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

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Abstract

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 genre ($p = 0.022$), were the factors with highest influence for biofilm production. There was also a strong correlation among catheterization and biofilm formation, despite being less significant ($p = 0.070$) than the former. In fact, some of the isolated bacteria were biofilm producer in all cases. Regarding resistance profile among bacterial isolates, the β-lactamic antibiotics presented the highest cases/percentages: ampicillin (32/55.2%), cephalothin (30/ 51.7%), amoxicillin/clavulanic acid (22/37.9%), although the carbapenemic group still represent a good therapeutic option (2/3.4%). Quinolones (nucleic acid synthesis inhibitors) also showed high resistance percentages. Furthermore, biofilm production clearly increases bacterial resistance. Actually, almost half of biofilm producing bacteria showed resistance against at least three different groups of antibiotics species. Bacterial resistance is often associated with catheterization. Accordingly, intrinsic (age and gender) and extrinsic (hospital unit, bacterial isolate and catheterization) 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 selecting a more effective antibiotic (among the susceptibility options suggested by the antibiogram) against biofilm producing bacteria. This approach reduces the putative bacterial resistance during the treatment, and the consequent need to adjust antibiotherapy.

Keywords: urinary tract infection; catheterization; biofilm; antibiotherapy; predictive model.

1. Introduction

Implantable medical devices help enhancing therapeutic results, saving human lives and improving life quality 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 microorganisms necessary to produce an infection (Guggenbichler *et al*., 2011). Nearly 50% of catheterized patients acquired infections after a short period of time (less than 7 days) depending on the type and location of device. In addition, 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 disease cause in industrialized countries, mostly due to the increase of evasive techniques such as catheters insertion and prostheses implantation, among others. According to a recent study, about 10% of the European population is hospitalized each year, among which 5% acquire at least one nosocomial infection. A mortality rate of 10% is also 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 bacterial associated to nosocomial infections respond only to a single antibiotic (PPCIRA, 2013).

In a report published by the National Nosocomial Infection Surveillance System, urinary tract infections are indicated as being among the most common causes of nosocomial infections, reporting that 97% of the cases were associated with urinary catheters (National System of Surveillance of Nosocomial Infection, 2002). Several biofilm producing agents (including bacteria, fungi and protozoa) have been related with these infections, and is known that biofilm presence is one of promoting factors,

assuming that 60% of infection processes derive from biofilm presence (Ponnusamy *et al*., 2012; Tamilvanan *et al*., 2008).

A consortium of microorganisms involving different groups might be found in the same biofilm, separated by interstitial spaces filled with surrounding fluid. The biofilm contains also water channels allowing the transport of essential nutrients and oxygen, contributing to the development of inner cells. The time required to form this biofilm on the device depends on the microbial consortium and the type of material, but in average a thick biofilm can be formed within 24 hours on the entire surface of polymeric devices (Tenke *et al*., 2012). Once the biofilm is formed, this will protect the pathogenic bacteria from action of antimicrobial agents and from the attack of immune response of the host (Guiton *et al*., 2010; 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 causing acute pyelonephritis, bacteremia, 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 its producing microorganisms allow an 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 biofilm development (Donlan & Costerton, 2002). Due to the high mortality rates and expensive costs for health care, it is urgent to establish strategies to prevent and eradicate the development of biofilms on urinary tract catheters (Dohnt *et al*., 2011).

Escherichia coli, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, MRSA (methicillin-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's the additional problem of the increased antibiotic resistance acquired by bacteria such as *Staphylococcus aureus*, *Enterococcus* species and different Gram negative bacilli (Guggenbichler *et al*., 2011).

Despite some available studies on biofilms production in nosocomial infections (Singhai *et al*., 2012; Niveditha *et al*., 2012), the association with variables such as age, gender, hospital unit, bacteria, or catheterization, is not clear. Accordingly, the aims of this study were to: i) 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 contribution of the studied variables as predictors of biofilm onset, allowing a more effective selection of the required antibiotic treatment.

2. Materials and Methods

2.1. Bacterial isolates

A retrospective study was conducted during 5 months (February to June, 2013). During this period, 1370 urine specimens from patients with presumed urinary infection (with or without catheter) that were attended in the Clinical Pathology unit of Centro Hospitalar de Trás-os-Montes e Alto Douro (CHTMAD) were collected and the urine was inoculated onto Cystine Lactose Electrolyte Deficient (CLED) (Biomerieux; Marcy l´ Etoile, France) medium with calibrated loops to determine the Colony Forming Units (CFU). From positive samples (enumeration > $1x10^5$ CFU/ml) the microorganisms were isolated for further characterization. From all positive samples, 156 bacterial isolates

were obtained. This study, duly approved by the Ethics Committee, was conducted in the CHTMAD, which is a public institution with 182 beds located in Chaves, North of Portugal.

2.2. Isolates identification and antimicrobial susceptibility testing

Microorganism's identification and susceptibility tests were performed using MicroScan panels (MicroScan®; Siemens Medical Solutions Diagnostics, West Sacramento, CA) by microdilution plate method. The interpretation criteria were based on Interpretive Breakpoints as indicated in Clinical and Laboratory Standards Institute (CLSI) Document M100-S18 (Performance Standards for Antimicrobial Susceptibility Testing, 2008) and the report of the *Committee of L'Antibiogramme de la Société Française de Microbiologie* (CA-SFM) (Comité de L'Antibibiogramme De La Société Française de Microbiologie, 2008).

2.3. Detection of biofilm formation

The detection of biofilms was done by the tube adherence method. The investigation of the biofilm production was performed on the basis of 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 (0,5 McFarland) in borosilicate test tubes with 10 ml of Muller Hinton broth and incubated under aerobic conditions at 37 °C, during 24 h (Tielen *et al*., 2011; Hassan *et al*., 2011). Then, the supernatants were discarded and the tubes were stained with 2 ml of violet crystal Sigma-Aldrich (Spruce Street; St. Louis, USA) for 5 min, washed with distilled water 3 times and dried (Al-Mathkhury *et al*., 2011; Hassan *et al*., 2011). A positive result was defined as the presence of a layer on the stained material which adhered to the inner wall of the tubes.

The exclusive observation of a stained ring at the liquid-air interface was considered as negative. This method does not require high technological preparation, it is not expensive and it allows a suitable effectiveness level of biofilm screening, especially for thick biofilms, which were the main concern in this work. In fact, this methodology was previously reported as allowing the same results when compared to scanning electron microscopy (Singhai *et al*., 2012; Christensen *et al*., 1982).

2.4. Predictive model

Predictive models might be built based on regression techniques, clustering, decision trees or neural networks. In either case, the predictive model file might be used to generate predictive scores in other datasets. In the scoring process, data is transformed in 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 related with 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.

3. Results and Discussion

Biofilm development on urinary catheters is a problem often underestimated. However, this factor highly promotes the urinary tract infection, which leads to high mortality rates, prolonged treatments and causes 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). Ages above 65 years and belonging to the female genus were previously reported as risk factors for bacteriuria (Guggenbichler *et al*., 2011; Niveditha *et al*., 2012). Also, the use of indwelling devices (more than 14 days), such as catheters, highly increases the risk of getting a urinary tract infection (Niveditha *et al*., 2012).

In our study, during covered period, from the 1370 requests of urine bacteriological analysis, 156 patients exhibited urinary tract infection. The mean age of patients was 64 years (6 months - 97 years), they were predominantly female (71.2%) and were submitted to bladder catheters in 23.1% (36 patients) of the cases; *E. coli* was the microorganism isolated in most cases (100; 64.1%) corroborating the results from previous studies (Eshwarappa *et al*., 2011; Ronald, 2002; Niveditha *et al*., 2012), followed by *K. pneumoniae* (33; 21.2%) and *P. mirabilis* (9; 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 in higher percentages (>60%); this difference might result from the fact that these studies were performed only in patients with bladder catheters, while in present study we included all the positive cases, among which only 36 patients (23.1%) were submitted to bladder catheters. Soto *et al*. (2007) performed a similar study to the one

described herein, in which the biofilm production was detected in 46% of the cases, a value also closer to the one obtained in the present work.

The average age for patients with biofilms was slightly higher (66.8 years, 6 months to 97 years); however, there was no statistically significant correlation (p = 0.260) among age and biofilm production variables. The fact of being a woman seemed to have a significant influence for biofilm production (p = 0.022), since 35 (60,3%) of the 58 patients with biofilm development, were isolated from woman, which is actually in agreement with previous results (Niveditha *et al*., 2012). On the other hand, only 18 (31%) of the 58 patients were catheterized; thereby, the statistical correlation among 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 it was previously pointed out (Niveditha *et al*., 2012; Tenke *et al*., 2006; Reid *et al*., 1992). Some complex features typical from biofilm producing bacteria promote antibiotic resistance, leading to infection processes related to the applied catheters (Singhai *et al*., 2012). Mechanisms responsible for resistance may be the following: (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 specialty with the highest percentage of biofilm producing bacterial isolates was Emergency (31; 53.5%), followed by Nephrology Appointment (13; 22.4%), despite the reduced statistical correlation between this two variables (p = 0.744).

On the first stage, urinary catheters may be colonized by a single pathogen such as *S. epidermidis*, *E. coli*, *E. faecalis*, or *P. mirabilis*. However, over time, microbial diversity might increase, comprising 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 bacteria (**Table 1**), species like *P. mirabilis, M. morganii, Citrobacter freundii, K. oxytoca* and *A. baumanni*, were always biofilm producers, confirming the previous information. It is possible that some microorganisms (in particular *Proteus*) change pH values by producing urease, which hydrolyzes 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 biofilms production. Urease producing microorganisms in urinary catheters are usually *P. mirabilis*, *K. pneumoniae*, *M. morganii*, *P. aeruginosa* and *P. vulgaris* (Tunney *et al*., 1999; Stickler *et al*., 1993a); this specification also meets our results, according to the detected statistical significance between bacterial species and biofilm production (p <0.001).

Regarding resistance profile among bacterial isolates (**Table 2**), β-lactam antibiotics presented the highest percentages: ampicillin (36; 62.1%), cephalothin (33; 56.9%), amoxicillin/clavulanic acid (22; 37.9%), although the carbapenemic group still represent 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 with the above described mechanisms, as in the case of biofilm producing *P. aeruginosa*, for which ciprofloxacin had a delay in penetration time (21 minutes compared with the usually required 40 seconds) (Suci *et al*., 1994).

After carbapenemics, 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 matter of concern, since bacteria might be up to

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

The 58 producing biofilm 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 microorganisms. Almost half of biofilm producers are simultaneously resistant to at least 3 different groups of antibiotics. Co-trimoxazole (44.9%) and ciprofloxacin (41.8%) (**Table 2**) showed high resistance percentages, even in non-producing biofilm 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. The pharmacokinetic and pharmacodynamic studies for antibiotics applied to infections are conducted using parameters such as minimum inhibitory concentration (MIC) for bacteria isolated from clinical samples, which behave as planktonic bacteria. Two crucial data are still missing in order to establish the best treatment for bacteria in biofilm: it is known that the MIC of these bacteria are always higher than in planktonic, however it is not known to what extent; on the other hand, the presence of more than one bacterial species in the same biofilm is an aggravation factor.

These limitations led us to consider the need of developing 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 variables inherent (age and gender) to the 156 patients with bacterial isolates 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 obtained model was provided as supplementary material, since it would be impracticable to be presented in results and discussion section due to its complexity. The model was validated by using the default training sample partition size of 50% and the default seed value of 2000000. The overall model quality was also verified (**Figure 1**). The obtained value (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 obtained classification table (**Table 3**) to verify correct prediction rates. The classification table is split into a training sample and a testing sample. The training sample was used to build the model, which was then applied to the testing 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 testing sample. Since the testing sample response rate is higher than 90%, the obtained model should be able to predict if a patient is prone to develop biofilm with at least 90% of probability.

Nevertheless, it should be clarified that its application range is limited to the population to which patients belong. To achieve a transnational, or even transregional, 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 whom have 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 scoring wizard (SPSS) that allows different scoring functions (*i.e.*, the types of "scores" available for the selected model). For the binary logistic 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 straightly a "yes" (value 1) or "no" (value 0) as answer. After applying the model to the testing dataset, it was verified that all patients were correctly classified as producing (positive) or not (negative) biofilm in agreement with the observed in hospital environment.

Hence, and despite the limitation of the 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 studied population, allowing to define the most suitable and effective antibiotic therapy, 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 preventing or decreasing biofilm formation, but this subject is still at basic research stage.

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

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Conflict of Interest

The authors have no conflicts of interest.

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

Bacterial isolate	Biofilm positive (%)	MDR* in biofilm positive isolates	Biofilm non-producers (%)	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

*A multidrug-resistant (MDR) organism was defined if resistant to all tested antimicrobials in 3 or more classes of antimicrobial agents (penicillins/cephalosporins, carbapenems, aminoglycosides and quinolones) not including tetracyclines or colistin (Sanchez et al., 2013).

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

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%)

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

		Predicted*					
		Training sample			Testing sample		
		Response recoded (1=positive, 2= negative)		Percentage correct	Response recoded (1=positive, 2= negative)		Percentage correct
Observed		Positive	Negative		Positive	Negative	
Response recoded	Positive	9	16	36.00	10	18	35.71
(1=positive, 2= negative)	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 actual positive responses is 100.00% for training sample and 90.91% for testing sample. This is greater than the specified minimum probability of 0.90 or 90.00%. This suggests that the obtained model could be used to identify a set of patients that would have at least 90% chances of bacterial biofilm developing.

Table 4. Dataset used to evaluate the obtained predictive model.

Age group	Gender	Bacterial isolate	Catheterization	Clinical service	Predicted value
80 to 89 years	Male	*Escherichia coli*	Yes	Nephrology appointment	1
70 to 79 years	Male	*Escherichia coli*	No	Internment medicine	0
80 to 89 years	Female	*Escherichia coli*	No	Emergency	0
40 to 49 years	Female	*Escherichia coli*	No	External appointment	0
60 to 69 years	Female	*Klebsiella pneumoniae* ESBL	No	Internment medicine	0

Figure 1. Overall quality for the obtained predictive model. A good model
above 0.5; when this value is lower it indicates that the model is no better t
prediction.