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Molecular characterization of nosocomial infections:

an Italian (KPC-producing *Klebsiella pneumoniae*) and a Spanish

(*Clostridium difficile* ribotype 027) experience

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For those I love

Thesis overview

During the last decade, *Clostridium difficile* and *Klebsiella pneumoniae* represented two of the most emblematic cause of nosocomial outbreaks, especially following the spread of epidemic variants, able to produce virulent factors and to present higher resistance against several antimicrobial classes.

The rapid identification of dangerous pathogens circulating in nosocomial environment has been made possible by the contribution of molecular methods in support to the classical diagnostic techniques.

The principal objective of this work is to highlight the effectiveness of different molecular typing techniques in the characterization of nosocomial infections, evidencing the genetic relatedness among isolates and the transmission routes of strains involved in epidemic events.

In the first part of the work (*Chapter one*), the analysis of the principal molecular and phenotypic features of clinical isolates of KPC-producing *K. pneumoniae* collected in a 27-months period at Ospedale di Circolo e Fondazione Macchi (Varese, Italy) is reported.

Of the 16 isolates analyzed, 7 were involved in an outbreak occurred the Intensive Care Unit (ICU). We investigated the presence of genes involved in carbapenemases resistance (i.e. bla_{KPC}, bla_{IMP}, bla_{NDM}, bla_{VIM}, and bla_{OXA}), the expression of genes for virulence factors (pili/fimbriae, capsular antigen, hypermucousviscosity protein, and siderophores), and mutations leading to colistin resistance.

We also made a phylogenetic analysis adding all the 16 genomes to 319 genomes that represent the global diversity of *K. pneumoniae* strains.

We found that all isolates analyzed belong to clonal complex CG258. This finding is not surprising, considering previous reports that showed the worldwide diffusion and high prevalence of this clonal group among carbapenem-resistant *K. pneumoniae* strains. Interestingly, three of the four previously identified groups of Italian isolates of CG258 were found circulating in the hospital, suggesting that several entrance events of the clones may occur over the study period.

About resistance genes, we showed that all 16 genomes express *bla*$_{KPC}$ genes and none of them had other known carbapenem-resistance genes. Interestingly, 10 of the sixteen isolates, which were colistin-resistance, presented IS5-like transposons in *mgrB* gene, conferring resistance to this drug.

The second part of the work (*Chapter two*) aimed to identify and characterize all the clinical isolates of *Clostridium difficile* ribotype 027 (here, briefly CD027) collected during a 20-months period in the Hospital General Universitario Gregorio Marañon (Madrid, Spain).

The main objective was to characterize the epidemiological links among the CD027 clinical isolates and to defining their transmission routes. To better understand the epidemiological relationships between the strains, all cases identified since January 2014 were added to the analyses. Besides, presence of genetic markers (i.e. mutation in *gyrA* gene) characterizing the evolution and spread of specific epidemic lineages of CD027 was investigated.

During a 20-months period (January 2014-August 2015), 132 first episodes of *C. difficile* ribotype 027 has been detected, distinguishing 9 different subtypes (MLVA-types), organized in 5 different clonal complexes (CC) and 4 unique MLVA-patterns. Specifically, one of the five clonal groups (named MLVA-type 1) was responsible of an outbreak which involved 111 patients, and quickly spread in the hospital from October 2014. MLVA typing analysis showed the close genetic relationship of all the strains, suggesting the evolution from a common ancestor. All CD027 isolates carried a specific mutation in *gyrA* genes, indicating the presence of high transmissible clones belonging to CD027 lineages, but this do not provide more information on their dissemination course.

Moreover, the clonal complexes showed different capacity of spread, which was evidenced by the dissemination of the only CC of MLVA-type 1 leading to the epidemic event.

For this reason, the second objective of *Chapter two* aimed to evaluate the possible correlation between the transmissibility of the analyzed CD027 clinical isolates and the ability to sporulate. An *in vitro* protocol has been performed for the evaluation of sporulation rate of epidemic CD027 strain comparing them with those of the ribotype 001, with the objective of determining whether the increased transmissibility of CD027 is due to the greater ability to release endospores into the environment. Comparison between

strains belonging to the same ribotype was performed, to evaluate the possible association between sporulation rate and transmissibility.

We found that CD027 strains possessed higher capacity (p=0.005) to produce spores respect to isolates belonging to CD001 but surprisingly their germination ability was significantly lower (p=0.0008) compared to that of CD001, leading to reflect on the adaptability of this pathogen to the environment and the complexity of the mechanisms regulating pathogenic capacity.

Once characterized the rapid spread of an outbreak due to a particular CD027 clone (MLVA-type 1), a further aim was to highlight the possible differences between the efficiency of sporulation and germination of strains belonging to the same ribotype but with different transmission characteristics, underlying the possible association between the ability of sporulation and the strain transmissibility.

Moreover, analysis showed non-significant strain-to-strain variability between *C. difficile* isolates belonging to the ribotype 027; in particular, comparison was conducted between highly transmissible MLVA-type 1 isolates and the other MLVA-types, showing that neither in sporulation rate (p value =0.72) nor in germination rate (p value=0.24) significant differences exist.

Our findings suggest that further studies preferably by analysis of the transcriptome must be performed to clarify the features involved in CD027 pathogenicity.

This work shows how is possible, by application of molecular typing, to identify pathogens responsible for epidemic situations and to determine the presence of virulence factors o drug resistance genes associated with pathogenicity of strains. Besides it is possible to obtain useful and additional information as the genetic relationships between analyzed strains and their transmission route.

In conclusion, it is showed that active surveillance and characterization of circulating strains at local and national level is crucial to prevent the spread of more virulent variants, with benefits for the patient and the health system.

CHAPTER 1

Whole genome characterization of a nosocomial outbreak due to carbapenem-producing *Klebsiella pneumoniae*

1.1 Introduction

1.1.1. General features of *Enterobacteriaceae*

Enterobacteriaceae are members of a family of Gram-negative rod-shaped bacteria of which the most known members are *Escherichia coli, Klebsiella spp, Proteus spp, Enterobacter spp, Serratia spp, Citrobacter spp, Morganella spp, Providencia spp* and *Edwardsiella spp*, which are widespread in the environment and largely disseminated in the long-term care facilities and hospitals, where they may cause a broad range of infections. Principal risk factors for the acquisition of infections due to Enterobacteria are the extended use of antibiotics, the underlying diseases presented by the patients, the old age and long hospital stay.

For these reasons, the ability to trace the circulating nosocomial pathogens which spread rapidly causing large outbreaks has become a major responsibility of the Clinical Microbiology Laboratory, giving the necessity to avoid consequences as largest hospitalization of the patient; poorest clinical outcome and necessity of additional or specific treatment for eradicate the infection, leading to increase in sanitary costs.

Enterobacteria are Gram-negative bacilli, asporigenous, mobile for the presence of peritrichous flagella, or non-mobile, and present, almost constantly, pili; variants of non-mobile species normally are to be considered in normally mobile strains.

Gram negative bacteria structure is constituted by an inner cytoplasmic membrane, the cell wall and the outer membrane. This latter is critical in the pathogenesis and selection of mechanism of resistance to antimicrobial drugs, interacting directly with the external environment (fig 1).

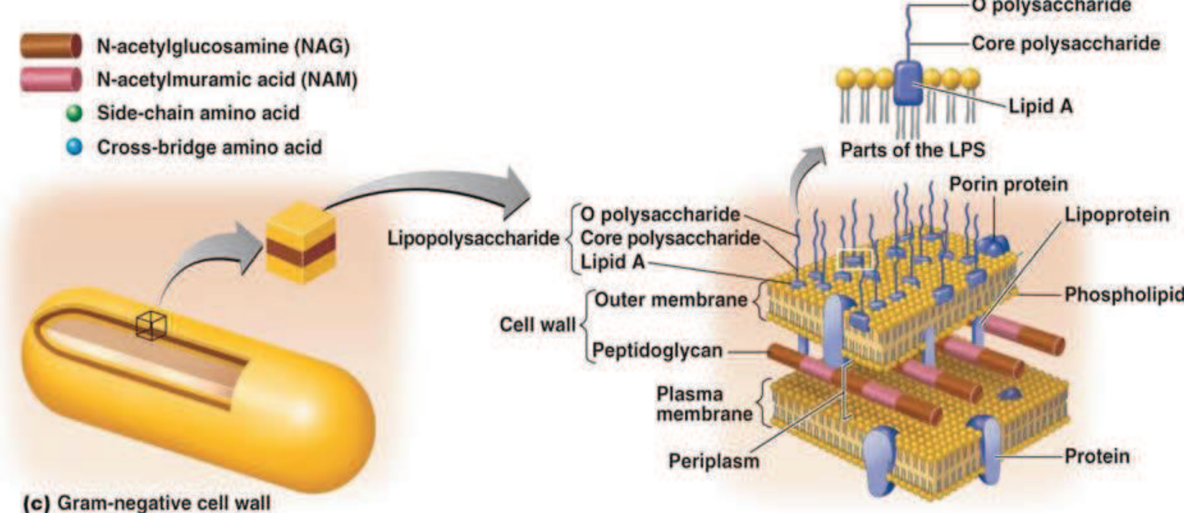


Figure 1. Structure of Gram negative cell wall

The outer membrane is characterized by the presence of numerous molecules of lipopolysaccharide (LPS know as bacterial endotoxin).

LPS consist of three main regions:

- lipid A, that is the bacterial endotoxin which plays the toxic action on the organism;
- oligosaccharide core, also called *common portion*;
- O antigen, which is a polysaccharide chain consisting of 20 to 50 repeat units. Generally can contain up to eight sugars, some commune (as galactose, glucose, and mannose) and other specific of bacterial species (colitose, abequose or paratose). It represents the outer portion and so the one exposed to the immune system (fig 2).

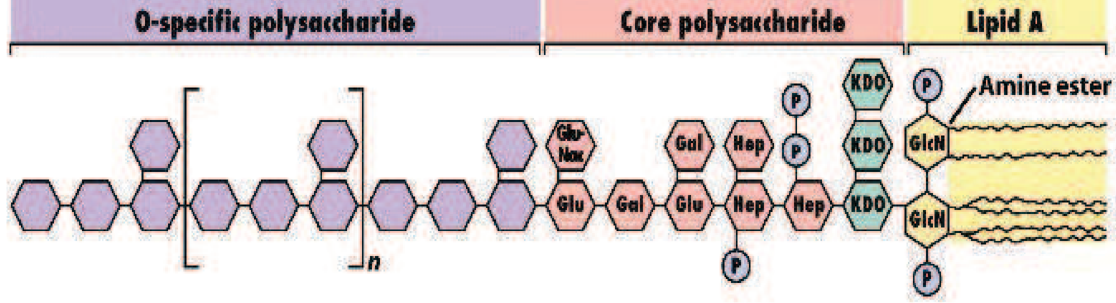


Figure 2. Lipopolysaccharide structure

Another important antigen in Gram negative bacteria is the K antigen, which is situated more superficially than O-antigen. It is mainly constituted by acidic polysaccharides or present as mucous layer; sometimes it assumes the dimensions of a capsule well developed (for example, in *Klebsiella-Enterobacter-Serratia* group, KES).

The K antigen prevents agglutination of bacteria when tested with sera containing anti-O, because acts masking the O-antigen and preventing a sufficient approach of bacterial cells required for the binding of bivalent antibody molecule.

Finally, the flagellar proteins, denominated H antigens, allow bacterial motility and exist in a large variety of antigenic types; possess proteic nature and are sensible to high temperature.

It is necessary to evaluate this antigen characteristic in the serological identification of several Enterobacteria.

1.1.2 *Klebsiella pneumoniae* infection

Klebsiella pneumoniae (KP) isolates are ubiquitous in the natural environment. They are found in surface water, sewage and soil and on plants, furthermore they colonize the mucosal surfaces of mammals such as humans, horses or swine.

In healthy people, the predominance of colonization by *K. pneumoniae* reaches mostly in the colon, and in the pharynx, while the skin is colonized only transiently. Therefore, *Klebsiella* spp. is frequent in human fecal material.

Recent data showed that *K. pneumoniae* bloodstream infection isolation rates are 1.5 times greater during the warmest months of the year. These rates most likely reflect increased fecal carriage in humans, which in turn is a reflection of increased organisms in the environment during warm months. This has important implications since colonized patients have a fourfold-increased risk of infection over non-carriers.

Classical studies show that there subspecies of *K. pneumoniae* involved in severe disease: *Klebsiella rhinoscleromatis* and *Klebsiella ozaenae* are involved in specific chronic infectious diseases, respectively

rhinoscleroma and atrophic rhinitis (ozena). These diseases occur most frequently in the tropical areas of the world.

Another subspecies of *K.pneumoniae* involved in sever diseases is *Klebsiella granulomatis*, which is the causal agent of inguinal granuloma (chronic genital ulcers).

K. oxytoca strains carrying a chromosomally encoded heat-labile cytotoxin have been increasingly recognized as a cause of antibiotic–associated hemorrhagic colitis [1].

The most frequent infectious processes caused by *Klebsiella pneumoniae* are represented by:

i. Community-acquired pneumonia: *Klebsiella pneumoniae* it is involved in a small percentage of cases of community-acquired pneumonia. This type of pneumonia is more common in Africa and Asia than in Europe and the United States, and occurs mostly in patients with co-morbidities such as alcoholism, diabetes and respiratory disease. The lung infection is common in hospitalized patients or admitted to long-term care facilities, due to the increased rate of colonization of the pharynx, considering the mechanical ventilation as prevalent risk factor [2]. From a clinical point of view, there are the typical production of purulent sputum, and evidence of a disease in the airways. Initially, the pulmonary involvement is very extensive and very frequently reflects the classic infiltrated involvement of fissures. Necrosis lung and pleural effusion may occur with the progression of the disease (fig 3).

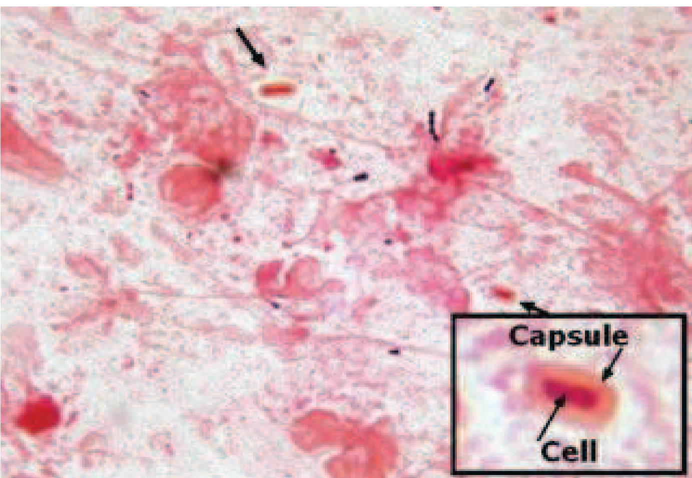


Figure 3. The image represents a cytological section of lung infection due to *Klebsiella pneumoniae*. In detail, a *K. pneumoniae* cell, presenting an evident capsule

ii. Urinary tract infection (UTI): *Klebsiella pneumoniae* is responsible for 1-2% of the episodes of UTI in healthy adults, while it is involved in the 5-17% of complicated UTI, including those associated with the presence of bladder catheter [3]

iii. Intra-abdominal infections: the spectrum of intra-abdominal infections caused by *K. pneumoniae* is comparable to that supported by *E. coli*, even if it is isolates with lesser frequency in these clinical manifestations.

The new variant “hypervirulent” *Klebsiella pneumoniae* (hvKP) have emerged in the past decade as a most dangerous pathogen, and was first reported in the Pacific areas [4] and, later, also in the USA [5], Canada [6], and Europe [7-10], leading to increased rate of intra-abdominal infection due to this pathogen, mainly in patients presenting liver abscesses caused by community-acquired infections or those who have no history of hepatobiliary disease traced to other organisms

iv. other infectious diseases: patients with devitalized tissue, such as diabetic ulcers, pressure ulcers and burns, as well as immune-compromised patients are predisposed to cellulite and soft tissue infections. *Klebsiella spp* also causes: surgical site infections, osteomyelitis and occasional contiguity with soft tissue infected, hematogenous endophthalmitis (particularly in combination with the presence of liver abscess), nosocomial sinusitis, neonatal and post-neurosurgical meningitis [11-13].

1.1.3 Treatment of infection due to Enterobacteria

The early initiation of appropriate antibiotic therapy, especially in severe infections, is associated with improved outcomes. When antibiogram profile is available, considering that the previous empirical treatment includes broad-spectrum drugs, it is important to continue the specific treatment with the most appropriate short-spectrum agent. This management avoids the selection of resistant bacteria and the potential super-infection by these, reduces costs and maximizes the usage duration of the antimicrobial agents currently available.

Generally, the most reliable drugs in the treatment Enterobacteria infections are amikacin, cefepime (resistant to β-lactamase AmpC), piperacillin-tazobactam, tigecycline and polymyxins (showing *in vitro* activity against the carbapenemases-producing strains) and carbapenems (molecules more effective, particularly against ESBL-producing strains).

The emergence of *Klebsiella pneumoniae* strains which present resistance to third generation cephalosporins is a growing phenomenon. This increasing incidence of multidrug resistant bacteria is mediated by acquisition of plasmids containing genes coding for ESBLs enzymes (resistance to beta-lactamic antibiotics) and leading to resistance against others drugs as aminoglycosides, tetracyclines and trimethoprim-sulfamethoxazole, resulting in the difficulty or the inability to treat the patients.

Consequently, in severe infections due to ESBL-producing *K.pneumoniae*, it is reasonable to set empiric therapy including amikacin or carbapenems. However, the use of these drugs has led to the selection of strains producing carbapenemases enzymes, able to confer resistance to those drugs.

The treatment is therefore very challenging, since carbapenemases-producing strains are often pan-resistant. In these cases, the optimal choice of therapy is uncertain, including tigecycline, polymyxin B and polymyxin E (colistin), which seems to be the most active agents *in vitro*, although in recent years an increase of colistin-resistant strains is registered.

1.1.4 Antibacterial drugs

Enterobacteria are particularly prone to present resistance against many antibacterial drugs, which may be natural or be resulting from the selection of particular variants.

Therapeutic choices must necessarily be guided by the determination of drug susceptibility through *in vitro* tests.

Drugs activity and mechanisms of resistance

Antibacterial drugs act through different pathways, with highly selective toxicity:

a) Inhibition of protein synthesis: the antibiotics that act on protein synthesis are mainly aminoglycosides, macrolides, tetracyclines, chloramphenicol and lincomycin. The mechanisms of action by which these perform their function act primarily by inhibiting the activity of bacterial ribosomes (different from those of the eukaryotic cells) and of all the initial complex necessary for the synthesis of the peptide chain, leading to a stop or a misreading or bacterial mRNA and the inability to produce proteins

b) Inhibition of nucleic acid synthesis. In this case, depending on the compound used, synthesis of DNA or RNA will be inhibited. Drugs inhibiting DNA synthesis are quinolones, fluoroquinolones and trimethoprim (TMP), which act on nucleic acid precursors or on DNA gyrase. Rifampicin stops mRNA synthesis, as it acts on the DNA-dependent RNA polymerase as showed

c) Inhibition of bacterial cell wall synthesis and function. All β-lactam antibiotics selectively inhibit bacterial cell wall synthesis. Initially, the drug interacts with the PBPs (Penicillin-Binding Proteins), involved in the transpeptidation reaction, so that peptidoglycan synthesis is blocked and the bacterial cell undergoes lyses or fragmentation (fig 4) [14].

Acquired resistance to drugs results from biochemical processes encoded by bacterial genes. Antimicrobial resistance arises by alteration of the binding sites of the antimicrobial agent and/or alteration of the membrane permeability or porin channels, via activation of efflux pumps, which extrude the drug from the cell before it can bind to its target (fig 4).

Drug-susceptible bacteria become resistant to antibiotics after mutation and selection (so-called "vertical evolution") or obtaining genetic information from other bacteria (for example plasmids; in this case we speak of "horizontal evolution") [15].

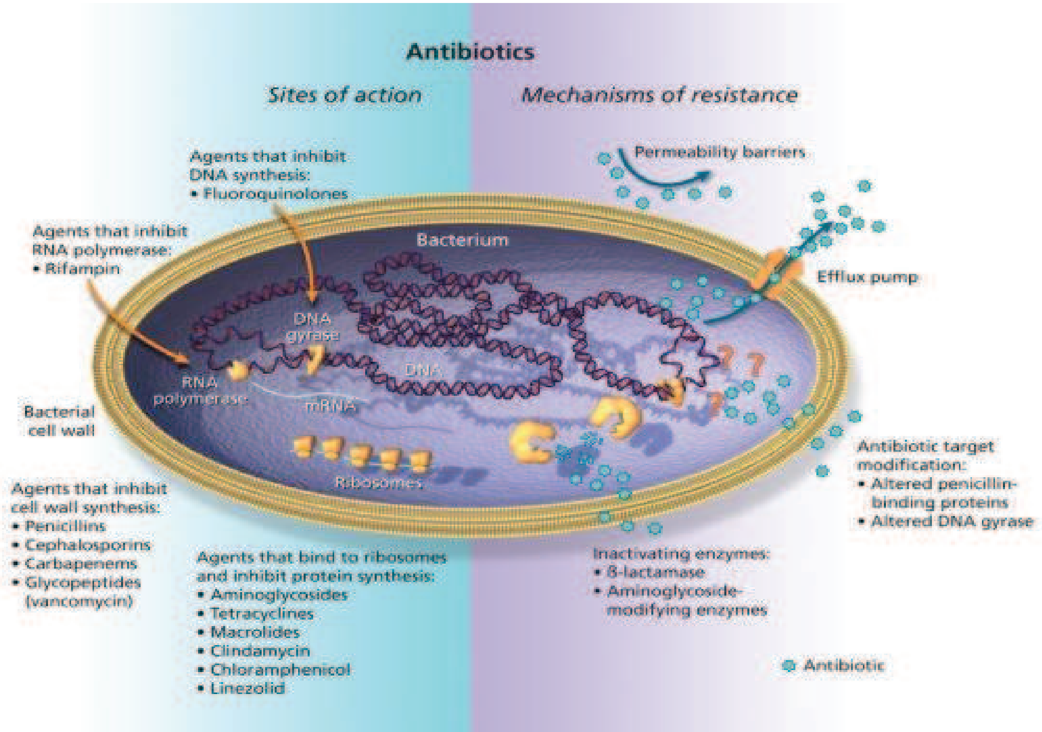


Figure 4. Schematic representation of action site of antibiotic (left) and drugs mechanism of resistance in bacteria (right); source [14]

Beta-lactamic drugs and beta-lactamases

Until the late 90s, efforts were focused on the study of microorganisms expressing mechanisms of resistance against different drugs but especially to beta-lactam antibiotics, representing about 50% of antimicrobial drugs used in humans.

The β-lactam antibiotics date back to the 1940-50s when only two β-lactam agents were known: penicillin G and penicillin V, that were the first β-lactam antibiotics introduced into clinical practice. It was not until the 1960s that semi synthetic penicillin was developed followed by semi synthetic cephalosporins and other β-lactam antibiotics. Today, class of β-lactam antibiotics include penicillins, cephalosporins, carbapenems, and monobactam, which act blocking the bacterial membrane formation, through inhibition of transpeptidase, the enzyme responsible for the formation of peptidoglycan. It constitutes the outer layer of the cells and it is composed by a polymer of *N*-acetyl-glucosamine (NAG) and *N*-acetyl-muramic acid (NAM) are cross-linked via the peptide chain.

Penicillin-binding proteins (PBPs) are transpeptidases (TP) that catalyze the formation of a cross-linked polymer of NAM-NAG, using D-ala dimers [16, 17].

The β-lactam antibiotics act miming the D-ala-D-ala component of the cell wall, which is the normal TP enzymatic substrate. The PBPs mistakenly attacks the carbonyl group of the β-lactam ring creating a covalent interaction in which PBPs remain bound and cell wall synthesis is blocked (fig 5).

In general, β-lactams account for greater than 60% of all antimicrobial consumption, but heavy usage has selected strongly for resistance [18, 19] .

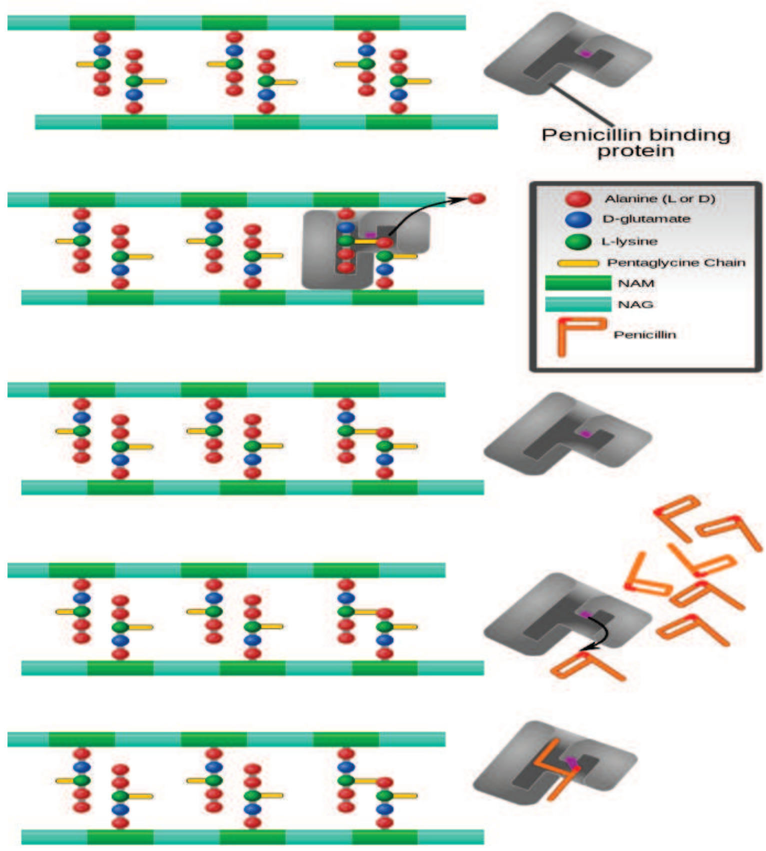


Figure 5. Mechanism of action of beta-lactam antibiotic: the PBP binds the peptide side chains and forms the cross-link with the expulsion of one D-Alanine; then it dissociates from the wall once the cross-link has been formed. Beta-lactam is added to the system and enters in the active site of the PBP. Drug reacts (covalently and permanently) with the active site and inhibits interaction between PBPs and chain, blocking wall synthesis (source, https://en.wikipedia.org/wiki/Penicillin)

Resistance to this type of antimicrobial agents is due to the expression of β-lactamase and extended spectrum β-lactamase (ESBL). These enzymes act by hydrolyzing the β-lactam ring of penicillins and cephalosporins, and may be mediated by plasmids or chromosomal genes.

Over 400 different β-lactamase produced by many bacterial species have been described in the literature. The classification of these enzymes is complex, but can be divided into two main schemes:

a) functional classification of Bush, Jacoby and Medeiros: divides β-lactamase in 4 functional groups and in different subgroups (indicated by letters of the alphabet) based on enzyme activity against different substrates and their susceptibility to inhibitors (clavulanic acid, sulbactam, tazobactam) [20];

b) molecular classification of Ambler: groups the β-lactamase in four molecular classes (A, B, C and D) based on their primary structure [21, 22].

Extended-Spectrum β-lactamases (ESBL)

Enzymes belonging to the ESBL class confer resistance to penicillins, cephalosporins, to the third generation, to aztreonam and sometimes fourth-generation cephalosporins. They are inhibited *in vitro* by clavulanic acid and other inhibitors of the β-lactamase (sulbactam, tazobactam) and do not hydrolyze carbapenems or the cephamycins, such as cefoxitin and cefotetan.

With the exception of OXA-type enzymes, which are class D enzymes, the ESBLs are mainly of the Ambler class A and can be divided into three groups: TEM, SHV, and CTX-M types. Most are located on plasmids which allow an efficient and rapid transmission of resistance [23-26].

The production of ESBL not always determines MIC values above the clinical resistance breakpoints, as in relation to the different hydrolytic activity of the enzymatic variants, as for the amount of enzyme produced, and as the variable ability of β-lactam antibiotics to cross the outer membrane of bacteria.

In these cases, despite the apparent susceptibility *in vitro*, the extended-spectrum cephalosporins may fail in the treatment of infections caused by ESBL-positive. Based on these considerations, there is an international consensus to interpret carefully all ESBL-producing strains, introducing a two-steps laboratory assay, constituted by a first screening and a subsequent confirmatory test.

AmpC β-lactamase

The first bacterial enzyme reported to hydrolyze penicillin was the AmpC β-lactamase of *E. coli*, although it was not given this name since the 1940s.

AmpC enzymes are grouped into Ambler class C and shows its activity against penicillins, expanded spectrum cephalosporins of third generation (such as cefotaxime, ceftriaxone, ceftazidime), cephamycins (cefoxitin and cefotetan), and aztreonam. The full spectrum of activity occurs when there is overexpression of the enzyme.

Unlike ESBLs, AmpC β-lactamases are not inhibited by the commonly used inhibitors as clavulanic acid, sulbactam, and tazobactam, but may be inhibited by cloxacillin or boronic acid.

Some strains of *Escherichia coli, Klebsiella spp., Salmonella spp., Enterobacter aerogenes*, and *Proteus mirabilis* have acquired plasmids containing genes of the β-lactamase AmpC, of which the most common enzymes belong to the families of CMY, FOX, and DHA.

The action of AmpC enzymes is given by its overproduction but may exist concurrently with other mechanisms such as the alteration of the membrane permeability or of the porins functionality, for which, as for ESBLs, the assessment of the sensitivity tests is to be carefully evaluated.

Carbapenem drugs and carbapenemases

The increase of resistance to penicillins and cephalosporins led some pharmaceutical companies to screen soil actinomycetes for searching new antibiotics. After several years, in 1980s, industries made available the first carbapenem: imipenem [27].

Carbapenems are a class of β-lactam antibiotics with an exceptionally broad spectrum of activity and, like beta-lactams. These drugs has been the main treatment choice, especially for severe infections caused by ESBL- and AmpC-producing *Enterobacteriaceae* [28].

Resistance to carbapenem is due mainly to the production of carbapenemases, which are enzymes able to make ineffective treatment with penicillins, cephalosporins, aztreonam, cephamycins and carbapenem,

although occasionally it may be due to the presence of a β-lactamase associated with a reduction of the permeability of the bacterial membrane.

Carbapenemases are divided into two principal molecular groups: metallo-carbapenemases and serine-carbapenemases (distinguished by hydrolytic mechanism at the active site) [20].

The first are inhibited by EDTA and contain at least one zinc atom at the active site; the second contain serine at the active site and are sensible to clavulanic acid and tazobactam, but not to EDTA [29-31] (table 1).

Molecular Class	A	B	D
Functional group	2f	3	2d
Active site	Serine	Zn^{++}	Serine
Aztreonam hydrolysis	+	-	-
EDTA inhibition	-	+	-
APBA inhibition	+	-	-

Table 1. Representation of principal characteristic of the carbapenemases, grouped according to Ambler classification

The major family of class A serine carbapenemases include KPC enzymes, while the most diffused and studied acquired metallo beta-lactamases include IMPs, VIMs, SPM-1, GIM-1, AIM-1, SIM-1, NDM-1 and DIM-1 families [32-36].

The emergence of carbapenems resistance constitutes a major clinical problem, since these antibiotics represent the reference drugs for the therapy of invasive infections due to multiresistant (MDR) gram-negative enterobacteria [35, 37, 38].

The carbapenem-resistant *Enterobacteria* represent a significant danger to public health, because they are frequent cause of hospital-acquired infections; it was observed an increased rate of mortality; resistance to carbapenems may be transmitted to other microorganisms through plasmids [39, 40].

For these reasons, it is essential to improve the preventive measures and implement specific guidelines as the most effective in the diagnosis of these microorganisms.

Automated systems for the determination of drug susceptibility can be unreliable in the detection of carbapenemases, in particular those which confer resistance to imipenem and meropenem. So, the presence of a high MIC for carbapenems should provide for the execution of additional tests: can be applied molecular methods, such as PCR for the identification of resistance genes, or phenotypic assays, as the modified Hodge tests [41, 42].

Focus on most diffused carbapenemases

KPC-type

This denomination refers to *Klebsiella pneumoniae* carbapenemases (KPC), because are most commonly expressed by this bacterium. KPC-type carbapenemases are included in molecular class A and functional group 2f (Ambler class); can hydrolyze different antimicrobial agents such as aminoglycosides, fluoroquinolones, and β- lactams including penicillins, carbapenems, cephalosporins, and aztreonam [43] [44] and are inhibited by clavulanic acid and tazobactam [45].

To date, several KPC gene variants have been reported and classified from KPC-2 to novel KPC-16 and KPC-17, grouped into $bla_{\text{KPC-2-like}}$ (including alleles 2, 4, 5, 6, 11, and 12) and $bla_{\text{KPC-3-like}}$ (including alleles 7, 8, 9, 10, and 13) [46, 47]. Interestingly original KPC-1 was found identical to KPC-2 [48, 49], while the KPC-2 to KPC-11 genes are characterized by non-synonymous single nucleotide substitutions within four codons [47]. The KPCs are predominantly found in *K. pneumoniae*; however, they have also been found in many other *Enterobacteriaceae* including *Salmonella species* [34], *Escherichia coli* [50-53] and others, such as in many non-*Enterobacteriaceae* including *Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains [54-56].

Although the first case of isolation of KPCs was in America, these enzymes are now widely distributed worldwide [57] (fig 6).

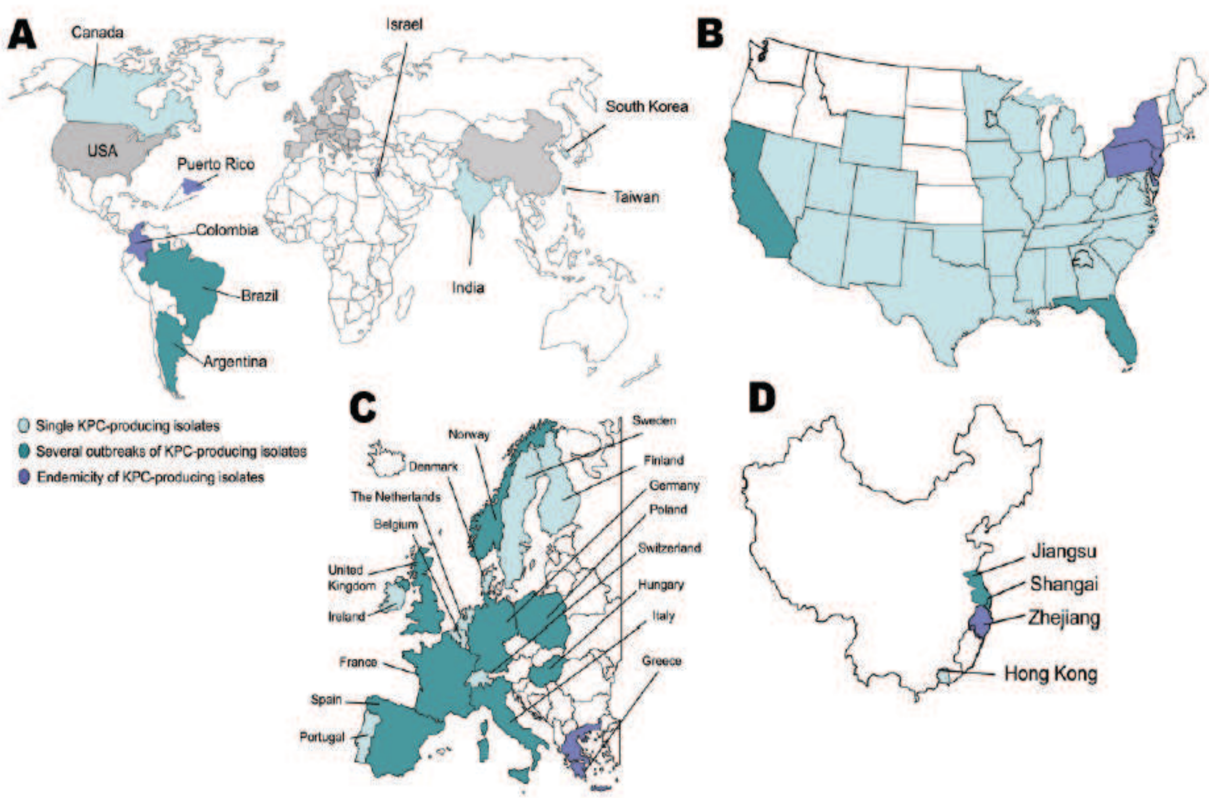


Figure 6. Global spread of KPC enzyme: a) worldwide distribution; b) spread in USA; c) European dissemination; d) distribution in China. In blue, single KPC-producing isolates; in green, detection of outbreaks due to KPC-producing isolates; in purple, endemic KPC-producing strains (source [57])

Infections produced by KPCs have frequently nosocomial spread and are associated with treatment failure and high mortality; for this reason, combination therapy is recommended for the treatment of KPC infections [58].

VIM-type

Verona Integron-encoded (VIM), called in this way because was first isolated in Verona, Italy, in 1997 is a metallo-β-lactamase belongs to the Ambler Molecular class B (metallo carbapenemases) [59].

These enzymes hydrolyze carbapenems and extended-spectrum beta-lactams, but are not inhibited by beta lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam [60].

VIM-dependent resistance production is considered a serious problem because of the rapid spread of this enzyme; moreover treatment options of VIM-producing organisms are considered limited, although combination therapy seems to be the best treatment [61-63] (fig 7).

IMP-type

IMP is a metallo beta-lactamase widespread globally and transmitted by transposons or plasmids. The first issues of IMP-1, the most popular IMP variant, date back to 1999, when it was isolated from *P. aeruginosa, K. pneumoniae* and *S. marcescens* and was associated with resistance to carbapenems [64]. Always during those years, there was a great spread of strains of *K. pneumoniae* IMP-producing that had the ability to induce strong resistance to carbapenems [65, 66].

Today the spread of this variant is expressed globally, similarly to VIM (fig 7), and to date most than 50 IMP-like variant are known (Lahey Clinic.: http://www.lahey.org/studies/) [67]

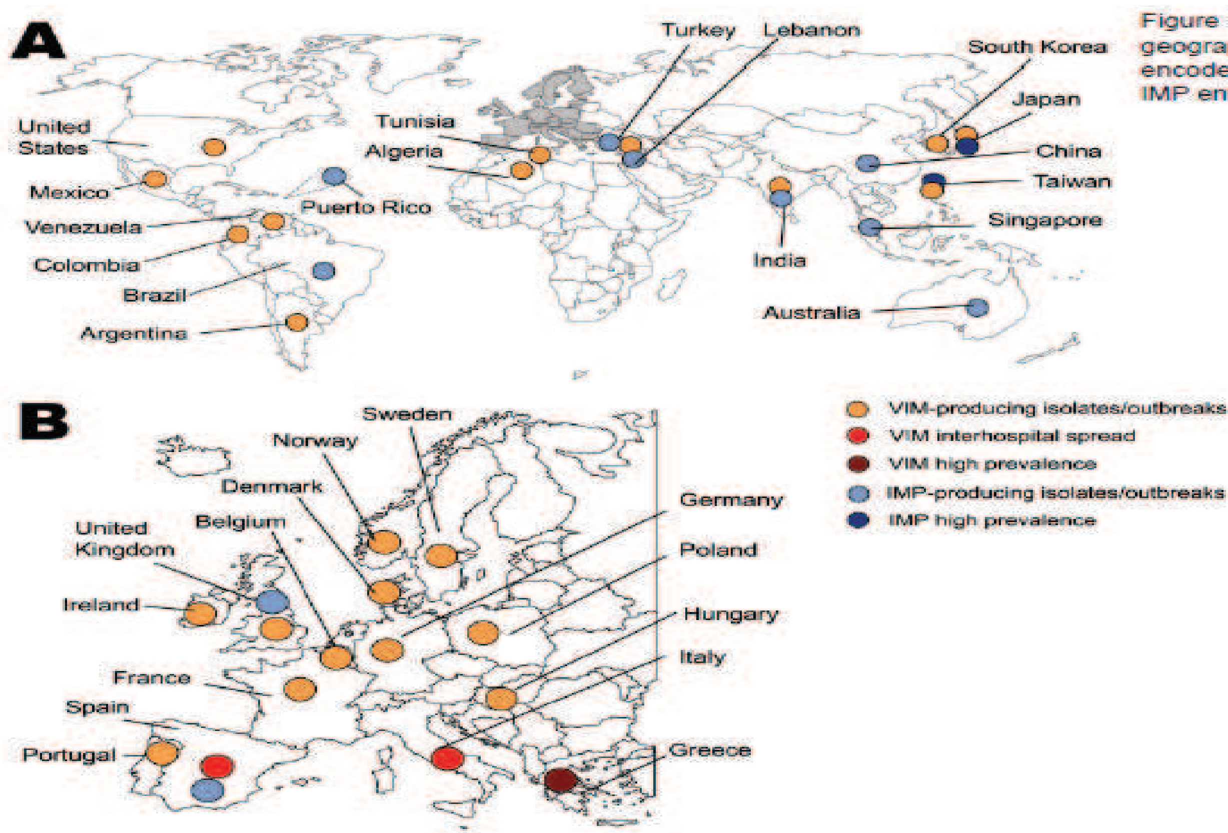


Figure 7. Geographical distribution of VIM and IMP enzymes (a) worldwide and (b) in Europe. About VIM-producing strains distribution: in yellow, VIM-producing nosocomial outbreaks; in red, VIM interhospital spread, and in purple, high prevalence of VIM-producing isolates. About IMP-producing isolates, in light blue, presence of nosocomial infection due to IMP-producers spread, and in blue prevalence of IMP enzyme diffusion [57]

OXA-type

Oxacillin-hydrolyzing enzymes (OXA) are part of the class D of the serine beta-lactamase. They were identified for the first time in Scotland, in a patient colonized by *Acinetobacter baumannii* presenting resistance to cephalosporins [23, 45]. The enzyme involved was named OXA-23 and, to date, more than two hundred variants of OXA-like enzymes are known, capable of hydrolyzing extended-spectrum cephalosporins and, in some cases, also confer low levels of resistance to carbapenems [68-70].

Among oxacillinases, OXA-48 is a plasmidic resistance gene widespread in the family of Enterobacteriaceae and confers resistance to different classes of drugs (cephalosporins, penicillins, β-lactam and even carbapenems) [68, 71, 72]. Moreover, *K. pneumoniae* OXA-48-producing is widespread worldwide [73-75].

NDM-type

The New Delhi Metallo-β-lactamase (NDM-1) is one of the β-lactam enzymes more recently identified and more quickly spreading worldwide: was isolated from a patient of Indian origin in Sweden and to date exist 16 NDM variant (NDM 1-16; Lahey Clinic.: http://www.lahey.org/studies/)[32].

In accordance to all beta-lactamases, also NDM is commonly expressed by *K. pneumoniae* and even by *Enterobacteriaceae* family at global level [76-78]. Curiously, the peculiarity of this enzyme is that has been recently shown to be associated with the highly successful, *E.coli* virulent clone, ST131 [79, 80].

1.1.5 Drug resistance in *Klebsiella pneumoniae*

K. pneumoniae carbapenemases

K. pneumoniae is major nosocomial pathogens, capable of rapidly spreading in hospitals [81], considering that infections caused by this bacterium are difficult to eradicate, especially due to the frequent carriage of drug resistance determinants: β-lactams and extended-spectrum cephalosporins (ESC), other classes of drugs such as aminoglycosides and occasionally also polymyxin B and colistin.

In 1996, a new strain of *K. pneumoniae* resistant to carbapenems was identified in New Carolina and was designed as carbapenemase-producing *K. pneumoniae* (KPC) [48].

Although the first hospital outbreak was described in a centre in New York, reports are now showing that KPC producing bacteria are disseminated worldwide [44, 82-88].

Genetic determinant of resistance to beta-lactam, moreover, have now been detected in several other members of the *Enterobacteriaceae* family and *Pseudomonas aeruginosa* [45, 89, 90]. Spread of carbapenemase-resistance genes among different bacterial species is possible thanks to the localization of them on plasmids and their association with transposons and insertional elements.

Different genes encoding for carbapenemases are bla_{KPC}, followed by bla_{NDM} (New Delhi metallo- β -lactamases), bla_{VIM} [91-96], and $bla_{OXA\text{-}48}$ [61, 97].

Since the first isolation of a clone of KPC in the United States, 13 genetic determinants of these enzymes have been identified. Determinants KPC-2 and KPC-3 were described for the first time in the USA [98] and seem to be the most frequent in Europe, with wide dissemination in Italy [99-101].

Colistin resistance

Colistin is considered as the last resort for treatment of severe infections caused by MDR Gram-negative bacteria because of its toxicity. However, in recent years the spread of genetic variants resistant to colistin is increasing [102, 103]. In *K. pneumoniae*, resistance to polymyxins and other cationic antimicrobial peptides is mediated by alterations of the lipopolysaccharide (LPS), with subsequent decrement of the affinity of polymyxins to the target. At the base of the mechanism regulating colistin resistance, relevant genetic factors include the *mgrB* gene, the *pmrC* gene (aminotransferase acting for the LPS synthesis) and the *pmrHFIJKLM* operon [104, 105]. Recent evidences suggest that insertional inactivation of the *mgrB* gene in *K. pneumoniae* represents the presumptive mechanism responsible for the emergence of colistin resistance via alteration of specific signaling pathway.

Normally the upregulation of the *pmrHFIJKLM* operon via the PhoQ/PhoP signaling pathway leads to modulation of the bacterial survival mechanism and to the LPS synthesis via activation of the *pmC* gene [108] (fig 8). During stress condition, MgrB mediates the negative feedback of the PhoQ/PhoP pathway, but if this protein results altered, PhoQ/PhoP signaling pathway cannot be inhibited and possible alteration on the LPS structure may occur. This means that the drug cannot recognize the LPS target, causing bacterial resistance [106, 107].

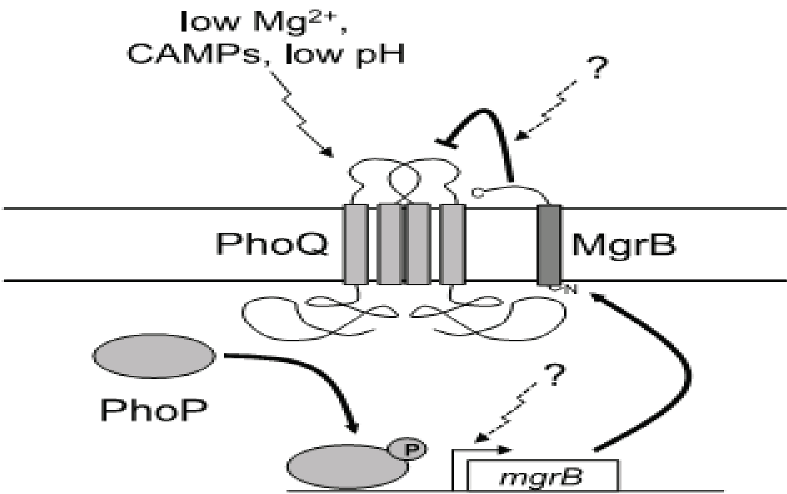


Figure 8. Schematic representation of MrgB function mediating negative feedback in the PhoP/PhoQ system. PhoQ stimulation (low extracellular magnesium, low pH or cationic antimicrobial peptides, CAMPs) leads to higher production of phosphorylated PhoP, which lead to increasing transcription of *mgrB* gene. MgrB inserts in the inner membrane and represses PhoQ, resulting in decreased PhoP phosphorylation [107]

1.1.6 *K. pneumoniae* virulence factors

The expression of virulence factor is a prerequisite for pathogenic bacteria, because it makes them capable of infecting effectively the host.

Innate and humoral immunity are the main mechanisms of defense against infections mediated by Enterobacteria. If these mechanisms are congenitally absent or temporarily compromised, susceptibility to infection and severity increase.

The mechanism of action of pathogenic Enterobacteria is complex and includes several co-factors involved in the development and evolution of infectious process: capsular antigens, siderophores and superficial adhesion molecules (fig 9).

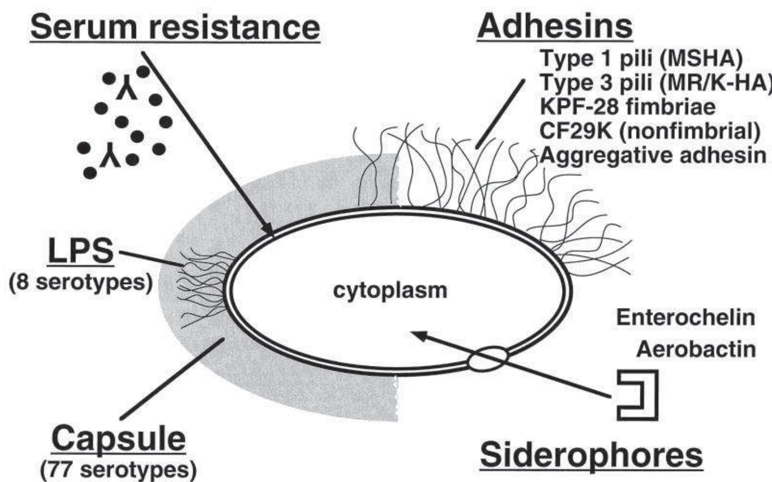


Figure 9. Representation of main virulence factors in *K. pneumoniae;* source, [81]

Capsular antigens

The extra cellular pathogens are able to resist to the bactericidal activities immune cells through surface elements as the lipopolysaccharide (LPS) and other elements present in the bacterial capsule.

The molecular mechanism at the basis of this activity would seem to consist in inhibiting or in the absorption of several immune system components.

In *Klebsiella spp*. are known more than a hundred different serotypes of the capsule; just think that already in 1998 were 77 known antigens [81].

It seems that great differences in virulence exist between strains that express specific capsular types and those that do not express them. For example, it was shown that strains of *K. pneumoniae* expressing capsular antigens K1 and K2 are particularly virulent in liver murine models, while isolates with the same mutant serotype showed lower virulence [109-113].

Maybe, the ability to resist to external environment is due to the production of a massive polysaccharide capsule. Studies have shown that overexpression of genes (*rpmA* and *magA*) encoding for this mucous viscous phenotype is associated with enhanced virulence [114-116].

Adhesion molecules

The initial and fundamental stage of infection is represented by the adhesion process, by which microorganisms adhere to the guest mucosa. The adhesive properties of Enterobacteria are mediated by several types of pili. These, also known as fimbriae, are filamentous and non-flagellar projections of bacterial surface.

These structures reach up to 1-2 micrometers in length and 2-8 nm in diameter and are composed of polymeric globular protein subunits (pilin) with a molecular mass of 15-26 kDa.

The direct link between the bacteria and host cell is mediated by molecules called adhesins or fimbriae that are differentiated primarily on the basis of their ability to agglutinate erythrocytes of different animal species. In fact, depending on whether the agglutination reaction can be inhibited by D-mannose, the adhesins are categorized as mannose-sensitive hemagglutinin (MSHA o type-1) or mannose-resistant (MRHA o type-3).

Klebsiella pneumoniae presents on the cell surface both type of adhesins [117-119]:

a) Type 1 fimbriae

Type 1 fimbriae are expressed by the majority of enterobacterial cells and are mannose-sensitive molecules. Several studies, aimed to characterize these molecules in *E. coli* and in *K. pneumoniae,* showed an high similarity in composition and gene cluster regulation [118]. Their main role is allow bacteria to adhere to the epithelium of the host leading, mainly, to urinary tract infections (UTI). Moreover, the type 1 fimbriae are also able to bind soluble glycoproteins rich in mannose, such as the Tamm-Horsfall protein, which allow them to mediate bacterial colonization of both the respiratory and urogenital tract [120, 121].

Type 1 fimbriae are archetypal and are encoded by a gene cluster *fimACDEFH*, where adhesive components are rapresented by *fimA* and *fimH* [121]

b) Type 3 fimbriae

The adhesin of type 3 are able only to agglutinate erythrocytes treated with tannin, for this reason defined as resistant to mannose. Moreover, although the commonly definition is "mannose-resistant *Klebsiella*-like hemagglutination" (MR/K-HA), this type of fimbriae is synthesized from many strains of Enterobacteria.

The main function of type 3 fimbriae seems associated with the formation of biofilm. Type 3 fimbriae are able to bind to different human cells: the endothelial cells, the epithelium of the respiratory and urinary tract. In the kidney, this type of pili allows the bacterial adhesion to the membrane of renal tubules, of the capsules of Bowman and of renal vessels [122, 123].

Genes involved in the synthesis of type 3 fimbriae are included in a cluster (*mrkABCDF*) containing protein subunits (*mrkA* gene), minor subunits (*mrkF* gene), genes coding for chaperone (*mrKB* gene), usher-protein (*mrkC* gene), and adhesins (*mrkD* gene) [123].

Phylogenetic analysis has shown that genes may be located at the chromosomal level, but also transferred between bacteria through plasmids and transposons [124, 125].

Transport of iron

The capacity to capture and metabolize iron is crucial for pathogen surviving. The growth of bacteria in the tissues of the human organism is limited not only by the host defense mechanisms but also by the low availability of iron, since it is linked to host proteins that may have deposit activity (i.e. hemoglobin, ferritin, hemosiderin and myoglobin) or transport activity (i.e. lactoferrin and transferrin). The effect of an iron supply on the pathogenesis has been demonstrated for *K. pneumoniae* using a animal model: after parenteral administration of iron, the susceptibility to infection with *K. pneumoniae* increases dramatically, so it is suggested that only microorganisms that are able to ensure the presence of intracellular iron can survive and replicate in the host tissues [126, 127] (fig 10).

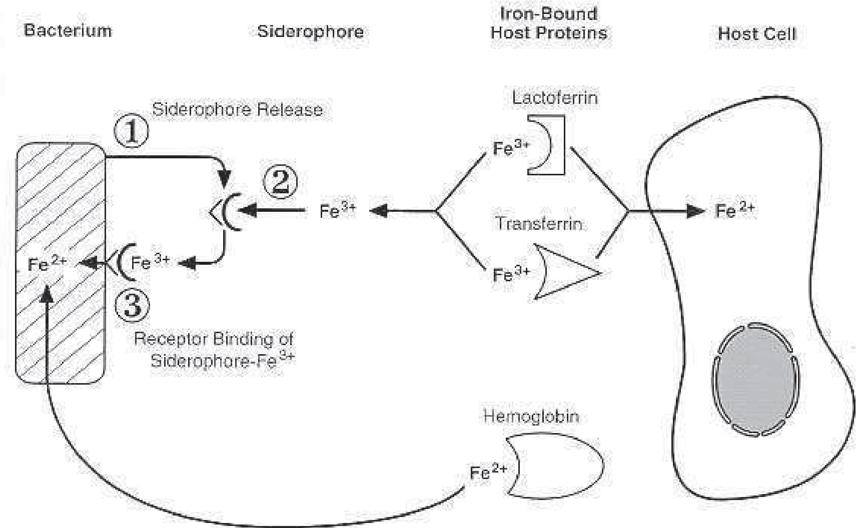


Figure 10. Mechanism of action of bacterial siderophores. Iron is usually bound to chelant protein (hemoglobin, myoglobin) expressed by the host cell. For its survival, bacteria have developed strategies concerning the release molecules called siderophores (1), which bind the ion with high affinity (2) and allow its subsequent entry into the bacterial cell (3) http://intranet.tdmu.edu.te.ua/data/cd/disk2/ch007.htm

Bacteria have developed strategies to sequester iron from infected tissues producing siderophores that are molecules able to capture host iron [128]. Different siderophores are proper of the *Enterobacteriaceae* family, of which the encoding genes are located in "pathogenicity islands" [128].

Under conditions of iron deficiency the *Enterobacteria* are able to synthesize siderophores belonging to two different chemical groups: "phenolate-type siderophores" and "hydroxamate-type-siderophores" (PTS and HTS, respectively).

The PTS are the most common group of siderophores, of which the best known representative of this group is the enterobactin or enterochelin, which has, compared to other siderophores, higher affinity to iron.

In the HTS class, ferrochromes (synthesized only by fungi), ferrioxamins and aerobactin are included; in particular, the aerobactin, unlike from enterobactin, is known to be necessary for bacterial virulence and surviving having different metabolic advantages. For example, it seems to have greater stability and solubility and, while enterobactin is hydrolyzed by an esterase after transferring the iron, the aerobactin can be reused at every metabolic cycle [127].

Data on the incidence of the aerobactin production in *Klebsiella spp* suggest that this siderophore could play a central role in its pathogenesis, but isolates of *Klebsiella pneumoniae* not capable of synthesizing aerobactin, are absolutely able to use exogenous aerobactin as the unic source of iron. This is a great advantage mainly in mixed infections, because these aerobactin-producers strains can gain an advantage over other bacteria [129, 130].

Although aerobactin play an important role in bacterial surviving, however *K. pneumoniae* is mainly known for the high production of the siderophore enterobactin (Ent). This siderophores is encoded by genes carried on the transposon *EntABCDEF* and possesses higher affinity for the iron respect other siderophores [131]

Unlike the aerobactin, the enterobactin functionality is compromised by the lipocalin2 (lcn2), a molecule produced by the host tissues that competes with the iron for the siderophore binding site causing, once bound, its inhibition [132]. To obviate this problem, it seems that the bacteria are capable of producing several "alternative siderophores", as salmochelin (glycosylated enterobactin) or yersiniabactin, which is structurally different from enterobactin.

Yersiniabactin (Ybt) initially was reported in the genus *Yersinia*. Subsequent studies have shown that this siderophore, as well as others commonly produced by various species of *Enterobacteriaceae,* is codified by genes carried on HPI (high-pathogenicity islands) [133]. In particular, in *K. pneumoniae*, yersiniabactin is encoded by YbtAS genes and regulated by iron-repressible genes irp1 and irp2 [134]. The yersiniabactin could have a key role in the survival of virulent strains of *K. pneumoniae*, but to date little is known with respect to the precise role played by the siderophores in the host organism, although a recent study affirmed that that the expression of these molecules is specific and tightly regulated in the various host tissues. In fact, different siderophores may coexist in the same host tissue and a specific combination of siderophores may exist in different host tissues, suggesting that the siderophores may have an important role in modulating host cellular pathways and determining the bacterial survival during infection (fig 11) [135].

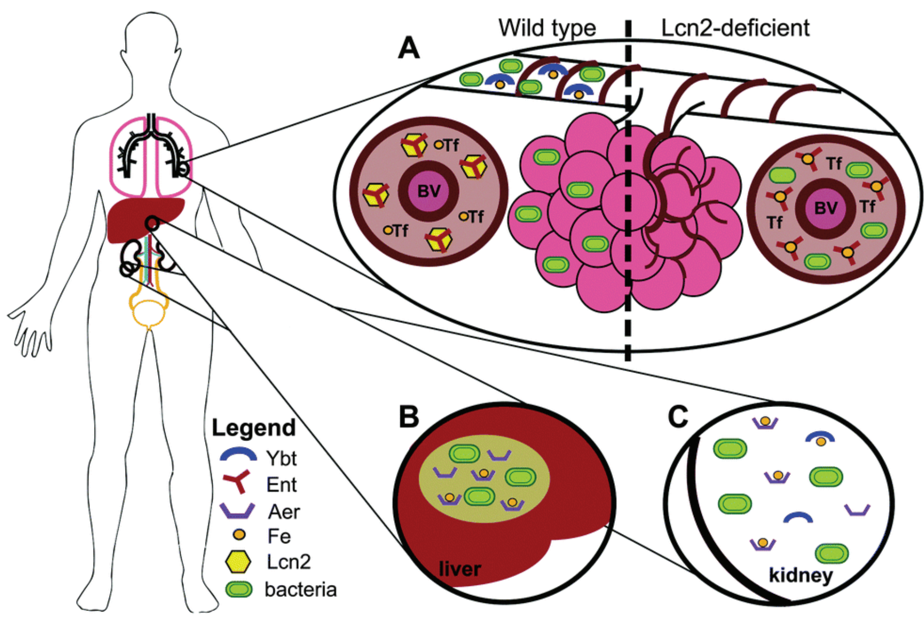


Figure 11. Bacteria can express a number of molecules with high affinity to the iron (siderophores). These may act specifically in different tissue, allowing bacteria to survive and infection to progress. In particular, it is showed a) in the lung lcn2 may capture enterobactin, but in absence of lcn2, enterobactin bind the iron allowing bacterial replication, while ybt may bind iron; b) in the liver, presence of aerobactin is prevalent; c) in the kidney, there is the presence of aerobactin and yersiniabactin. Ybt, yersiniabactin; ent, enterobactin; aer, aerobactin; Fe, iron; lcn2, lipocalin. Source [135]

1.1.7 Laboratory diagnosis of infection due to *Enterobacteriaceae*

In biological materials taken from a site of extra-intestinal infection, the identification of *Enterobacteriaceae* is less specific than that from anatomic sites considered sterile (such as blood), because the clinical suspicion of infection is intended to characterize any bacterium is present. For the bacterial growth the use of nonselective and enriched (blood, serum, etc) culture media is suitable for the development of the largest possible number of bacterial species. Each colony, consisting of Gram-negative bacteria that are negative to test of the indo-phenol oxidase and positive to catalase reaction (a part of *Shigella dysenteriae*), it must be considered as potentially generated by Enterobacteria.

Enterobacteria grow well in the common culture media, showing generally similar morphology, which may not be a critical method for the correct identification.

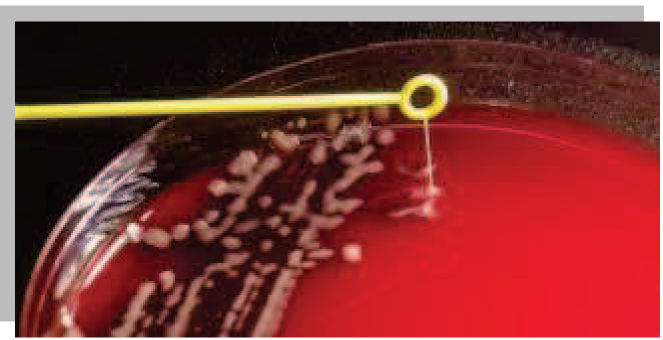


Figure 12. Imagine representing a hypermucousviscous *K. pneumoniae* strain, which appears sticky and forms mucous extensions of capsular component

The colonies of bacteria of the *Klebsiella-Enterobacter-Serratia* (KES) group, as with evident capsule, have mucosal appearance. In particular, the hypervirulent variant of *Klebsiella pneumoniae* may presents a typical viscosity of the colonies (fig 12).

The medium on which normally can be observed the growth of *Klebsiella spp* is the MacConkey agar (BD, Heilderberg, Germany), differential for Gram negative bacteria, in which colonies of discrete size, of color pink or whitish with mucous appearance and weakly lactose fermenting are observed. The preparation of MacConkey is based on the knowledge that bile salts are precipitated by acids and certain enteric bacteria can ferment lactose whereas others do not possess this ability, while bile salts and crystal violet inhibit the growth of Gram positive organisms. It is ideal for the analysis of clinical specimens containing mixed microflora, for example samples from urine, respiratory apparatus, wounds, etc., as it allows making an initial identification of *Enterobacteriaceae* and other Gram-negative fermenting and non-fermenting lactose.

Following the recent spread of bacteria showing high resistance to beta-lactamases and carbapenemases, it is necessary to improve the efficiency of screening methods used, also considering that, for both ESBLs that for carbapenemases, not always the identification of a certain value of MIC discriminates certainly for the presence of a resistant or sensitive strain. For example, the susceptibility assay, especially in the case of ESBLs, not always determines MIC values above the clinical breakpoints for resistance, as there is a lot of

variability due to the hydrolytic activity of the different variants, the amount of enzyme product and the variable ability of β-lactam antibiotics to cross the outer membrane of the bacteria. In these cases, despite the apparent sensitivity *in vitro*, the treatment may fail.

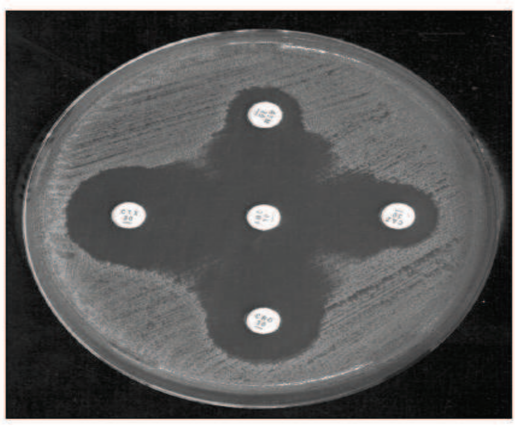
The protocol for the identification of ESBL and carbapenemases production in microorganisms isolated from clinical samples is strongly adjusted both by Clinical Laboratory Standard Institute (CLSI) that by European Committee on Antimicrobial Susceptibility Testing (EUCAST), for epidemiological purposes and control of infections, even if to date there is not still a phenotypic screening method for resolving all cases.

The detection of ESBL is performed in two steps, with an initial screening test to identify isolates potentially ESBL productors and a subsequent confirmation test, targeted to distinguish ESBL-producing isolates from those resistant to cephalosporins or presenting others mechanisms of resistance.

CLSI guidelines suggested testing as indicators of ESBL production drugs belonging to the cephalosporins class (i.e. cefotaxime, ceftazidime, ceftriaxone, cefpodoxime and aztreonam), considering that reduced susceptibility to one or more drugs indicates resistance against antibiotics. But because of different ESBL enzymes may have a different hydrolytic activity against the various drugs; there is no single indicator drug capable of detecting the totality of the ESBL-producing isolates.

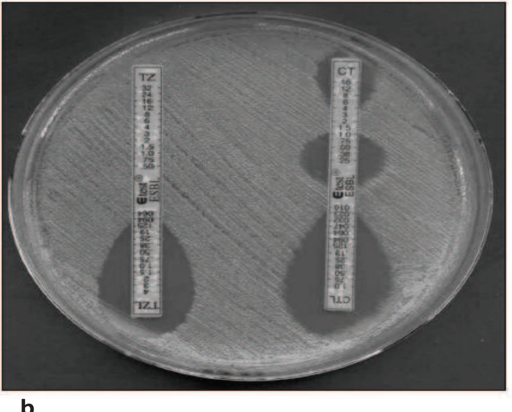
The confirmation test of ESBL production can be done by phenotypic test based on demonstration of the synergy between clavulanate and cephalosporins (ESBL are inhibited by clavulanate acid, tazobactam and monobactam), with possible different methods: the test of "double discs" consists in a classic agar diffusion assay where discs of a cephalosporin alone are positioned around a disc of amoxicillin-clavulanate placed in the middle of the plate, to assess the recovery of the activity of β-lactam in the presence of the inhibitor. The test is positive when it appears a distortion of the halo surrounding the β-lactam ring in the proximity of the disc of amoxicillin-clavulanate (fig 13) [136]. The combination test, another confirmatory test, is performed positioning on the plate discs containing the cephalosporin alone and a combination of cephalosporins and clavulanate acid. The halos of the discs related to the drug alone or the drug with clavulanic acid are compared and the sample is considered positive for ESBL if the diameter of inhibition is ≥5mm. Finally, the method of combination with E-test provides at the same time the MIC of a

cephalosporin (ceftazidime or cefotaxime) alone and in combination with clavulanic acid. Even in this case, ESBL production is confirmed if the clavulanic acid reduces the MIC of cephalosporin at least 3 fold dilutions (fig 13).



a

a) Confirmation of ESBL production by testing the double disc in *Klebsiella pneumoniae* producing SHV-12. Top, ATM, aztreonam (30 mg/mL); bottom, CRO, ceftriaxone (30 mg/mL) left, CTX, cefotaxime (30 pg/mL) right, CAZ, ceftazidime (30 pg/mL); at the center, AMC, amoxicillin + clavulanate (20+10 g/mL).



b

b) Confirmation of ESBL production by test combination (E test) in *Klebsiella pneumoniae* producing SHV-12. Left E test: top, TZ, ceftazidime (MIC> 2 mg/mL); bottom, TZL, ceftazidime + clavulanic acid (MIC 0.5 mg/mL); right E test: top, CT, cefotaxime (MIC 2 mg/mL); bottom , CTL, cefotaxime + clavulanic acid (MIC 0.094 mg/mL)

Figure 13. Two examples of confirmatory sensibility test performed to detect the presence of β-lactamases in *K. pneumoniae* strains. a) "double discs" method; b) combination E-test assay

In some cases, due to the difficulties in the interpretation of the test, for final confirmation of the presence of a gene coding for a ESBLs and characterization of the type and the variant enzyme produced, it has suggested the application of methods of molecular biology.

Also in the identification of carbapenemases-producing strains, it is recommended to apply both screening and confirmatory tests. The clinical significance in the diagnosis and treatment of carbapenemases is due to the fact that, virtually, a producer strain carbapenemase presents resistance to all beta-lactams, and possibly other medications. Furthermore, treatment of the patient can be difficult and the infection is generally associated with high mortality.

Furthermore, as previously mentioned, these mechanisms may be combined with others (alteration in efflux and/or membrane permeability, presence of beta-lactamases etc), leading to the presence of a great number of phenotypic variants. Besides, some strains may present decreased susceptibility to carbapenems due to the combination of production of ESBLs enzymes or AmpC enzymes combined with porins and membranes alterations.

The presence of carbapenemases for reduced susceptibility in respect of meropenem (MIC ≥0.25 ug/mL; diameter of the inhibition halo ≤25 mm) is an indicator of possible carbapenem resistance strains and it is preferable to use it than imipenem and ertapenem whereas the MIC values of producing strains carbapenemases may be in the cut-off and meropenem has good sensitivity and specificity in detection of them. If this drug is not available to testing, it is possible to use imipenem, while the use of ertapenem increases susceptibility but reduces the specificity and therefore further tests to confirm the result are required.

Confirmatory tests commonly in use in Clinical Microbiological Laboratory are the synergy test and the Hodge test. The first is performed by combination of carbapenem and specific inhibitors of carbapenemases, in order to assess the recovery of the activity of the drug in the presence of the inhibitor.

The confirmatory test is based on the knowledge that carbapenemases of class B are inhibited by dipicolinic acid, as the class A by the boronic acid and that cloxacillin inhibits the AmpC enzymes helping to differentiate between the overproduction of AmpC with loss of porins or the production carbapenemases. The table below showed the algorithm used to characterize presence of carbapenems in *Enterobacteriaceae* (fig 14).

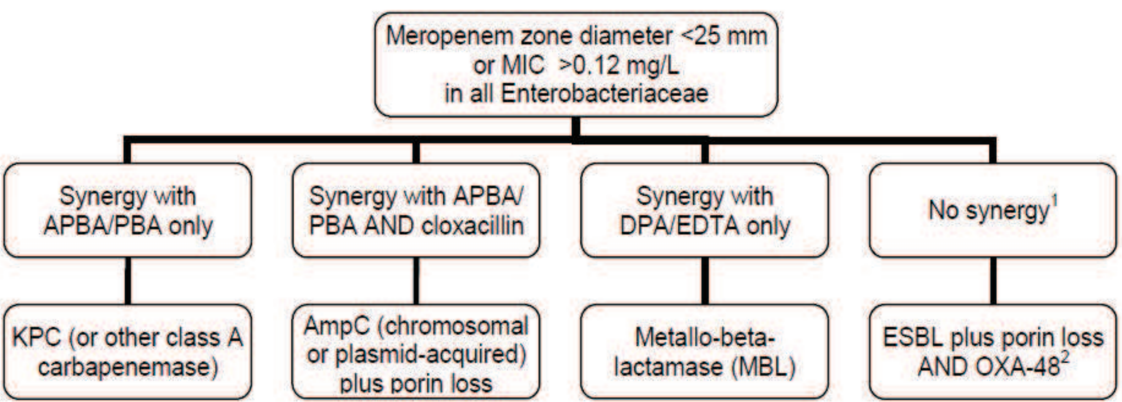


Figure 14. Schematic representation of diagnostic algorithm to carbapenemases detection, EUCAST guidelines (http://www.eucast.org/resistance_mechanisms). [1] combination of MBL and KPC may present high resistant isolates in absence of synergy. They are easiest to be detected by molecular methods; [2] presence of high temocillin resistance strains may indicate presence of OXA-48 production, it is suggest to consider it in absence of synergy with inhibitor of A and B classes of carbapenemases. APBA, aminophenyl boronic acid; PBA, phenyl boronic acid; DPA, dipicolinic acid; EDTA, ethylenediaminetetraacetic acid.

The Hodge test or MHT (Modified Hodge Test) is based on the reduction of activity of the carbapenem tested against a sensitive strain that serves as an indicator [137].

Of this test we have been proposed numerous methodological variations, but the most common protocol provides for: the preparation of a suspension of 0.5 McFarland in saline water of *E. coli* ATCC 25922 (indicator strain) and its confluence spread on plate of Mueller-Hinton agar; positioning in the middle of the plate a meropenem disc; deposition of 1-2 colonies of the strain to be tested in the proximity of the antibiotic and spread on a straight line in the direction of the edge of the plate (or inoculated by cutting in the surface of the agar) [137]. The test is considered negative if the zone of inhibition of the control strain is not a clear indentation at the inoculum of the strain tested. The test is considered positive whether the zone of inhibition of the control strain present a clear indentation at the inoculum of the strain tested.

The test is to define doubt in the presence of faded or minor alterations of the halo. The test Hodge does not allow the distinction between the different classes to which belong the carbapenemases, but shows only the presence of hydrolytic activities.

1.1.8 Molecular typing

To date, with the relentless spread of more virulent bacteria and with the increase of antibiotic resistance, the ability to trace the origin of pathogen isolates, to describe the genetic relatedness among them or to highlight the virulence factors and resistance genes, is imperative [138].

The use of molecular methods plays a crucial role in the identification, characterization and control of infections, with an integrated approach together with clinical methods [139, 140]. In recent years, molecular typing allow to implement an effective control of infectious diseases, resulting in the rapid detection and characterization the etiologic agent and the activation of specific surveillance programs [139].

Typing methods, a part of being accessible, rapid, easy to apply and as possible economic, may present so called “performance” criteria, such as discriminatory power, reproducibility, and typeability [141]. In particular, typeability refers to the ability of the method to assign a type to all isolates tested with that method in an elevated percentage of success; discriminatory power is the ability of a method to assign different types to two unrelated strains analyzed "randomly" within a population of a given species. For example, typing methods investigating polymorphisms in multiple sites of the whole genome have more probability to have a greater discriminatory power than the methods that investigate a single locus. Finally, reproducibility is refers to the ability of a typing method to often assign the type of one strain tested in independent occasions, separated in time and/or space. The reproducibility may be affected by various elements in a process, such as because of changes in microbial growth condition, the methods used in the extraction DNA, in the reagents used, different types of equipment, diversity in the observation and interpretation of results [141].

For this reason, sometimes it is not possible to compare results obtained from different laboratory. A molecular technique aimed to typing isolates cannot be separated from the adequate knowledge of its interpretation criteria, which vary depending on the methods. Guidelines have been proposed, for example, for the interpretation of patterns obtained by molecular techniques as Pulsed Field Gel Electrophoresis (PFGE), PCR-ribotyping and amplification with arbitrary primers (RAPD).

Numerous are the molecular techniques used to date, and it is very important to make a correct choose of the methods [142]. Although numerous molecular techniques exist, the most widely used are:

- Restriction Fragment Length Polymorphism (RFLP): based on analysis of fingerprinting obtained starting from the digestion of the DNA with restriction enzymes with rare cutting sites and a subsequent electrophoretic run on agarose gel. It was used for the first time in years 80s for the typing of viruses, fungi and bacteria. Its usefulness lies in the detection of changes (insertions, deletions, etc.) in the bacterial genome [143-146];
- PFGE is one of the most diffused and well characterized molecular typing techniques [147]. It is used for molecular characterization of many pathogens transmitted with food or pathogen causing nosocomial outbreak [148-151]. This method is considered for many laboratories as the "gold standard" for its good discriminatory power and easiness in application. It is based on the enzymatic digestion of the whole genomic DNA with restriction enzymes that perform a few cuts (i.e. *HindIII*), so it is possible to generate a number of fragments very small but with large dimension between 40kb and 600kb that are separated by the use of an electrophoretic system with pulsed electric field, in which the orientation of the field electricity is periodically changed to order to obtain a better separation of fragments. The fragments generated can be analyzed and compared through the use of dedicated software (such as BioNumerics and GelCompare, Applied Maths) that identify the locations of the bands, and so calculate the molecular weight of the DNA fragments using a mathematical matrix that analyzes similarities of the profiles [145, 152];
- certainly by its discovery, the Polymerase Chain Reaction (PCR) has been and is the technique most widely used in molecular biology laboratories. Thanks to the PCR and to the newer method based on this approach can be amplified many genes, including the most relevant to the study of pathogenic virulence factors related to pathogenicity and transmissibility of the bacteria and the genes of antibiotic resistance [153, 154]. For several years it has been introduced as an alternative to common PCR, called Real-time PCR, which allows to increase the susceptibility and reliability of the results obtained with the PCR and is based on measurement of the fluorescence signal that is generated at each cycle of amplification, by which method it was possible to reduce the processing

time and increase the efficiency and specificity of the final result. Various are PC-based techniques [155, 156], but the frequently used are Randomly Amplified Polymorphic DNA-PCR (RAPD-PCR) and Arbitrarily Primed PCR (AP-PCR) are part: the gene fragments are amplified using primers that recognize accidentally complementary sequences, leading to the final production of fragments of different length and specific to each strain. The fragments are often very variable and polymorphic, allowing, therefore, carrying out an effective investigation of the intraspecific variability of pathogens [157-159]

- among the methods based on sequencing of bacterial DNA, one of relief is the Multi-Locus Sequence Typing (MLST) [147]. It is one of the most used molecular methods for epidemiological investigations and studies on molecular evolution of many pathogenic and non-pathogenic bacteria, especially when there is necessary the typing of a microbial species with a high rate of genetic recombination. It is based on the detection of the polymorphisms present in the "housekeeping" genes, which code for enzymes that catalyze important metabolic functions [160, 161]. A variant of classical MLST is the Multilocus Ribosomal Sequence Typing (rMLST) that allows to evaluate the molecular change of the genes that encode bacterial proteins of the ribosomal subunits and, compared to MLST, had higher discriminatory power [162]. Thanks to the good reproducibility and transferability of data MLST was possible develop databases accessible to all which allow the identification of the strains in question [163, 164]. The disadvantage of this technique is the necessity of a genetic analyzer to sequence all the products obtained with relative high cost;
- Single-Nucleotide Polymorphisms (SNPs) analysis has the advantage of lead to the identification of polymorphisms in specific DNA sequences present, in according to the change of a single base nucleotide (SNPs). Initially used for the study of the genetics of eukaryotic cells, it is now largely worked to the characterization and differentiation of bacterial strains.

The necessity to investigate more thoroughly and to eliminate possible limitations due to the variability of the methods and to the difficulty of interpretation, allowed to go up to the ideation of a methodology of massive sequencing. Is the case of the Whole Genome Sequencing, which detects

precise genetic changes, occurred in bacterial genomes also analyzing mutations at single polymorphic locus and presents more vantages as the highest discriminatory power respect to other methods [122, 165].

After the success of the Human Genome Project, it has been evidenced the necessity to study extensively the single alteration of whole genome [166-169], to better understand genomic rearrangements involved in diseases process. Since that moment there was an exponential development of new sequencing technologies aimed to meet the requirements of speed, optimum relationship cost/efficiency and broad applicability [170-172].

Certainly, most of the applications of massive sequencing were about the study of human diseases, such as rare diseases or cancer [165, 173-175], but subsequently the benefits of this innovative technology have been applied to other areas, as the study of pathogens involved in hospital-acquired infections or zoonotic transmission or related to food [165, 176, 177].

The ability to sequence numerous bacterial genomes and interpret the resultant sequence information in near "real-time" is the basis of whole genome sequencing (WGS) technologies, mostly because it is possible to support clinical information and make more rapid diagnosis and prevention [178-180].

There were different platforms engaged the use of next generation sequencing. The most known from earlier years of 2000s were (454) GS FLX sequencer (Roche, Branford, USA), Illumina Genome Analyzer™ (Illumina Inc., San Diego, CA, USA) and Solid Sequencer™ (Thermo Fisher Scientific Inc., USA) [181-183] (table 2).

All platforms used the same basic workflow with small variations; this workflow consists in producing a library of DNA fragments (originated from a variety of front-end processes), in the preparation of a reaction amplification via PCR and in the sequencing of the products obtained. The big change compared to the classical methodology (Sanger sequencing) it is that, for all platforms, the products (clonally amplified or separated in flow cells) can be sequenced in parallel and massively, whereas previously it was necessary to analyze the fragments of different length by electrophoresis with individual reactions.

With the technology of next generation sequencing it is possible to carry out repeated cycles of amplification (through the action of a DNA polymerase or iterative cycles of ligation of oligonucleotides) that allow to obtain hundreds of Mb (millions of base pairs) or Gb (billion pairs of bases), which are sequenced in parallel.

Platform			
	Roche 454	**Illumina**	**SOLiD**
Sequencing chemistry	Pyrosequencing	Polymerase-based sequencing-by-synthesis	Ligation-based sequencing
Amplification approach	Emulsion PCR	Bridge amplification	Emulsion PCR
Paired ends/separation	Yes/3kb	Yes/200bp	Yes/3kb
Mb/run	100 Mb	1300 Mb	3000 Mb
Time/run (paired ends)	7h	4 days	5 days
Read length	250 bp	32-40 bp	35 bp

Table 2. Comparation of metrics and performance of the three most diffused platforms to perform next Generation Sequencing Analysis; adapted by [184]

In particular, in the Roche 454 system, specific adaptors are bound to the extremities of DNA fragments and the PCR reaction is performed by emulsion PCR where the beads in the solution bind one of the adaptors linked in the extremities of the DNA fragments allowing extension of the single strands of DNA. When amplification cycle ends, products are analyzed by pyrosequencing method (https://www.youtube.com/watch?v=rsJoG-AulNE). This platform has the disadvantage to be highly expensive and to present high error rate during sequencing reaction (table 2).

In SOLiD system (Sequencing by Oligo Ligation and Detection), the amplification reaction takes place with emulsion chemistry as in 454 platform, but the sequencing protocol is different and it is known as sequencing by ligation. The process starts with binding of beads on the amplification products and with the

preparation of a solution containing a ligase enzymes and “di-bases probes”, fluorescently labeled with 4 different dyes, where each dye represents the 16 combinations of di-nucleotides sequences. The ligase adds to each single DNA strand the complementary di-bases probes with emit a fluorescence signal based on the sequences bound (educational video at https://www.youtube.com/watch?v=nlvyF8bFDwM). Respect to 454 platform, SOLiD system is more accurate and allows to analyzed a higher quantity of data [181, 185] (table 2).

Finally, Illumina platform is based on sequencing by synthesis scheme (SBS, https://www.youtube.com/watch?v=womKfikWlxM).

The solid substrate, on which amplification is performed, takes the name of "flow-cell" and it is constituted by eight lanes where two types of synthetic oligonucleotides, complementary to the specific adapters of DNA fragments, are attached.

The sample DNA is fragmented and at each extremity a specific amplification primer and a adaptor sequence (complementary to the synthetic oligonucleotides of the flow cells) are added.

DNA fragments bind with one extremity the flow cell, so that complementary strand is synthesized and denatured, and the remaining single strand binds the other synthetic oligonucleotide, forming a bridge (fig 15). Amplification reaction synthesizes the complementary strand, leading to the formation of a double-stranding bridge that is subsequently denatured, resulting in two complementary single strands. This process is repeated a number of times, resulting in millions of amplified product bound to the flow cells.

In this phase, the reverse strands are washing up and the forward strands are used for sequencing; fluorescent nucleotides are added to form the complementary strand and signal emitted are read as fluorescence with a process called “synthesis by sequencing” (SBS). When sequencing of the forward strand ends, a new bridge is formed and amplification cycles are performed, at the end of which the sequencing of the reverse strand is carried out in the same way mentioned above (fig 15).

For the analysis of fragments, forward and reverse sequences obtained are coupled and linked to similarity clusters, forming contigs which are aligned to reference genomes [186] or assembled together using advanced computational techniques if no reference genome is available (*de novo* sequencing).

The reassembling phase requires a comparison with the genomes deposited on the database, as well as a high level of preparation especially to assemble properly similar fragment of genome and repetitive sequences [186, 187].

The advantage of this platform is the innumerable amount of information obtained with a new sequencing chemistry, but presents the disadvantage to shows increased errors rate with the greater reads length.

In the last years, platforms based on ion semiconductor sequencing are used, which have a sequencing chemistry similar to pyrosequencing, however with the detection of the release of ion (H^+) instead of pyrophosphate. This new system seems to be more cost-effective and time-efficient [188].

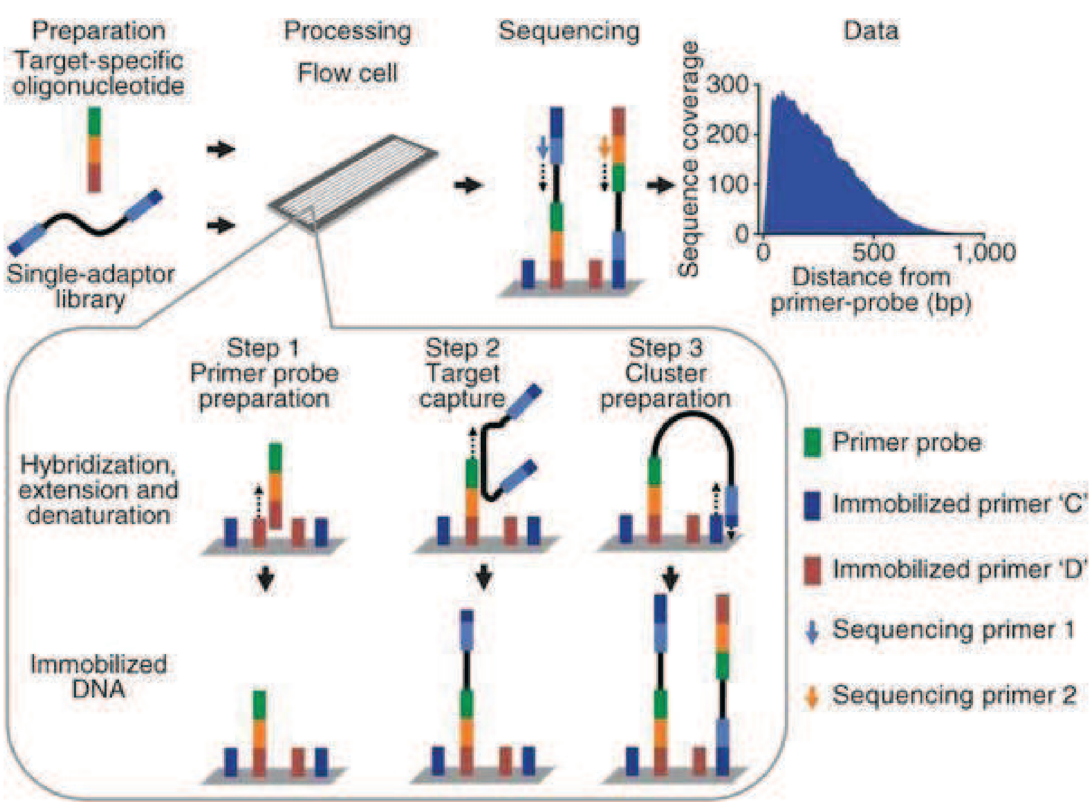


Figure 15. Schematic representation of amplification and sequencing reactions by "polymerase-based sequencing-by-synthesis" performed by Illumina platform (MiSeq System)

1.2 Shortcoming of the literature and aim of the study

Following the global spread of Multi-Drug Resistant Carbapenemases-producing *K. pneumoniae* (KPC-Kp) it is essential that the sanitary structures may act on prevention and control of infections.
Worldwide dissemination of *K. pneumoniae* carbapenemase-producing is due mainly to clonal group 258 (CG258), which divided into four subclades [189]. Several studies focused on the characterization of this pathogen [190], but many points have been not totally clarified. In our study, we addressed some of them.

a) In Italy, the dissemination of the GC258 and its transmission route has been elucidated only recently [99, 191]. Because of the lack of literature, the description of genetic relatedness of these KPC-Kp lineages at the local/regional level is poor.

b) During the last years, typing methods made possible the development of an integrated approach between phenotypical and molecular characterization [192, 193]. It is supposed that this progress is reflected in a more rapid and efficient identification of hospital infections. Different techniques are available but effectiveness in clinical practice must be depth and reinforced [194, 195].

c) *K. pneumoniae* may produce a number of pathogenicity features (virulence factors or genes implicated in antibiotic resistance) which are involved in the rapid dissemination and increased disease severity [196]. In the presence of epidemic events, these factors may promote the dissemination of more virulent variants. To date, only a few notions had been published evaluating the effect of convergence of virulence factors and resistance genes leading to the selection of epidemic isolates [196, 197].

The emergence of carbapenems- and colistin- resistant *K. pneumoniae*, for which there are even less treatment options, has been described recently. Following the increased incidence of this pathogen in our institution, the aim of the work was to assess a phylogenomic study of the collected clinical isolates to trace the origins and transmission routes of them.

Moreover, we aimed to compare genomes all of the strains collected with 319 publicly available genomes, representing the global diversity of *K. pneumoniae* to tracing phylogenic features of local isolates and to verify if increased rate of KPC *K. pneumoniae* in a single center was due to a single clone spread, characterizing the expression of high risks factor (virulence factors and genes of antimicrobial resistance).

1.3 Materials and methods

1.3.1 Infections due to *K. pneumoniae* and bacterial isolates

During a 27-months period (January 2011-March 2013), all the cases of infection due to carbapenem-resistant *K. pneumoniae* identify at the Ospedale di Circolo and Fondazione Macchi (Varese, Italy) were subjected to the study. During this time, a total of 16 non-duplicated isolates of *K. pneumoniae* were investigated. Seven of the clinical cases occurred in the intensive care unit (ICU) and were suspected to be part of a single epidemic event started in February 2013.

Specifically, the first isolate obtained from each patient was included in the study. Different *K. pneumoniae* isolates were obtained subsequently from each patient both for clinical reasons (e.g., spread of infection to other bodily sites), or in the course of surveillance studies.

Anatomical sites, from which the samples were taken, were multiple, as indicated in table 3: urine, blood, bronchoalveolar lavage, sputum, tracheal aspirate, and wound specimens. During the outbreak period, ICU patients were screened every three days for surveillance using swabs (direct to nasal, inguinal, armpit, and rectal sites).

Species identification and antibiotic susceptibility tests were performed with the FDA approved Phoenix® Automated Microbiology System (Becton Dickinson, Sparks, MD). Additional phenotypic assays were performed using the Etest (BioMerieux, Marcy l'Etoile, France) on Mueller Hinton Agar II (BD) plates according to clinical breakpoints from the European Committee for Antimicrobial Susceptibility Testing (EUCAST).

1.3.2 Molecular assays for drug resistance genes and virulence factors

Pure cultures of *K. pneumoniae* were isolated on MacConkey agar and subsequently bacterial DNA was extracted following a lysozyme pretreatment (Sigma-Aldrich, Milan, Italy) by extraction with the QiAmp

DNA Blood Mini Kit (Qiagen, Milan, Italy). Confirmatory species identification was performed via amplification by PCR and direct sequencing of the 16s rRNA gene. PCR was performed using AmpliTaq Gold® with Buffer I (Applied Biosystems, Life Technologies, Monza, Italy) using master mixes with a final volume of 50µL, prepared according to manufacturer's directions. PCR primers were synthesized by Sigma-Genosys (Haverhill, UK). Published primers and thermal protocols were used as previously described [198]. Analysis of DNA fragments was performed by electrophoresis in 1.5% gel made of standard agarose (Standard Low-mr Agarose, Bio-Rad Laboratories, USA) in TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.3) containing GelRed™ (10,000x in water; Biotium, DBA Italy, Segrate, Italy). Subsequently, PCR products were purified and sequenced on an ABI Prism 310 genetic analyzer (Life Technologies). Comparison of sequences was made with those of reference in GenBank (www.ncbi.nlm.nih.gov/blast/). PCR assays for detecting antimicrobial resistance genes [199] and virulence factors were performed according to published protocols. Genes coding for adhesion fimbriae, enterobactin and yersiniabactin siderophores were searched for; in detail: *fimH* gene coding for type-1 fimbriae [118]; *mrkA* gene coding for the major subunit protein and *mrkD* gene coding for the adhesin of type-3 fimbriae [122, 200]; e*ntE* gene coding for synthase subunit E and e*ntB* gene coding for isochorismatase of enterobactin siderophore synthesis [201, 202]; y*btS* gene coding for salicylate synthase of yersiniabactin siderophore synthesis; genes related to siderophore yersiniabactin, *irp-1* and *irp-2* [134]. Direct sequencing was performed as reported above.

1.3.3 Whole genome sequencing analysis

Whole genome DNA was sequenced using an Illumina MiSeq platform (Illumina Inc., San Diego, CA), with a 2x250 paired end run, after Nextera XT paired-end library preparation. Sequencing reads were assembled using the MIRA 4.0 (Mimicking Intelligent Read Assembly) software [203] with accurate *de novo* settings. MIRA is a multi-pass DNA sequence data assembler for whole genomes and allow manipulating contigs obtained from the different next generalization sequencing platforms. Genome assemblies were deposited to the EMBL database under accession number PRJEB7661.

1.3.4 Core SNP detection and phylogeny

Whole genome sequences of the 16 isolates were added to a previously characterized database of 319 genomes of *K. pneumoniae* isolated worldwide [204]. Single nucleotide polymorphisms (SNPs) were detected using an in-house pipeline based on the Mauve software [205], using the genomic sequence of the published NJST258_1 as reference [204]. Mauve software allows performing genome-wide phylogenetic analysis, constructing multiple genomes alignment in the occurrence of large-scale evolutionary events, recognizing recombination events and genomic rearrangements. Each genome was individually aligned to the reference one and alignments were merged with Perl scripts (Practical Extraction and Reporting Language) to obtain a global alignment. Core SNPs are variations in the single nucleotide flanked by at least one identical nucleotide on both sides present in all analyzed genomes [206], were detected. To obtain a Maximum likelihood phylogeny, all core SNPs were merged in a multialignment file using the RAxML software [207] with specific parameters, i.e. generalized time reversible (GTR) model and 100 bootstraps (computer-based method for assessing the precision of almost any statistical estimate).

Besides, mining of the genes, coding for antibiotic resistance and virulence factors, of which the preliminary screening was previously performed using classical PCR assays, was determined using BLAST-based alignment on a specifically designed database, i.e. BIGSdb-Kp [208].

All hits were manually checked and genes requiring specificity for a particular variant (e.g., *bla*KPC vs. *bla*OXA-48) were required to present 100% identity with the database sequences. BLAST searches and filters were also used to test the presence of yersiniabactin genes (*ybtS*, *ybtA*, *irp1*, and *irp2*) in all genomes used for the global phylogeny analysis. Analysis of the presence of deletions within the *mgrB* gene (a supposed determinant of colistin resistance) was performed by manually corrected BLAST search [108].

1.3.5 Core genome and *in silico* MLST

MLST profile was obtained *in-silico* by analyzing appropriate gene variants on each genome using an in-house Python script (another specific programming language used to analyze data on genomic sequences).

MLST profiles of analyzed samples are compared with those of reference deposited in a specific database:

(http://bigsdb.web.pasteur.fr/perl/bigsdb/bigsdb.pl?db_pubmlst_klebsiella_seqdef_public&page_downloadAlleles).

Core genome MLST (cgMLST) analysis was performed using the BIGSdb software and database [209], which allow to compare genomic sequences of the 16 strains analyzed with those of other deposited strains.

In this work, cgMLST profiles made of allelic variants at 694 loci were obtained for 219 genomic sequences of the clonal group 258 (CG258), comprising the 16 investigated genomes. Core genome MLST profiles were used to produce a similarity tree of all 219 genomes using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method.

1.3.6 Outbreak reconstruction

The transmission route of the clinical isolates involved in the ICU outbreak was reconstructed by combining core SNPs and the dates of samples collection, applying the SeqTrack implemented in the Adegenet R library [210]. Unlike the software used in phylogenetic analysis where the relatedness among samples is obtained reconstructing hypothetical ancestors, the SeqTrack system considers sampled in the same group ancestor and descendants strains, analyzing the relationships between all the existing samples. SeqTrack reveals as a more efficient algorithm respect the phylogenetic approaches, especially for the reconstruction of transmission routes of outbreaks.

The chain of transmission of the epidemic strain was then obtained using the R library Outbreaker [211].

1.4 Results

1.4.1 Species identification and antimicrobial susceptibility

In this work, the first isolate obtained from each single patient has been investigated. Biochemical approach and molecular assays allow identifying the species. The seven cases recognized in February 2013 were suspected to belong to a single outbreak, started when a 69 years old man recognized as the patient zero (indicated as KpVA-8 in figure 16) has been transferred to the ICU from a nearby hospital with an already diagnosis of infection due to KPC-producing *K. pneumoniae.* During his stay in the ICU, infection extends to other six patients, with a total of seven clinical isolates collected and defined as epidemic. In the figure 16, it is possible to observe the time-frame of ICU permanence of each patient involved in the outbreak and the date of first isolation of *K. pneumoniae* strains.

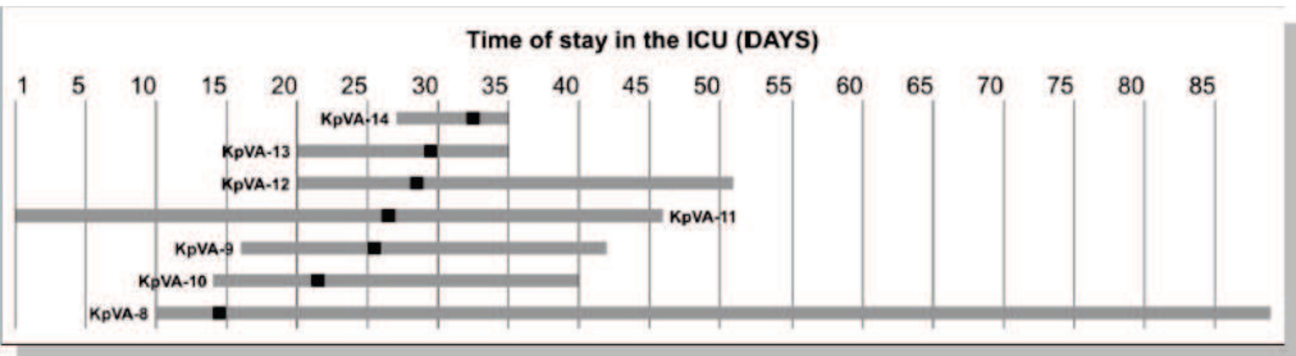


Figure 16. Time-frames of ICU permanence of the seven patients involved in the outbreak. Horizontal bars represent the length of stay of each patient; black squares indicate date of first isolation of *K. pneumoniae* for each patient

The other isolates are defined as sporadic, being identified as punctual and unrelated infections. Five out of the 16 isolates were obtained from blood cultures (3 of 7 among patients involved in the ICU outbreak) (table 3) while the others from different bodily sites.

Phenotypic assays detected resistance to imipenem, meropenem and ertapenem in all 16 clinical isolates, so that were thus classified as carbapenem-resistant. The seven epidemic isolates were also resistant to

colistin, but susceptible to aminoglycosides (gentamicin, amikacin, tobramicin). Only three out of 9 sporadic isolates presented resistant to colistin (table 4). A short description of the investigated isolates is reported in table 3 while results of minimal inhibitory concentration (MIC) values related to E-test assays for each isolate are reported in table 4.

Patient	Clinical status	Date of isolation	Source	Sequence Type
KpVA-4	Sporadic	01/11/11	B	ST-258
KpVA-5	Sporadic	01/28/11	B	ST-258
KpVA-6	Sporadic	03/14/11	B	ST-258
KpVA-7	Sporadic	05/03/11	B	ST-258
KpVA-1	Sporadic	10/31/12	SP	ST-512
KpVA-2	Sporadic	01/30/13	BAL	ST-258
KpVA-8	Epidemic	02/01/13	BAL	ST-512
KpVA-10	Epidemic	02/08/13	BAL	ST-512
KpVA-9	Epidemic	02/12/13	BAL	ST-512
KpVA-11	Epidemic	02/13/13	B	ST-512
KpVA-12	Epidemic	02/15/13	TA	ST-512
KpVA-13	Epidemic	02/16/13	WS	ST-512
KpVA-14	Epidemic	02/19/13	BAL	ST-512
KpVA-3	Sporadic	03/14/13	B	ST-258
KpVA-15	Sporadic	03/24/13	U	ST-512
KpVA-16	Sporadic	03/24/13	U	ST-512

Table 3.Clinical isolates involved in the outbreak. Description of clinical status, date of isolation of the sample analyzed, sequence type (ST), and source. B, blood; SP, sputum; BAL, bronchoalveolar lavage; TA, tracheal aspirate; WS, wound swab; U, urine

	KpVA-1	KpVA-2	KpVA-3	KpVA-4	KpVA-5	KpVA-6	KpVA-7	KpVA-8	KpVA-9	KpVA-10	KpVA-11	KpVA-12	KpVA-13	KpVA-14	KpVA-15	KpVA-16
Antimicrobial agents	**MIC (mg/L)**															
Ampicillin	R>8	R>8	R>8	R>16	R>16	R>16	R>16	R>8	R>8	R>8	R>8	R>8	R>8	R>8	R>8	R>8
Amoxicillin-clavulanate	R>8/2	R>8/2	R>8/2	R>16/8	R>16/8	R>16/8	R>16/8	R>8/2	R>8/2	R>8/2	R>8/2	R>8/2	R>8/2	R>8/2	R>8/2	R>8/2
Ceftazidime	R>8	R>8	R>8	R>16	R>16	R>16	R>16	R>8	R>8	R>8	R>8	R>8	R>8	R>8	R>8	R>8
Cefotaxime	R>4	R>4	R>4	R>32	R>32	R>32	R>32	R>4	R>4	R>4	R>4	R>4	R>4	R>4	R>4	R>4
Aztreonam	R>16	R>16	R>16	R>16	R>16	R>16	R>16	R>16	R>16	R>16	R>16	R>16	R>16	R>16	R>16	R>16
Ertapenem	R>1	R>1	R>1	R>1	R>32	R>1	R>1	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>1	R>1
Imipenem	R>8	R>8	R=8	R>32	R=8	R=32	R=8	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>8	R>8
Meropenem	R>8	R>8	R=12	R>32	R=32	R>32	I=6	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>8	R>8
Ciprofloxacin	R>1	R>1	R>1	R>2	R>2	R>2	R>2	R>1	R>1	R>1	R>1	R>1	R>1	R>1	R>1	R>1
Levofloxacin	R>2	R>2	R>2	R>4	R>4	R>4	R>4	R>2	R>2	R>2	R>2	R>2	R>2	R>2	R>2	R>2
Amikacin	R>16	R>16	R=48	R=48	R=64	R=48	R=64	S=1	S=1	S=1	S=1	S=1	S=1	S=1	R>16	R>16
Gentamicin	S=2	S=2	S=1.5	S=0.5	S=1.5	S=1	S=2	S=0.25	S=0.25	S=0.25	S=0.25	S=0.25	S=0.25	S=0.25	S=4	S=2
Tobramicin	R>4	R>4	R=12	S=0.5	R=16	R=16	R=24	S=0.38	S=0.38	S=0.38	S=0.38	S=0.38	S=0.38	S=0.38	R>4	R>4
Colistin	R>4	S≤1	S=0.19	S=0.125	S=0.19	S=0.38	S=0.19	R=8	R=8	R=8	R=8	R=8	R=8	R=8	R>4	R>4

Table 4. Antimicrobial susceptibility values (MIC) profiles of the 16 investigated clinical isolates. S, susceptible; I, intermediate; R, resistant

1.4.2 Drug resistance determinants and virulence factors

Isolates were subjected to PCR assays to detect drug-resistance and virulence factors genes. The bla_{KPC} gene was detected in all isolates (fig 17), while other carbapenem resistance genes (bla_{NDM}, bla_{IMP-1}, bla_{OXA-48}, bla_{VIM}) were not detected.

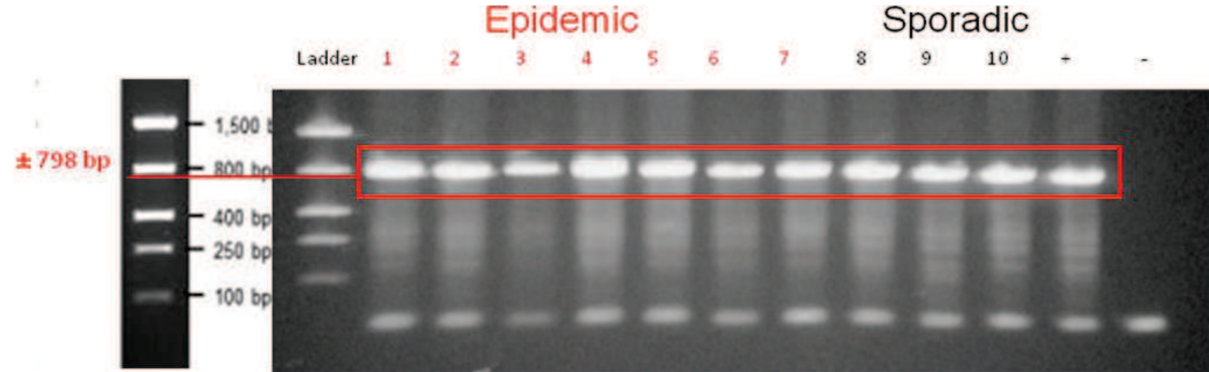


Figure 17. PCR assay for detection of *bla*$_{KPC}$ gene (798 bp). Sample 1-7, ICU epidemic KPC-Kp isolates. Samples 8-10, sporadic KPC-Kp isolates; +, positive control *K.pneumoniae* ATCC®BAA-1705; -, negative control "reference strain" *K.pneumoniae* ATCC®-700603

All isolates were positive for the presence of genes coding for type-1 and type-3 fimbriae (*fim* and *mrk* operons, respectively) as well as for the enterobactin siderophore that is harbored in the *ent* operon (table 5). Three of the nine sporadic isolates carried *ybtS* and the iron-repressible *irp1* and *irp2* genes for yersiniabactin [130, 201, 212].

1.4.3 Whole genome sequencing and characterization

Whole genome sequences were obtained for all 16 *K. pneumoniae* isolates and genes coding for drug resistance determinants and virulence factors were investigated on assembled genomes; moreover strains were characterized by *in silico* for MLST (table 3). MLST analysis enabled to identify two groups of isolate: six isolates, in particular, KpVA-2, KpVA-3, KpVA-4, KpVA-5, KpVA-6, and KpVA-7, were of ST258 and 10 isolates, together with the epidemic ones, belonged to ST512. The two Sequence Types differ for a single nucleotide, thus are considered belonging to the same clonal group, the CG258 [190, 191].

cgMLST analysis was performed on the same genomic dataset used for SNPs phylogeny (219 genomes of *K. pneumoniae* belonging to CG258) (fig 19). The resulting UPGMA tree is largely coherent with that resulting from the SNP-based phylogenomic analysis. Specifically, in both analyses, the main subdivisions of CG258 [213] are clearly detectable, while the 16 genomes presented in this work are clustered in four monophyletic groups, one of which corresponding to the seven strains involved in the epidemic.

Confirming the results obtained by molecular analysis, all strains encoded the bla_{KPC} gene. In three of six isolates belonging to ST258 the bla_{KPC} variant 2 (KPC2) was found, while the remaining 13 isolates coded for the bla_{KPC} variant 3 (KPC3). Samples were also investigated for the presence of any beta-lactamase gene and it was found that all 16 isolates presented bla_{SHV} genes, while bla_{TEM} genes were detected in 13 of the 16 genomes, being absent only in KpVa-2, KpVA-3 and KpVA-4 (table 5) [190].

The three sporadic isolates belonging to ST258 and possessing the $bla_{KPC}2$ variant also presented unique virulence profile. In fact, these strains, the so called KpVA-2, KpVA-3 and KpVA-4, harbored the *irp1, irp2,* y*btA* and *ybtS* genes, that were not detected in the other strains. These four genes encode yersiniabactin, a virulence factor expressed by *Yersinia* and other Enterobacteria, including *K. pneumoniae* [133, 190]. All 16 strains analyzed were positive for the presence of the *mrk* and *fim* operons, coding for fimbrial genes [117, 214], and the *Ent* operon coding for enterobactin [201], consistent with previous results showing that these genes are highly conserved in *K. pneumoniae* [122, 190]. None of the isolates coded for *rmpA* and wzy-K1 (*magA)* genes, which are hypermucoviscosity associated genes [190, 215, 216].

Genes related with colistin resistance were also investigated. The entire operon of *pmr* genes was highly conserved among the 16 strains, including locus *pmrB*, which has been indicated as a colistin resistance determinant [217]. All colistin-resistant strains presented a deleted variant of the *mgrB* gene interrupted by IS5-like transposons, as indicated in figure 18 and table 5.

a

Sample ID	Type	mgrB	Colistin susceptibility
KPCVA1	Sporadic	Del	R
KPCVA2	Sporadic	Wt	S
KPCVA3	Sporadic	Wt	S
KPCVA4	Sporadic	Wt	S
KPCVA5	Sporadic	Wt	S
KPCVA6	Sporadic	Wt	S
KPCVA7	Sporadic	Wt	S
KPCVA8	Epidemic	Del	R
KPCVA9	Epidemic	Del	R
KPCVA10	Epidemic	Del	R
KPCVA11	Epidemic	Del	R
KPCVA12	Epidemic	Del	R
KPCVA13	Epidemic	Del	R
KPCVA14	Epidemic	Del	R
KPCVA15	Sporadic	Del	R
KPCVA16	Sporadic	Del	R

b

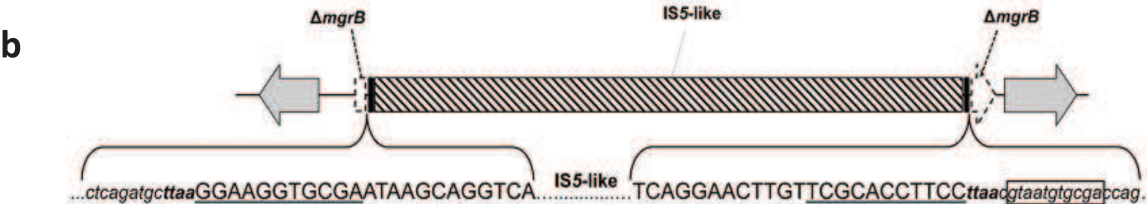


Figure 18. a) colistin-resistant strains carried the deletion in the *mgrB* gene via insertion of a IS5-like element; b) graphic representation of the genetic interruption of *mgrB* gene by IS5-like element

	KpVA-1	KpVA-2	KpVA-3	KpVA-4	KpVA-5	KpVA-6	KpVA-7	KpVA-8	KpVA-9	KpVA-10	KpVA-11	KpVA-12	KpVA-13	KpVA-14	KpVA-15	KpVA-16
Antibiotic Resistance Determinants																
***bla*KPC allele**	2	3	3	3	3	3	2	3	3	3	3	3	3	2	3	3
***bla*VIM**	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
***bla*NDM1**	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
***bla*IMP**	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
***bla*OXA48**	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
***bla*SHV**	12	11	11	11	11	11	12	11	11	11	11	11	11	11	11	11
***bla*TEM**	No	1	1	1	1	1	No	1	1	1	1	1	1	No	1	1
***mgrB* insertion**	No	No	No	No	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
Virulence determinants																
fimACDEFH	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
mrkABCDF	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
rpmA	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
magA	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
EntABCDEF	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No	Yes	No	No
ybtA/S	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No	Yes	No	No
Irp1/2	Yes	No	No	No	No	Yes	No	No	No	No	No	No	No	Yes	No	No

Table 5. Table indicating the virulence factors and resistance determinants detected in the 16 clinical isolates of *Klebsiella pneumoniae* analyzed in this study

1.4.4 Global core SNP phylogeny

A global genome phylogeny of *K. pneumoniae*, including the 16 isolates investigated in this study, was obtained by adding the *de novo* assembled genomes to a previously constructed database of 319 isolates [204]. Phylogeny was obtained in order to contextualize these strains within the previously sequenced *K. pneumoniae* isolates. The 16 novel genomes clustered in 5 monophyletic groups on the global tree (fig 19) and were fitting within CG258.

Interestingly, these 16 isolates belong to three of the four previously identified groups of Italian isolates of CG258 [204]. The relatedness among the investigated clinical strains was evaluated through the inclusion of them in the global phylogeny.

KpVA-2, KpVA-3, and KpVA-4, the three yersiniabactin-positive sporadic isolates, clustered together in a clade comprising 9 additional Italian strains and two American strains, all presenting the yersiniabactin genes [211]. Thirty-nine additional isolates, belonging to different sequence types and scattered on the global *K. pneumoniae* phylogeny, were also coding for yersiniabactin.

The genomes of the seven isolates collected in the ICU and hypothesized to belong to a single epidemic event, clustered together in a single, well supported, phylogenetic clade (fig 19). This result confirmed the starting hypothesis of a single clone being responsible for the seven infections that occurred in the ICU.

When analyzing the number of SNPs differentiating the isolates, the seven strains belonging to the investigated outbreak presented an average of 20 SNPs per genome, if compared among them.

Interestingly, a similar average number (27 SNPs per genome) differentiates strains KpVA-1, KpVA-15 and KpVA-16 that are also grouped in a single clade, but have been sampled over a longer time span (about five months). This could indicate a difference in the measured pace of the molecular clock between the two clusters. Multiple hypotheses could explain the observed situation, such as the presence of different environmental conditions, or a conservative pressure by purifying selection.

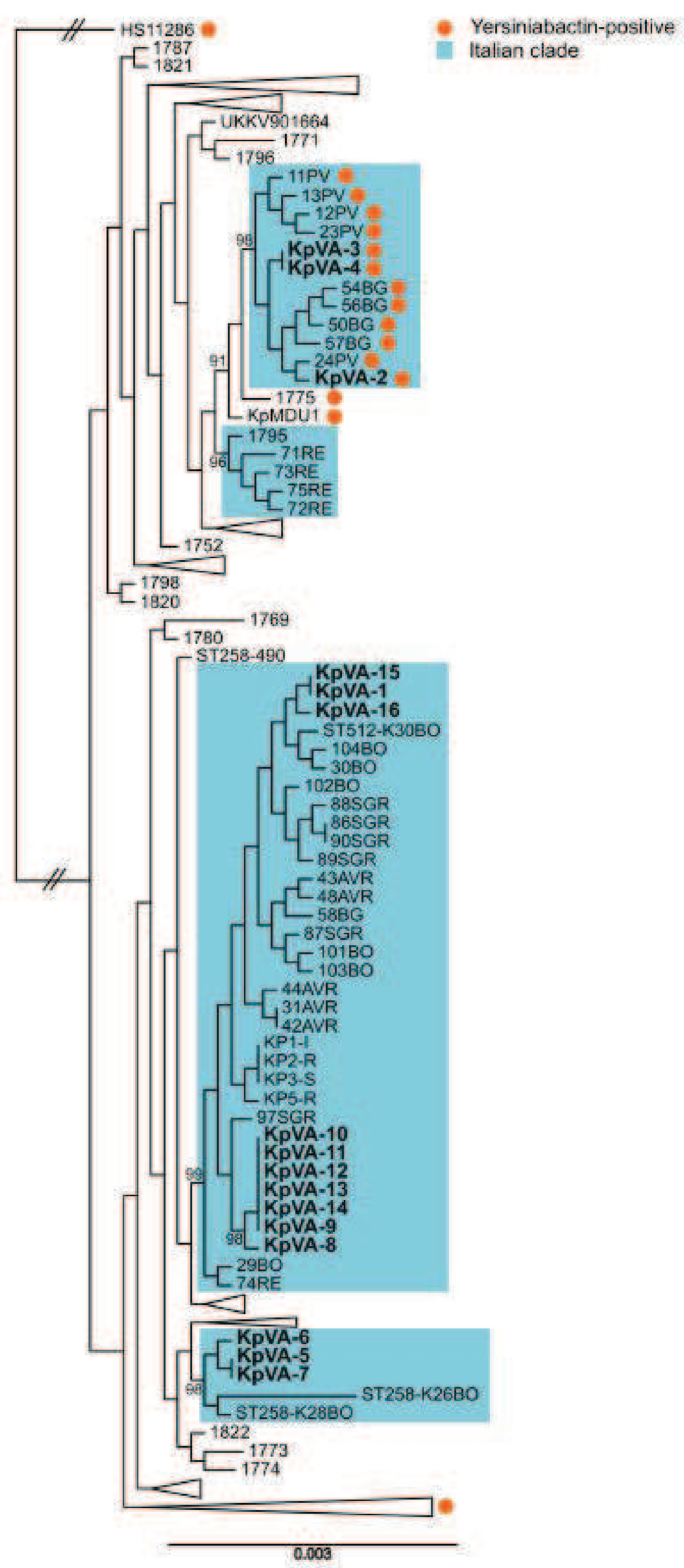


Figure 19. Schematic representation of phylogenetic relationships between isolates of *Klebsiella pneumoniae*. The 16 novel isolates investigated in this study are highlighted in bold. In the blue boxes, the four clades encompassing Italian isolates are evidenced. Triangles represent coherent monophyletic clades of isolates from other countries. Orange dots indicate the presence of yersiniabactin (*ybtA-S*) genes

1.4.5 Outbreak reconstruction

Core genome SNPs and isolation date of the epidemic strains were used to build a genomic network aimed to define genomic relatedness among them in the epidemic context.

The analysis showed a star-like topology centered on the isolate obtained from patient zero (KpVA-8), suggesting that infection do not spread linearly but multiple events of contagion probably took place, all starting from patient zero and infecting six ICU patients (fig 20).

This fits with the fact that the patient zero (KpVA-8) was hospitalized for over two months in the ICU and that the stay of the remaining infected patients was concomitant with his presence (fig 16).

Besides, the position of the beds in the ward is not related to transmission route (i.e., patients in beds closer to the bed of patient zero where not infected prior to patients in beds that were more distant from patient zero’s bed). Anyway, the result thus confirms that isolate KpVA-8 was at the origin of the outbreak.

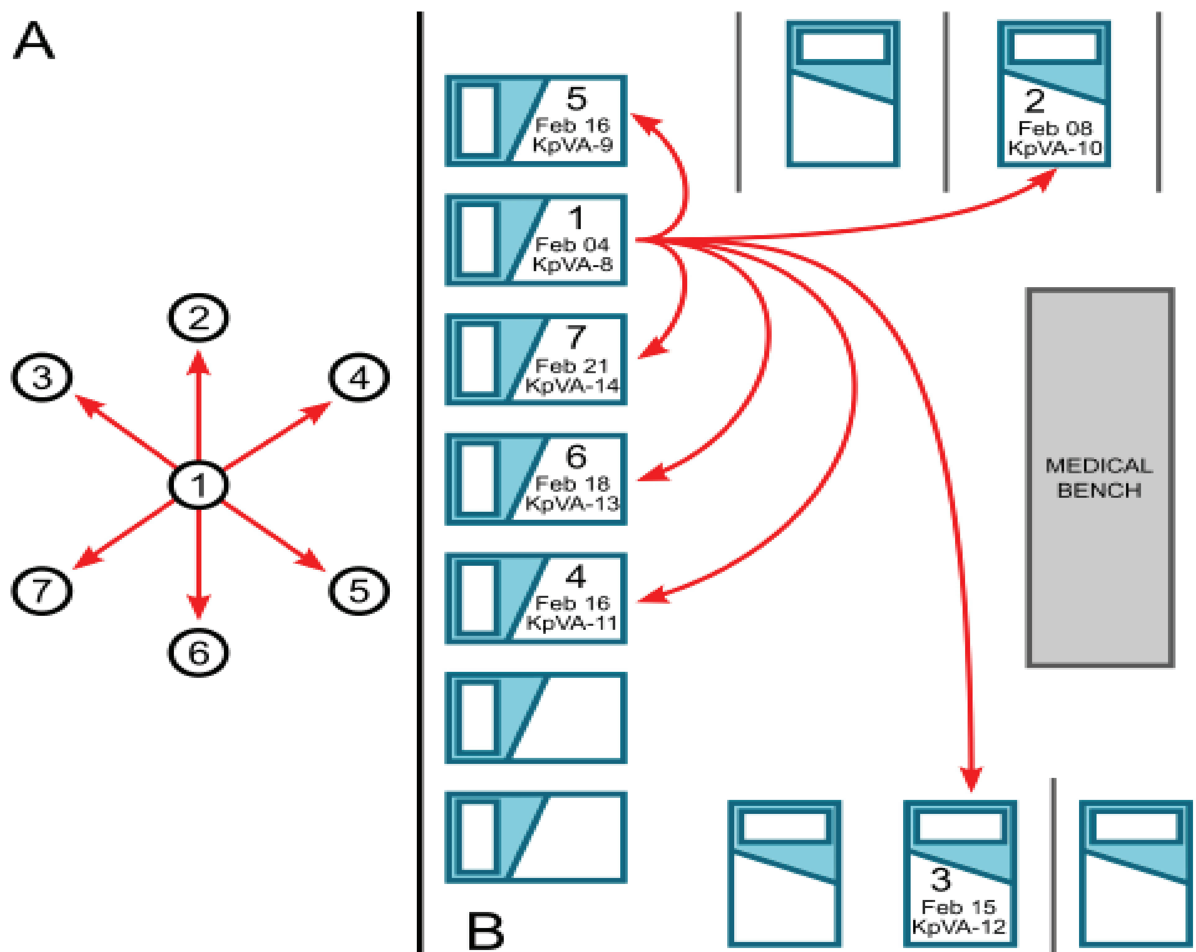


Figure 20. Representation of star-like diffusion pattern of the KPC-Kp outbreak. A) isolates belonging to a single outbreak event that occurred in February 2013. The star-like topology was obtained using the software Outbreaker. Numbers in bold indicate the temporal order of contagion. B) Graphic representation of the bed-to-bed spread of infection on ICU ward

1.5 Discussion

This work aimed to characterizing, with the use of molecular techniques, the genomic characteristics of 16 multidrug resistant *K. pneumoniae* clinical isolates, collected in the Ospedale di Circolo e Fondazione Macchi (Varese) during a period of 27-months.

The objective was to identify the possible presence of an outbreak; to elucidate the epidemiological relationships among the clinical isolates, and to place them in the context of the global phylogeny of *K. pneumoniae*.

Whole genome analysis allow defining that all 16 isolates belong to clonal complex CG258, the most prevalent KPC-producing *K. pneumoniae* lineage. About presence of resistance genes, all 16 genomes express bla_{KPC} genes and none of them had other known carbapenem resistance genes.

Prevalent presence of clonal complex 258 is not surprising, considering that previous studies showed the worldwide diffusion and high prevalence of KPC isolates of this clonal group among the carbapenem-resistant *K. pneumoniae* strains [189, 204, 218].

Sensitivity assays against different classes of antibiotics have been performed on all the samples, which unsurprisingly result being carbapenem resistant, but the most significant difference observed was presence of resistance to colistin in some of these. In fact, 10 of the analyzed samples showed MIC of 4 mg/L or higher, thus above the EUCAST MIC breakpoint. Molecular analysis showed the presence of the IS5-like transposons in the *mgrB* gene. Deletion of this gene leads to alteration in the lipopolysaccharide biosynthesis and subsequently to resistance to colistin, which target it [107, 108].

The obtained results appear to support this causative link, considering that none of the six colistin-sensitive strains presented the aforementioned insertion.

The global phylogeny of *K. pneumoniae* reveals that all the 16 isolates belong to three of the four previously characterized Italian clades of the CG258, which are probably representative of four different events of KPC dissemination in Italy, between 2008 and 2010 [204].

It is suggested that currently three of this four clades are circulating in Italy, because we were able to detect them in a single center hospital during a period of 27-months.

About virulence genes, all analyzed isolates possessed *fim, ent*, and *mrk* operons but lacked of genes coding for hypermucousviscosity proteins (*rpmA* and *magA* genes).

The only virulence factor that seems to be otherwise present in the samples is the yersiniabactin. In fact, in only three sporadic strains, the four genes responsible for yersiniabactin synthesis (*ybtA, ybtS, irp1*, and *irp2*) were present. Yersiniabactin has been known to provide advantages in bacterial metabolism and multiplication, particularly in mixed infections and under iron-deprived conditions and especially in pulmonary infections, according to recent studies [130, 201, 212]. Interestingly, through the global analysis of the CG258, it appeared that, apart from these three novel strains, other Italian isolates of the same subclade and two Americans strains constituted a monophyletic group and were positive for the presence of yersiniabactin genes. Moreover, the results suggest that these genes, once acquired before diversification in specific subclades, have been maintained since.

Moreover, we aimed to identify the presence of a nosocomial outbreak, occurred in the ICU in February 2013. According to the whole genomes analysis, seven of the total analyzed strains appeared to be genetically related in a monophyletic cluster and were confirmed to belong to a single outbreak. When analyzing the number of SNPs differentiating the isolates, the seven strains belonging to the investigated outbreak presented an average of 20 SNPs per genome, if compared among them.

We were also able to obtain the transmission route of the epidemic event, which was represented by a star-like diagram, originating from patient zero.

The seven epidemic isolates could not be discriminated from sporadic strains based on a specific pattern of the presence/absence of specific virulence factors and also the drug resistance profiles were highly similar. This shows that the dissemination of the outbreak was not related to genes conferring a specific advantage to the epidemic clone.

In conclusion, the availability of classical molecular methods and whole-genome sequencing analysis allow to easily and accurately characterizing multiresistant pathogens circulating in the hospital, which represent the major cause of outbreaks spread. The prosperity of data from genome sequencing allows reconstruction of the relatedness among clinical isolates collected in a single hospital and allows identifying the presence of an outbreak; moreover analyzed isolates were placed in overall global phylogenies,

highlighting, through comparison with deposited genomes, the presence of common characteristics that might give specific selective advantages. The possibility to introduce the bacterial genomics into clinical settings will allow the reconstruction of the routes of transmission and of the causes of nosocomial infections, with estimation of the relative roles of human- and microbe-related factors.

CHAPTER TWO

2.1 Introduction

2.1.1 General features of *C. difficile*

Clostridium difficile is the leading cause of nosocomial diarrhea, mainly associated with the use of antibiotic, and increasing cause of community-acquired diarrhea.

C. difficile owes its name to the Greek "kloster" (*κλωστήρ*, spindle) and to the Latin "*difficile*" and belongs to the phylum *Firmicutes* comprising the Bacillus and Clostridia classes. *Clostridiaceae* family belongs to the latter class, and contains the *Clostridium* genus (*sensu strictu*) and others (for example, *Anaerobacter*, *Oxobaxter* etc).

Clostridium genus includes gram-positive and anaerobic bacteria, many of which are capable of producing endospores, (dormant cell forms able to survive in extreme environmental conditions) [219] (fig 21).

Clostridium spp are ubiquitous microorganisms, which can be isolated from the soil, water, sewage system, and made part of the normal flora of the gastrointestinal tract of animals and humans.

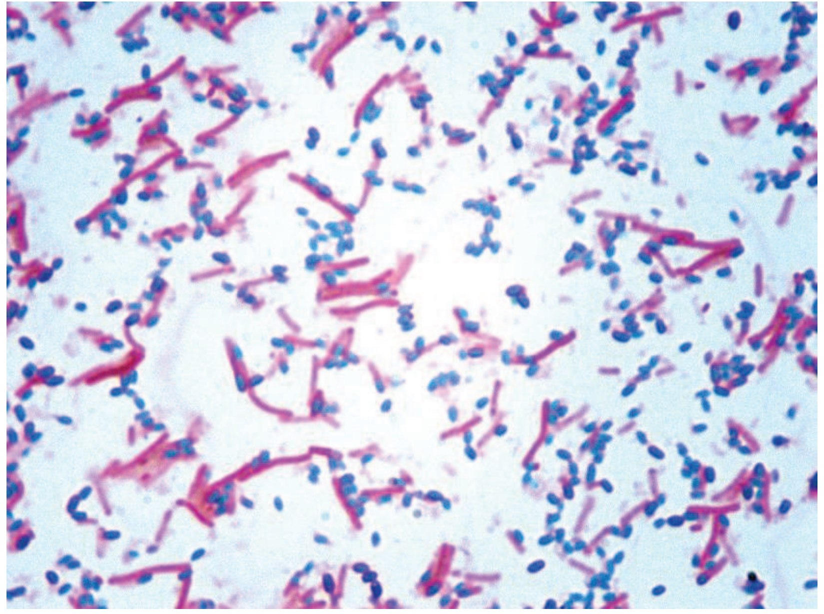


Figure 21. A differential stain of *C. difficile* spores. Malachite green staining methods allows identifying endospores (spherical shaped, in green) and vegetative cells (elongated cells, in red).

C. difficile was first reported in 1935, when the species was isolated from fecal samples of asymptomatic infants and, due to the difficulty of growing in conventional media, was named *Bacillus difficilis* [220]. Subsequently, studies demonstrated the ability of this microorganism to produce toxins, which caused respiratory arrest and occasionally death in the animal models used [221] while other studies were showing evidences of a correlation between antibiotic-associated diarrhea [222].

In the same years, *Bacillus subtilis* were renamed *Clostridium difficile* and the cytopathic effect of the toxins produced was increasingly associated with the onset of colitis, of which one of the most severe manifestations was the formation of pseudomembranes, leading to pseudomembranous colitis (PMC). Interestingly, the first cases of this disease were erroneously attributed to *Staphylococcus aureus* and *Candida albicans* [223] and only in 1978 PMC was definitively related to toxigenic *C. difficile* infections [222].

2.1.2 *Clostridium difficile* clinical disease

CDI is frequently associated with long hospital stay and antibiotic treatment; even if cases of community-acquired or community-associated diarrhea are increasingly reported (25%), and in these cases the patients do not normally show "traditional" risk factors for the CDI onset. For this reason, to distinguish between different types of CDI onset, a health-care onset case is associated with the health institution when symptoms start at least 48 hours after the admission in the healthcare setting, while it is considered as community-onset even the symptoms occurs within 4 weeks after discharge. If patient presents symptoms within 48 hours of admission and without previous hospitalization in the last 12 weeks, CDI is considered community-acquired (figure 22).

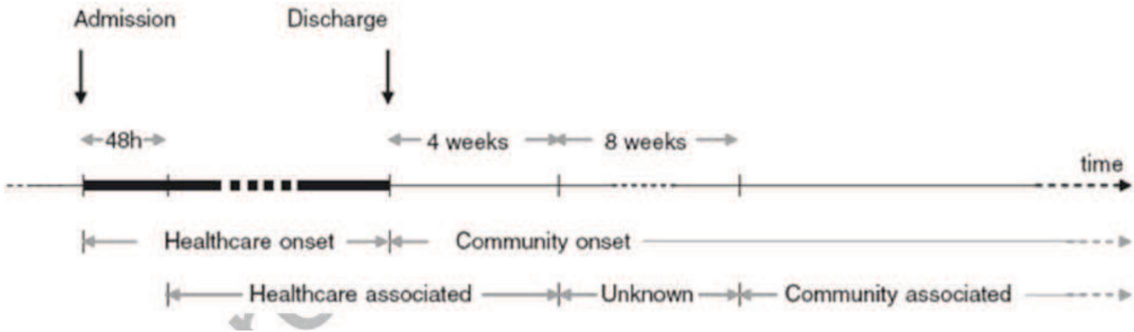


Figure 22. Graphical representation of the classification of community-onset, healthcare-onset and community-acquired *C. difficile* infection, source [224]

A patient is to be considered symptomatic when presenting ≥3 depositions of diarrhea during the 24 hours, with the presence of unformed stools appearing liquid (value 5 and 7 of Bristol scale), greenish and smelly. The severity of the CDI episode can be classified according to the clinical manifestations into mild, severe and severe-complicated CDI and may range from uncomplicated colitis to toxic megacolon, sepsis and death [225].

In the case of mild to moderate CDI, diarrhea is the principal feature and normally it is not accompanied by systemic manifestations, whereas severity is defined as a CDI episode with severe systemic symptoms as fever, abdominal pain, leukocytosis ≥15.000 cells/uL and other symptoms. In cases in which the patient shows symptoms not directly deducible as vomiting without diarrhea, fever of unknown origin, accompanied by diagnostic tests proving alterations of the intestinal wall (i.e. colon distension) and antibiotic treatment, it is any way possible to suspect *Clostridium difficile* infection.

One severe but infrequent (about 3% of all cases) clinical manifestation of CDI is toxic megacolon, which has a mortality of 50% and in which common symptoms are absence of diarrhea, leukocytosis (in severe cases, may arrive to >50000cells/uL), abdominal pain, fever, severe inflammatory response with nausea, dehydration, tachycardia, lethargy, and shock [226].

Doubtless, PMC is one infrequent but well known clinical manifestation of CDI: it was described for the first time in 1893 [223] and was commonly associated to the excessive use of antibiotic [227], while, although rare, cases of extra-intestinal CDI are described which are due in particular to surgical procedures,

manipulation of the intestinal tract or combination of other states of illness, which can lead to leakage or intestinal perforation [228].

One of the distinguishing characteristics of CDI is the frequency of relapses, which is around 15-30%, especially in patients with severe disease, subjected or less to antibiotic treatment.

The recurrences may be due to relapse or reinfection, distinguished by molecular typing of the causative strain: relapses usually occur in the first 10-14 days from the term of treatment and are due to the activity of the strain causing the first episode, while reinfection are caused by another strain and may occur in larger time periods as it depends on the time of exposure and on the contact between contaminated environment and patient [229] (table 6).

CDI disease category	Laboratory and clinical signs	Risk factors
Mild to moderate	Diarrhea; no systemic signs; leukocyte count <15.000cell/μL; serum creatinine <1.5 times baseline	Antimicrobial treatment; proton pumps inhibitors; long stay in hospital; chemotherapy; chronic kidney disease
Severe	Systemic symptoms and/or leukocyte count ≥15.000 cells/ μL and/or serum creatinine ≥1.5 times baseline	Infection due to more virulent strain (i.e. *C. difficile* ribotype 027); advanced age
Severe-complicated	Systemic symptoms and hypotension, paralytic ileum or toxic megacolon	Recent surgery; inflammatory bowel diseases (IBD); intravenous treatment with immunoglobulin; and all the above
Recurrence	New episode in <8 weeks from successful completing CDI treatment	Patients ≥65 years; underlying diseases; comorbidity; increased initial severe disease; concomitant use of proton pumps inhibitors and/or antibiotic treatment

Table 6. CDI classification based on disease severity, adapted by [226]. For each degree of disease severity, the main clinical manifestation and risk factors are described

Infection due to *Clostridium difficile* has, therefore, great clinical importance in several respects: it is the most known leading cause of nosocomial diarrhea, its incidence and relapses rates are increasing with the dissemination of epidemic strains (i.e. CD027); patients are forced to prolonged hospital stays. This may be translated in a high sanitary cost apart from a decline in the quality of life of the patient [230], for this reason a rapid, sensitive and efficient diagnosis is imperative with the main objective to identify presence of outbreaks and/or polyclonal spread of higher transmitted pathogen, aiming to limit their transmission between patients.

2.1.3 Risk factors for CDI acquisition

The main risk factors associated to the development of *C. difficile* infection are the antibiotic treatment, the use of proton pumps inhibitors because they lead to dysbiosis and weakened of the microbiota of the gut, favoring colonization and proliferation of *C. difficile* cells. Also other factor, as well as the underlying diseases and long stay in hospital, may affect immune response and so favoring the disease progression.

Probably, the principal risk factor for CDI progression is the large use of broad- spectrum antibiotics prescribed to the patient, leading to debilitation of the normal intestinal flora with the subsequent proliferation of opportunistic bacteria such as *C. difficile*. This bacterium presents resistance against a large number of drugs and so may survive and generate the production of toxins responsible for the onset of the disease.

Third generation cephalosporins, fluoroquinolones and clindamycin, used to treat a large number of diseases, had been ever considered the antibiotic giving the greatest risk factors for the onset of CDI [231] for their broad activity against many commensal bacteria.

The use of proton pumps inhibitors, which reduce gastric acid secretion and are prescribed for a wide range of diseases including acid reflux and peptic ulcers, probably it related to the higher ability of the *C. difficile* spores to cross the stomach, once ingested, despite the gastric acids produced here. The hypothesis is that,

because of the increased gastric pH, the spores pass more easily from the stomach into the gut, when they can germinate and lead to inflammation and disease [232].

Other risk factor related to the patient are the older age, the long stay in hospital, the hospitalization in the intensive care unit or in contact with symptomatic patients, and the underlying diseases which make the immune system weaker against possible infections. In particular, several studies show an increase of CDI rate in patients >65 years, although incidences are also increasing in youngest population. The combination of the old age with other factors, such as antibiotic treatment and hospital admission, is crucial to confer high risk to the patient in developing CDI [233].

For these reasons, it proves crucial the introduction of control measures as environmental hygiene (decrease transmission) as patients care (a greater awareness in the use of antibiotics).

Prevention starts with the introduction of good hygiene habits that the medical stuff must follow such as hand washing with soap and water when going to visit another patient and the other and the use of gloves to prevent the spread of spores. Furthermore, it would be appropriate to reduce the use of broad spectrum antibiotics, as far as possible, to avoid making sensitive the patient.

In the case of symptomatic patients, it is necessary to proceed to the isolation of this or, in case of low availability of rooms and/or in case of outbreak, it is appropriate to create specific cohorts. The rooms of symptomatic patients must undergo further cleaning with sodium hypochlorite or sporicidal products, knowing that the common detergents do not eliminate the spores of *C. difficile*. Medical staff (and family members of patients if allowed entry) should use gloves and disposable gowns to enter in the rooms in isolation in order to avoid the greater spread of spores in the environment.

Finally, each new case should be promptly notified to the group for hospital infection control to ensure rapid infection survey and management [234].

2.1.4 Treatment of CDI

First line antimicrobials used in CDI treatment are metronidazole and oral vancomycin, although nowadays a good range of treatment is available for the patient, as biotherapy (fecal microbiota transplantation or probiotics) or new treatment options, such as monoclonal antibodies or vaccines (several awaiting approval by the FDA) (table 7).

Metronidazol is the drug most widely used for the treatment of mild episodes of infection due to *C. difficile*. It is a nitroimidazole that displays bactericidal activity towards both protozoa and many anaerobic bacteria with a unique metabolic pathway. When administered orally, metronidazole is absorbed rapidly and almost completely, with only 6%–15% of the drug excreted in stool [235].

On the other hand, its disadvantages are the higher failure and recurrences rates compared to vancomycin in severe cases, so that therapy must be targeted towards eligible patient with a good probability to be cured.

FDA-approved	Off-Label Options	New Drugs in development	Biotherapeutics
Metronidazole	Rifaximin (Xifaxan, Salix)	LFF571 (Novartis)	Fecal microbiota transplantation
Vancomycin	Nitazoxanide	Surotomycin (CB-183, 315, Cubist)	VP20621 (ViroPharma)
Fidaxomicin (Dificid, Cubist)	Tigecycline (Tygacil, Pfizer)	SMT 19969 (Summit)	Probiotics
		Cadazolid (ACT-179811, Actelion)	
		Oritavancin (LY333328, The Medicines Company)	
		Cholate meta-benzene sulfonic derivative	

Table 7. Treatment options for *C. difficile* infections; adapted by[236]

Vancomycin, unlike the metronidazole, appears to be more effective in the treatment of the first episode of moderate to severe CDI or in cases of metronidazole therapy failure. It is a drug of choice for the treatment of CDI cases in pregnant women because lack of systemic absorption. Given to its hydrophilic nature, vancomycin it is not able to cross the intestinal wall, so that it is necessary to administer it orally [235].

Both vancomycin that the metronidazole are believed highly effective drugs in the treatment of mild CDI, also if studies demonstrate that the rate of failures associated to metronidazole in treatment of severe CDI is significantly higher than in vancomycin [237].

A detailed prospective study done by Zar *et al* showed that treatment of mild *C. difficile* associated disease (CDAD) with metronidazole or vancomycin resulted in clinical safety of the patients (90% versus 98%, respectively), while among the patients with severe CDAD, treatment with vancomycin was more effective than those with metronidazole (97% versus 76%, respectively) [238].

Rifaximin (Xifaxan, Salix) is a semi synthetic derivative of rifamycin approved for the treatment of travelers' diarrhea also used off-label for irritable bowel syndrome and hepatic encephalopathy. It shows its effectiveness mainly in the treatment of CDI recurrences. It is a drug particularly indicated for the treatment of bowel microbial infections, given to its low systemic absorption, estimated at a maximum of about 1%. These important pharmacokinetic properties allow to the antibiotic to reach the intestinal tract at high concentrations and to exert the therapeutic action directly *in situ* [239].

Although is a promising treatment option, difficulties in using rifaximin are the increased number of high resistant isolates (however infrequent)and the lack of guidelines on interpretation of *in vitro* susceptibility tests [240].

Fidaxomycin (FDX; Dificid, Cubist), a member of a new class antibiotics targeting bacterial RNA polymerase, shows high activity against *C. difficile* and a short activity spectrum against other intestinal microorganisms. In 2011, pivotal trials demonstrate that fidaxomycin respect to vancomycin was not inferior showing similar high cure (92.1% and 89.8%, respectively) and recurrence (13.3% versus 24%) rates, associated with its ability to preserve the normal gut microbiome and completely resolve the underlying CDI status [241, 242].

Other possible therapeutic options are: nitazoxanide, with acts interfering with the anaerobic metabolism of some bacteria, similarly than metronidazole [243]; intravenous tigecycline, possible adjunctive treatment for patients who had failed to respond to metronidazole and vancomycin [244]; ramoplanin which acts against vancomycin-resistant enterococci as well as *C. difficile* [245]; cadazolid, a antimicrobial used for treatment of multidrug resistant gram positive bacteria, showed a high activity against *C. difficile* and lower ability to induce resistance and toxicity [246, 247]; and surotomycin (CB-183,315, Cubist) that seems to have good potency against several strains of *C. difficile* being also able to minimize intestinal microbiota alteration and leading to a rapid recovery of the normal flora [248].

In recent years, patient showing treatment failure or recurrent CDI have been treated with fecal microbiota transplantation (FMT).

The first published cases of FMT use were about veterinarians who performed fecal transplantation to treat horses with diarrhea, infusing stool from healthy donors into the rectum of the sick animals. In fact, treatment was based on the ability of the donor's microflora to reintroducing a healthy intestinal microbiota in the host, reestablishing colonization resistance against *C. difficile* and other pathogenic bacteria [249].

The application of bio-therapy is useful in the treatment of CDI, especially in recurrent cases, leading the intestine to a state of equilibrium, considering that the administration of antibiotics is effective but still acts by destabilizing the gut environment.

The FMT may be administered by nasoduodenal/gastric tube, colonoscopy, enema or capsules; following different protocol which includes vancomycin pre-treatment or bowel lavage (fig 23) [250, 251].

Despite the proven scientifically benefits, the FMT is located in the middle of a strong scientific debate about possible adverse effects (AE) against the receiving patient. However, it appears that the adverse effects are less than the benefits received and that the biggest problem remains, however, the higher cost of therapy respect antibiotic treatment [252].

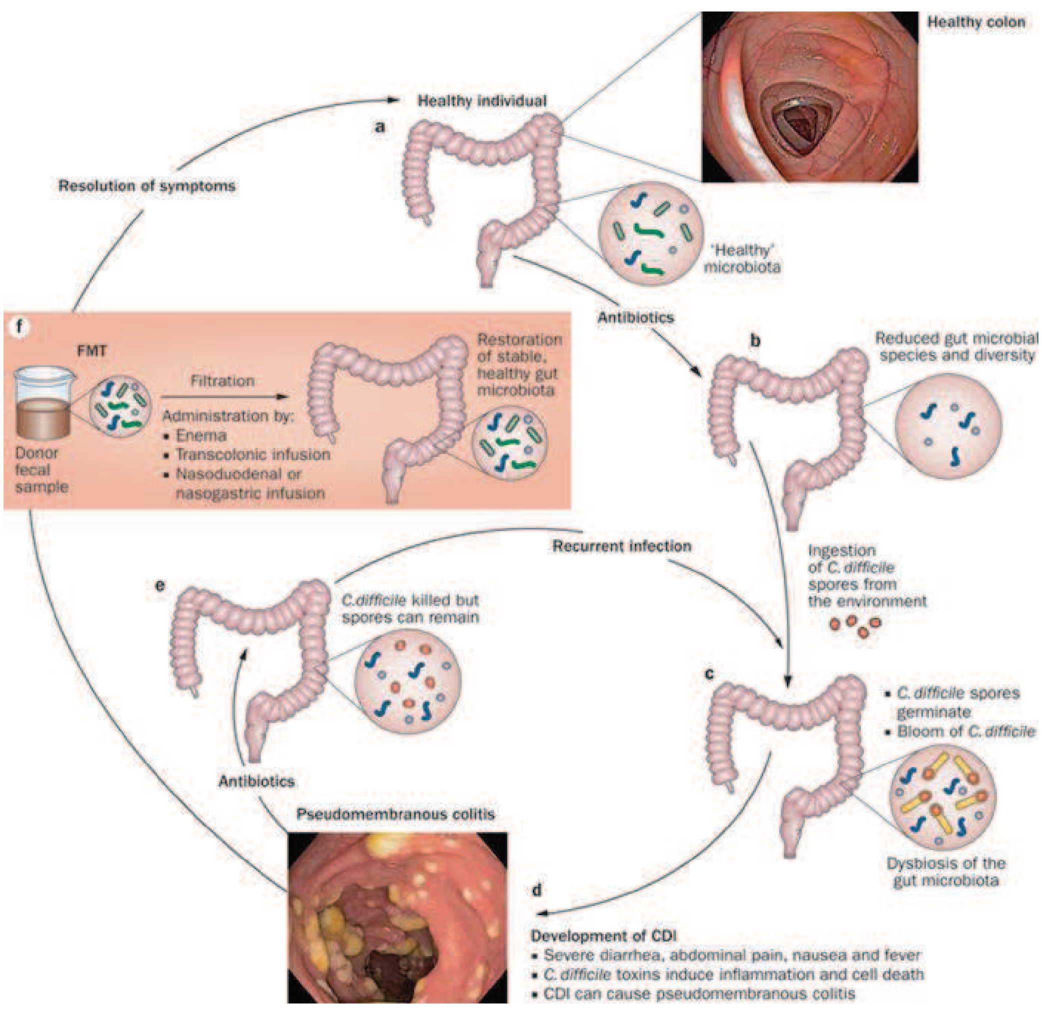


Figure 23. Infection process of *C. difficile* and utility of Fecal Microbiome Transplantation (FMT) to restore normal bacterial flora of patient [253]

2.1.5 *Clostridium difficile* virulence factors

Toxigenic *Clostridium difficile* strains are known to express different virulence factors, of which the mainly characterized are A, B and binary toxins (causative of disease progression) and spores (involved in the transmission), in addition to other less described factors of pathogenicity such as fimbriae, a polysaccharide layer and hydrolytic enzymes.

C. difficile sporulation and germination

Clostridium difficile isolates are capable of producing spores, whose casual ingestion by the patient, for contact with the contaminated surfaces, represents the first step for the transmission of the disease.

The spores are able to survive in extreme environmental conditions and are resistant to different chemical and physical agents, so that when they are released in the environment by a symptomatic patient, may contaminate the surfaces of the healthcare environment (bed linen and stainless steel, for example) and also the hands of the medical staff [255].

Sporulation is the adaptive process by which a bacterial species forms metabolically dormant, highly stress-resistant endospores and is divided into different phases: asymmetrically division of the cell into prespore and mother cell compartments; double-membrane prespore formation; synthesis of peptidoglycan cortex; spore coat assembly and release of mature spore in the surrounding environment.

Structurally, spores of *C. difficile* seem to be composed similarly to those of *B. Subtilis*, although many differences are showed to exist. Spore structure consists of a number of concentric layers, which are, from the inner to the outer: the inner core, which contains bacterial DNA, RNA and several enzymes and provides for DNA [256]; the inner membrane; the cortex, which differs in composition from that of vegetative cells, to ensure to the spores a strong resistance to environmental stimuli [257]; the protein coat, that seems may have an important role in generate inflammatory processes, being able to resist to different chemical agents and to play a key role in recognizing germination stimuli [258] (fig 24).

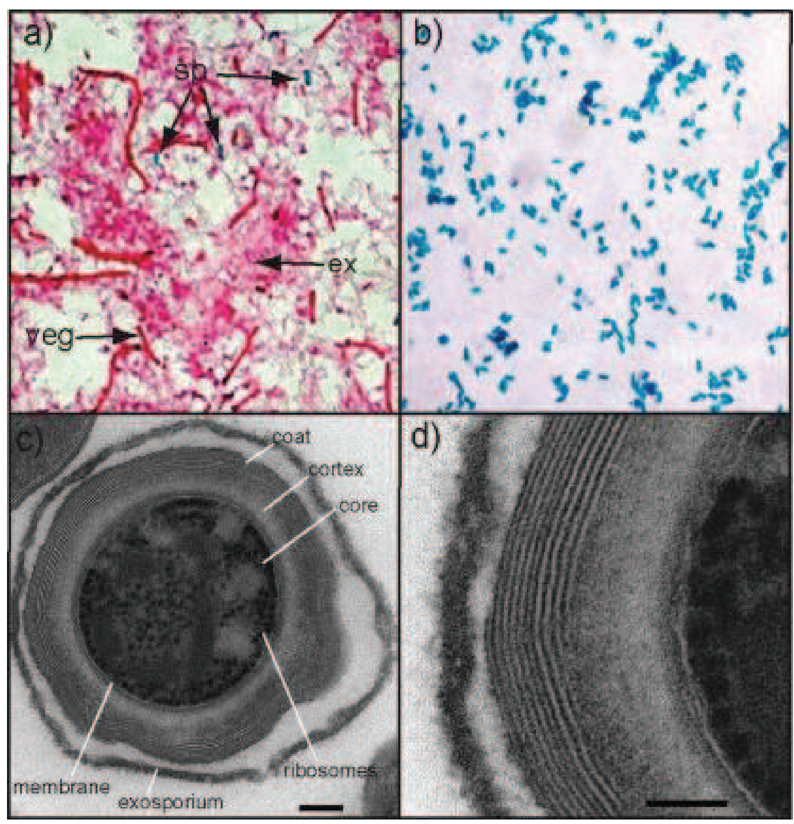


Figure 24. *C. difficile* spores staining and structure. a) endospores staining; sp: spores, veg: vegetative cells; ex:extracellular matrix; b) malachite green spores staining; c) imagine of a the ultrastructure of a spore by electron transmission microscopy; d) magnified section of outer surface of the spore [258]

In the last years, it was suggested that higher transmission of epidemic strains (i.e. *C. difficile* ribotype 027) may depend on the ability of these strains to produce a higher amount of spores.

In 2008, a study by Akerlund *et al*, performed to evaluate the sporulation rate of several isolates of *C. difficile* 027, showed that CD027 strains sporulated more than other ribotypes and that, in particular, *C. difficile* 027 strains collected from USA outbreaks presented higher sporulation and toxin production rates than all the others [259]. Therefore with the years, other studies have attempted to make a contribution on the higher virulence of *C. difficile* ribotype 027 [260, 261], but the results are contradictory [262].

However, the real conclusion of these studies is unclear, considering that to date there is not an *in vitro* method that may be considered standard. In fact, although several studies have used the same reference strains, conflicting results had been obtained. This could be due to growth variability of the bacteria, to the use of different culture media or to the restricted number of samples [263].

It is also necessary to consider that also at the molecular level, the sporulation signaling pathway of *Clostridium difficile* has not been yet understood, so that it is necessary to use *Bacillus subtilis* as model [264].

A recent review claimed that, although the model of *B. subtilis* is universally accepted, it is not possible to hide the presence of substantial differences in *C. difficile* signal transduction, mainly about the regulatory mechanisms relative to factors (called sigma factors) involved in synthesis of asymmetrical septum from mother cell and spore coat assembly [265].

In conclusion, although the discrepancy of studies published, the hypothesis that epidemic strains (i.e., CD027) have a greater capacity to sporulate is generally accepted.

While sporulation mechanism allows cell to produce spores under prohibitive conditions, the germination is the event in which the spore can mature in a metabolically active cell, able to release toxins, leading to the onset of the disease.

As for sporulation, also *B. subtilis* germination pathway is used as model of *C. difficile*, although it was demonstrated that the latter lacks of the germinant receptors expressed by *B. subtilis* and other species of *Clostridia,* suggesting that other unknown regulation mechanisms are involved.

Germination process is characterized first by the interaction of the germinant receptors with specific effectors molecules called germinants. At this point, a signaling cascade is activated that leads to metabolic activation of the spore: the water content of the spore increases, the core expands and enzymatic activity, metabolism and spore outgrowth occur [256].

In particular, for the germination begins, spore has to recognize specific germinants, which in human gut are represented by the bile salts. These are produced by the liver and then transported to the intestine, when they have different functions, including that of regulating bacterial colonization. The primary bile salts produced by the liver consist mainly of cholate and chenodeoxycholate conjugated with either taurine or glycine, leading to synthesis of glycochenodeoxycholate, taurochenodeoxycholate, glycocolate and taurocholate. Along the digestive tract they are metabolized, reabsorbed and recycled in a high percentage (95%). Unconjugated primary bile salts are taken up by a small percentage of bacterial species present in

the colon, which transform cholate and chenodeoxycholate in the secondary bile salts deoxycholate and lithocholate, respectively [266] (fig 25).

This knowledge lead to identify the possible mechanism by which starts the infection process mediated by *C. difficile*: ingested spores resist to the gastric acids of the host stomach and arrive to the jejunum, where a high concentration of nutrients and primary biliar salts (such as taurocholate) is present. Primary bile salts stimulate the germination of spores in vegetative cells, which go into the anaerobic environment of the cecum: here, they can survive together with other bacteria species which are metabolizing the cholate derivatives into secondary bile salts (i.e. deoxycholate) preventing outgrowth of *C. difficile* cells.

In the case of imbalance of intestinal microflora (for example, following administration of antibiotics), the protection of the gut is compromised and high amounts of primary bile salts are produced, causing the proliferation of vegetative *C. difficile* cells which acts releasing toxins and starting the inflammatory process [267, 268] (fig 25).

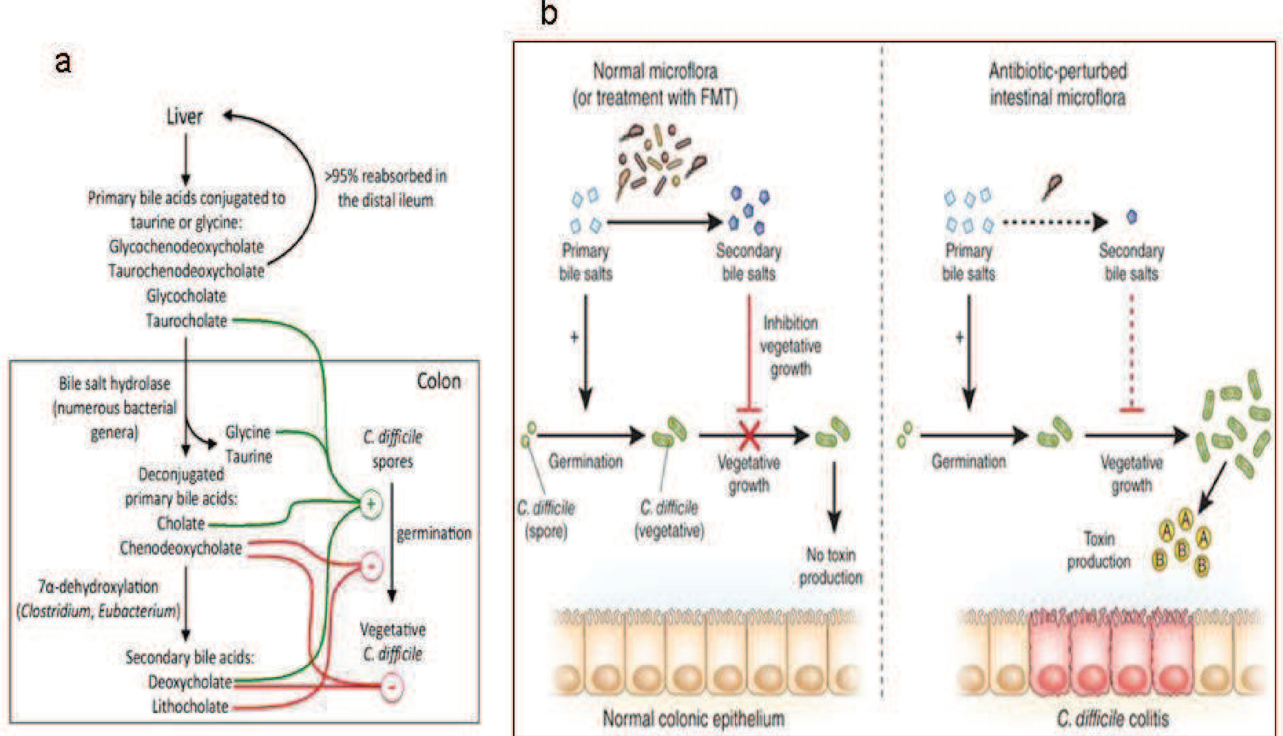


Figure 25. Germination in *C. difficile* may be enhanced or inhibited by production of primary and secondary bile salts, respectively. (a) bile salts synthesis and effect on *C. difficile* cells in (b) normal or perturbed gut [269]

Aside from play a fundamental role in starting the germination of *C. difficile* cells, the taurocholate has been used, since 1982, for the production enriched culture medium for the recovery of *C. difficile* [270, 271].

In fact, the culture media previously formulated, for example, the CCFA (with cefoxitin, cycloserine and fructose) were not adequate to *C. difficile* growth because they presented inhibitory effects against the bacterium. Consequently, modifications of these media were performed as the preparation based on horse serum, the addition of mannose instead of fructose and, especially, that of sodium taurocholate to promote germination of the *C. difficile* spores [272].

The possibility of using selective media containing taurocholate (i.e. ChromID™ *C. difficile* agar, BioMérieux, Marcy l'Etoile, France) for *C. difficile* growth can have several applications, such as to favor the identification of the pathogen in diagnostic laboratory, but also for studies of collected samples in the evaluation of the environmental contamination, due to the release of spores [273].

Toxin A (*tcdA*) and toxin B (*tcdB*) of *Clostridium difficile*

C. difficile infection (CDI) is caused by the toxigenic variants, whereas the non-toxigenic strains are not involved in pathogenicity. Infection cycle begins with the ingestion of *C. difficile* spores excreted from symptomatic patients, which may contaminate the environment for large periods. Spores are able to resist many physical and chemical stresses and, once ingested, can arrive in the anaerobic environment of gut, passing through the stomach without being affected from gastric acidity. In the gut of the patient, the spores can germinate in response to specific stimuli (i.e. primary bile salts), and mature into vegetative cells.

Mature cells adhere to the intestinal epithelium, through specific surface proteins (SlpA complexes), colonizing this environment. Subsequently, these can proliferate and can produce several types of toxins that alter the integrity and permeability of the intestinal epithelial cells, triggering various inflammatory processes responsible for the different clinical manifestations of the CDI (fig 26). Normally, the invasion of the intestinal mucosa by *C. difficile* occurs when competition of other microorganisms is not balanced and

when the intestinal microbiota is altered, for example after administration of broad spectrum antibiotics. *C. difficile* may also remain part of the intestinal flora for long time, exercising its pathogenic action later, when the alteration of the intestinal microbiota occurs. All these processes are very complex and factors related to both host and the microorganism may be involved.

Toxigenic strains are causative of the disease and produce two types of toxins called toxin A and toxin B, which are encoded respectively by *tcdB* and *tcdA* genes, located along with several regulatory genes in the Pathogenicity Locus (PaLoc) (fig 27).

In addition to toxins A and B, some strains of *C. difficile* are able to produce a third toxin, which is known as binary toxin and possessing ADP-ribosyl transferase activity. Binary toxin is encoded by *cdtA* and *cdtB* genes that are located on a locus called CdtLoc, which is located away from the PaLoc and presents important differences respect this latter (fig 27). The biological function of the binary toxin is not well understood, but it seems that may produce the disruption of the microtubules involved in cell rounding.

The function of the toxins A and B have been most thoroughly characterized; in particular, toxins destroy the layer of mucin deposited over the intestinal epithelium and subsequently recognize specific receptors on gut cells, specifically the toxin A binds to the apical side, while the toxin B to the basement side of them. Toxins can trigger, thus, their function both altering the cytoskeleton and causing a strong inflammatory response based on the release of interleukins (IL-1b, IL-6, and IL-8) and mediators of the immune response (neutrophils and polymorphonuclear leukocytes).

It is assumed that toxin A may have a prevalent role in the disease progression, being involved directly in causing both the disruption of the tight junctions of intestinal cells, leading to increased permeability on the lumen of the gut, than attracting the principal mediators of the immunitary response to the inflammation site. Once the damage in the mucosal membrane is done by action of the polymorphonuclear leucocytes and other immunomodulatory mediators, toxin B may diffuse to the lumen and causes further damages. The intestinal cells damage leads to the release of mucus and cells debris which favors the formation of pseudomembranes, a rare but severe disease presentation.

Given the imbalance of membrane permeability, resulting in electrolytic imbalance and the extended damage to the epithelium, typical symptoms (mainly colitis-associated diarrhea) of *C. difficile* infection (CDI) generally occurs. A synthetic scheme of CDI cycle is showed in figure 26.

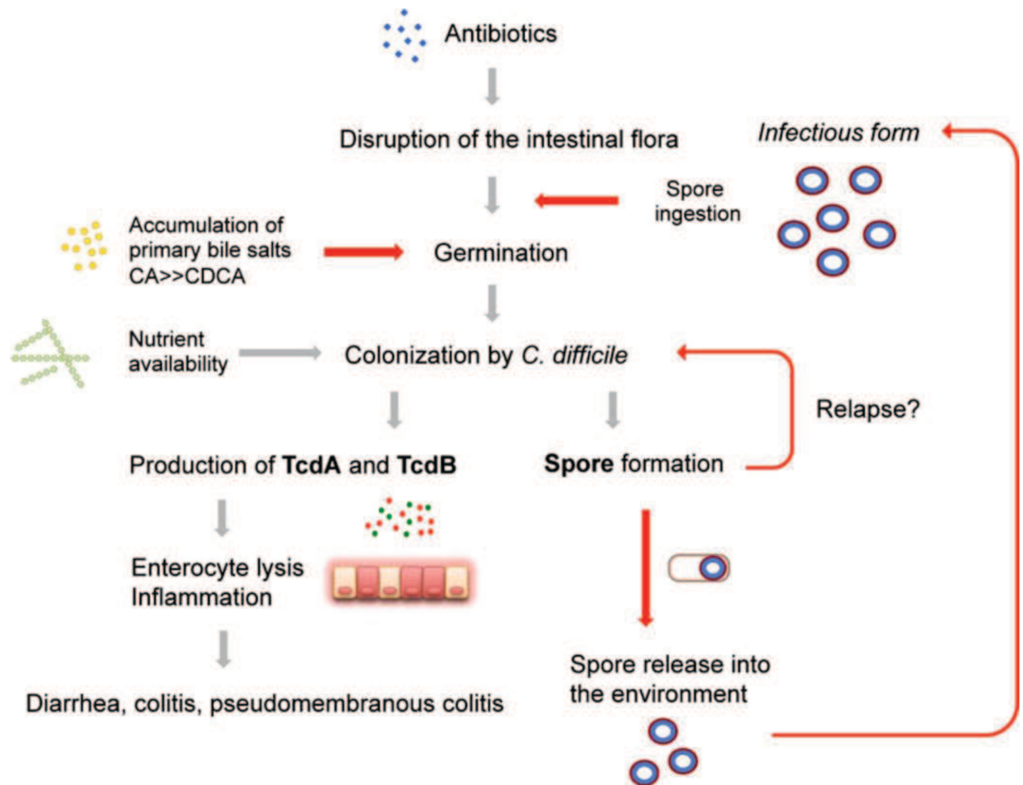


Figure 26. Representation of *C. difficile* infection cycle, comprising the spore-mediated contamination of the environment and colonization of patient mediated by their ingestion; cells maturation and production of toxins leading to the inflammation process and appearance of symptoms. During the diarrhea, spores are released and cycle starts again. Red arrows indicate events of the infectious cycle that are linked to spores or their germination. CA, cholate; CDCA, chenodeoxycholate [254]

Not all variants of *Clostridium* species are capable of producing toxins, in fact only toxigenic isolates carried the pathogenicity locus (PaLoc) and are considered the cause of the infection onset. Although the majority of toxigenic strains are capable of producing both the A that the B toxins, however there are isolate lacking of genes coding for toxin A (i.e. *C. difficile* ribotype 017, which is A-B+ isolate). In these cases, the mechanisms that lead to damage of the intestinal epithelial layer are generated by the high amounts of

toxin B released, unlike most of the strains that release both types of toxins and where toxin A plays a key role in intestinal inflammation.

Toxins A and B belong to the family of Large Clostridial Toxin (LCT) together with lethal toxin (TcsL) and hemorrhagic toxin (TcsH) of *Clostridium sordellii* and alpha toxin (Tcnα) of *Clostridium novyi*.

C. difficile toxins are high molecular weight glucosyltransferases (308 kDa and 270 kDa, respectively) which display a high degree of similarity to each other at the amino acid level (63%) and consequently are similar in the structural organization, which is composed by three main domains: a receptor binding domain, a catalytic/enzymatic domain and a translocation domain (fig 27) (231).

At the N-terminus, the greatest similar region between the two toxins, is located the catalytic site. It acts by acts mono-glucosylation of the Rho-GTPase elements, thus activating pathway which lead to the alteration of intestinal permeability and inflammation of intestinal epithelium.

The translocation domain allows to the toxigenic complexes to penetrate the membrane during the infectious process [274, 275], while the C-terminal binding domain facilitates interaction with the host receptors [276].

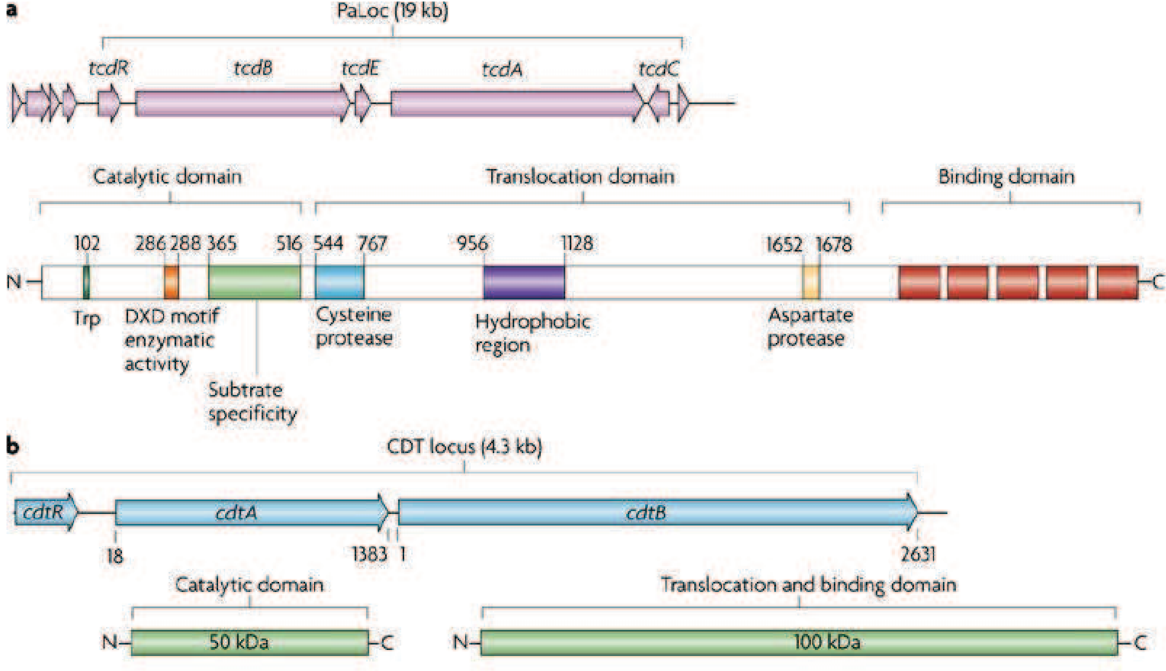


Figure 27. Schematic representation of: a) pathogenicity locus (PaLoc), harboring genes for synthesis and regulation of A and B toxins. In detail, domains of toxins structure, consisting in enzymatic, translocation, and binding domain; b) CDT locus (CdtLoc), harboring genes for binary toxin synthesis (*cdtA/cdtB*) genes and the regulator *cdtR* gene [277]

The first step of pathogenic pathway consists in a specific interaction between toxin A and B and the receptors located on the intestinal host cells. Toxins are so translocated into the cytosol into an early endosome, by which subsequently translocate into the cytosol. Through autoproteolysis, the glucosyltransferase domain is released. At this point, the interaction between glucosyltransferase domains and the target GTPases leads to the activation of specific signaling pathways which have as consequence the alteration and disorganization of the actin cytoskeleton of the intestinal epithelium (fig 28). Effects of the toxigenic processes are: the rupture of the tight junctions of the intestinal cells and increased permeability of the cell membrane; increased accumulation of fluid in the intestinal lumen; and activation of inflammatory responses, through the stimulation of the secretion of cytokines (as interleukins IL-1, IL-6, and IL-8) [278]

In particular, during active CDI, the IL-8 is involved in the recruitment and activation of neutrophils, which are present in high amounts in inflammation sites, causing the formation of pseudomembranes [279].

The ability to produce a toxins-associated cytopathic effect seems to be correlated to the amount of toxins A and B produced. Deletions in the regulator gene (*tcdC*, negative feedback) are hypothesized to lead to an over expression of *tcdA* and *tcdB* genes (coding for toxin A and B, respectively), with subsequent release of a great amount of toxins and relative increase on cytopathic effect [280].

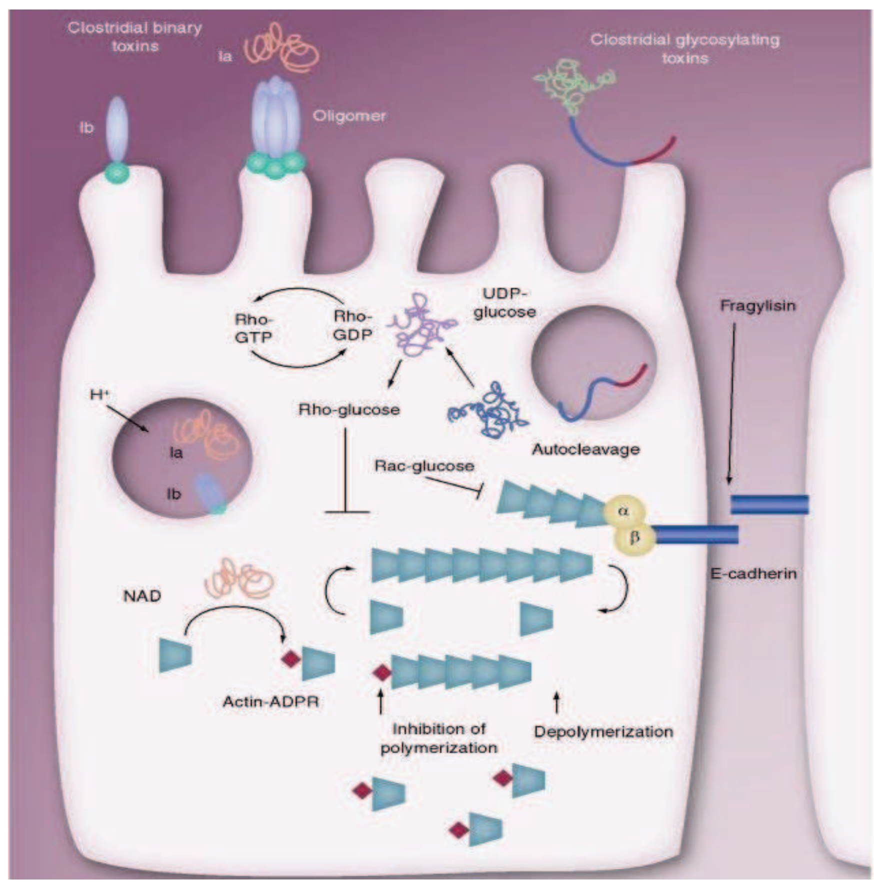


Figure 28. Intracellular pathway of *C. difficile* toxins. The first step is internalization of toxin structures via specific receptor-mediated endocytosis; subsequently, at low pH, toxins translocate from early endosomes to cytosol when, enzymatic domain is released and the toxins are able to recognize the target GTPases, leading to cytopathic effects [278]

Pathogenicity locus (PaLoc)

The pathogenicity locus (PaLoc) is a 19.6 kb region located in the same chromosomal integration site of all toxigenic strains and carried genes coding for toxin A (*tcdA*) and toxin B (*tcdB*) and three important open reading frames (*tcdC, tcdE, tcdR*) for the regulation of toxins expression (fig 27).

It is interesting that in non-toxigenic strains the PaLoc is replaced by a 115 bp non-coding sequence [281, 282], for what reason it is unclear how toxigenic isolates acquired this region. This evidence has long been debated by various scientists, suggesting that the locus had been incorporated by one or more unrelated strains through a process of horizontal gene transfer (HGT), which explains why in several phylogenetic clades may exist together toxigenic and non-toxigenic strains [283, 284].

In particular, *tcdR* gene encodes for a 22kD protein which acts as positive transcriptional regulator of toxin expression, by activation of promoter-specific transcription through binding of the RNA-polymerase [285].

The *tcdE* gene is a 501 bp open reading frame (ORF) located between *tcdA* and *tcdB* genes that encode a hydrophobic protein presenting homology with phage holins. It was assumed that this regulator may act as a lytic protein, forming membrane pores to facilitate toxins release [286].

Finally, *tcdC* gene is the most debated and characterized ORF of the PaLoc, mainly because mutations within this region seem to have important effects on the amount of toxins produced. In fact this gene encodes for a 26kD membrane-associated protein, which has no homology to any described regulatory protein and acts as negative regulator of *tcdA* and *tcdB* product [280]. For this reason, strains which possess deletions in *tcdC* gene are associated with toxin overproduction: for example, *C. difficile* NAP1/BI/CD027 harbors a single-bp deletion in position 117nt and the 18bp deletion on *tcdC* gene, which are believed to be the cause of higher pathogenicity in this epidemic strains, although this suggestion is now questioned (257)[287].

Binary toxin

In addition to toxins A and B, several subsets of *C. difficile*, such as epidemic strains ribotype 027 and ribotype 078, may express the binary toxin (CDT), whose biological role in the pathogenesis of *Clostridium difficile* infection has not yet been clarified [288]. Gene encoding for binary toxin (*cdtA* and *cdtB* genes) and a regulator gene (*cdtR*) are carried by the CdtLoc, a chromosomal region located at distance from the PaLoc [289].

The *cdtA* and *cdtB* genes encode for the enzymatic (CdtA) and binding (CdtB) components of binary toxins, which act similarly to toxins A and B, i.e. inducing destruction of the actin cytoskeleton and depolymerization of the microtubules with consequent formation of protrusions, facilitating adhesion of bacterial cells to host gut (fig 29) [290].

In CdtLoc, also a regulator gene (*cdtR* gene) is present, which similarly to as already written for the PaLoc acts as a negative regulator and, in the case of mutations, it can cause the altered production of toxins [291].

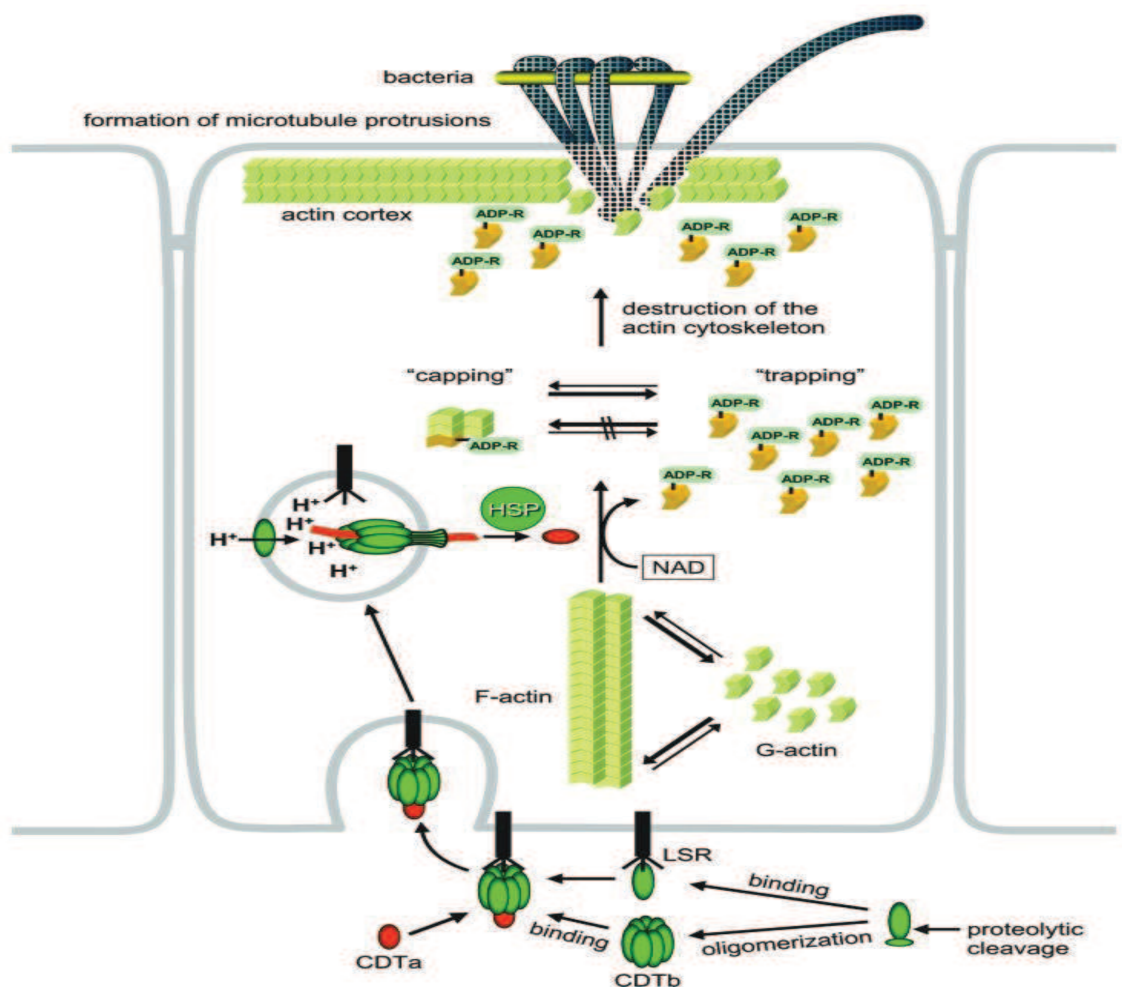


Figure 29. Schematic representation of the binary toxin mechanism of action [289]

Toxinotyping

The genetic variability on PaLoc genes allows the existence of numerous variants of the *C. difficile* strains given by the differential expression of toxins A and B (also considering the existence of strains producing binary toxins, although its role in the pathogenesis it is already undefined); so that the introduction of a method to differentiate them it was necessary. Toxinotyping is the technique of choice to classify toxigenic strains on the basis of PaLoc structural variations using a molecular method (i.e. RFLP-PCR) to analyze the

possible changes (i.e. deletions, single nucleotidic polymorphism, insertions) occurring in the 19 kb region of the *C. difficile* pathogenicity locus [292]. These variations, conserved at protein level, confer to the strains differences in antibody reactivity, targeting of intracellular small GTPases proteins and consequently a change in the effects on cells.

The method is based on the amplification of 10 specific regions of the PaLoc. In particular, for each toxin gene the fragments obtained correspond to the catalytic domain (PCR fragments A1 and B1), the putative domain (PCR fragments A2 and B2) and repetitive domain (PCR fragments A3 and B3). Considering that amplifying all the 10 PaLoc regions in very hard-working and that the most variable fragments are the B1 covering the 5'-end of *tcdB* and A3 covering the 3'-end of *tcdA,* in the most of the cases it is possible to assign a toxinotype group for the tested samples only according to the patterns obtained by the B1 and A3 analysis. In the case that new restriction patterns are observed in these regions, identifying a new toxinotype, the analysis of all ten regions is performed.

So, to each strain is assigned a toxinotype based on the comparison of the profile obtained with that of a strain of reference, the VPI 10463: for example, profiles similar to what of the reference (A+B+CDT-) are denominated toxinotype 0; up to 2015, 32 different toxinotypes had been described (named from toxinotype 0 to toxinotype XXXII) which main features can be found at the following page (http://www.mf.uni-mb.si/tox/) [293, 294] [295].

Considering all the possible combination of the three toxins, it is possible to obtain seven principal groups: A+B+CDT-; A-B+CDT-; A-B+ CDT+; A+B-CDT+; A+B+CDT+; A-B-CDT+, and A-B-CDT- (no-toxigenic strains).

The analysis of strains producing toxins A and B is obtained by genes amplification and/or detection of toxins production, whereas the presence of binary toxin CTD is confirmed by only the detection of the intact *cdtA* and *cdtB* genes located in a different locus (CdtLoc) (table 8).

Several toxinotypes may belong to the same toxins production group, as shown in Table 8, but to the same toxinotype only strains with identical PaLoc pattern belong. For example, different toxinotypes belong to the A-B+CDT- profile (VIII, X, XVI, and XVII) but the majority in diffusion and importance is the toxinotype VIII, which spread worldwide and of which most than 100 different circulating strains are known.

In conclusion, molecular diagnostic techniques are essentially based on the detection of genes coding for toxins A and/or B; for this, strains with alterations in PaLoc resulting in production of toxins variants could not be detected or detected but not differentiated. The usefulness of toxinotype is to identify these variants, although with a time-consuming method that which is beyond the necessity for a clinical laboratory and appropriate only for reference and research settings [293].

Toxin production type	Toxinotype	Molecular background
A+B+CDT –	0 minor types I, II, XII, XIII, XVIII, XIX, XX major type XXI	CDT –: absence of entire or large part of CDT locus
A+B+CDT+	major types III, IV, V, VI, VII, IX, XIV, XV, XXII, XXIII minor type XXIV	CDT+ : presence of full length CDT locus
A – B+CDT –	VIII	A – : nonsense mutation at aa position 47 in *tcdA* gene
	some 0-like strains	A – : mechanism unknown
A – B+CDT+	X	A – : rearrangement in PaLoc and large deletion causing probably changes in regulation and low or no transctiption of truncated *tcdA*
	XVI, XVII, some V-like strains	A – : mechanism unknown
A+B – CDT+	IX-like	B –: mechanism unknown
A – B – CDT+	XIa, XIb	A – , B – : only small nonfunctional part of PaLoc present
	some strains without PaLoc	A – B – : no PaLoc
A – B – CDT –	PaLoc and CDT locus negative strains	Complete absence of *tcd* and *cdt* genes

Table 8. Representation of several toxinotypes expressing different toxigenic patterns [293].

Other virulence factors

Fimbriae are molecules which allow bacteria to adhere to the host tissue and are mainly involved in in favor adhesion to the intestinal epithelial layer, during the first phase of the inflammatory process.

In 1998, Borriello et al demonstrated, using an *in vivo* model, that the adhesion to the host epithelium increased in strains which express pili o fimbriae [296]. Although their importance is known, scarce literature is published at regard.

The outer wall of *C. difficile* is covered by a polysaccharidic external layer that hypothetically would favor a higher resistance to the bacterium; however, also in this case, it seems that there are not demonstrations of a correlation between the increased virulence of some strains and the presence of this coat [297].

At last, it seems that some *C. difficile* strains may release many hydrolytic enzymes involved in destruction of epithelial gut wall, helping the bacterial cell to adhere to the epithelium. In 1990, a study showed that

several highly virulent strains were able to produce enzymes (hyaluronidase, chondroitin-4-sulphatase and collagenase) with high hydrolytic activity, but there was not observed an association with enlarged virulence, although highly virulent strains were more active than less virulent strains [298].

2.1.6 Epidemiology of *C. difficile*

Clostridium difficile is part of the intestinal flora in 1-3% of healthy individuals and more than 20% of hospitalized adults, who acquire the bacteria through contact with contaminated surfaces. In fact, symptomatic patients release large amounts of spores in the environment which can then be transferred on different surfaces where they resist for a large time and may be transferred to other surfaces also by medical staff and so be ingested from patients [299].

The presence of *Clostridium difficile* was also observed in foods (meat, salad) and in several animal species, although in the latter case the role of the bacterium in generating the disease is not entirely clear.

Infection due to *Clostridium difficile* is the most common cause of nosocomial diarrhea and has a significant clinical importance because of its high morbidity and mortality and because of the prolonged stay of patients in hospital, causing a significant increase in health care costs [300].

Considering that normally the CDI occurs in already hospitalized patients, cost of patients care increase because of the need of a specific treatment to eradicate *C. difficile* from the host gut, rigorous hygiene and environmental decontamination. A recent review showed that estimated healthcare costs for the treatment and management of CDI cases in a one year period in the USA were rising by $ 433 million to $ 797 million a year, considering that the per patient episode cost goes from an extra € 4067 to an extra € 9276 [300].

Our understanding of *C. difficile* microbiology and epidemiology is changing rapidly, considering that the various ribotypes seem to spread differently in the world also demonstrating a changing epidemiology.

Greater importance was given to the molecular epidemiology of *Clostridium difficile* since the early 2000s, when an increasing rate in frequency of severe cases in Canada and USA has been reported, related to the dissemination of epidemic *C. difficile* CD027/NAP1/B1 (PCR Ribotype, CD027; North America Pulse-field type, NAP1; Restriction Endonuclease Analysis, REA-type B1). This variant was able to produce toxin A, toxin B, and also binary toxins [301, 302], initially associated to higher virulence but whose role in the pathogenesis remains to be clarified. Following the spread on several outbreaks in the same period, it was observed that the proportion of patients with CDI who died a month after diagnosis gone from 4.7% in 1991–1992 to 13.8% in 2003 [303, 304].

Since that time, nosocomial outbreaks involving strains of *C. difficile* ribotype 027 were characterized at global level [305] (fig 30) but in Europe the increased incidence of CDI cases cannot be associated only with the emergence and spread of CD027, as other variants were already widespread.

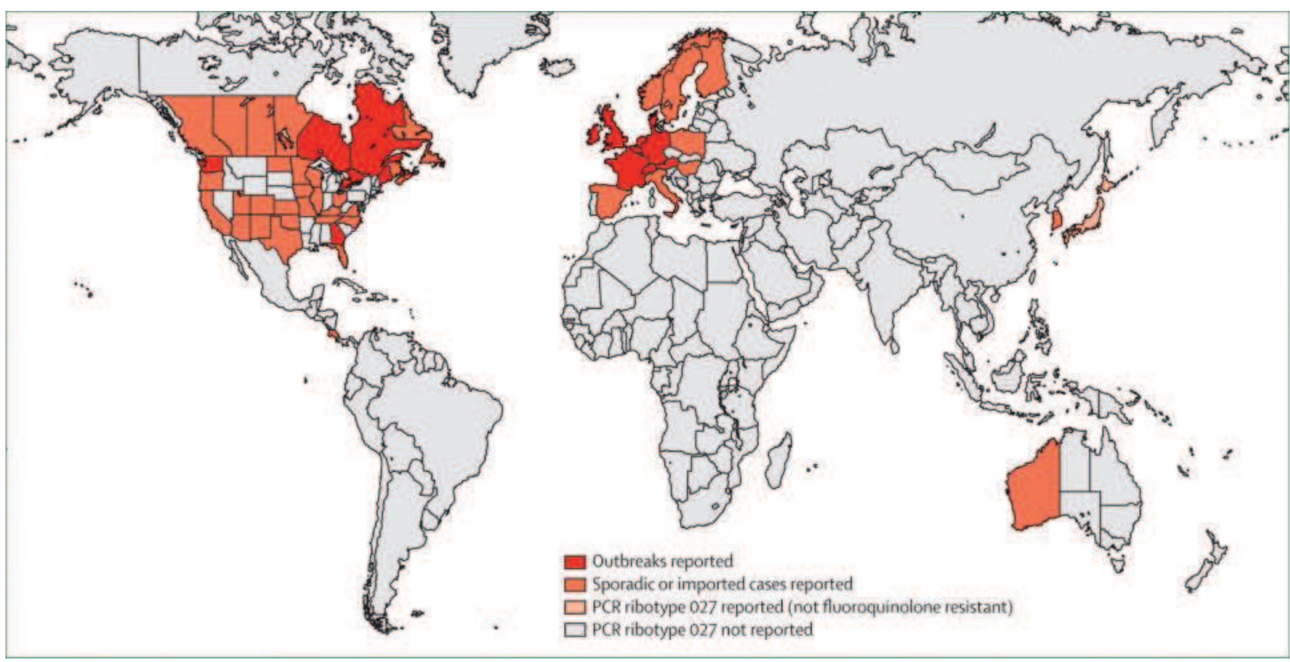


Figure 30. Representation of the global spread of CD027 strain. In red, the outbreaks detected; in orange, sporadic/imported cases; in pink, fluoroquinolones-susceptible CD027 cases; in grey, areas CD027-"free" [305]

In fact, a questionnaire-based study carried out in 2008, aimed to evaluate effectiveness of CDI diagnosis and to detect the circulating variants of *C. difficile* in Europe, has showed that the incidence of CDI, varying between the participating hospitals, was of 4.1 cases/10000 patients-day and that the distribution of the

circulating ribotypes widely differed depending to the country, among which the predominant were the ribotypes 014/020 (16%), 001 (9%), 078 (8%) [306]. Although outbreaks due to CD027 in UK and Ireland were reported, this study revealed that only 5% of the cases were due to this variant, as represented in the figure 31.

Another recent study, involving 468 European hospitals, showed that CDI incidence, respect the previous study, increase from 4.1 cases/10000 patients-day to 7.0 cases/10000 patients-day and that to date CD027 is the prevalent ribotype in the European territory, with a greater incidence than that observed in 2008 (18% versus 5%, respectively).

A part of increased incidence, interestingly it must be noted that higher incidence of CD027 cases seems to be shifted from UK and Ireland to Germany and Eastern Europe (Hungary, Poland, and Romania), where only sporadic cases were reported: this phenomenon of reduction in CDI cases in country where several outbreaks had been already spread is likely to be associated to the improvement in surveillance and control methods adopted in UK and Ireland [306, 307].Furthermore, also the diversity of the other ribotypes across Europe changed compared with finding from the study performed in 2008: 138 ribotypes compared to the previous 20 ribotypes have been detected, with increase of cases due to ribotype 001 (from 9% to 11%) and decrease of 014 (from 16% to 7%), highlighting the importance to perform molecular epidemiology studies to trace the changes in the movement of these variants.

In Spain, up to the year 2014, the incidence of CDI cases was around 3.8 cases/10000 patients-day and the frequency of CD027 infections, according to the last two multicenter national studies, seems to be of 3.5% (2013) considering that only few unrelated cases have been detected and considered as sporadic, whereas other ribotypes (as 014, 078/126, 001/072, and 106) are the most commonly diffused [308, 309], similarly to the general situation in Europe.

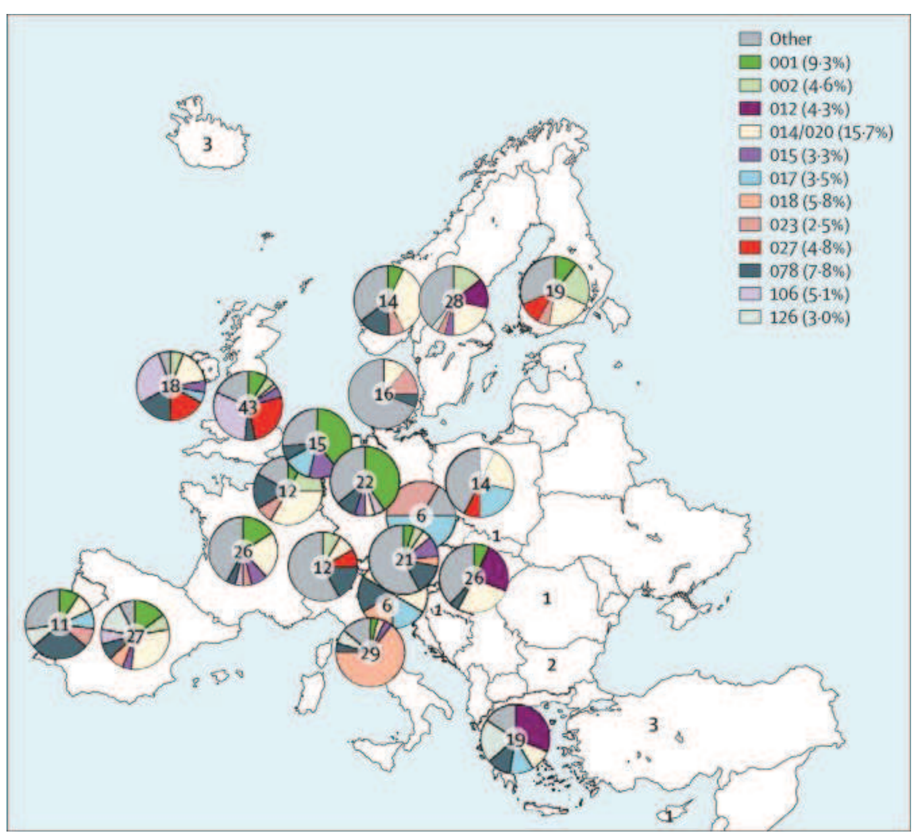


Figure 31. Representation of the most diffused *C. difficile* ribotypes in Europe in 2008, source [306]. It is possible to note the high frequency of ribotype 014/020, followed by 001 and 078 in most of the European countries. *C. difficile* ribotype 027 has been reported mainly in UK and Ireland, where caused outbreaks

2.1.7 Laboratory diagnosis of CDI

The laboratory diagnosis is of great importance in the detection of toxigenic strains of *Clostridium difficile*. For the proper execution of this, first, it is required that the sample arrives at the laboratory in adequate conditions, i.e. liquid stools in sterile containers without transport media or sporicidal substances (such as formaldehyde) to avoid the death of the pathogen and the degradation of toxins and spores.

Considering that the majority of the tests are based on detection of toxins which are sensitive to temperatures, to perform a good diagnosis it is necessary that the sample is kept at 2-8°C for a maximum of 48 hours or frozen, to avoid alteration of the toxigenic component.

Several studies have demonstrated a widespread underdiagnosis of CDI cases, due to the combination of lack of clinical suspicion and the low sensitivity of the diagnostic methods used. In particular, a multicenter

study carried out in Spain in 2013, showed that, although the improvement compared to study performed in 2008, only 50% of the CDI cases is properly diagnosed, because of the insensitive diagnostic tests (15.5%) or lack of clinical suspicion (35 %) [308].

Even at European level the situation is similar: during a recent (2014) European, multicenter, prospective, biannual, point-prevalence study of CDI infection in hospitalized patients (EUCLID), it was showed that only two-fifths of the participating hospital used adequate diagnostic methods and that the underdiagnosis rate of CDI cases was about 23%.

For this reason, it is suggested to analyze all samples constituted of liquid stools, independently by age of the patients (excluding kids <2 years), kind of clinical request and origin of the sample (nosocomial or community diarrhea) [308, 310].

Method	Sensibility	Specificity	Response time	Advantages	Disadvantages
Cytotoxicity assay	65-85	>97	2-3 days	Low sensibility; high specificity	Experience with cell cultures; long response time
Culture	>90	80-90	2-3 days	Good sensibility	Hard-working; long response time; also no toxigenic strains (only culture); possibility to ensure anaerobiosis condition
Culture + toxins detection (from pure colonies)	>90	>95	2-4 days	High sensibility and specificity	
GDH (EIAs) detection	60-90	85-95	Few minutes	Good sensibility; easy and rapid	Lack of specificity for toxigenic strains (need complementary test)
Toxins A and B detection	50-85	90-95	Few minutes	Moderate sensibility; easy and rapid	Only 60% cases diagnosed
Nucleic acid detection	>90	>97	1-3 hours	High sensibility and specificity; rapid	High cost; variable laboriousness

Table 9. Representation of principal features of laboratory diagnosis methods for the detection of toxigenic *C. difficile*

Nowadays there are many diagnostic methods used in the identification of infection due to toxigenic *C. difficile*, both as “classical” (cytotoxicity assay and toxigenic culture) and rapid tests (i.e. enzyme immunoassays, EIAs) for the detection of the toxins as well as molecular assays PCR-based for amplification of toxin genes. To date, a reference methods it is not present, because of the disadvantages presented by all the cited above, but in the last years toxigenic culture, followed by toxicity detection, starts to be accepted as gold standard [235].

The cytotoxicity assay was first methods used as gold standard for different years and is based on the detection of cytopathic activity of B toxin exerting its effect on the structure of the cytoskeleton and on the cells microfilaments. The test is performed by incubation of filtered fecal suspension on a mammalian cellular monolayer, which is constituted by cell lines of Vero (kidney epithelial cells of African green monkey), HeLa (human cervical cancer cells), CHO (Chinese hamster ovary cells) and MRC-5 (human fetal lung fibroblast cells). Cytotoxicity was confirmed after 24-48h observing the cytopathic effect as morphological changes and rounding of the cells, following the neutralization by an antitoxin (*C. difficile* or *C. sordellii* antitoxin) (fig 32). This assays presents a high specificity (>97%) but also limitations such as the need of expertise in maintaining the monolayer cell line and the interpretation of the cytopathic effect, which, in addition to being partially subjective, requires relatively long time (several readings at 24, 48 and 72 hours) [311]. Besides, its sensibility is not so good (65-85%) and does not give additional information about the toxigenic strain (table 9).

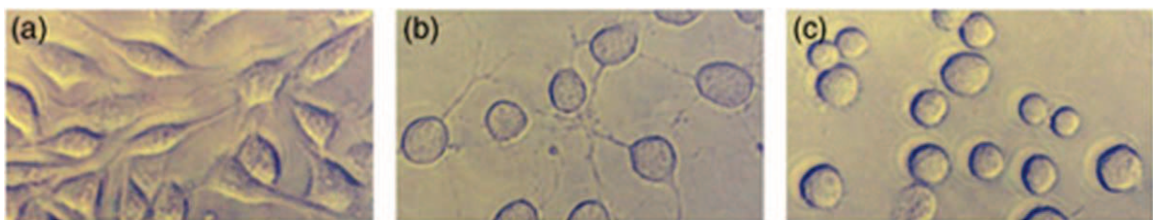


Figure 32. Cytopathic effect caused by *C. difficile* toxins. (a) Morphology of untreated cells; (b) *C. difficile* type of CPE is characterized by the remaining long protrusions and is caused by ordinary toxinotype 0 strains and most of the variant toxinotypes as well; (c) *C. sordellii* type of CPE is characterized by complete cell rounding [293]

Enzymatic Immuno Assay (EIAs) are the most used in the laboratory for the simplicity and rapidity and may combines in the same time the detection of glutamate dehydrogenase (GDH, expressed by most of the strains of *C. difficile*) together with toxin detection. EIA assays for the detection of A and B toxins present a sensibility of 50-85% but a higher specificity (90-95%), although only the 60% of cases is detected using only this test. Instead, the rapid tests for the detection of only GDH have higher sensitivity (60-90%), as this enzyme is produced by *C. difficile* strains both toxigenic and not toxigenic. This feature is the principal disadvantage of this technique, because GDH positivity cannot be used as the unique assay to validate diagnosis but must be associated with confirmatory tests for the identification of toxigenic strains (molecular assays or EIAs that combine the GDH and Toxins A/B detection) [312, 313] (table 9).

Molecular tests are the latest methods introduced in the diagnostic laboratory and present a great specificity (>97%), sensitivity (>90%) and rapidity (results between 45 minutes and 3 hours). Their main disadvantage is to be very expensive, even if the cost can be considered amply repaid by the rapid diagnosis of CDI cases and also from rapid isolation of patient and his decrease in hospital stays, which effects result in lower costs for the hospital [314].

The majority of the methods provide the possibility of analyzing the sample directly, while in other cases intervention of the staff is required for manipulating the sample at the first phases. With regard to the target genes, typically the presence of toxin B (*tcdB* gene) is detected (to not exclude the A-B + strains) and in some cases it is possible to detect also the genes encoding for the binary toxin (*cdtA/cdtB* genes), and *tcdC* deletion nt117 (tcdCΔ117) , which is a typical feature of CD027 and allow to rapidly detect this strain [315, 316].

Toxigenic cultures are based on isolation of *C. difficile* isolates in pure culture followed by detection of toxins by any of the methods above mentioned. Stool is pretreated with absolute ethanol or with a thermal shock (80°C for 15 minutes), to eliminate possible accompanying flora present in the samples, considering that *C. difficile* spores can resist to this kind of external stresses.

Subsequently, sample is cultured in selective media as CCFA (cycloserine, cefoxitin, fructose agar) and CCEY (cycloserine, cefoxitin, egg yolk agar). Nowadays these are substituted by media enriched with blood and antibiotics or no selective media as Brucella agar, Schaedler agar or chromogenic (selective and differential medium) agar. The latter allows identifying *C. difficile* colonies by color changes. At this point, *C. difficile* colonies are identified for the characteristic appearance and odor, and the detection of the toxins is performed. The main advantages of toxigenic culture are the higher sensitivity and specificity, although, as for cytotoxicity assay, the method requires a long response time and the possibility to perform culture in anaerobe conditions.

None of the techniques already described can be considered alone a technique cost-effective for the CDI diagnosis: molecular methods are rapid but have a high cost in order to be introduced in all laboratories moreover, detecting the DNA of the microorganism, carries patients or no viable bacteria; EIA technique for the detection of GDH alone or together with toxins are quick and specific but possess a limited sensitivity; finally, the cytotoxicity test and the toxigenic culture techniques are laborious and present an inconvenience relating to the time (48-72 hours for a result). For this reason, it is suggested to use diagnostic algorithms consisting of a screening assay (EIA assays for the identification of the GDH/toxins) and at least one confirmatory test: for example, tests based on the initial detection of the GDH/toxins detection may give different results: in presence of GDH+/Toxins+, normally the presence of toxigenic C. *difficile* is detected; if GDH-/Toxins- , the sample is considered negative for presence of *C. difficile*. In cases of discordant results as GDH+/Toxins- or GDH-/Toxins+, it is necessary to perform a confirmatory test (molecular assay for detection of toxins genes or cytotoxicity assay/toxigenic culture to confirm the presence of toxigenic *C. difficile* isolates). Initially, some international guidelines indicated confirmatory methods as the cytotoxicity assay and toxigenic culture, but, because of late diagnosis, they have been replaced by molecular methods. In this case, despite having high cost, molecular techniques allow to obtain a rapid diagnostic response and their cost (if used in a algorithm) is lower respect than when they are used as the unique diagnostic method [317] (fig 33).

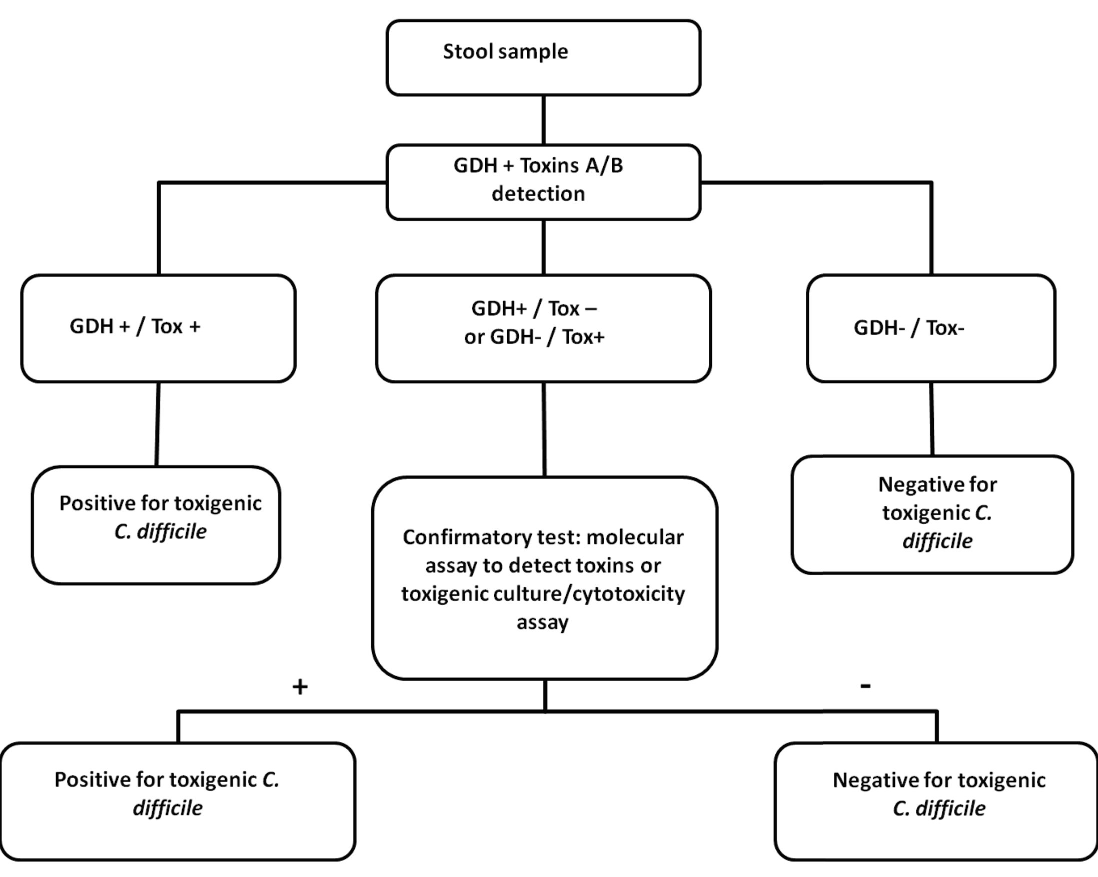


Figure 33. Schematic representation of a diagnostic algorithm used for toxigenic *C. difficile* identification

2.1.8 Molecular typing

In recent years, the molecular typing of bacteria has had great success due to its ability to identify spread of nosocomial outbreaks due to dangerous pathogens, allowing distinguishing strains involved and the changing epidemiology. Molecular assays also help to distinguish when a patient recurrence is due to the same (relapse) or different strain (reinfection).

The current limit in the molecular characterization of *C. difficile isolates* is that not all the Laboratory of Clinical Microbiology possesses tools and appropriate staff to carry out studies of molecular epidemiology. For this reason, it is very useful and important the installation of centers of reference on a regional or national scale, such as, for example, the national reference center of Leeds (UK), to which many samples are sent for typing and sub-typing by molecular assays (*Clostridium difficile* Ribotyping Network, CDRN, https://www.gov.uk/government/collections/clostridium-difficile-ribotyping-network-cdrn-service).

There are several methods that allow molecular analysis of *Clostridium difficile* strains which are changing over the time and possess different characteristics and principles, but are generally considered as valid the methods that possess enough discriminatory power, good reproducibility to allow comparation between different laboratories and typeability to discriminating the majority of the strains of a population; moreover, it would be appropriate that presented also quick and easy execution and simplicity in interpreting the results.

The main molecular methods can be classified into two groups, depending on whether they are based on analysis of bands or sequences, with specific advantages and disadvantages (table 10): band-based methods such as Restriction Endonuclease Analysis (REA), Pulsed-Field Gel Electrophoresis (PFGE), capillary or conventional PCR ribotyping and multilocus variable-number tandem repeat analysis (MLVA) or sequence-based methods such as multilocus sequence typing (MLST) and whole genome sequencing (WGS) [318].

A molecular typing method is adequate if it presents specific requirements, such as having a good power of discrimination and typeability (that is the ability to characterize unequivocally the majority of the samples analyzed); or a good reproducibility and transportability, i.e. the ability to achieve the same results in different moments and different laboratories and it should also be economical, fast and simple to apply (table 10).

Method	Target	Discriminatory power	Typeability	Transportability	Ease of interpretation	Technical simplicity	Cost/sample
			Band-based methods				
REA	Whole genome; HindIII restriction	+++	+++	+	+	+	+
PFGE	Whole genome; *SmaI* restriction	++	++	++	++	+	++
PCR-ribotyping	PCR ISR of 16S-23S	+++	+++	++	++	++	+
MLVA	Whole genome; VNTR	++++	++	+++	+++	+	+++
			Sequence-based methods				
MLST	Sequencing of 7 housekeeping genes	+++	+++	++++	+++	++	+++
WGS	Whole genome; SNPs	++++	+++	+++	+	+	++++

Table 10. Main features associated to molecular typing techniques used for *C. difficile* isolates characterization, adapted by [318]. + poor, ++ moderate, +++ good; ++++ excellent

Restriction Endonuclease Analysis (REA)

This method was first used by Devlin et al in 1987 and it is used mainly in North America, but, in recent years, it has been replaced by other methods with ease of interpretation [319].

The REA method is based on the cut in restriction sites from high frequency restriction enzymes such as *CfoI* or *HindIII* of the genomic DNA of the microorganism, and the consequent creation of banding patterns. Numerous DNA fragments of different length are produced, which are subsequently separated by agarose gel electrophoresis. The band profile obtained is examined in comparison to those of the reference strains, obtaining a REA identity.

Although possessing good discriminatory power and typeability, it has the great disadvantage of being laborious and difficult to interpret, so techniques with greater transportability are preferred to use (table 10).

Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed field gel electrophoresis (PFGE) was developed in 1984 [320] and was the most diffused molecular methods in USA, also recommended by the CDC, although in the last years it has been replaced by methods with higher discriminatory power and reproducibility (table 10).

Specifically, it s based on initial phase of digestion of *C. difficile* genomic DNA with an infrequent cutting restriction enzyme, for example *Sma*I, producing large size fragments. These are separated by agarose gel electrophoresis, characterized by alternately pulsed, perpendicularly oriented electrical fields, which enables, via the application of periodic changes in the direction of the electrical field, to separate and resolve fragments of different sizes on the gel. The pulse time of migration is linearly increased during the run so that progressively larger fragments are able to migrate forward through the gel, resulting into specific banding patterns, which are compared with those of reference strains and named with specific nomenclatures (for example NAP1 refers to North America Pulsotype 1 i.e. *C. difficile* ribotype 027)[321, 322](fig 34).

PFGE presents the advantage to be a already known methods, because many laboratories perform it for the molecular typing of other bacterial species, so with the same tools and similar protocols it is possible to characterize different microorganisms.

One of the most important limitations of PFGE applied on *C. difficile* regards the frequent degradation of its DNA, which does not allow obtaining band patterns with good resolution; furthermore, PFGE is a laborious technique which also requires a long time to yield results, a parte that the lack of a reference methods and universal nomenclatures make difficult the inter-laboratory exchange of data [321].

PFGE was the most common technique in USA for many years, but recently, more laboratories are using the "European" combination based on the application of ribotype associated MLVA, which presents a higher discriminatory power.

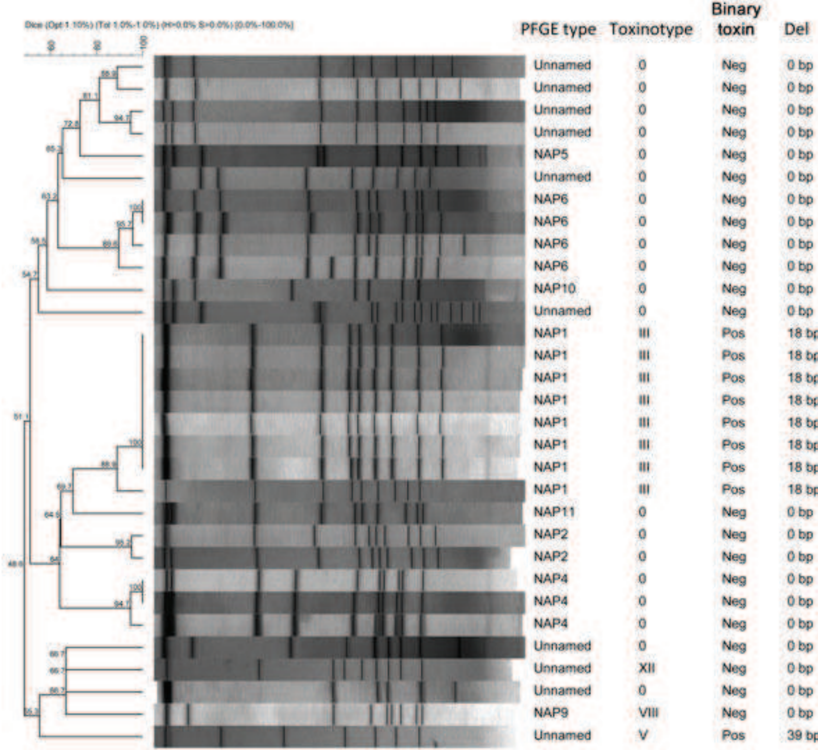


Figure 34. Representation of PFGE bands pattern of *C. difficile* isolates, which are correlated with analysis performed by toxinotype, detection of binary toxins and deletion in *tcdC* gene. Source CDC Atlanta, dispatch; Volume 17, Number 10—October 2011

PCR-ribotyping

PCR-ribotype is a typing method used principally in Europe, particularly in the United Kingdom, where it was for many years the gold standard to genotyping strains of *C. difficile.*

The method is based on the amplification by PCR of specific inter spacer regions (ISR) of conserved 16S-23S genes (coding for ribosomal RNA) and the subsequent gel-based analysis of the banding patterns obtained. These ISR (size ranging from 238 bp to 566 bp) are present in different positions, numbers and sizes within the genomes of *C. difficile* strains, for which their amplification and analysis allows differentiating within them [323].

In 1999, Stubbs et al published one of the most important studies about the discriminatory power of PCR-based ribotyping: 2,030 isolates from different sources were analyzed, resulting in identification of 116 different ribotypes, which had been used to construct a library where subsequently other samples were

compared [324]. Currently, the procedure indicated by Stubbs *et al* is the most used, although different others have been published [324, 325].

The patterns obtained must be analyzed with specific software that allows building a dendrogram of similarity, through image recognition and phylogenetic analyzes, because to date more than 500 ribotypes are known (all conserved in the Leeds library; https://www.gov.uk/government/collections/clostridium-difficile-ribotyping-network-cdrn-service), so that it is necessary to have appropriate tools for the comparison of investigated samples with the reference strains that not all laboratories possess.

Ribotyping presents a higher discriminatory power and simplicity in application, respect of PFGE and REA analyses, but has several limitations as the reproducibility between different laboratories and the reliability of the patterns obtained (table 10).

To overcoming the inter-laboratory problems to compare typing results, a variant of the PCR-based ribotype was performed: the capillary-electrophoresis ribotyping is based on the use of primers associated with fluorophores and the subsequent analysis of the amplification product by a genetic analyzer. Surely, this technique has greater discriminatory power and a more objective analysis of profiles, but is more expensive, and it is also necessary to have a sequencer available; in any case, the problem related to ribotypes nomenclature assignation continues to be evident [326, 327].

Multilocus Variable-number Tandem-Repeat Analysis (MLVA)

MVLA is a highly discriminatory molecular typing method, based on PCR amplification and fragment analysis of repeated short sequences in tandem, and mainly used to subtype strains of the same ribotype; to discriminate outbreaks and identify possible routes of transmission between patients.

It is based on variable number tandem repeat (VNTR) analysis, where VNTR are short sequence repeat (SSR) motifs dispersed in the genome. Considering that the number of repetitions in each locus varies in different strains of *Clostridium difficile*, the method consists in amplifying at least 7 loci of interest with primers labeled with fluorescent dyes and the amplified product are analyzed with a genetic analyzer (the same used to sequence DNA) to obtain the size of every locus. The number of repetitions of each locus is

calculated comparing the size of the fragment obtained with the size of the same fragment in the presence of only one repetition (considering also the number of base pairs of each repetition) [328]. To perform the analysis many softwares are available as GeneMapper® (Applied Biosystems, Carlsbad, CA, USA) or BioNumerics® (Applied Maths, Belgium); alternatively, it is possible to sequence the fragments for the different loci and count the number of repetitions, but it is a laborious and more expensive way. A code of seven numbers (seven loci) is assigned to each sample.

Once the number of repetitions on each locus is obtained, for each sample the difference of the amount of tandem repeat loci and the number of loci variants (summed tandem repeat difference or STRD) is calculated and on its basis a graphic representation through a Minimum Spanning Tree is created, which associates MLVA patterns on the basis of the smallest STRD [329].

In particular, it is considered that strains with STRD ≤2 are part of a clonal complex (are identical), while those with 2<STRD≤10 are genetically related; finally if there is a STRD>10 know samples are to be considered genetically unrelated.

One of the first scheme for MLVA application was published in 2006, by Marsh *et al*, which analyzed 86 clinical isolates of *C. difficile* (20 of which were reference toxigenic isolated) with MLVA and REA in order to confirm the susceptibility of the new technique and characterize isolates representing already the most common REA groups identified. Interestingly, direct comparison between MLVA and REA typing showed that MLVA discriminates with high specificities isolates belonging to the same epidemic cluster, highlighting the genetic correlation between them, concluding that MLVA could be a very useful tool in *C. difficile* genotyping for investigations of nosocomial infections [329].

van den Berg et al, as in previous cited study, used the MLVA technique to characterize recent outbreaks due to *C. difficile* ribotype 027 and to discriminate against severe infections due to the onset of a new strain RT017 (toxin A negative, toxin B positive). Also in this case, the conclusion of the study was that application of MLVA to *C. difficile* isolates was easy to perform and may give further evolutional information on pathogens spreading during a outbreaks situation [330].

Subsequent studies observed that two MLVA loci (F3 and H9, accordingly to van den Berg definition) were not discriminative, as they were invariable and that, regarding typing of RT078, another locus (A6, van den

Berg definition) is a null allele and for several other loci the PCR settings had to be optimized; however, the protocol of van den Berg is the most widespread and used to apply MLVA analysis [331].

In the last years, alternative schemes of MLVA methods were performed: the modified-MLVA (MMLVA) and the extended-MLVA (eMLVA). In the first case, instead of having seven loci, the number is reduced to five for the elimination of F3 and H9, by adding the PCR detection of toxins (*tcdA*, *tcdB* and deletion of *tcd*C) [332].

In the second case, the number of loci is increased to 15, with the advantage of a greater discriminatory power and allowing to grouping strains by ribotypes, but this also increases the cost and manual difficulty, leaving it as a second choice to "classical" MLVA with 7 loci [334].

The biggest disadvantage of this methods is the need of a genetic analyzer to perform analysis of data obtained, but, despite this, MLVA presents numerous advantages, such as a high transportability and ease of interpretation (table 10), moreover of being a high discriminative method (in fact allows subtyping elements belonging to the same ribotype), defining the route of transmission between patients, adding data on possible phylogenetic relationship between samples and allowing to study the evolution of them in a defined environment (like a hospital can be).

Multilocus Sequence Typing (MLST)

The MLST method was one of the first genomic methods applied mainly in typing of *Enterobacteria* and other microorganism as *Staphilococcus aureus*, having the advantage to examine long term bacterial population genetics and epidemiology.

The most recent application of MLST is the molecular typing of *C. difficile* isolates, performed by PCR-based amplification and sequencing of DNA fragments of seven housekeeping genes (conserved genes); for each sequence variants an allelic number is assigned; the combination of seven allelic numbers is called Sequence Type (ST). The definition of the ST of each sample is obtained, in a simple and objective way, by comparison with those archived in public databases available on Internet. According to the latest actualization (October 2015), 326 ST of *C. difficile* have been deposited

(http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_cdifficile_seqdef).

MLST has a power of discrimination quite similar to that of the ribotype, but with the difference that it is a technique more objective (analysis of sequences), simpler and with the advantage of allowing compare the results between different laboratories (table 10). Furthermore, many laboratories are more familiar with this technique, thanks to its application in respect of different bacterial species.

The higher limit for which probably ribotype continues to be the most used respect MLST is the high cost of sequencing multiple targets.

One important discovery obtained by MLST was the phylogeny reconstruction of *C. difficile* strains which revealed that *C. difficile* diversified into at least five well separated lineages during evolution, and possibly a sixth monophyletic lineage [335].

Whole Genome Sequencing (WGS)

During the last decade, a revolution in terms of high-throughput sequencing methodology occurred with the spread of WGS that replaced, in part, the conventional typing techniques.

WGS can detect precise genetic changes occurred in bacterial genomes also analyzing mutations at single polymorphic locus, allowing to define epidemiological lineages, chain of transmission of infection and also deduct profile MLST, overcoming also many of the limitations of conventional techniques, enabling to share the results with other laboratories, with the ability to store the entire sequence in specific databases.

Specifically, conventional typing methods have the disadvantage of not being able to identify very similar high-incidence isolates, while the WGS possess a highest discriminatory power that allows analyzing genomic mutations caused by SNPs (Single Nucleotide Polymorphisms) and clarifying the chain of transmission in space and time of each strain involved in outbreaks.

The first WGS characterization of *C. difficile* was performed in 2010, analyzing thirty isolates of different collections of US and Europe to determine both macro and microevolution of the species. This and other subsequently studies demonstrated that *C. difficile* has a highly dynamic genome, and it was possible to

discriminate isolates into 25 distinct genotypes based on SNP analysis, occupying distinct evolutionary lineages and harboring unique antibiotic resistance genes [336, 337].

Despite the high discrimination power, typeability and transportability of WGS, the main disadvantages of remain the necessity to have adequate bioinformatics tools and staff with expertise in data analysis, a part of the related cost, mainly due to the sequencing of the samples. However, the possibility to extrapolate MLST, PFGE, resistance gene, toxins gene sequences and other data from the same test could balance the cost-benefit analysis.

2.2 Shortcomings of literature and aim of the work

Clostridium difficile infection (CDI) due to CD027 strains is associated with increased transmission, higher severity of the disease and important public health problem regarding costs and management.
During the last decade, much has been discovered and published on CD027 dissemination, but despite the large number of publications, it possible to make some observations:

a) In Europe, the real incidence of *C. difficile* infection is probably underestimated because of the low susceptibility of diagnostic methods used by most of the hospitals, of the lack of correct clinical suspicion, and of the absence of national surveillance programs for infection control [307, 308]

b) Despite being described, large outbreaks spread globally, in Spain, according to data shown by the scarce published studies about molecular epidemiology of *C. difficile*, it seems that until 2014 ribotype 027 was very infrequent (3.5% in 2013), with a prevalence of ribotypes 014/020, 078/126 and 001/072. However, since 2007, in the Hospital General Universitario Gregorio Marañon, an active surveillance monitoring for CD027 is performed and thanks to which only some sporadic cases were identified [308, 338]

c) To date, in Europe, CD027 is the main ribotype detected, although it seems that its incidence though its incidence has declined in countries where so far was the main ribotype (UK and Ireland) to move to Central and Eastern Europe (Germany, Hungary, Poland and Romania) [306, 307]

d) The epidemiology of CDI is changing, but it is unclear if the increased incidence is due to a poly- or mono-clonal factor. There are relatively few studies that describe possible changes of molecular epidemiology of the spread of CD027.

e) Higher transmission of epidemic strains (i.e. CD027) is associated with increased sporulation [339]. Several studies have been interested in measuring the *in vitro* ability of sporulation of CD027 and the possible correlation with greater transmission and environmental persistence, but published data presented contradictory results [263];

The main objective of this study is the application of molecular typing methods for the characterization of strains of *Clostridium difficile* belonging to ribotype 027, following the observation of an increased frequency in infection due to this variant in the Hospital General Universitario Gregorio Marañon (Madrid, Spain) from 2014.

The second aim is to investigate, with an *in vitro* method, whether the increased transmission of CD027 isolates may be related with increased spore production and higher ability to germinate.

Molecular characterization of *C. difficile* ribotype 027 in a single Spanish hospital

2.3.1 Background

In Spain, until 2014, the incidence of *Clostridium difficile* 027 was much lower than in many European countries. During the three national studies performed between 2011 and 2013, only a few sporadic cases considered unrelated were detected [308, 338].

Since 2007, in the Hospital General Universitario Gregorio Marañon (HGUGM) a system of active surveillance has been introduced, which has allowed to identify, in the last year, a significant increase in cases of CDI due to ribotype 027.

This first part of the study has the aim to identify and characterize through the use of molecular methodology, all isolates of CD027, highlighting genetic relationships among them and the presence of one or more clones disseminated in the HGUGM.

2.3.2 Materials and methods

Study design

All *C. difficile* clinical isolates recognized as presumptive CD027 from Gene Xpert® *C. difficile* (Cepheid, Sunnyvale, CA, USA) assay or as GDH+/Toxins+ through screening with rapid tests (EIAs, C. DIFF Quik Check Complete®, Alere, Atlanta, GA, USA) have been sent to the laboratory of molecular bacteriology to perform confirmatory tests.

Multiplex PCR assays for the presence of toxin A, B, and binary genes (*tcdA*, *tcdB*, *cdtA/B*, respectively) are performed. All binary toxin-positive samples were further subjected to PCR-ribotype to confirm the presence of CD027 clinical isolates.

At this point, PCR assays are performed for the detection of the mutations in the DNA gyrase genes (in particular, *gyrA* and *gyrB*, which are molecular marker of epidemic lineages of CD027) and the analysis of the sequence of *tcdC* gene (CD027 counts with a single 1-bp deletion at nucleotidic position 117 and a deletion of 18-bp).

After this, CD027 samples are subjected to subtyping by MLVA analysis, to detect genetic relatedness among them (fig 35).

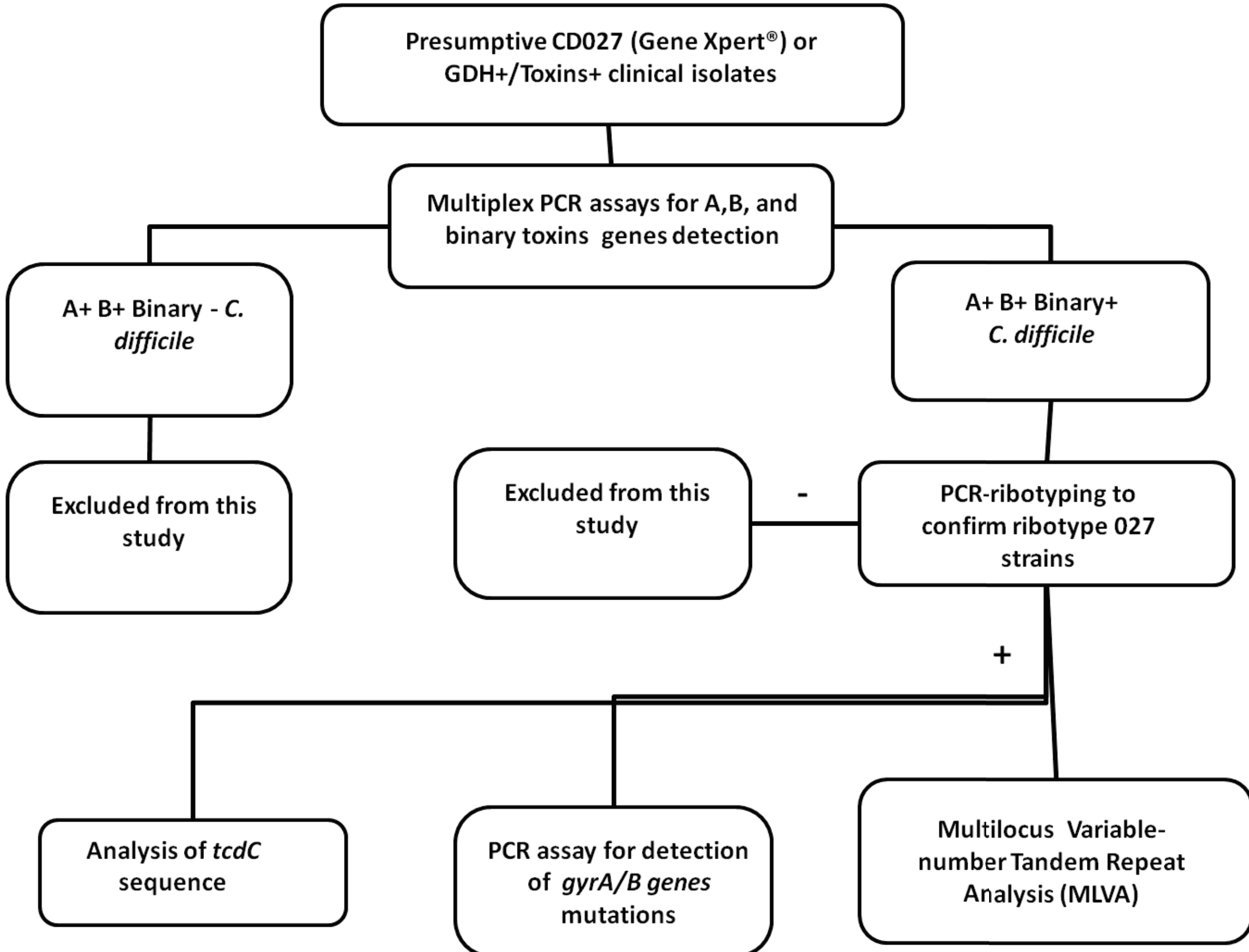


Figure 35. Schematic representation of the workflow used in this study. All presumptive-CD027 and all the *C. difficile* clinical isolates positive for A, B and binary toxins (by EIA test) are confirmed to be toxigenic strains by Multiplex PCR assay; subsequently, binary toxin-positive isolates are confirmed to belong to ribotype 027 by PCR-ribotyping. All confirmed CD027 isolates were further analyzed by sequencing of *tcdC* gene, PCR assay for the detection of mutations in *gyrA* and *gyrB* genes and MLVA, aimed to characterize genetic relatedness among them.

Bacterial isolates

In this study, we included all CD027 isolated from unformed stools sent to the Microbiology Laboratory of HGUGM in a period of 20 months (January 2014-August 2015), independently from the clinical suspicion.

To isolate *C. difficile*, stool samples were cultured on an agar plate CLO (BioMérieux, Marcy l'Etoile, France) at 35-37°C for 48h under anaerobic conditions. Colonies growth compatible with *C. difficile* were subcultured in Brucella agar (Becton-Dickinson) in the same condition described above. The pure cultures obtained were tested for detection of toxins A/B and GDH (C.DIFF Quik Chek; Alere, Atlanta, GA, USA).

All the GDH+/Toxins+ clinical isolates were sent to the laboratory for performing molecular typing.

When an increased incidence of CD027 was noted and the beginning of a outbreak suspected, samples were immediately processed by the Gene Xpert® and all presumptive CD027 sent to the laboratory for molecular typing.

Molecular assays

a) DNA extraction

Chromosomal bacterial DNA was extracted from pure cultures of toxigenic *C. difficile* in Brucella agar using Chelex resin according to the manufacturer's instructions (InstaGene Matrix, BioRad Laboratories): in detail, 1-2 pure colonies of *C. difficile* were resuspended in 200µL of Chelex resin. The suspension is mixed on a vortex and then incubated at 56°C for 30 minutes in a thermoblock to obtain the lysis of the bacteria. Subsequently, the samples have been incubated at 100°C for 8 minutes in a water batch and later centrifuged at 14000 rpm for 3 minutes, obtaining the separation of cellular fragments (pellet) from nucleic acid (supernatant), of which 50µL are taken to perform molecular studies.

b) Multiplex-PCR for toxins detection

Multiplex-PCR assays for the detection of the A, B and binary toxins genes (*tcdA, tcdB,* and *cdtA/cdtB*, respectively) were performed as previously described with modifications, using the primers showed in (table 11) [340, 341].

For each PCR reaction a master mix with a final volume of 50µL has been prepared, containing 25µL of Multiplex Master Mix (PCR kit, Qiagen, GmbH, Germany), 20µL of a suspension of oligonucleotides (table 12), and 5µL of the DNA of the investigated sample. To each PCR reaction, were added a negative control (5µL of nuclease-free H_2O) and a positive control (*C. difficile* ribotype 027, A^+ B^+ $Binary^+$).

The amplification reaction was performed in a GeneAmp PCR 9700 thermocycler (Life Technologies, California, USA) and had the sequent thermal protocol: 1x(94°C 10') + 35x(94°C 50''+ 54°C 40''+ 72°C 50'') + 1x(72°C 3') + (4°C ∞).

Primers	Target	Sequence	Concentration	Size (bp)	Reference
tcdA- F	*tcdA* (deletion at 3')	5' AGA TTC CTA TAT TTA CAT GAC AAT AT 3'	5 µM	365bp (complete) 110 (deleted)	[341]
tcdA-R		5' GTA TCA GGC ATA AAG TAA TAT ACT TT 3'			
tcdA-F3345	*tcdA* (conserved gene)	5' GCA TGA TAA GGC AAC TTC AGT GGT A 3'	10 µM	629bp	[342]
tcdA-R3969		5' AGT TCC TCC TGC TCC ATC AAA TG 3'			
tcdB-F5670	*tcdB*	5' CCA AAR TGG AGT GTT ACA AAC AGG TG 3'	5 µM	410bp	
tcdB-R6079A		5' GCA TTT CTC CAT TCT CAG CAA AGT A 3'			
tcdB-R6079B		5' GCA TTT CTC CGT TTT CAG CAA AGT A 3'			
cdtA-F739A	*cdtA*	5' GGG AAG CAC TAT ATT AAA GCA GAA GC 3'	5 µM	262bp	
cdtA-F739B		5' GGG AAA CAT TAT ATT AAA GCA GAA GC 3'			
cdtB- F617	*cdtB*	5' TTG ACC CAA AGT TGA TGT CTG ATT G 3'	5 µM	221bp	
cdtB-R878		5' CGG ATC TCT TGC TTC AGT CTT TAT AG 3'			
PS13	16S rDNA Control	5' GGA GGC AGC AGT GGG GAA TA 3'	5 µM	1062bp	
PS14		5' TGA CGG GCG GTG TGT ACA AG 3'			

Table 11. Primers used in this study to perform Multiplex-PCR for toxins detection of the *C. difficile* clinical isolates

The amplification products were separated by agarose gel electrophoresis. Gel has been prepared with standard agarose (Standard Low-mr Agarose, Bio-Rad Laboratories, USA) at a concentration of 2% in TBE

buffer 1X, using the RealSafe Nucleic Acid Staining (Real Life Science-Solution, Durviz, Valencia, Spain) with a final concentration of 10µL/mL.

Prior to gel loading, tubes containing 8uL of the amplified product and 2µL of the loading dye (Blue/Orange 6X Loading Dye, Promega, Madison, USA) were prepared and a total of 10µL of the final mixture were loaded in the gel, along with negative and positive controls and with the marker of known molecular weight (in this case with bands spaced every 100bp; DNA Ladder 100bp, Invitrogen, CA, USA), which serves to compare the expected size of the bands of the sample with those of reference. Electrophoresis was conducted at 50V during 40 minutes, at the end of which the gel was observed with a UV transilluminator.

c) PCR-ribotyping

The toxigenic *Clostridium difficile* A+B+Binary+ strains and the presumptive CD027 isolates were further subjected to ribotyping to confirm them as CD027 strains.

The protocol performed as previously published by Stubbs *et al* [324] (table 12), was based on the use of primers complementary to the 3'-end of the gene coding for the 16S rRNA and the 5'-end of that of the 23S, allowing to amplify the Internal Transcribed Spacer Regions (ISR) between these genes.

Target	Primers Sequence	Concentration
Site 1445-1466 of 16S	5'CTG GGG TGA AGT CGT AAC AAG G 3'	0.2 µM
Site 20-1 of 23S	5' GCG CCC TTT GTA GCT TGA CC 3'	0.2 µM

Table 12. Primers used in *C. difficile* PCR-ribotyping method [324]

The PCR Master Mix with a final volume of 25uL, consisted of: 3.5 µL of each primer, 12.5 µL of Multiplex Master Mix (PCR kit, Qiagen, GmbH, Germany), 0.5 µL of nuclease-free H_2O (to reach the required volume), and 5 µL of the DNA of the investigated sample. To each PCR reaction, were added a negative control (5µL of nuclease-free H_2O) and positive controls (*C. difficile* ribotype 027 and ribotype 001).

The amplification reaction was performed in a GeneAmp PCR 9700 thermocycler (Life Technologies, California, USA) and following the sequent thermal protocol: 1x(94°C 10') + 35x(94°C 1'+ 55°C 1'+ 72°C 2') + 1x(72°C 10') + (4°C ∞).

At the end of the PCR reaction, to each sample 3µL of Loading Dye (Blue/Orange 6X Loading Dye, Promega, Madison, USA) were added and of the resulting mixture, 12µL are cast into the agarose gel. This is composed of a specific agar, the MS-8 (Agarose MS-8, Laboratorios Conda, Torrejon de Ardoz, Madrid, Spain), of which chemical composition was made to ensure an efficient separation of small DNA fragments and PCR products.

The gels are prepared at 3% of agarose MS-8 in TBE 1X buffer, using as staining the ethidium bromide at a concentration of 5µL/100mL. For each gel, a genetic marker is added (DNA Ladder 100bp, Invitrogen, CA, USA), which is subsequently used for the normalization of the profiles obtained. The electrophoretic run takes place at 150V during 4h30m; when the operation is finished, the gel is visualized by a UV transilluminator, with which it is possible to take a digital photo, used for the sequent analysis of banding patterns obtained with the specific software BioNumerics v5.0 (Applied Maths, Belgium).

Profile analysis is based on the comparison between the tested samples and the reference strains, specifically CD027 isolates belonging to the library of the European reference center (Leiden University Medical Centre, Leiden, Netherlands).

The interpretation of the results involves the use of a similarity dendrogram using UPGMA (Unweighted Pair Group Methods using Arithmetic averages) criterion for band patterns comparison.

d) Sequencing of *tcdC* gene

Clostridium difficile ribotype 027 has alteration on *tcdC* gene, consisting of a single 1-bp deletion at nucleotidic position 117 and a deletion of 18-bp.

All the confirmed CD027 isolates were subjected also to the analysis of the *tcdC* gene sequence by products amplification via PCR reaction, using primers flanking *tcdC* (showed in table 13) and sequencing, as described by Spigaglia and Mastrantonio [343].

Primers	Primers Sequence	Concentration
***tcdC* C1**	5`TAA ATT AAT TTT CTC TAC AGC TAT CC 3’	5 µM
***tcdC* C2**	5’ TCT AAT AAA AGG GAG ATT GTA TTA TG 3’	5 µM

Table 13. Primers used in *tcdC* analysis, source [343]

The PCR reaction was performed preparing the master mix with a final volume of 50µL for reaction, consisting of: 5µL of each primer, 0.4µL of dNTPs [0.8mM], 5µL of PCR Buffer 10X without $MgCl_2$ (Roche, Basel, Switzerland), 0.5µL of $MgCl_2$ [25mM] (Roche, Basel, Switzerland), 1.5µL of Taq DNA polymerase [5 U/µL] (Roche, Basel, Switzerland), 5µL of investigated sample DNA and 23.1µL of nuclease-free H_2O (to reach the required volume). To each PCR reaction, were added a negative control (5µL of nuclease-free H_2O) and a positive control (*C. difficile* ribotype 027).

The amplification reaction was performed in a GeneAmp PCR 9700 thermocycler (Life Technologies, California, USA), following the sequent thermal protocol: 1x (94°C 5’) + 30x(94°C 1’+ 50°C 1’+ 72°C 1’) + 1x(72°C 10’) + (4°C ∞).

Electrophoresis in agarose gel (2% standard agar in TBE 1X, as described above) have been performed to verify the presence of the amplified products, were subsequently purified before sequencing reaction with a purification kit (High Pure PCR Product Purification Kit, Roche Diagnostics, GmbH). Sequencing was performed using the genetic analyzer Abi-Prism 3100 (Applied Biosystems, Carlsbad, CA, USA) located in the central unit of sequencing of the HGUGM.

Sequences obtained have been aligned with BioEdit software, version 7.0.0, and compared to the sequence of wild-type reference strain *C. difficile* 001 ATCC® 9689™.

e) MultiLocus Variable-number Tandem Repeat Analysis

All strains confirmed to belong to ribotype 027 (ribotyping and presence of the specific deletions at *tcdC* gene), were further subtyped by MLVA.

Protocol used was that previously described by Van Den Berg *et al* [330], in which primers had been designed to targeting seven regions with short tandem repeats spread over the genome designated A6, B7, C6, E7, F3, G8 and H9 (table 14).

The forward primers were labeled with different fluorophores (FAM or 6-carboxyfluorescein; NED or 2'-chloro-5'-fluoro-7',8'-fusedphenyl-1,4-dichloro-6-carboxylfluorescein; VIC or 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxylfluorescein; and HEX or 6-hexachlorofluorescein), as shown in table 15, with the purpose of amplifying the 7 loci by 4 different multiplex PCR reactions which can be separed by multicolor capillary electrophoresis and identified by the genetic analyzer, in order to obtain the size of each fragment and allow its subsequent analysis by specific software.

Locus	Tandem repeat sequence	Primer forward (5' -> 3')	Primer reverse (5' -> 3')
A6	AAGAGC	FAM-TTAATTGAGGGAGAATGTTAAA	AAATACTTTTCCCACTTTCATAA
B7	ATCTTCT	FAM-TAATACTAAACTAACTCTCTAACCAGTAA	TTATATTTTATGGGCATGTTAAA
C6	TATTGC	HEX- GTTTAGAATCTACAGCATTATTTGA	ATTGGAATTGAATGTAACAAAA
E7	ATAGATT	FAM- TGGAGCTATGGAAATTGATAA	CAAATACATCTTGCATTAATTCTT
F3	TTA	HEX-TTTTTGAAACTGAACCAACATA	ACAAAAGACTGTGCAAATATACTAA
G8	TAAAAGAG	NED- TGTATGAAGCAAGCTTTTTATT	AATCCAGCAATCTAATAATCCA
H9	TCTTCTTCC	VIC- GTTTTGAGGAAACAAACCTATC	GATGAGGAAATAGAAGAGTTCAA

Table 14. Primers used to perform PCR reaction for MLVA assay; source [330]

In detail, the PCR reaction has been set up, preparing a master mixes with a final volume of 50µL, containing a quantity of primers with final concentration of [0,4µM], 25µL of Multiplex Master Mix (PCR kit, Qiagen, GmbH, Germany), 5µL of DNA sample and an amount of nuclease-free H_2O to reach the final volume. A total of one single PCR for G8 locus and three PCR duplexes for others loci (A6-H9; B7-F3; C6-E7) have been performed.

The thermal protocol provides the sequent amplification cycles: 1x (95°C 15') + 35x(94°C 30''+ 51°C 30''+ 72°C 30'') + 1x(72°C 10') + (4°C ∞) and has been performed in GeneAmp PCR System 9700 thermocycler (Life Technologies, Carlsbad, CA, USA).

The amplification products were separated by agarose gel electrophoresis at 150V for 20 minutes. Gel and the samples to be loaded have been prepared as described above.

Once verified the presence of the amplification products, the fragments were analyzed by multicolor capillary electrophoresis with genetic analyzer Abi-Prism 3100 (Applied Biosystems, Carlsbad, CA, USA) and the results were processed by the GeneMapper software v4.0 (Applied Biosystems, Carlsbad, CA, USA) which assigns the fragment sizes and repeats number of each locus, comparing the size of the fragment obtained with the size of the same fragment in the presence of only one repetition (it is necessary to create a library of references for each of the 7 loci to compare the samples).

Phylogenetic relatedness among the isolates has been defined using the summed tandem repeat difference (STRD) or the difference of the amount of repetitions that each strain has in all of its loci. Briefly, if the STRD between two samples is ≤2 means that they belong to the same clonal complex, however, if 2<STRD≤10 samples are genetically related even though presenting a little "genetic distance". Finally, if STRD>10 isolates do not possess genetic correlation even among representative of the same ribotype.

For graphical representation of the correlation between different MLVA-types a Minimum Spanning Tree (MST) has been created from the analysis of the STRD of the investigated strains.

f) PCR assay for detection of mutations in DNA gyrase genes

In addition, PCR assays and direct sequencing were performed to detect point mutations in *gyrA* and *gyrB* genes, as already described by [344]. In particular, mutations in *gyrA* gene are used as genetic marker to identify and follow the dissemination of two more virulent lineages of CD027, evolved spontaneously from a common ancestor during the years of the large epidemics occurred in Canada and USA in early 2000s [336].

Primers used, amplifying part of the quinolone resistance-determining region (QRDR) are shown in table 15:

Primer	Sequence	Concentration	Size (pb)
gyrA-F	5'-AATGAGTGTTATAGCTGGACG-3'	5μM	390 bp
gyrA-R	5'-TCTTTTAACGACTCATCAAAGTT-3'		
gyrB-F	5'-AGTTGATGAACTGGGGTCTT-3'	5μM	390 bp
gyrB-R	5'-TCAAAATCTTCTCCAATACCA-3'		

Table 15. Primers used in PCR assay to detect mutation in gyrase A and gyrase B genes; source [344]

The PCR reaction was prepared starting from a solution with final volume of 50μL containing 5μL of each primers, 0,4μL of dNTPs solution at a concentration of [0,4μM], 5μL of PCR Buffer 10X without $MgCl_2$ (Roche, Basel, Switzerland), 4μL of $MgCl_2$ concentrated at [25mM] (Roche, Basel, Switzerland), 1μL of Taq DNA polymerase with a concentration of [5 U/μL] (Roche, Basel, Switzerland), 5μL of investigated sample DNA and 24,6μL of nuclease-free H_2O (to reach the required volume). To each PCR reaction, a negative control (5μL of nuclease-free H_2O) and a positive control (*C. difficile* ribotype 027) have been added. The reaction has been performed in GeneAmp PCR System 9700 thermocycler (Life Technologies, Carlsbad, CA, USA) with the following thermal protocol: 1x (95°C 5') + 35x(94°C 1'+ 53°C 1'+ 72°C 1') + 1x(72°C 10') + (4°C∞).

Amplicons have been separated with gel-based electrophoresis, purified and sequenced.

The sequences obtained have been aligned with BioEdit software (http://www.mbio.ncsu.edu/bioedit/page2.html), and compared to the sequence of the drug susceptible strains *Peptoclostrium difficile* 630 (NCBI Reference Sequence: NC_009089.1) and to those of the strain *C. difficile* ATCC® 9689™ (CD001) used as wild-type sequences.

2.3.3 Results

From January 2007 to August 2014, 6513 clinical isolates of *Clostridium difficile* have been analyzed by molecular assays at the Hospital General Universitario Gregorio Marañon (Madrid, Spain).

Of these, 977 (15%) of the isolates has been identified as $A^{+}B^{+}$Binary^{+} where *C. difficile* ribotype 027 constituted the 19.96% (195/977) of the binary toxin-positive isolates and the 2.9% of all the isolates analyzed.

Considering that up to 2014, only 9 unrelated and sporadic cases have been isolated, the remaining 186 clinical isolates have been detected between January-2014 and August 2014. The 9 sporadic cases were unrelated and corresponding to 5 patients, constituting only the 0.25% of all the toxigenic isolates analyzed.

The 186 CD027 clinical isolates belonged to 132 patients hospitalized in different wards of the same hospital, with a consequent total of 132/186 first episodes and the remaining 54/186 isolates representing recurrences of CDI due to *C. difficile* ribotype 027 (fig 36).

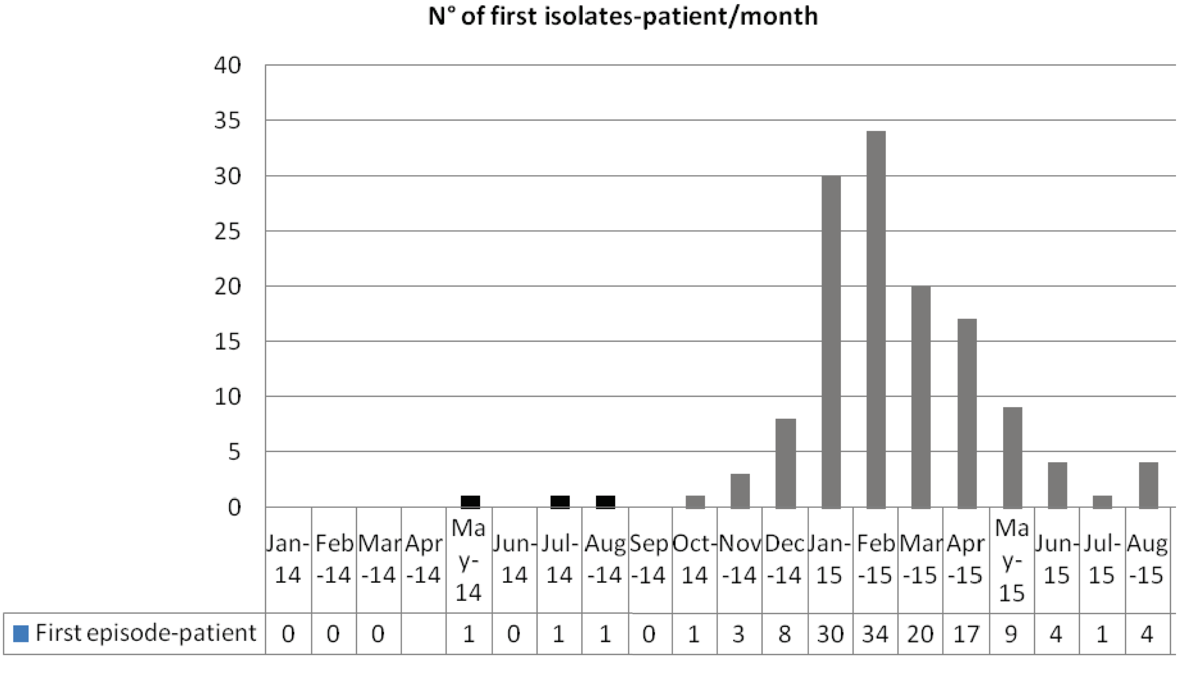


Figure 36. Representation of increased rate of CD027 episodes during the study period (January 2014-October 2015). Only the first episode for each single patient has been considered. In black, CDI cases considered previous to the outbreak. In gray, the CDI cases belonging to the outbreak.

Molecular analysis by means of MLVA has been performed on the first episodes for the single patient (i.e. 132 patients-isolates), with the purpose of showing the phylogenetic relationships among them and so has

allowed to identify 9 different sub-types (defined MLVA-types), which were organized in different clonal complexes (CC).

In particular, as showed in figure 37, the Minimum Spanning Tree (MST) resulting from the MLVA analysis highlighted the presence of 5 different clonal complexes (shaded colored areas) and 4 unique MLVA patterns. In particular, 84% of the isolates (111/132) belong to the same expanded CC (red shaded area) sharing the same MLVA pattern, which was considered the responsible of a large outbreak spread in the hospital from October 2014.

The remaining strains belong to other clonal complexes, specifically: 2/132 isolates belong to MLVA patterns called type 2 and other 2/132 to the type 7; 6/132 strains fit into the MLVA-type 8; other 5/132 strains were of the MLVA-type 3. As already mentioned, the other MLVA-types were present as unique patterns (fig 37).

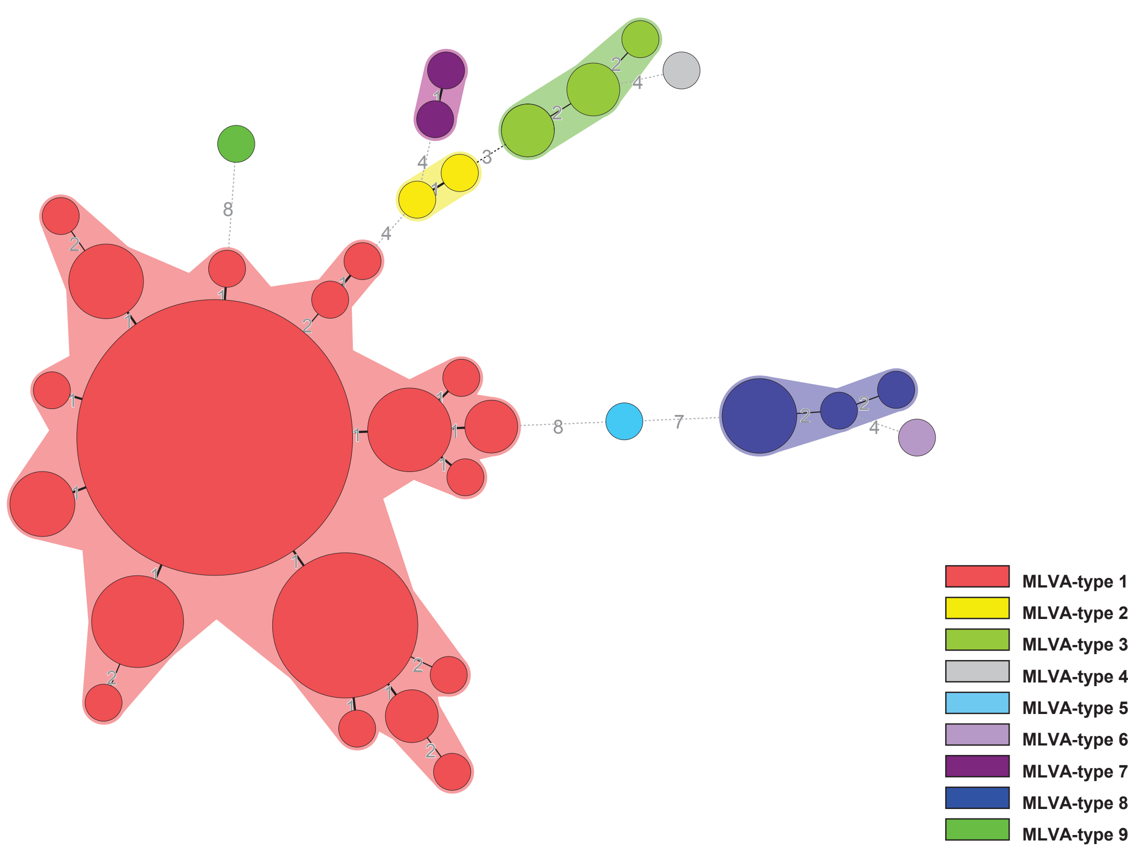


Figure 37. Minimum Spanning Tree (MST) representing spreading of 132 isolates grouped for MLVA-type. Each circle represents either a unique isolate or more isolates that have identical MLVA pattern. Shaded areas represent clonal complexes. The numbers between circles represent the STRD between strains

Lines between circles indicate the distance (in STRDs) between the clonal complexes and it is known that, by definition, two samples belong to the same clonal complex if STRD≤2; samples are genetically related if 2<STRD≤10 and isolates do not possess genetic correlation if STRD>10 even among representative of the same ribotype. All the CD027 isolates analyzed in this work, as showed in fig 38, were genetically correlated (shaded area) presenting all STRD≤10.

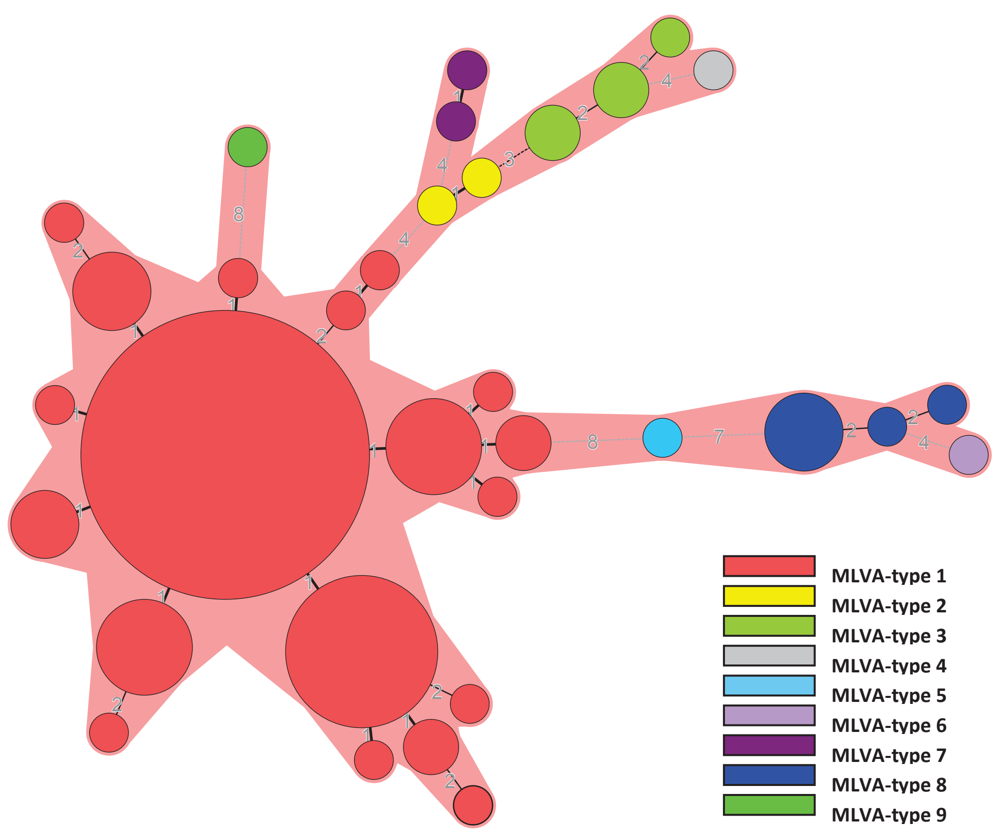


Figure 38. Minimum Spanning Tree (MST) representing all the CD027 isolates grouped using a STRD≤10. Each circle represents either a unique isolate or more isolates that have identical MLVA pattern (same color). Shaded area represent isolates with STRD≤10, which are considered as genetically related

With regard to the search for possible mutations in the genes coding for the DNA gyrase A and B in the CD027 isolates subject of the study, all samples, regardless of the pattern of MLVA, showed the same mutation on the *gyrA* gene (figures 39), but none has been detected in the *gyrB* gen .

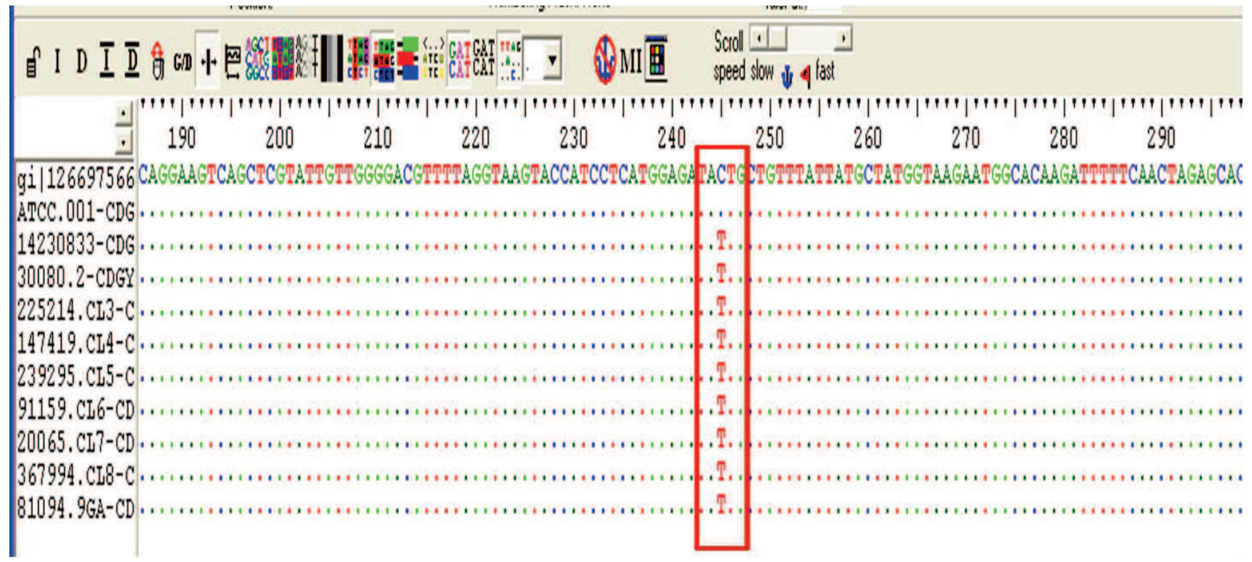


Figure 39. Alignment of the *gyrA* sequences of the nine CD027 MLVA-types with those of wild-type reference strains: *Peptoclostridium* 630 (ribotype 012) and ATCC 9689 (ribotype 001). It is highlighted the presence of the nucleotidic substitution C245T on the *gyrA* gene of CD027 strains

This *gyrA* deletion consists in the nucleotidic C245T switch, corresponding to the aminoacidic substitution Thr82Ile (figure 40), defined as marker for high transmitted lineages of CD027.

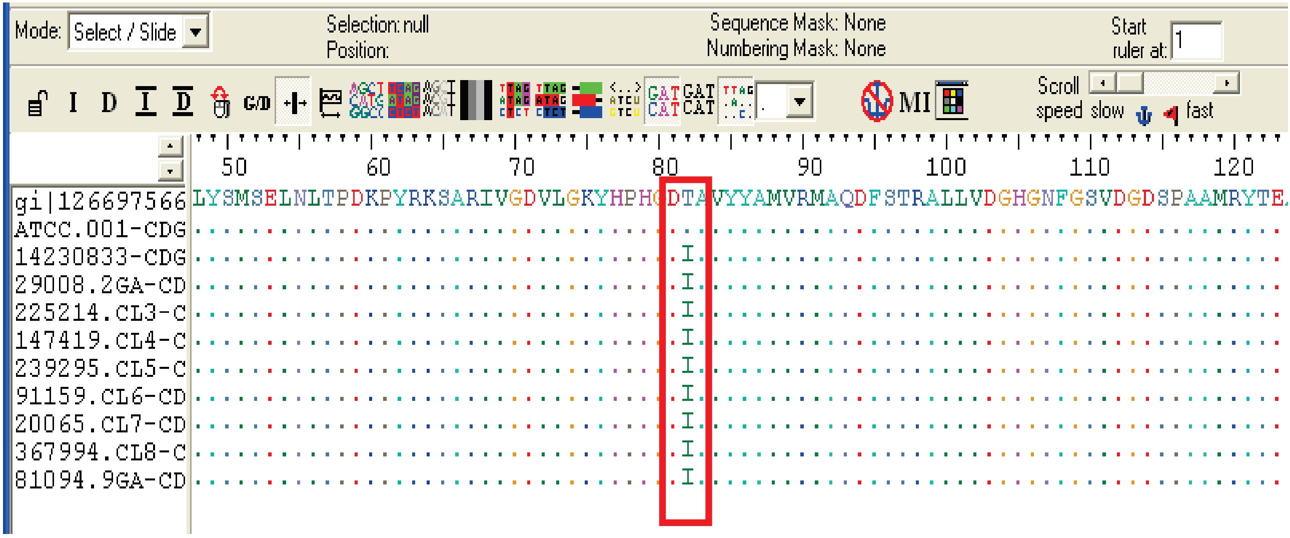


Figure 40. Alignment of the *gyrA* aminoacidic sequences of the nine CD027 MLVA-types with those of wild-types Peptoclostridium 630 (ribotype 012) and the reference strain ATCC 9689 (ribotype 001). It is highlighted the presence of the aminoacidic substitution Thr82Ile on the *gyrA* gene of the CD027 strains

2.3.4 Discussion

The global spread of *C. difficile* strains characterized by higher transmissibility and virulence is known globally [305]. In Europe, several outbreaks have occurred due to CD027 strains, whose frequency is increasing and a higher incidence is now detected in countries (such as Germany and Eastern Europe) where before only sporadic cases had been described [307].

In Spain, according to the few national epidemiological studies carried out until 2014, only a few sporadic and unrelated cases had been described [308, 338].

The HGUGM hospital, since 2007, makes an active search for CD027 isolates, thanks to which, by the beginning of the year 2014, has been highlighted the increased frequency of CDI caused by this pathogen.

Recently, a shift in the dissemination of this pathogen from UK and Ireland to the Central and Eastern Europe (where only sporadic cases were detected) has been described; similarly this may happen in Spain, showing a continue changing epidemiology respect the diffusion of this pathogen.

This study showed that the current frequency of binary toxin-positive *C. difficile* actually is around 15% over the number of total toxigenic *C. difficile* strains analyzed, but especially it must to be noted the increase in the incidence of CD027 strains, which in a year period have reach the 2.9% of the toxigenic isolates, considering the few cases reported in previous years (9 clinical isolates belonging to 5 patients) which constituted the 0.25% of the total number of toxigenic *C. difficile*.

The analysis of the 132 CD027 isolates, has allowed us to confirm the presence of a different subtypes spreading in the hospital, comprising 5 different clonal complexes. Among these, the presence of a majority complex (111/132 isolates) explains the presence of a monoclonal outbreak that has expanded rapidly in the hospital from October 2014. The simultaneous presence of these highly related clones (STRD≤10) justifies the increased frequency of CDI cases due to CD027, but their transmission ability is different and this is demonstrated by the fact that, apart from the most representative, the majority of clonal complexes have a contained expansion and were composed by a number between 2 and 6 strains, while others MLVA-types are present as isolated cases.

The results of this study suggest that the transmissibility of the strains may depend on factors such as the capacity to contaminating the environment. The hypothesis is that the greater ability of several strains to sporulate, cause a higher environmental contamination and consequently the ability to be transmitted from one patient to another, especially during an epidemic.

Besides, given their genetic relatedness, it is possible to assert a probable common origin from the same ancestor, unlike the strains isolated in previous years that seem to have no correlation.

The analysis of mutations in the genes encoding the DNA gyrase subunities (*gyrA* and *gyrB* genes) has a dual purpose: to give additional information about the presence of resistance towards fluoroquinolones (used in the treatment of different diseases and related to the selection of resistant *C. difficile* strains) [345], but especially to highlight the presence of high transmittable CD027 lineages. In fact, studies concerning phylogenomic evolution have shown that two CD027 lineages evolved from a common ancestor (sensitive to fluoroquinolones), acquiring spontaneously the same mutation on *gyrA* gene, and then spreading out with different intensity [346]. So, this mutation may be used as genetic marker for the study of transmission route of epidemic CD027 isolates and to discriminate more virulent clones from others. In our case, it was not possible to identify differences between strains, because all samples tested presented the same mutation, i.e. an already described substitution in *gyrA*, consists in the nucleotidic C245T switch, corresponding to the aminoacidic switch Thr82Ile, whereas presented a wild-type *gyrB* gene. Therefore, it was not possible to associate this genetic trait to a greater transmissibility of the clones CD027.

In conclusion, molecular typing (i.e. MLVA typing) provides important information about phylogenetic relationships among strains and help distinguishing the transmission course of different clones.

In particular, considering the large and rapid dissemination of CD027 isolates in our hospital, it is convenient to assume that epidemiology may change not only for the presence of different ribotypes, but also for the entrance of different clones belonging to the same ribotype from different sources, having different features and transmission ability.

This work shows that the active monitoring combined with molecular typing of toxigenic was crucial to recognize and control the spread of strains with higher virulence and transmissibility, so that it is

recommended the introduction of national surveillance systems, molecular epidemiology issues or reference centers to monitor the variation of the dissemination of the circulating strains.

These results are a first step towards more deep studies on the characterization of *C. difficile*, through technologies such as whole genome sequencing (WGS), targeted to distinguish the temporal evolution of the strains, their kinetic of transmission and the possible periods of CD027 entrance in Spain.

May sporulation have a role in *C. difficile* ribotype 027 virulence?

2.4.1 Background

Clostridium difficile is the leading cause of nosocomial diarrhea, mainly associated with the use of antibiotic, and increasing cause of community-acquired diarrhea.

Infection due to *Clostridium difficile* (CDI) has great clinical importance in several respects because its incidence and relapses rates are increasing with the dissemination of epidemic strains (i.e. CD027), and patients are forced to prolonged hospital stays. This may be translated in a high sanitary cost and a decline in the quality of life of the patient [230].

On the basis of higher transmissibility of several strains there is possibly the greater capacity to produce spores, contaminating the hospital environment. In fact, symptomatic patients release large amounts of spores in the environment which can then be transferred on different surfaces where they resist for a large time and may be transferred to other surfaces also by medical staff and so be ingested from patients [299].

Several studies have suggested that, apart from improved production of toxins, also an increased ability to sporulate is the basis of the intensified virulence of epidemic strains (i.e. CD027) [259], but results are often contradictory [262, 347], placing a limit on defining CD027 transmissibility capacity.

Moreover, to date, despite the multiplicity of molecular and *in vitro* techniques, the mechanisms by which *C. difficile* implements processes for the sporulation and germination have not yet been elucidated [265, 348].

In the first part of the work, the phylogenetic relationships between CD027 clinical isolates widespread in Hospital General Universitario Gregorio Marañon (Madrid, Spain) have been evaluated, emphasizing the ability of transmission of a particular clone responsible of an outbreak. The main objective of this part of the work was to determine, with an *in vitro* method, the spore production and germination rate of epidemic CD027 clinical isolates compared with endemic CD001 strains.

2.4.2 Materials and methods

Bacterial isolates

In this study, we selected 25 *C. difficile* ribotype 027 isolates and 25 *C. difficile* ribotype 001 (prevalent ribotype in the hospital) has been investigated. Among the 25 isolates of CD027, there is at least one representative of each MLVA-type. Bacterial isolation and molecular typing have been performed as described in the methods section of the first aim of teh work (section 2.3.2).

Sporulation and germination assay

In vitro assays had been optimized starting from existing protocols, with modifications [349]. Sporulation rate, expressed as the proportion of the cell population in spore form, was measured starting from pure *C. difficile* cultures isolated in Brucella agar (BD, Heilderberg, Germany)and incubated for 5 days (120hours) at 37°C under anaerobic conditions and then for 24 hours at 4ºC, to stimulate maximum spore production [350].

Three 10-fold serial dilutions of each sample were prepared, starting from a 0.5 McFarland suspension and cells count (spore and vegetative cells) was performed by optical microscopy using a counting chamber (Neubauer chamber, Celeromics, Valencia, Spain). When observed by optical microscopy, the spores appear as highly reflective spherical bodies if present in free form or wrapped in the end of the cell during its sporulation phase (sporangium). Vegetative cells, however, appear with an elongated shape and show less refractive capacity.

The sporulation rate was calculated as: number of spores per mL/number of total cells per mL (x100). Total cells number is considered as the count of both spore and vegetative cells.

The germination has been defined as the efficiency of the spore to complete their maturation and outgrowth as vegetative cells and it was determined by heating 1mL of each 10-fold dilution of *C. difficile* isolates (used for sporulation assay) at 70ºC for 10 min to kill all vegetative cells. An aliquot of 100µL from each dilution and from the original untreated suspension was inoculated in Brucella Agar (BD) and

incubated in anaerobic conditions at 35-37°C for 48 hours. After incubation, germinated spores were enumerated as the CFUs growth. Spores count was performed by optical microscopy using a counting chamber (Neubauer chamber, Celeromics, Valencia, Spain) and the germination rate was calculated as number of CFU per mL/ spores per mL (x100).

Data analysis

All analyses were carried out using GraphPad Prism 5.0 software (GraphPad Software Inc, La Jolla,CA, USA). Quantitative variables were expressed with the median and interquartile ranges (IQRs). Comparisons were performed by using non-parametric Mann-Whitney tests. A (two-tailed) p value <0.05 was considered significant.

2.4.3 Results

Sporulation and germination rates of epidemic isolates of *C. difficile* ribotype 027 were compared to those of isolates belonging to ribotype 001, using an *in vitro* method.

When isolates were grouped by ribotype, difference in sporulation rate was significant (p=0.005): median value of sporulation in CD027 isolates was of 81.92% (IQRs 76.27-83.67) while those of CD001 strains was 76.63% (IQRs 69.31-80.31) (fig 41).

The statistical analysis showed non-significant strain-to-strain variability in isolates of the same ribotype: comparison between sporulation rate of CD027 isolates of MLVA-type 1 (median sporulation rate of 82.70%, IQRs 74.82-83.67) with those of the others MLVA-types (median sporulation rate of 81.50%, IQRs 78.67-95.87) does not present statistical significance (p value=0.72) (fig 42).

Besides, we found that CD027 isolates showed statistically significant (p=0.0008) lower germination rate (median value of 0.0091%, with IQRs 0.0011 to 0.0486) compared to the ribotype 001 (median value of 0.0384% with IQRs 0.0195-0.1679) (fig 43).

When the capacity of germination among CD027 isolates belonging to the different MLVA-types was compared, any significant difference was not found (p value=0.24), similarly to what observed for the sporulation rate. In detail, germination rate of CD027 MLVA-type 1 isolates was of 0.0083% (IQRs 0.0063-0.0359) and that of the others representative MLVA-types has a median germination rate of 0.00899% (IQRs 0.00172-0.0348) (fig 44).

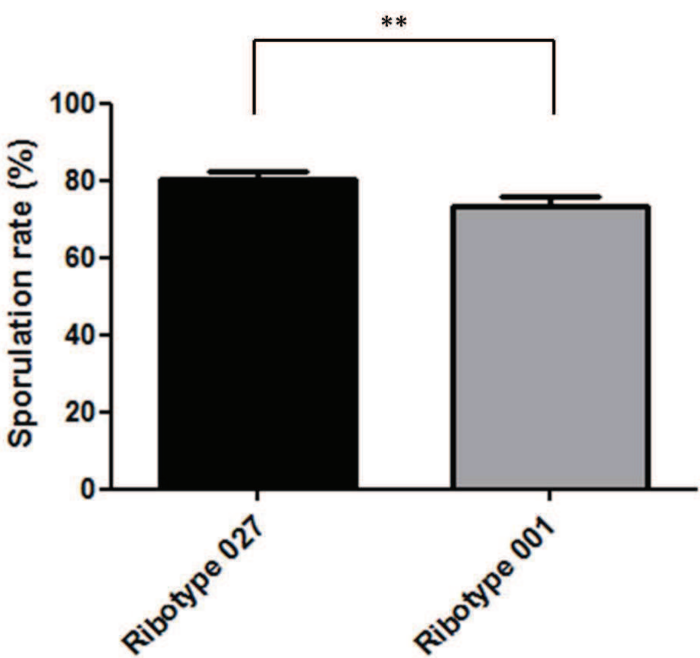


Figure 41. Sporulation rate of *C. difficile* ribotype 027 (n=25) versus *C. difficile* ribotype 001 (n=25) isolates (81.92% vs. 76,. %, respectively). Bars indicate standard error. P value=0.005.

(*, p <0.05; **, p<0.01; ***, p<0.001)

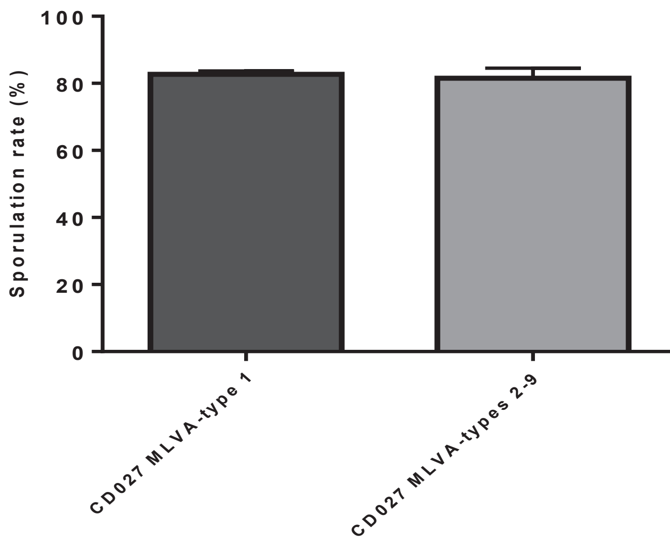


Figure 42. Comparison of sporulation rates of *C. difficile* ribotype 027 isolates of the MLVA-type 1 versus strains representing the others MLVA-types (clones 1-9). Bars indicate standard error. Analysis has not shown significant difference between sporulation rates of the groups (82.70% vs. 81.50%; p value=0.72)

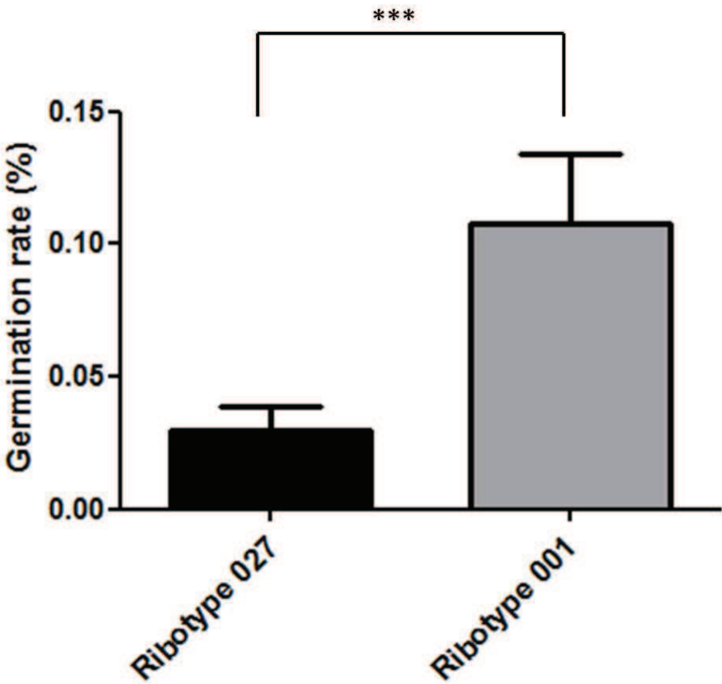


Figure 43. Germination rate of *C. difficile* isolates. It is highlighted the lower efficiency of germination of CD027 respect to CD001 (0.0091% vs. 0.0384%). Bars indicate standard error. P value=0.0008. (*p <0.05; **, p<0.01; ***, p<0.001)

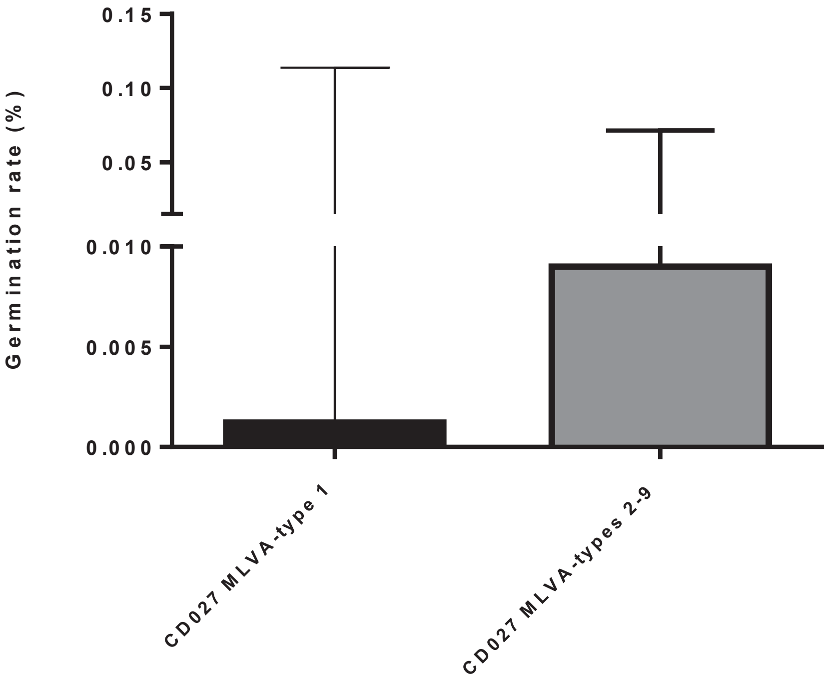


Figure 44. Comparison of germination rates of *C. difficile* ribotype 027 isolates of the MLVA-type 1 versus strains representing the others MLVA-types (clones 1-9). Bars indicate standard error. Analysis has not shown significant difference between sporulation rates of the groups (0.0083% vs. 0.00899%; p value=0.24)

2.4.4 Discussion

Since the identification of the multiple CDI outbreaks caused by *C. difficile* ribotype 027, there has been great interest in elucidating the mechanisms contributing to the more virulent phenotype, particularly those involved in transmission: spores are inactive cellular forms with great resistance to physical and chemical agents, contaminating the environmental surfaces for large periods and transmitted between patients [261].

In this part of the work, an *in vitro* sporulation assay allowed showing significant differences in between CD027 isolates and endemic CD001 strains, but gave contradictory results than the germination ability of CD027 isolates compared to those of CD001, this being much lower than expected.

At this regard, it is necessary to say that various studies were aimed to measuring the efficiency of sporulation of CD027 strains and results obtained lead to the generally accepted theory that this strain,

considering its higher virulence, may produce higher levels of spores than other *C. difficile* types [259, 260]; it is necessary to say that because of the variability of the methods used and especially in the absence of a gold standard assay, the consequent results are often discordant [263].

In this case, the rapid spread of *C. difficile* ribotype 027 isolates in the HGUGM, compared to that of strains belonging to endemic ribotype 001 ($p=0.005$), may be associated with increased release of spores into the environment, emphasizing the importance of this factor in ensuring greater transmission of the pathogen [351].

Besides, it is interesting to note that, within the same ribotype, the major circulating subtype (MLVA-type 1) does not appear to possess greater ability to sporulate respect the others clones and this does not explain its rapid and extensive spread in the hospital, considering that increased transmission is commonly associated to higher release of spores in the environment [352].

By one side the production of spores allows the pathogen to spread, by the other, it is necessary that the spore can mature in cells able to produce toxins, which allow the inflammatory process to start. This phenomenon is the known as germination and probably occurs *in vivo* under physiological stimuli [349, 353].

Interestingly, germination efficiency of the analyzed CD027 isolates showed lower values than CD001 strains (0.0091 versus 0.0384, $p=0.0008$, respectively) and unexpectedly did not correlate with higher sporulation rate, also considering the rapid spread of this so called “more virulent” variant.

Moreover, also analysis of germination efficiency between isolates belonging to same ribotype, particularly comparison between the prevalent MLVA-type 1 versus the others (0.0083 versus 0.00899, respectively), does not produce statistical significance ($p=0.24$).

This result could be due to a limit in the method, because we have not used media enriched in germinants (for example, taurocholate), considering that the role of these molecules in stimulating the germination of *C. difficile* has not been completely clarified [350, 354]. So, it was preferred to evaluate the samples in a state of "baseline germination", to have an analysis as less influenced by the variability of growth of the isolates under stimulation.

The finding suggests that multiple mechanisms may regulate germination responsiveness and adaptability to the environment. We propose that, to better understand complex mechanisms, it may be preferable to analyze the bacterial transcriptome under different conditions (i.e. reverse-transcriptional assays) [265, 350].

In conclusion, the study aimed at elucidating possible relationships between the higher transmission of *C. difficile* strains presenting rapid spread in the hospital environment and the sporulation rates. Isolates of CD027 were shown to have an increased spore production compared to isolates of the ribotype 001; this may in part explain why CDI cases due to this pathogen have increased with greater intensity in our hospital, but, considering the absence of differences in strains belonging to the same ribotype, the reason why one of the clones has been more transmittable than the others remain to be clarified.

This finding may suggest that, most likely, the synergistic effect of different factors (greater sporulation, fine germination control, inability to control environmental contamination, and possibly higher toxins production) has helped the CD027 phenotype to spread. These hypotheses give a hint to better understand the transmissibility of epidemic strains, and to elucidate their role in disease severity. Further studies could address the correlation between the ability of sporulation of specific ribotypes and the evaluation of environmental contamination, because, although this is an area of great debates, until today there are not key studies about this [352].

3. Concluding remarks

The spread of the higher transmissible and resistant pathogens made it necessary to devise strategies for their identification and prevention as accurately and as quickly as possible. Phenotypic characterization and clinical diagnosis of pathogens possibly involved in nosocomial outbreaks are today increasingly supported by typing molecular methods (i.e. PCR-ribotyping, MLVA, PFGE, and WGS).

In conclusion, this work demonstrates that:

1. Worldwide dissemination of carbapenem-producing *K. pneumoniae* (KPC-Kp) has been reported globally, but molecular tracing of is required also at a local scale, to understand epidemiological relationships among strains and to place them in a global context.

   In fact, little information is published about the molecular epidemiology of KPC-Kp in Italy, especially in the local level. According with the few published studies, it seems that MDR variants of Kp-KPC are already spread nationwide, increasing the incidence of infections.

2. The increasing incidence of infection due to particular virulent pathogen it is not always associated only to a single clone. In fact, molecular analysis of KPC-Kp isolates spread in the Ospedale di Circolo e Fondazione Macchi (Varese, Italy) showed the contemporary presence of strains belonging to three of the four previously characterized Italian clades belonging to CG258, suggesting the variability in Italian epidemiology.

3. The higher frequency of *K. pneumoniae* infections registered in the Ospedale di Circolo e Fondazione Macchi was due to the polyclonal dissemination of highly resistant isolates. In particular, a unique clone of colistin-resistant KPC-Kp belonging to ST512 was the responsible of an

outbreak involving 7 patients in the ICU of the hospital. The other clones, considered as sporadic cases, did not present the same capacity of dissemination.

4. The increased transmissibility of virulent strains of *K. pneumoniae* is generally associated with increased expression of pathogenic factors (pili/fimbriae, siderophores, hypermucousviscosity) and the expression of resistance genes. In this work, genomic analysis does not find a correlation between the increased transmissions of the epidemic clone with the specific expression of pathogenicity factors. Besides, all strains carried the bla_{KPC} gene conferring carbapenems resistance.

5. Detection of specific pathogenic factors does not always correlate with a clear phenotypic advantage of a more virulent clone. However, in this study, identification of the yersiniabactin (*ybtA/S*) genes only in a specific monophyletic clade of KPC-Kp strains having different origin suggests that some genetic features may be used as phylogenetic markers to obtain additional information about the genetic relatedness, the evolution and the global dissemination of these pathogens.

6. Though spread of *C. difficile* ribotype 027 (CD027) has been reported globally [189, 305], molecular tracing of is required also at a local scale, to understand epidemiological relationships among strains and to place them in a global context. In fact, in Spain, up to 2014, although three national studies were published, only few sporadic and unrelated cases of CD027 have been described. An active surveillance to identify and characterize CD027 isolates is necessary, to limit the spread of these dangerous pathogens.

7. The increasing incidence of infection due to particular virulent pathogen it is not always associated only to a single clone. The increasing incidence of *Clostridium difficile* ribotype 027 in the Hospital General Universitario Gregorio Marañon (HGUGM, Madrid, Spain) has been due to the concurrent

presence of nine different MLVA-types, which evolving from a common ancestor and spread independently in the hospital. Interestingly, the higher frequency of CD027 cases has been associated to one of these clones that represented the 84% of the clinical isolates, showing elevated transmission capacity and causing an outbreak involving 111 patients.

8. The increased transmissibility of virulent strains of *C. difficile* may be associated to the higher capacity to produce spores, which lead to an increased environmental contamination. This work demonstrated that CD027 strains have greater ability to sporulate compared with endemic ribotype 001; this could explain the high transmissibility of this virulent *C. difficile* variant. No differences were found between the different MLVA-types belonging to the ribotype 027; so that it is no possible explain why the majority clone had greater ability to spread respect all the others. This suggests that further studies are necessary.

9. Detection of mutations in the DNA gyrase A (*gyrA*) gene of *Clostridium difficile* ribotype 027 is not only correlated to selection of resistant strains, but mainly it can be used as phylogenetic markers to obtain additional information about the genetic relatedness, the evolution and the global dissemination of these pathogens.

Briefly, this work demonstrates the usefulness of molecular techniques in support of diagnostic methods applied to identify and characterize promptly nosocomial infections due to virulent pathogens, also allowing to characterize the presence of clones circulating concurrently (e.g. by WGS and/or MLVA subtyping), the genetic relationships between them, their transmission route and their phylogenomic evolution.

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5. *Curriculum vitae*

Dates : January 1st, 2013 - CURRENT
Employment: PhD student
Name and address of institution #1: Laboratory of Microbiology and Virology, Department of Biotechnology and Life Sciences, University of Insubria and Ospedale di Circolo-Fondazione Macchi, Viale Luigi Borri, 57, 21100 Varese, Italy
Name and address of institution #2: Departamento de Microbiologia y Enfermedades Infecciosas, Hospital General Universitario Gregorio Marañon, Universidad Complutense, Madrid, Calle del Doctor Esquerdo, 46, 28007 Madrid, España
Research activities: Molecular and phenotypic characterization of nosocomial infection with reference to virulence factors and determinants of drug resistance

Dates : 1 January 2012 - December 31, 2012
Employment: Research fellowship
Name and address of institution: Istituto Clinico Humanitas IRCCS, Via Manzoni 31, 20089 Rozzano, Italy
Research activities: Inflammatory Bowel Diseases (IBD): role of the pathway sphingosine 1-phosphate in endothelial cells.

Dates : October 1 2010-1 September 2011
Employment: Experimental thesis work
Name and address of institution: Department of Medical Biotechnology and Translational Medicine , University of Milan, Via Vanvitelli 32, 20129 Milan, Italy
Experimental activity: Polymorphism of "clock genes" and genes involved in metabolism and transport of irinotecan in colorectal carcinoma.

Dates : April 2009 - September 20, 2009
Employment: Training in experimental pharmacology
Name and address of institution: Department of Medical Pharmacology, University of Insubria, Via Dunant, 21100 Varese, Italy.
Activities: Thesis project concerning the localization of dopamine receptors in human lymphocytes by scanning electron microscopy.

EDUCATION
Dates : October 1 2009-5 October 2011
University of Milan , Via Vanvitelli , 20129 Milan
Principal subjects: Biology and anatomy, molecular biology, microbiology, recombinant DNA methods, biochemistry. Diagnosis and pathogenesis of neoplastic disease.
Title awarded: Master Degree in Medical Biotechnology and Molecular Medicine

Dates: October 1 2006-1 October 2009
University of Insubria, Varese, via Dunant , 21100 Varese, Italy
Principal subjects: Theoretical and practical aspects of general biology, molecular biology, recombinant

DNA techniques, microbiology, nanotechnologies, applied biomedicine.
Title awarded: Bachelor Degree in Biomedical Biotechnology

Publications

- **Onori R et al**; Tracking nosocomial *Klebsiella pneumoniae* infections and outbreaks by whole genome analysis: small-scale Italian scenario within a single hospital; J Clin Microbiol.; 2015 Jul 1. pii: JCM.00545-15

Courses and scientific meetings

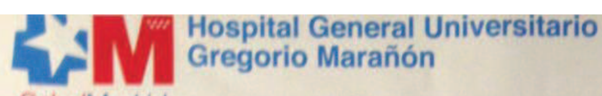
- Certificate of participation to "2nd Conference of translational medicine" (SIICA); November 2012, Congress Center Humanitas, Rozzano (MI)
- Certificate of participation to "3rd Meeting of Milan Meets Immunology (MMI); November, 2012, National Cancer Institute, Milan
- Certificate of participation to "Exploring the mRNA World"; November, 2012, Ca'Granda IRCCS Foundation, Policlinico, Milan (Italy)
- Certificate of participation to "Liver Immunology", AISF-SIICA Meeting; December 2012, Congress Center Humanitas, Rozzano (Milan, Italy)
- Certificate of participation to "Using New Technologies to Study the Genetics Disease", Illumina, Advances in Genome Science; September 2013, Novara (Italy)
- Speaker and chairman at the meeting "Italian young minds at work", November 2013; Desenzano del Garda (Brescia, Italy): "Molecular analysis of bacteria responsible of hospital outbreaks"
- Certificate of participation to “Malaria and parasitic infections in Italy”; December 2013, Circolo Hospital (Varese, Italy)
- European Society Clinical Microbiology and Infectious Diseases (ESCMID): junior member 2014/2015
- Attendance/CME Certificate - 13th ESCMID Summer School, Sigtuna (Sweden), 5-12 July 2014 – Oral presentation “Molecular investigation of hospital infections caused by multidrug resistant *Klebsiella pneumoniae*”.
- Scientific contribute to “42nd National Congress of the Italian Society of Microbiology”, 28 September-1 October 2014, Turin (Italy)- contribute published in the journal “New Microbiologica”, 2014, 37:63
- Certificate of participation to “I Gram-negativi MDR e XDR: dalla prevenzione alla terapia”; October 3, 2014; Università degli studi di Pavia (Italia)

- Participation to “Rusconi Lecture- Talassemia: il lungo percorso dal paziente al genoma- D.ssa MD Cappellini”; October 29, 2014; Villa Toeplitz (Varese, Italy)

- Participation to “II Reuniòn del Grupo Español de Estudio de la Infeccìon por *Clostridium difficile*”; February 12, 2015; Fundacìon Ramòn Areces (Madrid, España)

- Participation to “International Symposium. Microbiology: transmission”; May 7-8, 2015; Fundacìon Ramòn Areces and Fundaciòn General CSIC (Madrid, España)

Certificates of attendance



El Dr. D. Emilio Bouza Santiago, Catedrático de la Universidad Complutense de Madrid y , Jefe del Servicio de Microbiología Clínica y Enfermedades Infecciosas del Hospital General Universitario "Gregorio Marañón", de Madrid

CERTIFICA QUE:

Dña. Raffaella Onori, Estudiante de Doctorado de Medicina Sperimentale e Oncologia, dell'Universitá dell'Insubria, Ospedale Di Circolo, Fondazione Macchi, Varese (Va, Italy), ha realizado una estancia en el Servicio que yo dirijo desde el 1 de Enero hasta 31 octubre de 2015.

Lo que certifico a petición del interesada en,

Madrid, a 31 de octubre de 2015

Gregorio Marañón
Servicio de Microbiología Clínica

Fdo.: Prof. Bouza Santiago
Catedrático de Microbiología
Jefe de Servicio de Microbiología Clínica
y Enfermedades Infecciosas

Doctor Esquerdo, 46
28007 Madrid
www.hggm.es

Hospital General Universitario Gregorio Marañón

SaludMadrid

Comunidad de Madrid

To: Ufficio Post-Lauream,
Università dell'Insubria, Via Ravasi 2. 21100. Varese. Italy

From:
Clinical Microbiology and Infectious Diseases Dpt
Hospital General Universitario Gregorio Marañon
C/Dr. Esquerdo 46. 28007 Madrid. Spain.
Tf: +34-915868793

Dr Sir/Madam,

I am writing this letter in support of Miss Raffaella Onori, candidate to the final exam of Doctoral Thesis in Experimental Medicine and Oncology.

Miss Onori has been working in our Department for almost a year- January 2015-October 2015- and has always demonstrated great ability to learn and to participate in the everyday teaching and working activities.

She has been working in our institution during the period, showing good attitudes in performing several methodologies for molecular typing of.

She has acquired great experience in the Laboratory of Molecular Bacteriology and has contributed during these months in the control of 027 *Clostridium difficile* outbreak and in the typing of the isolates.

She is reliable, intelligent and a great asset for any Clinical Laboratory. It is for me a pleasure to back her up and I am happy to have the opportunity to work with her everyday.

I remain available for further information,

Sincerely yours,

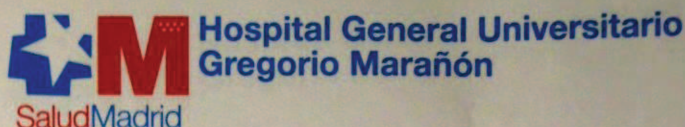
Madrid, October 31st 2015

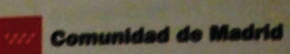
Hospital General Universitario Gregorio Marañón
SaludMadrid
Comunidad de Madrid
Servicio de Microbiología Clínica

Prof. Patricia Muñoz Garcia
University Complutense of Madrid
Section Chief Clinical Microbiology and Infectious Diseases

Doctor Esquerdo, 46
28007 Madrid
www.hggm.es

CÓD.: 3026201





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From:
Clinical Microbiology and Infectious Diseases Dpt
Hospital General Universitario Gregorio Marañon
C/Dr. Esquerdo 46. 28007 Madrid. Spain.
Tf: 0034-915868793

Dr Sir/Madam,

I'm writing this letter in support of Miss Raffaella Onori, candidate to the final exam of her Doctoral Thesis in Experimental Medicine and Oncology.

Miss Onori worked without any difficulty in our Laboratory of Molecular Bacteriology, showing good intellectual capacity, adaptability and reliability.

She has been working in our institution during the period January 2015-October 2015 in Clotridium difficile, showing excellent attitudes in performing several methodologies for molecular typing of *Clostridium difficile*.

I consider Raffaela a valuable element and excellent professional and fully capable for her defense of her Doctoral Thesis.

I remain available for any further information that you may request,

Sincerely,

Madrid, October 31st 2015

Hospital General Universitario Gregorio Marañón
Comunidad de Madrid
Servicio de Microbiología Clínica

Prof. Emilio Bouza Santiago
University Complutense of Madrid
Chief of Clinical Microbiology and Infectious Diseases

Doctor Esquerdo, 46
28007 Madrid
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6. Acknowledgments

I wish to express my gratitude to Professor Antonio Toniolo for giving me the opportunity to realize this PhD project and for remembering me to work more and more. I would like to thank Professor Emilio Bouza, for allowing me to spend a year at the Service of Clinical Microbiology and Infectious Diseases who heads with great professionalism.

It was a journey of great personal and intellectual growth and I'm grateful for every moment spent.

Special thanks to the excellent working group on *Clostridium difficile* of the Microbiology Department of Hospital General Universitario Gregorio Marañon, especially to Mercedes Marin, for her expert guidance in *Clostridium difficile* molecular typing, and for all the time spent to helping me in improving my work; to Elena Reigadas, to give me the opportunity to appreciate and exceed my limits; and to Luis Alcalà, for his amazing experience, for sharing with me his knowledge and having supported my theories (rather strange), and also for giving me lessons on ... diagnosis of *Clostridium difficile*, but also of life!!!

I would like to thank all my colleagues, and all the people working at the Laboratory of Microbiology and Virology, University of Insubria and Ospedale di Circolo-Fondazione Macchi (Varese) and at the Departamento de Microbiologia y Enfermedades Infecciosas, Hospital General Universitario Gregorio Marañon (Madrid) for the to welcome me warmly and for the happy times spent together. Thank you! I learned something from each of you...every single day!

I would like to express my sincere gratitude to Professor Bandi and to all the colleagues of Department of Biology and Biotechnology of the University of Pavia.

I would manifest my thankfulness to Davide, Stefano and Francesco for giving me the opportunity to learn about bioinformatics, for showing me what it means to work in a synergistic group, for the endless patience and sympathy.

Thanks to all the people who have taken a special place in my life:

Gianluca, you're definitely the sweetest and good friend (and colleague) I have. You've offered me your help unconditionally, and you made me feel your love every single day, even at 1618.9 km away! Thank you, I could not say more!

Alberto, you have been an unparalleled colleague and mentor. You taught me that destiny is to be earned, with the action and reasoning.... and with a coffee at 10:30 in the morning! I will be forever grateful to you!

Dori, you made me feel at home! Thanks for the advices, for the smiles, the breakfast together and for making me believe again in the goodness of the world!

Cris, Yure, and Marta… in such a short time, a so beautiful friendship, improvised weekends and a lot of happiness! Thank you so much! I will never forget this time!

Thanks to all the people who made unforgettable the last three (and more) years! I cannot write much, but… I'm lucky to have met all of you!

Sara, you took care of all I left behind when I had to leave Italy. I cannot thank you enough, I guess... Graziella, Edo, Giuli, Andre, Fede e Marco, without you I would not have began this journey, so…thanks for believing! Thanks to Tiger Blues, S.U.R.F., R.C.R., for give me the possibility to feel myself alive. Thanks to those who believed and who's not; to those who is no longer there; to those who have never been...

Infine, voglio ringraziare coloro per cui ho continuato a camminare, nonostante le cadute…

Mamma, Lori e Skay, siete tutta la mia vita. Vi amo infinitamente e mi mancate ogni giorno che passa. Mamma, mi hai insegnato con il tuo sacrificio, che il lavoro nobilita, per quanto umile possa essere, e che non bisogna mai arrendersi. Io ti ringrazio perchè hai rinunciato alla tua vita per dare luce alla mia, per cui ti prometto che i tuoi sforzi non andranno perduti. Sarò felice. Troverò la mia strada. Intanto ti dedico le vittorie di ogni giorno; in fondo, sono arrivata fin qui! Grazie a te, mamma! Grazie al tuo grande cuore!... Ti voglio un mondo di bene!

Lori, sei la migliore sorella che potessi mai desiderare! Grazie di esistere! Non ti fermare, esigi sempre il meglio, perchè tu sei il meglio! Ti voglio bene!

Grazie a Zio Enzo, saggio quotidiano consigliere. Non sono mai stata sola. Ogni singolo giorno, come il genio della lampada, tu sei apparso in mio soccorso! Grazie dei tuoi consigli, anche se poi faccio di testa mia!

Dedico tutti i miei successi a Voi! A mamma, Lori, Skay, zio Enzo, zio Pino e zia Esterina, zia Angi e zio Sasá, nonno e nonna, Vivi, Ale, Raffa Jr, Luigi e Lucia!! Siete il tesoro piú prezioso, la mia gioia piú grande, il mio piccolo mondo felice!! Vi amo con tutto il cuore! Grazie!

Raffaella