on 2,039 isolates collected during the 2012–2013 period in 16 hospitals across the whole country concluded that 69% of them were MRSA, with 75% being resistant to macrolides, 36–39% to fluoroquinolones, 38% to gentamicin, 23% to cotrimoxazole, and 3% to vancomycin.[2] This is in very good agreement with our own data, except that we did not detect any vancomycin resistance. This could be due to the too small number of isolates in our study and their collection

from a single institution (the study referred to above[2] did not specified whether resistance to vancomycin was concentrated or not in a single or a few hospitals). Our observations add to this large study direct information about the resistance profile of MRSA to additional drugs. It further demonstrates the multidrug-resistant character of most of these strains, especially those belonging to *spa*-types t189 and t437, which will make the treatment of the corresponding infections quite difficult. However, since all clinical isolates were collected in a single hospital, we cannot generalize our conclusion to the country or to the region where the situation still remains uncertain.

Biofilm formation is a frequently encountered feature for clinical isolates of *S. aureus*. In the present study, there

was a marked difference between the reference strain ATCC25923 and the collected clinical isolates, all of which were from persisting or recurrent infections. A previous study suggests that strains isolated from device-related infections or skin infections could be higher biofilm producers[17] than those collected from other infection types, but this was not confirmed in this study. Another study suggests that isolates from recurrent prosthetic joint infections form more biofilms and persist longer in bone cells than initially collected isolates,[3] but, again, this was not seen here since our strains were isolated from different fluids and/or sites of infections. Thus, biofilm formation seems to be a hallmark of isolates originating from persistent infections. Conversely, there was no difference in the degree of phagocytosis and intracellular survival between the reference strain ATCC25923 and the clinical isolates. Thus, intracellular hiding, by itself, does not seem critical for persistence of the infection.

A key and probably most important observation made in this work is that bacteria encased in biofilms, and, to some extent, those having taken refuge intracellularly, were less susceptible to the antibacterial activity of moxifloxacin. Yet, this fluoroquinolone is among the best antibiotics we found so far to act against bacteria in biofilms[5,18] or in phagocytes.[4,19] To our knowledge, the present study is one of the first to examine concomitantly the activity of this antibiotic against the same clinical isolates tested in these two models. Noteworthy, the loss of activity we observe is marginally affected by the susceptible or resistant character of the strain in biofilms, while the difference reaches significance intracellularly.

The concentration used, which corresponds to the average human C_{max}, is, however, 6–7 $\log_2$ dilutions higher than the MIC of susceptible isolates and still 1–2 $\log_2$ dilutions higher than that of the resistant ones. For biofilms, our data therefore suggest that bioavailability at the site of infection is probably a key determinant for modulating the expression of activity in biofilms, in line with the concept that minimal biofilm eradicating concentrations are usually far above MIC values.[20] Conversely, for intracellular bacteria, we previously showed that the level of cellular accumulation of fluoroquinolones is not predictive of their intracellular activity,[21] rather indicating that the cellular environment is probably the main factor modulating bacterial responsiveness.[22]

Thus, we conclude that, beyond resistance, both the capacity of *S. aureus* to maintain itself in biofilms and to survive intracellularly even in the presence of an active antibiotic strongly contribute to treatment failure. Although there was a correlation between the losses of activity of moxifloxacin seen in these two models when considering individual isolates, it must be noted that moxifloxacin was systematically less active in biofilm than intracellularly. Methods for assessing biofilm formation and intracellular survival of clinical isolates could be implemented in clinical microbiology laboratories if using high-throughput assays[23] to help predicting the risk factor of persistence and/or recurrence independently of resistance.

Acknowledgments

Disclosure Statement

Funding Information

Supplementary Material

Supplementary Figure S1
Supplementary Figure S2

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MDR-2019-0282 - SUPPLEMENTARY MATERIAL

Antibiotic resistance, biofilm formation, and intracellular survival as possible determinants of persistent or recurrent infections by *Staphylococcus aureus* in a Vietnamese tertiary hospital. Focus on bacterial response to moxifloxacin.

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Figure S1 :

Growth rate of clinical isolates *vs.* ATCC 25923 in MHB-CA. The $OD_{620\,nm}$ was followed over time in culture initiated at an $OD_{620\,nm}$ = 0.005. No major difference was observed among isolates, which grew at a slightly higher rate than ATCC 25923.
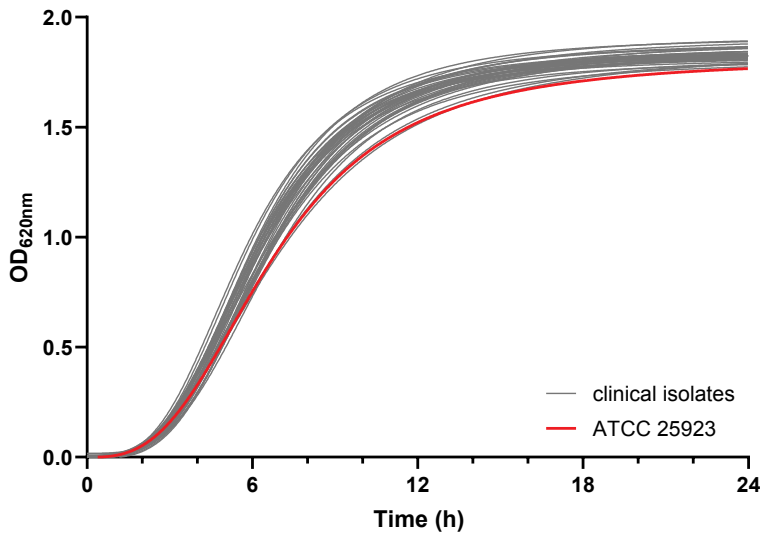
Figure S2: biofilm formation and intracellular infection of THP-1 monocytes by the reference strain ATCC 25923 and selected clinical isolates categorized according to their *spa*-type (t437, t189 or other). A: CFU counts in 24 h biofilms. B: post-phagocytosis inoculum in THP-1 cells exposed during 1 h to serum opsonized *S. aureus* (ATCC 25923 and 10 isolates), after elimination of extracellular bacteria using 100 x MIC gentamicin. C: intracellular growth of the same isolates after 24 h of incubation in the presence of 1 x MIC gentamicin (to avoid extracellular contamination).