Typing of the pilus-protein-encoding FCT region and biofilm formation as novel parameters in epidemiological investigations of Streptococcus pyogenes isolates from various infection sites

Thomas Köller,1† Andrea Guido Oreste Manetti,2† Bernd Kreikemeyer,1 Cordula Lemcke,1 Immaculada Margarit,2 Guido Grandi2 and Andreas Podbielski1

1Institute of Medical Microbiology, Virology and Hygiene, University Hospital, Schillingallee 70, D-18057 Rostock, Germany
2Novartis Vaccines and Diagnostics, Via Fiorentina 1, 53100 Siena, Italy

Streptococcus pyogenes is an important human pathogen for which an association between infection site and selected epidemiological or functional markers has previously been suggested. However, the studies involved often used strains with an insufficiently defined clinical background and laboratory history. Thus, the major goal of the present study was to investigate these relationships in 183 prospectively collected, well-defined, low-passage isolates from a North-East German centre for tertiary care. For each isolate the clinical background (91 respiratory, 71 skin and 21 invasive isolates) and antibiotic-resistance pattern was recorded. All isolates were classified according to their emm type, antibiotic-resistance and PFGE pattern (SmaI restriction analysis of genomic DNA). As novel discriminatory methods we performed a PCR-based typing of the pilus-protein-encoding FCT region (FCT) and biofilm-formation phenotyping in various culture media. Forty-one isolates were found to be resistant to at least one of the tested antibiotics. emm typing revealed emm28, emm12, emm1, emm4, emm89 and emm2 as the most frequent types in our collection. The novel FCT typing showed isolates encoding FCT types 4 and 2 to be the most common. Overall 113 strains with unique combinations of emm and FCT types, antibiotic-resistance and PFGE patterns were identified. The majority of all isolates revealed an association of biofilm-formation capacity with growth media. Comparing all results for potential associations, no correlation could be established between the anatomical site of isolation and the emm or the FCT type. There was no relationship between biofilm formation and emm type, antibiotic-resistance or PFGE patterns. However, a novel association between biofilm formation and FCT type became obvious among strains from our collection.

INTRODUCTION

Streptococcus pyogenes[group A streptococci (GAS)] is a very common and predominantly human pathogen causing a wide variety of diseases. The most frequent diseases are mild suppurative throat and skin infections (estimated 727 million cases per year worldwide). Severe invasive diseases (estimated 663,000 cases per year) like meningitis, necrotizing fasciitis and the streptococcal toxic shock syndrome occur more rarely (Carapetis et al., 2005; Cunningham, 2000; Bisno, 1991; Bisno & Stevens, 1996; Wahl et al., 2007; Stevens, 1996). S. pyogenes also causes non-suppurative post-infection sequelae (1.1 million cases per year) such as rheumatic fever, acute glomerulonephritis and obsessive compulsive neurological disorders (Carapetis et al., 2005; Bisno, 1991; Potter et al., 1978; Bessen et al., 1989; Nordstrand et al., 1999; Couser, 1999). Rheumatic fever, as well as the invasive infections, result in striking annual morbidity and lethality rates (estimated 1.78 million cases and 517,000 deaths per year) (Carapetis et al., 2005; Cunningham, 2000; Wahl et al., 2007; Stevens, 1996).

Serological differentiation of the surface attached, antigenically variable, major virulence factor M protein allowed the discrimination of up to 80 different serotypes

†These authors contributed equally to this paper.

Abbreviations: CLSI, Clinical and Laboratory Standards Institute; GAS, group A streptococci.
A table of isolate information and figures of FCT-typing and biofilm-assay data are available as supplementary material with the online version of this paper.
among the GAS (Lancefield, 1928, 1962). More recently, genotyping of the M-protein-encoding emm gene was established (Podbielski et al., 1991, 1994). This method currently permits the identification of more than 200 S. pyogenes emm genotypes (emm types) as listed in the S. pyogenes emm sequence database of the Center for Disease Control and Prevention (http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm; Li et al., 2003). Recent studies performed in industrialized countries similarly demonstrated emm1, emm3, emm12 and emm28 as the most prevalent emm types associated with around 50% of all clinical cases (Wahl et al., 2007; Brandt et al., 2001; Luca-Harari et al., 2008; Vlaminckx et al., 2004).

Genes encoding virulence factors and regulators, e.g. the major fibronectin-binding proteins F1 and 2 (prfF1, prfF2), the pilus-associated proteins (cpa, fctA, fctB) and the RofA/Nra regulators (rofA, nra), are located in the FCT region of the S. pyogenes genome (Bessen & Kalia, 2002; Ramachandran et al., 2004; Nakata et al., 2005; Mora et al., 2005). The encoded factors were shown to play a role in matrix protein binding and global virulence regulation in selected emm-type strains (Manetti et al., 2007; Abbot et al., 2007; Podbielski et al., 1999; Kreikemeyer et al., 1995, 2005, 2007). This region is variable in both gene content and sequence. Currently nine different FCT types can be distinguished (Kratovac et al., 2007; Falugi et al., 2008). These FCT types have been demonstrated to include different subsets of emm genotypes. It is still not known whether this association consistently applies to multiple strains of given emm types or if the association could be extended to the anatomical sites from which the isolates originated.

Biofilms are known to be the most ubiquitous form of life among microorganisms and are of special significance in medicine (Hall-Stoodley et al., 2004; Parsek & Singh, 2003; Donlan & Costerton, 2002; Cvitkovitch et al., 2003). Bacteria forming biofilm structures show increased resistance against environmental influences compared to planktonic cells of the same species (Donlan & Costerton, 2002; Baldassarri et al., 2006; Conley et al., 2003; Gualtieri et al., 2006). S. pyogenes was shown to form mono-species biofilms in vivo and under in vitro conditions. The ability to build a biofilm apparently varies between individual strains and, so far, has not been associated with the above-mentioned typing schemes (Potter et al., 1978; Conley et al., 2003; Baldassarri et al., 2006; Akiyama et al., 2003; Lembke et al., 2006; Takemura et al., 2004). Courtney and co-workers recently showed that the complex formation of M protein with lipoteichoic acid is crucial for surface hydrophobicity and biofilm formation (Courtney et al., 2009). Furthermore the pilus-associated genes were demonstrated to be involved in biofilm formation in the M1 SF370 strain belonging to the FCT 2 type (Manetti et al., 2007). However, the association between pilus and biofilm formation has not been studied on a broader scale.

The present study investigated a collection of 183 S. pyogenes isolates prospectively isolated from 2001 to 2006 in the University Hospital of Rostock (Germany). Their emm and FCT types, as well as their PFGE and antibiotic-resistance patterns, were assessed to classify them and to get information about their population diversity. We compared these data for all isolates to figure out relationships between the screened features. In addition, we measured biofilm formation with selected isolates from the identified clonal strains using different culture media. When comparing this biological function with the above-mentioned features, new associations could be established. They appear to be representative for a multitude of strains of given emm or FCT types rather than for the clinical background of such strains. Moreover, on the basis of this study we stress the necessity for rigorous standards to determine (i) how isolates should be collected, (ii) how the clinical background is recorded and (iii) which parameters need to be determined to perform epidemiological studies with S. pyogenes.

METHODS

Isolates. The S. pyogenes isolates were collected prospectively from patients of the University Hospital, Rostock (Mecklenburg Vorpommern, Germany), from 2001 to 2006. The hospital functions as a centre for tertiary care with 1070 patient beds and serves approximately 500 000 inhabitants. The patients represented three groups: (i) patients suffering from pharynx infections, including tonsillitis and tonsillar abscesses, (ii) patients suffering from skin infections at any location, including local abscesses, and (iii) patients with invasive diseases, i.e. the presence of bacteria at normally sterile anatomical sites. The S. pyogenes isolates were included in the collection according to the following criteria: (i) the originating persons had to be inpatients of the University Hospital, Rostock, (ii) the aetiopathology and class of infection had to be thoroughly stated in the accompanying documents, (iii) patients were not immunocompromised, (iv) isolates had to have been identified and processed in the diagnostic laboratory of the University Hospital, (v) S. pyogenes had to be the only pathogen in the specimen, (vi) only the first isolate per patient was included in the collection.

All isolates were differentiated as β-haemolytic streptococci of serological group A using the BBL Streptococcal enzyme latex test agglutination kit and the API 20 Strept biochemical differentiation scheme (bioMérieux) according to the manufacturers’ protocols. Isolates were aliquoted in Todd–Hewitt broth supplemented with 0.5% yeast extract [Todd–Hewitt yeast (THY) medium; Oxoid] and 20% (v/v) glycerol, after a maximum two passages on Columbia sheep blood agar in the diagnostic laboratory of the University Hospital, Rostock, and were subsequently stored at – 80 °C. All subsequent analytical procedures were performed by starting a pre-culture in THY broth using a storage culture aliquot to exclude selection effects due to extensive passaging (37 °C, 5% CO₂/20% O₂.). For molecular analysis cells were cultured in THY broth at the above described conditions until an OD₆₀₀ of 0.6 was reached. For biofilm analyses, cells were cultured in C medium (Manetti et al., 2007), tryptic soy broth (TSB; Oxoid), THY medium (Oxoid) and brain heart infusion broth (BHI) medium (Oxoid) for 24 h at 37 °C, 5% CO₂. If not stated otherwise, all different batches of media and the diagnostic reagents were purchased from Becton Dickinson.

Antibiotic-resistance test. The antibiotic susceptibility of all isolates was assessed by agar diffusion tests according to Clinical and Laboratory Standards Institute (CLSI) directives M100-S16 and
M2-A9 (CLSI, 2006a, b). Briefly, all isolates were suspended in 100 µl 0.9% NaCl to reach a final OD_600 of 0.5. Subsequently isolates were plated on Mueller–Hinton agar supplemented with 5% sheep blood. Antibiotic discs contained penicillin (10 µg), vancomycin (30 µg), erythromycin (15 µg), clindamycin (2 µg), levofloxacin (5 µg), tetracycline (30 µg) and gentamicin (30 µg). Antibiotic discs were stamped on prepared plates using a BD disc dispenser. The plates were incubated for 18–24 h at 35 °C in 20% O_2/5% CO_2 atmosphere. Inhibition zones were evaluated and interpreted according to CLSI standards.

**PFGE.** PFGE analysis was performed according to established protocols (Miranda et al., 1991) with minor modifications. Briefly, cells were cultured in 5 ml THY medium overnight. Approximately 10^7 S. pyogenes cells were transferred into casting wells and covered with agarose (Plaque GP agarose; Biozym). The resulting blocks were then exposed to mutanolysin, lysozyme (both Difco) and phagelysin C (kindly provided by Vincent Fischetti, Rockefeller University, New York, USA, and Daniel Nelson, University of Maryland, Rockville, MA, USA) at 37 °C for 5 h. The liberated genomic DNA was digested overnight using Smal restriction enzyme. After digestion the blocks were cut into plugs of 3 x 5 x 1 mm and embedded into 1% running gels with 0.5% Tris/borate/EDTA buffer (pH 8). The PFGE was performed with a CHEF-DRIII electrophoresis system (Bio-Rad Laboratories) for 22 h using the following settings: initial switch time, 5 s; final switch time, 35 s, voltage, 5.5 V cm⁻¹. Gels were stained with ethidium bromide, and images captured with the Gel Jet imager (Intas Science Imaging Instruments) and analysed with GelCompar II software using the UPGMA algorithm with Dice coefficients (Applied Maths) (Chiou et al., 2004).

**DNA extraction and FCT typing.** Purification of chromosomal DNA from GAS was performed according to the protocol of Cleary et al. (1988). The emm typing was performed according to established instructions using ‘all emm’ primer sequences (Beall et al., 1996; Podbielski et al., 1999). PCR products were purified with the PCR-clean-up system (Promega) and sequenced on both strands. Sequencing was repeated on two independent occasions using the ABI PCR-clean-up system (Promega) and sequenced on both strands. DNA extraction and FCT typing was performed for each strain on two independent occasions (biological replicates) with each representative isolate (data not shown). Experiments with C medium and BHI broth were repeated on four independent occasions. Results for the two latter media are shown as mean values for each representative isolate. Moreover, selected isolates of each FCT type were checked on five independent occasions (biological replicates) using at least three different medium batches (Supplementary Fig. S2 available with the online journal).

## RESULTS AND DISCUSSION

### Clinical data

During the study period 2001–2006, 183 out of a total number of 217 S. pyogenes isolates fulfilled the inclusion criteria of this study and were included in the strain collection. The annual distribution of the collected strains was as follows: 2001, 19; 2002, 32; 2003, 40; 2004, 30; 2005, 41; 2006, 21. The isolates originated from upper respiratory tract infections (91 strains, 50%), skin infections (71 strains, 39%) and invasive diseases (21 strains, 11%) (Supplementary Table S1 available with the online journal). Of the patients 109 (60%) were male, 74 (40%) female. With respect to age distribution for all cases the peak incidence was for patients from 0 to 10 years. The incidence sharply decreased in the middle decades of age and only slightly increased again for patients above 70 years (Fig. 1). With respect to infection sites, the rate of upper respiratory tract infections decreased whereas skin and invasive infection rates increased for elderly patients (Fig. 1).

Several epidemiological conclusions can be drawn from the data above. First, the clinical background of our collection (Fig. 1), reflects the regional ratio between S. pyogenes upper respiratory tract, skin and invasive infections leading to admission in a tertiary care hospital. Second, within the local population S. pyogenes infections affected more men than women. This difference was predominantly due to a higher rate of skin infections in our male patients. This finding is consistent with most recent observations from several European countries (Lamagni et al., 2008).

Third, with respect to the age of our patients, the incidence of all S. pyogenes infections decreased over life time. This observation is different from other studies that predominantly included patients suffering from invasive diseases (Darenberg et al., 2007; Ekelund et al., 2005; Luca-Harari et al., 2008). The relatively small number of invasive disease cases among our patients also showed a positive association with increasing age. According to the present investigation, the number of pharyngitis patients sharply drops above the age of 40, while skin infections become predominant. Finally, there could be regional differences in the age distribution, since, similar to the present, data a prevalence removing the staining solution, the wells were washed twice with PBS. Remaining crystal violet firmly associated to the cells was dissolved with 1 ml aqueous 1% SDS solution shaking for 10 min. Quantification was achieved by diluting the solution 1:10 in ddH_2O and subsequently measuring the OD_600 (Manetti et al., 2007). Analyses with TSA and THY medium were performed in triplicate on two occasions (biological replicates) with each representative isolate (data not shown). Experiments with C medium and BHI broth were repeated on four independent occasions. Results for the two latter media are shown as mean values for each representative isolate. Moreover, selected isolates of each FCT type were checked on five independent occasions (biological replicates) using at least three different medium batches (Supplementary Fig. S2 available with the online journal).
peak in patients below 40 years was also observed in two other Dutch and German studies (Vlaminckx et al., 2004; Wahl et al., 2007).

emm typing

emm typing identified 25 different emm genotypes in 183 isolates (Table 1, Fig. 2). The most frequently identified emm types were emm28 (24 %), emm12 (11 %), emm1 (11 %), emm4 (8 %), emm2 (7 %) and emm89 (7 %). Altogether, these six emm types accounted for 68 % of the isolates. The diversity and frequency of identified emm types among our isolates resembles those from comparable studies in Germany, Denmark, Sweden and Poland (Darenberg et al., 2007; Ekelund et al., 2005; Luca-Harari et al., 2008; Reinert et al., 2004; Szczypa et al., 2004; Wahl et al., 2007). Eight out of the eleven most frequently found emm types in those studies were also among the ten most frequent emm types in our study. This illustrates a well-known regional geographical stability of certain emm types over a limited time period. Over the sampling period of 6 years, no change of the prevailing emm types was observed. Compared to other studies (Colman et al., 1993), this unexpected result could be due to the small sample size or the short sampling period. Different from former studies, we could not demonstrate an association between isolation sites and emm types among our strains (Fig. 2). This result is beyond doubt for clonal isolates from different infection sites. However, for the majority of individual strains the result could have been affected by the sampling size in the present study.

Antibiotic resistance

Analysis of the antibiotic-susceptibility profiles of all the isolates showed 41 of 183 (22 %) isolates to be resistant against one or more antibiotics (Table 2). The most frequent unique resistance traits were those directed against erythromycin (ten isolates), tetracycline (eight isolates), levofloxacin (six isolates) and gentamicin (six isolates). Less frequently we identified combined antibiotic resistance traits against erythromycin/tetracycline (five isolates), erythromycin/levofloxacin (three isolates), levofloxacin/tetracycline (two isolates) and gentamicin/levofloxacin (one isolate) (Table 2). The recorded erythromycin resistance rate of 9.8 % was below the 14 % rate recently reported for juvenile and adult Germans (Reinert et al., 2003, 2004; Sauerermann et al., 2003). This could be due to a sampling area mostly in the western and southern parts of the country in the three studies. Correspondingly, erythromycin resistance rates among patients from geographically less distant countries such as Denmark and Poland ranged between 3 and 12 % (Luca-Harari et al., 2008; Szczypa et al., 2004, 2006).

The small but noticeable levofloxacin-resistance rate among our isolates is remarkable. This resistance trait was extremely rare in European isolates during the sampling period (0–0.2 %) (Biedenbach et al., 2006; Cantón et al., 2002; Sauerermann et al., 2003). Locally higher rates were associated with the occurrence of specific GAS emm6 clones (Alberti et al., 2005; Orscheln et al., 2005). Among our 11 levofloxacin-resistant strains, only 2 carried the emm6 gene and all isolates belonged to different

Table 1. emm types identified in the present clinical S. pyogenes strain collection

<table>
<thead>
<tr>
<th>emm type</th>
<th>No.</th>
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<tbody>
<tr>
<td>emm28</td>
<td>44</td>
</tr>
<tr>
<td>emm12</td>
<td>21</td>
</tr>
<tr>
<td>emm1</td>
<td>20</td>
</tr>
<tr>
<td>emm4</td>
<td>15</td>
</tr>
<tr>
<td>emm2</td>
<td>13</td>
</tr>
<tr>
<td>emm89</td>
<td>14</td>
</tr>
<tr>
<td>emm6</td>
<td>8</td>
</tr>
<tr>
<td>emm77</td>
<td>8</td>
</tr>
<tr>
<td>emm75</td>
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<td>emm118</td>
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<td>emm78</td>
<td>6</td>
</tr>
<tr>
<td>emm58</td>
<td>14</td>
</tr>
<tr>
<td>emm3</td>
<td>2</td>
</tr>
<tr>
<td>emm44</td>
<td>2</td>
</tr>
<tr>
<td>emm94</td>
<td>2</td>
</tr>
<tr>
<td>st794</td>
<td>2</td>
</tr>
<tr>
<td>emm109</td>
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</tr>
<tr>
<td>emm49</td>
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<tr>
<td>emm67</td>
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</tr>
<tr>
<td>emm81</td>
<td>1</td>
</tr>
<tr>
<td>emm83</td>
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</tr>
<tr>
<td>st3765</td>
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</tr>
<tr>
<td>stG61</td>
<td>1</td>
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clonal categories, indicating different and independent mutation or selection events as a basis for the observed prevalence of levofloxacin resistance and strain diversity.

As expected, all isolates were found to be susceptible to penicillin and vancomycin under the tested conditions. Depending on the infection site, we detected different proportions of resistant isolates (Fig. 3a). While 33% of all isolates obtained from patients with respiratory diseases showed at least one resistance trait, only 19% of the invasive isolates and 10% of all skin isolates did so. Of note, our pharyngitis isolates displayed a threefold higher erythromycin-resistance rate than the simultaneously collected skin isolates, while the clonal relatedness between the resistant isolates was equally low in both groups (Supplementary Table S1 available with the online journal). When comparing isolates from invasive diseases and asymptomatic pharyngeal carriers, Baldassarri and co-workers made a similar observation (Baldassarri et al., 2007). In both studies, a co-segregation of macrolide-resistance genes and site-specific, but varying, virulence traits as described by Creti et al. (2005), Haller et al. (2005), Robinson et al. (2006) and Spinaci et al. (2004) could be a possible explanation.

The proportions of isolates resistant to one or more antibiotics were found to vary among different emm types (Table 2). For instance only 14% of all emm28 and emm12 isolates displayed any antibiotic resistance. The percentages for the emm1, emm4 and emm77 were 35, 47 and 50%, respectively (Table 2).

**PFGE typing**

PFGE typing performed for all isolates resulted in the identification of 94 PFGE band patterns (Supplementary Table S1 available with the online journal). Seven isolates were not assignable because no band pattern could be achieved using the protocol described in Methods. According to the commercial analysis program, 82 isolates showed a similar or identical PFGE band pattern to other isolates.

The diversity of PFGE patterns varies between the different emm types and within the same emm type. Among the 44 emm28 isolates we observed 21 different PFGE patterns. Thus 50% of the isolates shared their PFGE pattern with another isolate (i.e. 50% of emm28 isolates are clonally related) (Supplementary Table S1 available with the online journal).

**Table 2. Antibiotic-resistance analysis**

The number of strains with specific antibiotic-resistance traits is shown in relation to their emm type. All, specific antibiotic-resistance traits among all tested S. pyogenes isolates; emm type, absolute numbers and ratios of resistant isolates relative to their emm types; resistant, percentage of antibiotic-resistant isolates among all tested streptococci or strains of a given emm type.

<table>
<thead>
<tr>
<th>Antibiotic resistance</th>
<th>emm type</th>
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<tbody>
<tr>
<td></td>
<td>All emm28 emm12 emm1 emm4 emm89 emm2 emm6 emm77 emm78 emm58 emm94 st794 emm81</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>10 1 2 4 1 1 1 1</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>8 1 2 2 1 1 1 1</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>6 1 1 2 2 2 2 2</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>6 1 1 1 1 1 1 1</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>0 0 0 0 0 0 0 0</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0 0 0 0 0 0 0 0</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>0 0 0 0 0 0 0 0</td>
</tr>
<tr>
<td>Erythromycin/tetracycline</td>
<td>5 1 1 1 1 1 1 1</td>
</tr>
<tr>
<td>Gentamicin/levofloxacin</td>
<td>3 1 1 1 1 1 1 1</td>
</tr>
<tr>
<td>Levofloxacin/tetracycline</td>
<td>2 1 1 1 1 1 1 1</td>
</tr>
<tr>
<td>Total</td>
<td>41 6 3 7 7 2 3 4 1 1 1 1</td>
</tr>
<tr>
<td>Resistant (%)</td>
<td>22% 14% 14% 35% 47% 14% 15% 38% 50% 17% 75% 50% 50% 100%</td>
</tr>
</tbody>
</table>
For the 21 emm12, 20 emm1 and 13 emm2 isolates we found a lower diversity of 5 PFGE patterns (76% clonally related strains), 6 PFGE patterns (70% clonally related strains) and 4 PFGE patterns (69% clonally related strains), respectively. A higher diversity was detected for 15 emm4 and 14 emm89 isolates with 10 PFGE patterns (13% clonally related strains) and 7 PFGE patterns (46% clonally related strains), respectively.

Isolates with frequently detected emm types typically displayed one major PFGE pattern (majority of isolates). The other PFGE patterns were only found for one or a few isolates. For instance, 14 of 42 emm28 isolates displayed the PFGE pattern 7, while only 4 isolates were found to share the PFGE pattern 4. Of note, isolates associated with the same clonal group were found to originate from different infection sites. This indicates that even genetically identical S. pyogenes showed poor tissue preferences.

**FCT typing**

Most recently, the major fibronectin- and collagen-binding proteins encoded by the FCT region were suggested to be involved in S. pyogenes tissue specificity (Abbot et al., 2007; Lizano et al., 2007; Manetti et al., 2007). Arguments are based on their specific functions, variable presence and sequence in different GAS strains, as well as a temperature-dependent expression profile consistent with skin infection conditions (Nakata et al., 2009).

Several attempts to demonstrate such correlations have been made by screening clinical S. pyogenes isolates for the existence of selected FCT region genes (Kreikemeyer et al., 2004; Baldassarri et al., 2007; Musumeci et al., 2003). Overall these attempts did not result in clear conclusions concerning the role of individual FCT region factors for infection targeting. Consequently, instead of identifying selected FCT genes the FCT types were determined for 183 isolates. The most frequent FCT type was FCT 4 detected in 105 isolates. The other FCT types were found as follows: FCT 1, 11 isolates; FCT 2, 22 isolates; FCT 3, 10 isolates; FCT 5, 15 isolates; FCT 6, 14 isolates; FCT 7, 0 isolates; FCT 8, 0 isolates; FCT 9, 6 isolates (Fig. 4). No FCT type showed an above-average association rate for a specific infection site (Fig. 4). Obviously no simple correlation exists between the presence of certain FCT region types and the anatomical site from where GAS isolates originate. Since the expression of the FCT region factors is controlled by several regulators in an FCT type-or even strain-dependent fashion, and involves other peculiar regulatory mechanisms (Nakata et al., 2005, 2009; Kreikemeyer et al., 2007), correlating the expression levels with anatomical sites could perhaps give a better clue.

FCT types 3 and 4 included the highest quantity of different emm types (8 and 12 emm types, respectively, Fig. 4b). Furthermore, we identified some emm type strains that had not been FCT-typed by Kratovac et al. (2007) (emm78, FCT 4; st794, FCT 3 and FCT 4; stG61, FCT 6). With respect to clonal groups as defined by identical PFGE patterns and emm types, again the majority of such clonal groups clustered in FCT type 4. Of note, we found five isolates that were associated with other FCT types than the majority of isolates with the same emm type (emm28 – 2 × FCT 2, emm75 – FCT 4, emm12 – FCT 3, emm118 – FCT 1). Also the isolates of the newly defined emm type st794 belonged to FCT types 3 and 4 (Fig. 4b).

Another still unanswered question is what is the potential contribution of the FCT region factors to S. pyogenes persistence after an acute infection? This question cannot be directly addressed using our data, since our collection did not include strains from asymptomatic carriers. However, GAS growth in superficial and intra-tissue mono-species biofilms has been used as an alternative explanation for antibiotic therapy failure and persistence (Baldassarri et al., 2006; Conley et al., 2003).

**Classification of strains**

Next, an assignment of isolates was carried out by integrating all previously reported results. The number of isolates – according to different collection criteria – showing distinct properties for at least one of the three typing parameters (emm type, PFGE type and FCT type) was 95. This corresponds to 52% of the complete strain collection. If antibiotic resistance was added as a
distinction criterion, the total number of unique isolates increased to 113, i.e. 62% of the total number of isolates. A total of 66 isolates were recognized to have characteristics identical to one of the 113 distinct strains and thus were regarded as clonally related to one of these distinct isolates (Supplementary Table S1 available with the online journal). Four isolates could not be assigned to either group because of incomplete typing results. According to the now defined classification directives we identified 40 of 41 antibiotic resistant isolates as distinctive. This shows a much stronger strain diversity of resistant isolates (only 2.5% clonally related strains) compared to susceptible isolates [73 distinct isolates, 65 clonally related isolates (47% clonally related strains)].

**Biofilm formation**

A role of the FCT 2-encoded pilus proteins in biofilm growth was recently demonstrated (Manetti et al., 2007). For this reason, and in order to establish biofilm testing as a novel epidemiological marker for *S. pyogenes*, we tested the biofilm formation capacity of representative isolates. We found sufficient data to support a type-specific function of FCT-region factors in biofilm development and/or maturation. However, this correlation was superimposed in the majority of cases by culture medium effects (Fig. 5). In each of the earlier publications on *S. pyogenes* biofilm formation, different culture media were used. In this study we tested C medium, THY, TSB and BHI media to compare their influence on biofilm formation measured using a plate assay after 24 h incubation periods. In the following section only the results for C medium and BHI broth are shown (Fig. 5a). Biofilm growth was tested for each of the 183 isolates. Within the typical range of strains with the same FCT type, the clonal isolates formed biofilms like the corresponding distinct strains. In order to exclude statistical bias due to clonal isolates, only the results obtained with the 113 distinct isolates are shown in this paragraph.

Biofilm formation experiments using C medium generally showed smaller biofilm masses for the majority of isolates than experiments using BHI medium, except for FCT 1 strains (see below). Results for strains cultured in THY medium or TSB were generally ranged in-between the results from the other two media.

As already seen for the FCT types, biofilm formation behaviour was found to be independent from the
anatomical site of isolation. Also the antibiotic-resistance patterns could not be associated with a special capacity to form ‘in vitro' biofilms (Fig. 3b). We found selected isolates to tolerate higher antibiotic concentrations of penicillin, erythromycin and tetracycline added to mature ‘in vitro' biofilms independent from their individual antibiotic-resistance profile (unpublished data). These results indicate the lack of genetic linkage between genes responsible for biofilm formation and conventionally determined antibiotic resistance, and simultaneously, the protection against defined antibiotic activities among biofilm-organized cells. Our data differ from those of Baldassarri et al. (2006) who demonstrated the ability to grow large biofilm masses was associated with a lack of erythromycin resistance in planktonic bacteria. When looking at the results of these authors, strains without protein F1 genes were more strongly associated with biofilm-formation capacity, indicating that Baldassarri and colleagues could have detected a special subset of S. pyogenes strains (Baldassarri et al., 2006).

However, we identified an association between several FCT types and corresponding biofilm-formation behaviour. FCT 1-associated strains were the only ones that showed biofilm formation irrespective of the culture media used (Fig. 5a). This observation should be kept in mind when interpreting the, so far, single transcriptome data on S. pyogenes biofilms obtained with an emm6 FCT type 1 strain (Cho & Caparon, 2005). In that study, Caparon and co-workers found the emm gene but no FCT region genes to be markers for biofilm formation. For a more representative view on the biofilm-relevant GAS transcriptomes, isolates from the much more frequent FCT types 3 and 4 should be analysed. Yet, biofilm formation and maturation is a multi-step process that obviously follows different rules in various S. pyogenes strains. Thus, even if FCT region factors obviously contribute to this feature, a complementary function is exerted by other factors such as M proteins in subsets of S. pyogenes strains. Experimental support for such a complementary function was most recently provided by work from Courtney et al. (2009). They proved that complexes of M protein with lipoteichoic acid contribute to biofilm formation (Courtney et al., 2009).

Strains belonging to FCT types 2, 5 and 6 (Fig. 5a) displayed biofilm development depending on the type of culture medium used for the assay. Representatives of FCT type 9 formed weak biofilm in all tested media (Fig. 5a).

While all strains with FCT types 1, 2, 5, 6 and 9 showed similar biofilm-formation rates for a given FCT type, strains with FCT types 3 and 4 displayed a heterogeneous biofilm growth within their FCT groups (Fig. 5a). One group of FCT 3 type strains (emm12, emm15, emm3, emm83, emm94) formed biofilm when cultured in BHI medium but not in C-medium, while the other group (emm49, emm67, st794) were poor biofilm formers in both media (Fig. 5b). For FCT 4 type strains the biofilm formation capacities were even more heterogeneous,
displaying different combinations of high and low biofilm masses when grown in different media (Fig. 5bii, right).

Based on the limited number of isolates studied here the association between FCT types and biofilm-formation capacity was stronger than any association between emm type and biofilm. For example, emm28 strains belonging to FCT 2 type behaved like other FCT 2 type strains, while all emm28 strains belonging to FCT 4 type collectively showed a different biofilm formation behaviour (Fig. 5c). A similar observation was made for the emm75 strains and their association to FCT types 4 and 9, the emm12 strains and their FCT types 3 and 4, and the emm118 strains associated to FCT types 1 and 4 (Fig. 5c).

Overall, the majority of the tested strains formed biofilms depending on culture conditions. Only 11 FCT 1 type strains were found to consistently build biofilms independent of the employed media. Moreover only a minority of three FCT type 3, two FCT type 9 and eight FCT type 4 strains showed no biofilm formation under any culture condition.

On the basis of our data we suggest that C medium and BHI medium are the best media for comparative GAS biofilm investigations. This information should be very useful for other groups who would like to include biofilm formation as one parameter for future epidemiological studies. After elucidation of FCT types it is now clear which medium should be used. Our study has unequivocally demonstrated that the biofilm feature of GAS is stable, reliable and independent of biological replicate experiments and different medium batches (Supplementary Fig. S2 available with the online journal).

The major goal of the present investigation was to assess the association between established genetic traits (emm genotyping, PFGE and antibiotic resistance), novel genetic traits (FCT typing), functional features (biofilm formation capacities) and clinical background in a thoroughly defined collection of S. pyogenes hospital isolates. In conclusion, the rigorous inclusion criteria, as well as the thorough genetic and functional characterization of the strain collection, guaranteed that only isolates with an infectious background were taken into consideration. The thorough typing was particularly necessary since a strain collection from one hospital has a higher chance to predominantly include clonal isolates. An even number of samples per year as well as the identification of 113 distinct isolates (according to the typing criteria) among the different 183 isolates (whole strain collection) demonstrates the low degree of clonality in our clinical S. pyogenes collection. This further points towards the extent of diverse infectious contacts even in a comparatively small population of potential hosts. Determination of the clonality status of each isolate and the normalization of the analyses by operating with only one representative isolate per identified strain is a clear advantage over retrospective studies. Those previous studies often relied on strain collections sampled outside the analysing laboratory, frequently under varying criteria over the sampling period, not devoid of copy strains and with incomplete clinical background information. Furthermore, in our collection the passage numbers after sampling were recorded, which makes the interpretation of functional data, such as antibiotic resistance or biofilm formation, more reliable. In summary, our data indicate that FCT region typing extends the panel of methods for characterization of S. pyogenes strains. Moreover, biofilm formation capacity in defined media could be exploited as a new epidemiological marker.

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