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1. EXECUTIVE SUMMARY

TROCAR members have agreed on the definition of 'high-risk clones' (HiRiC): "High-risk, resistant clones are those bacterial clones that associate: Mechanisms of resistance to antibiotics of critical clinical importance and 2a) the ability to be transmitted with high efficiency among hospitalised patients or 2b) with particular ability to produce severe or invasive infections; or 2c) the ability to efficiently colonize human hosts during long periods of time."

The combined effort of the consortium members has produced an inventory of strains for MRSA, VRE, *A. baumannii*, *P. aeruginosa* and ESMAC-BLs-producing Enterobacteriaceae (Extended-spectrum, metallo- and acquired AmpC beta- lactamases) according to their epidemiological impact and multidrug-resistant profiles. The data produced under the scope of the TROCAR project enabled to construct an unparalleled catalogue of well-characterised MRSA and MSSA isolates widely disseminated in Europe. These data are accessible and can be used by local health-care authorities to be acquainted of MRSA epidemiology in their own and neighbouring countries and to act on local infection control. The geographical abundance of *S. aureus*/MRSA clones has been displayed on a web-based mapping tool using a Google interface (publicly available at http://www.spatialepidemiology.net/srl-maps/). We took a step forward in the understanding of the origins (time and space) and clinical relevance of MRSA clades and clonal lineages with particular public health importance. Moreover, we identified highly discriminative targets that may help to distinguish specific strains and clades within specific high-risk clones, which can be effective in the management of MRSA outbreaks.

VRE has been investigated in depth. Identification of AsrR in *E. faecium* (with no homologue in *E. faecalis*) provides a significant resource combining, for the first time in *E. faecium*, a global transcriptomic approach and a deep phenotypic study and places AsrR as a key regulator modulating opportunistic traits and pathogenesis. Comparison of genomes of VRE with different epidemic strengths identified blocks of genes that are interesting markers for epidemicity.

During the development of the project, main research interest turned to carbapenemases, including detection and characterisation of new carbapenemase genes and associated HiRiC in Enterobacteriaceae. Different TROCAR studies have shown VIM and KPC-producing Enterobacteriaceae in different countries, although special relevance should be given to the NDM-producing *E. coli* and *K. pneumoniae* isolated in UK. Most of these genes are plasmid-mediated and some of them have been selected for full sequencing. Several contributions on ESBLs and or AmpC producers were also published from the TROCAR project. The potential link between antimicrobial resistance acquisition and virulence has also been investigated.

In order to better understand the current trend towards MDR and carbapenem-resistant *A. baumannii* and *P. aeruginosa* representatives of widespread MDR clones of *P. aeruginosa* producing MBLs/or another emerging carbapenemases and of *A. baumannii* producing carbapenemases of clinical significance were identified and characterised. Several novel efflux pumps were also characterised in *Acinetobacter* spp. Whole genome sequencing and annotation of several MDR strains of *P. aeruginosa and A. baumannii* of clinical importance are still in progress and will allow to perform comparative genomics between epidemic and non-epidemic isolates, providing insights into novel genetic structures that explain the dissemination ability of these HiRiC.

2. PROJECT CONTEXT AND OBJECTIVES

The health care systems of most European countries are based on a continuum from acute care hospitals to the community through other health care facilities. Such a framework provides the perfect opportunity for the widespread dissemination of high-risk resistant clones or genetic resistant elements. High-risk resistant clones are those bacterial clones that associate: 1) mechanisms of resistance to antibiotics of critical clinical importance and 2a) ability to be transmitted with high efficiency among hospitalised patients or 2b) with particular ability to produce severe or invasive infections; or 2c) ability to efficiently colonize during long periods of time human hosts. Methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* spp. (VRE), Extended-spectrum, metallo- and acquired AmpC beta-lactamase producing Enterobacteriaceae (ESMAC-BL), multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are the paradigm of resistant bacteria with a great potential for spread. In addition, selection, dissemination and evolution of antibiotic resistant genes and their genetic environmental location have been less studied and become essential to better design overall control strategies. The selection of the most appropriate strategies to control the dissemination of these microorganisms is under debate largely because of the lack of a definition of the specific traits of the epidemic strains.

This proposal is therefore motivated by **three main questions**:

1. Are certain resistant strains more epidemic than others? Are certain strains more prone to persist in the human environment? If so, why?

2. Do epidemic and persistent strains have specific virulence, physiological, colonization, or transmission-facilitating traits that non-epidemic strains do not have?

3. What is the origin and mechanisms of acquisition of these fitness-increasing traits in resistant bacteria? Might the elucidation of these mechanisms provide new insights for prediction and intervention?

TROCAR will harness the collective potential of several leading European research groups for advanced molecular investigations into the natural history and evolutionary trajectories of new and highly virulent multi-drug resistant strains of bacterial pathogens, causing particular concern to hospitals and the community, and will provide the scientific and public health community in Europe with advanced state-of-the-art research lines, for interventions and progress towards goals for the strategic control of these pathogens.

The driving concept of this project is to investigate the fundamentals of the epidemiology of new highly virulent multiresistant strains of MRSA, VRE, ESMAC-BL-producing Enterobacteriaceae, multidrug-resistant *P. aeruginosa* and *A. baumannii* will focus on three major strategic aims:

1. **The definition of the major high-risk resistant clones** based on an appropriate representative collection and new clinical strains obtained during molecular epidemiological studies, in hospitals and laboratories collaborating with their respective National Excellence Laboratories in European countries **using a standardised protocol**.
2. The **promotion of collaborative European research** to investigate, by **genomic and proteomic approaches**, specific traits associated with virulence, transmission, persistence and resistance of epidemic clones in comparison with non-epidemic clones as well as resistance determinants and their genetic location in horizontal gene transfer units and their genetic environment.
3. The development of **bioinformatics tools** to fully exploit the genomics data and allow the rapid identification of resistant strains with heightened epidemic potential.

By combining the outputs of the project it will be possible to provide tools for monitoring the spread of key community and nosocomial pathogens, to provide the scientific basis for an early warning system when isolates of a particular epidemicity appear in the community and nosocomial settings, to create a knowledge base for combating epidemicity and virulence, to characterise specific genetic elements carrying genes encoding ESCMAC-BL, to identify potential reservoirs of antibiotic resistance genes amongst community and nosocomial pathogens involving multi-drug resistance and to establish the bioinformatic tools and infrastructure necessary to achieve the ultimate TROCAR goal:

"to recommend novel control measures to limit or prevent the spread of highly virulent multi-drug resistant clones: from molecule to preventive action"

Primary objective

TROCAR aims to define genotypic or phenotypic traits of highly virulent multiresistant strains of methicillin-resistant *S. aureus,* vancomycin-resistant *Enterococcus* spp., ESMAC-BL producing Enterobacteriaceae, multidrug-resistant *P. aeruginosa* and *A. baumannii* for the better design of control strategies.

Specific objectives

The Epidemiology and Data Exchange platform: Relational Analysis and Portal to the TROCAR State-Of-The-Art Research Line.

1. To establish, maintain, and consolidate the coherence of existing European and national network structures of antimicrobial resistance surveillance and reference service with targeted organisms and resistance genes.

2. To establish the necessary criteria for the selection and inclusion of high risk clones (HiRiC) of MRSA, VRE, ESMAC-BL producing Enterobacteriaceae and multi-drug resistant *P. aeruginosa* and *A. baumannii* into the state-of-the-art research line (WP3-7).

3. To create an inventory of strains of bacterial pathogens with emerging public health importance currently co-circulating in European hospitals and communities.

4. To analyse the geographical abundance, migration patterns and evolutionary trajectories of high risk clones (HiRiC).

Comparative genomics and proteomics of established and emerging MRSA epidemic clones.

5. To set up a catalogue of MRSA clones circulating in Europe.

6. To investigate the phenotypic and genotypic characteristics of the *S. aureus* isolates circulating in Europe

7. To perform a comparative genome analysis of the most successful MRSA clones.

8. To identify new highly discriminative targets for the rapid differentiation of MRSA clones.

Deciphering vancomycin-resistant *Enterococcus faecium*

9. To harmonize molecular typing of VRE clones in Europe.

10. To perform a comparative genome analysis of VRE clones to identify factors of enhanced virulence.

11. To analyse the structure and composition of vancomycin resistance plasmids.

12. To carry out a comparative proteomic analysis of VRE clones to identify differences and characteristics of epidemic and non-epidemic isolates.

13. To establish an animal model for infection.

Extended-spectrum, metallo- and acquired AmpC beta-lactamase (ESMAC-BL) emerging threats in *Enterobacteriaceae*: from clones to genes.

14. To establish a surveillance data collection on ESMAC–BLs producing Enterobacteriaceae in Europe.

15. To identify the emergent successful clones (High-risk clones, HiRiC) and mobile elements harbouring ESMAC-BL genes. Gene architecture.

16. To perform a genomic analysis of selected plasmids harbouring ESMAC–BLs genes. Coalescence studies (evolutionary trajectories of clones, genetic elements and genes).

17. To characterise the physiology, ecology, and virulence of ESMAC–BL producing organisms.

18. Rapid detection of ESMAC-BL producing organisms.

Genomics, drug resistance and physiology of MDR *Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains.

19. To identify representatives of widespread MDR clones of *P. aeruginosa* producing metallo-β-lactamases and of *A. baumannii* producing carbapenemases of clinical significance and those which are representative for new strains of *P. aeruginosa* and *A. baumannii* of clinical concern due to their MDR phenotypes, virulence, tenacity and spreading propensity.

20. To analyse of the resistome of the selected strains for resistance determinants accounting for clinically relevant resistance traits and characterization of the genetic context.

21. To investigate the uncommon/still unclear mechanisms for clinically relevant resistance traits to novel or last-resort anti-gram-negative agents.

22. To perform a comparative genomic analysis of some MDR strains/R-plasmids, selected on the basis of their clinical importance and epidemiological impact.

23. To analyse the virulence traits of MDR *A. baumannii* strains and interaction with biofilm. Secretome and surfome analysis of the epidemic vs. non-epidemic *A. baumannii* strains by a proteomic approach.

In addition, computational biology and evolutionary analysis has been performed to establish web-based databases for the integration and management of the molecular data generated by the project and to examine the evolutionary dynamics and transmission of mobile elements in

pathogens, and to compare the phylogenetic consistency of different plasmid genes. The extension and development of models to elucidate the conditions under which plasmids are predicted to spread.

3. MAIN S&T RESULTS/FOREGROUNDS

The Epidemiology and Data Exchange platform: Relational Analysis and Portal to the TROCAR State-Of-The-Art Research Line.

We established, maintained, and consolidated the coherence of existing European and national network structures of antimicrobial resistance surveillance and reference service for targeted organisms and resistance genes through annual workshops and consultations with National Expert/Reference Laboratories. These National Expert/Reference Laboratories were identified through a bottom up query exercise among a minimum of 20% of all microbiological diagnostic laboratories in all EU Member States and accession countries. This query identified the existing about reference structure and the relevant expert laboratories for different pathogens. We were thus confident to invite the correct contact points. During annual meetings to which we invited the decision makers of all National Expert/Reference, all participants agreed to carry out European-wide structured surveys to identify the occurrence of bacterial clones with particular public health importance (High risk clones). As proof of principle (that these envisaged structured surveys would succeed) the initial emphasis was but on the determination of the genetic population structure of *Staphylococcus aureus* that cause invasive (blood stream) infections in European hospitals.

In the following years two additional consultations lead to address future surveys for carbapenemase-producing Enterobacteriaceae (CPE). It was however pointed out that a large effort to build the necessary diagnostic capacity would need to be made first before a structured survey could explore the occurrence and spread of CPE in hospitals at European continental scales.

During these annual consultations with National Expert/Reference Laboratories all participants concluded that the decisive criterion for the definition of High Risk Clones and the eventual inclusion of pathogens into the state-of-the-art research line would be their epidemiological and clinical behaviour i.e. their abundance and expansion dynamics and their disease causing potential. It was therefore agreed, to set-up a European strain collection and populate this repository with samples that are representative of pathogens that cause life-threatening disease in hospitals that are geo-demographic representative for all participating countries. As a proof of principle it was decided to

prospectively collect clinical isolates of *Staphylococcus aureus* (that cause blood stream infections) using a standard sampling frame, and characterise these isolates at national level and save them a European strain repository.

2,986 isolates of *Staphylococcus aureus* from patients with blood stream infections were collected in a period of six moths in 450 hospitals in 25 European countries. All isolates were characterised using DNA sequence-based methods at the respective National Expert/Reference Laboratories and subsequently submitted for storage in a European strain collection. Clone identification was repeated using different genomic methods and antibiotype confirmed centrally before storage.

A web site was set up including a highly interactive interrogation platform that makes it easy to follow High Risk Clones through time and space across Europe (www.spatialepidemiology.net/srl-maps/). With this innovation, stakeholders such as clinicians, microbiologists or epidemiologists can identify the geographic spread and abundance of High Risk Clones of *Staphylcoccus aureus* at European continental scales. What became clear using this first time representative mapping of the occurrence of particular isolates was that methicillin-resistant clones (MRSA) had a fundamentally different spreading behaviour than their susceptible counterparts (MSSA).

Comparative genomics and proteomics of established and emerging MRSA epidemic clones.

Phenotypic and genotypic characteristics of MRSA isolates circulating in Portugal: one of the European countries with the highest MRSA prevalence.

As an example of local MRSA epidemiology we studied in particular the case of Portugal, one of the countries with the highest nosocomial MRSA frequencies in Europe. The epidemiology of nosocomial MRSA in Portugal had been well documented between 1990 and 2006. The molecular characterization of MRSA collected from different hospitals in this country allowed the identification of several clonal replacements overtime, suggesting that the epidemiology of MRSA is highly dynamic and that continuous surveillance is demanded. Nonetheless, the actual scenario on MRSA epidemiology in hospitals in Portugal was not known until the beginning of these studies performed under TROCAR.

The most recent relevant change in MRSA epidemiology was its emergence as a pathogen in patients with no previous contact with the hospital. Community-associated MRSA (CA-MRSA) was reported for the first time in Australia among aborigine's communities, and in a few years became a worldwide problem. Nowadays CA-MRSA are epidemic in the USA and are becoming important in

some European countries. However, no information existed on the prevalence and epidemiology of CA-MRSA in Portugal.

The molecular characterization of MRSA collected from blood infections in different hospitals in Portugal by state-of-the-art typing techniques showed that presently the most common MRSA clonal lineages in Portugal are CC22 (EMRSA-15, ST22-IVh) and CC5 (variants of New York/Japan and paediatric clones, ST105-II and ST5-IV). Among MRSA isolates of CC22 a high diversity in the number of different *spa* types was observed, whereas isolates belonging to CC5 showed a high number of different SCC*mec* types. The results suggest that the two clones probably used different strategies of evolution.

The screening of MRSA in different populations in the community, namely those attending health-care centres and long-term care facilities, and public buses showed that EMRSA-15 clone was also extensively disseminated in these settings. In the community, EMRSA-15 isolates were able to persist in inanimate surfaces and to colonize and cause infection, mainly in elderly.

The comparison of MRSA isolates with their susceptible counterparts collected in the same geographic location and timeframe either in the hospital or in the community showed that very closely related isolates associated to the most common hospital and community clonal lineages (CC22 and CC5) were found between the two populations. The results suggest the frequent *in loci* SCC*mec* acquisition and loss in these specific genetic backgrounds. This current situation contrasts to previous reports, wherein contemporary MSSA and MRSA populations in Portugal were not correlated and might be a phenomenon associated to the nature of the presently established clonal types in Portugal.

Altogether the results obtained under the scope of this project suggest the extensive dissemination of a single major high-risk clone in Portugal (ST22-IVh) from the hospital into the community. The high prevalence of nosocomial MRSA in Portugal (>50%) may explain the spill over of MRSA into the community. But EMRSA-15 clone appears to carry several characteristics that might have also contributed to its epidemicity and survival in the community environment. This includes the carriage of a small sized SCC*mec* type, the low number of antibiotic resistant determinants, a higher growth rate (Tavares et. al, unpublished), increased fitness, higher resistance to desiccation and an improved capacity to form biofilm. Moreover, its epidemicity and adaptability could be even increased by its capacity to acquire and lose mobile genetic elements, such as SCC*mec* (this study), the Panton-Valentine Leukocidin (PVL) and the arginine catabolic mobile genetic element.

Assessment of MRSA virulence potential

In order to understand the reasons lying behind the higher epidemicity of certain high risk MRSA clones, phenotypic characteristics of these clones were determined and compared using *in vitro* and *in vivo* assays.

Several adhesins and invasins are involved in the adherence to epithelial cells surface and invasion of tissues, and are possibly involved in the epidemic capacity of MRSA strains. To test the ability of different MRSA strains to bind to eukaryotic extracellular matrix proteins (ECM) well-established *in vitro* assays were performed. The target molecules were collagen (Cn) type IV, fibronectin (Fn), vitronectin (Vn), laminin (Lm), fibrinogen (Fg) and plasminogen (glu). In addition, the capacity for plasminogen activation of the same strains was tested. Some differences were observed in the selected five MRSA/MSSA study strains in the preliminary assays on the binding to ECM proteins. However, no conclusions could be drawn without repeating experiments with a larger strain collection and a defined hypothesis.

Another possible reason for epidemicity is the resistance to several unfavourable environmental conditions, such as desiccation and skin fatty acids. In order to test if different strains present different levels of resistance to dissection, epidemic MRSA (ST8, ST22, ST45, ST247, ST254, ST239, ST225, ST228) and MSSA (ST15, ST30, ST45, ST25) were subjected to slow drying on a polyacetate surface up to 96 h. However, no difference in resistance to desiccation within isolates was observed.

The production of carotenoids by *S. aureus* has been found to confer resistance against oleic acid, which has been reported to be the predominant bactericidal unsaturated fatty acid naturally present in staphylococcal abscesses and on the skin surface. To understand if resistance to oleic acid could contribute to the epidemicity of widely disseminated HA-MRSA, *in vitro* survival was assessed by exposing planktonic cells in the late log-phase to 0.1% emulsified oleic. There were no differences in oleic acid resistance for representatives of HA-MRSA (ST8, ST22, ST225, ST228, ST45, ST247, and ST239) and MSSA (reference strain 8325, ST15, ST25, and ST45).

With the objective of comparing the pathogenic potential of different MRSA and MSSA strains, *in vivo* assays in *Manduca sexta* and *Caenorhabditis elegans* models were performed. Some differences in the pathogenic potential between strains in the *M. sexta* model were observed. However, no conclusions could be drawn without repeating experiments with a larger strain collection and a defined hypothesis.

Overall, the phenotypic assays performed showed that MRSA isolates may have different abilities to colonize and to invade the host as suggested by differences in binding to extracellular surface matrix proteins and pathogenic potential in *vivo* models.

Identification of the genetic basis for MRSA epidemicity

In an attempt to characterise the genetic basis for epidemicity, resistance and virulence differences already highlighted from epidemiological and experimental data and to identify new targets for rapid differentiation of clones, several isolates of high public health relevance were selected for whole-genome sequencing and analysed using different methodologies. The whole genome sequencing was obtained for strains belonging to MLST sequence types ST45, ST80, ST15, ST121 and ST59, which are widely disseminated clonal types frequently associated with MRSA infection. The data obtained were filtered using a threshold SNV (Phred Value) of >=30, and a coverage value of >= 9 and all sites with ambiguities were excluded. The total number of SNPs in coding regions against the reference genome after filtering varied between 9,340 (ST15) and 23,925 (ST59).

In order to carry out genomic comparisons of these strains, 999 "homology groups" corresponding to orthologous genes were identified that were present in all five newly sequenced strains and showed some variation (SNPs) in at least one of them. Of these 999 genes, about 25% (n=252) contain SNPs which give rise to stop codons. Most of these stop codons were present in only one or two of the strains and, in fact, almost all of them were found within strains belonging to ST121 and ST59. The fact that the related ST59/ST121 pair share these nonsense SNPs supports the hypothesis that these SNPs represent substantial loss of gene function and not artifacts. However, we observed no differences between the numbers of stop codons in each gene function category (non-essential *vs* essential genes) suggesting that stop codons did not result from relaxed selection. Moreover, the analysis of dN/dS, Ti/Tv and the ratio of GC enriching versus AT enriching mutations demonstrated reduced selective constraint on ST59 and ST121 strains. The impact of loss of gene function on the epidemicity of ST59 and ST121 is not known, but this loss of gene function through pseudogenisation was previously shown to have a marked impact on virulence. Moreover, the observed differences in selective constraint between different lineages can implicate variation in lifestyles and adaptation to different niches, and may therefore also indicate changes in virulence over longer evolutionary time-scales.

Due to its virulence potential and genetic particularities, a more detailed study of the population structure of *S. aureus* clonal complex CC121 was carried out. The analysis of 154 isolates belonging to CC121 collected in the five continents between 1950 and 2009 was performed by mutation discovery at 115 genetic housekeeping loci. In addition, we pyro-sequenced the genomes from ten representative isolates. The genome-wide SNPs that were ascertained revealed the evolutionary history of CC121, indicating at least six major clades (A to F) within the clonal complex and dating its most recent common ancestor to the pre-antibiotic era. The toxin gene complement of CC121 isolates correlated with their SNP-based phylogeny. Moreover, we found a highly significant association of clinical phenotypes with phylogenetic affiliations, which is unusual for *S. aureus*. All isolates evidently sampled from superficial infections (including staphylococcal scalded skin syndrome, bullous impetigo, exfoliative dermatitis, conjunctivitis) clustered in clade F, which included the European epidemic fusidic-acid resistant impetigo clone (EEFIC), whereas isolates from deep-seated infections (abscess, furuncle, pyomyositis, necrotizing pneumonia) were disseminated in several clades, but not in clade F. Community-associated MRSA and MSSA from Cambodia were extremely closely related, suggesting the few MRSA belonging to this CC probably arose in the region.

The whole genome sequencing of strains belonging to ST22 and ST225, which represent more than ¾ of all MRSA from German hospitals and are highly frequent in Central Europe, was also performed. By using mutation discovery at 269 genetic loci (118,804 base pairs) within an international isolate collection, we ascertained extremely low diversity among European ST225 isolates, indicating that a recent population bottleneck had preceded the expansion of this clone. In contrast, US isolates were more divergent, suggesting they represent the ancestral population. While diversity was low, however, our results demonstrate that the short-term evolutionary rate in this natural population of MRSA resulted in the accumulation of measurable DNA sequence variation within two decades, which we could exploit to reconstruct its recent demographic history and the spatiotemporal dynamics of spread. By applying Bayesian coalescent methods on DNA sequences serially sampled through time, we estimated that ST225 had diverged since approximately 1990 (1987 to 1994), and that expansion of the European clade began in 1995 (1991 to 1999), several years before the new clone was recognized. Demographic analysis based on DNA sequence variation indicated a sharp increase of bacterial population size from 2001 to 2004, which is concordant with the reported prevalence of this strain in several European countries. A detailed ancestry-based reconstruction of the spatiotemporal dispersal dynamics suggested a pattern of frequent transmission of the ST225 clone among hospitals within Central Europe. In addition, comparative genomics indicated complex bacteriophage dynamics.

The most rapidly spreading and tenacious clone in Europe is currently ST22 (EMRSA-15). In order to understand the reasons for its epidemicity, we have reconstructed the extraordinary expansion of this clone from next-generation sequencing data, and investigated the genetic changes associated with its emergence. The results showed increasing antimicrobial resistance over time, and how this has been influenced by country-specific drug use regimens. We also noted that the molecular genetic basis of 99.8% of resistance phenotypes can be identified from the sequence data, and that the emergence of fluoroquinolone resistance in EMRSA-15 in the English Midlands during the mid-1980s appears to have played a key role in triggering pandemic spread to the rest of the UK, Europe and beyond.

Identification of highly discriminative targets for the rapid typing of MRSA clones

There are already multiple targets for the reliable delineation of the major clonal complexes within the *S. aureus* population (e.g. MLST, PFGE). It is much more challenging to identify targets that distinguish between sub-lineages within clones. This is especially important in outbreak situations, when a particular clone predominates within a specific geographic region. In these cases traditional typing methods are useless because they are unable to distinguish between different isolates within each clone, impeding the inference of the recent patterns of transmission within or between hospitals. The whole-genome sequencing data generated in this project enabled the identification of potential high discriminative targets that can be used for this purpose. To identify high discriminative targets for MRSA typing we considered the whole genome sequence of strains ST80, ST15, ST121 and ST59. The discovery of genes likely under positive or diversifying selection was performed by combining PAML and scripts developed under this study to analyse the five genomes. A total of 92 genes were found to be under positive selection. These included several noteworthy virulence genes encoding adhesins, transport and iron uptake.

Moreover, we took advantage of the genome-wide SNP data gathered for *S. aureus* ST239 isolates from four hospitals in Turkey and exploited the variation present in their phage sequences to design rapid PCR-based typing tools. We tested 21 different primer pairs based on two phages (ΦSA1 & ΦSA3), each primer encompassed at least two SNPs. We showed that the variation in PCR patterns was consistent with the variation based on core SNPs – that is to say, that it was possible to distinguish the isolates from Izmir from isolates from Istanbul/Ankara. However, it was more difficult to distinguish between isolates from the latter two cities. This study showed how NGS data,

combined with highly variable phage sequences can be used as the basis for tailor-made cheap and rapid typing procedures for local epidemiology.

The comparison of whole genome sequencing and clinical data of isolates belonging to CC121 showed that specific sub-lineages inside this main lineage have specific clinical properties. These results suggested that genome-wide SNPs may be powerful discriminatory markers to distinguish between sub-lineages within CC121. Moreover, all CC121 genomes harboured a 41-kilobase prophage that was dissimilar to *S. aureus* phages sequenced previously, which can be used as a target to discriminate this clonal lineage from others.

Deciphering vancomycin-resistant Enterococcus faecium.

The CC17 clonal complex

The first vancomycin-resistant enterococci (VRE) were isolated in Europe and later in the United States about twenty years ago (Leclercq et al., 1988). Nowadays, the vast majority of VRE are *E. faecium*. Already two thirds of all *E. faecium* from US intensive-care units (ICUs) are vancomycin-resistant. The situation in Europe is quite diverse with countries of very low (< 1% VRE) and very high prevalence (> 20 %) of VRE (EARSS surveillance network: http://www.earss.rivm.nl). Using various molecular typing methods a distinct number of ecovars was identified within the species *E. faecium* allowing to differentiate between typical poultry, swine, cattle, human commensal and hospital strain types. Also a subpopulation of highly-virulent, hospital-adapted, epidemic strain types exists – the so-called clonal complex CC-17. Isolates of this complex are characterized by distinct markers, such as ampicillin resistance, possession of virulence markers [*esp* and *hyl* genes] and high-level fluoroquinolone resistance, but not necessarily vancomycin resistance. The model predicts dissemination of epidemic, hospital-adapted strain types that acquire determinants of vancomycin resistance to a later stage by horizontal gene transfer.

The first task of this work package was to identify the VRE types associated with outbreaks. Indeed, although the MLST database (http://www.mlst.net/) includes a large number of VRE isolated all over the world, there is little information on the sporadic or epidemic character of the isolates. Partners 4, 6 and 12 provided in the deliverable 4.1 a list of Sequence-Types (ST) associated with outbreaks in European hospitals, mostly from Germany and France (obtained from the National Reference Centres), but also from the literature and from the MLST data bank. They all belonged to the CC17 complex, thus confirming previous findings. Although the provided list of STs associated

with hospital outbreaks is not exhaustive, it includes 25 different STs (all belonging to CC17). ST17, ST18, ST78, ST117, ST192 and ST280 are the most prevalent in large outbreaks.

Since determination of ST types in routine is expensive and labour-consuming, Partner 12 designed an easy PCR reaction based on the detection of insertion element IS*16* generally present in hospital-associated *E. faecium* strains and absent from human commensal, livestock- and food-associated *E. faecium* strains. This technique allows predicting the epidemic strengths or supposed pathogenic potential of a given *E. faecium* isolate identified within the nosocomial setting (see deliverable 4.4). Also, a MALDI-TOF technique was developed by Partner 12 that allowed differentiation between CC17 and non-CC17 isolates, based on the entire spectrum. However, type-specific peaks were not identified.

Partner 4 has tested several typing methods for VRE either sequence or PCR-based, including an automated rep-PCR method. Variability of genes encoding surface proteins (*acm*) or containing repeats (homologue of *clfA*, clumping factor gene of *Staphylococcus aureus*) has been exploited to study this issue. However, none of the studied techniques were enough discriminative or reproducible in comparison to pulsed field gel electrophoresis (PFGE) and Multilocus-Sequence Typing (MLST). Partner 12 had previously studied MLVA that was also found less discriminatory than MLST and PFGE for hospital-adapted clonal types. Therefore, we could not propose alternative techniques and fulfil the objective 4.1 (harmonization of typing techniques). For further studies in the TROCAR program, the MLST technique was used for typing and choice of strains to be analysed.

Finally, and despite previous studies (confirmed by the data collected during the TROCAR program) clearly showed that the CC17 clones are hospital-associated, the reasons for this adaptation are not fully understood and many clonal VRE spreading in German and French hospitals lack the virulence markers identified so far. Also, the reasons for differences in the epidemic strengths of clones within the CC17 complex are not understood. Objectives of the TROCAR programs attempted to answer these questions.

Design of an experimental model to test epidemic strengths of VRE

To test epidemic strengths of VRE, Partner 4 has developed an experimental model (task 4.5). The *Galleria mellonella* infection model previously described for *E. faecalis* was used to test the virulence of VRE. The innate immune system of *G. mellonella* shares a high degree of structural and functional homology to that of humans and there is also substantial correlation between mice and *Galleria*

models. Interestingly, *E. faecalis* was lethal for the larvae whereas *E. faecium* did not kill the larvae where it could survive and multiply for several days. Since colonization and persistence rather than lethality is the major feature of *E. faecium* and accounts for its striking capacity to massively colonize healthy carriers and patients, to overcome the host defences and to spread, leading to major outbreaks, this model was adapted to discriminate strains according to their ability to survive in the insects.

This model appeared to be a suitable and convenient surrogate model to study *E. faecium* survival. Despite their low virulence, the ability of *E. faecium* to overcome host-immune responses and multiply within the host system was confirmed by monitoring bacterial CFU following infection. *G. mellonella* is proposed as a simple, rapid and cost-effective non-mammalian model system that can be used to unravel the colonisation capabilities of *E. faecium* strains as well as to identify suspected virulence factors in the adhesion or in the response to host defences during the infection process. Interestingly, two of the strains belonging to some CC17 clonal complex strains were significantly more able to colonize the larvae than other CC17 isolates and non-CC17 isolates.

E. faecium, born to colonize

It is remarkable that among microorganisms provoking hospital outbreaks, *E. faecium* appears to be a leader as compared to the other enterococcal species, including *E. faecalis*. This may be related to the major capacity of *E. faecium* to heavily colonize patients and spread. By contrast, *E. faecium* infections are much less frequent. For instance, 209 clustered cases due to VRE in hospitals have been notified in France since 2001. The ratio of infection/colonisation was 0.3 (generally 0.05-0.1 in the literature). However *E. faecium* is pathogenic in patients with severe risks factors. Generally, massive colonization precedes infection in patients. The reasons for these colonizing capacities are not fully understood. Probably, accumulation of antimicrobial resistance traits in *E. faecium* is a major factor leading to selection of this microorganism under antibiotic selective pressure. In addition, Partner 4 has identified an oxidative stress-sensing regulator modulating *E. faecium* opportunistic traits. Oxidative stress serves as an important host/environmental signal that triggers a wide range of responses in microorganisms. This regulator, called AsrR (Antibiotic and Stress Response Regulator), belongs to the MarR family. The MarR family of transcriptional regulators (*S. aureus, P. aeruginosa*) sense oxidative stress and regulate bacterial responses. In *P. aeruginosa*, OspR (homologue of AsrR), was found to play key roles in antibiotic resistance and virulence regulation.

The AsrR regulator used cysteine oxidation to sense the hydrogen peroxide which resulting in its

dissociation to promoter DNA. Transcriptome analysis showed that the AsrR regulon was composed of 181 genes, including representing functionally diverse groups involved in pathogenesis, antibiotic and antimicrobial peptide resistance, oxidative stress, and adaptive responses. Consistent with the upregulated expression of the pbp5 gene, encoding a low-affinity penicillin-binding protein, the *asrR* null mutant was found to be more resistant to β-lactam antibiotics. Deletion of *asrR* markedly decreased the bactericidal activity of ampicillin and vancomycin, which are both commonly used to treat infections due to enterococci, and also led to over-expression of two major adhesins, Acm and EcbA, which resulted in enhanced *in vitro* adhesion to human intestinal cells. Additional pathogenic traits were also reinforced in the *asrR* null mutant, including greater capacity than the parental strain to form biofilm *in vitro* and greater persistance in *G. mellonella* colonization and mouse systemic infection models. Despite overexpression of oxidative stress-response genes, deletion of *asrR* was associated with a decreased oxidative stress resistance *in vitro*, which correlated with a reduced resistance to phagocytic killing by murine macrophages. Interestingly, both strains showed similar amounts of intracellular reactive oxygen species. Finally, a mutator phenotype and enhanced DNA transfer frequencies were observed in the *asrR* deleted strain. These data indicate that AsrR plays a major role in antimicrobial resistance and adaptation for survival within the host, thereby significantly contributing to the opportunistic traits of *E. faecium*. A global picture of the AsrR role is depicted in Figure 1. In this model, under stress oxidative conditions, the bacteria would display i) diminished susceptibility to penicillins, vancomycin and to cationic antimicrobial peptides, ii) increased adhesion, biofilm formation and host-colonization, iii) a mutator phenotype and enhanced DNA transfer frequencies and, unexpectedly, iv) decreased susceptibility to oxidative stress both *in vitro* and in murine macrophages. The first three conditions would promote *E. faecium* host-adaptation. Part of this study was done in collaboration with the group of R. Willems and W. Van Schaik (ACE program).

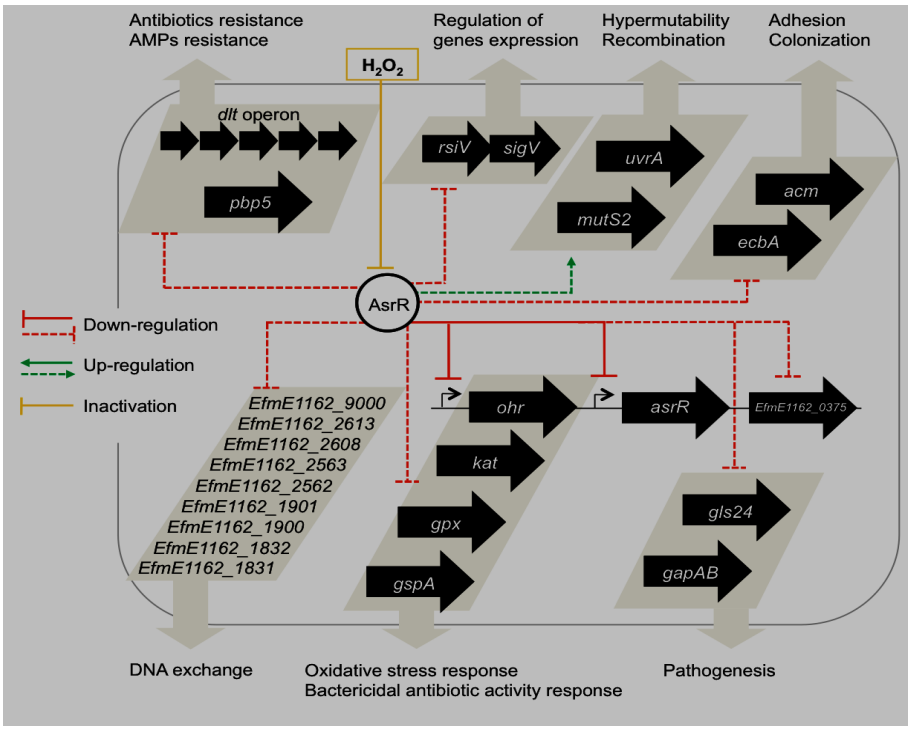

Figure 1. **Proposed model for the role of AsrR in *E. faecium***. Direct or indirect AsrR mediated up-regulations (green arrows) and down-regulations (red lines), H_2O_2 inactivation (red line), and interactions among transcriptional regulatory pathways (black dotted arrows) are indicated. Characterized *ohr* and *asrR* promoter region are indicated (black arrows). AsrR appears as a global repressor, inactivated by oxidative stress, of genes involved in important steps during the infection/colonization process.

In a future project, the expression of AsrR will be checked in strains with various epidemic strengths.

Identification of genetic factors or structures potentially involved in virulence

To identify genetic factors or structures potentially involved in virulence, two approaches presented below were used: the potential role of vancomycin-resistance plasmids and comparison of genomes of VRE with different epidemic strengths.

a. Role of plasmids in epidemicity:

Partner 4 has selected two strains of VRE. The first one 08-115 was responsible for a large outbreak in Strasburg, France, whereas the other one, 08-140, was a sporadic isolate obtained during this outbreak. Both contained large plasmids, the first one bearing a *vanA* operon and the second one a *vanB* operon. After *in vitro* conjugation to the susceptible, non-hospital-associated *E. faecium* HM1054, two corresponding transconjugants were obtained. A proteome analysis was performed by

Partner 1 and Partner 4 studied the in vitro oxidative stress response of the transconjugants, as well as their behaviour in the *G. mellonella* experimental model (tasks 4.3 and 4.4). Comparison of the proteomes and of proteins possibly involved in virulence did not show significant differences between the transconjugants. In particular, aggregation promoting protein and putative secreted antigen SagBb were detected in both. The responses of the two transconjugants to the oxidative stress and their behaviour in the *G. mellonella* model were similar.

For these two strains, the results would suggest that the background of isolates might be more important that the plasmid content to explain differences in the epidemic strength of the CC17 strains (in the absence of antibiotic selective pressure). However, further studies need to be performed with other isolates. Since plasmid studies were also an objective of the PCRD program ACE, no further studies were performed on this topic.

b. Comparative genome analysis of VRE clones to identify factors of enhanced virulence:

Partners 4 and 12 have selected three isolates to be investigated on the basis of different epidemic strengths: (a) a ST18 human sporadic isolate (major type in the CC17 complex) from Germany, multiply resistant to antibiotics; (b) a ST18 human isolate multiply resistant to antibiotics and involved in a large outbreak in France; and (c) a ST18 human sporadic isolate, multiply susceptible to antibiotics, the *E. faecium* AR-EM 40, isolated in a healthy carrier in Germany. Strains have been sequenced by Partner 15 and analysed by Partner 4

Comparison of the three sequenced genomes showed that 6 DNA blocks were present in the multiply antibiotic resistant strains but missing in the *E. faecium* AR-EM 40 sporadic strain. The 6 blocks were also identified in the contigs of the genomic sequence of the multiple antibiotic resistant strain of *E. faecium* DO (available in Genbank database) and mapped in the genome of the epidemic Australian strain of *E. faecium* Aus0004 (entirely annotated and available in GenBank database) (Figure 2). In the Australian strain, blocks appeared to be fragmented in the chromosome.

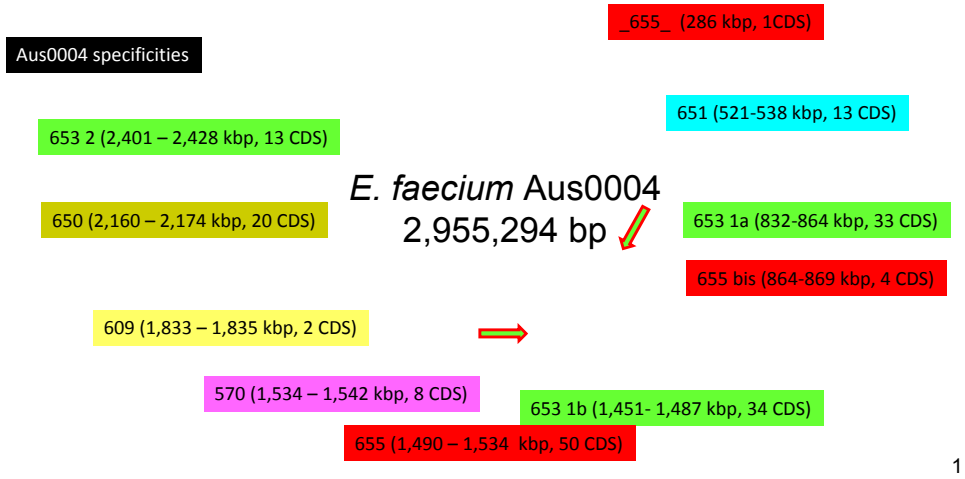

Figure 2. **Mapping of the 6 blocks missing in *E. faecium* AR-EM 40 in the chromosome of the Australian epidemic and multiply antibiotic resistant *E. faecium* Aus0004**. Each block is highlighted by a specific colour. Some blocks are fragmented. The number of Open-Reading-Frames is indicated in parenthesis.

Analysis of the blocks showed several genes that could confer higher virulence or colonization strength. In particular, genes encoding oxidative stress response proteins (in particular, Manganese containing catalase), a virulence-associated protein E, and some proteins of unknown functions were identified. The absence of the coding gene in the sporadic strain and their presence in the epidemic strains was confirmed by PCR and re-sequencing. However, no striking differences were found between the genomes of the two epidemic and sporadic multiply antibiotic-resistant strains. Since these genes could constitute markers for high-risk clones, we have searched for their presence in eight *E. faecium* isolates from various origins. Most of these genes were detected by PCR in *E. faecium* strains responsible for outbreaks. In contrast, they were not detected in two commensal isolates.

Epidemicity of a new type of vancomycin resistance

Partner 4 has characterized a new vancomycin resistance type in *E. faecium*, called VanN, that was transferable to other enterococci. The mechanism of resistance has been elucidated (low affinity for vancomycin of peptidoglycan precursors due to modification of the D-Alanine end in D-Serine) (Lebreton et al., 2011b). Consistently with the objectives of the program, we wished to evaluate the potential hazard raised by this new vancomycin-resistance type. Although resistance to vancomycin was transferable, the strain belonged to an animal ST type, was not multiply resistant and no other

VanN-type isolate was isolated since the first description. So, we can confidently predict that, currently, this type of strain does not present a high epidemic hazard.

Extended-spectrum, metallo- and acquired AmpC beta-lactamase (ESMAC-BL) emerging threats in Enterobacteriaceae: from clones to genes.

Collection of HiRiCs - ESMAC-BLs Enterobacteriaceae

During the development of the project, main research interest turned to carbapenemases, including detection and characterization of new carbapenemase genes and associated HiRiC in Enterobacteriaceae. Nevertheless, several contributions on ESBLs and or AmpC producers were also published from the TROCAR project.

As examples of these contributions, the evolution and spread of a multi-drug resistant (MDR) *Proteus mirabilis* clone in Europe were described. This MDR clone was detected in at least three EU countries (Poland, Italy and Greece). The bla_{CMY} gene associated with this isolate was demonstrated to be originated from a single IS*Ecp1*-mediated mobilization-transfer integration process, followed by the spread and evolution over time in a large geographic area (D'Andrea et al. Antimicrob Agent Chemother 2011; 2011; 55: 2735-42). In addition the molecular epidemiology of CTX-M-15 producing enteroagregative *Escherichia coli* recovered from stool samples from travellers returning from India was investigated (Guiral et al. Emerging Infect Dis 2011;17: 1950-3).

The current situation of carbapenemase producing Enterobacteriaceae (updated Feb-2012) was published as a review (Cantón et al. Clin Microbiol Infect 2012; 18:413-3) in which information on this issue was included with special attention to the spread of HiRiC in Europe. During the development of the TROCAR project a shifting epidemiology of *Klebsiella pneumoniae* producing carbapenemase (KPC) enzymes was noted. KPCs enzymes rapidly emerged in Italy associated with ST258-*K. pneumoniae* HiRiC. A rapid spread was also demonstrated in a multicenter study (Giani et al. J Clin Microbiol 2009; 47:3793-4). In this study, the emergence of colistin resistance within this clone was also demonstrated, affecting one fourth of the studied isolates. Contrary to the Italian situation, the emergence and maintenance of KPC enzymes in Spain were associated with non ST258-*K pneumoniae* HiRiC but in clones representing the local clonal pool (Curiao et al. J Antimicrob Chemother 2010; 65:1608-14, Ruiz-Garbajosa et al. manuscript submitted). Unlike Greece, Italy or Israel, the international *K pneumoniae*-ST258 lineage is not currently circulating as a major vector of

KPCs in the UK (Livermore et al. manuscript in preparation). Interestingly, KPC enzymes increased in prevalence in Greece over VIM enzymes although *K. pneumoniae*-ST258 is not longer the unique clone involved (Giakkoupi et al. J Antimicrob Chemother 2011; 66:1510-3; Papagiannitsis et al. Int J Antimicrob Agents 2012; 39:178-8).

Other TROCAR publications also described increasing importance of KPC carbapenemases. Cross border transmission of KPC producing Enterobacteriaceae was shown within EU countries as well as importation from countries outside EU (Cuzon et al. Int J Antimicrob Agents 2012; 39:448-9; Poirel et al. J Antimicrob Chemother 2011; 66:675-6; Brink et al. J Clin Microbiol 2012; 50:525-7; Potron et al. J Antimicrob Chemother. 2012; 67:242-3). Interestingly, integration of bla_{KPC} genes was observed in *E. coli* HiRiCs, such as ST131 (Naas et al. Antimicrob Agents Chemother 2011; 55:4933-4). Dispersion of KPC enzymes was also demonstrated in the environment (Poirel et al. Antimicrob Agents Chemother. 2012; 56:1662-3).

Other carbapenemases that deserved attention of TROCAR research were the metallo-beta-lactamases. Some of these enzymes were associated with specific clones, such as the new VIM-27 enzyme that was associated with *K. pneumoniae* ST147 (Papagiannitsis et al. Antimicrob Agents Chemother 2011; 55:3570-2). Moreover, in addition to hospitalized patients they have been detected in the community in colonized patients but with previous health care system contact (Gijón et al. J Clin Microbiol 2012; 50:1558-63). NDM producing Enterobacteriaceae were also detected in EU but nowadays to a lesser extent than OXA-48 producers (Cantón et al. Clin Microbiol Infect 2012; 18:413-3; Poirel et al. J Antimicrob Chemother 2012; 67:1597-606; Nordmann et al. Trends Microbiol 2011; 19:588-596). In Spain OXA-48 enzyme was first detected in a *K. pneumoniae* ST101 HiRiC with porin loss (Pitart et al. Antimicrob Agents Chemother 2011; 55: 4398-401). In Italy, the OXA-48 carbapenemase was shown to be encoded by a novel Tn1999 transposon derivative (Gianni et al. Antimicrob Agent Chemother 2012; 56:2211-13) and in France associated with the spread of specific clones previously detected in Morocco (Potron et al. Clin Microbiol Infect 2011; 17:E24-6; Poirel et al. J Antimicrob Chemother 2011; 66:1181-2).

Full sequencing and annotation of plasmids harbouring $bla_{ESMAC\text{-}BL}$ *in endemic strains or that have disseminated among multiple strains*

Different plasmids were selected for full sequencing within the TROCAR project. Specific publications are in preparation or sequences are being further analysed *in-silico* in comparison with

other sequenced plasmids of the same incompatibility groups. The following plasmids were selected for sequencing:

- a CTX-M-1 encoding IncN plasmid epidemic in Italy;
- an IncI1 plasmid harbouring *sul3* gene associated with bla_{ESBL} genes in Spain;
- a TEM-4 encoding IncF(k) persistent plasmid isolated in different Enterobacteriaceae in Spain,
- a CTX-M-14 IncN encoding plasmid identified in different Enteobacteriaceae in Spain,
- a highly transmissible plasmid encoding VEB-1 and VIM-1 in *Proteus mirabilis* from Greece (Papagiannitsis et al. Antimicrob Agents Chemother 2012; 56:4024-5)
- a KPC-2 encoding non-typable plasmid (pKP1433, GenBank accession number, pKP1433 Kp-1433 JX397875) isolated from an ST340 *K. pneumoniae* HiRiC in Greece
- a VIM-1 encoding non-typable plasmid (pKP1780) isolated from a VIM and KPC producing ST147 *K. pneumoniae* strain Kp-1780 in Greece.
- an OXA-48 encoding plasmid isolated in France.

Physiology and virulence traits contributing to the persistence and dissemination of ESMAC–BL organisms and their corresponding genes

The interest of this deliverable was mainly on ESBL producing Enterobacteriaceae. CTX-M-15 producing enteroagregative *E. coli* isolates from India were investigated. A clear predominance of phylogenetic groups D and B2 was observed as well as ST38 causing intestinal disease. The $bla_{CTX\text{-}M\text{-}15}$ gene was located either chromosomally or in plasmids, potentially associated with IS mobilization (Guiral et al. Emerging Infect Dis. 2011: 17:1950-3). Moreover, contribution of CTX-M-15 plus porin loss in phenotypic expression of OXA-48 in ST101-*K. pneumoniae* associated with an outbreak was also studied (Pitart et al. Antimicrob Agents Chemother 2011; 55:4398-401).

In addition, fitness cost of different CTX-M plasmids is being studied by *in vitro* competition assays (Rossolini et al. ongoing). Moreover, *in vitro* selection models for the emergence of CTX-M variants with resistance to β-lactam-β-lactamase inhibitors combinations were developed and published (Aida et al. Antimicrob Agents Chemother 2011; 55:4530-6).

In relation with carbapenemases, the mechanisms of *in vivo* emergence of colistin resistance in KPC producing *K. pneumoniae* is currently under investigation (Rossolini et al. on-going). The detection of $bla_{NDM\text{-}1}$ gene on a 300-kb plasmid also containing CTX-M-15, TEM-1 and a truncated DHA-1 and a full length ISAba125 found in NDM-*Acinetobacter* suggested horizontal gene transfer to

Enterobacteriaceae from *Acinetobacter* of the NDM-1 beta-lactamase gene (Solé et al. Antimicrob Agents Chemother 2011; 55:4402-4).

Additionally, a series of studies on fluoroquinolone resistance have also been performed. Increased expression of AcrAB/TolC in norfloxacin-resistant *E. coli* mutants caused by point mutations within *soxR* eventually lead to an increased expression of the transcriptional activator SoxS and allowed characterization of a new member of the *marA-soxS-rob* regulon, the *mdtG* gene, encoding a putative transport protein (Fábrega et al. Antimicrob Agents Chemother 2010; 54:1218–25). The comparison of isogenic norfloxacin-resistant and susceptible *E. coli* strains found overexpression of *ompN* in the resistant strain. This gene is positively regulated by SoxS but not by Rob or *marA* and is co-transcriptionally expressed with *ydbK* (FEMS Microbiol Lett 2012: 1–7). Moreover, heterogeneity of quinolone target gene mutants upon exposure to ciprofloxacin in *Yersinia enterocolitica* was also studied (Int J Antimicrob Agents; 2011: 38:550-2).

New tools enabling the rapid and simple detection of resistance genes in current and emerging circulating ESMAC–BL– Enterobacteriaceae

Main interest was on carbapenemases. These tools were implemented at different levels:

a) Chromogenic and selective culture media (Giani et al. Rapid detection of intestinal carriage of *K. pneumoniae* producing KPC carbapenemases during an outbreak. J Hosp Infect 2012; 81:119-22; Nordmann et al. J Clin Microbiol. 2012 Feb 22),

b) Molecular test based in microarrays (Cuzon et al. J Antimicrob Chemother 2012; 67:1865-9 Naas et al. J Clin Microbiol 2011; 49:1608-13) and real-time PCR protocols (Naas et al. Antimicrob Agents Chemother 2011; 55:4038-43; Poirel et al. Diagn Microbiol Infect Dis 2011; 70:119-23). These tools have also been evaluated by other TROCAR partners.

Genomics, drug resistance and physiology of MDR Pseudomonas aeruginosa and Acinetobacter baumannii strains

This WP focused on two opportunistic pathogens, *Acinetobacter baumannii* (Ab) and *Pseudomonas aeruginosa*, that are frequently responsible of nosocomial infections in intensive care

unit patients. Multidrug resistant (MDR) bacteria have increasingly been reported during the last decade and carbapenem resistance is now observed worldwide in these species, leading to limited therapeutic options. Several mechanisms are responsible for resistance to carbapenems. The carbapenem-hydrolyzing β-lactamases are either metallo-β-lactamases (MBLs), oxacillinases (carbapenem-hydrolyzing class D β-lactamases [CHDLs]) or more rarely class A enzymes. In order to better understand the current trend towards MDR and carbapenem-resistance in these species our work has focused on: the epidemiology of carbapenem resistance, on the underlying genetic features supporting carbapenem resistance, on natural resistance genes and their potential role in MDR, on recently acquired novel resistance genes and their potential to disseminate, and on the role of the environment as a reservoir for the dissemination of these resistance genes.

Most of the objectives could be reached in time and several points are in a process to be completed in the next few months. Several representatives of widespread MDR clones of *P. aeruginosa* producing MBLs/or another emerging carbapenemase and of *A. baumannii* producing carbapenemases of clinical significance were identified in several participating countries and were further characterized. The resistome and the genetic elements suppporting these genes have been characterized and revealed several novel resistance genes, DNA recombination systems, insertion sequences, transposons, and resistance islands (mostly in *A. baumannii*).

Acinetobacter baumannii

Several epidemiological investigations of *A. baumannii* have suggested increasing prevalence of OXA-carbapenemases in Europe, with however different resistance determinants involved. Increasing trends have also been observed in neighbouring countries, especially of the North African continent, from where frequent patient transfers to European hospitals occur. In Spain an *A. baumannii* clone (Ab41) has been found to be widely spread in that country. In Italy, $bla_{\text{OXA-58}}$ and $bla_{\text{OXA-23}}$ represented 75% and 25%, respectively of the carbapenem-resistance genes found in carbapenem-resistant *A. baumannii*. Finally, the occurrence of carbapenemase-producing Acinetobacter spp. in dairy Cattle from France is another illustration of the extend of the problem, suggesting that the food chain may also be involved in the dissemination of these MDR bacteria.

The genetics of carbapenem-resistance was extensively studied and several novel genetic vehicles have been identified (plasmids, IS, transposons) and likely are at the origin of resistance gene mobilization. A novel PCR-based replicon-typing scheme (the Ab PCR-based replicon typing [AB-PBRT] method) was devised to categorize the Ab plasmids into homogeneous groups on the basis of

the nucleotide homology of their respective replicase genes. Finally resistance islands (AbaRs), known to carry many resistance genes at the origin of MDR, have been identified in most multidrug resistant *A. baumannii* from worldwide origin. Two epidemiologically unrelated OXA-40 producing *A. baumannii*, the first identified in Italy, revealed a common plasmid named pABVA01, carrying a novel DNA module likely involved in mobilization by a novel site-specific recombination mechanism.

The reservoir of carbapenem-hydrolysing oxacillinases, which remains unknown for many resistance determinants, has been further investigated. Except for OXA-23, where *A. radioresistens* is the progenitor, the other progenitors are still unknown. Several *Acinetobacter* species intrinsically possess chromosomal CHDL genes: Ab (bla_{OXA-51}), *A. radioresistens* (bla_{OXA-23}), and *A. lwoffii* ($bla_{OXA-134}$). Three novel chromosomal CHDL genes have been identified in *A. johnsonii*, in *A. calcoaceticus* and in *A. haemolyticus*, respectively, $bla_{OXA-211}$, $bla_{OXA-213}$ and $bla_{OXA-214}$ genes. These enzymes hydrolyse carbapenems in a similar manner to OXA-23, and thus could have to the potential to spread in a near future, given that mobilizing genetic elements are present in the bacteria.

Besides OXA-carbapenemases, several novel resistance determinants were identified in *A. baumannii*. A point mutant derivative of the naturally produced AmpC enzyme that has increased hydrolysis activity towards ceftazidime, cefepime, and aztreonam, several ESBLs genes (PER-like) and several non-OXA carbapenemases (GES variants and NDM-1), illustrating the ongoing evolution towards multi-drug resistance of *A. baumannii*. The contribution of non-enzymatic resistance mechanisms, such as efflux systems, have also been addressed, as they participate to the overall high resistance background. They play major roles in resistance to tigecycline and other non ß-lactam antibiotics. Several novel efflux pumps have been characterised: an AdeABC-like pump in *A. nosocomialis*, and a major facilitator superfamily efflux pump associated with chloramphenicol resistance in *A. baumannii.* For colistin resistance, *pmr* mutations are currently in investigation in *P. aeruginosa, and in vitro* colistin-resistant *A. baumannii* and *P. aeruginosa* mutants are currently under molecular characterization.

Fast and accurate identification of genomic species from the *A. baumannii* complex is difficult based on phenotypic tools and requires the use of heavy molecular techniques. A rapid and accurate identification of genomic species from the *A. baumannii* (Ab) group by MALDI-TOF MS has been developed. As MALDI-TOF MS is increasingly used in microbiology laboratories, this work is an important contribution in *Acinetobacter* identification. Finally, the effect of biofilm formation on the survival of *A. baumannii* on dry surfaces has also been investigated, showing that the biofilm-producing *A. baumannii* strains can survive longer than the ones that did not.

Pseudomonas aeruginosa

Carbapenem resistance in *P. aeruginosa* isolates involves several mechanisms, such as porin modifications, efflux pump overexpression, and acquired carbapenem- hydrolyzing ß-lactamases (carbapenemases). Most of the carbapenemases identified in *P. aeruginosa* are metallo-ß-lactamases, but the Ambler class A ß-lactamases of the GES-type and very recently KPC-type have also been reported. KPC carbapenemases were initially described in *Klebsiella pneumoniae* in 2001 in North Carolina and have since rapidly emerged and disseminated worldwide, in enterobacterial species in particular. KPC-producing *P. aeruginosa* isolates were reported first in 2006 from Colombia and subsequently in Puerto Rico, in Trinidad and Tobago, and very recently in China. The rapid spread of KPC enzymes in Enterobacteriaceae has been linked to the genetic elements carrying the bla_{KPC-2} gene: plasmids of different sizes harbouring a Tn*3-like* transposon, Tn*4401*. In order to understand the epidemiological potential of KPC in *P. aeruginosa*, KPC-2-producing *P. aeruginosa* isolates from the USA and from Colombia have been molecularly characterized. These isolates were multidrug resistant, and in most cases, the *bla*KPC-2 genes were carried by plasmids of different sizes and were associated with Tn*4401b* or a new structure containing only part of the Tn*4401* sequence. In Colombia, KPC-2 producing *P. aeruginosa* isolates are identified in many different places, suggesting an important dissemination.

In an initial Italian nationwide survey on acquired MBLs in *P. aeruginosa*, an overall prevalence of 1.3% was observed, even though with some regional variability. VIM-1 was the most prevalent MBL, followed by VIM-2 and IMP-13. Recent data showed an alarming emergence in Italy, and in many European countries as well as in Argentina, of one IMP-13 producing clone, demonstrating the propensity to international dissemination. In the latest nationwide survey on acquired MBLs in *P. aeruginosa* in Italy, the overall prevalence raised to 7.5%, with still VIM-1 being the most prevalent, followed by VIM-2 and IMP-13. A *Pseudomonas mosselii* clinical isolate producing the VIM-1 MBL, isolated in Genova, Italy, in 1994, may represent the earliest known VIM-producing strain suggesting that influx of the bla_{VIM-1} MBL gene in the clinical setting started at least in the early 1990s. Finally, *Pseudomonas otitidis*, a novel *Pseudomonas* species recently linked to otic infections in humans, including acute otitis externa, acute otitis media, and chronic suppurative otitis media, was shown to constitutively produce a novel subclass B3 metallo-beta-lactamase (MBL), that was named POM (after *P. otitidis* metallo-beta-lactamase), and which is active on carbapenems and other beta-lactams.

Annotated genomic sequences.

Whole genome sequencing and annotation of several MDR strains of *P. aeruginosa* (VIM-1, SPM-1 and KPC-2) and *A. baumannii* (OXA-23, OXA-40) of clinical importance is still in progress. These data are a pre-requisite for performing comparative genomics between epidemic and non-epidemic isolates. These results will provide insights into novel genetic structures carrying resistance determinants and new information on the linkage between resistance and virulence genes in clinically relevant MDR strains. Preliminary results on KPC-2 carrying plasmid in *P. aeruginosa* indicate a novel plasmid backbone. In addition several novel genomic islands have been identified and are likely linked to the epidemic/MDR phenotype (work in progress).

Bioinformatic and evolutionary analysis of the genome-wide SNP data for bacterial pathogens

Although ambitious when originally written, the sample sizes of the TROCAR data, (5-10 strains per species) were relatively modest compared to data that came available during the course of the project for 100s of isolates. Because of this, the work for WP7 thus considered these data alongside the TROCAR genomes. For example, a key deliverable of WP7 was the establishment of a community-oriented database with user-friendly interrogation tools (akin to the highly successful model previously developed for MLST data (http://www.mlst.net)). Such a system has been developed in collaboration with colleagues at Imperial College, London, the Sanger Institute, and partner 8 of TROCAR using a large (>400 isolate) genome-wide dataset of >450 isolates of *S. aureus* from a pan-European survey.

With respect to understanding the emergence and spread of high-risk clones (HiRiC), and the phenomenon of "clonal replacement", the analysis carried out in WP7 took a dual perspective. Firstly, we considered how population level effects associated with the epidemiology of different clones might impact on the efficiency of selection. This re-phrases the question "what are the genetic changes responsible for the success of new emergent clones" to "what are the genetic changes responsible for the decline of old clones". We found that two of the sequenced genomes (SA09_1872 and SA07_2971) correspond to a single lineage associated with inefficient purifying selection and a high frequency of pseudogenes. This lineage is thus predicted to be less fit than the lineages

corresponding to the major MRSA clones currently which have been circulating throughout Europe over the last few years (e.g. EMRSA-15). We have also re-analysed the genome-wide SNP dataset for the HA-MRSA clone ST239 and noted that the accumulation of deleterious mutations in this clone might also be associated with its replacement in Europe and, more recently, other parts of the world. We also used a combination of dedicated scripts and existing methods (e.g. PAML) to detect genes under positive selection. From the five sequenced *S. aureus* genomes, we detected such 92 such genes, and many of these are promising candidates for new virulence genes. We used a similar method to examine positive selection within references genomes USA300 and MRSA252, and the highly divergence genome MSHR1132 ((Genome Biol Evol. 2011; 3: 881–895) showed strong evidence that different lineages have very different adaptive paths. This supports the view that the major clonal complexes in the *S. aureus* population represent adaptation to different niches.

One of the key mechanisms for the acquisition of resistance or virulence is the horizontal transfer of DNA. A large part of the work in WP7 was focused on examining the extent and impact of recombination on the core genome and the horizontal acquisition of accessory genes and mobile genetic elements. Regarding the former, we note that blocks of core DNA acquired by recombination tend to show a high preponderance of synonymous changes, which weakens the possibility that they are likely to be a great selective consequence. We also quantified the rate of recombination within the core genome using a new Bayesian methodology (BratNextGen) with colleagues at the University of Helsinki. We show significant variation in recombination rates between different phylogenetic groups, illustrating an interplay between epidemiological factors (transmission and bottlenecks) and molecular dynamics (recombination and mutation). We also note that not all accessory genes are gained and lost at the same rate. Some genes are very stable, and defined the “core” genome for major clonal complexes, whereas others (such as phage) tend to be much more dynamic, such that no two isolates are identical. The ranking of accessory genes and mobile elements in terms of their rapidity of gain and loss will shed light on those elements most likely to change the fitness of clones (either positively or negatively) over the very short term, and those which might be responsible for the adaptive differences between the major clonal groups in the *S. aureus* population as a whole. In the end, it is necessary to consider both long-term and short-term patterns of change in order to understand, and possible predict, where new clones of clinical significance will emerge.

4. POTENTIAL IMPACT

Socio-economic impact and the wider societal

A close collaboration, meeting and communication structure between National Expert/Reference Laboratories could be established. This structure continues to be the basis for important collaborative initiatives across all EU Member States, EFSA, accession- and candidate countries an will generate important contributions to the understanding of the spread and dynamics of High Risk Clones well beyond the lifetime of the TROCAR initiative. The decision to identify High Risk Clones on the basis of their abundance among isolates that cause life-threatening infections and to identify these clones in a systematic fashion using a prospective and representative sampling frame led to the first European strain repository which will be the stepping stone for future systematic research into the emergence and evolution of High Risk Clones. Moreover, our initiative created the critical capacity to identify clones of public health importance by DNA-sequence-based techniques at the national level. The initiative was instrumental to harmonise all identification/typing approaches across Europe and therefore allows National Reference Laboratories to inform and interrogate a single central database about the occurrence and spread of High Risk Clones with epidemic potential. This database can be accessed via World Wide Web (www.spatialepidemiology-net/srl-maps) and informs clinicians, microbiologist and public health stakeholders about he geo-temporal dissemination of High Risk Clones in European Hospitals.

Our spatial geographic analysis led to the appreciation that antibiotic resistant *Staphylococcus aureus* (MRSA) in contrast to susceptible clones (MSSA) are mainly confined to regional health care collectives being transmitted between different hospitals through patient movements as a result of between hospital referrals and the health care seeking behaviour of European citizens. This also led us to develop a novel 5-level staging system that is able to capture the level of epidemicity at national level in different European countries and can serve as a warning system for hospital repatriation. Finally, our novel approach to identify extant pathogens with important public health potential (High Risk Clones) by active surveillance using structured surveys improves our ability to deliver critical isolates to state-of-the-art service lines for detailed molecular analysis about the genetic and phenotypic features. It was however slightly unfortunate that analogous system could not be accomplished for the carbapenemase-producing Enterobacteriaceae since our TROCAR collaborators were determined to concentrate their efforts on problems that they had identified upfront.

One of the cornerstone elements of TROCAR was the improvement of the knowledge of the resistome and specific features of the HiRiC clones of *A. baumannii*, *P. aeruginosa* and (Extended-spectrum, metallo- and acquired AmpC beta- lactamases) ESMAC-BLs-producing enterobacteriaceae. Whole-genome sequencing and annotation of several MDR strains of *P. aeruginosa* (VIM-1, SPM-1 and KPC-2) and *A. baumannii* (OXA-23, OXA-40) of clinical importance as well as specific plasmids carrying genetic resistant determinants is still in progress. These data are a pre-requisite for performing comparative genomics between epidemic and non-epidemic isolates. These results will provide insights into novel genetic structures carrying resistance determinants and new information on the linkage between resistance and virulence genes as well as ability to disseminate in clinically-relevant MDR strains.

In addition, new tools enabling the rapid and simple detection of carbapenemase producing *Enterobacteriaceae* and their corresponding genes were implemented at different levels including chromogenic and selective culture media and molecular test based in microarrays and real-time PCR protocols.

TROCAR project shall contribute to:

- Create background knowledge for combating epidemicity and virulence
- Provide the scientific basis for an early warning system when isolates of a particular epidemicity appear in the community and nosocomial settings.
- Provide tools for monitoring the spread of key community and nosocomial pathogens.
- Characterise specific genetic elements carrying genes encoding ESCMAC-BL
- Identify potential reservoirs of antibiotic resistance genes among community and nosocomial pathogens involving multi-drug resistance.

Dissemination activities and exploitation

The TROCAR partners have been fully committed to ensure the maximum possible exploitation of the Project results in both national and international levels.

The dissemination of the main objectives of TROCAR started with the development of Project hallmarks such as the logo, a flyer and the website. A batch of flyers was distributed among the different TROCAR partners who disseminated it thought their colleagues and scientific national entities. The TROCAR website (http://trocarproject.eu) was launched in February 2009 and it contained an open and a closed part (Intranet). The closed part, with a restricted access, has been used for internal communication among the partners within the Consortium. The website played the major role in the dissemination of the Project objectives, activities and results generated. The flyer as well as all peer-reviewed publications have been uploaded into the website. The website will be kept operational until the end of 2012.

Peer-reviewed publications have also played an important role in the dissemination of the results. A total of 140 peer-reviewed publications and more than 120 communications (including oral presentations, posters, press releases, workshops and conferences) have been achieved in the TROCAR Consortium. The Consortium is also evaluating the possibility of preparing a monographic issue from the Multiple Drug Resistance Journal. This has been an invitation from the editor-in-Chief (Dr. A. Tomasz).

In addition, in order to disseminate information about TROCAR we used the established communication channels (conferences, newsletter, website etc.) to the scientific community in large and the industry. Exhibitions and demonstrations at large events were also used to foster dissemination. TROCAR booths were prepared for the European Congress of Clinical Microbiology and Infectious Diseases (the largest event in the field of Clinical Microbiology and Infectious Diseases, exceeding 9,000 participants). These booths included large posters, brochures and two scientists from TROCAR were there to answer as many questions as the visitors requested. TROCAR booth was also serving as a collaboration platform for companies at the 20th ECCMID 2010 in Vienna, Austria and 21st ECCMID 2011 in Milan, Italy. During these two meetings the TROCAR booth become an area for discussion in the industrial exhibition to inform possible industrial partners about the progress of the research and to provide opportunities for the exchange of views with industry (TROCAR Innovation Lounge). The TROCAR booth was well attended by potential collaborators.

In addition, TROCAR throughout one its partners (ESCMID) used its professional community consisting of some 28,000 microbiologist and clinicians as a platform for TROCAR to disseminate its

information. E.g. ESCMID published information about TROCAR on the website as well as an article in the ESCMID Yearbook 2010/2011. Additionally, ESCMID provided the infrastructure for TROCAR to distribute information about the project during the ECCMID 2011 and 2012 (mainly distribution of TROCAR leaflet and TROCAR conference announcements):

- 21st European Congress of European Clinical Microbiology and Infectious Diseases, 7-May 2011, Milan, Italy (ECCMID 2011)

- 22nd European Congress of European Clinical Microbiology and Infectious Diseases, 31 March – 3 April 2012, London, UK (ECCMID 2012).

A further major achievement was the organization of two Conferences as a platform to present and discuss the result of the TROCAR project:

- ESCMID Conference on Diagnosing Infectious Diseases: Future and Innovation, 24-26 October 2011, Venice, Italy.

- Conference on Multidrug Resistant High Risk Clones (MDR-HiRiC): Features, Epidemiology and Detection, 5-6 June 2012, Barcelona, Spain.

The main objective of this later conference was to disseminate all results generated from the TROCAR Project. In this sense we have to highlight that there was no registration fee and we provided up to 37 travel grants, therefore attracting a lot of young scientist who were very enthusiastic with the project.

5. PROJECT DATA

The TROCAR website (http://trocarproject.eu) was launched in February 2009 and it has two parts an open and a closed part (Intranet). The closed part, with a restricted access, has been used for internal communication among the partners within the Consortium. The website played the major role in the dissemination of the Project objectives, activities and results generated and it will be available until the end of this year 2012. We are currently updating the website to provide direct links to the PDF files of all open-access publications.

6. SCIENTIFIC ADVISORY COMMITTEE FINAL REPORT ON THE EVALUATION OF THE TROCAR PROJECT

In terms of the quality of science and the number of publications (140, during the 3 years funding period), the TROCAR project is clearly a success story. The publications appear in high impact professional Journals and provided a truly novel and comprehensive update on the nature of the most important drug-resistant pathogens in Europe. TROCAR was clearly a good investment of European Union funds.

The project produced updates on drug-resistant bacterial diseases concentrating on the most important pathogens such as carbapenamase-producing Enterobacteriaceae (WP2 and WP5); MRSA (WP3 and WP2); vancomycin-resistant *Enterococcus faecium* (WP4); multidrug resistant strains of *Pseudomonas* and *Acitenobacter* (WP6). The aim of WP7 was to develop web-based tools to manage and explore the large number of sequence data to be produced by the project. A collaboration with ESCMID (WP8) was also incorporated into the project mainly as a means to serve as a forum to disseminate information generated by TROCAR.

The collaboration of virtually all European countries by providing clinical isolates and also relevant epidemiological/clinical information has allowed the TROCAR investigators to use modern molecular probes and produce an update on the nature of the most important drug-resistant pathogens in Europe – with a level of resolution – that has not been possible before. The application of sophisticated molecular typing techniques has allowed the TROCAR investigators to try to identify the genetic basis of the "epidemic success" of the most important clones of drug-resistant *E. coli* and *Pseudomonas*, *E. faecium*, *Acinetobacter*, and *S. aureus*. In fact the most impressive aspect of this project is the successful combination of both "horizontal" (epidemiology, treatment options) as well as "vertical" (molecular characterization) coverage of issues, each one of which represents key concerns of the infectious diseases community worldwide. This raises the issue of how to bring the results of TROCAR to the attention of a broader-international community of experts? While the TROCAR project involved scientists associated with research centres and hospitals from eight European countries; the multi drug-resistant clones are not confined by continental barriers and identical issues and challenges are faced by the infectious diseases communities of the USA, South America, Asia, and Australia as well.

One way of sharing the key findings of TROCAR with the international scientific community would be to arrange a publication of the contribution to the Final TROCAR Symposium held in Barcelona in June 2012. This would provide the TROCAR project a well-deserved publicity but also would be a real service to the international infectious diseases community. I strongly suggest that the TROCAR considers this option.

The TROCAR Project was able to enlist some of the top scientific talent from Europe to produce one of the most comprehensive updates on multidrug resistant pathogens. Besides the scientific quality of its collaborators the success of TROCAR have clearly benefited from the congenial and effective leadership of the entire Project by Jordi Vila and his staff.

August 23 2012

Alexander Tomasz

7. PEER-REVIEWED PUBLICATIONS GENERATED FROM THE TROCAR PROJECT

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