Colistin heteroresistance, mechanisms, diagnostic methods, and therapeutic options: A review

Razieh Dehbanipour[1], *Vala Taghi Zadeh Maleki*[2], *Zohreh Ghalavand*[3,*]

Abstract

The heteroresistance phenotype refers to the presence of bacterial subpopulations with reduced antibiotic susceptibility compared with the main population. Mathematical modelling and computer simulations suggest that heteroresistance can lead to negative treatment outcomes and finally, treatment failure. Due to the low frequency and resistance level of resistant subpopulations, detection of heteroresistance phenotype in the diagnostic laboratory is problematic. Routine laboratory tests do not have the ability to accurately detect heteroresistance, but on the other hand, specific methods are time consuming and expensive. The emergence of colistin heteroresistance is a public health concern that threatens human health. Colistin heteroresistance to date has been reported in eight pathogens including *Acinetobacter* spp., *Klebsiella* spp., *Enterobacter* spp., *Pseudomonas* spp., *Escherichia coli, Salmonella enterica* serovar Typhimurium (referred to as *Salmonella Typhimurium*), *Neisseria meningitidis* and *Stenotrophomonas maltophilia.* The growing emergence of colistin heteroresistance worldwide underscores the crucial need for coordinated global action to combat it. Understanding the mechanisms of colistin heteroresistance can help to provide better guidelines for reducing antibiotic resistance and to achieve new therapeutic approaches. Our review showed that the prevalence of colistin heteroresistance strains varies in different countries. It seems that the use of different treatment strategies, especially combination therapy, can be effective in reducing the incidence of resistant subpopulations. Also, the use of new generation diagnostic methods can have a significant impact on treatment. Our findings in this review are needed to raise the awareness of microbiologists and specialists to the colistin heteroresistance mechanisms and to achieve effective treatment.

Keywords **Colistin, resistance, heteroresistance, MDR, gram-negative bacteria, population analysis profiles.**

Introduction

Antibiotic resistance is a global health challenge that threatens the achievements of modern medicine. According to the Centers for Disease Control and Prevention (CDC), infections caused by antibiotic-resistant pathogens result in 23,000 deaths in the United States each year.[1] It is estimated that antibiotic resistance will cause 10 million deaths worldwide annually by 2050 and is therefore a serious threat to human health.[2] Because the development and application of new antimicrobial agents and treatment strategies may take a long time, it is worthwhile for health institutions to focus on the effective use of available antibiotics and prevent the spread of unrestrained antibiotic resistance.

In the last few decades, many studies on antibiotic resistance in various pathogens have been conducted, which has led to our better understanding of the mechanisms of antibiotic resistance. Acquired resistance is a resistance mechanism, which refers to mutation or horizontal transfer of a resistance gene, resulting in a predictable increase in phenotypic resistance. In fact, this indicates a correlation between

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bacterial genotype and bacterial phenotype.[3] However, phenotypic heterogeneity in terms of antibiotic susceptibility may be exhibited in subpopulations of a seemingly isogenic bacterial isolate. Heteroresistance denotes coexistence of susceptible and resistant strains in the same clinical sample, which makes it difficult to classify bacteria as susceptible or resistant.[4]

Therefore, since colistin heteroresistance is a challenging problem and no comprehensive study has been conducted on it, we focus in this review on colistin heteroresistance as an example of population heterogeneity. We discuss the impact of heteroresistance on the efficacy of treatment and the diagnostic methods and difficulties in detection of heteroresistance in pathogens. Next, we examine the prevalence and different mechanisms of colistin heteroresistance as well as treatment recommendations in gram negative pathogens based on all available studies.

Review criteria

This review provides an overview of the colistin heteroresistance research. Published works before 2025 on colistin heteroresistance studies were identified using the following search terms "heteroresistance", "heteroresistant", "colistin heteroresistance", "population analysis profiles" and "population analysis profiling" in Medline, PubMed, Scopus and Google Scholar. All original studies evaluating the prevalence, mechanism, diagnostic methods and therapeutic options of colistin heteroresistance in pathogens were eligible for review. Studies written in languages other than English were excluded.

Definition of heteroresistance

Heteroresistance was first described in the 1940s for *Haemophilus influenzae*.[5] Heteroresistance broadly refers to the presence of subpopulations that exhibit reduced antibiotic susceptibility compared with the main population.[4] It should be noted that, heterogeneity in resistance could be generated from different origins (polyclonal heteroresistance) or a single clone (monoclonal heteroresistance). In summary, polyclonal heteroresistance could be the result of mixed infections with different bacterial genera/species (susceptible and resistant bacteria) or the emergence and increase of rare spontaneous resistant mutants, in a population of susceptible bacteria, under antibiotic pressure (during antibiotic treatment). Alternatively, monoclonal heteroresistance is considered as the differentiation of a single clone into two susceptible and resistant populations (Figure 1).[6]

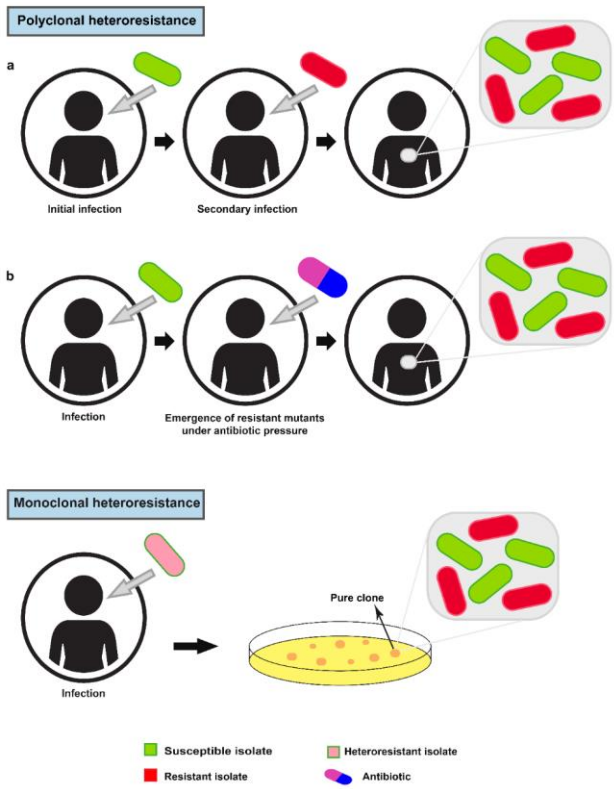

Figure 1. Clonality of heteroresistance. Polyclonal heteroresistance: a) Polyclonal heteroresistance can result from mixed infections caused by the entry of susceptible and resistant bacteria during initial and secondary infection. b) The emergence and increase of rare resistant mutants in a population of susceptible bacteria during antibiotic treatment can lead to polyclonal heteroresistance. Monoclonal heteroresistance: Monoclonal heteroresistance is caused by differentiation of a single clone into susceptible and resistant populations (in the absence of antibiotic pressure). Unlike polyclonal heteroresistance, culturing a purified clone can identify the monoclonal heteroresistance phenotype.

The specific mechanisms of monoclonal heteroresistance formation are two: phenotypic or genetic basis. An example of phenotypic monoclonal heteroresistance has been reported in *Enterobacter cloacae*. It was shown that, in a murine model of infection, heteroresistant *E. cloacae* subpopulations survive colistin treatment and lead to treatment failure. This occurrence was dependent on the histidine kinase *pho*Q, part of the *pho*P-*pho*Q two-component system which activates by limiting Mg^{2+} concentrations and results in modifying the lipopolysaccharide component of the outer membrane and eventually resistance to colistin.[7]

By contrast, in genetic basis of monoclonal heteroresistance, often gene amplifications lead to increased resistance genes copy number, which results in higher expression levels and eventually decreased susceptibility. It is worthwhile to note that, because of instability of gene amplifications, resistant subpopulations can revert to susceptibility in the absence of antibiotic pressure. However, in polyclonal heteroresistance cases, resistance-causing mutations are maintained even in the absence of antibiotic pressure and give rise to genetically distinct cell lines.[8]

Of note, analysis of cultures from purified clones can detect monoclonal heteroresistance phenotype while not being able to detect polyclonal heteroresistance. In fact, in cases of polyclonal heteroresistance, depending on which populations (susceptible or resistant) the purified clone originated from, the result of antimicrobial susceptibility tests can be considered as fully susceptible or fully resistant.[6]

Another example of population heterogeneity has been described as persistence. This phenomenon similar to heteroresistance enable bacteria to survive antibiotic treatment, although persistent subpopulations do not have the ability to grow in the presence of antibiotics.[8] The difference between resistance, persistence and heteroresistance is shown in Figure 2.

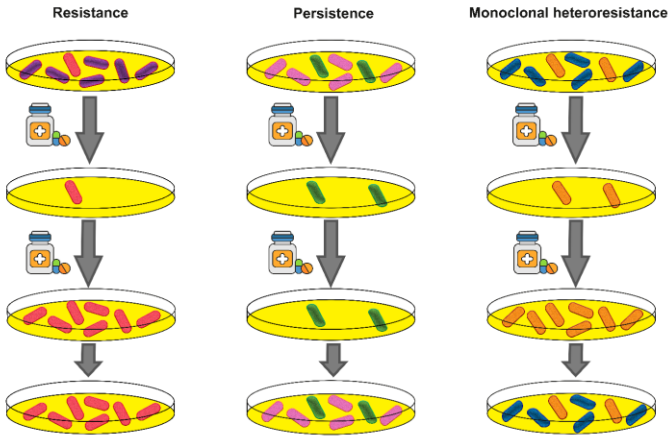

Figure 2. Difference between resistance, persistence and heteroresistance. Resistance: Stable genetic changes lead to the development of resistant cells that can survive and grow in the presence of antibiotics. These resistant cells then expand and eventually form a new population. Persistence: persister cells, similar to heteroresistant cells, can survive antibiotic treatment but lack the ability to grow in the presence of antibiotic. After stopping antibiotic treatment, persister cells can return to the susceptible phenotype. Heteroresistance: heteroresistant cells, similar to resistant cells, have the ability to survive and grow in the presence of antibiotics. However, because resistance in monoclonal heteroresistant cells is unstable, they return to susceptible phenotype after cessation of antibiotic treatment.

Another noteworthy point about heteroresistance is its stability. The results of various studies show that heteroresistance is stable or unstable (Figure 3). The unstable heteroresistance refers to whenever resistant subpopulations return to susceptible phenotype in the absence of antibiotic pressure during subculturing. Stable heteroresistance includes cases in which resistant subpopulations maintain the resistance phenotype even in the absence of antibiotic pressure.[9,10] Stable heteroresistance is caused by frequent mutations (for example, insertions, deletions and single nucleotide polymorphisms). If these resistance mutations confer low fitness cost, they are more likely to be stable in the absence of antibiotic selective pressure.[9] Various studies have demonstrated that

efflux and influx of antibiotics play important role in resistance in subpopulations with stable heteroresistance. For example, in *P. aeruginosa* clinical isolates, stable imipenem heteroresistance was linked to overexpression of the efflux pump *mex*AB,[11] while it was observed that in *E. coli* fosfomycin-heteroresistant isolates, lack of the *uhpT* gene (encoding hexose-6-phosphate transporter involved in fosfomycin influx) was associated with stable and homogenous nonsusceptibility to fosfomycin.[12]

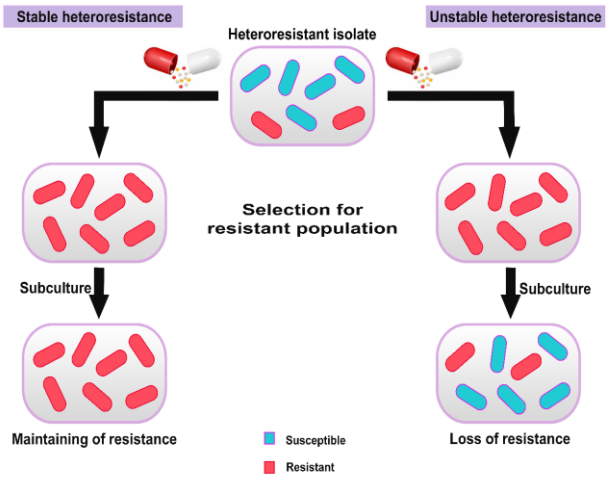

Figure 3. Stability of heteroresistance

The mechanisms involved in unstable heteroresistance (more common type of heteroresistance) can be divided into two main categories. The first mechanism is through stable resistance mutations with high fitness costs. The high fitness costs of these mutations may be the main factor in their reversibility during growth in the absence of antibiotic selective pressure. In fact, during several generations of pathogen growth in the absence of antibiotic, selection of compensatory mutations leads to a reduction in fitness cost and thus a reduction in resistance.[9,13,14] The selection of increased fitness and susceptibility through compensatory mutations has been shown in different bacteria such as *K. pneumoniae*, *E. coli* and *S.* Typhimurium.[9] Of note, it has been found that in imipenem-heteroresistant multidrug-resistant (MDR) *A. baumannii* isolates, insertion of *ISAba1* into promoters of a β-lactamase-encoding gene (bla_{ADC-29}) is associated with unstable heteroresistance phenotype.[13]

The second mechanism consists of tandem gene amplifications that are mechanistically unstable and costly. The stability of amplifications is affected by frequent unequal crossing overs.[15-17] On the other hand, the fitness costs of tandem gene amplifications are associated with their reduction in copy number (reduction of resistance) in the absence of selective pressure.[18] In colistin-heteroresistant *S.* Typhimurium isolates, resistance was linked to amplification of chromosomal regions including the *pmrD* gene, which is involved in increasing colistin resistance by up-regulation of proteins involved in modification of lipid A. Indeed, increasing the copy number of resistance genes seems to be associated with the heteroresistance phenotype.[19] It should be noted that mathematical modeling has showed that unstable amplifications may evolve into stable resistance mutations in response to growth under antibiotic pressure.[20] Similarly, evaluation of *A. baumannii* isolates recovered from cerebrospinal fluid (CSF) samples of a patient with meningitis has shown that colistin heteroresistance has evolved to complete resistance phenotype during a five-day treatment period.[21]

Heteroresistance and treatment failure

Whether the presence of resistant subpopulations is associated with treatment failure is still unclear. Numerous studies have linked treatment failure to heteroresistance in different pathogens. Several studies have shown an association between heteroresistant vancomycin *Staphylococcus aureus* (hVISA) and instances such as persistent bacteremia, prolonged hospital stays and increased mortality.[22,23] Another study on the epidemiological and clinical features of carbapenem heteroresistant *A. baumannii* showed similar results.[24] A retrospective study on a patient who experienced recurrent episodes of peritonitis indicated that treatment failure was associated with heterogeneous vancomycin-resistant *Staphylococcus epidermidis*.[25] Similarly,

treatment failure has been linked to carbapenem heteroresistance in *A. baumannii*.[26]

Although the mentioned studies suggest that heteroresistance can lead to treatment failure, others show the opposite of this hypothesis. Several cohort studies on hVISA have observed no correlation between heteroresistant phenotypes and treatment failure.[23,27,28] Similar results have been found for colistin heteroresistant *A. baumannii* isolates.[29] These discrepancies between studies may be due to variations in sample sizes and the identification methods, which makes it difficult to compare independent studies. However, it should be noted that pharmacodynamic and mathematical modelling confirms the impact of heteroresistance on the efficacy of antibiotic treatment.[9,30]

Another issue discussed about heteroresistance is the possibility of an association between previous colistin exposure and the frequency of resistant subpopulations. Although the results of some studies indicate a link between prior exposure to colistin and a higher proportion of resistant subpopulations,[31] in some studies the existence of this association has been questioned.[32] Therefore, on the basis of these observations, further research is required to fully understand the parameters associated with heteroresistance.

Heteroresistance detection methods

Although bacterial resistance to antibiotics can be assessed by various methods, heteroresistance becomes a health crisis because routine laboratory tests are unable to detect the presence of resistant subpopulations. Population analysis profile (PAP) test is considered as gold standard for identifying heteroresistance but it is time-consuming, costly and therefore not routinely performed in the microbiology laboratory.[4] In PAP method, after inoculation of bacteria at a variety of antibiotic concentrations in Mueller-Hinton (MH) medium, the frequency of the resistant subpopulation of cells is determined (Figure 4).[33] Although the PAP test is the most reliable method, other methods are currently used to identify heteroresistance such as disc diffusion, broth microdilution, E-test, agar screen, agar dilution, VITEK 2, BACTEC 960 liquid media system and molecular detection methods such as line probe assays.[6]

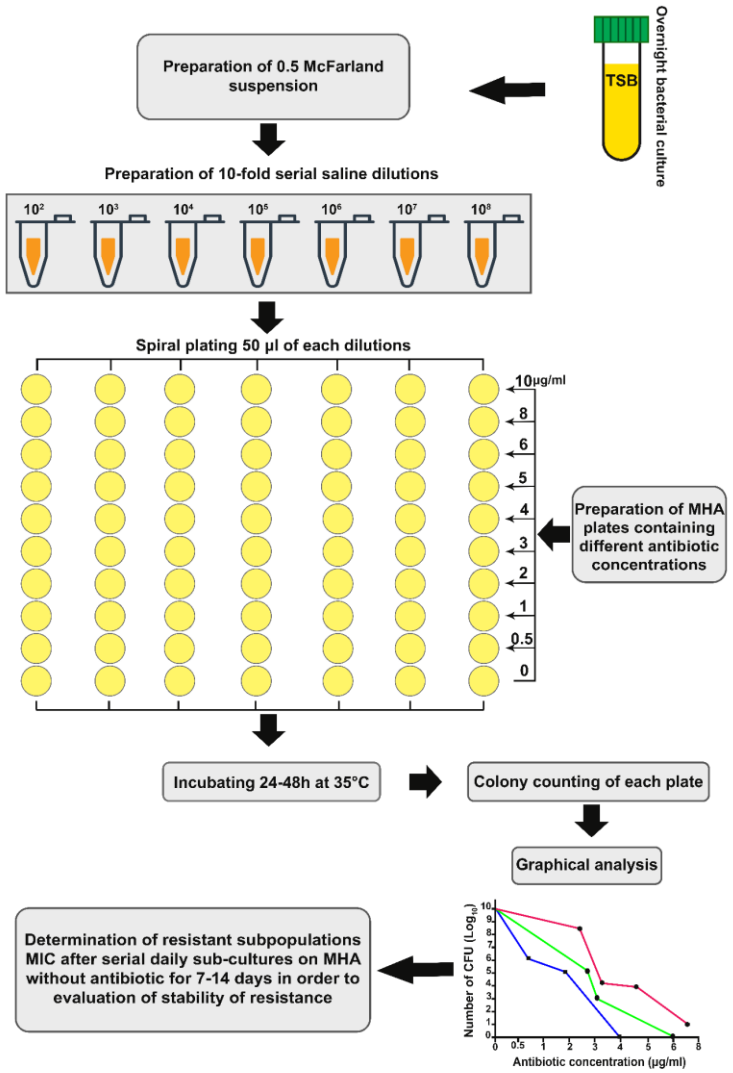

Figure 4. Schematic representation of population analysis profile (PAP) test protocol to identify heteroresistance. After preparing MHA plates containing different antibiotic concentrations, serial dilutions of bacterial suspension are spread on the plates. The colonies of each plate are then counted after one day of incubation at 35°C. Finally, a graphical analysis of the results is performed. To evaluate the stability of the heteroresistance phenotype, a colony from the highest antibiotic concentration is selected and cultured on MHA without antibiotic for 7 to 14 days, and then the MIC is determined using the agar dilution method.

Comparison of these different methods for detecting colistin heteroresistance in *A. baumannii* showed that VITEK 2 is not a proper method.[34]

However different methods mentioned above seem to have poor sensitivity and poor specificity compared to the PAP method for identification of resistant subpopulations. In addition, there are some new generation methods such as droplet digital PCR, whole genome sequencing and line probe assays that allow detection of genes and mutations involved in resistance. However, the use of these new methods is limited to some pathogens including *Helicobacter pylori* and *Mycobacterium tuberculosis* and cannot be applied to all antibiotics.[6,35,36]

Since the identification of heteroresistance phenotype is a prerequisite for choosing an appropriate treatment, it seems that the development of diagnostic tools can help achieve a successful treatment.

Heteroresistance in clinical isolates

Heteroresistance has been observed in a variety of pathogens, and against diverse antibiotics worldwide. Heteroresistance has been detected in both Gram-positive and Gram-negative bacteria such as staphylococci, enterococci, *Clostridioides difficile*, *E. coli*, *P. aeruginosa*, *A. baumannii*, *Klebsiella* spp., and others.[9] One of the most extensively studied cases is heteroresistance to vancomycin. Vancomycin heteroresistance in *S. aureus* was first observed in Japan and was then reported from numerous studies on staphylococci.[37] Additionally, the results of a meta-analysis covering 91 studies, showed an increase of the hVISA prevalence from 4.68% in studies before 2006 to 7.01% in 2014. In this study, most of the reports of hVISA prevalence belonged to Asia.[38] Heteroresistance phenotype has also been observed against carbapenems. In a cohort study of *A. baumannii* in Spain, 24% were heteroresistant to meropenem and 20% to imipenem.[14] Another study displayed imipenem heteroresistance (25%), ertapenem heteroresistance (17.2%) and meropenem heteroresistance (3.9%) among *E. coli* isolates in China.[39]

Furthermore, the emergence of heteroresistance to last-resort antibiotics such as polymyxins (polymyxin B and colistin) has caused concern. Numerous studies have been performed to identify polymyxins heteroresistance in pathogens, including *Klebsiella* spp., *Enterobacter* spp., *Pseudomonas spp.*, *Acinetobacter spp.*, *E. coli*, *S.* Typhimurium.[9,40] It should be noted that the variations between the results of studies investigating heteroresistance prevalence may be due to different definitions of heteroresistance and different detection methods.

Colistin resistance and heteroresistance

After the isolation of colistin (polymyxin E) from *Paenibacillus polymyxa* subsp. *colistinus* in 1947 by Koyama, this antibiotic was first approved by the US Food and Drug Administration in 1959.[41] Colistin was an appropriate choice for treatment of Gram-negative bacterial infections due to its bactericidal activity through bacterial cell membrane destruction. Indeed, colistin has the ability to bind to anionic groups on the lipid A of lipopolysaccharide (LPS) in Gram-negative bacteria through its cationic residues. However, the use of polymyxins was questioned in the 1970s due to reports of nephrotoxicity and neurotoxicity and was limited to treatment of diseases such as cystic fibrosis.[42]

The increase in infections caused by Gram-negative bacteria presents a serious problem worldwide. Gram-negative bacteria have the ability to exhibit different resistance phenotypes such as MDR, extensively drug-resistant (XDR), and pan-drug-resistant (PDR) through multiple resistance mechanisms. The emergence and rapid spread of these superbugs (strains of bacteria that are resistant to most of available antibiotics commonly used for treatment) and the lack of new treatment strategies is considered a formidable menace. According to the above mentioned, despite their toxicity, older antibiotics such as colistin were recommended as "last resort" since the mid-1990s.[43]

Unfortunately, the emergence of colistin resistance in Gram-negative pathogens such as *P. aeruginosa*, *A. baumannii* and *K. pneumoniae* has

become a global concern in recent years. Colistin resistance in Gram-negative bacteria occurs through various mechanisms.[44] A study showed 57% and 22% colistin resistance rate in *K. pneumoniae and P. aeruginosa* isolates.[45]

Heteroresistance to colistin was first detected in *A. baumannii* isolates in 2006.[46] The emergence of the colistin heteroresistance phenomenon has caused confusion in the diagnostic and treatment levels. In fact, the presence of clinically undetected colistin heteroresistance subpopulations leads to treatment failure, prolonged length of hospital stays and even the death of a patient.[47-49] According to the studies to date, colistin heteroresistance has been observed in different species including *Acinetobacter* spp., *Klebsiella* spp., *Enterobacter* spp., *Pseudomonas* spp., *E. coli*, *S.* Typhimurium, *N. meningitidis* and *S. maltophilia.* In the following section, studies on colistin heteroresistance in these pathogens are discussed.

Colistin heteroresistance in Gram-negative pathogens

1. *Acinetobacter* spp.

Among the various species of the genus *Acinetobacter*, *A. baumannii* is clinically the most important species. *A. baumannii* is one of the most successful Gram-negative bacteria responsible for a spectrum of nosocomial infections.[50] The ability of this pathogen to acquire resistance to commonly used antibiotics has led to the widespread prevalence of resistant isolates since the 1970s and it has caused global concern.[51] According to the Infectious Diseases Society of America *A. baumannii* is one of the 6 antibiotic resistant pathogens responsible for a high mortality rate among patients.[52] Although carbapenems have been known as a viable treatment option for multiple resistant *A. baumannii*, in recent decades studies have shown an upward trend in resistance to carbapenems.[51,53] According to the European Antimicrobial Resistance Surveillance Network (EARS-Net) in 2018, the rates of carbapenem resistance among *A. baumannii* isolates was more than 30%.[54] Increased resistance to carbapenems in MDR *A. baumannii* strains led to colistin being considered as a last resort treatment option.[51] However, after the first report of colistin resistant *A. baumannii* in Czech Republic in 1999,[55] the results of studies in different countries indicate the emergence of colistin resistance, mainly associated with monotherapy.[31] A study of 514 *A. baumannii* isolates collected from various sites across the USA and Puerto Rico showed 5.3% resistance to colistin among the isolates.[56] Based on the World Health Organization Regional Offices reports from 2000 to 2017, colistin resistance in clinical isolates of *A. baumannii* in Europe and South-East Asia was 1.8% and 6.7% respectively.[57]

1.1. Colistin heteroresistance in *A. baumannii*

Colistin heteroresistance in *A. baumannii* was fist described by Li et al.[46] in 2006 and raised an alarm over dangerous rates of colistin resistance. Colistin heteroresistance is defined as the presence of colistin resistant subpopulations within a susceptible main population. In fact, these subpopulations have the ability to grow in the presence of colistin due to their higher minimum inhibitory concentration (MIC ≥ 2- to 8-fold) than the main population (MIC ≤ 2 mg/L).[4,6] In recent years, there have been several reports of colistin heteroresistance in *A. baumannii* from different parts of the world (Table 1). A number of studies have demonstrated that the emergence of colistin resistant subpopulations is associated with prior exposure to colistin. A report has shown that increased consumption of colistin in a university hospital of Argentina has led to high prevalence of colistin heteroresistance in *A. baumannii* strains.[58] It is suggested that there are two types of colistin-heteroresistance in *A. baumannii* isolates. Type I has a typical heteroresistance phenotype that can be identified by gold standard method, PAP. Since colistin resistant subpopulations of this type became resistant after exposure to high colistin concentrations, it was suggested that treatment with high colistin concentrations may lead to the emergence of colistin resistance. In contrast, type II colistin-heteroresistant *A. baumannii* isolates could not survive in high

colistin concentrations in PAP. Additionally, these findings indicate the instability of resistance in resistant subpopulations in both types of colistin heteroresistance. Based on these observations, it seems important to differentiate between the two types of heteroresistance because different treatment strategies must be considered for each type.[59]

1.2. Mechanisms of heteroresistance

Although extensive studies have not been performed to investigate the mechanisms of colistin heteroresistance, the results of studies indicate the importance of the three main mechanisms. Mutations of *lpxA*, *lpxC*, and *lpxD* in *A. baumannii* might be the cause of colistin heteroresistance. *lpxA/C/D* are involved in the synthesis of the lipid A component of LPS, therefore any mutation within these genes can lead to loss or decrease in production of LPS.[76] It should be noted that such mutations may lead to increased bacterial susceptibility to several antibiotics such as carbapenems, rifampicin and ampicillin/sulbactam, by increasing the permeability of the outer membrane and thus better access to target sites for these antibiotics.[77,78] Furthermore, genetic changes in the *pmrA* or *pmrB* gene may lead to colistin heteroresistance. Such mutations can lead to upregulation of *pmr*CAB operon and the addition of phosphoethanolamine (PEtN) to lipid A through activation of *pmr*AB two-component system. Following these interactions, the negative charge of the outer membrane of *A. baumannii* and thus the binding of colistin reduces.[79] C.H. Rodriguez et al.[67] observed that *A. baumannii* isolates carrying mutations in the *lpxC* and *pmrB* genes presented slow growth. They believe that the slow growth of colistin resistant subpopulations can lead to misdetection in microbiology laboratories.

Also, it has been suggested that efflux system overexpression is associated with colistin heteroresistance in *A. baumannii*. Therefore, it can be concluded that the use of efflux pump inhibitors as adjuvants along with colistin treatment can resensitize *A. baumannii* to colistin.[80]

1.3. Heteroresistance detection

The detection of resistant subpopulations by the traditional susceptibility testing methods is difficult. That is why colistin heteroresistant isolates are usually misdiagnosed as colistin sensitive. Several reasons have been suggested for the failure to identify colistin heteroresistant isolates, including low proportion of colistin-resistant subpopulations, slow growth of colistin-resistant subpopulations and MIC values close to breakpoint values.[67] This misdiagnosis, especially in the case of colistin as a last-line treatment option for MDR *A. baumannii*, can lead to ineffective treatment initiation, loss of golden treatment time and even increased patient mortality.

Although the gold standard method for identifying colistin heteroresistance is PAP,[4] there are several methods for detection of colistin heteroresistance, each with advantages and disadvantages over the other. However, there are not many studies available comparing different diagnostic methods to identify colistin heteroresistant isolates of *A. baumannii*. In 2007, Lo-Ten-Foe et al.[34] examined and compared several techniques (VITEK 2, E-test, agar dilution and disk diffusion) with broth microdilution, introduced as a reference method by the Clinical and Laboratory Standards Institute (CLSI), for identifying colistin heteroresistance in *A. baumannii*. The results of their study showed that although VITEK 2 is a reliable and an easy-to-use tool, it is better to be cautious in interpreting the results obtained from genera that typically have colistin heteroresistant subpopulations. In such genera, alternative and appropriate methods should be used to identify resistant subpopulations. The study suggests that if Iso-Sensitest agar is used in the disk diffusion and E-test methods instead of the Mueller-Hinton agar, the identification of resistant colonies within the colistin inhibition zone (known as colistin heteroresistant) is more accurate. The results of the agar dilution test showed high levels of agreement with the broth microdilution method. Of note, agar dilution test can detect colistin heteroresistant subpopulations of *A. baumannii*

Table 1. Heteroresistance to colistin in *Acinetobacter* spp. isolates

Author (year)	Country	Number of samples	Method for determination of resistance	Prevalence of resistance (%)	Prevalence of heteroresistance (%)	Prior colistin treatment (%)
Li J (2006)[46]	Australia	16	Broth microdilution	0	94	0
Hawley JS (2008)[60]	USA	19	Broth microdilution	0	100	37
Hernan RC (2009)[48]	Argentina	28	Agar dilution	0	46.4	NR
Yau W (2009)[61]	Western Pacific Region	30	Broth microdilution	3.3	23	NR
Dudhani RV (2010)[62]	Australia	2	PAP	0	50	0
Rodriguez CH (2010)[63]	Argentina	14	Agar dilution	7.14	42.8	NR
Herrera ME (2011)[64]	Argentina	75	Unclear	Unclear	19	Unclear
Vidaillac C (2012)[65]	France	2	Broth microdilution	0	100	NR
Rodriguez CH (2014)[58]	Argentina	129	Agar dilution	NR	95	NR
Juhász E (2017)[40]	Hungary	76	Agar dilution	2.6	20	NR
Li H (2017)[66]	China	29	Broth microdilution	0.5	31	NR
Srinivas P (2018)[29]	USA	24	Broth microdilution	0	83	4
Rodriguez CH (2019)[67]	South America	165	Broth microdilution	17.85	11	NR
Ezadi F (2019)[68]	Iran	44	Broth microdilution	5.6	21	NR
Çağlan E (2019)[69]	Turkey	14	Broth microdilution	28	21	NR
Nicoloff H (2019)[9]	Sweden	10	E-test, PAP	0	0	NR
Chen L (2020)[70]	China	576	Broth microdilution	0	2	0
Sacco F (2021)[71]	Italy	51	Broth microdilution	11.76	11.76	NR
Jo J (2023)[72]	Korea	7	PAP	0	100	NR
Kon H (2023)[73]	Italy	173	PAP	0	67.1	NR
Zulfiqar A (2024)[74]	Pakistan	130	PAP	0	31.5	NR
Pakzad I (2024)[75]	Iran	22	PAP	0	27.2	NR

NR – not reported; **PAP** – population analysis profile test.

regardless of the use of Mueller-Hinton agar or Iso-Sensitest agar.[34]

In 2019, Sherman et al.[33] reviewed and compared three efficient methods for detection of colistin heteroresistance in *A. baumannii* including disc diffusion, E-test and PAP. As stated in the study by Lo-Ten-Foe et al.,[34] in the E-test technique, observation of colonies within the

inhibition zone ellipse indicates a heteroresistant isolate. However, if the frequency of resistant subpopulations in heteroresistant isolates is low, no colony will be seen within the zone of clearing and therefore this technique will be ineffective in these cases. In addition, because E-test strips are expensive, this method may not be ideal as a routine laboratory diagnosis of heteroresistance. In contrast to E-test strips, colistin discs are cost effective and can be used to detect heteroresistance. It should be noted that E-test and disc diffusion are a non-quantitative methods.[33] Additionally, compared to PAP, the sensitivity of the disk diffusion and E-test is 22.9% and 54.2%, respectively; while the specificity of these methods is 100%.[81]

PAP is a reliable, quantitative and reproducible method for detecting heteroresistance. However, one of the disadvantages of this method is that it is time consuming and requires more materials than other techniques.[33]

In addition, there are two methods, including time-kill assay and resistant colony restreak, for differentiating between heteroresistance and other forms of resistance such as stable mutation or persister cells. Persisters, mutant subpopulations of a bacterial strain, does not have the ability to grow in the presence of antibiotics, unlike heteroresistant subpopulations. Therefore, time-kill assay method examines the ability of bacteria to grow in the presence or absence of colistin and thus confirms the heteroresistance of the isolate. On the other hand, in resistant colony restreak method, a single colony obtained from a PAP plate or from within the inhibition zone on a disk diffusion or E-test assay palate is transferred to a broth media in the absence of colistin. The decrease in the frequency of the resistant subpopulation after culture in the broth media confirms the colistin heteroresistance of the isolate.[33]

1.4. Therapeutic options

In addition to what was revealed about type I colistin-heteroresistant *A. baumannii* isolates, several studies have confirmed the conversion of colistin heteroresistant *A. baumannii* to colistin resistant during treatment.[31] High nephrotoxicity risk of colistin consumption has complicated the situation. However, there are several recommendations to confront with these problems. Combination therapy can be one of the solutions of interest. A study on two mouse infection models showed that amplification of colistin-resistant *A. baumannii* subpopulations was linked to monotherapy with colistin.[62] Rodriguez et al.[63] have shown that using rifampicin or imipenem with colistin has a synergistic effect on heteroresistant *A. baumannii* isolates and can help prevent the development of resistance. They also reported a 22-year-old case which developed fever and rise of CSF after surgery for meningioma and received various treatments and became resistant to colistin 48 hours after colistin administration. However, after 5 days of starting combination therapy with colistin and rifampicin, CSF cultures were sterilized and the patient was successfully treated.[48] Nonetheless, in the study by Gedik et al., conducted on 51 patients infected with colistin-only sensitive *A. baumannii* (heteroresistance was not checked), it was found that there was no significant difference between outcomes of colistin monotherapy and colistin combined therapy among patients with bloodstream infections and ventilator-associated pneumonia (VAP). However, they noted that colistin monotherapy may result in the emergence of heteroresistant strains.[82]

Another way is to use polymyxin B instead of colistin. Aggarwal et al.[83] has recommended that polymyxin B could be used instead of colistin because of the high toxicity of colistin at their research recommended dose. Indeed, colistin nephrotoxicity is completely dose-dependent.

The third potential solution to the problem is to use antioxidants. The results obtained from Arslan et al. research have suggested that luteolin (a common flavonoid in different types of plants) can be used as an inhibitor of high nephrotoxicity, together with colistin. However, confirmation of this finding requires certain clinical studies.[84] It seems that confronting the emergence of colistin resistance requires the development of new therapeutic strategies.

2. *Klebsiella* spp.

A member of ESKAPE pathogens, rod-shaped Gram-negative *K. pneumoniae* is the most important species of the genus *Klebsiella* and the leading cause of community- and hospital-acquired infections. The ability of this pathogen to produce extended-spectrum β-lactamases (ESBLs) has led to resistance to β-lactam antibiotics since 1983.[85] However, carbapenems seemed to be a good choice for treatment of infections caused by ESBL-producing pathogens until carbapenem-resistant strains were first reported in 1993.[86] The global prevalence of carbapenem-resistant *K. pneumoniae* is associated with treatment failure and high mortality rate and thus has revived the use of colistin for critically ill patients.[87] With the start of colistin administration for the treatment of MDR *K. pneumoniae*, there were reports of colistin resistance from around the world and posed a serious threat to public health. Mutations in two-component regulatory systems and insertional inactivation of the *mgrB* regulator seem to be the most common colistin resistance mechanism in *K. pneumoniae* isolates.[88]

2.1. Colistin heteroresistance in *K. pneumoniae*

Since colistin is often the last resort for *K. pneumoniae* infections, reports of colistin heteroresistant *K. pneumoniae* in the last two decades are worrisome (Table 2).[89-92] The inability of routine susceptibility tests to identify colistin heteroresistant isolates is challenging for clinical laboratories and leads to treatment failure.[47] In the study by Poudyal et al.,[89] it was found that out of 16 colistin-susceptible isolates based on MICs, 15 isolates displayed colistin heteroresistance based on the PAP method. In addition, Band et al.[47] have reported instance of treatment failure result from undetected colistin heteroresistant *K. pneumoniae* which indicates that the usual colistin susceptibility tests are unreliable. The association between colistin exposure and the emergence of resistant subpopulations in *K. pneumoniae* has not been extensively investigated. However, Kim et al.[93] demonstrated that exposure to colistin increases MIC and causes diverse amino acid substitutions in *pmrB, phoPQ* and *mgrB* genes. Although Barragán-Prada et al.[32] found that direct colistin selective pressure was not the only cause of the emergence of colistin resistance in *K. pneumoniae*, similar to Seo et al.[94] they suggest that evaluation of colistin susceptibility should be done carefully, even in patients not exposed to colistin.

Interestingly, some colistin resistant subpopulations from the same parental strain have shown different amino acid substitutions.[91,93] On the other hand, it has been reported that some colistin resistant subpopulations have no mutations in the genes responsible for resistance.[93] These observations suggest that the mechanisms of colistin resistance in *K. pneumoniae* are not yet fully understood and further studies are needed.

2.2. Mechanisms of heteroresistance

The deciphering of colistin heteroresistance mechanisms in *K. pneumoniae* was first performed in 2015 by Jayol et al.[106] They observed that amino acid substitutions in protein PhoP (a part of the PhoPQ two-component system) were involved in the development of colistin resistance. In following years, other instances of mutations in the *phoPQ* gene were reported from different countries.[91,92,95] *phoPQ* mutations lead to the overexpression of *pmrE* gene and *pmrHFIJKLM* operon (involved in 4-deoxyaminoarabinose (LAra4N) synthesis) and *pmr*C gene (involved in phosphoethanolamine (pEtN) synthesis). The addition of LAra4N and pEtN to lipid A increases the positive charges of the LPS and finally reduces the affinity of LPS to colistin.[107] Of note, the PhoP/PhoQ system is negatively regulated by *mgr*B. *mgr*B is a regulatory transmembrane protein and is produced upon activation of the *pho*PQ signaling system. Since the *pho*Q activates *pho*P by phosphorylation, *mgr*B can repress *pho*P phosphorylation by interaction with the sensor kinase *pho*Q. Therefore, alterations in *mgr*B can lead to up-regulation of the *pho*PQ system and then to colistin resistance by up-regulation of the *pmrHFIJKLM* operon.[108] Mutations in two-component regulatory systems, *pmr*AB, is another mechanism involved in

Table 2. Heteroresistance to colistin in *Klebsiella* spp. isolates

Author (year)	Country	Number of samples	Method for determination of resistance	Prevalence of resistance (%)	Prevalence of heteroresistance (%)	Prior colistin treatment (%)
Poudyal A (2008)[89]	Australia	22	Broth Microdilution	27.27	93.75	NR
Meletis G (2011)[90]	Greece	20	Broth Microdilution	20	75	40
Halaby T (2016)[95]	Netherlands	13	Broth Microdilution	53.84	38.46	100
Juhász E (2017)[40]	Hungary	140	Agar dilution	0.7	48.6	NR
Barragán-Prada H (2018)[32]	Spain	30	Broth Microdilution	70	33.33	66.66
Band VI (2018)[47]	USA	2	Broth Microdilution	0	100	NR
Wozniak JE (2019)[96]	USA	265	Broth Microdilution, E-test	0	0.37	NR
Cheong HS (2019)[91]	Korea	252	Broth Microdilution	5.1	1.3	NR
Nicoloff H (2019)[9]	Sweden	10	E-test, PAP	0	0	NR
Morales-León F (2020)[92]	Chile	60	Broth Microdilution	0	13	NR
Band VI (2020)[97]	USA	286	PAP	9.4	8.4	NR
Wang Y (2022)[98]	China	98	PAP	2	71.9	NR
Sánchez-León I (2023)[99]	Spain	10	PAP	0	100	NR
Sánchez-León I (2023)[100]	Spain	9	PAP	0	100	NR
Weng Y (2023)[101]	China	455	PAP	0	6.2	NR
Rajakani SG (2023)[102]	Europe	16	PAP	0	18	NR
Wang T (2023)[103]	China	2	PAP	0	2	NR
Afyoncu E (2024)[104]	Turkey	154	PAP	16.23	0	NR
Braspenning AJMM (2024)[105]	Europe	288	PAP	0	37.5	NR

NR – not reported; **PAP** – population analysis profile test.

colistin resistance in *K. pneumoniae*. PmrAB has the same mechanism of action as the PhoPQ.[91]

Additionally, a study of colistin-resistant subpopulations among ESBL-producing *K. pneumoniae* isolates showed that in addition to mutations in *phoPQ* and *mgrB*, mutations in the *yciM* and *lpxM* genes may also play a role in colistin resistance. Because mutation in *yciM*, a gene involved in LPS biosynthesis, leads to decreased susceptibility to colistin in *Escherichia coli*, probably also causes colistin resistance in *K. pneumoniae*. Moreover, *lpxM* is responsible for the acylation of lipid A in Enterobacteriaceae and thereby mutation in this gene is involved in colistin resistance.[95] Silva et al.[109] demonstrated for the first time that biofilm formation may be associated with the emergence of colistin heteroresistance in *K. pneumoniae*. Sato et al.[110] have reported that acquisition of colistin heteroresistance may be associated with disrupting mutation in the DNA repair enzyme MutS in *K. pneumoniae*. Albeit plasmid-mediated colistin resistance in the form of mcr-1 has received a lot of attention,[111] no *mcr-1* plasmid-mediated gene have been found in studies on colistin heteroresistant *K. pneumoniae* isolates.[32,92,112]

2.3. Heteroresistance detection

Usually, methods other than PAP are not able to reliably identify resistant subpopulations because the proportion of heteroresistant cells is normally low. In the study by Meletis et al.[90] among the 20 *K. pneumoniae* clinical isolates that were classified as colistin-susceptible by routine susceptibility testing, 12 isolates contained resistant subpopulations. A study by Seo et al.[94] also showed that disc diffusion and E-test methods were not able to detect colistin heteroresistance compared to the PAP method. Additionally, Poudyal et al.[89] observed that detection of colistin heteroresistance by broth microdilution is not reliable.

2.4. Therapeutic options

Unfortunately, there is not much information on the treatment of heteroresistant *K. pneumoniae* isolates. However, it is suggested that colistin monotherapy and long dosage intervals may be problematic for the treatment of colistin-heteroresistant *K. pneumoniae* strains.[89] Cheong et al.[91] demonstrated that meropenem combined with colistin allowed rapid eradication of colistin-heteroresistant *K. pneumoniae* isolates. In addition, it has been shown that combination regimens in colistin-heteroresistant and fosfomycin-susceptible *K. pneumoniae* isolate had a more favorable effect than colistin monotherapy, while in colistin-heteroresistant and fosfomycin-resistant *K. pneumoniae* isolate no significant difference was seen.[113] In fact, it is necessary to examine the resistance patterns of the isolates before initiating combination therapy. Further investigations on therapeutic options for colistin-heteroresistant *K. pneumoniae* infections is warranted.

3. *Enterobacter* spp.

The opportunistic *Enterobacter* spp., a member of the ESKAPE group of significant bacterial pathogens in humans, have the ability to cause infections in the respiratory, urinary, blood and gastrointestinal tracts.[52,114] The emergence of multidrug resistance and carbapenem resistance in *Enterobacter* spp. due to several mechanisms caused colistin to be reconsidered as last line treatment option.[115] However, resistance to colistin has emerged recently as a consequence of chromosomal mutations and plasmid-mediated *mcr-1* gene in *Enterobacter* spp. and has sounded the alarm.[116,117]

3.1. Colistin heteroresistance in *Enterobacter cloacae*

Colistin-heteroresistant *E. cloacae* first reported in 2007 and it was suggested that exposure to colistin induced resistance.[34] Subsequently, the second report of heteroresistance to colistin in *E. cloacae* was reported from the United States in 2014 and demonstrated that treatment with colistin has led to an increase in the frequency of the resistant subpopulations.[118] Additionally, the isolation of carbapenemase-producing *Enterobacter* strains with heteroresistance to colistin is worrisome because colistin is considered as the last resort for

treatment of MDR infections.[119,120] The characteristics of studies on colistin heteroresistance in *Enterobacter* spp. are summarized in Table 3.

3.2. Mechanisms of heteroresistance

The major mechanism of colistin heteroresistance in *E. cloacae* is associated with the *arnBCADTEF* operon. Lipid A modification enzymes are regulated by PmrAB and PhoPQ two-component systems which directly activate *arn* expression in Enterobacteriaceae.[125] However, the results of studies show that unlike *E. coli*, *Salmonella enterica* or *K. pneumoniae*, colistin heteroresistance in *E. cloacae* involves a model of PmrAB-independent *arn* regulation. Indeed, PhoP can induce L-Ara4N biosynthesis through binding directly to the *arnB* promoter and PmrAB is unessential for colistin resistance in *E. cloacae*.[126] Additionally, Huang et al.[127] found that the presence of a new small transmembrane protein, encoded by the *ecr* gene, increases the expression of PhoP and the *arnBCADTEF* operon and thus contributes to colistin heteroresistance in *E. cloacae* complex. They also found that three genes *phoP*, *ded*A (encoding an inner membrane protein of the *ded*A family) and *tol*C (encoding part of the AcrAB-TolC efflux pump) are involved in colistin heteroresistance. This is the first report of a correlation between the *ded*A gene and heteroresistance.[127] It was also previously found in a study that the AcrAB-TolC efflux pump proteins induction is triggered by a *soxRS* regulator. It has been suggested that cell stress produced by colistin leads to the overexpression of *soxRS* and thus overexpression of the AcrAB-TolC efflux pump in colistin heteroresistant *Enterobacter* strains.[122] Outer membrane proteins (OMPs) analysis in colistin-susceptible and its colistin-heteroresistant *Enterobacter asburiae* counterpart confirms the role of the altered cell wall in resistance.[128] Association between the cluster membership and the heteroresistance phenotype to colistin in *E. cloacae* is debatable. A study reported that colistin heteroresistance in *E. cloacae* appeared cluster-dependent based on partial sequences of the *hsp60* gene, while another study suggested that reduced colistin susceptibility occurs sporadically in this pathogen.[114,121]

3.3. Heteroresistance detection

Similar to the microorganisms mentioned above, in *Enterobacter* spp. PAP is described as gold standard method for detection of colistin heteroresistance. However, studies show that other methods have the ability to detect resistant subpopulations. Lo-Ten-Foe et al.[34] demonstrated that agar dilution, E-test, disk diffusion (all on Iso-Sensitest agar instead of MH agar) and broth microdilution methods have the ability to identify resistant subpopulations in *E. cloacae* while the VITEK 2 displayed low sensitivity and seemed to be unreliable in the identification of heteroresistance. However, another study showed that the broth microdilution method was not a reliable method for detecting heteroresistance.[121] In a study by Band et al.,[97] 93.2% of colistin heteroresistant isolates were classified as colistin susceptible by routine laboratory diagnostic methods. It has also been shown in *E. cloacae* that misclassification of a heteroresistant isolate as susceptible has led to colistin treatment failure.[7]

3.4. Therapeutic options

Although no specific study has been performed on the treatment of *Enterobacter* spp. colistin heteroresistant isolates, a study by Napier et al.[118] suggests that treatment of heteroresistant isolates with colistin leads to an increase in resistant subpopulations and induction of cross-resistance to the host antimicrobial lysozyme. These findings highlight the importance of identification of heteroresistance in microbiology laboratory before initiating treatment and the importance of combination therapy in colistin heteroresistant isolates.

4. *Pseudomonas* spp.

P. aeruginosa, well-defined species of the genus *Pseudomonas*, is a Gram-negative nonfermenting bacillus which has the ability to cause severe infections such as bloodstream infections, urinary tract infections, pneumonia and surgical site infections.[129] Unfortunately, improper use of antibiotics in recent years has led to spreading

Table 3. Heteroresistance to colistin in *Enterobacter* spp. isolates

Author (year)	Country	Number of samples	Method for determination of resistance	Prevalence of resistance (%)	Prevalence of heteroresistance (%)	Prior colistin treatment (%)
Lo-Ten-Foe JR (2007)[34]	Netherlands	15	Broth Microdilution	26.66	40	100
Guérin F (2016)[121]	France	124	Broth Microdilution	35.48	57.25	NR
Juhász E (2017)[40]	Hungary	50	Agar dilution	2	16	NR
Telke AA (2017)[122]	Laos and Nigeria	4	MALDI-TOF	100	50	NR
Mashaly G (2021)[120]	Egypt	49	Broth Microdilution	14.28	34.69	NR
Band VI (2021)[97]	USA	74	Broth Microdilution	2.7	21.62	NR
Sato T (2022)[123]	Japan	59	PAP	20.3	18.64	NR
Fukuzawa S (2023)[124]	Japan	138	PAP	27.5	27.5	NR

antimicrobial resistance in *P. aeruginosa*.[130] so for this reason carbapenem-resistant *P. aeruginosa* is on the "critical" group by the World Health Organization and is in urgent need of new antibiotics.[131] Because multidrug resistant *P. aeruginosa* has shown high susceptibility to colistin, colistin has been described as the last-line antibiotic for the treatment of MDR/XDR infections.[130] Over past years, reports of colistin-resistant *P. aeruginosa* outbreaks and plasmid-borne colistin resistance were published and caused public health concern worldwide.[132,133]

4.1. Colistin heteroresistance in *P. aeruginosa*

Co-existence of MDR and susceptible strains has been reported from a single *P. aeruginosa* isolate obtained from a cystic fibrosis patient in 2010. [134] Then in 2011 the first colistin heteroresistance in *P. aeruginosa* was reported by Bergen et al.[135] In a study by Juhasz et al.,[40] the prevalence of colistin heteroresistance among 152 *Pseudomonas* spp. clinical isolates from blood cultures was 27%. In addition, in a recent study of 143 colistin-susceptible carbapenem-resistant *P. aeruginosa* isolates, colistin heteroresistance was observed in up to 26% of the samples.[136] In the most recent study in 2024, Banerjee et al.[137] reported 1% colistin heteroresistance in *P. aeruginosa*.

4.2. Mechanisms of heteroresistance

The mechanisms involved in colistin heteroresistance in *P. aeruginosa* are examined only in one study. Of note, no significant difference was observed between the colistin resistance and colistin heteroresistance mechanism. Alterations of the PmrAB regulatory system was the main mechanism of heteroresistance in this study.[138]

4.3. Heteroresistance detection

Unfortunately, no specific studies have been performed on diagnostic methods of heteroresistance in *Pseudomonas* spp. Considering that routine susceptibility tests are not able to accurately identify the heteroresistance phenotype, the PAP test is still known as the gold standard. Lack of information in reviewing and

comparing different diagnostic tests of heteroresistance highlights the need for further studies to introduce available and cost-effective identification methods.

4.4. Therapeutic options

Studies reveal that monotherapy leads to the emergence of colistin resistance, so combination therapy has been suggested to prevent this problem.[139,140] Colistin combined with meropenem and doripenem has shown a beneficial effect in patient care especially at the commencement of therapy. It has been suggested that colistin may improve bactericidal activity of meropenem and doripenem by affecting on membrane permeability which increases their access to the periplasmic space and penicillin-binding proteins. Thus, this finally reduces the amount of bacteria in the face of the immune system.[135,141]

5. Other Gram-negative pathogens

Limited studies have been performed on colistin heteroresistance in other Gram-negative bacteria. The prevalence of colistin heteroresistance among *E. coli* clinical isolates was reported in 2015 and 2017.[40,97] The first report for colistin-resistance and heteroresistance mechanisms in *E. coli* demonstrated that substitutions in *pmrB* and upregulation of the *pmrCAB* operon are mainly involved in colistin heteroresistance, while the presence of the *mcr-1* gene contributed to resistance to colistin. Results of that study suggest that carbonylcyanide m-chlorophenylhydrazone (CCCP) action has a significant effect on colistin-heteroresistant isolates.[142] Then another study confirmed the role of mutations in *pmr*B and also showed that mutations in *pho*Q were associated with colistin heteroresistance in *E. coli* isolates in swine.[143] Additionally, it has been found that the plasmid-mediated Kluyvera-Like *arnBCADTEF* operon is a new colistin heteroresistance mechanism in *E. coli*.[144]

Results of a study revealed that over-expression of the *pmrD* gene conferred colistin resistance in *S.* Typhimurium and proposed that the variable copy number of the *pmrD* gene in subpopulations can explain the heteroresistance phenomenon.[19] Also, the first identifications of colistin heteroresistance in *N. meningitidis* and *Citrobacter freundii* were reported in 2019 and 2024, respectively.[145,146] The heteroresistance phenomenon has been described in *Stenotrophomonas maltophilia* too. Results of that study suggest that combination therapy should be used against *S. maltophilia* due to its ability to rapidly adapt to colistin.[147]

Conclusions

Labiaplasty, particularly through laser-assisted techniques, has proven effective for many women seeking relief from physical discomfort or dissatisfaction with labial appearance. While postoperative complications such as infection remain relatively uncommon, they can occur and should be proactively addressed through thorough preoperative screening, careful surgical technique, and structured aftercare. Despite increasing demand and high patient satisfaction, the field still lacks standardized definitions, classification systems, and large-scale outcome studies. To ensure safe, ethical, and patient-centered care, future research must focus on establishing evidence-based guidelines that balance functional outcomes, aesthetic goals, and psychological well-being.

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