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## Comparative antimicrobial susceptibility of biofilm versus planktonic forms of strains isolated from children with gastroenteritis

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**Abstract: Purpose.** In the present study 194 *S. enterica* strains, isolated from infected children, 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.

**Methods.** For the biofilm forming strains the Minimal Inhibitory Concentration for Bacterial Regrowth from the Biofilm (MICBR) of nine clinically applicable antimicrobial agents, was determined, and the results were compared to the respective MIC values of the planktonic forms.

**Results.** A hundred and nine *S. enterica* strains out of 194 (56%) belonging to 13 serotypes were biofilm forming. The biofilm forms showed increased antimicrobial resistance versus 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).

**Conclusions.** A remarkable shift of the MICBR50 and MICBR90 toward resistance was observed in the biofilm forms as compared to the respective planktonic forms. Further studies are required to elucidate the biofilm antimicrobial resistance mechanisms of the bacterial biofilms and their contribution to the therapeutic failure in infections with in vitro susceptible bacteria.

Response to Reviewers: Dear Mr. Alex van Belkum,

we have taken into consideration all the reviewer's comments and we have revised the manuscript accordingly. There is one exception with respect to the introduction comments g. and h. were we rewrote the text, but not in the proposed way because this would alter the essence. We have also corrected the references.

with best regards,

Prof. Stylianos Chatzipanagioto

1 **Concise Article**

2

3 **Comparative antimicrobial susceptibility of biofilm versus planktonic forms of**

4 ***Salmonella enterica* strains isolated from children with gastroenteritis.**

5

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## 31 Abstract

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33 **Purpose.** In the present study 194 *S. enterica* strains, isolated from infected children,  
34 belonging to various serotypes were investigated for their ability to form biofilms and the  
35 biofilm forms of the isolated strains were compared to their corresponding planktonic forms  
36 with respect to the antimicrobial susceptibility.

37 **Methods.** For the biofilm forming strains the Minimal Inhibitory Concentration for Bacterial  
38 Regrowth from the Biofilm (MICBR) of nine clinically applicable antimicrobial agents, was  
39 determined, and the results were compared to the respective MIC values of the planktonic  
40 forms.

41 **Results.** A hundred and nine *S. enterica* strains out of 194 (56%) belonging to 13 serotypes  
42 were biofilm forming. The biofilm forms showed increased antimicrobial resistance versus the  
43 planktonic bacteria. The highest resistance rates of the biofilm bacteria were observed with  
44 respect to gentamicin (89.9%) and ampicillin (84.4%) and the lowest rates with respect to  
45 ciprofloxacin and moxifloxacin (2.8% for both).

46 **Conclusions.** A remarkable shift of the MICBR<sub>50</sub> and MICBR<sub>90</sub> toward resistance was  
47 observed in the biofilm forms as compared to the respective planktonic forms. The  
48 development of new consensus methods for the determination of the antimicrobial  
49 susceptibility of biofilm forms seems to be a major research challenge. Further studies are  
50 required in order to elucidate the biofilm antimicrobial resistance mechanisms of the bacterial  
51 biofilms and their contribution to the therapeutic failure in infections with in vitro susceptible  
52 bacteria.

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54 **Key words:** Antimicrobial resistance; biofilms; planktonic bacteria; *Salmonella enterica*;  
55 gastroenteritis.

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## 58 Introduction

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

68 Although the mechanisms are poorly understood, there is evidence that the biofilm associated  
69 resistance should be related to modified nutrient environments, leading to suppression of  
70 growth rate within the biofilm, interaction between exopolymer matrices and the antimicrobial,  
71 as well as the development of biofilm / attachment specific phenotypes [5-8].

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

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

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

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91 **Patients and Methods**

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

97 Biofilm formation was detected by the use of silicone disks (Folio C6 0,25 mm,  
98 NOVATECH; new biotechnology for life ZI ATHELIA III-VOIE ANTIOPE 13705 LA CIOTAT  
99 CEDEX-FRANCE) modified as described previously [14]. The silicone disks were cut in  
100 similar size (4-5 mm) and weight (25-30 mg) through an in-house invented spacer  
101 construction and were placed into tubes, weighed at a scale and left overnight under UV  
102 irradiation for sterilization. Tubes containing 2,5 ml trypticase soya broth (TSB) were  
103 inoculated with *Salmonella* strains and incubated for 72 hours at 30° C. The content was then  
104 poured off and the tubes were washed 3 times with distilled water and air dried in a laminar  
105 flow for 24 hours. The tubes containing the silicone disks with the attached bacteria were  
106 weighed once more and the difference in weight showed the presence of biofilms.

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

114 The strains producing biofilms were further tested for their antimicrobial susceptibility by  
115 determination of the Minimal Inhibitory Concentration for Bacterial Regrowth from the biofilm  
116 (MICBR) using a modified broth macrodilution method according to the guidelines of the  
117 Clinical laboratory Standards Institute (CLSI) [15].

118 Silicone disks coated with the biofilm forming *Salmonella* strains were prepared in tubes as  
119 described above, omitting the last step (air-drying). Serial dilutions of the antimicrobials in

120 Mueller – Hinton broth, corresponding to the concentrations used for the MIC determination  
121 regarding the planktonic bacteria, were prepared and poured into the silicone disk containing  
122 tubes. The antimicrobial containing tubes were then incubated at 35° C for 24 h. The growth  
123 of planktonic bacteria was visualized by the development of turbidity in the medium. The  
124 MICBR was defined as the lowest concentration inhibiting the growth in the medium as  
125 observed by a complete clarity. An aliquot of the medium from the tubes with the lowest  
126 antimicrobial concentration showing a turbidity indicating bacterial growth, was subcultured in  
127 blood and Mc Conkey agar medium in order to check the purity of the grown *Salmonella*  
128 population.  
129 The statistical analysis was performed using the statistical package SPSS for Windows  
130 (version 15.0) in order to disclose any significant differences between the percentages of  
131 antimicrobial susceptibility of the planktonic and the biofilm bacterial forms. The analysis was  
132 done by applying an appropriate hypothesis test concerning the difference between the  
133 proportions of two samples. The normal approximation to the binomial distribution was used.

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## Results and Discussion

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140 The distribution of serotypes among the *S. enterica* isolates and their respective ability to form  
141 biofilms are shown in table 1. Biofilm formation was detected in 109 out of 194 *Salmonella*  
142 strains (56%) included in the study. For all the positive strains the difference in weight before  
143 and after incubation of the silicone disk was >50 mg, while in the negative strains the absence  
144 of biofilm formation was indicated by differences of less than 1 mg.

145 The antimicrobial resistance rates of the planktonic and the biofilm bacteria are given in table  
146 2. The biofilm forms showed increased antimicrobial resistance versus the planktonic  
147 bacteria. The highest resistance rates of the biofilm bacteria were observed with respect to  
148 gentamicin (89.9%) and ampicillin (84.4%) and the lowest rates with respect to ciprofloxacin  
149 and moxifloxacin (2.8% for both).

150 Since the P-value for all antimicrobials was less than 1%, all the differences were assumed to  
151 be statistically significant at 99.0% (at least) confidence level (table 2).

152 A remarkable shift of the MIC<sub>50</sub> and MIC<sub>90</sub> toward resistance was observed in the biofilm  
153 forms as compared to the respective planktonic forms (table 3). The MICBR<sub>50</sub> lay in the  
154 upper limit corresponding to susceptibility only for cefotaxime, imipenem and the two  
155 quinolones (ciprofloxacin and moxifloxacin), while the MICBR<sub>90</sub> was above this limit  
156 (intermediate or resistant) for all the antimicrobials tested.

157 In the present study a significant difference in antimicrobial susceptibility was found between  
158 the planktonic and biofilm forms of the *S. enterica* strains isolated from clinical cases with  
159 gastroenteritis, the biofilm forms showing increased resistance rates. Despite this statistically  
160 significant increase in resistance, quinolones and the broad spectrum  $\beta$ -lactams cefotaxime  
161 and imipenem showed the best antimicrobial activity against the biofilm forms, having  
162 MCBR<sub>50</sub>s in the level of susceptibility (table 3). Although the methodology followed regarding  
163 the antimicrobial susceptibility testing of the biofilm bacteria is not a standardized consensus  
164 one, the results arising are clear and more or less in agreement with previous reports,  
165 referring to *Salmonella*, as well as to other bacterial species (*Escherichia coli*, *Staphylococcus*  
166 *epidermidis*, *Pseudomonas aeruginosa*) using in-house biofilm formation and biofilm  
167 antimicrobial susceptibility detection methods [9, 16-19]. However the detection of biofilm  
168 formation as such, does not predict in all cases a possible clinical therapeutic failure (or  
169 clinical resistance). According to the hitherto reports, this phenomenon seems not to pertain  
170 to all bacterial species [9]. In veterinary infections caused by *Pasteurella multocida*,  
171 *Mannheimia haemolytica* and *Haemophilus somnus* no difference in susceptibility was found  
172 between the planktonic and the biofilm forms, and the treated animals responded well to most  
173 antimicrobials. This diversity in biofilm response to antimicrobials demonstrates the  
174 complexity in prediction of the therapeutic outcome, the latter probably depending on a variety  
175 of factors conditioned by the nature of the antimicrobial, the bacterial species properties and  
176 the specific biofilm features.

177 In the present report *Salmonella enterica* served practically as a very good model for the  
178 study of biofilm formation and antimicrobial susceptibility, first because about half of the strains  
179 (56%) were biofilm producers in vitro, and second because most of the strains in their  
180 planktonic forms bore antimicrobial susceptible phenotypes (table 2), thus giving conspicuous  
181 differences in the biofilm phenotypes.



182 Soon after the beginning of the “golden” antibiotic era in 1940, the emergence of the  
183 antimicrobial resistance arose, which incited intensive research and major developments in  
184 the field of the antimicrobial chemotherapy. However, despite the progress in the revelation of  
185 most of the antimicrobial resistance mechanisms, the discovery of new drugs and the  
186 improvement and standardization of the antimicrobial susceptibility testing using consensus  
187 methodologies, the problem of the antimicrobial “clinical resistance” resulting in many cases  
188 in therapeutic failure is still of major concern.

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

195 In all the hitherto published antimicrobial susceptibility studies dealing with biofilm bacterial  
196 forms, the applied methodology varies, because standardized techniques have not yet been  
197 established. However, all reported results are completely in agreement with each other,  
198 indicating that human pathogenic biofilm forming bacteria bear significantly increased  
199 antimicrobial resistance properties, compared to their corresponding planktonic forms. In the  
200 present study, testing of antimicrobial susceptibility (or resistance) referred to the ability of  
201 already formed biofilms to grow and generate planktonic forms. In other reports, biofilm  
202 resistance was found to be related to the low growth rates within the biofilm mass [21-24].  
203 This versatile reaction of biofilms against the antimicrobials is noticeably impeding the search  
204 for novel anti-biofilm acting antimicrobial agents [25].

205 The development of new consensus methods for the determination of the antimicrobial  
206 susceptibility of biofilm forms in biofilm forming bacteria seems to be a major research  
207 challenge in infectious disease therapeutics for the management of infections that are difficult  
208 to treat. The present study involving *S. enterica*, a biofilm forming microorganism, was  
209 conducted to demonstrate the ability of bacterial biofilms to escape in vitro the action of the  
210 commonly used antimicrobial agents. Further studies are warranted to elucidate the biofilm

211 antimicrobial resistance mechanisms and their contribution to therapeutic failure in infections  
212 with in vitro susceptible bacteria.

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**Table 1. Distribution of serovars and biofilm production among the *Salmonella enterica***

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**strains isolated from children with gastroenteritis.**

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<i>Salmonella enterica</i> serovar	number of strains	biofilm positive
<i>S. enteritidis</i>	143	78
<i>S. typhimurium</i>	27	17
<i>S. abony</i>	3	0
<i>S. oranienburg</i>	3	3
<i>S. II</i>	2	1
<i>S. muenchen</i>	1	1
<i>S. montevideo</i>	1	1
<i>S. lagos</i>	1	1
<i>S. goldcoast</i>	1	0
<i>S. blockley</i>	1	1
<i>S. thompson</i>	1	1
<i>S. newport</i>	1	1
<i>S. virchow</i>	1	1
<i>S. bardo</i>	1	0
<i>S. stanley</i>	1	0
<i>S. miami</i>	1	1
<i>S. haifa</i>	2	1
<i>S. bovismorbificans</i>	1	0
<i>rough strains</i>	1	0
<i>total</i>	194	109

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318 **Table 2. Antimicrobial resistance rates of planktonic and biofilm forms of *Salmonella***319 ***enterica* strains isolated from children with gastroenteritis. P-values refer to the results**320 **of performing a hypothesis test concerning the difference between the two proportions**321 **(using normal approximation).**

Antimicrobial	Planktonic	Biofilm	P
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

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337 **Table 3. MIC<sub>50</sub> and MIC<sub>90</sub> of the antimicrobials for the planktonic and MICBR<sub>50</sub> and**338 **MICBR<sub>90</sub> of the biofilm forms of *Salmonella enterica* isolates.**

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Antimicrobial	mg/l		mg/l		Breakpoints (susceptible)
	planktonic MIC <sub>50</sub>	biofilm MICBR <sub>50</sub>	planktonic MIC <sub>90</sub>	biofilm MICBR <sub>90</sub>	
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

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