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Propensity for biofilm formation by clinical isolates from urinary tract infections: developing a multifactorial predictive model to improve antibiotherapy

Maria José Alves,[1,2,3,4] João C. M. Barreira,[3,5] Inês Carvalho,[4] Luis Trinta,[4] Liliana Perreira,[4] Isabel C. F. R. Ferreira[3] and Manuela Pintado[1]

Correspondence
Isabel C. F. R. Ferreira
iferreira@ipb.pt
Manuela Pintado
mpintado@porto.ucp.pt

[1]CBQF-Escola Superior de Biotecnologia, Universidade Católica Portuguesa Porto, Rua Dr António Bernardino de Almeida, 4200-072 Porto, Portugal

[2]Centro Hospitalar de Trás-os-Montes e Alto Douro – Unidade de Chaves, Av. Dr Francisco Sá Carneiro, 5400-249 Chaves, Portugal

[3]Centro de Investigação de Montanha (CIMO), ESA, Instituto Politécnico de Bragança, Campus de Santa Apolónia, Apartado 1172, 5301-855 Bragança, Portugal

[4]Escola Superior de Saúde, Instituto Politécnico de Bragança, Av. D. Afonso V, 5300-121 Bragança, Portugal

[5]REQUIMTE/Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira no. 228, 4050-313 Porto, Portugal

A group of biofilm-producing bacteria isolated from patients with urinary tract infections was evaluated, identifying the main factors contributing to biofilm formation. Among the 156 isolates, 58 (37.2 %) were biofilm producers. The bacterial species ($P<0.001$), together with patient's gender ($P=0.022$), were the factors with the highest influence for biofilm production. There was also a strong correlation of catheterization with biofilm formation, despite being less significant ($P=0.070$) than species or gender. In fact, some of the bacteria isolated were biofilm producers in all cases. With regard to resistance profile among bacterial isolates, β-lactam antibiotics presented the highest number of cases/percentages – ampicillin (32/55.2 %), cephalothin (30/51.7 %), amoxicillin/clavulanic acid (22/37.9 %) – although the carbapenem group still represented a good therapeutic option (2/3.4 %). Quinolones (nucleic acid synthesis inhibitors) also showed high resistance percentages. Furthermore, biofilm production clearly increases bacterial resistance. Almost half of the biofilm-producing bacteria showed resistance against at least three different groups of antibiotics. Bacterial resistance is often associated with catheterization. Accordingly, intrinsic (age and gender) and extrinsic (hospital unit, bacterial isolate and catheterization) factors were used to build a predictive model, by evaluating the contribution of each factor to biofilm production. In this way, it is possible to anticipate biofilm occurrence immediately after bacterial identification, allowing selection of a more effective antibiotic (among the susceptibility options suggested by the antibiogram) against biofilm-producing bacteria. This approach reduces the putative bacterial resistance during treatment, and the consequent need to adjust antibiotherapy.

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INTRODUCTION

Implantable medical devices help enhance therapeutic results, save human lives and improve the quality of life of patients. However, these devices can be readily colonized by bacteria and fungi, since the presence of a foreign body will reduce the number of micro-organisms necessary to produce an infection (Guggenbichler *et al.*, 2011). Nearly 50 % of catheterized patients acquire infections after a short period of time (less than 7 days), depending on the type and location of the device. Patients catheterized for long periods (28 days) have a 100 % chance of developing infections (Dohnt *et al.*, 2011).

Infections suffered during medical care are the fourth leading cause of disease in industrialized countries, mostly

A supplementary model is available with the online version of this paper.

due to the increased use of invasive techniques such as catheter insertion and prosthesis implantation, among others. According to a recent study, about 10 % of the European population is hospitalized each year, 5 % of which acquire at least one nosocomial infection. A mortality rate of 10 % among the hospitalized individuals is estimated as being due to hospital infections (Guggenbichler *et al.*, 2011). The Portuguese situation is even more alarming. According to the latest report of the Program for Infections and Antimicrobials Prevention and Control, 70 % of the bacteria associated with nosocomial infections respond only to a single antibiotic (PPCIRA, 2013).

A report published by the National Nosocomial Infections Surveillance System indicated that urinary tract infections are among the most common causes of nosocomial infections, reporting that 97 % of the cases were associated with urinary catheters (National Nosocomial Infections Surveillance System, 2002). Several biofilm-producing agents (including bacteria, fungi and protozoa) have been linked with these infections, and it is known that biofilm presence is one of the promoting factors, assuming that 60 % of infection processes derive from biofilm presence (Ponnusamy *et al.*, 2012; Tamilvanan *et al.*, 2008).

A consortium of micro-organisms involving different groups may be found in the same biofilm, separated by interstitial spaces filled with the surrounding fluid. Biofilms also contain water channels allowing the transport of essential nutrients and oxygen, contributing to the development of internal cells. The time required to form a biofilm on the device depends on the microbial consortium and the type of material, but on average a thick biofilm can be formed within 24 h on the entire surface of a polymeric device (Tenke *et al.*, 2012). Once the biofilm is formed, it will protect the pathogenic bacteria from the action of antimicrobial agents and from attack by the immune response of the host (Guiton *et al.*, 2010; Regev-Shoshani *et al.*, 2011). This process can lead to chronic or recurrent infections hard to treat. If such urinary infections are not treated, there is the possibility of acute pyelonephritis, bacteraemia, chronic bacterial prostatitis, bacterial vaginosis, chronic renal infection, bladder cancer and, in some cases, death (Guggenbichler *et al.*, 2011; Guiton *et al.*, 2010; Tenke *et al.*, 2012).

The biofilm structure and the physiological characteristics of the micro-organisms that produce it allow intrinsic resistance to antimicrobial agents. The resistance mechanisms are usually based on the delayed penetration of the antimicrobial agent, changes in the microbial growth rate or other physiological alterations related to the development of the biofilm (Donlan & Costerton, 2002). Due to the high mortality rates and the expense of health care, strategies to prevent and eradicate the development of biofilms on urinary tract catheters urgently need to be established (Dohnt *et al.*, 2011).

Escherichia coli, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, MRSA (meticillin-resistant *Staphylococcus aureus*) and *Enterococcus* spp. are the bacteria involved in urinary infections with greater biofilm production rates (Stahlhut *et al.*, 2012; Wasfi *et al.*, 2012; Foxman *et al.*, 2012; Al-Mathkhury *et al.*, 2011; Bonkat *et al.*, 2013). Furthermore, there is the additional problem of the increased antibiotic resistance acquired by bacteria such as *S. aureus*, *Enterococcus* spp. and different Gram-negative bacilli (Guggenbichler *et al.*, 2011).

Despite some available studies on biofilm production in nosocomial infections (Singhai *et al.*, 2012; Niveditha *et al.*, 2012), the association with variables such as age, gender, hospital unit, bacterial species or catheterization is not clear. Accordingly, the aims of this study were: (i) to evaluate the capacity of bacteria isolated from patients with urinary tract infections to produce biofilms; (ii) to evaluate the association of different variables (age, gender, hospital unit, bacteria, catheterization) with biofilm formation; and (iii) to develop a model in order to understand the possible contribution of the variables studied in predicting biofilm onset, allowing more effective selection of the required antibiotic treatment.

METHODS

Bacterial isolates. A retrospective study was conducted over a period of 5 months (February to June 2013). During this period, 1370 urine specimens from patients with presumed urinary infection (with or without catheter) that attended the Clinical Pathology unit of the Centro Hospitalar de Trás-os-Montes e Alto Douro (CHTMAD) were collected and the urine was inoculated onto cystine–lactose electrolyte-deficient (CLED) medium (bioMérieux, France) with calibrated loops to determine the number of colony forming units (c.f.u.). The micro-organisms present in positive samples ($>1\times10^5$ c.f.u. ml^{-1}) were isolated for further characterization. In total, 156 bacterial isolates were thus obtained. This study, duly approved by the Ethics Committee, was conducted in CHTMAD, which is a public institution with 182 beds located in Chaves, north Portugal.

Isolate identification and antimicrobial susceptibility testing. Micro-organism identification and susceptibility tests were performed using MicroScan panels (Siemens) by a microdilution plate method. The interpretation criteria were based on interpretive breakpoints as indicated in CLSI (2008) and the report of the CA-SFM (Comité de l'Antibiogramme de la Société Française de Microbiologie, 2008).

Detection of biofilm formation. The investigation of biofilm production was based on the adherence of the biofilms to borosilicate test tubes, following a previous methodology (Christensen *et al.*, 1985) with minor changes. Each pure isolate was inoculated (to McFarland 0.5 standard) into borosilicate test tubes with 10 ml Mueller–Hinton broth and incubated under aerobic conditions at 37 °C, for 24 h (Tielen *et al.*, 2011; Hassan *et al.*, 2011). Then, the supernatants were discarded and the tubes were stained with 2 ml crystal violet (Sigma-Aldrich) for 5 min, washed three times with distilled water and dried (Al-Mathkhury *et al.*, 2011; Hassan *et al.*, 2011). A positive result was defined as the presence of a layer of stained material adhering to the inner wall of the tubes. A stained ring exclusively at the liquid–air interface was considered to be a negative result. This method does not require high technological preparation, it is not expensive and it allows a suitable level of effectiveness of biofilm screening, especially for thick biofilms, which were the main concern in this work. In fact, this methodology was previously

reported to give the same results as scanning electron microscopy (Singhai *et al.*, 2012; Christensen *et al.*, 1982).

Predictive model. Predictive models may be built based on regression techniques, clustering, decision trees or neural networks. In each case, the model file can be used to generate predictive scores in other datasets. In the scoring process, data are transformed in such a way that the model is expressed internally as a set of numeric transformations to be applied to a defined set of variables (the predictors specified in the model) in order to predict results from different datasets containing the same variables. The propensity to produce biofilm was assigned using different characteristics relating to patients and infection: gender, age, bacterial isolate, clinical service, catheter use. Biofilm production was used in the response field, considering its presence as a positive response value. Hence, a binary logistic regression model was obtained, in which the target predictive score had only two possible outcomes (biofilm presence or absence). The model was built using a dataset (156 patients) for which the outcome of interest (biofilm formation) was known. The model was then tested with a different dataset to validate its usefulness in predicting biofilm formation.

RESULTS AND DISCUSSION

Biofilm development on urinary catheters is a problem often underestimated. However, this factor greatly promotes urinary tract infection, which leads to high mortality rates, prolonged treatments and high costs in health care services (Dohnt *et al.*, 2011). The most serious urinary infections are caused by *E. coli*, which is responsible for nearly 90 % of the infections acquired in the community and 50 % of urinary nosocomial infections that have also been associated with biofilms (Soto *et al.*, 2007; Donlan & Costerton, 2002). Age above 65 years and belonging to the female gender have previously been reported to be risk factors for bacteriuria (Guggenbichler *et al.*, 2011; Niveditha *et al.*, 2012). Also, the use (for more than 14 days) of indwelling devices, such as catheters, highly increases the risk of getting a urinary tract infection (Niveditha *et al.*, 2012).

During the period covered by our study, from the 1370 requests for urine bacteriological analysis, 156 patients were found to exhibit urinary tract infection. The mean age of patients was 64 years (range 6 months to 97 years); they were predominantly female (71.2 %) and 23.1 % (36 patients) had bladder catheters. *E. coli* was the micro-organism isolated in most cases (100 patients; 64.1 %), corroborating the results from previous studies (Eshwarappa *et al.*, 2011; Ronald, 2002; Niveditha *et al.*, 2012), followed by *K. pneumoniae* (33 patients; 21.2 %) and *Proteus mirabilis* (9 patients; 5.8 %). Among the 156 isolates, 58 (37.2 %) were biofilm producers. In similar studies (Niveditha *et al.*, 2012; Reid *et al.*, 1992; Ponnusamy *et al.*, 2012), the biofilm-producing isolates were detected at higher percentages (>60 %); this difference might result from the fact that these studies were performed only in patients with bladder catheters, while in the present study we included all the positive cases, among which only 36 patients (23.1 %) had bladder catheters. Soto *et al.* (2007) performed a similar study to the one described herein, in which biofilm production was detected in 46 % of cases, a value closer to that obtained in the present work.

The mean age of patients with biofilms was slightly higher (66.8 years, range 6 months to 97 years); however, there was no statistically significant correlation (P=0.260) among age and biofilm production variables. Being a woman seemed to have a significant influence for biofilm production (P=0.022), since 35 (60.3 %) of the 58 patient samples showing biofilm development were isolated from women, which is in agreement with previous results (Niveditha *et al.*, 2012). On the other hand, only 18 (31 %) of the 58 patients were catheterized; the statistical correlation for both variables lay below 95 % (P=0.07). Despite lacking high statistical significance, the presence of a urinary catheter seemed to be a risk factor for biofilm production, as was previously pointed out (Niveditha *et al.*, 2012; Tenke *et al.*, 2006; Reid *et al.*, 1992). Some complex features typical of biofilm-producing bacteria promote antibiotic resistance, leading to infection processes related to the use of catheters (Singhai *et al.*, 2012). Mechanisms responsible for resistance may be: (i) delayed penetration of the antimicrobial agent through the biofilm matrix, (ii) altered growth rate of biofilm organisms and (iii) other physiological changes due to the biofilm mode of growth (Donlan & Costerton, 2002).

The medical department with the highest percentage of biofilm-producing bacterial isolates was Emergency (31 patients; 53.5 %), followed by Nephrology out patients (13; 22.4 %), despite the reduced statistical correlation with the two variables, gender and age (P=0.744).

As the first stage, urinary catheters might be colonized by a single pathogen such as *S. epidermidis*, *E. coli*, *Enterococcus faecali* or *Proteus mirabilis*. However, over time, microbial diversity might increase, to include additional species like *Providencia stuartii*, *K. pneumoniae* (Stickler, 1996), *Morganella morganii*, *Acinetobacter calcoaceticus* (Stickler *et al.*, 1993a) or *Enterobacter aerogenes* (Stickler *et al.*, 1993b). Despite *E. coli* being detected as the most common biofilm-producing bacterium (Table 1), species like *Proteus mirabilis, M. morganii, Citrobacter freundii, Klebsiella oxytoca* and *Acinetobacter baumanni* were always biofilm producers, confirming previous information. It is possible that some micro-organisms (in particular *Proteus*) change pH values by producing urease, which hydrolyses urea to ammonia (Tunney *et al.*, 1999). Ammonia increases pH value, promoting the precipitation of minerals, which in turn are deposited on catheters, causing mineral inlays and stimulating biofilm production. Urease-producing micro-organisms in urinary catheters are usually *Proteus mirabilis*, *K. pneumoniae*, *M. morganii*, *Pseudomonas aeruginosa* and *Pseudomonas vulgaris* (Tunney *et al.*, 1999; Stickler *et al.*, 1993a); this accords with the statistical significance between bacterial species and biofilm production (P< 0.001) that we detected.

Regarding resistance profile among bacterial isolates (Table 2), β-lactam antibiotics presented the highest

Table 1. Screening results for biofilm formation among the 156 urinary isolates

In each column, the first number is the number of bacterial isolates.

Bacterial isolate	Biofilm-positive (percentage)	MDR* in biofilm-positive isolates (percentage)	Biofilm non-producers (percentage)	Total
Acinectobacter baumannii	2 (100)	2 (100)	0	2
Citrobacter freundii	3 (100)	1 (33.3)	0	3
Enterobacter aerogenes	2 (50)	2 (100)	2 (50)	4
Escherichia coli	22 (22)	6 (28.6)	78 (78)	100
Klebsiella oxytoca	2 (100)	1 (50)	0	2
Klebsiella pneumoniae	10 (37.0)	5 (100)	17 (63.0)	27
Morganella morganii	3 (100)	3 (100)	0	3
Proteus mirabilis	9 (100)	7 (77.8)	0	9
Pseudomonas aeruginosa	5 (83.3)	1 (20)	1 (16.7)	6
Total	58	26	98	156

*An organism was defined as multidrug-resistant (MDR) if resistant to all tested antimicrobials in three or more classes of antimicrobial agents (penicillins/cephalosporins, carbapenems, aminoglycosides and quinolones) not including tetracyclines or colistin (Sanchez *et al.*, 2013).

percentages – ampicillin (36; 62.1 %), cephalothin (33; 56.9 %), amoxicillin/clavulanic acid (22; 37.9 %) – although the carbapenem group still represented a good therapeutic option (2; 3.4 %). Quinolones (nucleic acid synthesis inhibitors) also showed high resistance percentages (nalidixic acid 30, 51.7 %; norfloxacin 25, 43.1 %; ciprofloxacin: 21, 36.2 %).

These resistances might be related to the mechanisms described above, as in the case of biofilm-producing *Pseudomonas aeruginosa*, for which ciprofloxacin had a delay in penetration time (21 min compared with the usually required 40 s) (Suci *et al.*, 1994).

After carbapenems, gentamicin and tobramycin (both protein synthesis inhibitors) were the antibiotics with lowest resistance percentages (17.2 and 15.5 %, respectively); nonetheless, the detected resistance is a matter of concern, since bacteria can be up to 15 times more resistant to tobramycin when biofilm formation occurs (Hoyle *et al.*,

Table 2. Antibiotic resistance profiles for biofilm-positive and -negative isolates

In each column, the first number is the number of bacterial isolates and the numbers in parentheses are percentages.

Antibiotics	Resistance		
	Biofilm-positive (n=58)	Biofilm-negative (n=98)	Resistance of all isolates (n=156)
Amoxicillin/clavulanic acid	22 (37.9)	25 (25.5)	47 (30.1)
Ampicillin	36 (62.1)	67 (68.4)	103 (66.0)
Cephalothin	33 (56.9)	55 (56.1)	88 (56.4)
Cefazolin	26 (44.8)	33 (33.7)	59 (37.8)
Cefoxitin	23 (39.7)	25 (25.5)	48 (30.8)
Cefuroxime	24 (41.4)	25 (25.5)	49 (31.4)
Cefotaxime	18 (31.0)	21 (21.4)	39 (25.0)
Ceftazidime	16 (27.6)	21 (21.4)	37 (23.7)
Cefepime	13 (22.4)	21 (21.4)	34 (21.8)
Fosfomycin	14 (24.1)	7 (7.1)	21 (13.5)
Nalidixic acid	30 (51.7)	50 (51.0)	80 (51.3)
Ciprofloxacin	21 (36.2)	41 (41.8)	62 (39.7)
Norfloxacin	25 (43.1)	43 (43.9)	63 (40.4)
Nitrofurantoin	16 (27.6)	16 (16.3)	32 (20.5)
Gentamicin	12 (20.7)	17 (17.3)	29 (18.6)
Tobramycin	12 (20.7)	18 (18.5)	30 (19.2)
Co-trimoxazole	20 (34.5)	44 (44.9)	64 (41.0)
Piperacillin/tazobactam	7 (12.1)	10 (10.2)	17 (10.9)
Imipenem	2 (3.4)	1 (1.0)	3 (1.9)

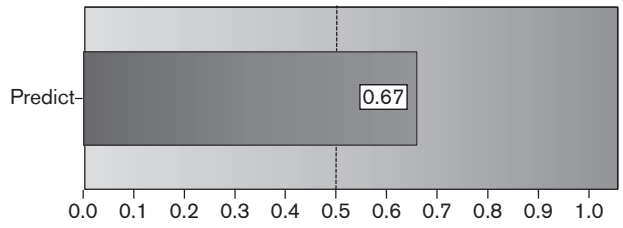

Fig. 1. Overall quality of the predictive model obtained. A good model has a value above 0.5; when this value is lower it indicates that the model is no better than random prediction.

1992). In fact, the so-called persistent cells are not resistant to antibiotics *per se*, acquiring resistance only when a biofilm is formed (Lynch & Robertson, 2008). Fosfomycin also appears to be an alternative therapy for these cases, despite the observed resistance (22.4 %). The quinolone group was second in terms of the highest percentages of resistance (>70 %), in line with the results obtained in a similar study (Niveditha *et al.*, 2012).

The 58 biofilm-producing isolates were multi-resistant in 44.8 % of the cases, compared with only 28.6 % among the 98 non-biofilm producers; this strongly indicates that biofilm production increases the resistance profile of the micro-organisms. Almost half of biofilm producers are simultaneously resistant to at least three different groups of antibiotics. Co-trimoxazole (44.9 %) and ciprofloxacin (41.8 %) (Table 2) showed high resistance percentages, even in non-biofilm-producing isolates, probably due to excessive and inappropriate use of these antibiotics, in particular in urinary tract infections.

Overall, microbial biofilms are a clinical reality closely linked to a variety of persistent infections that respond poorly to conventional antibiotherapy. Accordingly, biofilms greatly complicate the clinical use of antimicrobials. Pharmacokinetic and pharmacodynamic studies of antibiotics applied to infections use parameters such as minimum inhibitory concentration (MIC) for bacteria isolated from clinical samples which behave as planktonic bacteria. Two crucial pieces of information are still missing in order to establish the best treatment for bacteria in biofilms: it is known that MICs are always higher for bacteria when growing in biofilms than when planktonic, but it is not known to what extent; the presence of more than one bacterial species in the same biofilm is an aggravating factor.

These limitations led us to consider the need to develop a predictive model to anticipate the probability of biofilm occurrence according to different related variables. The file used to obtain a predictive model regarding the targeted outcome (biofilm formation) included factors inherent (age and gender) to the 156 patients from which the bacterial isolates were obtained and to the clinical condition (bacterial isolate, catheterization, clinical unit), all used as predictor variables, and, of course, information regarding the development of biofilm during antibiotic treatment (a value of 1 indicates 'biofilm-positive', while a value of 0 indicates 'biofilm-negative'), used in the response field. The model obtained is available in the online Supplementary Material since it is too complex to present here. The model was validated by using the default training sample partition size of 50 % and the default seed value of 2×10^6. The overall model quality was also verified (Fig. 1). The value obtained (0.67) indicates a good ability to predict the targeted outcome, but this result should be interpreted carefully since it only reflects a general measure of overall model quality. In fact, the model quality might even be considered 'good' if the correct prediction rate for positive responses does not meet the specified minimum probability. To overcome this limitation we should examine the classification table obtained (Table 3) to verify correct prediction rates. The classification table is split into a training sample and a test sample. The training

Table 3. Classification table with scores obtained for training and test samples

Response code 1, positive: 2, negative.

Observed		Predicted*					
		Training sample			Test sample		
		Response recoded		Percentage correct	Response recoded		Percentage correct
		Positive	Negative		Positive	Negative	
Response recoded	Positive	9	16	36.00	10	18	35.71
	Negative	0	45	100.00	1	52	98.11
Overall percentage		100.00	26.23	77.14	90.91	25.71	76.54

*For the cases predicted to have a positive response, the correct classification rate for positive responses is 100.00 % for the training sample and 90.91 % for test sample. This is greater than the specified minimum probability of 0.90 or 90.00 %. This suggests that the model obtained could be used to identify a set of patients that would have at least a 90 % chance of bacterial biofilm developing.

Table 4. Dataset used to evaluate the predictive model

Age group (years)	Gender	Bacterial isolate	Catheterization	Clinical service	Predicted value
80–89	Male	*Escherichia coli*	Yes	Nephrology out patient	1
70–79	Male	*Escherichia coli*	No	In patient	0
80–89	Female	*Escherichia coli*	No	Emergency	0
40–49	Female	*Escherichia coli*	No	Out-patient	0
60–69	Female	*Klebsiella pneumoniae* ESBL*	No	In patient	0

*Possessing an extended-spectrum β-lactamase.

sample was used to build the model, which was then applied to the test sample to evaluate the model effectiveness. The selected minimum biofilm formation probability was 0.9 (or 90 %). The correct classification rate for positive biofilm is 100.00 % in the training sample and 90.91 % in the test sample. Since the test sample response rate is higher than 90 %, the model obtained should be able to predict if a patient is prone to develop biofilm with at least 90 % probability.

Nevertheless, it should be made clear that its application range is limited to the population to which patients belong. To achieve a trans-national, or even trans-regional, dimension, the dataset used to build the predictive model should include patients from different countries or regions.

To evaluate the predictive ability, a dataset (Table 4) from patients who had not been used to obtain the predictive model was tested. The dataset included the same variables as those used as predictors (age, gender, bacterial isolate, catheterization, clinical service). The results were classified using the SPSS scoring wizard, which allows different scoring functions (i.e. the types of 'scores' available for the selected model). For the binary logistics model used in this study, the available functions are: predicted value, probability of the predicted value, probability of a selected value, and confidence. Due to its simplicity, we opted for the predicted value function, which gives a straight 'yes' (value 1) or 'no' (value 0) as the answer. When the model was applied to the test dataset, all patients were correctly classified as producing (positive) or not producing (negative) biofilm in agreement with the observations in the hospital environment.

Hence, and despite the limited number of cases (which ideally would be greater) used to obtain the predictive model, this result might have the potential to anticipate the formation of biofilm among the population studied, allowing choice of the most suitable and effective antibiotic therapy, and offering health advantages to the patients and financial benefits to the health care institutions.

Decreasing biofilm formation would be a different approach, but it seems rather difficult. More knowledge is needed regarding the molecular mechanisms that lead to the production of biofilms. Furthermore, genetic regulation and expression of genomic factors might allow prevention or decrease of biofilm formation, but these are still at the basic research stage.

Thus, the statistical approach might be useful, since after identifying the bacteria and considering certain characteristics of the patient, biofilm production can be predicted immediately. This might help prevent multibacterial urinary infection, especially in patients with bladder catheters, and enable more effective antibiotic therapies to be chosen; i.e. among the options given by the antibiogram, the clinician may select an antibiotic from the most advanced generation within a group, in the case of a biofilm-producing strain, thus reducing the possibility of appearance of new resistances. Once a biofilm has developed, the effectiveness of the selected antibiotic is lower and readjustment of the antibiotherapy is often required. Accordingly, anticipating biofilm formation in those cases where information is not available during identification of bacterial isolates might allow the selection of a more suitable antibiotic at the beginning of the treatment, thus avoiding the need to change antibiotics at a later stage.

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