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SHORT COMMUNICATION

Detection of Biofilm Production of *Yersinia enterocolitica* Strains Isolated from Infected Children and Comparative Antimicrobial Susceptibility of Biofilm Versus Planktonic Forms

A. Ioannidis · A. Kyratsa · V. Ioannidou · S. Bersimis · S. Chatzipanagiotou

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Abstract
Background and Objectives The ability of *Yersinia* species to produce biofilms has not been hitherto systematically studied, although there is evidence, that *Y. enterocolitica* is able to form biofilms on inanimate surfaces. The present study aimed to detect the production of biofilms by 60 clinical strains of *Y. enterocolitica* and to compare the antimicrobial susceptibility of planktonic versus biofilm-forming bacteria.
Methods *Y. enterocolitica* strains were collected from stool and blood cultures collected from β-thalassaemic children, with gastroenteritis and/or septicemia. The isolated bacterial strains were grouped by biotyping and serotyping and the antimicrobial susceptibility of the planktonic forms was investigated by MIC determination. Biofilm formation was detected by the use of silicone disks and for the biofilm forming strains the minimum inhibitory concentration for bacterial regrowth (MICBR) of 11 clinically important antimicrobials was determined. The presence of the *waaE,* a gene reported to be related with biofilm formation was investigated in all the strains.
Results All of 60 strains were positive for biofilm production by the use of silicone disks. The great majority of the biofilm forms were resistant to all the antimicrobials. In antimicrobial concentrations far higher than the CLSI breakpoints, bacterial regrowth from the biofilms was still possible. None of the strains bore the *waaE* gene.
Conclusions These results, indicate that biofilm formation by *Y. enterocolitica* might be an inherent feature. The presence of biofilms increased dramatically the MICBR in all antimicrobials. The way in which biofilms could contribute to *Y. enterocolitica* pathogenicity in humans is a matter needing further investigation.

A. Ioannidis
Department of Nursing, Faculty of Human Movement and Quality of Life Sciences, University of Peloponnese, Orthias Artemidos and Plataion, 231 00 Sparta, Greece

A. Kyratsa · V. Ioannidou · S. Chatzipanagiotou (✉)
Department of Biopathology and Clinical Microbiology, Aeginition Hospital, Athens Medical School, Vass. Sophias av. 72, 115 28 Athens, Greece
e-mail: chatlouk@hotmail.com; schatzipa@gmail.com; schatzi@med.uoa.gr

S. Bersimis
Department of Statistics and Insurance Science, University of Piraeus, Piraeus, Greece

1 Introduction

Yersinia enterocolitica is a common enteric pathogen in children and adults, causing clinical syndromes, varying from mild gastroenteritis to severe lethal septicemia [1–3]. The pathogen is widely distributed throughout natural environments, in aquatic and animal reservoirs [2, 4]. The bacterium adheres and penetrates the ileum, causing terminal ileitis, lymphadenitis and acute enterocolitis with secondary manifestations of erythema nodosum, polyarthritis and less commonly septicemia and endocarditis [3–5]. Septicemia caused by *Y. enterocolitica* is almost exclusively associated with patients with iron overload or those being treated with the iron-chelating agent deferoxamine [6, 7].

Virulence in *Y. enterocolitica* is a complex interplay between ecology, geographic distribution, biochemical and antigenic properties, chromosomal and plasmid encoded genes [8, 9].

In recent years one of the most researched virulence factors in microbes in general, is their ability to produce

biofilms. 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 (ESP) of carbohydrate or exopolysaccharide adhering to synthetic or biological surfaces [10–13]. The encased sessile microorganisms bear quite distinct properties from those growing independently, or as planktonic populations, in liquid media.

The most important property of the biofilm forms in clinical medicine is the enhanced resistance to antimicrobial agents, through protection by the ESP, leading to multidrug resistance and therapeutic failure. Although the mechanisms are poorly understood, there is evidence that they 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 [14–17].

The ability of *Yersinia* species to produce biofilms has not been hitherto systematically studied, although there is evidence based on studies with very limited number of strains, that *Y. enterocolitica,* as well *Yersinia pseudotuberculosis* are able to form biofilms on inanimate surfaces [18]. Previous studies report the presence in one strain of *Y. enterocolitica* serotype O:8, of a gene sharing high homology with the *waaE* gene, which might be involved in biofilm synthesis in some *Enterobacteriaceae*, like *Klebsiella pneumoniae* and *Serratia marcescens* [19]. For *Y. enterocolitica* there are few studies investigating the signal pathway and the concomitant induced gene expression, but not the biofilm production [20–23].

The present study aimed to detect the production of biofilms and its relation to the presence of the *waaE* gene, in clinical strains of *Y. enterocolitica*, isolated from infected β-thalassaemic children, and to compare the antimicrobial susceptibility of planktonic versus biofilm-forming bacteria.

2 Methods

2.1 Patients and Sample Collection

During a 6-year period (2006–2011), 60 *Y. enterocolitica* strains were collected from an equal number of β-thalassaemic children with gastroenteritis and/or septicemia admitted at Thriassio General Hospital of Elefsina in Attica Greece. Forty-six strains were isolated from stool cultures, 11 from blood cultures and 3 from both stool and blood cultures. The age of children ranged between 6 months and 3 years.

2.2 Culture and Identification of *Y. enterocolitica*

Primary stool and blood culture were performed using selective media for *Yersinia* (CIN agar, Oxoid, Hampshire, England) and Mac Conkey No3, as well as, non selective media. Agar plates were incubated at 22–26 °C for 48 h.

Identification of presumptive *Yersinia* colonies was performed by conventional bacteriological methods and by means of a commercially available identification system (API 20E, Biomerieux, Marcy l' Etoile, France).Isolates were then stored at −70 °C in 50 % glycerol.

2.3 Biotyping and Serotyping

The isolated bacterial strains were grouped by biotyping, as described by Wauters et al. [24]. Serotyping was performed by slide agglutination with commercially available specific O-antisera for most common serotypes O:3 and O:9 (Diagnostics Pasteur, Marnes la Coqquette, France) in the Mediterranean region [25].

2.4 Detection of Biofilm Formation

Biofilm formation was detected by the use of silicone disks (Folio C6 0,25 mm, NOVATECH; new biotechnology for life ZI ATHELIA III-VOIE ANTIOPE 13705 LA CIOTAT CEDEX-FRANCE) as described previously [26]. Briefly, silicone disks cut in similar size (4–5 mm) and weight (25–30 mg) were placed into tubes and left overnight under UV irradiation for sterilization. Trypticase soya broth (2.5 ml) was added to each tube and the tubes were inoculated with *Yersinia* strains and incubated for 72 h at 30 °C. The bacterial suspension was then poured off, the tubes containing the silicone disks were washed 3 times with distilled water and air-dried in a laminar flow for 24 h. The silicone disks with the attached bacteria were weighed once more and the difference in weight showed the presence of biofilms.

2.5 Detection of *Y. enterocolitica waaE* Gene

The presence of the *waaE* gene was investigated as described by Izquierdo et al. [18] using the set of primers WAAE Y1—forward (5′-GTCATGGGATCGAACGTC-3′) and WAAE Y2—reverse (5′-CTGTTGACCGACGAAGACTA-3′). The PCR conditions were as follows: 5 min at 95 °C (one cycle), 1 min at 95 °C, 1 min at 60 °C, 1 min at 72 °C (35 cycles), 5 min at 72 °C (one cycle), storing at 4 °C. The PCR product was electrophoretically visualized.

2.6 *Y. enterocolitica* Antimicrobial Susceptibility of the Planktonic and Biofilm Forms

Antimicrobial susceptibility of the planktonic bacterial forms was performed by determination of the minimal inhibitory concentration (MIC). MIC was determined using two methods: (a) the automated system VITEK 2 (bioMérieux SA, 69280 Marcy-l'Etoile, France) and (b) the standard broth dilution method according to guidelines of the Clinical Laboratory Standards Institute (CLSI) [27, 28]. The antimicrobials included were those of importance for the treatment of *Y. enterocolitica* in the clinical practice: cotrimoxazole, amicacin, netilmicin, gentamicin, tobramycin, imipenem, ceftazidim, aztreonam, cefepime, cefotaxime, ciprofloxacin.

The strains producing biofilms were further tested for their antimicrobial susceptibility by determination of the minimal inhibitory concentration for bacterial regrowth from the biofilm (MICBR) using a modified broth dilution method as described previously [27, 28]. Silicone disks coated with the biofilm forming *Yersinia* 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 of the planktonic forms, were prepared and poured into the silicone disk containing tubes. The antimicrobial containing tubes were then incubated at 35 °C for 48 h. The growth of planktonic bacteria was visualized by the development of turbidity in the medium. The MICBR was defined as the lowest concentration showing no 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 Mc Conkey agar medium in order to check the purity of the grown *Yersinia* population.

The results for the planktonic, as well as for the biofilm forms were assessed using the breakpoints given by the guidelines of the CLSI [27, 28].

2.7 Statistical Analysis

Statistical analysis was performed using the statistical package SPSS for Windows (version 20.0) 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.

3 Results

From the 60 *Y. enterocolitica* strains, 59 belonged to serotype/biotype O:3/IV and one strain to O:9/II. None of the strains bore the *waaE* gene but all of strains were positive for biofilm production by silicone disks methods—disks showing a difference in weight before and after incubation of >50 mg). Although there were quantitative differences in biofilm production among the various strains, we could not assess biofilm production in relation to the biofilm weight, as our method was not standardized for that purpose. This would require very accurate and reproducible experimental conditions (e.g. number of inoculated bacteria, silicone surface, etc.).

The antimicrobial resistance rates of the planktonic and the biofilm bacteria are given in Table 1. The great majority of the biofilm forms were resistant to all the antimicrobials. In antimicrobial concentrations far higher than the CLSI breakpoints, bacterial regrowth from the biofilms was still possible (Table 2).

Since the P value for all antimicrobials was less than 1 %, all differences were assumed to be statistically significant (Table 2).

4 Discussion

The predominance of the serotype/biotype O:3/IV among the *Y. enterocolitica* strains coming from the area of Attica, found in the present study, has been previously reported, and seems to be enduring [2, 12, 25].

The most remarkable finding was the ability of all these strains to form biofilms, under conventional incubation conditions, needing no special stress inducing factors (such

Table 1 Antimicrobial resistance rates of planktonic and biofilm forms of *Yersinia enterocolitica* strains isolated from infected children

Antimicrobial	Planktonic (%)	Biofilm (%)	P
Cotrimoxazole	0	100	<0.001
Amicacin	1.66	93.34	<0.001
Netilmicin	1.66	95	<0.001
Gentamicin	0	100	<0.001
Tobramycin	0	100	<0.001
Imipenem	0	98.34	<0.001
Ceftazidime	0	100	<0.001
Aztreonam	0	100	<0.001
Cefepime	0	100	<0.001
Cefotaxime	1.66	100	<0.001
Ciprofloxacin	0	100	<0.001

P values refer to statistical tests used in the study

Table 2 MIC_{50} and MIC_{90} of the antimicrobials for the planktonic and $MICBR_{50}$ and $MICBR_{90}$ of the biofilm forms of *Yersinia enterocolitica* strains

Antimicrobial	mg/l		mg/l		Breakpoints (susceptible)
	Planktonic MIC_{50}	Biofilm $MICBR_{50}$	Planktonic MIC_{90}	Biofilm $MICBR_{90}$	
Cotrimoxazole	≤20	≥2,560	≤20	≥2,560	≤40
Amicacin	≤2	≥128	8	≥128	≤16
Netilmicin	≤1	≥32	2	≥32	≤8
Gentamicin	≤1	≥128	≤1	≥128	≤4
Tobramycin	≤1	≥128	≤1	≥128	≤4
Imipenem	≤1	≥128	≤1	≥128	≤1
Ceftazidime	≤1	≥128	≤1	≥128	≤4
Aztreonam	≤1	≥128	≤1	≥128	≤4
Cefepime	≤1	≥128	≤1	≥128	≤8
Cefotaxime	≤1	≥128	≤1	≥128	≤1
Ciprofloxacin	≤0.25	≥32	≤0.25	≥32	≤1

as carbon, nitrogen or iron depletion or low oxygen concentration). These results, as well as those from previous reports, indicate that biofilm formation by *Y. enterocolitica* might be an inherent feature [29]. However, the regulation of biofilm production in *Y. enterocolitica* remains still an unsolved issue. The polypeptide WaaE, encoded by the gene *waaE* is active as a glycosyltransferase in the substitution of α-L-glycerol-D-manno-heptopyranose by β-D-glucopyranose during the lipopolysaccharide inner-core biosynthesis procedure in the *Enterobacteriaceae*, and is indirectly involved in biofilm formation regulation [18, 19, 30]. The expression of *waaE* has been reported in a strain of *Y. enterocolitica* serotype O:8 in previous studies. The gene was sharing high homology with the *waaE* gene, involved in biofilm synthesis in some *Enterobacteriaceae*, like *Klebsiella pneumoniae* and *Serratia marcescens* [18, 19].

The absence of *waaE* in our strains could be explained, either because the specific gene might not be necessary for the lipopolysaccharide inner-core biosynthesis in serotypes O:3 and O:9, or because the latter bear a related gene, which was not detected by the primer sequence used in our study.

According to our results it is not possible to draw any conclusions about any association of biofilm production with *Y. enterocolitica* virulence, because all the strains included in the study were biofilm producing and clinically relevant.

Regarding the antimicrobial resistance, just like in previous reports [31], the planktonic forms were well susceptible to the antimicrobials used in the clinical practice and included in the study, such as the aminoglycosides, cotrimoxazole, quinolones and some newer β-lactam antibiotics (Tables 1, 2). The presence of biofilms increased dramatically the MICBR in all antimicrobials. Although there are no standard procedures for the determination of MICBR, our results are in agreement with previous reported data, with respect to the role of biofilms in the increase of the bacterial antimicrobial resistance [32]. Moreover, they confirm that the experimental conditions used, led to biofilm synthesis. In imipenem, the increase of resistance, was in few strains less, compared to other β-lactam antibiotics. This might be due to the ability of the carbapenems, including imipenem, to inhibit various penicillin-binding proteins, practically acting this way against cells being in a metabolic stationary phase, like bacteria in mature biofilms [33, 34]. The relatively better activity of netilmicin against biofilm forms, with a lower MIC versus the other aminoglycosides, is difficult to explain, because the conditions prevailing in the biofilm micro-environment, with the low oxygen pressure and the concomitant reduction of the cellular respiration, as well as the inhibition of the penetration of the biofilm by the negatively charged aminoglycoside molecule are disfavouring the action of this antibiotic group [33].

Biofilm production belongs to the virulence factors of many microbial species, and in some cases, such as *Staphylococcus epidermidis* and *Pseudomonas aeruginosa,* to the most important ones for the expression of pathogenicity in infectious diseases [32, 35, 36]. In *Y. enterocolitica*, most of the studies regarding virulence focus on the complex mechanisms of enterocyte invasion, while biofilm formation has been given little attention. The way in which biofilms could contribute to *Y. enterocolitica* pathogenicity in humans is a matter needing further investigation. The bacterium owns features that might favour and initiate biofilm synthesis through exopolysaccharide production, such as the enhanced cell surface hydrophobicity and self agglutination [37]. A better understanding of biofilm formation by *Y. enterocolitica* various serotypes and biotypes, as well as by other *Yersinia* species, would contribute to a

better understanding of their pathogenicity in human infections.

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