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All'Amore,

All'amore autentico,

e alla sua massima
espressione, Mio Marito

"Tutto posso in Colui
che mi dà forza"

(Fil 4,13)

Index

Abstract

Carbapenems resistant *Enterobacteriaceae* infections are increasing worldwide representing an emerging public health problem. The application of phylogenetic and phylodynamic analyses to bacterial whole genome sequencing (WGS) data have become essential in the epidemiological surveillance of multi-drug resistant nosocomial pathogens.

In this study, *K. pneumoniae* KPC strains circulating within different wards of the University Hospital Campus Bio-Medico were collected and WGS applied.

These applications could help in the epidemiological surveillance of MDR pathogens to discern outbreak from non-outbreak strains in both community and hospital settings.

The aim was to infer the origin and the spread of *K. pneumoniae* nosocomial strains and to clarify the epidemiological transmission as so as the eventual reservoir in the hospital setting supporting the epidemiological surveillance and infections control strategies.

Among patients undergoing endoscopic retrograde cholangiopancreatography (ERCP), patients have investigated infections by KPC K. pneumoniae and endoscope were sampled to check the correct duedonoscope reprocessing.

A microbiological surveillance on duedonoscope was performed.

Between January 2012 and February 2013, twenty-one multi-drug resistant *K. pneumoniae* strains, were collected from patients hospitalized among different wards of the University Hospital Campus Bio-Medico. Epidemiological contact tracing of patients and Bayesian phylogenetic analysis of bacterial WGS data were used to investigate the evolution and spatial dispersion of *K. pneumoniae* in support of hospital

infection control. The epidemic curve of incident *K. pneumoniae* cases showed a bimodal distribution of cases with two peaks separated by 46 days between November 2012 and January 2013. The time-scaled phylogeny suggested that *K. pneumoniae* strains isolated during the study period may have been introduced into the hospital setting as early as 2007. Moreover, the phylogeny showed two different epidemic introductions in 2008 and 2009.

The results of the sampling performed highlighted a first multi-microbial contamination supported by MDR organism (K. pneumoniae, P. aeruginosa and A. baumanii) eliminated from the introduction of the new reprocessing protocols.

Due to the complex design of duodenoscopes, it is difficult an adequate disinfection of the channels. Duodenoscopes can be difficult to dry and a potential risk of cross contamination is higher using this type of endoscope. New duodenoscope designs should facilitate more efficient disinfection, FDA has identified design features that facilitate cleaning, disinfection and sterilization and reduce the likelihood of retaining debris.

Bayesian genomic epidemiology is a powerful tool that promises to improve the surveillance and control of multi-drug resistant pathogens in an effort to develop effective infection prevention in healthcare settings or constant strains reintroduction.

Introduction

1. Enterobacteriaceae

The Enterobacteriaceae family is the largest and most heterogeneous collection of Gram-negative bacilli of medical/biological importance. Members of this family are cause primary infections of the human gastrointestinal tract and are major causes of opportunistic infection (including septicemia, pneumonia, meningitis and urinary tract infections). The genera belonging to this family were classified based on biochemical properties, antigenic structure, DNA-DNA hybridization and sequencing of 16S rRNA. They are also dispersed in nature and can be found in plants, soil, water, normal microbiota in the intestinal tract of both man and other animals. Microbiological and medical importance stems from the development of infections, as well as pathogenicity and appearance of multi-resistant bacteria to antibiotics used in therapy (Farmer, Boatwright e Janda 2007) (Murray, Rosenthal e Pfaller 2010).

Most genus and species belonging to this family have the following properties: they are Gram-negative straight, of moderate size (0.3 to 1.0 x 1.0 to 6,0m), share a common antigen, do not form spores, have motile with 5 polar flagella in one pole or are without flagella, develop into peptone or meat extract supplement or without added sodium chloride as well as on MacConkey agar. They are facultatively anaerobic and most species grow well at 37°C, although some species grow better at 25-30°C. Some strains grow on D- glucose as the sole source

of carbon and energy, but other strains require vitamins and or amino acids. Acid is produced during the fermentation of D- glucose and other carbohydrates (Ewing, Farmer e Brenner 1980) (Holt JG 1994), reduce nitrate to nitrite, is catalase positive, oxidase negative and contains 39% - 59% guanine-cytosine (GC) in their DNA. Most of the microorganisms have fimbriae, also known as pili, which are subdivided into common fimbriae encoded by chromosomal genes and sex pili which is encoded by genes located on conjugative plasmids.

The common fimbriae are important for bacterial adherence to specific receptors on host cells, since the sexual pili facilitates gene transfer between bacteria (Farmer, Boatwright e Janda 2007) (Murray, Rosenthal e Pfaller 2010).

Enterobacteria make up about 80% of the strains of medically important Gram-negative bacilli and approximately 50% of all bacteria isolated in microbiology laboratories. These micro-organisms are responsible for about half of all cases of sepsis and more than 70% of cases of urinary tract infection, and a considerable percentage of intestinal infections, and may be associated with community and hospital infections. Can also cause infections of surgical wounds, abscesses, pneumonia, sepsis and meningitis (Farmer, Boatwright e Janda 2007).

The lipopolysaccharide (LPS) thermostable is the principal antigen of the cell wall and is composed of three components: the somatic polysaccharide O, located more externally, a central polysaccharide to common to all Enterobacteriaceae (enterobacter common antigen) and in the internal portion the lipid A. The central polysaccharide is important in the classification of the micro-organism as a member of the family Enterobacteriaceae, the polysaccharide O is crucial to the epidemiological classification of strains within species and lipid A, is responsible for endotoxin activity, an important virulence factor (Murray, Rosenthal e Pfaller 2010).

Enterobacteriaceae can cause opportunistic infections including septicemia, pneumonia, meningitis and infections of the urinary tract:

- Some members of the family, such as Salmonella species, Shigella species, etc., always cause diseases in humans.
- Other species, such as Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, etc., found as normal intestinal flora in humans can also cause infections in other sites of the human body.
- In addition to these, there is another group of Enterobacteriaceae

organisms, which are found as normal commensals in humans but become pathogenic when they acquire virulence factor genes through plasmids, bacteriophages, or pathogenicity islands. E. coli associated with gastroenteritis in humans is one such example.

1.1. Cultivation on blood agar

Organisms that usually colonize the gastrointestinal tract cultivated on blood agar reveal colonies that appear large, smooth, shiny, circular and raised which unlikely are hemolytic or pigmented.

Colonies of Escherichia coli are without hemolysis, but several strains isolated from infections are beta-hemolytic.

Mucous colonies of Klebsiella pneumoniae on blood agar are non-hemolytic (gamma-hemolytic).

Proteus mirabilis on blood agar has the ability to swarm (RAUSS phenomenon) over the surfaces of solid cultivation media (the spreading growth covers other organisms in the culture and thus delays other isolation) (Murray, Baron, et al. 2003) (Figure 1).

Fig 1. Enterobacteriaceae blood agar.

1.1.1. Cultivation on endo agar

Endo agar is a selective culture for the detection of coliform and other enteric microorganisms. This type of agar does not allow the growth of Gram-positive microorganisms due to the sodium sulfite/basic fuchsin combination. Endo agar permits the differentiation of lactose fermenters from the non-fermenters. Coliform

microorganisms ferment the lactose producing pink to rose-red colonies and similar coloration of the medium. The colonies of organisms which do not ferment lactose are colorless to faint pink against the pink background of the medium (Murray, Baron, et al. 2003).

1.2. The genus Klebsiella - General characteristics

The genus Klebsiella contains six species and three subspecies. There are four species related to humans and they include K. pneumoniae subspecies pneumoniae, ozaenae, and rhinoscleromatis; K. oxytoca; K. granulomatis and K. variicola. The other two species, K. singaporensis and K. michiganensis have been isolated from the soil and from a tooth brush holder respectively. The genus consists of over 77 capsular antigens (K antigens) and 8 somatic lipopolysaccharide (LPS, O antigens), leading to different serogroups (Hansen, Mestre, et al. 1999) (Parija, Coliforms 2012). The members of Klebsielleae have been classified into over 80 serotypes based on the capsular K antigens and somatic O antigens. All serotypes are of the same virulence (Parija, Coliforms 2012).

These well-developed polysaccharide capsules give the colonies their typical mucoid appearance (Podschun e Ullmann 1998).

Klebsiella species are non-motile, usually encapsulated rod-shaped bacteria, belonging to the family Enterobacteriaceae. These bacteria produce lysine decarboxylase but not ornithine decarboxylase and are generally positive in the Voges-Proskauer test as well as indole and urease tests. They are generally facultatively anaerobic, and range from 0.3 - 1.0mm in width and 0.6 - 6.0mm in length (Podschun e Ullmann 1998).

On MacConkey agar, the colonies typically appear large, mucoid, and red, with red pigment usually diffusing into the surrounding agar, indicating fermentation of lactose and acid production (Podschun e Ullmann 1998).

They can cause bacteraemia and hepatic infections, and have been isolated from a number of unusual infections, including endocarditis, primary gas-containing mediastinal abscess, peritonitis, acute cholecystitis, crepitant myonecrosis, pyomyositis, necrotising fasciitis, psoas muscle abscess, fascial space infections of the head and neck, and septic arthritis (NHS 2015) (Janda e Abbott 2006). Klebsiella are the normal inhabitant of the intestinal tract of human, animal, soil, water

and botanical environment (Ørskov 1984) (Brisse, Grimont e Grimont 2006) (Brooks, Butel e Morse 2007).

1.2.1. K. pneumoniae

K. pneumoniae, also known as Friedlander's bacillus, was first isolated by Friedlander in 1883, from fatal cases of pneumonia; and was named after Edwin Klebs, German pathologist. The species received the name for its ability to cause severe pneumonia (Qureshi 2017).

They are non-motile and non-sporing. They are arranged singly or in pairs. Freshly isolated strains show a well-designed polysaccharide capsule. The capsule is often conspicuous and can be made out even in Gram-stained smears as haloes around the bacilli, and is produced well when grown in media enriched with carbohydrates. The capsule can also be demonstrated by India ink preparation and Quellung's reaction. Accumulation of extracellular polysaccharides as a loose slime gives mucoid appearance to Klebsiella colonies.

They are fimbriated, and most strains possess one or more of three types of fimbriae: types 1, 3, and 6. They are nonmotile and nonsporing. They are lactose-fermenting, urease- positive, and indole-negative organisms; however, some strains of K. pneumoniae and K. oxytoca are exceptions. They do not produce hydrogen sulphide, and they are both VP and MR tests positive. They grow well on ordinary media, such as nutrient agar and MacConkey agar at 37°C, forming large, domeshaped, mucoid colonies. They produce lactose-fermenting red colonies on MacConkey agar.

Typical features for identification are the presence of fermentation of lactose, capsule production, lack of motility, no indole production, negativity in methyl red test, and typical pattern in decarboxylase assays (lysine +, arginine -, ornithine -).

The discrimination using gene sequence analysis (16S ribosomal RNA gene sequencing, complete genome or multilocus sequence typing, MLST) should be the routine in a clinical setting, with the support of matrix-assisted laser desorption/ionisation time of flight mass spectrometry (Mellmann, et al. 2009) (Angeletti, Dicuonzo, et al. 2015); this requires further evaluation.

The genus Klebsiella was originally divided into 3 main species based on biochemical reactions and medical importance into three species corresponding to the diseases they caused: K. pneumoniae, K.

ozaenae, and K. rhinoscleromatis. Based on DNA-DNA hybridization data K. ozaenae and K. rhinoscleromatis, taxonomically, are regarded as subspecies of K. pneumoniae (Podschun e Ullmann 1998) (Drancourt, et al. 2001) (Hansen, Aucken, et al. 2004) (Rosenblueth, et al. 2004) (Brisse, Passet e Grimont 2014).

Klebsielleae consists of invasive bacteria. They possess many virulence factors (Parija, Coliforms 2012):

1. Capsule is the principal virulence factor. The capsule prevents the bacteria from phagocytosis by polymorphonuclear granulocytes. The capsule also prevents bacterial death caused by bactericidal serum factors by inhibiting the activation or uptake of complement components, especially C3b.
2. Multiple adhesins are additional virulence factors. In the beginning step of the disease process, these adhesins help the bacteria to adhere to host cells, allowing to initiate the process.
3. LPS is another factor that prevents membrane damage and death of bacteria, like capsule. The LPS of the bacteria activates the complement, which causes selective deposition of C3b onto LPS molecules at sites away from cell membrane of the bacteria. This inhibits the formation of the membrane attack complex (C5b–C9), which is responsible for cell death of the bacteria.

Klebsielleae organisms cause a variety of clinical syndromes in humans. These are: community-acquired pneumonia, UTI, nosocomial infection, and bacteremia and sepsis (Parija, Coliforms 2012).

1. Community-acquired pneumonia is a severe condition with a fast onset and has often fatal outcome even if an early and appropriate antimicrobial treatment is use. Lobar pneumonia characteristically is associated with considerable mucoid inflammatory exudate of lobar or lobular distribution, involving one or more lobes of the lung. Necrosis and abscess formation are more frequent than in pneumococcal pneumonia. K. pneumoniae serotypes 1, 2, and 3 are usually associated with the condition. Patients present with an acute onset of high fever and chills, flu-like symptoms, and productive cough with abundant, thick, tenacious, and blood-tinged sputum. Blood culture is positive in about 25% of the cases.

2. UTI caused by K. pneumoniae is a common problem in patients with catheters careers. UTIs caused by K. pneumoniae is very similar clinically with those caused by common bacteria, such as E. coli.
3. K. pneumoniae are emerging important agents of nosocomial infections. The presence of invasive devices, contamination instruments, use of urinary catheters, and use of antibiotics greatly increases the likelihood of nosocomial infections in hospitalized patients. Furthermore, immune-depressed health status and treatment in an intensive care unit or nursing home are other factors. UTI, pneumonia, bacteremia, wound infection, cholecystitis, and catheter-associated bacteriuria are the common nosocomial infections associated with K. pneumoniae. Other rare nosocomial infections include cholangitis, meningitis, endocarditis, and bacterial endophthalmitis.
4. Klebsiella bacteremia and sepsis produce clinical manifestations similar to those caused by E. coli and other Gram-negative enteric organisms. In neonatal units, outbreaks of infection caused by extended-spectrum beta-lactamase (ESBL)-producing Klebsiella strains present a more serious problem and may be associated with high mortality. ESBL strains of Klebsiella show the following features: (a) these are extremely virulent, (b) they possess capsular type K55 antigen, and (c) they have a surprising ability to spread.

Diagnosis of K. pneumoniae infection is made by isolation of bacteria from clinical specimens obtained from possible sites (e.g., wounds, peripheral or central intravenous access sites, urinary catheters, respiratory support equipment) and by culture. Klebsiellae strains could be isolated from urine, blood, pleural fluid, and wounds. Serological tests are not used for K. pneumoniae identification.

1.2.1.1. Klebsiella pneumoniae genome

The genome sizes of the different K. pneumoniae strains range from 5.1 to 5.6 Mb. In addition, five plasmids have been determined for K. pneumoniae MGH 78578, pKPHS1 (122,799 bp, 49.5% GC content), pKPHS2 (111,195 base pair (bp), 53.3% GC content), pKPHS3 (105,974 bp, 52.5% GC content), pKPHS4 (3,751 bp, 52.2% GC content), pKPHS5 (3,353 bp, 42.8% GC content), and pKPHS6 (1,308 bp, 47.9% GC

content) (P. Liu, et al. 2012), while other strains ranged from one to three (Conlan, et al. 2016) (Fouts, et al. 2008) (Wu, et al. 2009).

1.2.2. Other Klebsiellae

Klebsiella rhinoscleromatis

Rhinoscleroma caused by K. rhinoscleromatis is a chronic inflammatory disease involving the nasopharynx. Infection with K. rhinoscleromatis has a worldwide distribution and is common observed in areas of southeastern Europe, Central America, and India. Patients present with a purulent nasal discharge with formation of crusts and nodules that may lead to respiratory obstruction. The bacilli are seen intracellularly in lesions, which can be isolated and identified by biochemical reactions. Diagnosis is by positive blood culture supplemented with histology. Rifampin is used for treatment of rhinoscleroma (Parija, Coliforms 2012).

Klebsiella ozaenae

Ozena, caused by K. ozaenae, is a chronic atrophic rhinitis characterized by necrosis of nasal mucosa and mucopurulent nasal discharge. It often occurs in elderly persons. Nasal congestion and a constant nasal bad smell are the common symptoms. However, unlike rhinoscleroma, nasal congestion is not a prominent feature. Patients may also complain of headache and symptoms attributable to chronic sinusitis. Identification of K. ozaenae is difficult due to wide variations in the biochemical reactions of isolated strains. Trimethoprim and sulfamethoxazole are used for treatment of ozena (Parija, Coliforms 2012).

Klebsiella oxytoca

K. oxytoca may be rarely isolated from clinical specimens. It is being increasingly isolated from patients with neonatal septicemia. The bacteria have also been associated with neonatal bacteremia, especially among premature infants and in neonatal intensive care units (Table 1) (Parija, Coliforms 2012).

Tab. 1. Important properties used for differentiation of Klebsiella species (Parija, Coliforms 2012). V means variable results in different species or strains.

Properties	***Klebsiella pneu-moniae***	***Klebsiella ozaenae***	***Klebsiella rhinoscler-omatis***	***Klebsiella oxytoca***
Indole	-	-	-	+
Urease	+	-	-	+
Citrate	+	V	-	+
ONPG	+	+	-	+
Malonate	+	-	+	+
Lysine decarboxylase	+	V (40%)	-	+
Ornithine de-carboxylase	-	-	-	-
MR methyl red	-	+	+	V (20%)
VP Voges-Proskauer	+	-	-	+

2. Antimicrobial drugs

Antimicrobial drugs (also termed anti-infective drugs) are a specific class of synthesis capable also in high dilutions of neutralizing or inhibiting microorganisms (Parija, Antimicrobial Agents: Therapy and Resistance 2012).

The term *antimicrobic* is used to define all antimicrobial drugs, independently of origin (naturally or chemically produced). The natural metabolic processes of some microorganisms produce the antibiotic as a compost that can neutralize or inhibit other microorganisms. Through chemical reactions is possible to generate antibiotic from colors or other organic compounds. Although division into these two categories has been traditional, they tend to overlap, because most antibiotics, termed semisynthetic antibiotics, are now chemically altered in laboratory.

Antimicrobial drugs vary in their spectrum of activities (Parija,

Antimicrobial Agents: Therapy and Resistance 2012) in two categories:

- Broad-spectrum or extended-spectrum antibiotics are active against a broader range of different microorganisms. For example, tetracyclines are active against a variety of Gram-positive and Gram-negative bacteria, Rickettsiae, Mycoplasmas, and even protozoa.
- Narrow-spectrum antibiotics are effective against one or very few microorganisms. For example, vancomycin is active against certain Gram-positive bacteria (such as Staphylococci and Enterococci) or griseofulvin, which is used only against fungal skin infections.

2.1. Mechanisms of action of Antimicrobial Drugs

There are two different categories of antimicrobial drugs (Parija, Antimicrobial Agents: Therapy and Resistance 2012):

- **Bactericidal drugs** kills bacteria and they are very helpful in (a) life-threatening situations, (b) endocarditis, (c) patients with low polymorphonuclear count (below 500/ L), and (d) conditions in which bacteriostatic drugs do not cause a cure.
- **Bacteriostatic drugs** inhibit the growth of bacteria, but does not kill them. They depend on the host defense mechanisms, such as phagocytes to kill the bacteria. Hence, these drugs are not used when the patient has limited neutrophils.

Antibiotics can act against bacteria through these following mechanisms:

1. Inhibition of cell wall synthesis
2. Inhibition of protein synthesis
3. Inhibition of nucleic acid synthesis
4. Alteration of cell membrane function
5. Inhibition of Folate synthesis

2.1.1. Inhibition of Cell Wall Synthesis

Penicillins, cephalosporins, carbapenems and vancomycin are antibiotics that act against bacteria by interfering with their cell wall synthesis; these antibiotics are bactericidal. Penicillins, carbapenems and cephalosporins are called β–lactam antibiotics because they have a

complete β-lactam ring (common chemical nucleus, aminopenicillanic acid) crucial for antimicrobial activity.

Penicillins act primarily against Gram-positive organisms. Other penicillins, such as ampicillin, amoxicillin, carbenicillin, ticarcillin, piperacillin etc., act against both Gram-positive and Gram-negative organisms (Walsh e Wencewicz 2016) (Parija 2012).

Cephalosporins differ from penicillins in having 7-amino-cephalosporanic acid instead of 6-aminopenicillanic acid in their structure. They are effective against a wide variety of bacterial pathogens (Table 2).

Tab. 2. List of cephalosporins used in clinical practice (Parija 2012).

	First generation	**Second generation**	**Third generation**	**Fourth generation**
Drugs	Cephalexin, cephalothin, cephradine, cephaloridine	Cefoxitin, cefaclor, cefamandole, cefuroxime, cefprozil, cefmetazole	Cefoperazone, cefotaxime, ceftazidime, ceftizoxime, ceftriaxone, cefixime	Cefpirome, cefepime
Anti bacterial activity	**Gram-positive bacteria**: Staphylococcus aureus, Streptococcus spp. except enterococci **Gram-negative bacteria:** Escherichia coli, Klebsiella, Haemophilus influenzae, Proteus mirabilis	First-generation spectrum + Enterobacter spp., Serratia spp., Proteus vulgaris, Citrobacter spp. + Gram-negative anaerobes	Second-generation spectrum + Pseudomonas aeruginosa, Neisseria gonorrhoeae including β-lactamase–producing strains	Third-generation spectrum + extended Gram-negative coverage including Citrobacter spp. and Enterobacter spp. resistant to third-generation cephalosporins

Carbapenems (such as imipenem) and monobactams (such as aztreonam) are structurally different from penicillins and cephalosporins (Figure 2).

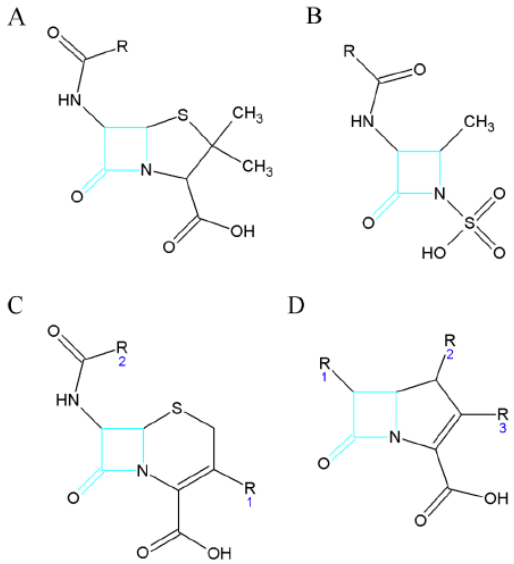

Fig. 2. General structures of the four groups of β-lactam antibiotics. A: penicillins, B: monobactams, C: cephalosporins and D: carbapenems. The main structure, the β-lactam ring (marked in light blue), is central in each molecule. Variable areas are marked with R.

Vancomycin is a glycopeptide, but its action approach is very similar to β-lactam antibiotics (Parija 2012).

Teicoplanin is a glycopeptide antibiotic extracted from *Actinoplanes teichomyceticus*, with a similar spectrum of activity to vancomycin (Parija 2012).

2.1.2. Inhibition of Protein Synthesis

Bacteria have 30S and 50S ribosomal units, whereas mammalian cells have 80S ribosomes. This difference permit to create a specific target, of the subunits of each type of ribosome, their chemical composition and their functional specificities, these antimicrobial drugs can inhibit protein synthesis in bacterial ribosomes without affect severely mammalian ribosomes (Parija 2012) Aminoglycosides and tetracyclines act on the 30S ribosomal subunits, whereas erythromycins, chloramphenicol, and clindamycins act on the 50S ribosomal subunits.

2.1.3. Inhibition of Nucleic Acid Synthesis

Quinolones, and rifampin are drugs that act by inhibition of nucleic acid synthesis.

Quinolones are synthetic analogs of nalidixic acid; they are a family of drugs that include ciprofloxacin, ofloxacin, and levofloxacin. They act as bactericidal inhibiting bacterial DNA synthesis by blocking DNA gyrase (enzyme that unwinds DNA strandsto replicate). Quinolones are effective against both Gram-positive and Gram-negative organisms. Rifampin inhibits bacterial growth by binding strongly to the DNA dependent RNA polymerase of bacteria. Therefore, it inhibits bacterial RNA synthesis. Rifampin acts in a different way in viruses; it blocks a late stage in the assembly of poxviruses.

2.1.4. Alteration of Cell Membrane Function

The cytoplasm of living cells is delimited by the cytoplasmic membrane, a selective permeability barrier. The cytoplasmic membrane carries out active transport functions, and thus controls the internal composition of the cell. If the functional integrity of the cytoplasmic membrane is disrupted, macromolecules and ions escape from the cell, and cell damage or death ensues. The cytoplasmic membrane of bacteria and fungi has a structure different from that of animal cells and can be more readily disrupted by certain agents. Consequently, selective chemotherapy is possible. Antifungal drugs act by altering the cell membrane function of the fungi. They show selective toxicity because cell membrane of the fungi contains ergosterol, while human cell membrane has cholesterol. Bacteria with the exception of Mycoplasma do not have sterols in their cell membranes, hence are resistant to action of these drugs.

2.1.5. Inhibition of Folate synthesis

Sulfonamides and trimethoprim inhibit folic acid synthesis acting in the folic acid metabolism. Sulfonamides are structural analogs of para-aminobenzoic acid (PABA). Due to structural similarity to PABA, sulfonamide competes with the latter during bacterial metabolism. Sulfonamides enter into the reaction in place of PABA and compete for the active center of the enzyme. As a result, nonfunctional analogs of folic acid are formed, preventing further growth of the bacterial cell. The inhibitory action of sulfonamides on bacterial growth can be

counteracted by an excess of PABA in the environment (competitive inhibition). Many other bacteria, however, synthesize folic acid, as mentioned above, and consequently are susceptible to action by sulfonamides. Sulfonamides are bacteriostatic drugs effective against a variety of Gram-negative and Gram-positive bacteria. Trimethoprim (3,4,5-trimethoxybenzylpyrimidine) is a bacteriostatic drug active against both Gram-positive and Gram-negative organisms. The compound inhibits the enzyme dihydrofolic acid reductase 50,000 times more efficiently in bacteria than in mammalian cells. This enzyme reduces dihydrofolic to tetrahydrofolic acid, leading to decreased synthesis of purines and ultimately of DNA. Sulfonamides and trimethoprim inhibit the synthesis of tetrahydrofolic acid, the main donor of the methyl groups that are essential to synthesize adenine, guanine, and cytosine.

Cellular target sites of antimicrobial drugs are listed in Table 3.

Tab. 3. Cellular target sites of antibiotics

Drug	Target site	Mechanism of action
β-lactams	Cell wall	Bactericidal, interfere with cross-linking of cell wall peptidoglycan molecules
Erythromycin, fusidic acid, tetracycline	Ribosomes	Bacteriostatic or bacteriocidal, interfere with translocation and attachment of t-RNA, thus inhibiting protein synthesis
Polyenes	Cytoplasmic membrane	Bacteriostatic, disrupt yeast cell membrane
Metronidazole, idoxuridine, acyclovir	Nucleic acid replication	Bactericidal, interfere with DNA replication

2.2. Resistance to Antimicrobial Drugs

The choice of a specific antimicrobial agent depends on antibiotics susceptibility patterns of isolated strains. Bacterial resistance to drugs is the condition in which the bacteria that were earlier susceptible to antibiotics develop resistance against the same antibiotics and are not

susceptible to the action of these antibiotics.

WHO identified the antibiotic-resistance as the third most important problem concerning the human health. Antimicrobial resistance occurs naturally over time, usually through genetic changes. However, the misuse and overuse of antimicrobials is accelerating this process. In many places, antibiotics are overused and misused in people and animals, and often given without professional oversight. Examples of misuse include when they are taken by people with viral infections like colds and flu, and when they are given as growth promoters in animals and fish. This creates an excessive selective pressure on the bacteria (WHO 2016).

It is important to make a clarification in term of definitions for acquired resistance (Magiorakos, et al. 2012):

- multidrug-resistant (MDR) is defined as non-susceptibility to at least one agent in three or more antimicrobial categories
- extensively drug-resistant (XDR) is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories). This is epidemiologically significant due not only to their resistance to multiple antimicrobial agents, but also to their ominous likelihood of being pandrug resistant.
- pandrug-resistant (PDR) is defined as non-susceptibility to all agents in all antimicrobial categories (i.e. no agents tested as susceptible for that organism).

Multidrug-resistant organisms (MDRO) are a worldwide emergency, and are seen more commonly in hospital-acquired infections, especially the intensive care units, than in community-acquired infections.

Common examples of such strains of bacteria showing drug resistance include hospital strains of S. aureus and Gram-negative enteric bacteria, such as Klebsiella pneumoniae and Pseudomonas aeruginosa. Resistance to multiple antibiotics is mediated by plasmid-carrying several genes that encode enzymes responsible for the resistance (Parija, Antimicrobial Agents: Therapy and Resistance 2012).

There are active surveillance programs on the microbiological monitoring of hospitalized patients and environments in order to identify those who are most likely to contract infections, describing the incidence of such infections over time, identifying any epidemics,

reservoirs and transmission mechanisms, to undertake antibiotic therapy aimed at assessing the effectiveness of sanitation interventions (Karchmer, et al. 2002).

2.2.1. Mechanisms of Antibiotic Resistance

There are many different mechanisms by which microorganisms might exhibit resistance to drugs.

Molecular Mechanisms: modified porin (A), efflux pump (B), modified target (C) and deactivating enzymes (D).

Genetic Mechanisms: mutation(1), transformation from free DNA(2), transduction from bacteriophage(3) and conjugation from plasmid(4).

- **Modified porin (A):** The primary function of these porins is to allow the passage of nutrients and other molecules in and out of the cell. This inadvertently allows the passage of several types of antibiotics. The loss of one or more of these porins can hinder or limit the ability of the antibiotic to enter the cell and lead to a reduction in susceptibility. When in combination with active efflux, high levels of resistance to multiple antibiotics can be achieved. In K. pneumoniae the loss of the OmpF homologue OmpK35 has been associated with cefoxitin resistance (Ananthan e Subha 2005).
- **Efflux pump (B):** Efflux pumps have been found to be responsible for conferring resistance to many groups of antibiotics including aminoglycosides, quinolones, etc. they are important mechanisms of antibiotic resistance for two reasons: they allow the bacterium to cope and survive in a stressful environment (in the presence of antibiotics) and concurrently, by delaying the death of the bacterium they increase exposure time to the antibiotics in which the bacterium may undergo mutations in order to achieve higher levels of resistance. Efflux pumps are increasingly implicated in the causes of antibiotic resistance within numerous clinically relevant bacterial species including K. pneumoniae and P. aeruginosa (Webber e Piddock 2003) (Borges-Walmsley, McKeegan e Walmsley 2003).
- **Modified target (C):** Certain bacteria produce modified targets against which the antibiotic has no effect. For example, a methylated 23S ribosomal RNA can result in resistance to erythromycin, and a mutant protein in the 50S ribosomal subunits can result in resistance to streptomycin. Penicillin resistance in S. pneumoniae

and enterococci is caused by the loss or alteration of PBPs.

- **Deactivating enzymes (D):** Bacteria produce enzymes that inactivate antibiotics. For example, penicillin-resistant staphylococci produce an enzyme β-lactamase that destroys the penicillins and cephalosporins by splitting the β-lactam ring of the drug. Gram-negative bacteria resistant to aminoglycosides, mediated by a plasmid, produce adenylating, phosphorylating, or acetylating enzymes that destroy the drug. Certain microorganisms develop an altered enzyme that can still perform its metabolic function, but is much less affected by the drug. For example, in trimethoprim-resistant bacteria, the dihydrofolic acid reductase is inhibited far less efficiently than in trimethoprim-susceptible bacteria.
- **Mutation (1):** Chromosome-mediated resistance occurs as a result of spontaneous mutation. This is caused by mutation in the gene that codes for either the target of drug or the transport system in the membrane of the cell wall, which controls the entry of drugs into cells. The frequency of chromosomal mutation is much less than the plasmid-mediated resistance. It varies between 10^7 and 10^9.
- **Transformation from free DNA (2):** Drug resistance is also mediated by transposons that often carry the drug resistance genes. Transposons are small pieces of DNA that move from one site of the bacterial chromosome to another and from bacterial chromosome to plasmid DNA.
- **Transduction from bacteriophage (3**): Bacteriophages are also capable of carrying plasmids, transposons and integrons (often carry the drug resistance genes), and so can facilitate their dissemination in this manner.
- **Conjugation from plasmid (4):** Plasmid-mediated resistance plays a very important role in antibiotics usage in clinical practice. This is because: A high rate of transfer of plasmids from one bacterium to another bacterium takes place by conjugation; Plasmids mediate resistance to multiple antibiotics; Plasmid-mediated resistance occurs mostly in Gram-negative bacteria.

For example, the genes code for enzymes like β-lactamases that destroy β-lactam ring (which is responsible for the antibactericidal action of β-lactam antibiotics, such as penicillins, carbapenems and cephalosporins).

2.2.2. Antibiotic Resistance of the *Enterobacteriaceae*

As soon as the penicillin was discovered, the β-lactam antibiotic resistance arose due to the production of an enzyme capable of hydrolase the β-lactam ring. In the 80s-90s *Enterobacteriaceae* β-lactam resistant strains have been found. These strains have been identified as Extended-Spectrum β-Lactamases (ESBL) species, responsible of severe infections since not sensible to antimicrobial therapy (Paterson e Yu 1999).

A wide range of β-lactams, aminoglycosides, quinolones, and other antibiotics are useful for treatment of Klebsiella infections.

Cephalosporins are widely used as monotherapy and in combination with aminoglycosides. Cephalosporins are not used for ESBL strains of K. pneumoniae. The carbapenems (imipenem, ertapenem, meropenem e doripenem), are effective against such ESBL strains (ESBLA and –M).

Aztreonam and quinolones are recommended for patients allergic to penicillin. Hand washing holds the key to prevent transmission from patient to patient via medical personnel. Contact isolation is useful for patients colonized or infected with highly antibiotic-resistant Klebsiella strains, such as ESBL-K. pneumoniae. Reduced membrane permability due to loss of porins and efflux pumps in combination with ESBL-production can however cause a carbapenem resistant phenotype. Carbapenemase-producing Enterobacteriaceae (CPE) hydrolyze also carbapenems, but not all CPE express high-level resistance. The use of a carbapenem in combination therapy against severe infections caused by CPE has shown improved outcome when carbapenem MIC-values are ≤ 4 and even up to 8 mg/L (Tumbarello M 2012) (Tangden 2015).

Resistance in Klebsiella pneumoniae – common intestinal bacteria that can cause life-threatening infections – to a last resort treatment (carbapenem antibiotics) has spread to all regions of the world (Patel e Bonomo 2011). K. pneumoniae is a major cause of hospital-acquired infections such as pneumonia, bloodstream infections, and infections in newborns and intensive-care unit patients. In some countries, because of resistance, carbapenem antibiotics do not work in more than half of people treated for K. pneumoniae infections (Kumarasamy, Toleman, et al. 2010).

2.3. Carbapenems

Carbapenems are β-lactam antibiotics, as are penicillins and cephalosporins, but differ from these other classes in their exact chemical structure. Carbapenem use has increased as a result of the rising resistance to cephalosporin antibiotics in Enterobacteriaceae (Escherichia coli, Klebsiella, Enterobacter, and related genera). This cephalosporin resistance is largely due to the spread of extended spectrum β lactamases (ESBLs), which hydrolyse cephalosporins (Hawkey e Jones 2009). ESBLs now occur in 10–12% of E. coli from bacteremia in the UK (Hawser, et al. 2011) and in 50–80% of those in India and China, with many ESBL producing strains also resistant to quinolones and aminoglycosides (Hsueh, et al. 2010). They possess the broadest spectrum of antibacterial activity covering most Gram-positive and Gram-negative aerobic and anaerobic bacteria and have an important role in the treatment against severe infections caused by ESBL-producing Enterobacteriaceae.

They are active against:

- Haemophilus influenzae
- Anaerobes
- Most Enterobacteriaceae (including those that produce ampC β-lactamase and extended-spectrum β-lactamase [ESBL], although P. mirabilis tends to have higher imipenem minimum inhibitory concentration (MICs)
- Methicillin-sensitive staphylococci and streptococci, including S. pneumoniae (except possibly strains with reduced penicillin sensitivity)

Most Enterococcus faecalis and many P. aeruginosa strains, including those resistant to broad-spectrum penicillins and cephalosporins, are susceptible to imipenem, meropenem, and doripenem but are resistant to ertapenem. However, meropenem and doripenem are less active against E. faecalis than imipenem. Carbapenems are active synergistically with aminoglycosides against P. aeruginosa. E. faecium, Stenotrophomonas maltophilia, and methicillin-resistant staphylococci are resistant. Many multidrug-resistant hospital-acquired bacteria are sensitive only to carbapenems. However, expanded use of carbapenems has resulted in some carbapenem resistance.

Imipenem and meropenem penetrate Central System F when meninges are inflamed. Meropenem is used for Gram-negative bacillary

meningitis; imipenem is not used in meningitis because it may cause seizures. Most seizures occur in patients who have Central nervous system (CNS) abnormalities or renal insufficiency and who are given inappropriately high doses.

Doripenem has a black box warning stating that when used to treat patients with ventilator-associated bacterial pneumonia, it has an increased risk of death compared with imipenem. Also, clinical response rates were lower with doripenem. Doripenem is not approved for the treatment of pneumonia.

2.3.1. Epidemiology of Klebsiella pneumoniae carbapenemases

The first strain recorded with a KPC enzyme, in 1996, was a K. pneumoniae isolate collected in a North Carolina hospital and submitted to the Centers for Disease Control and Prevention (CDC) through Project Intensive Care Antimicrobial Resistance Epidemiology (ICARE) (Yigit, et al. 2001).

Since their first description, (Yigit, et al. 2001) KPC enzymes have spread across countries and continents, although the exact epidemiology of their expansion varies by geographical location.

Bacteria producing these enzymes are generally only susceptible to a few antibiotics, and there is high mortality among patients with bloodstream infections caused by these organisms. Many bacteria with these enzymes remain susceptible to colistin, tigecycline, and one or more aminoglycoside, but some are resistant even to these drugs. Moreover, only a few drugs are in development against KPC-positive bacteria.

After the first case of KPC, a report describing KPC positive isolates from New York City hospitals from 1997 to 2001 have been described (Bradford, et al. 2004).

For the next few years, descriptions were largely confined to the north-eastern US, (Chiang, et al. 2007) (Pope, et al. 2006) (Hossain, et al. 2004) (Smith Moland, et al. 2003) but isolates have been detected in 40 states (C. f. CDC 2017).

In USA in 2012, fewer than 5% of short-stay acute-care hospitals reported infections with carbapenem resistant Enterobacteriaceae to the National Healthcare Safety Network of the CDC (C. f. CDC 2017) (C. f. CDC 2013).

The first known KPC enzyme in the UK was a KPC-4 variant in an

Enterobacter spp from a blood specimen in Scotland in 2003 (Livermor, et al. 2008); and the first recorded K pneumoniae producing a KPC enzyme was from Scotland too, in 2007 (Woodford, et al. 2008). KPC-positive isolates confirmed by the national reference laboratory remained rare and geographically scattered in 2008 and 2009, mostly cases with the source patients having travelled to Cyprus, Greece, or Israel (Public Health England 2011) (Roche, et al. 2009) (Morris, et al. 2012) (Health Protection Surveillance Centre 2017).

In Spain in 2009, eight cases colonised with KPC-3-positive K pneumoniae to occur in a health-care facility in Madrid, Spain (Curiao, et al. 2010). In late in 2009, a second outbreak was detected in Madrid, Spain, with three clonally related KPC-2-positive (Gómez-Gil, et al. 2010) (Poirel, Barbosa-Vasconcelos, et al. 2012).

The first clinical isolates with a KPC β-lactamase in France were from patient who have been travelling/admitted in hospital in endemic regions: New York, Israel, India and Greece (Naas, Nordmann, et al. 2005) (Dortet, et al. 2008) (Cuzon, Naas, et al. 2008) (Petrella, et al. 2008) (Potron, et al. 2012) (Naas, Cuzon e Gaillot, et al. 2011) (Rogers, Sidjabat e Paterson 2011).

From 2009 KPCs were detected from patients did not have any relevant travel history and several nosocomial clusters have been reported, with one of them associated with a contaminated endoscope (Cuzon, Naas, et al. 2012) (Naas, Cuzon e Babics, et al. 2010).

The first KPC-positive K pneumoniae isolates linked to Greece were identified in 2007 in France, United Kingdom and Sweden (Cuzon, Naas, et al. 2008) (Tegmark Wisell, et al. 2007). Afterwards in 2 years from the first exported cases, KPC-positive K pneumoniae had disseminated into most acute-care facilities in Greece (Maltezou, Giakkoupi, et al. 2009) (Pournaras, et al. 2009) (Souli, et al. 2010) (Kontopoulou, et al. 2010) (Zarkotou, et al. 2011) (Maltezou, Kontopidou, et al. 2014) (Mavroidi, et al. 2012) (Wernli, et al. 2011) (Van der Bij e Pitout 2012) (Giakkoupi, et al. 2011) (Giakoupi, et al. 2009) (WHONET 2017).

In Italy, the first KPC-positive K pneumoniae was isolated in 2008 from an inpatient with a complicated intraabdominal infection in Florence (Giani, D'Andrea, et al. 2009). The isolate had KPC-3 enzyme, with the corresponding gene located in transposon Tn4401, which has been described in Israeli ST258 isolates. After that KPC-2- positive K

pneumoniae strain were detected in Rome with no travel history (Fontana, et al. 2010).

Active surveillance was done in two hospitals in Padua from 2009 to 2011, and almost 200 cases were identified (Richter, et al. 2012).

The initial epidemiological pattern entailed dissemination of KPC-3-positive K pneumoniae ST258 and KPC-2-positive K pneumoniae ST14. Subsequently, the former lineage prevailed and spread from ICUs to medical, surgical, and long-term wards (Richter, et al. 2012). Simultaneously, seven clonally related KPC-3-positive K pneumoniae ST258 isolates were identified from wound cultures of different patients in a surgical ICU in Verona (Mazzariol, et al. 2012) and, horizontal transmission of colistin-resistant KPC-3-positive K pneumoniae was described in different wards of an acute general hospital in Palermo in 2011 (Mammina, et al. 2012).

KPC-positive K pneumoniae have spread rapidly and extensively in Italy, with a sharp increase reported by the EARS-Net surveillance system (E. C. ECDC 2017) for bacteraemia isolates, from 1–2% carbapenem resistance in 2006–09 to 30% in 2011, and by the Micronet surveillance network, (Sisto, et al. 2012) from 2% in 2009 to 19% in 2012. Infection control interventions at the national level are scarce, with only a few reports of local containment.

The European monitoring of K. *pneumoniae* KPC in the European countries from European Center for Disease Prevention and Control (ECDC) highlighted a great concern for the Italian situation. In Italy, the percentage of KPC ranged from 2% in 2006, to 35,7 % in 2015; this indicated Italy as the most affected countries of carabapenems resistant in Europe with Greece (Albiger, et al. 2015) (Figure 3).

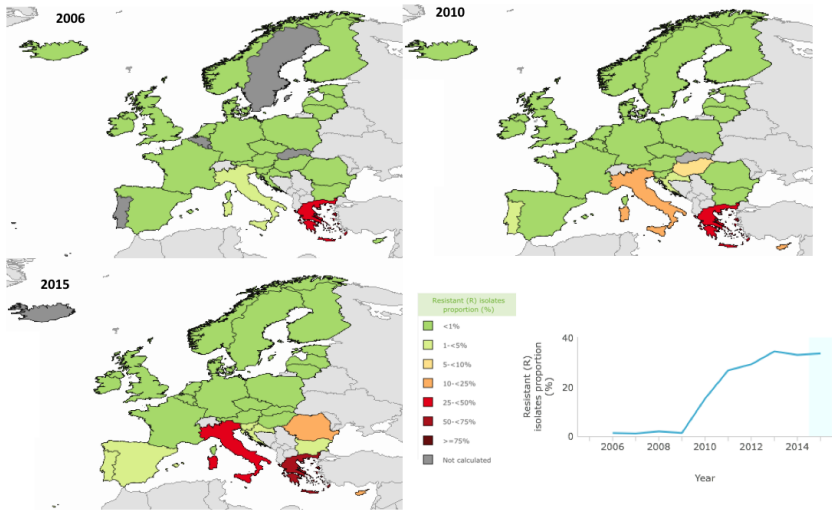

Fig. 3. Data from the ECDC Surveillance Atlas - Antimicrobial resistance. Percentage distribution of Klebsiella pneumoniae isolates not susceptible of carbapenems of European countries. In Italy 2% (2006), 15,9 % (2010), 35,7 % (2015). (https://ecdc.europa.eu/en/antimicrobial-resistance/surveillance-and-disease-data/data-ecdc)

2.3.2. Amber classes

The carbapenemases belong to the Ambler class A, B and D, divided in serine carbapenemases (Ambler class A or D) and metallo-carbapenemases (Ambler class B enzymes) (Table 4).

Tab. 4. Amber classes

Class of ESBL	Amber class	Enzymes	Phenotypic test	Areas of endemicity (Nordmann, Cuzon e Naas 2009) (Nordmann, Naas e Poirel 2011) (Miriagou, et al. 2010)
$ESBL_A$	A	CTX-M, TEM/ SHV-ESBL	Inhibited by clavulanic acid	Worldwide
$ESBL_M$	C	Plasmid-AmpC mostly CMY	Inhibited by cloxacillin	
$ESBL_{CARBA}$	A	KPC	Synergy with boronic acid	Greece, Italy, Israel, Northern America, China
	B	NDM, IMP	Synergy with EDTA	Indian subcontinent, the Balkans, Middle East
		VIM		Greece
	D	OXA-48	None available, termocillin R	Northern Africa, the Middle East

Class A, C and D β-lactamases have a serine residue in the catalytic site. Class A enzymes like class D are inhibited by clavulanic acid.

Class A enzymes include several proteins with distinctive catalytic activities: penicillinases (TEM-1 and SHV-1 that only hydrolyze penicillin), ESBLs (such as CTX-M) to carbapenemases like KPC.

CTX-M is a plasmid-encoded ESBL generally in K. pneumoniae, E. coli, and other Enterobacteriaceae worldwide; other Ambler class A ESBLs like TEM-3, did not originate from TEM or SHV, but likely acquired from Kluyvera spp. through Horizontal Gene Transfer (HGT) (Bonnet 2004). Insertion sequences (ISEcp1) and transposable elements such as Tn402-like transposons are associate with genes encoding CTX-M enzymes. These mobile elements (MGEs) may have been spread through plasmids or phage-like sequences (Poirel, Lartigue, et al. 2005). CTX-M enzymes have become the most prevalent ESBL worldwide and are responsible for a large proportion of cephalosporin resistance in E. coli and K. pneumoniae.

Five different families of class A carbapenemases have been reported, three are typically chromosomally encoded (IMI, imipenem-hydrolyzing enzyme, SME, Serratia marcescens enzyme, and NMC,

not-metallo-enzyme carbapenemase), and the remaining two (KPC and GES) are classically in plasmids or other MGEs (Queenan e Bush 2007).

Class B enzymes are also known as metallo- β-lactamases due to the use of a metal ion (most usually Zinc) as cofactor (instead of a serine residue) for the nucleophilic attack of the β-lactam ring. They are inhibited by the presence of ion-chelating agents such as EDTA and, similar to class A carbapenemases, they are active against some β-lactams, as carbapenems.

Metallo-β-lactamases efficiently hydrolyze cephamycins, aztreonam is typically a poor substrate. These enzymes were discovered about in 1940/50s translated by genes located in the chromosome of non-pathogenic bacteria. But, the situation dramatically changed in 1990s, when enzymes like IMP and VIM were progressively identified in clinical strains of Enterobacteriaceae, Pseudomonas spp. and Acinetobacter spp (Queenan e Bush 2007). Indeed, genes encoding these enzymes have been found as part of the accessory genome of pathogenic bacteria suggesting HGT. There are about 10 types of metallocarbapenemases, but most of the clinically important ones are: IMP, VIM, SPM and NDM.

The first IMP-type enzymes were described in Japan in the early 1990s in S. marcescens, and since then, more than 20 different subtypes have been described worldwide in Enterobacteriaceae, Pseudomonas spp., and Acinetobacter spp, among other organisms. The blaIMP genes have been found on plasmids and forming the class 1 integrons (Poirel, Pitout e Nordmann 2007). Regarding the VIM-type enzymes, they were first described in the late 1990s in Verona, Italy (Verona integron-encoded metallo β-lactamase) and have since spread worldwide. These enzymes were initially found in P. aeruginosa, but they have been found in different types of MGEs in bacterial species becoming a major concern worldwide. VIM-2 is the most widely distributed enzyme, identified in Europe, Asia, Africa, and the Americas (Cornaglia, Giamarellou e Rossolini 2011).

In 2008, a new carbapenemase was identified in a K. pneumoniae isolate recovered from a Swedish patient who had been previously admitted to a hospital in New Delhi, India. The enzyme was designated NDM-1, in reference to its origin (New Delhi Metallo β-lactamase) (Kumarasamy, Toleman, et al. 2010). NDM-1 shares little amino acid

identity with other members of the Ambler class B enzymes (e.g. 32% with VIM-1), but its hydrolytic profile is very similar to all of them. The blaNDM gene has been found in several types of plasmids transferable among different species of Gram-negatives, and it has also been associated with the presence of insertion sequences such as the ISAba125. In contrast to other genes encoding metallo-enzymes, blaNDM is not usually related to integron-like structures (Nordmann, Poirel, et al. 2011). Nevertheless, NDM-1 rapidly spread around the globe, becoming a prime example of how a resistance determinant can readily disseminate worldwide despite many efforts to avoid its transmission. Moreover, MGEs-containing genes coding for NDM enzymes generally carry multiple other resistance determinants such as genes encoding other carbapenemases (e.g. VIM-type and OXA-type enzymes), ESBL, AMEs, methylases conferring resistance to macrolides, the quinolone resistance Qnr protein, enzymes that modify rifampin and proteins involved in resistance to sulfamethoxazole, among others. Thus, the presence of NDM-1 is frequently accompanied by a multidrug-resistant phenotype.

The emergence of NDM-1 is particularly concerning because the blaNDM gene has shown to be readily transmissible among different types of Gram-negative organisms, spreading to many countries in a short span of time and becoming one of the most feared resistance determinants in several parts of the world (Cornaglia, Giamarellou e Rossolini 2011). In addition, in the Indian subcontinent (i.e. India and Pakistan), the blaNDM gene is not only extensively disseminated among nosocomial pathogens, but it is frequently found in community-associated isolates. Furthermore, several reports have found NDM-1 producing Gram-negative bacteria in the soil and drinking water for human consumption, suggesting that these genes may be disseminating through the human microbiota (Walsh, et al. 2011).

Class C β-lactamases confer resistance to all penicillins and cephalosporins (although cefepime is usually a poor substrate), including cephamycins. The most clinically relevant class C enzyme is AmpC, which is a cephalosporinase that is generally encoded on the chromosome (although the blaAMPc gene has also been found in plasmids) (Jacobs, Frère e Normark 1997) (Johnson, Fisher e Mobashery 2013).

Class D β-lactamases include a wide range of enzymes that were initially differentiated from the class A penicillinases due to their

ability to hydrolyze oxacillin (hence their name) and because they were poorly inhibited by clavulanic acid. Many OXA variants have been reported, including enzymes capable to degrade third generation cephalosporins (ESBLs) (e.g., OXA-11 from P. aeruginosa) and carbapenems (e.g., OXA-23 from A. baumanii). For example, OXA-48 (class D carbapenemase) was described in 2001 in Turkey from a MDR isolate of K. pneumoniae. OXA-48 and its variants are now widely spread in clinical isolates of K. pneumoniae and other Enterobacteriaceae, and have also been found in A. baumanii (Evans e Amyes 2014).

Even if Class D enzymes are prevalent in A. baumanii, they have been reported in other organisms, such as E. coli, Enterobacter spp., K. pneumoniae and P. aeruginosa, among others, enzymes like OXA-23 and OXA-58 currently being spread worldwide.

3. Nosocomial infections

Nosocomial is used to indicate any disease developed by patient under hospital care (Khan, Ahmad e Mehboob 2015); it is a patient infection acquired during hospital stay and occurring 48 hours after admission. Furthermore, healthcare *associated infections* (HAI) is used for the type of infections caused by long hospital stay and it could be a major risk factor for serious health issues leading to death (Brusaferro, Arnoldo, et al., Harmonizing and supporting infection control training in Europe 2015). About 75% of the burden of these infections is present in developing Countries (Obiero, Seale e Berkley 2015). Based on extensive studies in USA and Europe shows that HAI incidence density ranged from 13.0 to 20.3 episodes per thousand patient-days (Allegranzi 2011). The infection may be considered also from asymptomatic patients if these pathogens are found in the body fluids or at a sterile body site, such as blood or cerebrospinal fluid (Murray, Rosenthal e Pfaller 2010).

Infections that are acquired by hospital staff, visitors or other healthcare personnel may also be considered as nosocomial infections (Lolekha, Ratanaubol e Manu 1981).

The situations in which infections are not believed as nosocomial are:

- The infections that were present at the time of admission and become complicated, nevertheless pathogens or symptoms change

resulting to a new infection;
- The infections that are acquired trans-placentally due to some diseases like toxoplasmosis, rubella, syphilis or cytomegalovirus and appear 48 h after birth (Festary, et al. 2015).

HAI appeared before the origination of hospitals and became a health problem during the miraculous antibiotic era. Due to these infections, not only the costs but also the use of antibiotics increased with an extended hospitalization. This resulted in elevated morbidity and mortality.

The CDC estimated that the cost of events related to NIs was an average of $2,100, and varied from $680 for urinary tract infections to $5,683 for respiratory tract infections in the United States of America (Abramczyk, et al. 2003) (Kouchak e Askarian 2012).

NIs can be caused by any organisms but few organisms are particularly responsible for hospital-acquired infections.

National Healthcare Safety Network with Center for Disease Control (CDC) for surveillance has classified nosocomial infection sites into 13 types, with 50 infection sites, which are specific on the basis of biological and clinical criteria. The sites which are common include urinary tract infections (UTI), surgical and soft tissue infections, gastroenteritis, meningitis and respiratory infections (Raka, et al. 2006). A change regarding nosocomial infection sites can be easily detected with time due to the elevated use of cancer chemotherapy, advancement in organ transplantation, immunotherapy and invasive techniques for diagnostic and therapeutic purposes. The perfect example of this can be seen in the case of pneumonia as prevalence of nosocomial pneumonia increased from 17% to 30% during five years (Duque, et al. 2007).

The four main categories of nosocomial infections are (CDC. 2014):
- **Central Line-associated Bloodstream Infection (CLABSI**): A central line-associated bloodstream infection (CLABSI) is a severe infection that occurs when microorganisms (usually bacteria or viruses) enter the bloodstream through the central line. Hospital staff must follow a strict protocol when inserting the line to make sure the line remains sterile and a CLABSI does not occur. In addition is important to apply a rigorous infection control practice each time they check the line or change the dressing. The CLABSI symptoms are fever, red skin and soreness around the central line. If any

symptoms appear it is fundamental to test if there is an infection present. CLABSIs are deadly nosocomial infections with the death incidence rate of 12%–25% (C. f. CDC 2011).

- **Catheter-associated Urinary Tract Infections (CAUTI):** A urinary tract infection (UTI) is an infection involving any part of the urinary system (urethra, bladder, ureters, and kidney). UTIs are the most common type of HAI reported in nosocomial environment. Among UTIs acquired in the hospital, approximately 75% are associated with a urinary catheter. Between 15-25% of hospitalized patients receive urinary catheters during their hospital stay. The most important risk factor for developing a catheter-associated UTI (CAUTI) is prolonged use of the urinary catheter. Therefore, catheters should only be used for appropriate indications and should be removed as soon as they are no longer needed. According to acute care hospital stats in 2011, UTIs account for more than 12% of reported infections (CDC. 2017).
- **Surgical Site Infection (SSI):** A surgical site infection is an infection that occurs after surgery in the part of the body where the surgery took place. Surgical site infections can sometimes be superficial infections involving the skin only. Other surgical site infections are more serious and can involve tissues under the skin, organs, or implanted material. CDC provides guidelines and tools to the healthcare community to help end surgical site infections and resources to help the public understand these infections and take measures to safeguard their own health when possible. The incidence may be as high as 20% depending upon procedure and surveillance criteria used (Owens 2008).
- **Ventilator-associated Pneumonia (VAP):** ventilator-associated pneumonia is a lung infection that develops in a person who is on a ventilator. An infection may occur if germs enter through the tube and get into the patient's lungs. CDC provides guidelines and tools to the healthcare community to help end ventilator-associated pneumonia and resources to help the public understand these infections and take measures to safeguard their own health when possible. VAP is nosocomial pneumonia found in 9–27% of patients on mechanically assisted ventilator (Hunter 2012).

Nosocomial infection affects huge number of patients globally,

elevating mortality rate and financial losses significantly.

According to estimate reported of WHO, approximately 15% of all hospitalized patients suffer from these infections (Emily e Sydnor 2011). These infections are responsible for 4%–56% of all death causes in neonates, with incidence rate of 75% in South-East Asia and Sub-Saharan Africa (WHO. 2017). The incidence is high enough in high income countries i.e. between 3.5% and 12% whereas it varies between 5.7% and 19.1% in middle and low income countries.

The frequency of overall infections in low income countries is three times higher than in high income countries whereas this incidence is 3–20 times higher in neonates (Nejad, et al. 2011).

Nosocomial infections are caused by many microbes and each one can cause infection in healthcare settings. Bacteria are responsible for about ninety percent infections, whereas protozoans, fungi, viruses and mycobacteria are less contributing compared to bacterial infections (Gatermann, et al. 2005).

Three to seven percent of hospital-acquired bacterial infections are related to K. pneumonia, which is the eighth significant pathogen in healthcare settings (Lin, et al. 2015).

In hospital settings, K. pneumonia can be transmitted by person-to-person contact and especially when healthcare professionals do not wash or clean hands after checking a contaminated patient. Respiratory machines, catheters or exposed wounds can be the source of its transmission.

K. pneumoniae is reported to be transmitted through stool (77%), patients' hands (42%) and pharynx (19%) (Lin, et al. 2015). Resistance to ß-lactam antibiotics is a major cause of complications in nosocomial infections.

3.1. Control of nosocomial infections

MDROs make it extremely difficult to devise a proper plan and its implementation for control (Obiero, Seale e Berkley 2015). Guidelines for the sterilization and disinfection of invasive devices and medical instruments used for surgeries were developed as the infection rates tend to raise (Patterson 2009) (Rutala, Weber e SHEA 2010) (CDC. 2016). Lack of compliance with the guidelines, leads to the transmission of nosocomial infections.

CDC provides the methodology for surveillance of nosocomial

infections along with investigation of major outbreaks. Infection prevention and control guidelines have been developed but the implementation is not yet much known (Stone, et al. 2015). Training of healthcare professionals, especially nurses and medical doctor, is extremely important for the control and prevention of infection (Brusaferro, Arnoldo, et al. 2015) (Pegram e Bloomfield 2015).

To control the spread of nosocomial infectious there are three levels where it is possible to act (Khan, Baig e Mehboob 2017) (WHO. 2014):

- Environment: where the care is delivered such as inadequate hygienic conditions and waste disposal.
- Susceptibility and condition of the patient: such as the immunosupression intrinsic of the patients, or due to the protracted use of antibiotics, and lengthy stay in intensive care unit.
- The lack of awareness of such prevailing infections among staff and health care providers. This could be due to improper use of injection techniques, poor knowledge of basic infection control measures, inappropriate use of invasive devices (catheters) and lack of control policies (Chand Wattal 2014). In low income countries, these risk factors are associated with poverty, lack of financial support, understaffed health care settings and inadequate supply of equipment (Allegranzi 2011).

There is the need to develop new diagnostics and tools in healthcare institutes to contrast the evolving resistance (WHO., WHO's first global report on antibiotic resistance reveals serious,worldwide threat to public health 2014).

To better improve the hospital environment, there are numerous prevention campaigns, such as i.e. hand hygiene or the correct disinfection also for the patients (Figure 4).

Fig. 4. Prevention campaigns (CDC).

To act on the immunosuppression due from the long stay or antibiotic therapy there are guidelines about the correct use of the antibiotic therapy and to try to minimize the stay in intensive care unit.

Antimicrobial use should justify the adequate clinical diagnosis or an infection causing microorganism. CDC estimates that each year about 100 million courses of antibiotics is prescribed by office-based physicians, while approximately 50% of those are unnecessary (Colgan 2001). The selection of antimicrobials should be based upon the patient's tolerance in addition to the nature of disease and pathogen.

The aim of antimicrobial therapy is to use a drug that is selectively active against most likely pathogen and least likely to cause resistance and adverse effects (Ducel, Fabry e Nicolle 2002). Antimicrobial prophylaxis should be used when it is appropriate i.e. prior to surgery, to reduce postoperative incidence of surgical site infections. In case of immunocompromised patients, prolonged prophylaxis is used until immune markers are reinstate (Leekha e Edson 2011).

The unregulated use of antibiotics could cause antibiotic resistance that is responsible for the death of a child every five minutes in South-East Asia region. Drugs that were used to treat deadly diseases are now losing their impact due to emerging drug resistant microorganisms (Kavanagh, Calderon e Saman 2015). Self-medication with antibiotics, incorrect dosage, prolonged use, lack of standards for healthcare workers and misuse in animal husbandry are the main factors responsible for increase in resistance.

To improve the awareness of the nosocomial infection in the 70s were introduce in the hospital the National Nosocomial Infection Surveillance System (NNIS) coordinated from CDC.

Despite there is a large gap between the existence of guidelines and their actual implementation (Menegueti, et al. 2015), some studies emphasize the importance of early identification of asymptomatic carriers through the adoption of the guidelines (Munoz-Price, Poirel, et al. 2013) (Munoz-Price e Quinn 2013).

3.2. Surveillance of nosocomial infections

Surveillance can be interpreted as "the ongoing, systematic collection, analysis, and interpretation of health data essential to the planning, implementation and evaluation of public health practice, closely integrated with the timely dissemination of these data to those who need to know" (C. f. CDC 2012). As a part of infection control program, surveillance collect the data of infected individuals with their infection sites. Hospital staff can work on this data to control the infections by evaluating the efficacy of treatment. For this purpose, hospitals can adopt a strategy comprising of infection control practices (Kavanagh, Calderon e Saman 2015).

To eradicate nosocomial infections, the surveillance should be active coordinate by infection control center in each hospital using the guidelines provided from CDC or ECDC.

The efficient surveillance methods include data collection from multiple sources of information by trained data collectors; information should include administrative data, demographic risk factors, patients' history, diagnostic tests, and validation of data. Following the data extraction, analysis of the collected information should be done which includes description of determinants, distribution of infections, and comparison of incidence rates. Feedback and reports after analysis

should be disseminated by infection control committees, management, and laboratories keeping the confidentiality of individuals. The evaluation of credibility of surveillance systems is required for effective implementations of interventions and its continuity. Finally, the undertaking of data at regular intervals for maintenance of efficiency of surveillance systems should be made compulsory (Aitken 2001). A methodology for a surveillance approach is suggested by (Khan, Baig e Mehboob 2017) (Figure 5).

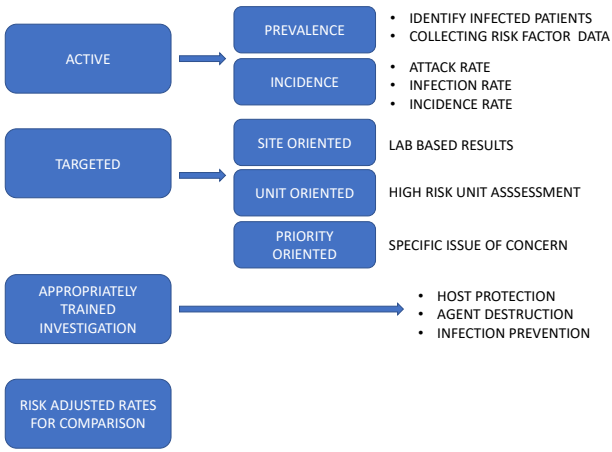

Fig. 5. Organization for efficient surveillance.

3.3. Duodenoscope-Related Infections

The devices used in clinical practice are real vehicles of nosocomial infection (Collins 2008).

Duodenoscopes (specialized endoscopes used for endoscopic retrograde cholangiopancreatography [ERCP]) is the main vehicles of patient-to-patient transmission and since 2014, these have been investigated for carbapenem-resistant Enterobacteriaceae infections by the Center for Disease Control (CDC) and the US Food and Drug Administration (FDA) consequently to outbreaks occurring across the United States (CDC. 2014) (Epstein, et al. 2014) (FDA. 2015) (FDA. 2015) (FDA. 2015).

During gastrointestinal endoscopy, endoscopes are exposed to millions of bacteria that constitute the gut flora. Unlike "critical" devices (such as laparoscopes), which require sterilization because they breach

sterile tissue planes, endoscopes are currently categorized as "semi-critical" devices (Spaulding 1971), which require mechanical cleaning followed by high-level disinfection (HLD). Flexible endoscopes are semi-critical devices that can't undergo a steam-sterilization (Figure 6).

Category	Patient Contact	Reprocessing Step	Expectation
Non-critical	Intact skin	Cleaning and/or low or intermediate level disinfection	Sterility is unnecessary for safe reuse
Semi-critical	Intact mucous membranes or non-intact skin	Cleaning followed by sterilization or high level disinfection if sterilization is not practicable	High Level Disinfection (HLD): should be free of microbes unless contaminated with high levels of bacterial spores
Critical	Bloodstream or normally sterile tissue or body space	Cleaning followed by sterilization	Free of all viable organisms

Fig. 6. Reusable device classification.

Manufacturers of endoscopes and automated endoscopes reprocessors provide instructions for use describing the requisite steps for reprocessing. They are obligated to show that their protocols result in a 6-log10 reduction in Mycobacterium, a surrogate marker for elimination of risk for scope-to-person transmission of infectious agents. In the absence of endoscope damage, automated endoscope reprocessor malfunction, or technical breaches in reprocessing, transmission of infectious agents via endoscopy has been exceedingly uncommon with standard endoscopes used for upper endoscopy and colonoscopy, which have relatively uncomplicated mechanical designs.

In contrast, duodenoscopes used for ERCP possess mechanical features on their distal (insertion) tip. These endoscopes have an elevator mechanism that enables the endoscopist to angle a catheter into the biliary ducts, facilitating such interventions as removal of bile duct stones, treatment of cholangitis, and sampling of tissue. Some duodenoscopes have O-rings, seals, and a wire cable that actuates the elevator, further complicating mechanical cleaning. Nevertheless, endoscopists have presumed that following manufacturers' reprocessing instructions will eliminate the risk for transmission of infectious agents during ERCP. Unfortunately, recent events suggest otherwise.

Microbial outbreaks by carabapenem-resistant Enterobacteriacae (CRE) linked to duodenoscope use in clinical practice represents an emerging problem (Verfaillie, et al. 2015) (Epstein, et al. 2014) (Wendorf, et al. 2015) (Kola, et al. 2015); these infections were similar to other ERCP-related infections dating back to at least the 1980s (Allen, et al. 1987). Nowadays there is an increased of this type of infections.

Endoscopes can be considered the main vehicles of patient-to-patient transmission of infections respect to any reusable medical device in healthcare due to the poor adherence to reprocessing guidelines.

The main manufactures involved in these infections are Olympus, Pentax and Fujifilm (Rubin e Murthy 2016).

Side-viewing duodenoscopes have a complex architecture for the presence of a recess under the elevator difficult to reach during the disinfection making the process inadequate for complete bacterial decontamination.

In several reports, endoscope and its elevator structure have been involved in bacterial infections consequently to ERCP procedure. The evidence of ERCP-related infections suggested that improved microbiological surveillance and endoscope design optimization could represent valid tools to assure patient safety (Verfaillie, et al. 2015) (Aumeran, et al. 2010) (Gastmeier e Vonberg 2014) (Alrabaa, et al. 2013) (Epstein, et al. 2014) (Humphries e McDonnell 2015) (Petersen 2015).

To contrast these CRE outbreaks in United States and Europe consequently to ERCP encouraged Food and Drug Administration (FDA) and the CDC to produce new guidelines for duodenoscopes cleaning, reprocessing with particular care for the elevator mechanisms and for microbiological surveillance. (CDC. 2015) (F. a. FDA 2016).

Recently, FDA concluded that effective duodenoscope reprocessing can be impaired by its own complex structure since related outbreaks occurred even in case of respect of manufacturer's instruction and guidelines (FDA. 2015).

The evidence that outbreaks were related to multidrug-resistant (MDR) microorganism contributed to exacerbate the actual panorama arousing attention from the scientific community (Gastmeier e Vonberg 2014) (Ben-David, et al. 2012).

The most frequent MDR pathogen involved in the described outbreaks was Klebsiella pneumoniae and duodenoscopes cultures were positive even after reprocessing in two-third of cases (US Senate 2016)

(FDA 2017). Antegrade sampling of all duodenoscopes did not identify microbial contamination. Only by culturing samples from the recess under the forceps elevator of duodenoscopes VIM-2-producing P. aeruginosa strain was detected. This means that the disinfection procedure used to clean endoscopes is not always effective, and that microbiological surveillance of an endoscope is not always able to reveal microbial contamination. A standardized protocol according to guidelines for duodenoscopes sampling and surveillance cultures is available (SFERD 2011).

This sampling technique involves the use of sterile saline solution to irrigate the channels of the endoscopes. Endoscopes are sampled by injecting the channels with 120mL of neutralizing pharmacopoeia diluent buffer and sodium thiosulfate using sterile connectors (CTINILS 2007).

FDA and CDC suggested additional preventive measures to decrease the risk of transmission of MDR pathogens and guarantee patient's safety. At this aim in August 2015 FDA provided a list of procedures improving duodenoscopes disinfection and surveillance such as by ethylene oxide sterilization, high-level disinfection achieved by liquid sterilants, microbiologic sampling of duodenoscopes and repeated cycles of high-level disinfection (U. F. FDA 2015). Sterilization (rather than HLD) of duodenoscopes might provide a greater microbial safety margin, but current duodenoscope materials are damaged by heat-based and some chemical-based sterilization methods. Some facilities have begun to use gas sterilization using ethylene oxide, but this involves carcinogens and is time-consuming, expensive, and not universally available. Disconcertingly, sterilization may be ineffective if mechanical disruption of biofilm on the endoscope is incomplete.

A biofilm is an assemblage of microbial cells that is irreversibly attached to a surface and enclosed in a matrix of exopolymeric substances (Donlan e Costerton 2002). A typical biofilm will contain around 85% polymeric substances and only 15% bacterial mass, and cells are located in matrix-enclosed "towers" and "mushrooms" (Chicurel 2000) (O'Toole, Kaplan e Kolter 2000).

Bacteria growing within biofilms have a number of characteristics that distinguish them from planktonic populations. The ability to form biofilms allows microorganisms to survive under conditions of drying and chemical and antibiotic exposure (Costerton, et al. 1995) (R. Patel

2005). Under adverse conditions, biofilms are capable of releasing their bacterial population into a planktonic state. During endoscopy, the environment provides optimal conditions for contamination and subsequent growth of biofilms.

Endoscope manufacturers recognize the demand for a solution and are discussing low-heat chemical sterilization options and development of duodenoscopes that are heat-resistant (autoclavable) or even partially or completely disposable.

Such options will not be available for years, so the CDC recommends that health care facilities consider establishing duodenoscope surveillance programs using an unvalidated protocol to identify and remove contaminated duodenoscopes from use until they are shown to be decontaminated (CDC. 2015).

Endoscope drying has been described as one of the most important steps in limiting bacterial proliferation and the risk of contamination during endoscope storage. This is particularly true for P. aeruginosa, which develops in a wet environment. The use of storage cabinets reduces microbial contamination of most endoscopes except for duodenoscopes (Saliou, et al. 2015).

To better advance the duodenoscope cleaning and disinfection, the complex architecture of the duedonoscope should be redesigned to facilitate more efficient disinfection, but enhancement in microbial contamination assessing is necessary (Verfaillie, et al. 2015).

Currently, no standard protocols have been adopted by the different healthcare facilities and prevention measure vary among them. In some cases, a microbiologic screening to identify colonized patients has been offered, in other cases deep sterilization by ethylene oxide has been added to the most common high level disinfection protocols. Furthermore, some facilities introduced duodenoscope microbiologic screening and quarantine if specific criteria are not met, following CDC indications (CDC., Interim Protocol for Healthcare Facilities Regarding Surveillance for Bacterial Contamination of Duodenoscopes after Reprocessing 2015).

Duodenoscopes are complex instruments that contain many small working parts. If not thoroughly cleaned and disinfected, tissue or fluid from one patient can remain in a duodenoscope when it is used on a subsequent patient. In rare cases, this can lead to patient-to-patient infection (FDA 2017).

After the Centers for Disease Control and Prevention (CDC) alerted the FDA to a potential association between multi-drug resistant bacteria and duodenoscopes, several manufacturer duodenoscopes sent Warning Letters describing violations (Olympus, Fuji, Pentax).

Regarding microbiologic testing, the CDC surveillance protocol provides aseptic sampling after duodenoscope processing following defined interval times, leaving the device in quarantine for 48-72 hours until absence of microbial growth has not been guaranteed (CDC. 2015).

Standardized protocols outlining bacterial growth levels and bacterial identification determine whether the endoscope can be safely used (U. F. FDA 2015).

Despite a seemingly extensive track record, no many data about this "culture and hold" approach are available because it was not largely adopted for lack of uniform and standardized procedures and for increased costs (Rutala e Weber, Reprocessing endoscopes: United States perspective 2004) (ASM 2015). As demonstrated in outbreaks where bacteria were not recovered from implicated duodenoscopes, a negative culture result does not eliminate this instrument as a potential source. Creating further uncertainty, the American Society of Microbiology recommended in April 2015 that clinical laboratories should not perform cultures from duodenoscopes given the lack of validated test characteristics for these cultures and concerns about some technical aspects of the CDC protocol (ASM 2015). Although some hospitals have chosen to perform endoscope cultures in their clinical laboratory despite these concerns, others have outsourced the testing, adding logistical complexity, cost, and increased endoscope turn-around time, or have chosen to forgo the testing entirely.

Additionally, the CDC protocol's undefined testing interval is problematic, causing potential variation among different hospital. It is not clear if the sampling should be performed after each endoscope use, or monthly.

There is the need to eradicate the possibility to have infection using these devices, especially improving the real-time microbial biofilm detection and identification using micro-probe able to visualize the possible biofilm presence even after adequate protocol of disinfection, drying and surveillance culture. This could increase the microbial contamination assessment.

4. Bioinformatics

Searching the definition of bioinformatics in Oxford English dictionary: "bioinformatics is conceptualizing biology in terms of molecules (in the sense of physical chemistry) and applying "informatics techniques" (derived from disciplines such as applied maths, computer science and statistics) to understand and organize the information associated with these molecules, on a large scale. In short, bioinformatics is a management information system for molecular biology and has many practical applications".

Due to the increasing amount of biological data (nucleotidic and proteic sequences and scientific publications), computers have become essential to biological research.

Bioinformatics is an interdisciplinary scientific field of life sciences. Bioinformatics research and application include the analysis of molecular sequence and genomics data; genome annotation, gene/protein prediction, and expression profiling; molecular folding, modeling, and design; building biological networks; development of databases and data management systems; development of software and analysis tools; bioinformatics services and workflow; mining of biomedical literature and text; and bioinformatics education and training (Abdurakhmonov 2016).

Between the numerous aims, the mains are (Luscombe, Greenbaum e Gerstein 2001):

- To organize in the simplest way to allow all the researchers to be able to search information and to submit new ones.
- To develop tools and resources that help in the analysis of data; requiring expertise in both computational sciences and in biology.
- To be capable to use these tools to analyze the data and be able to understand the data in a biologically meaningful way.

4.1. Next-Generation Sequencing

The genome sequence gives a general framework for assembling fragmentary DNA information into setting of biological structure and function (Lander 2011).

Next Generation Sequencing (NGS) technologies have continued to evolve — increasing capacity by a factor of 100–1,000 (Kircher e Kelso

2010)— and have integrated revolutionary innovations to tackle the complexities of genomes. These advances are providing read lengths as long as some entire genomes, they have brought the cost of sequencing a human genome down to around US$1,000 (as reported by Veritas Genomics) (Veritas Genetics 2016), and they have allowed the use of sequencing as a clinical tool (Wetterstrand 2016) (Veritas Genetics 2016).

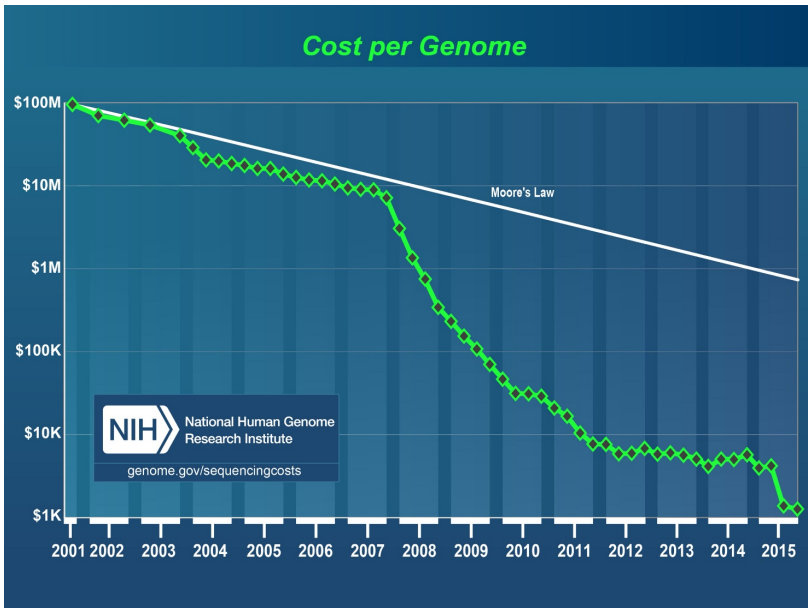

Fig. 7. Cost per Genome. (Courtesy: National Human Genome Research Institute: The Cost of Sequencing a Human Genome *https://www.genome.gov/27565109/the-cost-of-sequencing-a-human-genome/)*.

Each sequencing technology has its characteristics, but all machines share some common features (Mardis 2011). The initial preparatory steps are condensed and easy. After that the amplification of the library fragments is essential and sequencing reactions are performed and detected mechanically.

The silver lining of the new technologies is the possibility to analyze all the amount of data contained in a organism but existing problems are worsened or new problems arise. The related error rates (~0.1–15%) are higher and the read lengths generally shorter (35–700 bp for short-read approaches) (L. Liu, et al. 2012) than those of

traditional Sanger sequencing platforms, demanding an adequate checking of the results, especially for Single Nucleotide Polymorphisms (SNPs) detection and clinical applications.

For NGS, it is possible to recognize the second and third generation sequencing technologies (Roumpeka, et al. 2017); these are two main groups for characterize the NGS machineries:

- **Short-read NGS (second generation sequencing):** The Short-read sequencing approaches have two broad categories: sequencing by ligation (SBL) (SOLiD and Complete Genomics) and sequencing by synthesis (SBS) (CRT (Illumina, Qiagen), SNA (454, Ion Torrent)) (Goodwin, McPherson e McCombie 2016). Second-generation sequencing includes technologies such as Illumina and Ion Torrent that produce many millions of short reads (150–400 bp);
- **Long-read NGS (third generation sequencing)**: there are two main types of long-read technologies: single-molecule real-time sequencing approaches (PacBio and ONT) and synthetic approaches (Illumina synthetic long-read sequencing platform and the 10X Genomics emulsion-based system) that rely on existing short-read technologies to construct long reads in silico (Goodwin, McPherson e McCombie 2016). This kind of technology produces longer reads (6–20 kb) but fewer reads per run (typically hundreds of thousands).

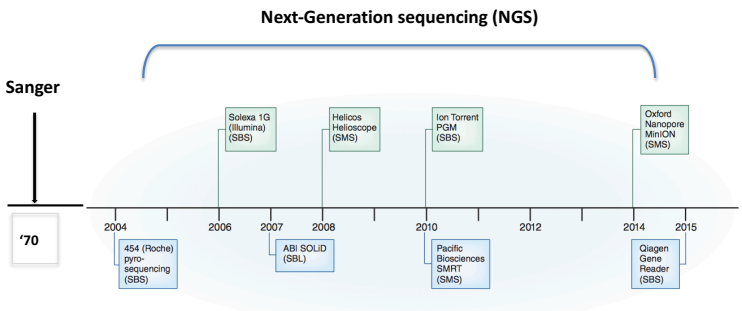

Fig. 8. Timeline on the sequencing methods.

Illumina technology uses the sequence-by-synthesis method. Short DNA fragments are attached to a glass slide or microwell and amplified to form clusters. Fluorescently labeled nucleotides are washed

across the flowcell and are incorporated complementary to the DNA sequence of the clustered fragment.

Fluorescence from the incorporated nucleotides is detected, revealing the DNA sequence. Illumina is almost certainly the leading sequencing technology in genomics labs. It offers the highest throughput, producing relatively short reads with length up to 300 bp, and with the lowest cost per-base. The Illumina output is compatible with the most applications for further study (van Dijk, et al. 2014).

In Ion Torrent technology, DNA fragments are attached to beads, and single beads are placed into micro-wells. Each one of the four nucleotides flows through the wells and gets incorporated into a complementary strand, and in doing so, releases an HC ion that can be measured as a voltage change. This process is repeated in multiple cycles. The Ion Torrent technology can finish a run in a significantly less time than other platforms and produces reads up to 400 bp length. However, it is not as widely used as Illumina technologies possibly due to the high rate of homopolymer errors (van Dijk, et al. 2014).

Pacific Biosciences is based on SMRT sequencing technology.

An engineered DNA polymerase is attached to a single strand of DNA, and these are placed into micro-wells called ZMWs. Each of these ZMWs contains a polymerization complex of a sequencing primer, the template and a DNA polymerase attached to the bottom. During polymerization, the incorporated phospholinked nucleotides carry a fluorescent tag (different for each nucleotide) on their terminal phosphate. The tag is excited and emits light which is captured by a sensitive detector (through a powerful optical system). At the end, the fluorescent label is cleaved off and the polymerization complex is ready for extending the strand (Buermans e Den Dunnen 2014). The PacBio sequencing platforms require a large amount of genomic DNA as input; however, the platforms are capable of very long reads 10–15 kb with some reads >50,000 bp (Goodwin, McPherson e McCombie 2016).

PacBio sequencing has a high raw error rate (15%) but this can be corrected to very high accuracy (Koren, et al. 2012) (Chin, et al. 2013).

Oxford Nanopore technologies also offer single-molecule sequencing. In nanopore sequencing, a single strand of DNA passes through a protein nanopore and changes in electric current are measured. This technology, DNA polymer complex, consists of a double stranded DNA and an enzyme which unwinds the double strand and passes the

single stranded DNA through the nanopore. As the DNA bases pass through the pore, there is a detectable disruption in the electric current and the order of the bases on the DNA stand is identified. In 2014, ONT released the MinION sequencing systems which, unlike the other technologies bulk sequencing installations, is a palm-sized device producing long reads in real time. At launch, the MinION read length was approximately 6–8 kb (Jain, et al. 2015) however, (Urban, et al. 2015) published a lab protocol which could improve the MinION reads length producing many reads even longer than 100 kb. Like PacBio, ONT technologies also have high systematic error rates (Ip, et al. 2015).

The main utility of NGS in microbiology is to replace conventional characterization of pathogens by morphology, staining properties and metabolic criteria with a genomic definition of pathogens. The genomes of pathogens define what they are, may harbor information about drug sensitivity and inform the relationship of different pathogens with each other which can be used to trace sources of infection outbreaks. Nowadays is common using NGS to reveal and trace outbreaks of MDRO in nosocomial environment (Chiu, et al. 2008) (Azarian, Cook, et al. 2015) (Azarian, Maraqa, et al. 2016).

Interestingly how routine microbiological surveillance cannot show that the cases of these pathogens in time before. NGS of the pathogens, however, allowed precise characterization of the pathogen isolates and revealed a protracted outbreak of these (Behjati e Tarpey 2013).

The newest instrument in the NGS landscape, the nanopore sequencer, is still in the process of finding its niche in the field. Nevertheless, researchers are capitalizing on its rapid library preparation time, real-time generation of data and its small size. Recently, researchers at the Stanley Royd Hospital in the United Kingdom used MinION sequencing to monitor an outbreak of Salmonella enterica. Using phylogenetic placement, the authors were able to unambiguously identify the serovar within 50 minutes after the start of sequencing, indicating that the MinION device is a viable platform for rapid pathogen profiling (Quick, Ashton, et al. 2015). Perhaps one of the most striking applications of MinION sequencing in the field was its use during the 2014 Ebola outbreak, which is outlined in Quick et al. (Quick, Loman, et al. 2016). Under the auspices of the European Mobile Laboratories in Guinea, the authors were able to monitor the transmission history

and evolution of the Ebola virus as the outbreak unfolded.

4.1.1. Genome Assembly

DNA sequencers sequence fragments of genomes, and assembly refers to the process of reconstructing in silico the original genome sequence from the smaller sequenced fragments.

Assembly of a single genome is a relatively complex procedure as repetitive elements, within genomes, make the assignment of reads to chromosomes non-trivial. There are some problems due to the genome assembly:

- Lots of short reads that are not ordered in any way
- Relatively high error rates versus sequence polymorphisms
- Repetitive regions
- Non-uniform coverage
- Compositional biases

There are two types of assembly:

- De Novo assembly: refers to reconstruction of the underlying sequence without a previously resolved reference sequence.
- Alignment-based mapping and assembly refers to reconstruction of the underlying sequence facilitated by alignments to a previously resolved reference sequences.

Even if there is a third approach: the hybrid one, where there is alignment of the reads to reference, the reads unaligned there is the De novo assembly remaining reads for identification of novel regions/genomes.

Before the assembly it is important to evaluate the initial control of the generated reads (FASTQ files) and this is followed by the filtering of the reads by quality (Altmann, et al. 2012).

De novo assembly is the process of merging overlapping sequence reads into contiguous sequences (contigs) without the use of any reference genome as a guide.

The workflow has been divided into five logical sections: assembly, ordering of contigs, annotation, genome comparison and typing.

The most efficient assemblers for short-read sequences are typically those that employ de Bruijn graphs to produce an assembly (Compeau, Pevzner e Tesler 2011).

The program most used for the De Novo assembly is the open-source program Velvet (Zerbino e Birney 2008), which includes in this package different tools.

The alignment-based mapping and assembly is used when a suitable reference sequence is available, index the reference genome sequence and search it efficiently. For this purpose, map-alignment sequence assembly approaches generally use a computing strategy called Burrows–Wheeler indexing to notably reduce compute time and memory usage.

Once the ordered set of contigs has been obtained, the next step is to annotate the draft genome. Annotation is the process of ' gene' finding, and can also include the identification of ribosomal and transfer RNAs encoded in the genome (Edwards e Holt 2013).

There are several published pipelines to perform the analysis from the raw reads to the results obtained to make sense to the results.

4.2. Phylogeny

Most recently, the application of next generation WGS and phylogenetic analysis to investigating bacterial pathogens has provided the resolution required to discriminate between genetically similar isolates, enhancing outbreak investigation as well as the study of pathogen emergence and spread (Le e Diep 2013).

Phylogeny is the process of branching of lineages in the evolution of life. Its reconstruction is essential for the systematic dedicated to rebuilding the relationship of evolutionary relatedness of taxonomic groups of organisms at all systematic levels (https://en.wikipedia.org/wiki). To investigate the evolution and relationships among genes and organisms, different kinds of data can be used. The classical way of estimating the relationship between species is to compare their morphological characters (Linnaeus 1758). Taxonomy is still based largely on morphology. The increasingly available molecular information, such as nucleotide or amino acid sequences, and single nucleotide polymorphisms (SNPs), can also be used to infer phylogenetic relationships. The use of molecular data, for inferring phylogenetic trees, has now gained considerable interest among biologists of different disciplines, and it is often used in addition to morphological data to study relationships in further detail.

According to evolutionary theory, all organisms evolved from one

common ancestor, going back to the origin of life. Different mechanisms of acquiring variation have led to biodiversity of today. These mechanisms include mutations, duplication of genes, reorganization of genomes, and genetic exchanges such as recombination, re-assortment, and lateral gene transfer. Of all these sources, mutations (i.e. point mutations, insertions and deletions) are more often used to infer relationships between genes. Basically, phylogenetic methods consider the similarity among genes, assuming that they are homologous (*i.e.* they share a common ancestor). Although it is assumed that all organisms share a common ancestor, over time the similarity in two genes can be eroded so that the sequence data themselves do not carry enough information about the relationship between the two genes and they have accumulated too much variation.

Therefore, the term homology is used only when the common ancestor is recent enough for the sequence information to have retained enough similarity for it to be used in phylogenetic analysis. Thus, genes are either homologous or they are not.

The similarity is a quantitative data; when two sequences are compared, one can always calculate the percentage similarity by counting the number of identical nucleotides or amino acids, relative to the length of the sequence. This can be done even if the sequences are not homologous.

Phylogenetic analysis establishes the relationships between genes or gene fragments, by inferring the common history of the genes or gene fragments. To achieve this, it is essential that homologous sites be compared with each other (positional homology).

The sequences under investigation are aligned such that homologous sites form columns in the alignment. Alignments are usually constructed with specific software packages that implement algorithms. Many popular algorithms start by comparing the sequence similarity of all sequence pairs, aligning first the two sequences with the highest similarity. The other sequences, in order of similarity, are added progressively. The alignment continues in an iterative mode, adding gaps when required to achieve positional homology. The resulting alignment can be improved by manual editing. Obtaining a good alignment is one of the most crucial steps towards a good phylogenetic tree. When the sequence similarity is so low that an alignment becomes too ambiguous, it is better to delete that gene fragment from the

alignment. Columns with gaps at the beginning and end of a sequence, representing missing sequence data for the shorter sequences, must be removed to consider equal amounts of data for all sequences, unless the software used can deal with such missing data. Often columns in the sequence alignment with deletions and insertions for most of the sequences are also removed from the analysis. For a reliable estimate of the phylogenetic relationship between genes, the entire gene under investigation must have the same history.

Recombination events within the fragment under investigation will affect phylogenetic inference.

Recombination outside the fragment of interest does not disturb the tree; and a different clustering of two consecutive fragments can even be used to investigate recombination. Genes originating from a duplication event recent enough to reveal their common ancestry at the nucleotide or amino acid level are called paralogous. Comparing such genes by phylogenetic analysis will provide information about the duplication event. Homologous genes in different species that have started to evolve independently because of speciation are called orthologous. Comparing such genes by phylogenetic analysis will provide information about the speciation event. Evolution under similar selective pressures can result in parallel or convergent evolution. When two enzymes evolved to have similar function, the similar functional requirements can result in a similar active site consisting of the same or similar amino acids. This effect can result in the two sequences having higher similarity than expected by chance, which can be mistaken for homology. Other events can result in a higher similarity of the two sequences than the similarity expected from their evolutionary history. Sequence reversals occur when a substitution reverts to the original nucleotide, multiple hits when a substitution has occurred several times at the same nucleotide, and parallel substitutions when the same substitution happened in two different lineages. Such events can lead to homoplasy in the alignment, and they can confound the linear relationship between the time of evolution and sequence divergence.

4.2.1. Phylogenetic trees

Evolutionary relationships among genes and organisms can be illustrated elegantly using a phylogeny, that shows which genes or

organisms are most closely related.

The various diagrams used for depicting these relationships are called phylogenetic trees because they resemble the structure of a tree and the terms referring to the various parts of these diagrams (i.e. root, branch, node, leaf) are also reminiscent of trees. External (terminal) nodes or leaves represent the extant (existing) taxa and are often called operational taxonomic units (OTUs), a generic term that can represent many types of comparable taxa (e.g. a family of organisms, individuals, or virus strains from a single species or from different species). Similarly, internal nodes may be called hypothetical taxonomic units (HTUs) to emphasize that they are the hypothetical progenitors OTUs. It is said monophyletic the properties of a set of taxa to be all the descendants of a common ancestor. A group of taxa with this property is called monophyletic cluster. It is said paraphyletic a group of taxa, phylogenetically closely related to each other, which is also derived from a common ancestor, but that do not include all descendants of that ancestor. The branching pattern that is, the order of the nodes is called the topology of the tree. An unrooted tree only positions the individual taxa relative to each other without indicating the direction of the evolutionary process. In an unrooted tree, there is no indication of which node represents the ancestor of all OTUs. To indicate the direction of evolution in a tree, it must have a root that indicates the common ancestor of all the OTUs. The tree can be rooted if one or more of the OTUs if one or more of the OTUs form an outgroup because they are known as, or believed to be, the most distantly related of the OTUs (i.e. outgroup rooting). The remainder then forms the in-group. The root node is the node that joins the in-group and outgroup taxa and thus represents their common ancestor. It is still possible to assign a root even in the case where there is no suitable outgroup available, for example, because all available outgroup taxa are related too distantly and the alignment with the outgroup is too ambiguous. Assuming that the rate of evolution in the different lineages is similar, the root will then lie at the midpoint of the path joining the two most dissimilar OTUs (midpoint root). When trying to root a tree, it is not advisable to choose an outgroup that is related too distantly to the ingroup taxa. This may result in topological errors because sites may have become saturated with multiple mutations, implying that information at these sites may have been erased. On the other hand, an outgroup that is

related too closely to the taxa under investigation is also not appropriate; it may not represent a "true" outgroup. The use of more than one outgroup generally improves the estimate of the tree topology. Midpoint rooting could be a good alternative when no outgroups are available, but only in the case where all branches of the tree have roughly similar evolutionary rates.

Phylogenetic analysis provides useful tools to calculate the time to the most recent common ancestor (TMRCA) for all the extant alleles/genes. Divergence time calculations are often used when investigating the origin of a species. Because of recombination, the genealogy relating the sequences of one gene can differ from the genealogies of other genes (Thorne e Kishino 2002). Hence, divergence times can vary among genes. Divergence times may also vary among genes because they can have different allelic copy numbers (i.e. autosomal vs. sex-linked vs. mitochondrial loci). Evolutionary rates differ over time and among genes. Between 1962 and 1965, Zuckerkandl and Pauling (Zuckerkandl e Pauling 1965) (Zuckerkandl e Pauling 1962) noticed that the genetic distance of two sequences coding for the same protein, but isolated from different species, seems to increase linearly with the divergence time of the two species. Since several proteins showed a similar behavior, Zuckerkandl and Pauling hypothesized that the rate of evolution for any given protein is constant over time. This suggestion implies the existence of a sort of molecular clock ticking faster or slower for different genes but at a constant rate for any given gene among different phylogenetic lineages. So, if a molecular clock exists and the rate of evolution of a gene can be calculated, this information can be used for dating the unknown divergence time between two species just by comparing their DNA or protein sequences. The molecular clock is not only a tool for estimating historical dates and rate of evolution; it can render phylogenetic reconstruction more accurate. Sequence data is often obtained by sampling individuals at different time points. During the sampling period, nucleotide substitutions may have accumulated if the sampling scheme extends into ancient times or when the organisms have evolved very fast during more recent times. The former case of measurably evolving populations (MEPs; (Drummond, Pybus, et al. 2003)) can be represented by well characterized vertebrate subfossil material from which ancient DNA is reliably amplified (e.g. (Barnes, et al. 2002) (Shapiro, et al. 2004)), while the

latter is frequently observed in RNA virus evolution. Models to test the assumption of rate constancy for MEPs should take into account the date of sampling. Instead of enforcing an *ultrametric* tree topology, the tips of the tree are now constrained at distances from the root that are proportional to the sampling times. In this model, the evolutionary rate is an additional parameter that rescales the times of the internal nodes into units of expected number of substitutions per site (Rambaut 2000). This implies that differences in sampling time are an important source of information to calibrate molecular clocks.

Indeed, to estimate divergence times, it is assumed that sequence divergence accumulates at a roughly constant rate over time; this assumption is referred to as the molecular clock hypothesis. When the molecular clock holds, all lineages in the tree have accumulated substitutions at a similar rate. However, the evolutionary rate is dependent on many factors, including the underlying mutation rate, metabolic rates in a species, generation times, population sizes, and selective pressure. Therefore, real molecular data frequently violates a strict molecular clock assumption. There are statistical tests that evaluate how the evolutionary rates along the branches in a tree deviate from a uniform rate. Relaxed molecular clock models have recently been developed, which can accommodate rate variation then estimating divergence data.

The ancestor-descendant relationship between species (or group of organisms) can be represented using a cladogram, which is not necessarily based on phylogenetic analysis. A phylogram depicts the phylogenetic relationships between a group of taxa with branch lengths representing evolutionary distances, which were inferred using a phylogenetic approach.

Reconstructing the phylogeny from nucleotide or amino acid alignments is not as straightforward as one might hope, and it is rarely possible to verify that one has arrived at the "true" conclusion. Although there are many methods available, none of them guarantees that the inferred phylogenetic tree is, in fact, the "true" phylogenetic tree.

The methods for constructing phylogenetic trees from molecular data can be grouped according to the kind of data they use, discrete character states or a distance matrix of pairwise dissimilarities, and according to the algorithmic approach of the method, either using a clustering algorithm usually resulting in only one tree estimate, or using

an optimality criterion to evaluate different tree topologies (Figure 9).

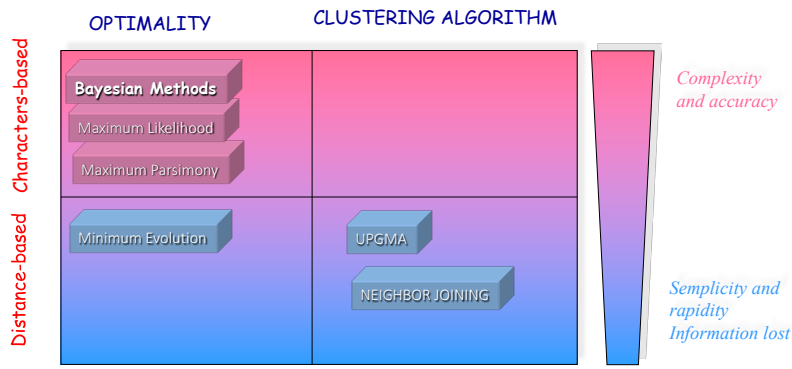

Fig. 9. Tree construction methods.

Character-state methods can use any set of discrete characters, such as morphological characters, physiological properties, restriction maps, or sequence data. When comparing sequences, each sequence position in the aligned sequences is a "character," and the nucleotides or amino acids at that position are the "states."

Usually, all character positions are analyzed independently; so, each alignment column is assumed to be an independent realization of the evolutionary process.

Character-state methods retain the original character status of the taxa and therefore, can be used to reconstruct the character state of ancestral nodes. In contrast, distance-matrix methods start by calculating some measure of the dissimilarity of each pair of OTUs to produce a pairwise distance matrix, and then infer the phylogenetic relationships of the OTUs from that matrix. These methods seem particularly well suited for rapidly analyzing relatively large data sets. Although it is possible to calculate distances directly from pairwise aligned sequences, more consistent results are obtained when all sequences are aligned.

Distance-matrix methods usually employ an evolutionary model, which corrects for multiple hits. When two sequences are very divergent, it is likely that, at a certain position, two or more consecutive mutations have occurred. Because of multiple events, two sequences are

related more distantly than can be deduced from the actual percentage differences. Mathematical models allow for correcting the percentage difference between sequences. This results in genetic or evolutionary distances, which are always bigger than distances calculated by direct comparison of sequences (also called *p-distance*). Distance methods discard the original character state of the taxa. As a result, the information required to reconstruct character states of ancestral nodes is lost. The major advantage of distance methods is that they are generally computationally inexpensive, which is important when many taxa have to be analyzed.

Tree-evaluation methods employ an optimality or goodness-of-fit criterion and examine different tree topologies for a given number of taxa in the search for the tree that optimizes this criterion. Maximum likelihood methods have the advantage of using a statistical criterion because they consider the probability that a tree gave rise to the observed data (i.e. the aligned sequences) given a specific evolutionary model. This allows the investigator to compare the relative support for different phylogenetic trees in a statistical framework. Unfortunately, an exhaustive search exploring all possible trees is usually not possible since the number of possible trees, and thus the computing time, grows explosively as the number of taxa increases; the number of bifurcated rooted trees for n OTUs is given by (2n−3)!/(2n−2(n−2))!. This implies that, for a data set of more than 10 OTUs, only a subset of possible trees can be examined. In this case, various heuristic strategies have been proposed to search the "tree space," but there is no algorithm that guarantees to find the best tree under the specified criterion.

Phylogenetic and population genetic inference (phylodynamics) based on genome-wide SNP data can resolve putative outbreaks, investigate their etiology, and provide spatiotemporal context during investigations (Gray, et al. 2011) (Harris, et al. 2010) (Lemey, Salemi e Vandamme 2009).

These methods can also be used in conjunction with gene identification to elucidate macro-level (population) transmission and understand pathogen success (Grenfell, et al. 2004). The utility of phylogenetics in understanding the emergence of pathogens is also well recognized (Grenfell, et al. 2004). While the field of genomic epidemiology is in its infancy, the application of these methods to investigate bacterial pathogens due to an increasing in frequency (Walker, et al.

2012) (Köser, Bryant, et al. 2013) (Snitkin, et al. 2012) (Köser, Holden, et al. 2012) (Prosperi, et al. 2013) (Diep 2012).

4.3. Multilocus sequence typing

Multilocus sequence typing (MLST) is a nucleotide sequence-based method that is for characterizing the genetic relationships among bacterial isolates (Enright e Spratt 1999) (Feil, et al. 2004) (Maiden, et al. 1998); that aims to be a robust and portable method for the characterization of bacterial isolates at the molecular level. It differs from many other former and current approaches to isolate characterization in that it is based explicitly on the population genetic concepts that underpinned the earlier technique of multilocus enzyme electrophoresis (MLEE) (Selander, et al. 1986) (Jolley, Chan e Maiden 2004).

MLST has the additional aims of providing a unified bacterial isolate characterization approach that generates data that can also be used for evolutionary and population studies of a wide range of bacteria, regardless of their diversity, population structure, or evolution (Urwin e Maiden 2003).

MLST was proposed in 1998 as a transferrable, universal, and conclusive method (Maiden, et al. 1998) was made possible by three advances in molecular microbiology: (a) improved knowledge of bacterial evolution and population biology (Levin, Lipsitch e Bonhoeffer 1999) (Musser 1996) (Spratt e Maiden, Bacterial population genetics, evolution and epidemiology 1999); (b) increasing availability and decreasing cost of high-throughput nucleotide sequence determination (M. Maiden 2000); and (c) developments in information technology, specifically the development of the Internet as an efficient, essentially instantaneous, and cost-effective means of information exchange (Chan, Maiden e Spratt 2001).

MLST was conceived as a tool for clinical microbiologists and epidemiologists (Urwin e Maiden 2003), although the successful exploitation of MLST data in studies of bacterial population genetics has somewhat obscured its utility in applied microbiology (Smith, Feil e Smith 2000) (Spratt, Hanage e Feil 2001). The design of MLST is that of a typing technique that exploits and contributes to population genetic analysis, rather than a technique for the study of microbial population biology that could be applied to routine typing. A number of features of MLST schemes are a result of the pragmatic priorities of routine typing

and need to be considered if the aims and objectives of the MLST project are to be fully appreciated.

The existent K. pneumoniae MLST scheme employs seven loci: rpoB (Beta-subunit of RNA polymerase B), gapA (Glyceraldehyde 3-phosphate dehydrogenase), mdh (Malate dehydrogenase), pgi (Phosphoglucose isomerase), phoE (Phosphoporine E), infB (Translation initiation factor 2), tonB (Periplasmic energy transducer) (Diancourt, et al. 2005). There are about 2944 profiles (ST) worldwide (http://pubmlst.org/kpneumoniae/).

5. Aim of the study

One of the most leading causes of hospital-acquired infections (HAIs), is CPE *strains*, often due to the presence of plasmid-encoded *K. pneumoniae* carbapenemase (KPC), increasing rate of morbidity and mortality (Giani, Pini, et al. 2013) (E. C. ECDC 2012) (Chung The, et al. 2015) (Girmenia, Serrao e Canichella 2016). The genetic diversity of K. pneumoniae strains circulating in hospital settings is not clear enough; the most common actions are antimicrobial susceptibility profiles or molecular genotyping methods such as pulsed field gel electrophoresis (PFGE) or MLST. But these methods, even providing information about the large-scale population structure of bacterial species, lack the discriminatory resolution to investigate epidemics on finite geographical or temporal scales (Girmenia, Serrao e Canichella 2016) (Maâtallah, et al. 2013), next-generation sequencing (NGS) has been applied at this aim. In this way, the transmission events could be reconstructed and adequate measure for hospital infection control could be improved (Halachev, et al. 2014). Among patients undergoing endoscopic retrograde cholangiopancreatography (ERCP), patients have investigated infections by KPC K. pneumoniae and endoscope were sampled to check the correct duedonoscope reprocessing.

In this study, *K. pneumoniae* KPC strains circulating within different wards of the University Hospital Campus Bio-Medico were collected and WGS applied. A microbiological surveillance on duedonoscope was performed.

These applications could help in the epidemiological surveillance of MDR pathogens to discern outbreak from non-outbreak strains in both community and hospital settings (Azarian, Maraqa, et al. 2016)

(Azarian, Cook, et al. 2015).

The aim was to infer the origin and the spread of *K. pneumoniae* nosocomial strains and to clarify the epidemiological transmission as so as the eventual reservoir in the hospital setting supporting the epidemiological surveillance and infections control strategies.

PARTE I

Outbreak investigation

1. Methoods

1.1. Sample collection and epidemiologic investigation

Between January 2012 and February 2013, twenty-one *Klebsiella pneumoniae* MDR and KPC strains were collected from inpatients of the tertiary care 280-bed University Hospital Campus Bio-Medico of Rome, Italy. The strains were collected from different culture type from different patients infected (Table 5).

Tab. 5. distribution of culture type for the strains collected.
SSI= Surgical Site Infection

Culture Type	
Bile	1
Blood Culture	5
SSI*	2
Ulcer	2
Urine Culture	11
Total	**21**

Patients were admitted in different wards distributed along four floors and two distinct hospital locations (East and West area) as in Figure 10.

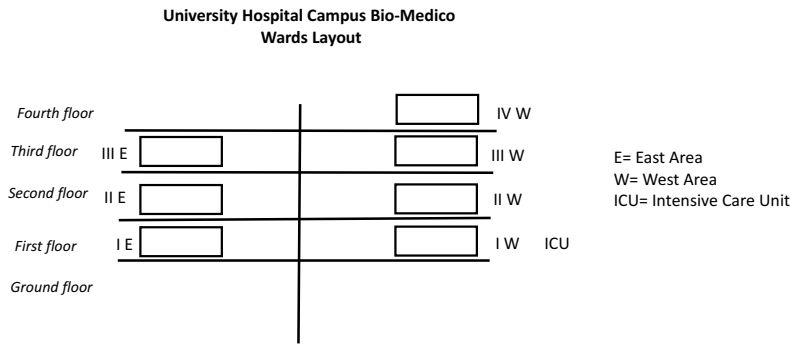

Fig. 10. Wards Layout of University Hospital Campus Bio-Medico (Cella et al. 2017).

Clinical samples were obtained from patients with signs of bacterial infections as part of routine clinical evaluation as assessed by the clinical team. *Klebsiella pneumoniae* strains were obtained from routine processing of clinical samples used for bacterial infection diagnosis. All methods were carried out in accordance with relevant guidelines and regulations in effect at the University Hospital Campus Bio-Medico of Rome. The study was approved by the local Ethical Committee (prot. 48/16 OSS ComEt CBM). Informed consent was obtained from all subjects at ward admission.

1.2. Identification and antimicrobial susceptibility tests of the isolates

Klebsiella pneumoniae isolates were identified by MALDI-TOF using the MALDI Biotyper 3.0 software version (Bruker Daltonics, GmbH, Bremen, Germany) (Angeletti, Dicuonzo, et al. 2015).

Fig. 11. MALDI-TOF-MS.

The identification consists of putting the sample, a single culture grown up not more of 24 hours on Blood Agar and MacConkey plate, creating a thin film, directly on one of 96 wells of the metallic plate of MALDI with an inoculation pipette of 0.1 μL.

after that in each well it is added 2 μL of matrix formed of: α-HCCA (alpha-cyano-4-hydroxycinnamic acid) in 50% of Acetonitrile (ACN) and 2,5% of Frifluoroacetic acid (TFA).

The plate needs to dry for few minutes (Figure 12).

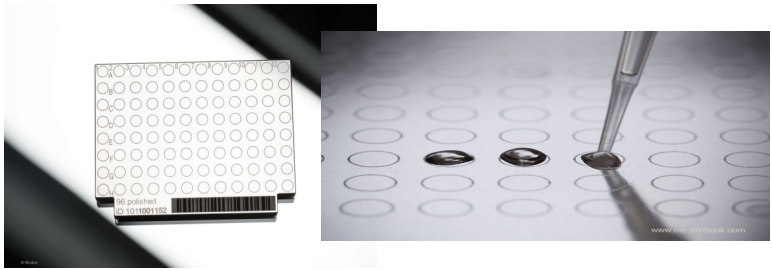

Fig. 12. MALDI-TOF metallic plate.

Once dry, the plate is inserted into the machine, and the "vacuum" reached, the microbial identification starts.

A set of mass spectra for each well is obtained. Each spectrum is compared with other reference spectra stored in the Bruker Taxonomy library within the software to identify each individual microorganism. For each spectrum, a score of compatibility with the reference spectrum is established so that the comparison between the two spectra is optimal. The score must have a value greater than 2, for lower values the species identification is unreliable because probably the machine identified only the genus of the microorganism.

Klebsiella pneumoniae antimicrobial susceptibility tests were performed by Vitek2 Compact (bioMérieux, Marcy l'Etoile, France) using cards AST-N202 and the resistant phenotype further confirmed with the Kirby-Bauer method according to Clinical Laboratory Standard Institute (CLSI) and European Committee for Antimicrobial Susceptibility Test (EUCAST) (Gherardi, et al. 2012).

Such AST-N202 cards consist of 64 wells containing 16 antibiotics present at different concentrations. The instrument detects and proceeds for each antibiotic the minimum inhibitory concentration (MIC),

that is the lowest concentration of an antimicrobial substance capable of inhibiting the growth of a bacterium. This is done in vitro by testing a standard concentration of microorganisms with a range of scalar dilutions of antibiotics.

Two tubes are prepared for each colony, of which only the first contains 3 mL of saline solution. Read the barcode, unique for each patient, the inoculum is prepared by draining the bacterial colony in the first test tube to reach a density of 0.5-0.6 McF measured through the densitometer "DensiCheck" provided by the bioMérieux manufacturer (Figure 13).

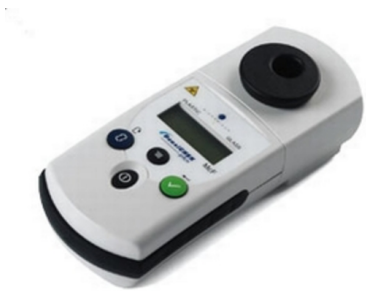

Fig. 13: DensiCheck.

Once reached the density, is inserted the AST-N202 card, specific to negative Gram-bacteria, in the second empty tube and read its barcode. The entire SMART Carrier Station (SCS) containing the inocules and the cards are inserted into the VITEK 2 machine.

Inside the instrument, the inoculum is then transferred to the empty cartridge containing the card and, after reaching the vacuum state of the machine, for capillary passage from the tube to the wells containing the antibiotics of the card. Individual cards separated from the entire media are then incubated.

Each colony from which the inoculum was obtained is associated with the specific patient from which it originated, compiling the information (name, surname, clinical source of origin, sample type, date), and specifying for each colony both the type of the card that the corresponding microorganism that infected it.The results are read the next day after incubation. For each patient, the instrument records the type of bacterium and the result of the antibiogram (Figure 14).

Fig. 14. VITEK® 2.

The antibiotics in the AST-N202 card used for K. pneumoniae were the following:

- Amikacin
- Amoxicillin / Clavulanic Acid
- Cefepime
- Cefotaxime
- Ceftazidime
- Ciprofloxacin
- Colistin
- Ertapenem
- Imipenem
- Meropenem
- Fosfomycin
- Gentamicin
- Piperacillin / Tazobactam
- Cotrimoxazole

The results of ASTs were interpreted according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing EUCAST (http.//eucast.org/clinical_breakpoint).

After that it was determined if the strains were sensible, resistant (multi, extensively or pan) (Magiorakos, et al. 2012).

The Hospital Infection Control Team and the investigators of the Clinical Pathology and Microbiology Unit performed a detailed epidemiologic investigation. Medical records were examined for epidemiologic data collection, such as dates of patient's ward admission and

discharge, bed assignment, diagnostic and therapeutic procedures, and microbiological laboratory results.

Two epidemic curves based one on the number of Klebsiella pneumoniae isolated in the temporal frame of the study and one on the number of isolates for each ward have been developed.

1.3. Whole-genome sequencing (WGS)

Bacterial DNA was extracted by the EZ1 DNA tissue kit (Qiagen, Dusseldorf, Germany) and whole genome sequenced by Next Generation Sequencing using Illumina MiSeq II sequencer (Library Preparation Kit: Nextera XT DNA Sample Prep Kit, Indexing: Dual Indexing Reagent Kits: MiSeq Reagent Kit v3, Analysis Workflow: Resequencing, Analysis Software: MiSeq Reporter) (Figure 15).

Fig. 15. Illumina MiSeq II sequencer.

The Illumina MiSeq II sequencer has a run time from 5 to 55 hours for run with a maximum of 25 million of reads for run. The maximum read length produced is 2x300bp. This sequencer is suggested for Small-genome sequencing, amplicon sequencing, targeted DNA and RNA sequencing. Using these features is important to calculate the minimum coverage desired to understand how many samples is possible to do in one run. In this case, there were performed two runs to have a significant coverage.

Sequencing reads from the isolates obtained were assembled using an established bioinformatics pipeline an optimized analysis pipeline implemented in the UF Galaxy platform (http://galaxy.rc.ufl.edu).

After demultiplexing, single FASTQ output files of raw reads were filtered by length threshold of 30 and quality threshold of 30 using Sickle v. 1.2.

De novo assemblies were constructed using the SPAdes (Bankevich, et al. 2012), and contigs ordered using the strain CAV1596 as reference sequence with Mauve v. 2.3.1 (A. Darling, et al. 2004) (Rissman, et al. 2009). The strain CAV1596 was selected as the best reference genome performing an analysis with PARSNP (Treangen, et al. 2014) and visualizing the output with Gingr (Treangen, et al. 2014) to identify the more appropriate reference genome.

Parsnp is designed to align the core genome of bacterial genomes shortly. Parsnp leverages contextual information provided by multi-alignments surrounding SNP sites for filtration/cleaning, in addition to existing tools for recombination detection/filtration and phylogenetic reconstruction. Gingr provides an interactive display of multi-alignments, variants and the phylogenetic tree estimated from the core genome alignment.

Sequences were then aligned with Progressive Mauve (Darling, Mau e Perna 2010). Progresive Mauve is the tool of mauve to perform genome multi alignment. Mauve uses anchoring

as a heuristic to speed alignment.

De novo assemblies were annotated with RAST (http://rast.nmpdr.org/), a fully automated service generating high-quality annotations for complete or nearly complete bacterial and archaeal genomes and used to generate a heat-map of gene expression (Aziz, et al. 2008). Annotations were confirmed using Prokka, which relies on external feature prediction tools to identify the coordinates of genomic features within contigs (Seemann 2014).

The pangenome as the entire gene set of all strains of a species was considered. It includes both: genes present in all strains (core genome) and genes present only in some strains of a species (variable genome).

Instead, core genome included the genes present across all strains of a species. It typically includes housekeeping genes for cell envelope or regulatory functions (Tettelin, et al. 2005). To perform the pangenome alignment was utilized Roary (Page, et al. 2015). Roary is a high-speed stand alone pangenome pipeline, which takes annotated assemblies in GFF3 format (produced by Prokka (30) and calculates the pangenome. Roary also performed the statistics on the Prokka outputs.

From here it was extracted the alignment including only the core genome.

Recombination was evaluated using Gubbins (Genealogies Unbiased By recomBinations In Nucleotide Sequences) on the core genome alignment. Gubbins is an algorithm that iteratively identifies loci containing elevated densities of base substitutions while concurrently constructing a phylogeny based on the putative point mutations outside of these regions. Simulations demonstrate the algorithm generates highly accurate reconstructions under realistic models of short-term bacterial evolution diversification of sequences through both point mutation and recombination and can be run in only a few hours on alignments of hundreds of bacterial genome sequences (Croucher, et al. 2014).

The quality single-nucleotide polymorphisms (hqSNPs) were based on the core genome shared by all isolates, i.e., regions in the accessory genome not conserved by all strains were omitted. The hqSNPs were extracted as variable sites using MEGA 6 (Tamura, et al. 2013) removing all ambiguous sites and gaps. It was calculated the pairwise genetic distances between all the strains using MEGA 6 (Tamura, et al. 2013).

1.4. Multilocus Sequence typing (MLST)

MLST was performed according to the protocol described by Diancourt and colleagues (Diancourt, et al. 2005) based on seven housekeeping genes: *gapA* (glyceraldehyde 3-phosphate dehydrogenase), *infB* (translation initiation factor 2), *mdh* (malate dehydrogenase), *pgi* (phosphoglucose isomerase), *phoE* (phosphorine E), *rpoB* (betasubunit of RNA polymerase) and *tonB* (periplasmic energy transducer). The MLST database used for *K. pneumoniae* is available at http://www.pasteur.fr.

1.5. Phylogenetic and phylogeographic analyses

Phylogenetic signal was assessed by likelihood mapping of 10,000 random quartets generated using TreePuzzle (Schmidt, et al. 2003). A likelihood map consists of an equilateral triangle containing dots representing the likelihoods of the three possible unrooted trees for a set of four sequences (quartets) randomly selected from the data set: the dots close to the corners (1, 2,3) or at the sides represent respectively tree-like (fully resolved phylogenies in which one tree is clearly better than the others) or network-like phylogenetic signals (three regions in which it is not possible to decide between two topologies). The central area (7) of the map represents a star-like signal (the region in which the star tree is optimal) (Figure 16) (Strimmer e von Haeseler 1997) (Salemi, et al. 2008).

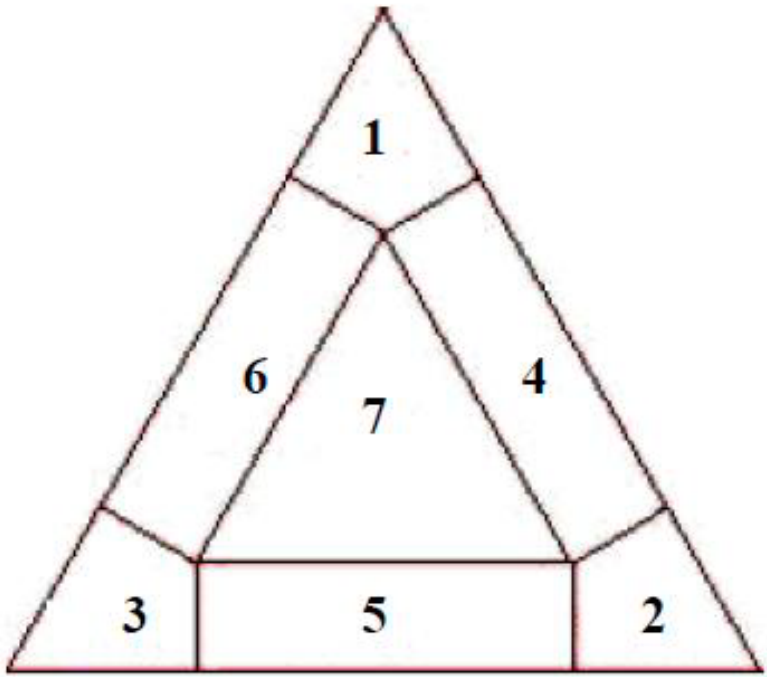

Fig. 16. Likelihood map example.

Several studies demonstrated that a percentage greater than 33% in the central area (7), suggests a tree topology not resolved and in this case the data is not trustable and suitable for a phylogenetic analysis. To be confident about the analysis the cut-off is ≥ 60% for the three corners and the central area less than 30-33%.

A transitions/transversions ratio vs. divergence graph as well as the Xia's test of substitution saturation were implemented in DAMBE (Xia e Xie 2001).

The aim of Xia test is to verify the substitution saturation, an increasing of this brings in a decreasing of sequences information.

If the sequences change a lot it is not possible to perform an accurate alignment and the dataset should be excluded from the analysis (Hedges, Moberg e Maxson 1990).

The transitions/transversions ratio vs. divergence graph is another way to test the substitution saturation. Substitutions where a purine is exchanged by a pyrimidine or vice versa (A↔C, A↔T, C↔G, G↔T) are called transversions (Tv), all other substitutions are transitions (Ts) (Lemey, Salemi e Vandamme 2009).

When resulting in an amino acid change, transversions usually have a larger impact on the protein than transitions, because of the more drastic changes in biochemical properties of the encoded amino acid. There are four possible transition errors (A ↔ G, C ↔ T), and eight possible transversion errors (A ↔ C, A ↔ T, G ↔ C, G ↔ T); therefore, if a mutation occurred randomly, a transversion would be two times more likely than a transition.

However, the genetic code has evolved in such a way that, in many genes, the less disruptive transitions are more likely to occur than transversions.

The HKY+I+G nucleotide substitution model was chosen as best-fitting model by using the hierarchical likelihood ratio test (Modeltest, implemented in PAUP*4).

The evolutionary substitution model becomes indispensable when using DNA or protein sequences to estimate phylogenetic relationships among taxa. Models of evolution are sets of assumptions about the process of nucleotide or amino acid substitution. They describe the different probabilities of change from one nucleotide or amino acid to another along a phylogenetic tree, allowing us to choose among different phylogenetic hypotheses to explain the data at hand. When the

model assumed is wrong, branch lengths, transition/transversion ratio, and divergence may be underestimated, while the strength of rate variation among sites may be overestimated. Simple models tend to suggest that a clade is significantly supported when it cannot be, and tests of evolutionary hypotheses can become conservative.

In general, models that are more complex will fit the data better than simpler ones just because they have more parameters.

The best-fit model of evolution for a particular data set can be selected using sound statistical techniques. Different approaches have been proposed, to select the best-fit model of evolution within a collection of candidate models, like hierarchical likelihood ratio tests (hLRTs), Akaike Information Criterion (AIC), Bayesian Infomation Criterion (BIC) or performance-based approaches.

Statistical support for internal branches of the Maximum Likelihood (ML) tree was evaluated by bootstrapping (1000 replicates) and fast likelihood-based sh-like probability (SH-aLRT). ML analysis was performed with IQ-TREE (Trifinopoulos, et al. 2016) and visualized in FigTree 1.4.0.

To check the reliability of branches in a certain tree, it is possible to use bootstrapping combining alignment subsampling and consensus trees to get support values on branches.

Bootstrap analysis is a widely used sampling technique for estimating the statistical error in situations in which the underlying distribution is either unknown or difficult to derive analytically (Efron e Gong 1983). The bootstrap method offers a useful way to approximate the underlying distribution by resampling from the original dataset.

First, the sequence data are bootstrapped, which means that a new alignment is obtained from the original by randomly choosing columns from it with replacements. Each column in the alignment can be selected more than once or not at all until a new set of sequences, a bootstrap replicate, the same length as the original one has been constructed. Therefore, in this resampling process, some characters will not be included at all in a given bootstrap replicate and others will be included once, twice, or more. Second, for each reproduced (i.e. artificial) data set, a tree is constructed, and the proportion of each clade among all the bootstrap replicates is computed. This proportion is taken as the statistical confidence supporting the monophyly of the subset.

Shimodaira and Hasegawa (Shimodaira e Hasegawa 1999) devised a valid test to assess a set of a posteriori selected trees when the maximum likelihood tree is among the tested trees (fast likelihood-based sh-like probability (SH-aLRT)).

Bayesian methods were applied on the SNPs core alignment.

Bayesian methods are character-state methods that use an optimality criterion, they employ the concept of likelihood, but by targeting a probability distribution of trees, they search for a set of plausible trees or hypotheses for the data. This posterior distribution of trees inherently holds a confidence estimate of any evolutionary relationship.

Posterior probabilities are obtained by exploring tree space using a sampling technique, called Markov chain Monte Carlo (MCMC). This sampling method starts by simulating a random set of parameters and proposes a new "state," which is a new set of parameters, by changing the parameters to some extent using random operators. In each step, the likelihood ratio and prior ratio is calculated for the new state relative to the current state. When the combined product is better, the parameters are accepted and a next step is proposed; if the outcome is worse, the probability that the state is rejected is inversely proportional to how much worse the new state is. After an initial convergence to a set of probable model/tree solutions ("burn-in", which needs to be discarded), it is hoped that this stochastic algorithm samples from the "posterior" probability distribution.

The frequency by which a particular tree topology is sampled is then proportional to its posterior probability. The results are usually presented as summary features of the samples, for example, the mean or median for continuous parameters, but for trees, a consensus tree or maximum a posteriori tree can be presented. Bayesian methods are computer intensive, but from a single MCMC, run support values for the clusters in a tree can be derived.

The evolutionary rate of the SNPs core genome alignment of K. pneumoniae was estimated by calibrating a molecular clock using known sequences sampling times with the Bayesian Markov Chain Monte Carlo (MCMC) method implemented in BEAST v. 1.8.2 (http://beast.bio.ed.ac.uk) (Drummond e Rambaut 2007) (A. Drummond, A. Rambaut, et al. 2005).

The concept of the molecular clock was introduced by Zuckerkandl and Pauling after the postulation of postulated the neutral theory of

evolution of Kimura (Kimura 1968) (Zuckerkandl e Pauling, Molecular disease, evolution, and genetic heterogeneity 1962) (Zuckerkandl e Pauling 1965). It was noticed that the genetic distance of two sequences coding for the same protein, but isolated from different species, seems to increase linearly with the divergence time of the two species. Since several proteins showed a similar behavior, Zuckerkandl and Pauling hypothesized that the rate of evolution for any given protein is constant over time. This suggestion implies the existence of a sort of molecular clock ticking faster or slower for different genes but at a more or less constant rate for any given gene among different phylogenetic lineages.

The information about the divergence time between two species (for example, estimated from fossil data) is known, then the rate of molecular evolution of a given gene can be inferred. An additional advantage of assuming a molecular clock is that it can render phylogenetic reconstruction much easier and more accurate.

In order to investigate the demographic history, independent MCMC runs were carried out enforcing both a strict and relaxed clock with an uncorrelated log normal rate distribution and one of the following coalescent priors: constant population size, exponential growth, non-parametric smooth skyride plot Gaussian Markov Random Field (GMRF), and non-parametric Bayesian skyline plot (BSP) (A. Drummond, A. Rambaut, et al. 2005), (Drummond, Nicholls, et al. 2002) (Minin, Bloomquist e Suchard 2008) with ascertainment bias correction (McGill, Walkup e Kuhner 2013) (Gray, et al. 2011). Marginal likelihoods estimates for each demographic model were obtained using path sampling and stepping stone analyses (Baele, Lemey e Vansteelandt 2013) (Baele, Li, et al. 2013) (Baele e Lemey 2008). Uncertainty in the estimates was indicated by 95% highest posterior density (95% HPD) intervals, and the best fitting model for each data set was by calculating the Bayes Factors (BF) (Baele, Lemey e Vansteelandt, Make the most of your samples: Bayes factor estimators for high-dimensional models of sequence evolution 2013) (Kass e Raftery 1995). In practice, any two models can be compared to evaluate the strength of evidence against the null hypothesis (H0), defined as the one with the lower marginal likelihood: $2\ln BF < 2$ indicates no evidence against H0; 2–6, weak evidence; 6–10: strong evidence, and > 10 very strong evidence. Chains were conducted for at least 100x 106 generations and

sampled every 10000 steps for each molecular clock model. Convergence of the MCMC was assessed by calculating the ESS for each parameter. Only parameter estimates with ESS's of >250 were accepted. The maximum clade credibility (MCC) tree was obtained from the trees posterior distributions, after a 10% burn-in, with the Tree-Annotator software v 1.8.2, included in the Beast package (A. Drummond, A. Rambaut, et al. 2005) (Drummond e Rambaut, BEAST: Bayesian evolutionary analysis by sampling trees 2007). Statistical support for specific monophyletic clades was assessed by calculating the posterior probability (pp>0.90).

The continuous-time Markov Chain (CTMC) process over discrete sampling locations implemented in BEAST (Drummond e Rambaut, BEAST: Bayesian evolutionary analysis by sampling trees 2007) was used for the phylogeography inference, by using the Bayesian Stochastic Search Variable Selection (BSSVS) model, which allows the diffusion rates to be zero with a positive prior probability. Locations considered were the different wards of the hospital and different hospital areas. Comparison of the posterior and prior probabilities of the individual rates being zero provided a formal BF for testing the significance of the linkage between locations. The MCC tree with the phylogeographic reconstruction was selected from the posterior tree distribution after a 10% burn-in using the Tree Annotator.

An epidemic curve based on the number of isolates falling within each phylogenetic clade during the study period has been developed.

1.6. R0 estimate

Using BEAST v1.8, the basic reproduction number (R0) was calculated for core genome SNPs alignment under a relaxed clock with Birth-Death Basic Reproductive Number demographic model (Stadler, et al. 2012). R0 is the basic reproductive number (infectivity) of a pathogen, i.e. the average number of secondary infections caused by each primary infected individual. In a pathogen population exponentially growing at rate r, where D is the average duration of infectiousness, it can be shown that if the pathogen is transmitted at the same rate during the total length of infection, then R0 = rD + 1 (Pybus, et al. 2001).

1.7. Duedonoscope disinfection protocol

Due to several outbreaks of patient infections have been reported following procedures with flexible endoscopes, mostly the duodenoscopes used for ERCP a microbial sampling surveillance of the endoscopic instruments was implemented.

Before the start of the microbial sampling surveillance these were the steps of the reprocessing in different levels (Table 6):

Tab. 6. Reprocessing step.

Manual disinfection	**Disinfection**	**Drying**	**Endoscopic storage**	**Endoscopic valves**	**Traceability**
Olympus multipurpose pipe cleaner and brushes are used, depending on the size of the channels. The brushes are disinfected in a dishwasher after each use (no autoclaved).	In ETD 3 Plus of Olympus with Cycle 2 (i.e. only cleaning and disinfection, without drying) for any type of endoscope.	External and internal drying of channels with forced medical air before storage in the closet in the evening.	No ventilated metal cabinets for all endoscopes.	All disinfected reusable after use in endoscope disinfectant (non-autoclaved).	Manual disinfection provides paper traceability while automatic disinfection is computerized.

From September 29, 2016, it has been started the microbiologic surveillance on all our endoscopes and on all water used rinsing the machines to sanitize the instruments, aimed to sample in this way:

- Every month every critical instrument (Duodenoscopes, Bronchoscopes, Linear Echoendoscopes).
- Twice a year on the other instruments.
- Approximately 6 endoscopes per week.

1.7.1. Sampling technique

The material required for an endoscope sampling:

- 1 luer-lock 20 ml syringe
- luer-lock 50 ml syringes
- Sterile gaps
- Ethanol
- 5 sterile barrels (urine test type) with a capacity of 200 ml
- Sterile gloves, mask, sterile lab coat
- 200 ml tampon dnp solution + 0.5% thiosulfate
- Electrical resistance to aspirating "sterile" air or using filter syringes
- Sterile stand
- Sterile fittings for endoscope channels
- A clamp

Sampling technique consists in several steps:

- Place the disinfected endoscope at least 12 hours before on a sterile cell.
- Engage the connectors on the endoscope channels.
- Pour Tampon DNP + 0.5% Thiosulfate into a sterile jar.

With sterile technique proceed to sampling the channels according to this order:

1. 20 ml to rinse the distal tip
2. 20 ml per auxiliary channel sampling (if present)
3. 50 ml for air / water channel sampling (25 ml + 25 ml by alternately clamping the channels)
4. 50 ml for biopsy channel sampling
5. 50 ml per Suction channel

In case the two-channel instrument proceeds to sampling the second biopsy channel with equally volume of buffer solution.

Here is the correct maneuver for collecting the sample of each channel:

- Disinfect the endoscope distal with sterile gauze and ethanol before sampling for each channel.
- Aspirate the required amount of solution from the sterile barrel.
- Inject the entire solution into the channel by keeping the endoscope's distal inside a sterile jar in which to collect the liquid.

- Aspirate part of the collected liquid and inject it again through the canal (motivation: the above-described maneuver allows to create mechanical turbulence inside the canal so as to allow particles to detach if there is biofilm).
- Transfer the fluid samples (instrument channel flush, sterile brush fluid) to 50-cc conical tubes.

Membrane Filtration

1. Set up membrane filtration equipment in a laboratory (e.g. sidearm filtering flask, vacuum, filter housings, gridded filters, sterile forceps, etc.).
2. The total volume needed to assay samples in duplicate on both blood and MacConkey agar plates is 40 mL at a minimum. Consider other volumes or dilutions depending on observed counts by the facility.
a) Blood agar: 2 – 10 mL.
b) MacConkey agar: 2 – 10 mL.
3. Filter the samples, making sure to rinse the filter housing liberally with a sterile buffered solution after each sample.
4. Place the gridded filter using sterile forceps, grid side up, on the agar plate; taking care to place the filter completely flat and removing any air bubbles or creases in the filter.
5. Add up to 0.5 ml of the remaining sample to TSB (5 mL) for enrichment in order to capture contamination below the detection limit .
6. Incubate at 35°C to 37°C; MacConkey agar for 18- 24 h (overnight), blood agar for 48 h, and TSB for 48 h.
7. Incubate at 35°C to 37°C; MacConkey agar for 18- 24 h (overnight) and blood agar for 48 h (two days).
a. Observe plates for suspect colonies.
8. Streak suspect colonies for isolation.
9. Work up pure isolates for characterization of “low- concern” bacteria, which represent flora from skin and the environment, and species identification of “high-concern” bacteria.
a) “Low-concern” bacteria include, but are not limited to, coagulase-negative staphylococci, micrococci, diptheroids, Bacillus spp. and other Gram-positive rods.
b) “High-concern” bacteria include, but are not limited to,

Staphylococcus aureus, Enterococcus spp., Streptococcus sp. viridians group, Pseudomonas aeruginosa, Klebsiella spp., Salmonella spp., Shigella spp. and other enteric Gram-negative bacilli.

If successfully disinfected, culturing should not detect any high-concern organisms; any quantity of high-concern organism (i.e., one colony or greater) warrants further remedial actions as should be reprocessed again with repeat post-reprocessing cultures obtained. The duodenoscope should not be used again until it has been demonstrated to be free of high-concern organisms and has an acceptable level of low-concern organisms. This is consistent with previous recommendations (Queensland Government 2017) (CDC 2008).

Small numbers of low-concern organisms (i.e., organism less often associated with disease and potentially a result of contamination of cultures during collection) might occasionally be detected (e.g., coagulase-negative staphylococci excluding Staphylococcus lugdunensis, Bacillus species, diphtheroids).

Positive cultures should prompt a procedure review to ensure adherence to the manufacturer's reprocessing instructions and to ensure cultures are being performed correctly. If a reprocessing breach is identified, appropriate facility personnel (e.g., infection prevention staff) should be notified and corrective actions should be immediately implemented.

1.7.2. Duodenoscope used in ERCP architecture

Among all the microbiologic surveillance, in this study only the duedonoscope involved in ERCP were considered.

Duodenoscopes are flexible, lighted tubes that are threaded through the mouth, throat, and stomach into the top of the small intestine (duodenum) (Figure 17). They are used during endoscopic retrograde cholangiopancreatography (ERCP), a potentially life-saving procedure to diagnose and treat problems in the pancreas and bile ducts. In the United States, duodenoscopes are used in more than 500,000 ERCP procedures each year.

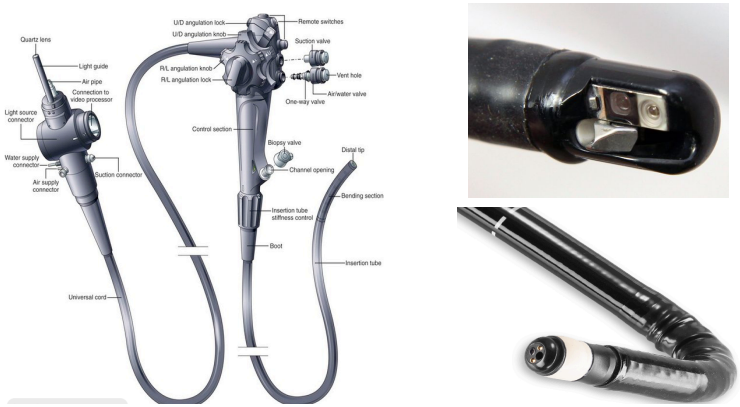

Fig. 17. Example of a duedonoscope.

Five instruments were considered in this study:

- VIDEODODEOSCOPE TJF Q180V (Olympus)
- VIDEODUODENOSCOPE TJF 145 (Olympus)
- VIDEODUODENOSCOPE TJF 160R (Olympus)
- CONVEX ECOENDOSCOPIC PROBE (Pentax)
- EDGE-3870T LINEAR PROBE (Pentax)

The sampling was performed on the duodenoscope channels (4) (Figure 18):

- Auxiliary channel
- Air/water tube
- Biopsy tube
- Suction tube

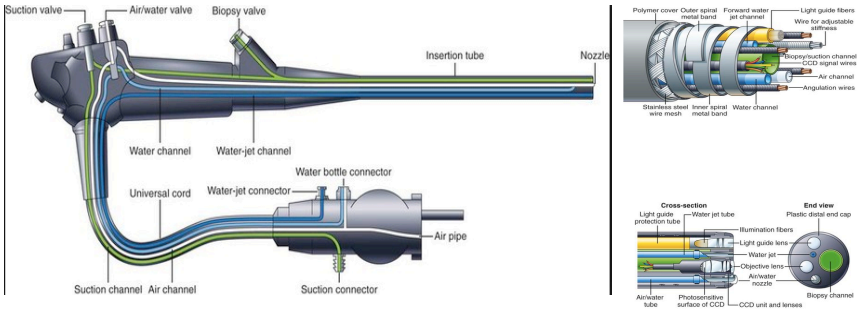

Fig. 18. Composition of a duodenoscope.

Unfortunately, some channels (i.e suction and biopsy) share the end part of the tube so a contamination is a real possibility.

It was performed an epidemiological analysis, mostly descriptive, analyzing the number of positive bilicolture, K.pneumoniae MDR strains.

2. Results

2.1. Study population and epidemiologic investigation

Klebsiella pneumoniae MDR and KPC strains included in the study were isolated from 21 patients with a median age of 76 years (70-81 years) and a sex distribution male: female of 13:8.

The patients were distributed in different hospital wards and hospital areas as in Figure 19 and 20.

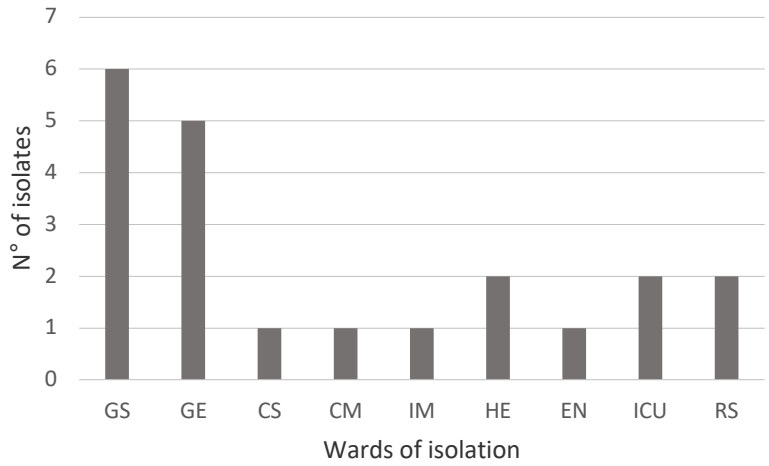

Fig. 19. Epidemic curves based on the number of Klebsiella pneumoniae isolated according to the hospital ward. GS= General Surgery; GE= Geriatrics; CS= Cardiac Surgery; CM= Clinical Medicine; IM= Internal Medicine; HE=Hepatology; EN= Endoscopy; ICU= Intensive Care Unit; RS= Reconstructive Surgery (Cella et al. 2017).

The two main representative wards for Klebsiella pneumoniae strains are General Surgery and Geriatrics with 6 and 5 isolates respectively.

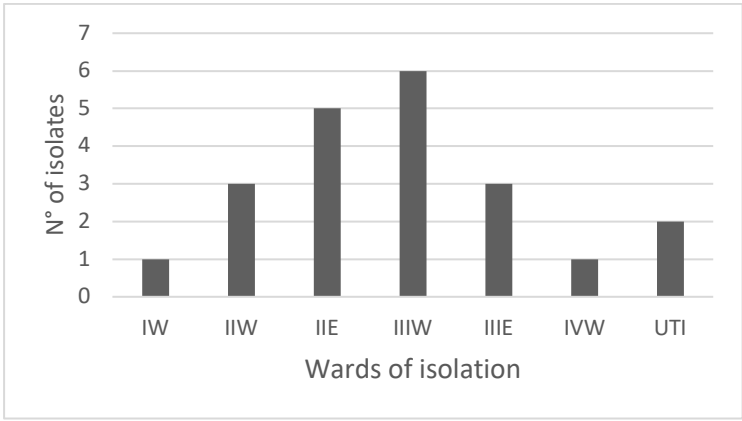

Fig. 20. Epidemic curves based on the number of Klebsiella pneumoniae isolated according to the hospital area (Cella et al. 2017).

The two main representative hospital area for Klebsiella pneumoniae strains are III West (W) and II East (E) with 6 and 5 isolates respectively.

The epidemic curve based on the number of isolates collected during the study period (Figure 21) showed two discrete periods of outbreaks (November 2012 and January 2013) separated by 46 days.

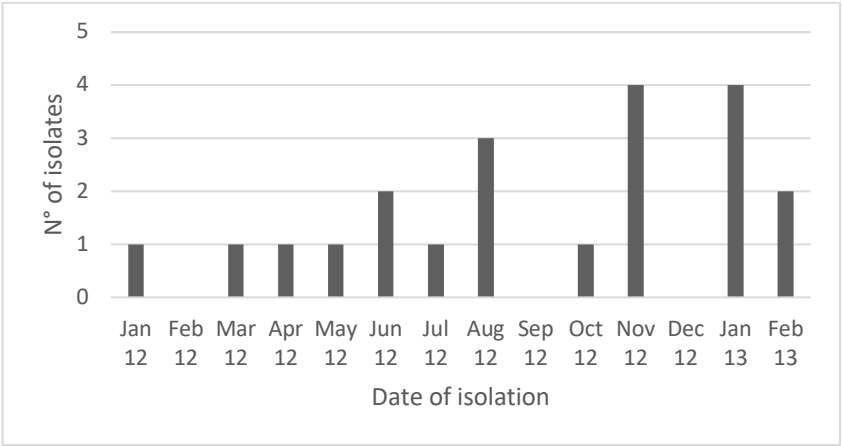

Fig. 21. Epidemic curves based on the number of Klebsiella pneumoniae isolated in the temporal frame of the study (Cella et al. 2017).

A timeline representing the K. pneumoniae MDR and KPC isolated in relationship with the ward and the length of stay in each ward was built (Figure 22).

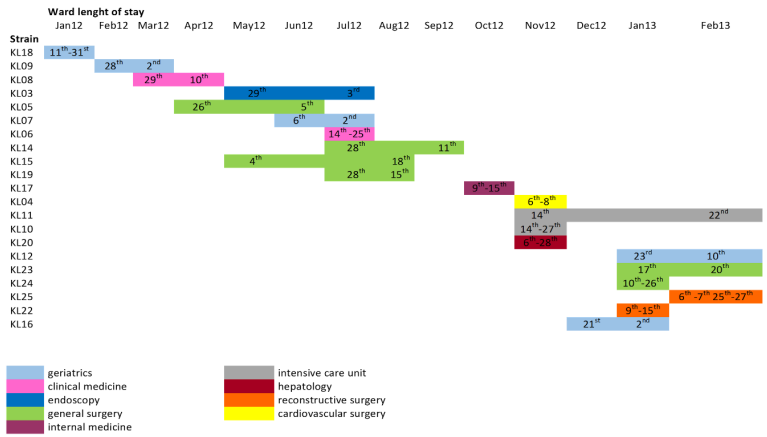

Fig. 22. Timeline representing the K. pneumoniae MDR and KPC isolated in relationship with the ward and the length of stay in each ward (Cella et al. 2017).

It showed that the general surgery was involved continuously between April and September 2012, whereas the geriatric ward was involved in three distinct periods separated by 60 and 120 days

respectively (January-March 2012, June-July 2012 and December 2012-February 2013), as in Figure 22.

2.2. Identification and antimicrobial susceptibility tests of the isolates

All of the 21 clinical isolates included in the study were identified as Klebsiella pneumoniae by MALDI-TOF MS with scores >2.0 (categorized as highly probable species identification) at the Biotyper software analysis.

AST profile determined by Vitek2 with the AST-N202 cards have been reported in the Table 7.

Tab. 7. Klebsiella pneumoniae isolates antimicrobial susceptibility test (AST).

Isolates	**Isolation date**	**AST**	**bla$_{kpc}$**
KL18	Jan 2012	Piperacillin/tazobactam >128 (R) Meropenem >8 (R) Imipenem >16 (R) Gentamicin 4 (I) Amikacin >16 (R) Ciprofloxacin >4 (R) Trimethoprim/sulfamethoxazole >4/76 (R) Colistin <1 (S)	3
KL09	Mar 2012	Piperacillin/tazobactam >128 (R) Meropenem >8 (R) Imipenem >16 (R) Gentamicin 4 (I) Amikacin >16 (R) Ciprofloxacin >4 (R) Trimethoprim/sulfamethoxazole >4/76 (R) Colistin <1 (S)	3
KL08	Apr 2012	Piperacillin/tazobactam >128 (R) Meropenem >8 (R) Imipenem >16 (R) Gentamicin 4 (I) Amikacin >16 (R) Ciprofloxacin >4 (R)	3

		Trimethoprim/sulfamethoxazole >4/76 (R) Colistin <1 (S)	
KL14	Aug 2012	Piperacillin/tazobactam >128 (R) Meropenem >8 (R) Imipenem >8 (R) Gentamicin 4 (I) Amikacin >16 (R) Ciprofloxacin >4 (R) Trimethoprim/sulfamethoxazole >4/76 (R) Colistin <1 (S)	3
KL19	Aug 2012	Piperacillin/tazobactam >128 (R) Meropenem >8 (R) Imipenem >8 (R) Gentamicin 4 (I) Amikacin >16 (R) Ciprofloxacin >4 (R) Trimethoprim/sulfamethoxazole >4/76 (R) Colistin <1 (S)	3
KL17	Oct 2012	Piperacillin/tazobactam >128 (R) Meropenem >8 (R) Imipenem >16 (R) Gentamicin 4 (I) Amikacin >16 (R) Ciprofloxacin >4 (R) Trimethoprim/sulfamethoxazole >4/76 (R) Colistin <1 (S)	2
KL05	May 2012	Piperacillin/tazobactam >128 (R) Meropenem >8 (R) Imipenem >8 (R) Gentamicin >4 (R) Amikacin >16 (R) Ciprofloxacin >4 (R) Trimethoprim/sulfamethoxazole >4/76 (R) Colistin <1 (S)	3
KL03	Jun 2012	Piperacillin/tazobactam >128 (R) Meropenem >8 (R) Imipenem >8 (R) Gentamicin 4 (I)	3

		Amikacin >16 (R) Ciprofloxacin >4 (R) Trimethoprim/sulfamethoxazole <1/19 (S) Colistin <1 (S)	
KL06	Jul 2012	Piperacillin/tazobactam >128 (R) Meropenem >8 (R) Imipenem >8 (R) Gentamicin >4 (R) Amikacin >16 (R) Ciprofloxacin >4 (R) Trimethoprim/sulfamethoxazole >4/76 (R) Colistin >16 (R)	3
KL20	Nov 2012	Piperacillin/tazobactam >128 (R) Meropenem >16(R) Imipenem >8 (R) Gentamicin <1 (S) Amikacin<2 (S) Ciprofloxacin >4 (R) Trimethoprim/sulfamethoxazole >4/76 (R) Colistin <1 (S)	3
KL10	Nov 2012	Piperacillin/tazobactam >128 (R) Meropenem >16(R) Imipenem >8 (R) Gentamicin <1 (S) Amikacin<2 (S) Ciprofloxacin >4 (R) Trimethoprim/sulfamethoxazole >4/76 (R) Colistin <1 (S)	2
KL16	Jan 2013	Piperacillin/tazobactam >128 (R) Meropenem >16(R) Imipenem >8 (R) Gentamicin <1 (S) Amikacin>64 (R) Ciprofloxacin >4 (R) Trimethoprim/sulfamethoxazole >4/76 (R) Colistin <1 (S)	3
KL22	Jan 2013	Piperacillin/tazobactam >128 (R) Meropenem >8 (R)	3

		Imipenem >8 (R) Gentamicin 4 (I) Amikacin >16 (R) Ciprofloxacin >4 (R) Trimethoprim / sulfamethoxazole >4 / 76 (R) Colistin <1 (S)	
KL12	Jan 2013	Piperacillin / tazobactam >128 (R) Meropenem >8 (R) Imipenem >8 (R) Gentamicin 4 (I) Amikacin >16 (R) Ciprofloxacin >4 (R) Trimethoprim / sulfamethoxazole >4 / 76 (R) Colistin <1 (S)	3
KL23	Jan 2013	Piperacillin / tazobactam >128 (R) Meropenem >8 (R) Imipenem >8 (R) Gentamicin 4 (I) Amikacin >16 (R) Ciprofloxacin >4 (R) Trimethoprim / sulfamethoxazole >4 / 76 (R) Colistin <1 (S)	2
KL24	Feb 2013	Piperacillin / tazobactam >128 (R) Meropenem >8 (R) Imipenem >8 (R) Gentamicin 4 (I) Amikacin >16 (R) Ciprofloxacin >4 (R) Trimethoprim / sulfamethoxazole >4 / 76 (R) Colistin <1 (S)	3
KL25	Feb 2013	Piperacillin / tazobactam >128 (R) Meropenem >8 (R) Imipenem >8 (R) Gentamicin 4 (I) Amikacin >16 (R) Ciprofloxacin >4 (R) Trimethoprim / sulfamethoxazole >4 / 76 (R) Colistin <1 (S)	3

KL04	Nov 2012	Piperacillin/tazobactam >128 (R) Meropenem >8 (R) Imipenem >8 (R) Gentamicin 4 (I) Amikacin >16 (R) Ciprofloxacin >4 (R) Trimethoprim/sulfamethoxazole >4/76 (R) Colistin <1 (S)	2
KL15	Aug 2012	Piperacillin/tazobactam >128 (R) Meropenem >8 (R) Imipenem >8 (R) Gentamicin 4 (I) Amikacin >16 (R) Ciprofloxacin >4 (R) Trimethoprim/sulfamethoxazole >4/76 (R) Colistin >16 (R)	3
KL11	Nov 2012	Piperacillin/tazobactam >128 (R) Meropenem >8 (R) Imipenem >8 (R) Gentamicin 4 (I) Amikacin >16 (R) Ciprofloxacin >4 (R) Trimethoprim/sulfamethoxazole <20 (S) Colistin >16 (R)	3
KL07	Jun 2012	Piperacillin/tazobactam >128 (R) Meropenem >8 (R) Imipenem >8 (R) Gentamicin 4 (I) Amikacin >16 (R) Ciprofloxacin >4 (R) Trimethoprim/sulfamethoxazole <20 (S) Colistin <1 (S)	3

All Klebsiella pneumoniae strains resulted MDR and KPC. 12/21 (57%) strains were susceptible only to colistin, whereas 9/21 (43%) showed a more variable or complex AST profile and 3/9 (33%) strains were colistin resistant.

All isolates carried blakpc type 3 except four that were type 2, (Table 8).

2.3. Whole-genome sequencing and genomic analysis

The twenty-one strains were sequencing using Illumina MiSeq II sequencer. The reads produced of the strains ranged from 458’899 to 1’773’414 bp (Figure 23).

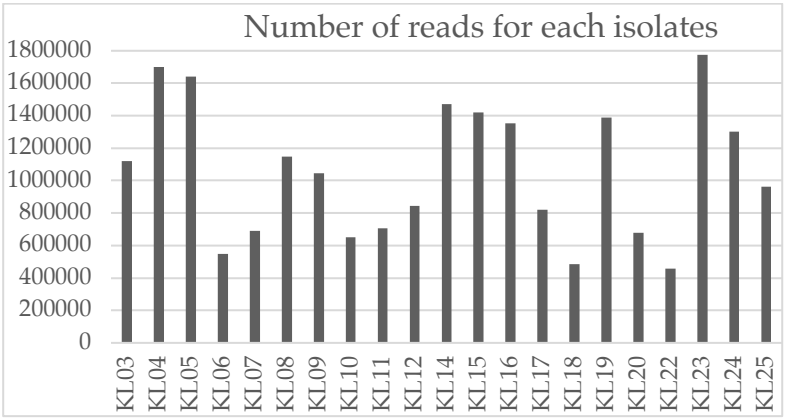

Fig. 23. Number of reads produced for each isolate (Cella et al. 2017).

The mean coverage obtained for the Klebsiella pneumoniae runs ranged from 30,47x to 119,28x (Figure 24). the mean coverage describes the average number of reads that align to, or "cover," known reference bases. The coverage level often determines whether variant discovery can be made with a certain degree of confidence at particular base positions. There is the recommendation of a value from 10× to 30× depth of coverage.

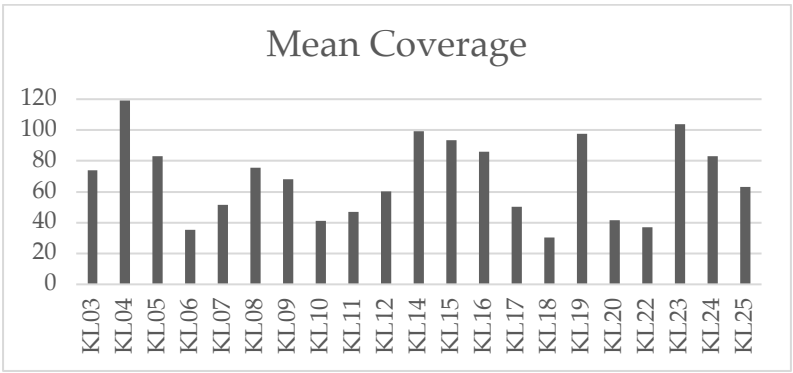

Fig. 24. Mean coverage of the reads produced for each isolate (Cella et al. 2017).

Analyzing the percentage of the reads mapped against the reference used, it has been found that this percentage ranged from 64,9% to 90,3%, this is possible due to the presence of different plasmids in the strains sequenced. The minimum percentage of reads mapped should be greater than 90% more or less (Figure 25).

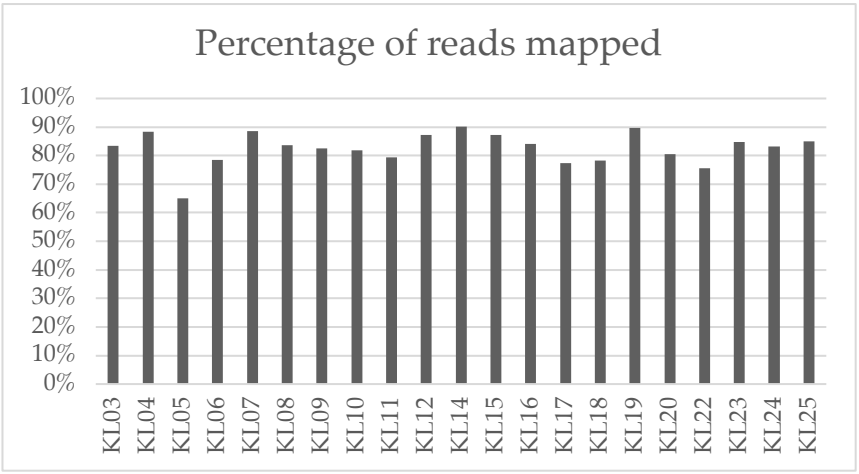

Fig. 25. Percentage of reads mapped against the reference sequence (Cella et al. 2017).

Moreover, the reads above 15bp were the most representative ones; the percentage of the reads above 15bp were from 89,8% to 96,5% (Figure 26).

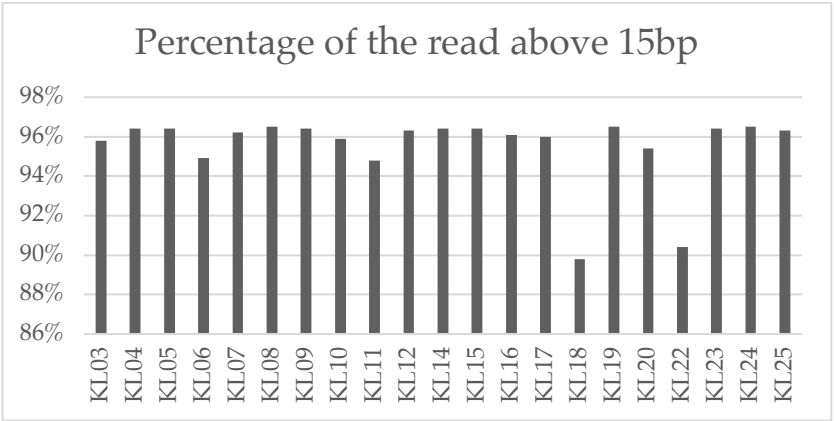

Fig. 26. Percentage of the reads above 15bp of the *K. pneumoniae* strains (Cella et al. 2017).

De novo assemblies were annotated with RAST removing the genome of the strain KL22 because it showed a very different expression than the other strains. In figure 50, there were two different groups of expression: one group (group I) included KL03, KL05, KL07, KL11 and KL18; and the other one (group II) included the remaining sequences (Figure 27).

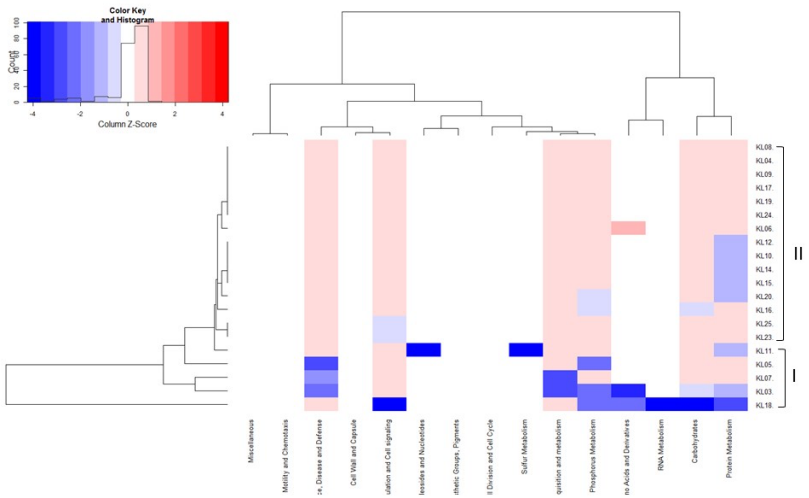

Fig. 27. Genetic expression of Klebsiella pneumoniae isolates. Group I and II are highlighted. In the X-axis genes divided by group are represented. Blue boxes represent underexpressed gene, whereas red boxed overexpressed genes. The white color corresponds to the "zero" value indicating absence of over/under gene expression (Cella et al. 2017).

Where the group I was under expressed the group II was over expressed, whereas for most of the gene groups all the sequences had a similar expression.

Annotations were confirmed using Prokka, the type of genes annotated are reported in Table 8.

Tab. 8. Annotation of the genomes with Prokka.

Core genes (99% <= strains <= 100%)	2686
Soft core genes (95% <= strains < 99%)	1126
Shell genes (15% <= strains < 95%)	1670
Cloud genes (0% <= strains < 15%)	587
Total genes	6069

Prokka identified a total of 6069 genes divided in the following categories: 2686 core genes (found in the 99% to 100% of the strains), 1126 soft core genes (found in the 95% to 99% of the strains), 1670 shell genes (found in the 15% to 95 % of the strains) and 587 cloud genes (found in the 0% to 15% of the strains).

Below the annotation of each Klebsiella pneumoniae isolates according number of contigs, bp length, number of annotated CDS, number of tRNAs and number of tmRNAs (Table 9).

Tab. 9. Annotation of the Klebsiella pneumoniae strains.

Strain	Contigs	Bases	Annotated Cds	tRNA	tmRNA
KL03	118	5519475	5220	77	1
KL04	140	5728441	5457	77	1
KL05	120	5788232	5519	71	1
KL06	153	5655030	5369	72	1
KL07	92	5565333	5268	76	1
KL08	169	5703883	5415	77	1
KL09	186	5750489	5464	78	1
KL10	175	5699291	5402	79	1

KL11	183	5626612	5347	77	1
KL12	129	5735016	5461	77	1
KL14	152	5721203	5448	75	1
KL15	149	5727878	5450	78	1
KL16	123	5633761	5360	77	1
KL17	143	5723684	5445	78	1
KL18	157	5443824	5182	71	1
KL19	136	5724773	5452	78	1
KL20	144	5655062	5344	77	1
KL22	160	3498357	3360	48	1
KL23	152	5688549	5414	77	1
KL24	141	5731909	5453	78	1
KL25	139	5690122	5420	72	1

All the strains are similar in terms of annotation according Prokka. Figure 28 showed the recombination analysis performed with Gubbins on the core genome alignment.

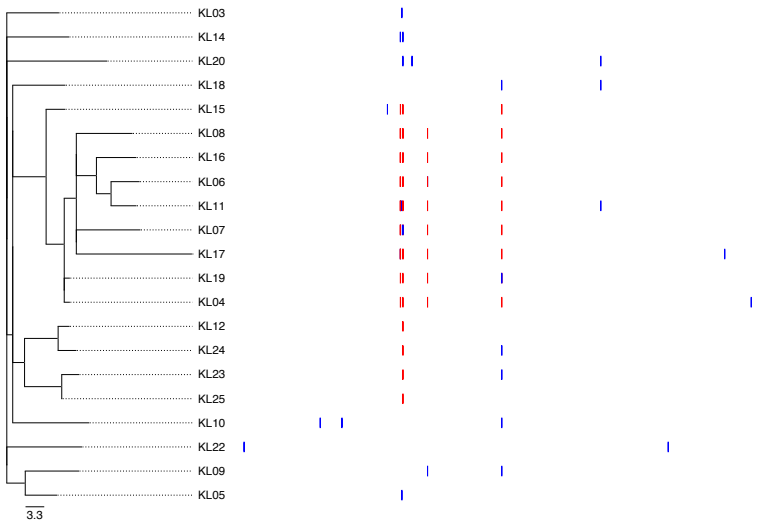

Fig. 28. Recombination analysis performed with Gubbins on Klebsiella pneumoniae core genome alignment (Cella et al. 2017).

For each isolate, blocks representing the regions identified as recombinant are indicated by colored blocks. Blue blocks are unique to a single isolate while red blocks are shared by multiple isolates through common descent. The horizontal position of the blocks represents their position in the alignment. The figure showed a light recombination along the alignment due to the possible translocation between the strains. In case of recombination events, Gubbins built a new SNPs alignment excluding the recombinant position.

The mean single nucleotide polymorphism difference was calculated using MEGA 6 (Figure 29).

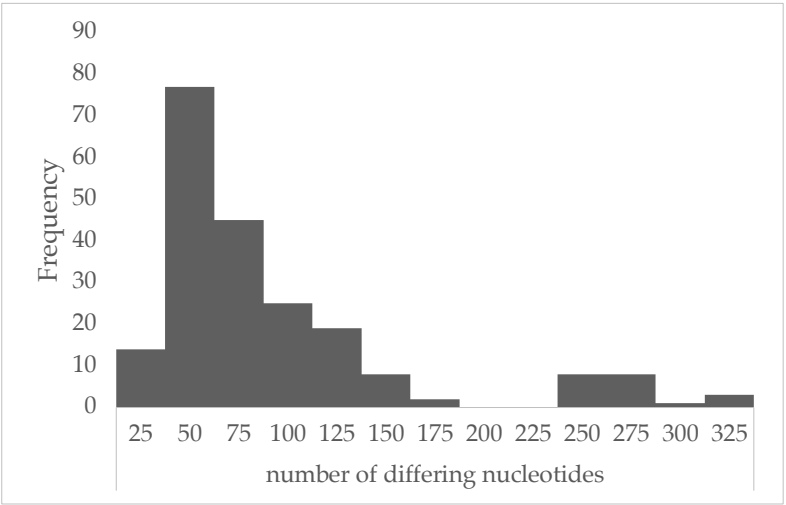

Fig. 29. Mean single nucleotide polymorphism (SNP) differences.

Klebsiella pnemomiae core genome SNPs alignment has 655 SNPs with an overall mean pairwise nucleotide difference of 80, with a median pairwise nucleotide difference of 36 (55-98).

2.4. Multilocus Sequence typing (MLST)

MLST was performed based on seven housekeeping genes: gapA, infB, mdh, phoE, rpoB and tonB. K. pneumoniae MDR and KPC strains resulted to be ST512 except one (KL05) ST650, different locus variants

belonging to the same clonal complex (CC258) Table 10. The two distinctive ST differ only for the gene profile of infB, giving a high similarity between these ST classes.

Tab. 10. MLST analysis.

Isolate	**ST**	Gene Profile						
		gapA	*infB*	*mdh*	*pgi*	*phoE*	*rpoB*	*tonB*
KL03	512	54	3	1	1	1	1	79
KL04	512	54	3	1	1	1	1	79
KL05	**650**	54	9	1	1	1	1	79
KL06	512	54	3	1	1	1	1	79
KL07	512	54	3	1	1	1	1	79
KL08	512	54	3	1	1	1	1	79
KL09	512	54	3	1	1	1	1	79
KL10	512	54	3	1	1	1	1	79
KL11	512	54	3	1	1	1	1	79
KL12	512	54	3	1	1	1	1	79
KL14	512	54	3	1	1	1	1	79
KL15	512	54	3	1	1	1	1	79
KL16	512	54	3	1	1	1	1	79
KL17	512	54	3	1	1	1	1	79
KL18	512	54	3	1	1	1	1	79
KL19	512	54	3	1	1	1	1	79
KL20	512	54	3	1	1	1	1	79
KL22	512	54	3	1	1	1	1	79
KL23	512	54	3	1	1	1	1	79
KL24	512	54	3	1	1	1	1	79
KL25	512	54	3	1	1	1	1	79

1.5. Phylogenetic and phylogeographic analyses

The phylogenetic signal analysis involves several levels of analysis;

this is the fundamental step of a phylogenetic analysis because otherwise without phylogenetic signal an analysis would be useless and uninformative. In phylogeny, it is important to have enough variability in the data to trace the evolutionary history of the microorganism/organism.

The phylogenetic signal was assessed first using a transition/transversion ratio vs. divergence graph performed with DAMBE (Figure 30).

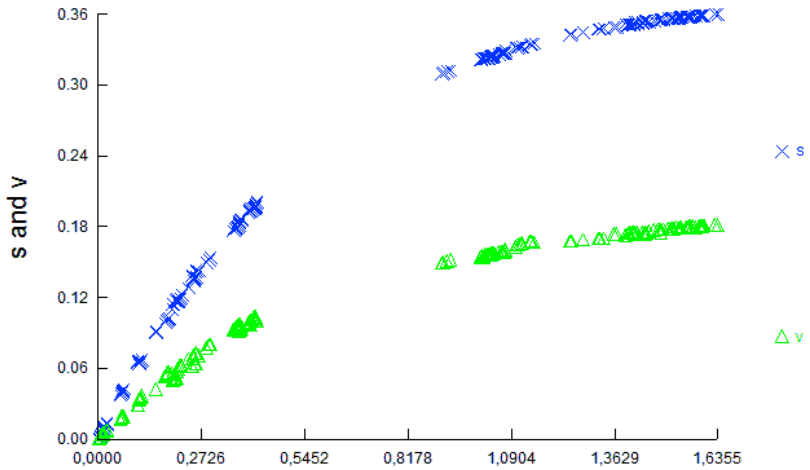

Fig. 30. Transitions/transversions ratio vs. divergence graph for the Klebsiella pneumoniae core genome SNPs dataset. X transitions; Δ transversion (Cella et al. 2017).

Generally, in the whole genome the ratio of transitions to transversions is typically around 2. In protein coding regions, this ratio is higher, frequently above 3. The higher ratio occurs because, especially when they occur in the third position of a codon, transversions are much more likely to change the encoded amino acid. The graph showed the ratio of transition (ts) was higher along all the genetic distance values.

The phylogenetic signal was also assessed using the Xia's test, Xia's method (X. Xia, Z. Xie, et al. 2003) is based on the concept of entropy in information theory, using the nucleotide frequencies as input. When sequences have experienced full substitution saturation, then the expected nucleotide frequencies at each nucleotide site are equal to the global frequencies. The test of substitution saturation can be done by

simply testing whether the observed H value in is significantly smaller than HFSS (full substitution saturation). If H is not significantly minor than HFSS, then the sequences have faced severe substitution saturation. This leads to a simple index of substitution saturation is defined as

$$Iss = H/H_{FSS}$$

As the sequences often will fail to recover the true phylogeny long before the full substitution saturation is reached, there is the need to find the critical Iss value (referred to hereafter as Iss.c) at which the sequences will begin to fail to recover the true tree. Once Iss.c is known for a set of sequences, then we can simply calculate the Iss value from the sequences and compare it against the Iss.c. If Iss is not smaller than Iss.c, then we can conclude that the sequences have experienced severe substitution saturation and should not be used for phylogenetic reconstruction.

The Xia's Test analysis performed with DAMBE showed little saturation ($p < 0.00$) (Table 11).

Tab. 11. Xia's test for substitution saturation: testing whether the observed Iss is significantly lower than Iss.c in the Klebsiella pneumoniae core genome SNP alignment. IssSym is Iss.c assuming a symmetrical topology. IssAsym is Iss.c assuming an asymmetrical topology.

Iss	**Iss.cSym**	**T**	**DF**	**P**	**Iss.cAsym**	**T**	**DF**	**P**
0,17	0,74	75,91	631	0,00	0,49	42,9	631	0,00

Note: two-tailed tests are used. Interpretation of results:
Iss < Iss.c & significant difference = little saturation;
Iss > Iss.c & significant difference = useless sequences;
Iss < Iss.c & non-significant difference = substantial saturation;
Iss > Iss.c & non-significant difference = very poor for phylogenetics.

Likelihood mapping analysis, to test the presence of phylogenetic analysis, reported 7.7% of star-like signal (phylogenetic noise) indicating overall that the data set contained enough information for reliable phylogeny inference (Figure 31).

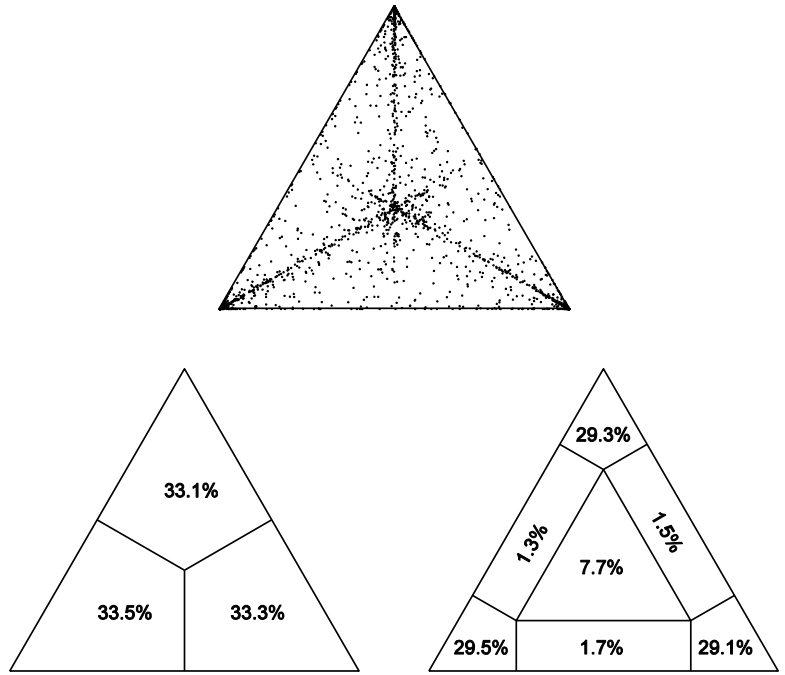

Fig. 31. Likelihood mapping of the Klebsiella pneumoniae core genome SNPs dataset. The dots inside the triangles represents the likelihood probabilities of the possible unrooted topologies for each quartet. Numbers indicate the percentage of dots in the center of the triangle corresponding to phylogenetic noise (star-like trees) (Cella et al. 2017).

All these analyses indicated that enough signal for phylogenetic inference existed (Figure 30, 31 and Table 11).

Maximum Likelihood tree of K. pneumoniae core genome SNPs alignment in Figure 32.

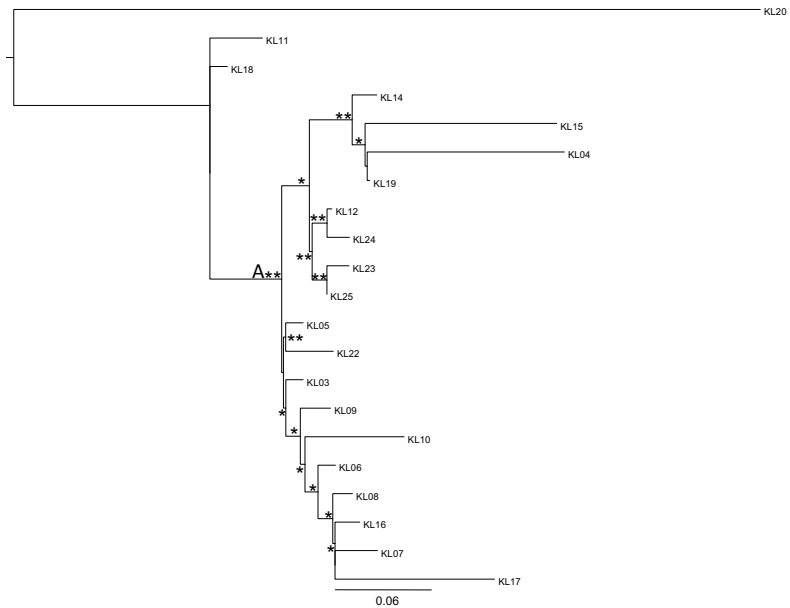

Fig. 32. ML of Klebsiella pneumoniae core genome SNPs alignment. * along the branches indicating a statistical value from bootstrap or sh-lrt analyses; ** indicating a statistical value from bootstrap and sh-lrt analyses (Cella et al. 2017).

KL20 strain appeared to be more divergent than the other strains (as confirmed from the annotation analysis); indeed, in the tree, this strain was considered as outgroup. A statistically significant clade was highlighted (clade A). Inside the clade A, there were identified subclades and clusters, statistically supported by bootstrapping and SH-aLRT analyses, highlighting different epidemic waves.

The phylogeographic analysis was performed on the hqSNPs of the core genome to investigate the evolution of the *K. pneumoniae*. The exponential growth demographic model with a relaxed molecular clock was selected as the most appropriate to describe the evolutionary history of *Klebsiella pneumoniae*. Molecular clock calibration estimated the evolutionary rate of the *K. pneumoniae* SNPs core genome alignment at 4.97×10^{-3} substitutions site per year (95% HPD $9.98\text{x}10^{-3}$ - $9.67\text{x}10^{-4}$) (Figure 33).

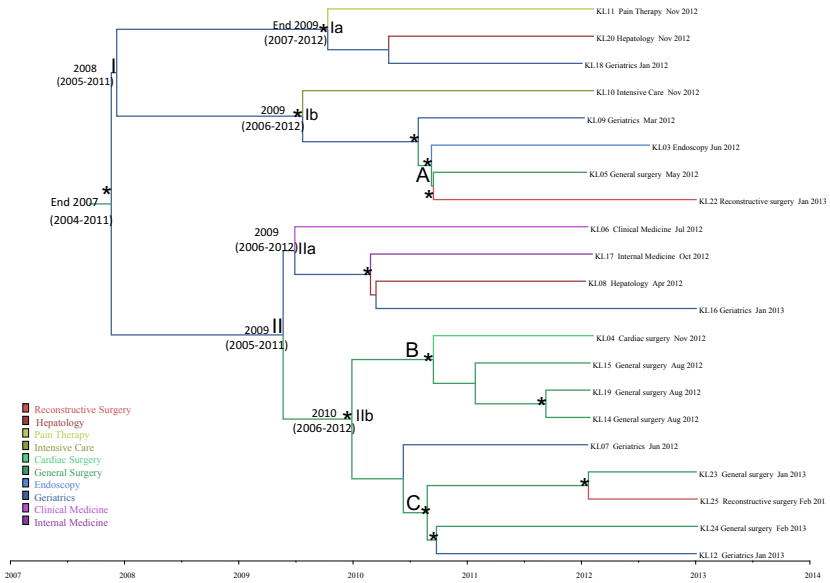

Fig. 33. Maximum clade credibility (MCC) tree with Bayesian phylogeographic reconstruction of Klebsiella pneumoniae isolates. Branches are scaled in time and colored according to the legend to the left where each color represents the geographic location of the sampled sequence (tip branches), as well as of the ancestral lineage (internal Branches) inferred by Bayesian phylogeography. Significant posterior probability support ($p \geq 0.9$) as indicated by an asterisk. Clade and clusters are highlighted (Cella et al. 2017).

The root of the time of the most common recent ancestor (tMRCA) corresponding to the end of 2007 (HPD 95% 2004 – 2011) and the most probable location for the MRCA was general surgery ward (in hospital area IIIW). The tree was composed by two major clades (clade I and II), both probably originated from geriatrics ward (hospital area IIIW). Clade I dated back 2008 (HPD 95% 2005-2011) instead clade II dated 2009 (HPD 95% 2005-2011).

Cluster Ia dated back to end of the year 2009 (HPD 95% 2007-2012) and the probable location for MRCA was geriatrics ward (hospital area IIIW) and this cluster included sequences from pain therapy, hepatology and geriatrics ward. Cluster Ib dated back to 2009 (HPD 95% 2006-2012) with geriatrics wards (hospital area IIIW) too as location; this cluster included sequences from intensive care, geriatrics, endoscopy, reconstructive surgery and general surgery wards.

The KL10 sequence belonged to a patient admitted in intensive care

ward, but in the three days before the culture request for suspected infection, he was in the geriatrics ward. In particular, inside this cluster there was a statistical significant group (labelled as A) including three sequences probable placed in general surgery (hospital area IIIW).

The cluster IIa dated back 2009 (HPD 95% 2006-2012) and possible location was geriatrics ward (hospital area IIE); this cluster included sequences from clinical medicine, internal medicine, hepatology and geriatrics wards.

The cluster IIb dated back 2010 (HPD 95% 2006- 2012) with general surgery ward as possible location and IIE as hospital area.

The KL23 sequence belonged to a patient admitted in general surgery ward, but in the six/five days before the culture request for suspected infection, he stayed in intensive care ward. The group labelled as B had as possible location general surgery ward in hospital area IIIE; it included sequences from general surgery and cardiac surgery wards sampled from August to November 2012.

The KL14 and KL19 sequences belonged to two patients admitted both in general surgery ward in the similar period in two different hospital areas (IIIE and IIE respectively). These patients in the four days before the culture request for suspected infection, were in intensive care ward in the same night in two beds next each other (ICU bed 05 and ICU bed 03 respectively).

The possible location of group labelled as C was general surgery ward in hospital area IIE; this cluster included sequences from general surgery, reconstructive surgery and geriatrics wards sampled in January and February 2013.

Based upon antimicrobial susceptibility testing results, 9/21 (43%) Klebsiella pnemomiae (KL05, KL03, KL22, KL16, KL10, KL20 and KL07) strains showed a more variable AST profile.

Strains KL05, KL03 and KL22 were closely related within the cluster 1b but different phenotype than KL10 and KL09 belonging to the same cluster.

Strain KL07 was different from the other strains into the statistically supported group C, in agreement with the AST profile. Strain KL20 appeared to be very close to strain KL18 without any statistically support in agreement with the different AST phenotype.

Three of nine (33%) strains (KL11, KL15 and KL06) were colistin resistant and interspersed in the tree each out of the proper subclade

(Figure 33).

Based upon the epidemic curve, Klebsiella pnemomiae strains clustering within the cluster IIb are the most represented. Since June 2012 their circulation continue up to February 2013 and interestingly these strains concentrate their circulation especially in August 2012 (Figure 34).

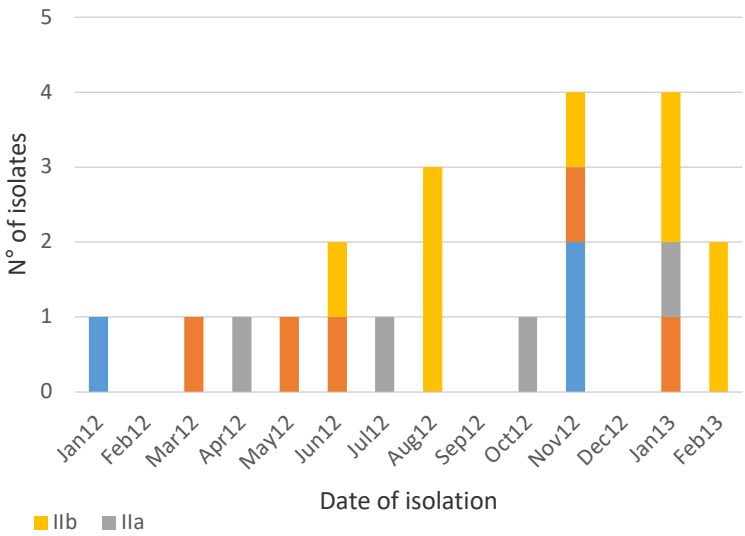

Fig. 34. Epidemic curve based on the number of isolates falling within each phylogenetic clade during the study period (Cella et al. 2017).

Figure 35 shows the Bayesian skyline plot for the effective population size of *Klebsiella pnemomiae* core genome SNPs alignment. There was an exponential growth from 2010 to the end of the same year. In 2011 started a light decrease followed by a plateau until 2013.

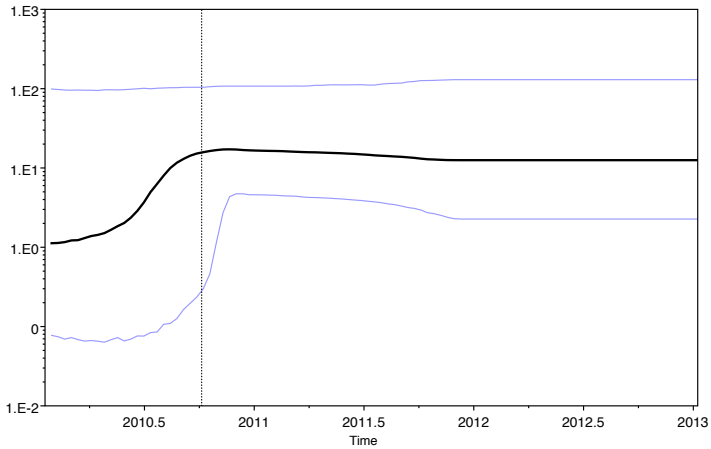

Fig. 35. Bayesian skyline plot (BSP) of the Klebsiella pneumoniae isolates. The effective number of infections is reported on the Y-axis. Time is reported in the X-axis. The colored lines correspond to the credibility interval based on 95% highest posterior density interval (HPD) (Cella et al. 2017).

The Bayesian analysis reported R_0 of 1.573 (95% confidence interval from 0.763 to 2.59).

2.6. Duedonoscope disinfection protocol and microbiological surveillance

The microbiological surveillance was divided in three steps: the first one between September 20, 2016 to October 9, 2016, the second one between October 10, 2016 to January 13, 2017 and the last one from January 14, 2017 to September 11, 2017.

During the first step of the surveillance there were performed 13 samplings on the five instruments, duedonoscopes.

It has been found 7 positive sampling with several MDROs (K. pneumoniae, P. aeruginosa, A. baumanii, E. coli) with a 53% of infected instruments (Table 12).

Tab. 12. First sampling description.

SAMPLING FROM SEPTEMBER 20, TO OCTOBER 9, 2016	**SAMPLING POSTIVE/ TOTAL SAMPLING**	**BACTERIA DETECTED 5 - 25 CFU**	**BACTERIA DETECTED > 25 CFU**
VIDEODODEOSCOPE TJF Q180V (Olympus)	**2** / 4	S.hemolyticus	K.pneumoniae (MDR), P.aeruginosa (MDR), E.faecalis, E.coli (ESBL)
VIDEODODEOSCOPE TJF 145 (Olympus)	**2** / 3	A. xylosoxidans, S.maltophilia	K.pneumoniae (MDR), P.aeruginosa (MDR), A.baumanii (MDR)
VIDEODODEOSCOPE TJF 160R (Olympus)	**2** / 3		K.pneumoniae (MDR), P.aeruginosa, S.maltophilia
CONVEX ECOENDOSCOPIC PROBE (Pentax)	**1** / 2		S.maltophilia, D. acidovorans
EDGE-3870T LINEAR PROBE (Pentax)	**0** /1		

After this sampling step some measures were implemented in the instruments sanitization (reprocessing) (Table 13).

Tab. 13. Reprocessing measures adopted after the first sampling.

Manual disinfection	**Disinfection**	**Drying**	**Endoscopic storage**	**Endoscopic valves**	**Traceability**
Used disposable pipe cleaner and brushes (Lorenzatto) or used reusable pipe cleaner sterilized in autoclave.	The duodenoscopes, the operative endoscopes and the bronchoscopes are disinfected with the cycle 1, which includes the addition of drying. The other endoscopes are disinfected instead with Cycle 2 (Olympus ETD 3 plus)	Medical air forced only after the last wash of the day before the storage in the closet.	No ventilated metal cabinets for all endoscopes.	Disposable valves are used on all duodenoscopes and during procedures of known infectious patients and with bleeding.	Manual disinfection provides paper traceability while automatic disinfection is computerized.

In the second step of the surveillance there were performed 31 samplings on the five instruments, duedonoscopes.

It has been found 3 positive sampling with none MDROs, only K. pneumoniae as important germ, with a 9 % of infected instruments (Table 14).

Tab. 14. Second sampling description.

SAMPLING FROM OCTOBER 10, TO JANUARY 13, 2017	SAMPLING POSITIVE/ TOT SAMPLING	BACTERIA DETECTED 5 - 25 CFU	BACTERIA DETECTED > 25 CFU
VIDEODODEOSCOPE TJF Q180V (Olympus)	**2** / 7	S.sanguinis	K.pneumoniae
VIDEODODEOSCOPE TJF 145 (Olympus)	**1** / 7		N.flavescens R.muciiaginosa
VIDEODODEOSCOPE TJF 160R (Olympus)	**0** / 7		
CONVEX ECOENDOSCOPIC PROBE (Pentax)	**0** / 3		D. acidovorans
EDGE-3870T LINEAR PROBE (Pentax)	0 /7		

After this sampling step, other measures were implemented in the reprocessing to improve the detection and sanitization (Table 15).

Tab. 15. Reprocessing measures adopted after the second sampling.

Manual disinfection	Disinfection	Drying	Endoscopic storage	Endoscopic valves	Traceability
By inhaling the inhaler attachments we inject in the accessory channel of duodenoscope TJF 145 and linear endosonographers 10 ml of proteolytic, diluted properly in water, closed distal tip and	New machines for sanitization Soluscope Series 4. Duodenoscopes, linear endoscopes and bronchoscopes perform a cycle 2 (2 detergents and 1 disinfection) and a cycle 4 (1 disinfection).	It is carried out at the end of each wash cycle from the new storage cabinets.	They are stored in storage cupboards each in a separate compartment separated from the others. The wardrobe, in	Disposable valves are used on all duodenoscopes and during procedures of known infectious patients and with bleeding.	All reprocessing machines are interfaced with each other through the ITsoluscope server, which allows you to keep all the data necessary for traceability

10 ml with open distal tip. It has been replaced the Proteoxim Plus enzymatic detergent with Aniosyme DD1. New semi-automatic washbasins allow traceability of all stages of the process.	All other instruments run a cycle 1 (1 cleansing + 1 disinfection)		addition to the drying, allows traceability of the disinfection times and traces the storage.		of the process.

After these big sanitization improvements, it has been performed the last sampling step. There were performed 62 samplings on the five instruments, duedonoscopes.

It has been found 20 positive sampling with none MDROs, only K. pneumoniae, P. aeruginosa as important microorganisms, with a 32 % of infected instruments (Table 16).

Tab. 16. Third sampling description.

SAMPLING FROM JANUARY 14, TO SEPTEMBER 11, 2017	SAMPLING POSITIVE/ TOTAL SAMPLING	BACTERIA DETECTED 5-25 CFU	BACTERIA DETECTED >25 CFU
VIDEODODEOSCOPE TJF Q180V (Olympus)	**1** / 7	E.faecalis, B. simplex	
VIDEODODEOSCOPE TJF 145 (Olympus)	**7** /18	S.epidermidis, N.macace, R.mucilaginosa, S.salivarius	K.pneumoniae, S. hominis, S.capitis, E.faecium
VIDEODODEOSCOPE TJF 160R (Olympus)	**5** / 18	K. Pneumoniae	K. Pneumoniae, P.aeruginosa, E. coli
CONVEX ECOENDOSCOPIC PROBE (Pentax)	**2** / 6	E. faecium, N. subflava	S.capitis, S. paucimobilis
EDGE-3870T LINEAR PROBE (Pentax)	**5** / 15	B. cereus, S. epidermidis, K.pneumoniae, E. faecium	S. maltophilia, E.coli, P. putida, A.junii

Even if the proportion of positive sampling is higher in the last step, the measures implemented are highly better. In this step it has been used Tampon DNP+0.5% Thiosulfate instead water in the manual disinfection, this is clearly a better compost to wash away the possible biofilm inside the duedonoscope channels. Interestingly no MRDO has been found after the first improvement in the sanitization.

It has performed also an epidemiological investigation to compare the number of positive bilicolture, Klebsiella pneumoniae and MDR strains detected (Figure 36).

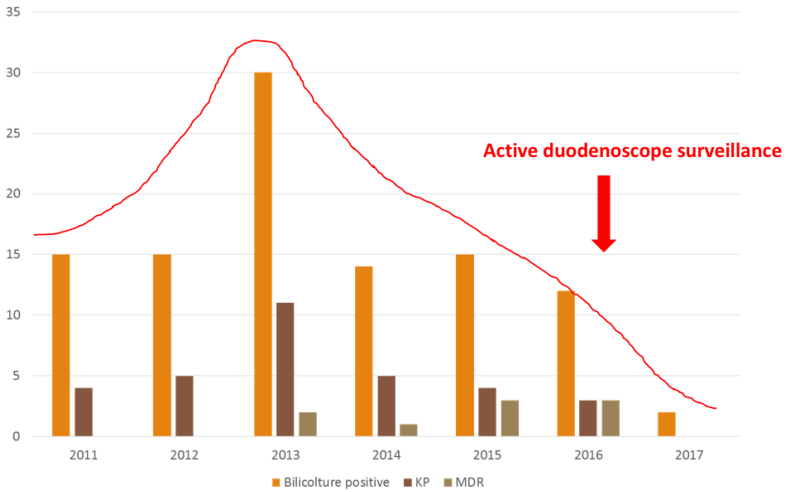

Fig. 36. Number of positive bilicolture, Klebsiella pneumoniae and MDR strains from 2011 to June 2017.

There was a pick of positive bilicolture, Klebsiella pneumoniae and MDR strains identified in 2013 with a rapid decrease of K. pneumoniae and MDR strains to 2016, until the MDR were not detected in 2017. There is a decreasing of the positive bilicolture first slowly then significantly.

3. Discussion

The combination of epidemiological analysis and high-resolution WGS has been shown to be valuable for nosocomial outbreaks investigation. NGS is becoming an important framework for clinical diagnostics. The NGS methodology has been recently used to characterize pathogens in different contexts (El-Herte, et al. 2012) (Archer, Rambaut, et al. 2010) (Kuroda, et al. 2010)

(Poon, et al. 2010) (Rozera, et al. 2009) (Archer, Braverman, et al. 2009) (Wang, et al. 2007) (Yin, et al. 2012), moreover the reasonable cost of the analysis make it the possibility to use also in a routine diagnostic setting (Kingsmore e Saunders 2011) and may be an important resource for nosocomial bacterial surveillance (Angeletti, Dicuonzo, et al. 2015). The study was designed providing a "snap shot" of Klebsiella pneumoniae in hospital setting combining data for surveillance with molecular ones. Moreover, Bayesian phylogenetic and phylogeographic analyses have been powerful tools used to follow the spread of Klebsiella pneumoniae in different wards and different time.

The epidemic curve based on the number of isolates collected during the study period (Figure 21) showed two different picks of outbreak (November 2012 and January 2013) separated by 46 days, making the investigators aware that an improvement in preventive measures need to be adopted.

The Bayesian tree dated the TMRCA indicating that strains isolated between 2012 and 2013 could be introduced in the hospital setting since the end of the year 2007. Moreover, the phylogenetic tree showed two different epidemic entrance in the hospital (2008 and 2009 years). Connecting epidemiological data with phylogenetic analysis was evident as ERCP performed in four patients were found in two different

groups A and B. Interestingly, within group A, strain KL09 isolated in a patient coming from a different hospital was admitted with documented infection in March 2012. The phylogenetic tree showed how this strain is a sort of outgroup that probably infected the other components of group A. In this group are included strains KL05 and KL03 from patients submitted to ERCP suggesting that after its introduction the strain circulation was maintained through the nosocomial endoscopic and post-surgery dressing procedures. Strain KL22 even if not receiving ERCP treatment or not admitted in the same ward it is clearly significantly related with the other three strains in group A probably for surgery or visit room sharing.

In group B, isolates KL14 and 19 were from patients admitted both in general surgery, in overlapping periods, but in two different hospital areas (IIIE and IIE respectively). These patients in the four days before the culture request were in ICU in the same night in two beds next each other (ICU bed 05 and ICU bed 03 respectively). In these last two patients, it was probable the man-to-man transmission as evident by the phylogenetic tree and confirmed by the epidemiological data. Anyway, we cannot exclude if fomites, or devices, or persons encountering these patients have been a sort of "bridge" to transmit the infection between them.

Phylogenetic analysis also revealed the presence of another statistically supported group (group C). In this group strains KL23 and KL25 were from patients that in overlapping period were admitted in different ward at the same floor but in different hospital areas (II East and II West). The tree topology showed a significant relationship between them suggesting that the strain could be the same probably acquired during the hospital stay. Another statistically supported cluster of group C was evident in the tree for strains KL12 and KL24. These isolates were from patients admitted in the same floor but in different hospital areas (II West and II East, respectively). In this case, the classical epidemiology does not help to clarify the way of transmission, whereas the phylogenetic analysis clearly suggests the same strain infecting these two patients. It is noteworthy, that patients of group C were in overlapping period hospitalized in the same floor, the second floor.

The Bayesian skyline showed an exponential growth of K. pneumoniae infections from 2010 reaching a plateau in 2013. This is in line

with the absence of microbiological surveillance at hospital admission looking for multidrug-resistant (MDR) bacteria until the year 2013. From the year 2013 a microbiological surveillance and the adoption of adequate preventive measures, such as contact isolation upon MDR bacteria detection, have been performed, showing the effectiveness of this policy. By reconstructing the demographic of bacterial population, it was also possible to estimate the R0 value for Klebsiella pneumoniae infections. Even if the colonization state, during which bacteria can transiently or persistently colonize an individual, can represent a bias for R0 values estimate, the R0 calculated for our strains, ranging from 0.763 to 2.59, does not suggest a clear indication that the outbreak is self-sustaining over the studied period being the lower limit range value <1.

The microbiological surveillance on duedonoscope revealed a dramatic problem in the reprocessing of these, as found also (Rubin e Murthy 2016) (Gastmeier e Vonberg 2014) (Patterson 2009).

In the first sampling step the percentage of contaminated instruments was 53% with several MDROs detected, this could be a serious vehicle in the nosocomial infections.

The percentage of contaminated instruments in the second sampling step collapsed down to 9% with no MRDOs identified indicating that the new measures put into practice were functional in the sanificatazion and the microbiogical identification. In the last sampling step the percentage of contamined instruments increased to 32% with no MDROs detected. Several measures have been put into practice, all of them very useful for the purpose, but in particular it has been replaced the water with Tampon DNP+0.5% Thiosulfate. Tampon DNP+0.5% Thiosulfate is a powerful compost to break off the potential biofilm formed inside the complex instruments which probably required a deeper procedure.

The results of the sampling performed highlighted a first multi-microbial contamination supported by MDR organism (K. pneumoniae, P. aeruginosa and A. baumanii) eliminated from the introduction of the new reprocessing protocols. But eliminating the 90% of the total outbreaks but the remaining 10% is due to: ruined channels in overused instruments (often the instruments are in use for several years, due to substantial cost of these), duodenoscopes complex design, growth of multi-microbial biofilm resistant to reprocessing protocol.

Due to the complex design of duodenoscopes, it is difficult an adequate disinfection of the channels. Duodenoscopes can be difficult to dry and a potential risk of cross contamination is higher using this type of endoscope (Verfaillie, et al. 2015). New duodenoscope designs should facilitate more efficient disinfection, FDA has identified design features that facilitate cleaning, disinfection and sterilization and reduce the likelihood of retaining debris (FDA 2017).

Combining the data from this study, the ones from the molecular epidemiology on KPC strains in this hospital (Angeletti, Lo Presti, et al. 2016) and the epidemiological results of the bilicolture it is possible to determinate a time frame (Figure 37).

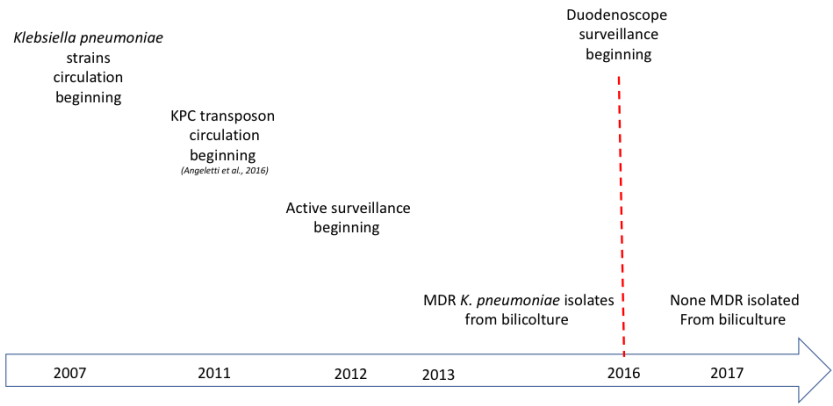

Fig. 37. Time frame of the preventive measures introduced in the hospital.

The absence of MDROs detected in the bilicolture and in the duedonoscope sampling is a clear sign of the importance of an active surveillance in the hospital with a real collaboration between all the figures inside the hospital (ward nurses and medical doctors, laboratory staff and nosocomial center members) for a rapid detection of the infections and the eradication of them.

In addition to the preventive measures realized since 2013, consisting in patient contact isolation upon MDR bacteria detection, some others measure should be improved based on the results of the present study. Microbiological surveillance of rectal, nasal and pharyngeal swabs could be included for all the inpatients at the admission time as

well as outpatients referred to endoscopic procedures such as ECRP in day hospital. Staff hand hygiene adhesion should be improved by collection of data of adherence and continuing education program; the microbiological surveillance should be extended to all the operative endoscope used at regular time interval depending of the instrument complexity.

In conclusion, our study showed the complex transmission and circulation dynamics of nosocomial strains. The cross-talk between classical and molecular epidemiology, when both are known, allowed us to accurately define the way of strains transmission. The molecular epidemiology based on phylogenetic analysis could represent a useful tool to support the classical epidemiology in the MDR pathogen surveillance. The two different approaches if adopted together could aid to trace exactly the way of transmission of the pathogen and perform a focused action plan in the wards where the transmission began.

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Carbapenems resistant Enterobacteriaceae infections are increasing worldwide representing an emerging public health problem. The application of phylogenetic and phylodynamic analyses to bacterial whole genome sequencing (WGS) data have become essential in epidemiological surveillance of MDR pathogens to discern outbreak from non-outbreak strains in both community and hospital settings.

In this study, K. pneumoniae strains circulating within different wards of university hospital were collected and WGS applied. Moreover a microbiological surveillance on duedonoscopes was performed to evaluate their reprocessing. The aim was to infer the origin and the spread of K. pneumoniae nosocomial strains and to clarify the epidemiological transmission as so as the eventual reservoir in the hospital setting supporting the epidemiological surveillance and infections control strategies.

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