



New Trends in Microbial Epidemiology: Can An Old Dog Learn New Tricks?

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Abstract

In historical terms, almost 50 years ago, all the knowledge coming from clinical microbiology was provided by classical methods, mainly based on phenotypic observations and culture-dependent methods. With the advent of DNA fragment analysis, PCR-based methods and Sanger sequencing, the dawn of the Era of Molecular Diagnosis began. Significant improvements were achieved, namely improved resolution concerning the identification of etiological agents was possible in a reduced timeframe between sample collection and results. To overcome some of the difficulties presented by molecular diagnosis, almost 20 years ago, massive parallel sequencing technology was introduced into epidemiological studies. Nowadays, it is possible to sequence the whole genome of a given microorganism (including information on antibiotic resistance, virulence factors, and evolutionary relationships) or to assess the complete diversity of microorganisms present in a given individual or in environmental samples (metagenomics). The present review covers the major outbreaks registered worldwide in the past 12 years and highlights the use of new technologies in source identification, outbreak detection, routes of transmission determination, pathogen evolution inference and mechanisms of pathogen adaptation to drug therapies.

Keywords: Clinical microbiology; Epidemiology; Genetics; Massive parallel Sequencing; Microbiome

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Introduction

In generic terms, the obligation of a clinical microbiologist is to retrieve pieces of information (e.g. species identification, metabolic capabilities, antigenic and staining properties, antibiotic resistance profiles, etc.) from pathogenic agents [1]. In clinical medicine, a rapid and accurate pathogen identification constitutes the cornerstone for correct diagnosis, effective treatment, recovery and wellbeing of the patients [2,3].

Before the 1970's, the diagnostic tools available for the detection of the causative agents of infectious diseases were restricted to phenotypic methods, in which the identification at the genus and/or species level (using culture-dependent techniques) was coupled with the determination of typical antigenic characteristics and/or antimicrobial resistance patterns [4].

Since the late 1980's, with the development of PCR-based techniques, a paradigm shift was observed inside clinical microbiology laboratories: the conventional phenotypic methods were replaced by molecular diagnostics based on DNA, either fragment analysis-based or PCR-based methods [e.g. Pulsed-Field Gel Electrophoresis (PFGE), Restriction Fragment Length Polymorphism (RFLP), Randomly Amplified Polymorphic DNA (RAPD), Arbitrarily Primed PCR (AP-PCR), Amplified Fragment Length Polymorphism (AFLP), Multiple-Locus Variable Number Of Tandem Repeats Analysis (MLVA), Single-Nucleotide Polymorphism (SNP), Multilocus Sequence Typing (MLST), Multi-Virulence-Locus Sequence Typing (MVLST), Real-Time Polymerase Chain Reaction (RT-PCR), and Sanger sequencing] [2,4]. Moreover, these new methods allowed an improvement on the taxonomical level identified, increasing resolution from the genus/species level previously obtained to isolates or strains. Comparing both phenotypic and molecular methods, the latter approaches allowed to reduce the turnaround time between sample collection and the result. However, these methods only provide limited information insufficient for outbreak and transmission investigation, failing to investigate fast evolving pathogens [3].

Since the early 2000's, the traditional Sanger sequencing has been complemented by Massive Parallel Sequencing technology (MPS) [also known as Next-Generation Sequencing (NGS)] [4]. Due to its elevated multiplexing capacity, this new method paved the way to different approaches: i) sequencing the whole genome (WGS) of a given microorganism (bacteria, fungi or virus), including the information on antibiotic resistance, virulence factors, and evolutionary relationships; ii) or sequencing all the microbial species present in samples from a given individual or environment (metagenomics) [1,3,5].

A major advantage of NGS over Sanger sequencing is its high throughput capacity. Nowadays hundreds millions of sequencing reactions can be performed in parallel, allowing to sequence an entire bacterial genomes in just one or two instrument runs. Also, a single protocol can be applied for all pathogens for both identification and typing purposes [6]. Moreover, NGS obviates the need for bacterial cloning of DNA fragments, depending only on the preparation of NGS libraries in a cell free system. Furthermore, the application of NGS allows to reduce both costs (depending on the genome length, usually less than US\$1000 per genome) and the turnaround time (a few hours). Additionally, there is no need for an *a priori* knowledge about the sequence of a particular gene/genome because these new technologies can read the DNA templates randomly distributed throughout the entire genome and next, *de novo* genome assembly can be applied. Finally, and particularly important in cases involving microorganisms in diagnostics, monitoring, and treatment, NGS overcomes the need for isolation and culture of the microorganism of interest, a particularly important aspect since many strains are unable to grow in culture media [7]. Moreover, NGS also allows to identify microorganisms present in minute concentrations that would be undetected using conventional methods. Therefore, combining these two last aspects, these technologies provide insights much closer to the true microbial diversity present in a given sample. The major disadvantage of the NGS is the shorter read length for each DNA template, at the present, only 35-500 base pairs (bp) can be obtained; while with Sanger sequencing sequences ranging from 1,000 to 1,200 bp can be easily obtained [3,8-11].

In 2014, NGS-based approaches were introduced in routine diagnostics, for investigation of outbreak and transmission, and for genotyping of highly-resistant microorganisms. Therefore, clinical microbiologists or infectious disease specialists frequently resort to NGS, in collaboration with molecular microbiologists and infection control professionals [3]. In fact, NGS has frequently been proven as suitable for studies concerning source identification, outbreak detection, transmission routes, pathogen evolution and dynamics of multi-drug resistant pathogens [3].

As reviewed in [12], a long list of examples of the application of NGS in studies related with outbreaks and transmission of pathogens can be presented: the cases of *Mycobacterium tuberculosis* (Canada, 2006) [13], *Acinetobacter baumannii* (United Kingdom, 2008) [14], swine-origin influenza A (H1N1) virus (America, 2009) [15], *Vibrio cholera* (Haiti, 2010) [16], Shiga toxin-producing *Escherichia coli* O104:H4 (Germany, 2011) [17], Coronavirus (Saudi Arabia, 2012) [18], avian-origin influenza A (H7N9) virus (China, 2013) [19], Poliovirus (Middle East, 2013) [20], Ebolavirus (Sierra Leone, 2014) [21], *Legionella pneumophila* (Portugal, 2014) [22], and Zika virus (Southern America, 2015) [23] (Figure 1).

Despite its Epidemiological applications, the study of

microorganism source, evolution and transmission routes is also related to Microbial Forensics. The knowledge gained through these aspects may serve as auxiliary evidence to clarify possible causes of death (e.g. toxicological changes, hospital-acquired infections, sudden infant death and shaken baby syndromes), to assist in human identification (skin, hair, and body fluids microbiomes), to allow the geolocation (soil microbiome), and to estimate postmortem interval estimation (thanatomicrobiome and epinecrotic microbial community) [24,25]. Also, genotyping studies have been proven useful both in cases of bioterrorism, such as the famous Amerithrax attacks in 2001 [26], and biocrime, such as the intended dissemination of human immunodeficiency [27] and hepatitis C viruses [28,29].

The present review explores relevant epidemic and pandemic cases, discussing how the most recent NGS techniques revolutionized the classical epidemiological studies.

Relevant Epidemic and Pandemic Cases

Tuberculosis (Canada, 2006)

Between 2006 and 2008, an outbreak of tuberculosis (etiological agent: *Mycobacterium tuberculosis*) registered in British Columbia (Canada). By the end of this period a total of 41 cases were confirmed (37 laboratory confirmed, and four clinically confirmed). RFLP analysis, and 24-loci mycobacterial interspersed repetitive unit-variable-number tandem repeats analysis (MIRU-VNTR) were performed. Identical genetic fingerprints were obtained for all the isolates included in this study, indicating that all isolates were genetically similar (clonal outbreak). Complementarily, 36 *M. tuberculosis* isolates were subjected to WGS. In this study, a total of 204 SNPs unique to the 36 isolates were identified and used for evolutionary analysis. The results revealed the co-occurrence of two distinct *M. tuberculosis* lineages, corresponding to two simultaneous tuberculosis outbreaks [13].

Acinetobacter baumannii hospital outbreak (United Kingdom, 2008)

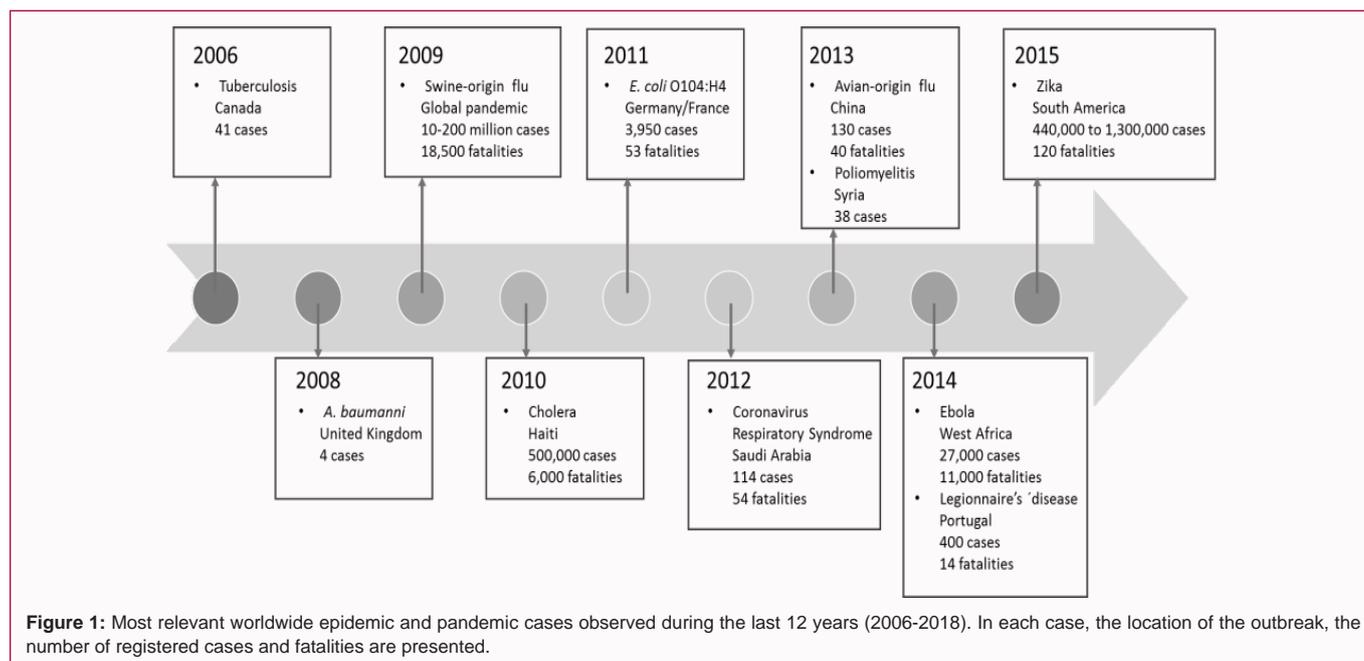
In 2008, an outbreak of multidrug-resistant *Acinetobacter baumannii* (MDR-Aci) was registered in the Selly Oak Hospital (Birmingham, United Kingdom). Over a five-week period, this hospital admitted four military patients, repatriated from Iraq and Afghanistan, and the hospital was colonized with strains of MDR-Aci. MDR-Aci was isolated from specimens from five patients (three military and two civilians) that shared the same critical care unit. All the isolates collected presented identical PFGE, VNTR, and antibiotic resistance profiles.

Whole-genome sequencing of the isolates identified several SNP that allowed to determine the transmission route within a small-scale outbreak and discriminated between alternative epidemiological hypotheses [14].

Swine flu (H1N1) (America, 2009)

In April 2009, a new strain of the H1N1 virus was identified in Mexico. A month after, the World Health Organization (WHO) declared this virus as a pandemic potential. In June, this virus was detected in 200 countries infecting between 10 and 200 million people, leading to 18,500 deaths [15,30-32].

In this case, viral genetic sequences derived from NGS were used for phylogenetic analysis [36-38], studies of virulence [39], and identification of genetic viral variants in targeted genetic regions associated with drug resistance [31], among others. The influenza A



virus genome is composed by 8 individual segments of single stranded RNA prone to genetic rearrangements. It may present high mutation rates and recombination events allowing the emergence of new viral strains with potential to create epidemic or pandemic outbreaks. Next, infection was likely caused by husbandry practices, where pigs co-habitat with other animals, such as humans, birds, and poultry, among others. Indeed, pigs act as reservoirs of several influenza virus (H1N1, H3N2 and H1N2), favoring genetic interchange between virus followed by genetic rearrangements, providing the genetic diversity that originated the H1N1 virus outbreak in 2009 - "mixing vessel" theory [33]. In fact, the H1N1 virus that caused the outbreak in 2009 presented genes with human (PB1), swine (HA, NA, NP, M, and NS), and avian (PB2 and PA) origins [15,34,35].

Cholera (Haiti, 2010)

In 2010, after a devastating earthquake that struck Haiti, an outbreak of cholera, a disease caused by *Vibrio cholerae*, resulted in more than 500,000 government-acknowledged cases and more than 6,500 deaths [36]. Since the beginning of this epidemic, the origin of the *V. cholerae* serotype was a matter of some controversy. In one hand, it has been proposed that the outbreak was a consequence of global climate change, being associated with the *V. cholerae* serotypes normally present in coastal waters. On the other hand, it has been suggested that this serotype has been brought from Nepal by peacekeepers [37].

Several approaches were used to identify the origin of this strain, ranging from classical microbiological methods such as PFGE, SNPs and antibiotic susceptibility testing to more recent WGST [16,38-40]. Applying MPS to sequence clinical isolates could determine the origin of a strain in South East Asia, likely introduced by human activity from humanitarian aid in response to the earthquake [16,36,41,42].

Shiga toxin-producing *Escherichia coli* O104:H4 (Germany, 2011)

Between May and July, 2011 an outbreak of gastroenteritis and the hemolytic-uremic syndrome was observed in Germany and in Southern France [43,44]. These pathologies were attributed to the ingestion of salad sprouts, shipped from Egypt to Germany and

France in December 2009, contaminated with Shiga-toxin-producing *Escherichia coli*, serotype O104:H4. However, the first suspects of disseminating this outbreak were (improperly) Spanish cucumbers (Shiga toxin-producing *E. coli* detected by PCR), resulting in an embargo to Spanish horticultural products (e.g., cucumbers, lettuce, and tomatoes). A total of 3,950 infection cases and 53 deaths were reported during this outbreak [17,45,46].

NGS was applied to determine the origin [47], evolution [46] and diversity [17] of this unusual *E. coli* serotype. Interestingly, the bacteria presented the pathogenic features typical of both enteroaggregative (genes *attA*, *aggR*, *aap*, *aggA*, and *aggC*, located in the virulence plasmid) and enterohemorrhagic *E. coli* (capacity to produce Shiga toxin- gene *stx₂* - and extended-spectrum β -lactamases) [45,47]. Comparing the German and French isolates, a greater genetic diversity was found in the French cases (19 SNPs vs 2 SNPs) [17]. The results allowed to confirm several theories associated with these two outbreaks, mainly a bottleneck effect on the diversity of the German isolates, existence of different mutation rates in the two populations, or an uneven distribution of diversity in the seed populations out setting each outbreak [17].

Middle East respiratory syndrome (Saudi Arabia, 2012)

Middle East Respiratory Syndrome (MERS), a severe form of pneumonia caused by a Human β -Corona Virus (HCoV), was reported for the first time in Saudi Arabia in September 2012 [48]. During one year, WHO reported a total of 114 cases of MERS-CoV (laboratory-confirmed) and 54 deaths dispersed by several countries from the Middle East (Saudi Arabia, Jordan, Qatar, and the United Arab Emirates) [49]. Moreover, a hospital-acquired outbreak was reported in four different health care facilities from the province of Al-Hasa (Saudi Arabia) [18].

NGS was used to study the sequence of the viral genome, trying to uncover its origin [48], evolution [48], diversity, reservoirs and transmission routes [50]. In terms of its origin and evolution, HCoV is a human corona virus closely related to corona viruses HKU4 and HKU5, previously isolated from bats [48]. Phylogenetic analysis of this virus revealed the existence of three distinct genotypes with

geographically disperse zoonotic reservoir [50]. Next, transmission routes within Saudi Arabia were compatible with the movement of animal reservoirs, animal products, and infected people [50].

Avian flu (H7N9) (China, 2013)

In late March 2013, a new virus was identified in China - avian influenza A H7N9 virus [51]. In less than a two months, this virus was responsible for more than 130 infection cases and 40 deaths dispersed by several provinces in West China [52]. Evolutionary analysis revealed that, similarly to what was been described for the H1N1 pandemic, the new H7N9 avian influenza A virus was caused by rearrangements from at least four independent lineages present in both poultry and migratory birds [19,52]. Whole-genome sequencing was performed in order to understand the evolutionary history of the virus [53], identify infected persons, domesticated and wild animals (i.e., pigs and birds) and infrastructures (aviaries, slaughterhouses and live animal markets) [54,55]. The genes H7 and N9 originated from virus circulating in migratory birds were transferred to domestic ducks and then to chickens in poultry markets. After genetic rearrangements with the H9N2 virus (genotype G57) emerged the H7N9 virus identified in humans during this outbreak [56].

Poliomyelitis (Middle East, 2013)

A Poliovirus outbreak was registered in the Middle East. First detected in Syria (2013) and later spread in to Iraq. A total of 38 cases have been officially confirmed (36 in Syria and 2 in Iraq) and a decline of the epidemic was only observed in the beginning of the following year. Due to the high infection rate of this disease, the WHO declared an emergency in the global public health to contain polio, estimating that for each confirmed case another 199 infected children are carrying and spreading the virus undetected, corresponding to a total of 7600 infected children [57,58]. Genetically related polioviruses from Pakistan were detected in sewage samples from nearby countries such as Egypt (December 2012), Israel, West Bank, and Gaza Strip (February - October 2013) [59,60].

Ebola (West Africa, 2013/4)

The Ebolavirus outbreak began in Guinea in December 2013 being only detected in March 2014. Since its initial detection, the virus spread to Liberia, Sierra Leone, Nigeria, Senegal, Mali, Spain, United Kingdom and United States. This outbreak was the most severe Ebola outbreak ever recorded, either in number of cases (almost 27,000) and deaths (almost 11,000) [61-64]. However, the WHO mentioned that the number of cases could probably be much higher than officially reported due to resistance from the involved governmental institutions to report on cases. Fortunately, several organizations, including the Economic Community of West African States (ECOWAS), the Control Center for Disease Prevention (CDC), and the European Commission donated funds and mobilized personnel to help fighting this outbreak; charities, including Doctors Without Borders, the Red Cross and Samaritan's Purse continue working in the area [63].

NGS technology was applied to study the origin and transmission dynamics [21], evolution and genetic diversity of this virus [62]. Results showed that the source of this Ebola outbreak was traced back to the prefecture of Guéckédou in the forested region and then spread to the nearby regions of Macenta and Kissidougou (southeastern Guinea). It has been suggested that a single transmission event occurred from the natural reservoir (fruit bats belonging to *Hypsignathus monstrosus*, *Epomops franqueti*, and *Myonycteris torquata* species) to a human,

followed by human-to-human transmission probably elicited by the social ceremonies of the funeral of the first victim [63]. After its spread, phylogenetic analysis revealed the sustained co-circulation of three different viral lineages: *i*) one lineage from Guinea, closely related to the earliest virus sampled during the epidemic; *ii*) a second lineage including virus repeatedly reintroduced from Sierra Leone; *iii*) a third lineage later coming from Guinea to Mali [62].

Legionnaires' disease (Portugal, 2014)

During the Legionnaires' disease outbreak (caused by *Legionella pneumophila* serogroup 1), registered in Vila Franca de Xira (near Lisbon, Portugal) in November 2014, more than 400 infection cases (377 laboratory-confirmed) and 14 deaths were reported [65,66]. A total of 49 possible sources were identified and the environmental samples collected included: domestic, industries with wet cooling systems, hospitals, malls, and public recreation facilities. Samples were analyzed by the Department of Water Microbiology, INSA - Instituto Nacional de Saúde Doutor Ricardo Jorge, being the source attributed to a wet cooling system from a local factory [67].

During this outbreak, two different approaches were implemented: in one hand the Sequence-Based Typing (SBT) according to the protocol recommended by the European Working Group for Legionella infections [67]. On the other hand WGS was used to identify the origin of the strain, to study its diversity [22], to compare between clinical and environmental strains for source attribution [68], to identify virulence factors, and to evaluate a possible person-to-person transmission [69]. Results showed that the outbreak was caused by a novel Sequence Type (ST) - ST1905 [22]. WGS was applied to compare the sequences of 1 environmental and 10 clinical samples and no nucleotide dissimilarities within the genome were found, fitting environmental samples with the origin of the outbreak [68]. In addition, the strain presents a ~65 kb pathogenicity island, probably imported via HGT from the strain Philadelphia-1, that carries numerous virulence factors, such as genes that mediate the oxidative stress resistance in vitro and in macrophages as well as resistance to several antimicrobial agents in other bacteria [69].

Zika virus (Southern America, 2015)

In December 2015, an outbreak of Zika virus was firstly registered in northeast region of Brazil and then spread not only to several countries from Southern America but also to North America and Asia. Two months after, the WHO declared a public health emergency. The transmission of this virus occurs via a bite of an infected *Aedes* mosquito (*A. aegypti*, *A. albopictus*, *A. africanus*, *A. luteocephalus*, *A. furcifer*, and *A. taylori*) [70]. Also, there is a strong evidence for person-to-person transmission, both sexual and congenital [71]. Only in Brazil, it has been estimated a total of between 440,000 to 1,300,000 Zika infections [71], associated to more than 5,600 suspected cases of microcephaly in newborns and 120 deaths attributed to congenital malformations [72].

NGS was used to study the origin [23,73], evolution [23,73], and diagnosis [74] of the virus. Evolutionary analyses indicated that the virus was introduced in the Americas between May and December 2013, as a single event, from an Asian (French Polynesia, 2013) lineage evolving and geographically dispersing throughout Asia and the Pacific Islands [23,73].

Conclusions

New disease outbreaks are appearing and rapidly spreading

to a global scale. This highlights the need for fast and accurate diagnosis to efficiently block the dissemination of infectious agents at a stage as early as possible. Although the classical microbiological methodologies remain as the gold standard for epidemiological studies, novel high-throughput technologies are paving the way to address major challenges in the field.

Since almost 20 years ago, the advances of NGS revolutionized the field of epidemiological research. Classical microbial techniques present several disadvantages: (i) They are time consuming, for instance, a routine tuberculosis diagnosis can require up to two months; (ii) Since they are culture-dependent methods, they can fail revealing the true microbiological diversity from a given sample once several microorganisms cannot grow in Petri dishes under laboratorial conditions.

When compared with classical microbial techniques, the major advantage of NGS is its high throughput capacity due to an extensive multiplexing capacity and parallel sequencing of millions of molecules. Nowadays, is possible, at an affordable cost and reduced turnaround times, either to sequence a complete genome from a microorganism (WGS) or to sequence selected regions from several microorganisms present at a given sample (microbiome). Indeed, nowadays NGS approaches are being applied to elucidate several topics associated to epidemic and pandemic outbreaks, such as the identification of the etiological agent, determination of the geographic origin and reservoirs, detection of genetic diversity and inference of its evolution, identification of the presence of virulence factors and antimicrobial resistance, trace of possible transmission routes, and even diagnosis. In a near future, NGS is expected to be regularly applied in public health to map and prevent the dissemination of disease outbreaks.

The presented epidemic and pandemic cases overall indicate several aspects that should be considered when studying epidemiological studies: (i) human populations are expanding and reaching forest areas, providing contacts between humans and animals that are natural reservoirs for many infectious diseases (as in the case of the Ebolavirus outbreak); (ii) every day, around 11.8 million passengers cross the skies in local or transcontinental flights, facilitating pathogen dissemination throughout the world (as in the epidemics of cholera in Haiti and Zika virus in Southern America); (iii) war situations in several locations of the world have interrupted vaccination programs and induced a massive migration of war refugees, allowing pathogens to move from its endemic regions (as in the case of the poliomyelitis outbreak in Syria). These are major aspects causing epidemics; however, many others could be presented. In any case, these aspects highlight the need for more rapid, accurate, affordable and cost-effective methods to identify and study the transmission routes of emerging novel pathogenic agents.

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