

# CARBAPENEM RESISTANCE IN *ACINETOBACTER BAUMANNII* IS ASSOCIATED WITH ENHANCED SURVIVAL ON HOSPITAL FABRICS

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The success of *Acinetobacter baumannii* as an emerging organism is probably linked to its high resistance to adverse environmental conditions. This study was conducted to analyze the association between some factors that may favor the dissemination of *A. baumannii* clinical isolates. A total of 47 clinical strains of *A. baumannii* were evaluated to carbapenem, the ability to produce biofilm, the susceptibility to some antiseptics, and the survival time on cotton fabrics. Most of the isolates were resistant to carbapenem (72.3%), produced biofilm (83%), and survived more than 7 (51%) days on fabrics. A significant association between decreased susceptibility to antiseptics containing chlorhexidine or triclosan and carbapenem resistance and survival on fabrics could be observed. The resistance to carbapenem was significantly associated with survival on fabric, but not with the ability to form biofilm. The survival of the isolates on fabric was not associated with the ability to produce biofilms. Characteristics, such as resistance to antibiotics, ability to form biofilm, and survival on dry surfaces, probably contribute to the proliferation of this organism when selected in the hospital environment and can partly explain its success as responsible for nosocomial infection.

**Keywords:** *Acinetobacter baumannii*, antiseptics, biofilm, antimicrobial resistance, survival

## Introduction

*Acinetobacter baumannii* is an opportunistic pathogen known for its ability to survive in hospital environments, particularly under humid conditions where it can form biofilms, thereby increasing its resistance to a wide variety of antibiotics [1, 2]. The majority of infections caused by *A. baumannii* are hospital-acquired, and the transmission occurs mainly through the hands of healthcare workers, contaminated

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clinical material and clinical environments, and also possibly through the air [3, 4]. The prolonged environmental survival of this pathogen and its high capacity to acquire resistance to multiple antibiotics makes its eradication difficult once it has become endemic [5]. In recent years, the spread of carbapenem-resistant *Acinetobacter* has worsened the worldwide epidemiological situation related to this pathogen [6]. Since *Acinetobacter* was included in the European Antimicrobial Resistance Surveillance Network in 2012, more than half of the countries involved in the study showed an increase for the period 2012–2015 with regard to combined resistance to the three antimicrobial groups: fluoroquinolones, aminoglycosides, and carbapenems [5].

In light of its ability to survive in the environment, the disinfection of materials, surfaces, and hands is particularly important to prevent the spread of *Acinetobacter* spp. and other organisms responsible for nosocomial infections. Unlike resistance to antibiotics, resistance to commonly used disinfectants appears to have no active role in the spread of these organisms [7]. In general, all disinfectants inhibit the growth of all organisms at the concentrations and contact times recommended by the manufacturers; however, minor deviations from the recommended procedures could have a determinate role in nosocomial cross-transmission [8].

Some studies have linked the ability of bacteria to produce biofilms with their resistance to harsh environmental conditions and difficulties in their eradication from hospital environments [1, 9]. Bacteria within biofilms show greater resistance to disinfectants, and several studies have established a significant correlation between biofilm formation and resistance to multiple drugs [10, 11]. The protection exerted by the extracellular matrix, phenotypic changes to the cells in biofilms, and other mechanisms still to be fully elucidated confers increased tolerance by the cells growing in biofilms to antibiotics and disinfectants [10, 12].

To better understand the characteristics of *A. baumannii* that contribute to its ability to cause nosocomial infections, this study aimed to determine the correlations between parameters of long-term survival on cotton fabrics, the ability to form biofilms, and susceptibility to antibiotics and hand antiseptics in clinical isolates.

## Materials and Methods

### *Bacterial strains and antibiotic resistance*

Non-repetitive clinical isolates of *A. baumannii* obtained from patients with hospital-acquired infections at the University Hospital in Malaga (Spain) were

used in this study. A 24-h culture in trypticase soy broth (TSB; Panreac, Spain) for each isolate was centrifuged and the sediment resuspended in phosphate-buffered saline (PBS; pH 7.4) to obtain a bacterial suspension adjusted to  $0.12 \pm 0.02$  at OD<sub>630</sub>. This bacterial suspension was 10-fold diluted in PBS to obtain a standard culture (SC) and maintained at 4 °C until use (<2 h). A series of 10-fold dilutions were prepared from the SC ( $\sim 10^7$  CFU/ml) and 100 µl aliquots were then concomitantly sprayed onto separate CHROMagar® *Acinetobacter* medium (CRMA) plates and CRMA plates supplemented with CR102 (CHROMagar, Paris, France). Trypticase soy agar (TSA) plates were used as a control and to quantify the number of viable bacteria in each dilution. Plates were incubated overnight at 37 °C. CRMA is a rapid medium for the selection and identification of *Acinetobacter* spp. [13], and CR102 is an antimicrobial selective supplement that can be added to CRMA (CRMA-CR102) to detect carbapenem-resistant *Acinetobacter* [14]. When *A. baumannii* strains only grew in CRMA, or their mean growth (CFU/ml) in CRMA-CR102 plates was less than three standard deviations that of the mean CRMA growth, the strains were classified as non-resistant to carbapenem.

#### *Antiseptic susceptibility testing*

The antiseptics used in this study were as follows: 0.4% chlorhexidine (CHX) and 70% vol/vol n-propanol (CHX 0.4%, Albus, Spain), 0.2% mecetronium and 45% vol/vol 2-propanol, and 30% vol/vol 1-propanol (STR; Sterillium®, Lab Bode-Chemie GmbH, Germany), 0.5% triclosan, 15.76% vol/vol 2-propanol, and 49.9% vol/vol ethanol (DRX; Daromix®, Lab Collado, Spain), and 70% ethanol vol/vol as hydroalcoholic gel (ANG; Aniosgel®, Lab Anios, France). As a neutralizing agent (NA), a commercial preparation (Scharlau Chemie, Spain) containing 0.1% peptone, 0.1% L-histidine, 0.3% lecithin, 0.36% monopotassium phosphate, 0.72% disodium phosphate, 0.43% sodium chloride, and 3% polysorbate 80 diluted in distilled water was used. The bactericidal activities of the antiseptics were measured by triplicate using the dilution–neutralization method against bacteria in suspension as follows: 100 µl of a SC were added to tubes containing doubling-dilutions in distilled water of antiseptic (900 µl). Tubes without biocide were used as a control. After 1-min incubation at room temperature, 100 µl of the mixture were mixed with 900 µl of NA and maintained for 10 min. The bacterial count (in CFU/ml) was subsequently determined on TSA by serial dilutions in TSB. To determine the bactericidal activities of the antiseptics, the mean number of viable bacteria (in CFU/ml) before and after exposure to a disinfectant was expressed as the log<sub>10</sub> value, and the logarithmic reduction (LR) was calculated as described in the UNE-EN 1040 [15]. Antiseptics were considered bactericidal when  $LR \geq 5$  within

the chosen contact time. The higher dilutions of antiseptics in which  $LR \geq 5$  for each strain were designated as the minimum inhibitory concentration (MIC) dilution.

### *Biofilm formation*

An aliquot of 100  $\mu$ l of SC for each of the strains was transferred to the wells of a sterile polystyrene flat-bottomed 48-well microtiter plate (Iwaki Co., Japan) each containing 900  $\mu$ l of TSB, and was incubated at 37 °C. After 24 h of incubation, the culture medium was pipetted out of the wells and replaced with fresh TSB. After incubation for another 24 h, the wells were decanted and washed with PBS to remove any bacteria that had not adhered to the wells and then were allowed to dry (30 min). Next, 900  $\mu$ l of 99.8% methanol were added to each well to fix the biofilm for 15 min, and later decanted and allowed to dry for another 30 min. The biofilm was then stained with 900  $\mu$ l of 0.5% crystal violet (w/v) for 15 min, and once decanted again quantified at 560 nm after solubilization with 900  $\mu$ l of 95% ethanol. TSB without bacteria was used as a negative control. Five replicates were carried out for each strain and the results [optical densities (OD)] were averaged. Biofilm production was interpreted according to previously established criteria [9, 16]: the cut-off absorbance value (ODc) was considered as three standard deviations above the mean OD of the negative control. The *A. baumannii* strains were thus classified into four categories: none ( $OD \leq ODc$ ), weak ( $ODc < OD \leq 2 \times ODc$ ), moderate ( $2 \times ODc < OD \leq 4 \times ODc$ ), and strong biofilm producers ( $4 \times ODc < OD$ ).

### *Survival on hospital fabric*

*A. baumannii* survival was tested on fabric commonly used in our hospital (100% smooth cotton) as follows. Autoclaved swatches ( $\sim 1 \text{ cm}^2$ ) were placed in petri dishes and 100  $\mu$ l of a previously prepared SC ( $< 2 \text{ h}$ ) were smoothly deposited onto the surface of the fabric and incubated at 25 °C and 40%–60% humidity, as previously described [17]. Viable counts were then determined at 0 days ( $< 5 \text{ min}$  after being inoculated) and 1, 3, 7, 14, and 21 days until the colony counts on TSA plates were  $\leq 25$ . For viable counts, each swatch was vortexed for 60 s in 5 ml of sterile 0.2% Tween-80 in PBS (PBS-Tween) and 100  $\mu$ l aliquots were directly plated onto TSA plates (undiluted) or after appropriate dilution in sterile PBS. Three swatches were used separately for each count, and three 10-fold dilutions were made for each swatch. TSA plates were incubated at 37 °C for 24 h for colony counting, and each assay was repeated thrice. The survival of each strain on the fabric at each time point was calculated from the mean number of viable colonies for each replicate.

### Statistical analysis

The  $\chi^2$  test or Fisher's exact test was used to compare qualitative variables, as appropriate. Analysis of variance of the survival on fabric at each time point was performed with regard to antibiotic resistance, biofilm formation, or the antiseptic MIC dilution. Differences of  $p < 0.05$  were considered statistically significant.

## Results

A total of 47 *A. baumannii* clinical isolates were included in this study. A SC containing an inoculum of  $\sim 10^7$  CFU/ml (range:  $3 \times 10^6$ – $2.6 \times 10^7$  CFU/ml) was obtained for each strain immediately before being used in any of the trials included in this study. All of the 47 *A. baumannii* isolates (100%) grew on CRMA plates and the counts did not show significant differences with respect to the inoculums grown on control TSA plates. Of the 47 strains studied, 34 strains (72.3%) showed growth on the CRMA-102 plates and were defined as carbapenem-resistant *A. baumannii* (CRAB); the remaining 13 strains (27.7%) were defined as non-resistant to carbapenem (non-CRAB) (Table I).

Most of the isolates (83%) were able to form some degree of biofilm, whether this was classified as weak, moderate, or strong (Table I). Approximately 45% of the isolates (21 strains) were classified as strong biofilm producers. The distribution of carbapenem resistance among the different strains was similar to that of biofilm formation.

Table II shows the antiseptic susceptibility patterns of *A. baumannii* isolates after 1 min exposure to each dilution of the tested antiseptics. All strains were inhibited after 1 min exposure by dilutions in sterile distilled water of up to 1:4 for

**Table I.** Biofilm classification based on microtiter plate method and susceptibility to carbapenem according to a method of screening using CRMA-102

	Resistance to carbapenem		Total (%)
	CRAB	Non-CRAB	
No biofilm	6	2	8 (17.0)
Weak biofilm	8	4	12 (25.5)
Moderate biofilm	6	0	6 (12.8)
Strong biofilm	14	7	21 (44.7)
Total (%)	34 (72.3)	13 (27.7)	47 (100.0)

*Note:* CRAB: carbapenem-resistant *A. baumannii*; Non-CRAB: *A. baumannii* non-resistant to carbapenem; CRMA-102: CHROMagar® *Acinetobacter* medium supplemented with CR102 to identify resistance to carbapenem.

**Table II.** Bactericidal activity of antiseptics for hand against *A. baumannii* isolates

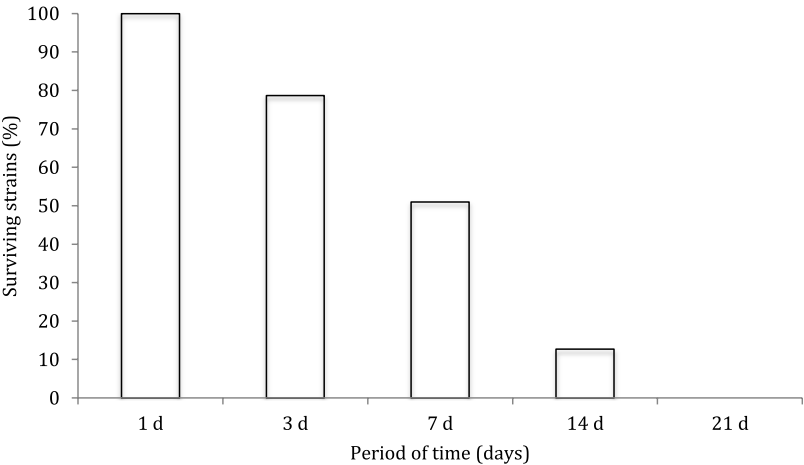
	Number of isolates according to MIC dilution obtained for each strain									RS	ANRS
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512		
ANG	0	0	18	25	4	0	0	0	0	18	29
CHX	0	0	0	15	32	0	0	0	0	15	32
DRX	0	0	0	0	0	1	14	14	18	15	32
STR	0	0	12	32	2	1	0	0	0	12	35

*Note:* MIC dilution: the higher dilutions of antiseptics in which LR $\geq$  5 for each strain; ANG: Aniosgel<sup>®</sup>; CHX: chlorhexidine 0.4%; DRX: Daromix<sup>®</sup>; STR: Sterillium<sup>®</sup>. RS: isolated showing reduced susceptibility to antiseptics (MIC dilution = 1:8 for ANG and STR, 1:16 for CHX or  $\leq$ 1:128 for DRX); ANRS: isolated with non-reduced susceptibility to antiseptics (MIC dilution:  $\geq$ 1:16 for ANG and STR, 1:32 for CHX or  $\geq$ 1:256 for DRX).

STR and ANG, up to 1:8 for CHX, and up to 1:32 for DRX. The antiseptic with the highest biocide activity at the lowest concentration was DRX.

Figure 1 shows the percentage of surviving *A. baumannii* strains on cotton fabrics under the test conditions described above. Approximately 50% of the strains survived for at least 1 week on the fabric samples, but only six strains (12.7%) survived up to 14 days and no strains grew after 21 days on the fabric.

To evaluate the association between antimicrobial resistance, susceptibility to antiseptics, the ability to form biofilm, and the persistence on cotton fabric samples, *A. baumannii* isolates were classified as follows: strains that produced no or weak biofilm were classified as scarce biofilm (SB) producers; strains that produced moderate or strong biofilm were classified as abundant biofilm (AB)



**Figure 1.** Survival of clinical isolates of *A. baumannii* on cotton fabrics

producers; strains susceptible only to the lowest dilutions tested with each antiseptic (Table II) were classified as isolates with reduced susceptibility (RS) to antiseptics, and strains susceptible to the highest dilutions tested were classified as isolates with non-RS to antiseptic (ANRS); finally, based on their survival on cotton fabrics, the isolates were classified as having either low persistence (survival <7 days, 17 strains, 36.2%) or more persistence (MP, survival  $\geq 7$  days, 30 strains, 63.8%).

The resistance of the strains to carbapenem was not associated with the degree of biofilm production, even when the ability to form biofilm was divided into the two groups, SB (weak or no biofilm, 20 strains, 42.5%) and AB (moderate and strong biofilm, 27 strains, 57.5%).

According to the susceptibility to each antiseptic, isolates with MIC dilution values  $\leq 8$  (ANG),  $\leq 16$  (CHX),  $\leq 128$  (DRX), or  $\leq 8$  (STR) were classified as RS for the respective antiseptic (Table II). Isolates with higher MIC dilution values were considered as ANRS for the corresponding antiseptic. RS to DRX and CHX was associated with resistance to carbapenem (DRX,  $p = 0.027$  and CHX,  $p = 0.002$ ). However, RS to STR or ANG was not associated with resistance to carbapenem. When comparing the MIC dilution values of the different antiseptics, only RS to CHX was associated with RS to DRX ( $p = 0.007$ ). Whereas, when comparing the susceptibility to antiseptics of the isolates with the ability to form biofilm, only RS to STR was associated ( $p = 0.036$ ) with a greater ability to produce biofilm (AB strains).

To compare the characteristics of survival on cotton fabric with resistance to carbapenem, higher survival (MP  $\geq 7$  days) of the isolates on fabric was associated ( $p = 0.017$ ) with resistance to carbapenem. However, the survival of the isolates on fabric was not associated with the ability to produce biofilm. When the survival on fabric and the susceptibility to antiseptics was compared for *Acinetobacter* strains, MP ( $\geq 7$  days) was significantly associated with RS to DRX ( $P = 0.002$ ) and to CHX ( $P = 0.037$ ), and was close to significance ( $p = 0.055$ ) with STR.

## Discussion

Several studies have investigated the rapid diffusion of antibiotic resistance in hospital environments as a consequence of the widespread use of antibiotics [18, 19], but no clear evidence has been found that the same thing happens with antiseptics and disinfectants [7, 20]. Unlike antibiotics, the use of disinfectants/antiseptics does not seem to have resulted in an increase in resistance, even after a prolonged period of use [20]. In this study, the antiseptics tested were effective at eliminating *Acinetobacter* isolates when used at the concentrations and according

to the procedures recommended by the manufacturer. When the strains of *Acinetobacter* included in the study were divided into two groups according to their susceptibility to the antiseptics tested, the groups of isolates with RS to CHX or DRX (more tolerant to antiseptic) were associated with resistance to carbapenem. The relationship between antibiotic resistance and RS to biocides is controversial [8, 21] and has only been established for the biocides chlorhexidine digluconate or benzalkonium chloride [22–24]. CHX and DRX, which include CHX digluconate and triclosan, respectively, are good substrates of efflux pumps, which in turn are involved in resistance to several antibiotics [23, 25]. This may suggest a competitive advantage of the organisms more resistant to these biocides.

The relationship between the ability to produce biofilm and resistance to antibiotics is a controversial issue [26]. Although the number of resistant biofilm-forming strains is high, some studies have observed that non-biofilm-forming strains are more resistant than biofilm-forming strains [1, 27], but these results differ from those of other studies in which the opposite is observed [26, 28]. In this study, 83% of the strains investigated had the ability to form biofilm, which was consistent with the results observed by Sechi et al. [29] and Krzyściak et al. [30] (80% and 81.9%, respectively). In addition, 85% of the biofilm-forming strains were also classified as carbapenem resistant; however, the biofilm-forming strains were not significantly more resistant than those that did not form biofilm.

Biofilm formation increases the survival rate of *A. baumannii* on dry surfaces and may contribute to its persistence in hospital environments, increasing the probability of causing nosocomial infections and outbreaks [1]. The persistence of *Acinetobacter* on dry surfaces over long periods of time is well known, and the observed differences in its ability to remain viable correspond more to the composition of the surface, environmental conditions, and the techniques used to determine its viability [30, 31]. In this study, the MP isolates were significantly more resistant to antibiotics and with RS to the antiseptics DRX and CHX. The relationship between biofilm formation and resistance to antibiotics, survival on dry surfaces, and tolerance to antiseptics/disinfectant is slowly being clarified. It is often stated that the general resistance of organisms to antimicrobial agents as well as their long-term survival on dry surfaces contributes to the epidemic spread of nosocomial pathogens [8, 32]. In this study, all tested antiseptics displayed good bactericidal activity against all *Acinetobacter* isolates at the concentrations of use. However, when the antiseptics were diluted to calculate the MIC, strains that displayed an RS phenotype to antiseptics, such as CHX or DRX, harbored resistance mechanisms that were shared in function with antibiotic resistance mechanisms. Therefore, strains that displayed resistance to antiseptics were more likely to be resistant to carbapenem and show greater persistence on cotton fabric.



It is known that *Acinetobacter*, like many other organisms, can persist on surfaces in hospital environments for prolonged periods of time, and that this duration can vary depending on the composition of the surface and factors such as humidity or temperature [31, 33]. In this study, the survival time of the isolates was within the range (3 days to 5 months), as previously described for this organism [31], although the test methods, surfaces, and environmental factors used in previous studies have been diverse. None of the isolates included in the study survived 21 days on the cotton fabric under the conditions studied (Figure 1). It has been shown that the survival of various organisms on cotton fabrics is lower than that on other materials such as plastics or wood, which may explain why longer survival periods were not detected [31, 34], although other environmental conditions and the method used may also have influenced the results of this study. A relationship between biofilm formation and the survival of *A. baumannii* was observed by Espinal et al. [1] demonstrating that that biofilm producers were more persistent on dry surfaces than non-biofilm producers; this was not possible to detect in this study. The lack of association between the ability to form biofilm and resistance to carbapenem and survival on cotton fabric could be related to the small number of non-biofilm isolates used in this study, as was suggested to be the case in another study [35], as those with weak or no ability to form biofilm showed a high frequency of resistance to carbapenem. MP strains on cotton fabric, however, were associated with carbapenem resistance and lower susceptibility to CHX and DRX. These characteristics could potentially facilitate the colonization of *Acinetobacter* strains that are more resistant to antibiotics and environmental conditions in the hospital environment.

The mechanisms involved in the resistance of *A. baumannii* to carbapenem or biofilm formation are complex and therefore it is difficult to establish a clear relationship between phenotypes and the genes involved in the formation of biofilm or susceptibility to antimicrobial agents [30].

The limitations of this study were that for clinical purposes, experiments with antiseptics were performed on bacteria in suspension, which may not be fully representative of the actual environmental conditions. In addition, this study was only performed with clinical isolates, so additional studies would be necessary to determine if the frequency of the characteristics analyzed (biofilm, tolerance to antiseptics, and resistance to antibiotics or survival) varies among environmental strains. Susceptibility to antibiotics was determined based on the growth of isolates on CRMA-CR102 plates, rather than estimating the respective MICs to the different antibiotics or analyzing the genes involved in resistance. However, the use of CRMA-CR102 has proven effective as a screening medium for the detection of CRAB, and carbapenem resistance has been associated with multidrug resistance [14]. Furthermore, in this study, resistance to carbapenem

was associated with lower susceptibility to antiseptics as common resistance mechanisms exist.

The results of this study showed that most of the clinical isolates of *A. baumannii* were able to form biofilm, were resistant to carbapenem, and could survive in a dry environment on cotton fabric, and an association was detected between the susceptibility to carbapenem and some antiseptics. The ability to form biofilm, drug and antiseptic susceptibility, and persistence are survival mechanisms for bacteria and their frequency among *A. baumannii* strains are influenced by in different environmental pressure. Studies that analyze all of these characteristics simultaneously should increase our understanding of the causes behind the proliferation of this opportunistic pathogen in hospital environments.

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### Conflict of Interest

None of the authors have any conflicts to report.

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