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ARTICLE

Comparative antimicrobial susceptibility of biofilm versus planktonic forms of *Salmonella enterica* strains isolated from children with gastroenteritis

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Abstract In the present study, 194 *Salmonella enterica* strains, isolated from infected children and belonging to various serotypes, were investigated for their ability to form biofilms and the biofilm forms of the isolated strains were compared to their corresponding planktonic forms with respect to the antimicrobial susceptibility. For the biofilm-forming strains, the minimum inhibitory concentration for bacterial regrowth (MICBR) from the biofilm of nine clinically applicable antimicrobial agents was determined, and the results were compared to the respective MIC values of the planktonic forms. One hundred and nine *S. enterica* strains out of 194 (56%) belonging to 13 serotypes were biofilm-forming. The biofilm forms showed increased antimicrobial resistance compared to the planktonic bacteria. The highest resistance rates of the biofilm bacteria were observed with respect to gentamicin (89.9%) and ampicillin (84.4%), and the lowest rates with respect to ciprofloxacin and moxifloxacin (2.8% for both). A remarkable shift of the $MICBR_{50}$ and $MICBR_{90}$ toward resistance was observed in the biofilm forms as compared to the respective planktonic forms. The development of new consensus methods for the determination of the antimicrobial susceptibility of biofilm forms seems to be a major research challenge. Further studies are required in order to elucidate the biofilm antimicrobial resistance mechanisms of the bacterial biofilms and their contribution to therapeutic failure in infections with in vitro susceptible bacteria.

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Introduction

Microbial biofilms are a major concern in human and veterinary medicine. They consist of growing microorganisms intimately associated with each other, producing an extracellular polymeric substance (EPS) consisting of carbohydrate (exopolysaccharide) adhering to synthetic or biological surfaces [1–4]. The encased sessile microorganisms bear quite distinct properties from those growing independently or as planktonic populations in liquid media. One of the most important properties of the biofilm-associated bacteria in clinical medicine is the markedly enhanced resistance to antimicrobial agents, through protection by the EPS, leading to multidrug resistance and therapeutic failure.

Although the mechanisms are poorly understood, there is evidence that the biofilm-associated resistance should be related to modified nutrient environments, leading to

suppression of growth rate within the biofilm, interaction between exopolymer matrices and the antimicrobial, as well as the development of biofilm-/attachment-specific phenotypes [5–8].

In comparative antimicrobial susceptibility studies, many common gram-negative and gram-positive bacterial pathogens produce biofilms showing significantly higher antimicrobial resistance rates than their planktonic state. Most of these studies have largely focused on *Staphylococcus aureus*, *S. epidermidis*, and *Pseudomonas aeruginosa* [9].

Little is known about the response to antimicrobials of the biofilm forms of the *Salmonella enterica* serotypes, which are, worldwide, the most common cause of acute, self-limited gastroenteritis, usually not requiring treatment [10, 11]. However, the majority of *S. enterica* strains are able to form biofilms and synthesize cell surface components, which help them survive in hostile or suboptimal environments [12], and express resistance to multiple antimicrobials. This ability contributes to their persistence in the host after the acute phase of infection (carrier state), the dissemination of the organism over a long period of time in the environment, and its transmission to new individuals [13]. Regarding these properties, *Salmonella enterica* could serve as a very good model for the study of the comparative response to antimicrobials between the planktonic and their respective biofilm forms.

The present study aimed to detect the production of biofilms by clinical strains of *S. enterica* serotypes isolated from children with gastroenteritis and to compare the antimicrobial susceptibility of planktonic versus biofilm-forming bacteria.

Patients and methods

During a three-year period (2006–2008), 194 *S. enterica* strains were collected from children with gastroenteritis, who were either hospitalized or who attended the outpatient clinic. The age of the children was balanced in range 1–14 years. The isolation and serological identification of *S. enterica* was performed by conventional methods.

Biofilm formation was detected by the use of silicone disks (Folio C6 0.25 mm, NOVATECH; New Biotechnology for Life, Z.I. Athélia III, Voie Antiope 13705 La Ciotat Cedex, France) modified as described previously [14]. The silicone disks were cut into similar size (4–5 mm) and weight (25–30 mg) through an in-house invented spacer construction and were placed into tubes, weighed on a scale, and left overnight under UV irradiation for sterilization. Tubes containing 2,5 ml trypticase soya broth (TSB) were inoculated with *Salmonella* strains and incubated for 72 h at 30°C. The contents were then poured off and the tubes were washed three times with distilled water and air-dried in a laminar flow for 24 h. The tubes containing the silicone disks with the attached bacteria were weighed once more and the difference in weight showed the presence of biofilms.

The antimicrobial susceptibility of the planktonic bacterial forms was performed by means of determination of the minimum inhibitory concentration (MIC). The MIC was determined using two methods: (a) the automatic VITEK 2 system (bioMérieux SA, 69280 Marcy-l'Etoile, France) and (b) the standard broth dilution method according to guidelines of the Clinical Laboratory Standards Institute (CLSI) [15]. The antimicrobials included were those of importance in clinical practice: ampicillin, coamoxiclav, cefuroxime, cefotaxime, gentamicin, imipenem, cotrimoxazole, ciprofloxacin, and moxifloxacin.

The strains producing biofilms were further tested for their antimicrobial susceptibility by the determination of the minimum inhibitory concentration for bacterial regrowth (MICBR) from the biofilm using a modified broth macrodilution method according to the guidelines of the CLSI [15].

Silicone disks coated with the biofilm-forming *Salmonella* strains were prepared in tubes as described above, omitting the last step (air-drying). Serial dilutions of the antimicrobials in Mueller–Hinton broth, corresponding to the concentrations used for the MIC determination regarding the planktonic bacteria, were prepared and poured into the tubes containing the silicone disks. The tubes containing the antimicrobial were then incubated at 35°C for 24 h. The growth of planktonic bacteria was visualized by the development of turbidity in the medium. The MICBR was defined as the lowest concentration inhibiting the growth in the medium as observed by a complete clarity. An aliquot of the medium from the tubes with the lowest antimicrobial concentration showing a turbidity indicating bacterial growth was subcultured in blood and McConkey agar medium in order to check the purity of the grown *Salmonella* population.

The statistical analysis was performed using the statistical package SPSS for Windows (version 15.0) in order to disclose any significant differences between the percentages of antimicrobial susceptibility of the planktonic and the biofilm bacterial forms. The analysis was done by applying an appropriate hypothesis test concerning the difference between the proportions of two samples. The normal approximation to the binomial distribution was used.

Results and discussion

The distribution of serotypes among the *S. enterica* isolates and their respective ability to form biofilms are shown in Table 1. Biofilm formation was detected in 109 out of 194 *Salmonella* strains (56%) included in the study. For all of

Table 1 Distribution of serovars and biofilm production among the *Salmonella enterica* strains isolated from children with gastroenteritis

Salmonella enterica serovar	Number of strains	Biofilm-positive
S. enteritidis	143	78
S. typhimurium	27	17
S. abony	3	0
S. oranienburg	3	3
S. II	2	1
S. muenchen	1	1
S. montevideo	1	1
S. lagos	1	1
S. goldcoast	1	0
S. blockley	1	1
S. thompson	1	1
S. newport	1	1
S. virchow	1	1
S. bardo	1	0
S. stanley	1	0
S. miami	1	1
S. haifa	2	1
S. bovismorbificans	1	0
Rough strains	1	0
Total	194	109

the positive strains, the difference in weight before and after incubation of the silicone disk was >50 mg, while in the negative strains, the absence of biofilm formation was indicated by differences of less than 1 mg.

The antimicrobial resistance rates of the planktonic and the biofilm bacteria are given in Table 2. The biofilm forms showed increased antimicrobial resistance compared to the planktonic bacteria. The highest resistance rates of the biofilm bacteria were observed with respect to gentamicin (89.9%) and ampicillin (84.4%), and the lowest rates with respect to ciprofloxacin and moxifloxacin (2.8% for both).

Since the *p*-value for all antimicrobials was less than 1%, all of the differences were assumed to be statistically significant at the 99.0% (at least) confidence level (Table 2).

A remarkable shift of the MIC_{50} and MIC_{90} toward resistance was observed in the biofilm forms as compared to the respective planktonic forms (Table 3). The $MICBR_{50}$ lay in the upper limit, corresponding to susceptibility only for cefotaxime, imipenem, and the two quinolones (ciprofloxacin and moxifloxacin), while the $MICBR_{90}$ was above this limit (intermediate or resistant) for all of the antimicrobials tested.

In the present study, a significant difference in antimicrobial susceptibility was found between the planktonic and biofilm forms of the *S. enterica* strains isolated from clinical cases with gastroenteritis, with the biofilm forms showing increased resistance rates. Despite this statistically significant increase in resistance, quinolones and the broad-spectrum β-lactams cefotaxime and imipenem showed the best antimicrobial activity against the biofilm forms, having $MICBR_{50}$ values at the level of susceptibility (Table 3). Although the methodology followed regarding the antimicrobial susceptibility testing of the biofilm bacteria is not a standardized consensus approach, the results arising are clear and more or less in agreement with previous reports referring to *Salmonella* as well as to other bacterial species (*Escherichia coli*, *S. epidermidis*, *P. aeruginosa*) using in-house biofilm formation and biofilm antimicrobial susceptibility detection methods [9, 16–19]. However, the detection of biofilm formation as such, does not predict in all cases a possible clinical therapeutic failure (or clinical resistance). According to the hitherto reports, this phenomenon seems not to pertain to all bacterial species [9]. In veterinary infections caused by *Pasteurella multocida*, *Mannheimia haemolytica*, and *Haemophilus somnus*, no difference in susceptibility was found between the planktonic and the biofilm forms, and the treated animals responded well to most antimicrobials. This diversity in biofilm response to antimicrobials demonstrates the complexity in the prediction of therapeutic outcome, the latter probably depending on a variety of factors conditioned by the nature of the antimicrobial, the bacterial species properties, and the specific biofilm features.

In the present report, *S. enterica* served practically as a very good model for the study of biofilm formation and antimicrobial susceptibily, first because about half of the strains (56%) were biofilm producers in vitro and second because most of the strains in their planktonic forms bore antimicrobial susceptible phenotypes (Table 2), thus, giving conspicuous differences in the biofilm phenotypes.

Soon after the beginning of the "golden" antibiotic era in 1940, the emergence of antimicrobial resistance arose, which

Table 2 Antimicrobial resistance rates of planktonic and biofilm forms of *Salmonella enterica* strains isolated from children with gastroenteritis. The *p*-values refer to the results of performing a hypothesis test concerning the difference between the two proportions (using the normal approximation)

Antimicrobial	Planktonic	Biofilm	*p*-value
Ampicillin	12.8%	84.4%	0.000
Gentamicin	0%	89.9%	0.000
Coamoxiclav	0%	51.4%	0.000
Cotrimoxazole	0.9%	63.3%	0.000
Cefuroxime	7.4%	63.3%	0.000
Cefotaxime	0.9%	23.8%	0.000
Imipenem	0%	7.3%	0.004
Ciprofloxacin	0%	2.8%	0.008
Moxifloxacin	0%	2.8%	0.008

Table 3 MIC_{50} and MIC_{90} of the antimicrobials for the planktonic and $MICBR_{50}$ and $MICBR_{90}$ of the biofilm forms of *Salmonella enterica* isolates

Antimicrobial	mg/l		mg/l		Breakpoints (susceptible)
	Planktonic MIC_{50}	Biofilm $MICBR_{50}$	Planktonic MIC_{90}	Biofilm $MICBR_{90}$	
Ampicillin	≤ 2	≥ 64	≥ 32	≥ 64	≤ 8
Coamoxiclav	≤ 2	32	4	64	≤ 8
Cefuroxime	4	32	8	64	≤ 8
Cefotaxime	≤ 1	8	≤ 1	64	≤ 8
Gentamicin	≤ 1	16	≤ 1	32	≤ 4
Imipenem	≤ 1	4	≤ 1	8	≤ 4
Cotrimoxazole	≤ 20	160	≤ 20	320	≤ 40
Ciprofloxacin	≤ 0.25	1	≤ 0.25	2	≤ 1
Moxifloxacin	≤ 0.25	1	1	2	≤ 1

incited intensive research and major developments in the field of antimicrobial chemotherapy. However, despite the progress in the revelation of most of the antimicrobial resistance mechanisms, the discovery of new drugs, and the improvement and standardization of antimicrobial susceptibility testing using consensus methodologies, the problem of the antimicrobial "clinical resistance" resulting in many cases in therapeutic failure is still of major concern.

In clinical practice, the MIC assay is the gold standard and the best way to select potentially effective antimicrobial agents for the rational treatment of infections [4, 20]. The setting of the antimicrobial breakpoints taking into consideration the pharmacokinetic–pharmacodynamic properties of the drugs, besides clinical trials, is based on the use of planktonic bacterial forms, a fact that does not correspond to the in vivo infectious disease pathogenesis, as, in some cases, other factors like the formation of biofilms might be involved.

In all of the hitherto published antimicrobial susceptibility studies dealing with biofilm bacterial forms, the applied methodology varies, because standardized techniques have not yet been established. However, all reported results are completely in agreement with each other, indicating that human pathogenic biofilm forming bacteria bear significantly increased antimicrobial resistance properties compared to their corresponding planktonic forms. In the present study, the testing of antimicrobial susceptibility (or resistance) referred to the ability of already formed biofilms to grow and generate planktonic forms. In other reports, biofilm resistance was found to be related to the low growth rates within the biofilm mass [2, 21–23]. This versatile reaction of biofilms against antimicrobials is noticeably impeding the search for novel antibiofilm-acting antimicrobial agents [24].

The development of new consensus methods for the determination of the antimicrobial susceptibility of biofilm forms in biofilm-forming bacteria seems to be a major research challenge in infectious disease therapeutics for the management of infections that are difficult to treat. The present study involving *S. enterica*, a biofilm-forming microorganism, was conducted to demonstrate the ability of bacterial biofilms to escape in vitro the action of the commonly used antimicrobial agents. Further studies are warranted to elucidate the biofilm antimicrobial resistance mechanisms and their contribution to therapeutic failure in infections with in vitro susceptible bacteria.

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