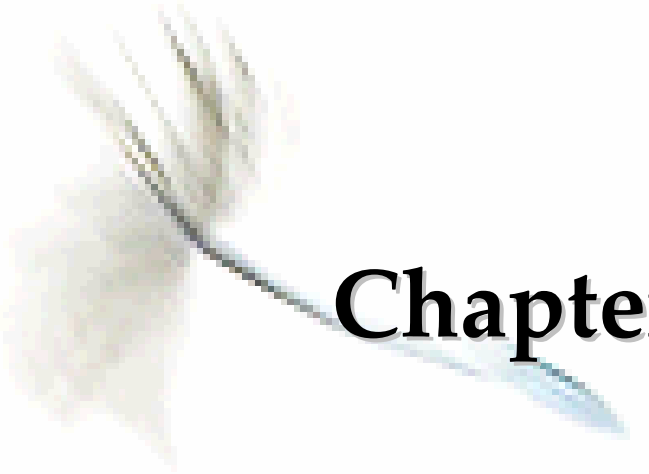

Chapter 9

General discussion

For many years *Enterococcus faecium* was considered a commensal of the digestive tract, which could sometimes cause opportunistic infections in severely ill patients, while *Enterococcus faecalis* was much more prevalent causing 80-90% of enterococcal infection. In the early 1980s, initial reports of increased infection rates and outbreaks due to ampicillin resistant *E. faecium* were published, which was followed by increased infection rates and outbreaks due to glycopeptide resistant enterococci (both *E. faecium* and *E. faecalis*) in the late 1980s. Based on molecular typing of a small number of vancomycin resistant *E. faecium* (VREF) isolates, clustering of hospital related isolates was observed. These strains were characterized by ampicillin and quinolone resistance. In addition, the presence of the variant *esp* gene was strongly associated with these outbreak strains. In 2001, Esp was described as a putative virulence factor in *E. faecalis*, and 1 year later Shankar and coworkers demonstrated that in *E. faecalis esp* was contained on a so-called pathogenicity island (PAI).

This thesis describes the molecular epidemiological studies of *E. faecium*, from a hospital wide (University Medical Center Utrecht, (UMCU)), nationwide (the Netherlands) and global perspective. Furthermore, a rapid identification scheme for enterococci and a rapid molecular typing scheme for *E. faecium* were developed. Finally, the chromosomal region encompassing the *E. faecium esp* gene was characterized.

The identification of enterococci is subject to a lot of controversies. Especially the discrimination between *E. faecium* and the low level vancomycin intrinsic resistant *E. casseliflavus* and *E. gallinarum* isolates is problematic. Rapid and correct identification of enterococci is a prerequisite to take appropriate infection control measures. The evaluation of different phenotypic methods described in chapter 2 revealed that the 100% reliable method doesn't exist as a result of a-typical reacting enterococci. Even with the proposed rapid identification scheme (4 hours), based on five phenotypic tests a few a-typical reacting isolates were found resulting in an accuracy of 92%. Raman spectroscopy, a phenotypic identification method under development, appeared to be a promising tool for the rapid identification of enterococci, but the reference database needs to be extended with other enterococcal species before the reliability of Raman spectroscopy in the identification of enterococci can be determined.

Molecular typing schemes based on multiple loci dispersed over the chromosome, like multi locus sequence typing (MLST) and multiple locus variable number tandem repeat analysis (MLVA), can be used to confirm species identification, but more importantly also to determine the genetic relatedness of

bacterial isolates. In chapter 3, MLVA was introduced as a novel rapid and cheap typing scheme for *E. faecium*, which was based on variation in numbers of tandem-repeats.

Most MLVA typing schemes developed for other bacterial species are based on small repeat units e.g. 12 bp, which need special equipment like a DNA sequencer for analysis of band sizes. Variation in numbers of small repeats is thought to result from "mistakes" by the DNA polymerase during replication, the so-called slip strand mispairing mechanism. Our goal was to develop a rapid typing method. Therefore large repeat units, easily detectable on agarose gels without special equipment, were chosen. Due to the size of the repeat units (between 121 and 279 bp) in the *E. faecium* MLVA typing scheme, it is unlikely that slip strand mispairing is the underlying mechanism in variation in numbers of repeats. Variation in large repeat units is probably a result of recombination events. The fact that recombination is an important mechanism for genetic diversification of *E. faecium* was also observed by MLST analysis on a set of 411 isolates (chapter 6).

Although a correlation in variation in tandem repeats and mutation/recombination in housekeeping genes seems unlikely, surprisingly, MLVA was highly congruent to MLST in recognizing the MLST-based hospital adapted CC17 isolates, formerly designated the C1- lineage (chapter 3). eBURST clustering of 127 MLVA profiles predicted MT-1 as the primary founder of this cluster. Despite the high congruence several different STs (predominantly ST17) were found among MT-1 isolates. But, vice versa, different MTs were found among ST17 isolates as well. Therefore, one could conclude that although the clock-speed of these genetic events is not known, they might be different for tandem repeats and housekeeping gene variation.

While MLVA is a relative new technique, this typing scheme was compared with Pulsed Field Gel Electrophoresis (PFGE), which is still considered the "gold standard" typing method in infection control programs in hospitals (chapter 4). There are, however, several pitfalls related to PFGE typing of *E. faecium* isolates. In *E. faecium* a high degree of genetic rearrangements due to the presence of mobile elements has been observed, resulting in DNA banding pattern polymorphisms and affecting PFGE pattern stability. Therefore, PFGE of *E. faecium* is only suitable to be used for short-term and local epidemiology like outbreak situations in a single hospital. For long-term or global epidemiology evolutionary related isolates are no longer recognized due to lack of stability of PFGE banding patterns. Furthermore, PFGE typing is labor-intensive, lacks standardized methods and criteria for interpretation of banding patterns, and is, therefore, not suitable for interlaboratory data exchange. For our study, described

in chapter 4, isolates collected from an US hospital within a 2-month period were included. In this hospital, VREF are endemic in the ICU and are therefore expected to be genetically highly related. In this study MLVA and PFGE were highly congruent implying that MLVA could replace PFGE for short-term epidemiology purposes.

In chapter 5 analysis of the up- and downstream regions of the *esp* gene revealed that, like in *E. faecalis*, *esp* of *E. faecium* is also contained on a putative pathogenicity island (PAI). Interestingly, except for one other gene, *ara*C, the PAIs of both species are different. Another important difference is the host range of the *E. faecalis* and *E. faecium esp* containing PAIs. Although the *E. faecalis* PAI was found enriched among clinical isolates, it has also been detected in isolates from non-hospitalized persons and in animal isolates. The *E. faecium* PAI has, so far, only been detected in outbreak related and clinical isolates, suggesting a role in nosocomial epidemicity of *E. faecium*. The reasons behind this difference in host range of the *E. faecalis* and *E. faecium* PAI is not known. Analysis of the partially sequenced PAI, including the *esp* gene among several isolates revealed a remarkable sequence heterogeneity with insertions, deletions and mutations, which reflects the enormous plasticity of the *E. faecium* genome.

The *esp* gene encodes the enterococcal surface protein (Esp) thought to be involved in adhesion and biofilm formation. Polymorphisms of Esp may influence tissue tropism or cell surface expression. The exact role of the *E. faecium* Esp is, however, still poorly understood due to lack of an *esp* knockout. Only very recently our research group succeeded in constructing an *esp* knockout, which will enable us to elucidate its role in *E. faecium* pathogenesis.

Several attempts have been made to clone and sequence the up- and downstream regions from the described PAI sequence (chapter 5) to determine the exact size and "ends" of the PAI, but due to mobile elements with high sequence similarity to several copies of similar mobile elements on the *E. faecium* genome, the borders of this PAI have not yet been identified.

MLST typing of bacteria is now a widely used technique for molecular epidemiological studies but can also be used to obtain insight in the population structure and evolution of bacteria. In chapter 6, MLST typing of 411 *E. faecium* isolates, including VREF and vancomycin susceptible *E. faecium* (VSEF) isolates from varies origins, revealed 175 sequence types (STs). Clustering based on the MLST profiles using the eBURST algorithm revealed a population structure, which was characterized by a large cluster with ST22 as predicted primary founder. Within this cluster a smaller complex with ST17 as predicted secondary founder, designated clonal complex 17 (CC17), included most of hospital

outbreak and clinical isolates and was characterized by ampicillin resistance and the presence of the PAI. In a recent review, this population structure, now based on 855 *E. faecium* isolates, was slightly changed and predicted ST26 as primary founder of the large cluster, though grouping of outbreak related and clinical isolates in CC17 remained unchanged (8). In addition to the clustering of clinical relevant isolates, host specific clustering of related STs was observed. For example, human community VREF isolates clustered together with VREF from pigs, suggesting colonization of pig VREF isolates in humans due to consumption of pig meat or direct transmission from animals to humans. Interestingly, human community isolates, ampicillin and vancomycin susceptible, probably representing the commensal population in humans, clustered apart from the main complex. Apparently, the hospital-adapted subpopulation did not directly evolve from the human commensal population.

eBURST predicted ST17 as founder of CC17. So far ST17 is the most globally dispersed *E. faecium* MLST type, identified in nosocomial outbreaks in North and South America, Asia, Europe and Australia and is comprised of PAI positive and negative isolates. Apparently, this clone was very successful in nosocomial spread. At the moment, there are 3 predominant single locus variants (SLVs) of ST17 identified: ST78, ST16 and ST18, respectively (8). ST78 and ST16 have caused documented hospital outbreaks worldwide (2,6,7,9-11,13) and are both PAI positive, while from ST18, although globally dispersed, no documented hospital outbreaks are known and the majority of these isolates are PAI negative (4). These findings contribute to the hypothesis that ST17 by acquisition of the putative pathogenicity island, other adaptive determinants and genetic diversification evolved to a successful clonal complex with increased capability of spread and virulence.

Although we have shown that recombination has played a major role in establishing genetic variation in *E. faecium*, it seems paradoxical that we are still able to detect CC17 as a distinct clonal complex. Therefore, we hypothesize that the emergence of this complex occurred relatively recent and that over time CC17 will fade away, resulting in an *E. faecium* population structure, which resembles a network of a frequently recombining population in which periodically successful clones may dominate.

An unexplained increase of bloodstream infections due to *esp* negative, ampicillin resistant *E. faecium* (AREfm) in our hospital in 2003 prompted the study described in chapter 7. This study showed an ecological replacement of *E. faecalis* by multiresistant CC17 *E. faecium*, illustrated by a decrease in total numbers of invasive enterococcal infections and increase in proportions of invasive AREfm. Furthermore, the ratio *E. faecium*/*E. faecalis* among bloodstream

isolates changed in favor of *E. faecium*, while point prevalence studies revealed high carriage rates of AREfm especially among haematology and nephrology patients. Risk factors for AREfm colonization were diabetes mellitus, three or more admissions in the preceding year and use of β-lactams and quinolones. In the UMCU, haematology patients receive ciprofloxacin prophylaxis during prolonged granulocytopenia, while imipenem is the empirical therapy for granulocytopenic fever. As AREfm are resistant to both groups of antibiotics, selection for AREfm can be explained.

To investigate whether the increase of invasive AREfm was restricted to the UMCU, a nationwide study was initiated (chapter 8). In this study, data on annual numbers of invasive ampicillin resistant enterococci from 26 laboratories in the Netherlands were analyzed and the *E. faecium*/*E. faecalis* ratio among bloodstream isolates provided by 10 laboratories, were determined. The average number of invasive AREfm per hospital increased between 1994 and 2005, and this increase was more pronounced in university hospitals. Proportions of AREfm also increased among bloodstream isolates, again predominantly in university hospitals. MLVA typing of all *E. faecium* isolates revealed spread of 4 types belonging to CC17 in three or more hospitals, including MT-159, which was found in at least 9 hospitals dispersed over the Netherlands in 2006. Furthermore, a sudden increase of *esp* positive isolates, within the most frequent MLVA types, was observed from 2004 on.

The observed increase of AREfm in the Netherlands raises the question, why AREfm replaced *E. faecalis*. An obvious explanation might be selective antibiotic pressure or lapses in infection control. As shown β-lactam antibiotics and quinolone use are risk factors for AREfm colonization. But in the UMCU, quinolone and β-lactam use remained stable over the last 10 years (unpublished data). Only cephalosporin use increased, but it seems unlikely that this has increased selection pressure for AREfm specifically, as all enterococci, including *E. faecalis*, are intrinsically resistant to these agents.

The spread of multiple subclones, all belonging to CC17, without an existing community-reservoir (chapter 7) can only be explained by cross-transmission. Many studies have documented the ability of enterococci to colonize the patients' skin and their prolonged survival in the inanimate environment, both facilitating patient-to-patient spread (3). As a result, standard hygienic measures are frequently insufficient to prevent hospital transmission of enterococci. In the UMCU, a nosocomial outbreak with complex-17 VRE was controlled by combining genotyping and preemptive isolation of patients suspected of carriage (9). In another study, improved environmental cleaning was associated with 31% reductions in VRE-acquisitions rates in ICU-patients (5).

Perspectives

For infection control measures rapid identification of outbreak related *E. faecium* is a prerequisite. The rapid phenotypic test panel can be used for identification of *E. faecium* within 4 hours, while MLVA typing can be used to rapidly determine the genetic relatedness of isolates against low costs. Further studies are necessary to determine whether Raman spectroscopy could be used to identify CC17 *E. faecium* isolates and outbreak related isolates.

For short-term epidemiology like recognition of outbreak related isolates, MLVA typing is a good alternative for PFGE typing. However, in our study (chapter 4), there were several examples of single locus variants of predominant MLVA types with similar PFGE banding patterns, suggesting genetic relatedness of these isolates. Therefore, we propose the following criteria for MLVA typing, as has been done for PFGE (12), for infection control programs in hospitals.

(i) **Indistinguishable**. Isolates are genetically indistinguishable if their MLVA profiles are the same. Though for definitive proof of cross-transmission, epidemiological data like sharing of rooms or overlapping periods of stay in a ward should always be taken into account. To confirm clonal spread of one MLVA type, we recommend to type representative isolates by MLST, as we consider MLST as the new "gold standard" typing technique to determine genetic relatedness among isolates. MLST results can then easily be compared to other worldwide documented outbreaks.

(ii) **Closely related**. Isolates are considered closely related if their MLVA profiles differ only in one of the VNTR loci, so-called single locus variants (SLV). For these isolates, the same considerations as for the indistinguishable isolates to provide proof of cross-transmission are recommended.

(iii) **Possibly related**. Isolates are considered possibly related if their MLVA profiles differ in two of the VNTR loci, so-called double locus variants (DLV). Only in cases of a strong suspicion of cross-transmission the genetic relatedness of these isolates should be confirmed by MLST.

(iv) **Unrelated**. Isolates are considered unrelated if their MLVA profiles differ in more than two of the VNTR loci.

Molecular epidemiological studies in the UMCU indicated horizontal transfer of the *E. faecium* PAI in addition to clonal spread of strains. This was illustrated by the simultaneous finding of isolates belonging to a single clone carrying and lacking this PAI. Vice versa, representative isolates of different clones carried the same PAI subtype. Acquisition of the PAI through horizontal gene transfer may improve the pathogenic properties of clones and increased prevalence, which in itself enlarges the chance of acquiring additional adaptive mechanisms further

enlarging the possibilities for spread. Cumulative acquisition of adaptive mechanisms has been called "genetic capitalism (1)" and reflects the process in which already successful clones only become more successful. Since adaptive genetic elements predominantly represent the accessory genome, addition of these elements to molecular typing schemes will probably gain insights in the mechanisms that contribute to the ecological success of particular clones and may improve the resolution of current typing scheme.

The big challenge resides in what to do with emergence of AREfm in hospitals. There are several options to address this problem; (i) we simply accept the increase of AREfm, while AREfm is already endemic on many wards and implementation of extensive infection control measures, as done during the 2000 VRE outbreak (9) is too expensive or (ii) we improve environmental cleaning as described by Hayden *et al.* (5) to determine whether a similar reduction in AREfm acquisition rates can be obtained as was observed for VRE acquisition rates or (iii) we can try to replace the AREfm by restoring normal microbiota by use of probiotics. In hospitalized patients the residual microbiota is often significantly altered through the detrimental effect of antibiotics. In the UMCU two prospective studies will be performed to determine the last two possibilities. Allowing a further increase in the prevalence of AREfm implies an increased use of vancomycin, linezolid and daptomycin to treat AREfm infections, which might select for either VREF or already described linezolid resistant AREfm and continues the evolutionary rat race, further shaping the selective advantage of multiple antibiotic resistant or virulent bacterial clones.

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