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Adherence of *Neisseria gonorrhoeae* to surface of SiHa cells (human cervical carcinoma cell line)

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WHAT DO WE KNOW SO FAR ABOUT GES CARBAPENEMASES, AND WHAT THREAT DO THEY POSE?

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Abstract. Carbapenemases, classified as bacterial enzymes, have the ability to hydrolyze carbapenems – important broad-spectrum antibiotics. This work attempts to summarize the information on the diversity of Guiana Extended-Spectrum (GES) subgroup of carbapenemases, and highlights the serious threat posed by infections caused by bacteria capable of producing these enzymes. The structure, functional characteristics, classification of different types of GES carbapenemases and diagnostic methods are discussed in detail. There are 59 GES-type carbapenemases, which have different amino acid sequences of the protein chains as well as activity against various antibiotics. Currently, bacterial strains with antibiotic resistance of the GES type are treated with: cefiderocil belonging to the cephalosporins, eravacycline belonging to the tetracyclines, lefamulin belonging to the pleuromutulins, colistin, fosfomycin, nitrofurantoin, tobramycin, amikacin, imipenem with relebactam, meropenem with waborbactam, ceftazidime with avibactam and plazomycin. In addition, the following drugs are under study: durlobactam with sulbactam, taniborbactam and cefepime with enmetazobactam This paper aims to summarize the current knowledge on GES-type carbapenemases, their diagnosis and treatment.

Keywords: antimicrobial resistance, carbapenemases, Guiana Extended-Spectrum, carbapenemases, carbapenemase-producing strains

1. Introduction

Gram-negative bacteria continue to pose a severe threat to the health and lives of people around the world due to their constantly increasing drug resistance. Carbapenems, strong antibiotics from the beta-lactam group used to treat severe infections caused by these microorganisms, have been named antibiotics of last resort. However, carbapenemase enzymes produced by bacteria can inactivate carbapenems, thus significantly complicating the effective treatment of infections (Chmielewska and Leszczyńska 2019).

Carbapenemases are bacterial enzymes that hydrolyze the β-lactam bond present in carbapenems. These beta-lactamases also can deactivate penicillins, cephalosporins, and monobactams, which shows how broad their spectrum of action is (Queenan and Bush 2007).

Until the early 1990s, all carbapenemases were described as species-specific, but this view was incorrect because different classes of enzymes began to be detected in various bacterial species. The genes encoding these bacterial enzymes are often found on plasmids, which makes them easy to transfer. Plasmids can be transferred by conjugation, which promotes the rapid spread of resistance. Moreover, carbapenemases belong to different classes of β-lactamase enzymes, reflecting their evolutionary diversity, which results from selective pressure induced by antibiotics, leading

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Table I
A comparison of the main features of selected GES-type carbapenemases. Some of the GES-type antibiotic resistances are ESBL-type enzymes, which do not have carbapenemase properties. Some of them do have carbapenemase activity.

Enzyme	Mutation	Gene location	Microrganism	Year and country of identification	Referencesm
GES-1	A170G	Plasmid	*K. pneumoniae*	1998, France	Poiler *et al.* 2000
GES-2	G170N	Plasmid	*P. aeruginosa*	2000, South Africa	Poiler *et al.* 2001
GES-4	G170S	Plasmid	*K. pneumoniae*	2002, Japan	Queenan and Bush 2007
GES-5	G170S	Chromosomal	*P. aeruginosa*	2007, Spain	Viedma *et al.* 2009
GES-6	G170S	Plasmid	*K. pneumoniae*	2004, Greece	Queenan and Bush 2007
GES-11	G243A	Plasmid	*A. baumannii*	2008, France	Moubareck *et al.* 2009
GES-14	G170S, G234A	Plasmid	*A. baumannii*	2008, described in Belgium	Mabrouk *et al.* 2017
GES-16	Gln38Glu, G170S	Plasmid	*S. marcescens*	2011, Brazil	Escandón *et al.* 2017
GES-18	G170S, V80I	Plasmid	*P. aeruginosa*	2010, Belgium	Bebrone *et al.* 2013
GES-20	A165S	Chromosomal	*P. aeruginosa*	2011, Mexico	Garza-Ramos *et al.* 2015

to the acquisition and modification of resistance genes. Thus, what was once a problem of clonal dispersal has now become an interspecies problem on a global scale (Queenan and Bush 2007).

2. Carbapenemases classification

Carbapenemases represent the most diverse family among beta-lactamases. The classification of β-lactamases considers two criteria: a functional one, based on enzymatic activity, developed by Bush and Jackoby (2010), and a molecular one, based on amino acid homology, developed by Ambler et al. (1991). The first system distinguishes four functional groups (marked with numbers from 1 to 4), and carbapenemases occur mainly in group 2f, susceptible to inhibition by β-lactam inhibitors, and group 3, including metallo-β-lactamases (MBLs), which are inhibited by ethylenediaminetetraacetic acid (EDTA), but are not susceptible to β-lactam inhibitors. The second division, showing the evolutionary relationship of β-lactamases, distinguishes four groups differing in molecular structure (marked with letters A to D). The hydrolytic mechanisms involving serine are present in classes A, C, and D, while carbapenemases from class B have zinc in their active sites (Mammeri *et al.* 2005; Paterson and Bonomo 2005).

Class A includes *Klebsiella pneumoniae* carbapenemase (KPC), Sulfhydryl variable-5 (SHV-5), Sulfhydryl variable-38 (SHV-38), cefotaximase-33 (CTX-M-33), Imipenemase/Not Metallo-β-lactamase Carbapenemase-A (IMI/NMC-A), Broad-spectrum *Klebsiella* Carbapenemase-1 (BKC-1), *Serratia fonticola* Carbapenemase-1 (SFC-1), *Serratia marcescens* Enzyme (SME), Frankfurt Resistance-Imipenem (FRI), Formosa Lactamase Class C (FLC) and the Guiana Extended-Spectrum (GES) β-lactamases, which were initially identified as the ESBL family (Queenan and Bush 2007; Bonnin *et al.* 2021). ESBLs are extended-spectrum β-lactamases. The ESBL-producing strain is capable of hydrolyzing penicillins, cephalosporins (except cephamycins), and monobactams but is susceptible to carbapenems and β-lactamase inhibitors (Chmielewska and Leszczyńska 2019). Over time, GES variants were discovered and characterized by low but measurable hydrolysis of imipenem – an antibiotic belonging to the carbapenem group, ultimately separating a new subgroup (Queenan and Bush 2007). The name GES comes from where this enzyme was discovered – French Guiana (Poirel *et al.* 2000). The main features of selected GES-type carbapenemases are compared in Table I.

3. General characteristics of GES carbapenemases

GES carbapenemases are relatively rare compared to other carbapenemases, e.g. class A (KPC) or class B (MBLs). The bacteria most often found to be able to produce GES carbapenemases are *Pseudomonas aeruginosa, Acinetobacter baumannii,* and *Enterobacterales* (including *Klebsiella pneumoniae, Escherichia coli,* and *Serratia marcescens*). These bacteria are responsible for infections of the urinary tract and respiratory system, such as pneumonia, especially in hospitalized patients. Penetrating the bloodstream, they can cause bacteremia or sepsis. Infections caused by bacteria producing GES carbapenemases also include skin, surgical wounds, soft tissue infections, infections related to medical devices, and peritonitis.

Genes determining the resistance of GES-type carbapenemases are encoded on plasmids, and ESBLs are encoded on plasmids and integrons. Ellington et al. (2020) wrote that these genes are inherited by

horizontal gene transfer (conjugation, transduction, and transformation) between genera and species. The GES carbapenemase genotype may confer an ESBL-like resistance phenotype to bacteria with low resistance to ertapenem and meropenem. GES-type antibiotic resistance, or more specifically GES-1, was first discovered in 1998 in a strain of *Klebsiella pneumoniae* in France (Poirel *et al*. 2000). It was found to confer resistance to cephalosporins and penicillin, but not carbapenemase activity. There are many variants of GES, and it is worth noting that some variants confer resistance to carbapenems, unlike ESBLs. When describing resistance, it is worth mentioning that in the case of ESBL resistance, in addition to resistance to β-lactam antibiotics, there may also be resistance to other antibacterial drugs such as aminoglycosides, trimethoprim, sulfonamides, chloramphenicol, tetracyclines, and fluoroquinolones. The cause is that the genes encoding ESBL are located on transposons and plasmids close to other antibiotic resistance genes and are commonly co-transferred between bacteria (Mammeri *et al*. 2005; Paterson and Bonomo 2005; Hawkey *et al*. 2018; Pablo-Marcos *et al*. 2023).

4. Omega (Ω) loop

The Ω loop is a fragment of Ambler class A β-lactamases, which include GES carbapenemases. It significantly impacts the substrate selectivity of these enzymes because it is located in their active center. A component of this loop is Glu166, an amino acid playing a key role in the two-step catalytic cycle of β-lactam antibiotic hydrolysis (Egorov *et al*. 2019).

In addition to Glu166, the loop also includes Asn170 and both these amino acids are involved in the adhesion of the water molecule necessary for the deacylation of the antibiotic (Levitt *et al*. 2012).

5. GES-1

This variant was first discovered in *K. pneumoniae*, although it may also occur in other species. The active site of the GES-1 carbapenemase contains several key residues. These include serine (Ser70), which is a nucleophilic residue that attacks the beta-lactamase ring; lysine (Lys73), which stabilizes the transition state during hydrolysis; glutamate (Glu166), which is involved in the deacylation step of the enzymatic reaction; and serine (Ser130), which contributes to substrate binding and catalysis. The GES-1 active site lacks Asn170 (the ligand for hydrolytic water), which is replaced by glycine, making the enzyme unable to hydrolyze imipenem (Poirel *et al*. 2001; Smith *et al*. 2007).

6. Characteristics of individual GES carbapenemases

Currently, there are several dozen types of enzymes classified as GES. It should be noted that we classify some of the GES-type antibiotic resistances as ESBL-type enzymes, which do not have carbapenemase properties, but not all, some of them do have carbapenemase activity. The properties of GES enzymes that can inhibit the action of carbapenems are presented below. GES carbapenemases are classified based on the differences in amino acid composition. These differences are substitutions of one to three amino acids, determining substrate specificity. GES-type beta-lactamases, which, according to Ambler's classification, belong to class A, represent a large and diverse group of enzymes. As of 2023, they include as many as 54 lactamases, of which at least 20 are classified as carbapenemases, but not all have been fully characterized. Some of the main variants of the GES carbapenemases that are currently best known are described below (Tanabe *et al*. 2023). Due to the existence of a large number of GES-type carbapenemases and editorial limitations, the article presents those that are the best studied and provide the most helpful information (U.K. Health Security Agency, 2024).

6.1. GES-2

The carbapenemase active site of GES-2 exhibits a similar distinguishing pattern to that observed in GES-1, with serine (Ser70), lysine (Lys73) and glutamate (Glu166) all playing a comparable role. However, a critical mutation resulted in the conversion of glycine-to-asparagine (Asp170), which is responsible for increasing the enzyme activity (Poirel *et al*. 2001).

A characteristic feature of this carbapenemase is that it has a canonical asparagine at position 170, unlike GES-4, -5, and -6, which have a Gly170Ser substitution at this position. In this enzyme, a hydrolytic water molecule positions itself between Ser70 and Glu166 and is also bound to Asn170. The presence of GES-2, like GES-4, -5, and -6, causes a decrease in bacterial susceptibility to imipenem. Research conducted by Frase et al. (2011) showed that blocking this carbapenemase with tazobactam (at a concentration of 4 μg/ml) changed the MIC (minimum inhibitory concentration) for piperacillin from > = 128 μg/ml (resistance) to 1 μg/ml. Based on this, it can be concluded that tazobactam, in combination with carbapenems, can be used in therapy against bacteria with GES-2 resistance. The dissociation constant for the noncovalent complex of GES-2 and tazobactam was in the nanomolar range, indicating the high affinity of tazobactam for GES-2. Additionally, studies have shown that the mentioned inhibitor has a rapid onset of enzyme inhibition (Frase *et al*. 2011).

6.2. GES-4

The GES-4 carbapenemase active site exhibits a similar function to that of GES-1, with the presence of serine (Ser70), lysine (Lys73) and glutamate (Glu166). However, critical mutations have occurred, resulting in the conversion of glycine to serine (Ser170), which is responsible for increasing the enzyme activity. Additionally, the conversion of alanine to valine (Val173) affects the substrate specificity and efficiency of the enzyme (Wachino *et al.* 2004; Barlow and Tenover 2024).

Research conducted by Vourli et al. (2006) showed that after exposure *K. pneumoniae* 78–01 strain with GES-4 and SHV-5 resistance to clavulanic acid in combination with imipenem or ceftazidime, the susceptibility to these antibiotics was partially restored. The gene encoding GES-4 (*blaGES-4*), in the form of genomic cassettes, is located in the variable regions of class 1 integrons, which are carried by plasmids, and this enables horizontal transfer of this resistance between bacteria (Bebrone *et al*. 2013).

6.3. GES-5

The active center of the GES enzyme 5, a carbapenemase, is structured similarly to other class A β-lactamases. It contains a catalytic serine residue in its active site, crucial for its hydrolytic activity. This serine is part of a conserved sequence motif (Ser70-X-X-Lys73) that plays an essential role in the enzyme's ability to hydrolyze β-lactam antibiotics (Smith *et al*. 2012).

The study performed by Kotsakis et al. (2010) showed that this enzyme has the highest carbapenemase activity, which is associated with the presence of serine at position 170. The Gly170Ser substitution increases the ability to hydrolyze cefoxitin and imipenem but also causes a decrease in activity towards ceftazidime and aztreonam. The presence of serine at position 170 changes the structure of the enzyme, namely in the Ω loop and results in improved catalytic properties of the enzyme against carbapenems and cephamycin; it also increases the resistance to β-lactamase inhibitors (Poirel *et al*. 2018).

IR-GES-5 refers to an integron-associated GES-5 (Guiana Extended Spectrum) β-lactamase enzyme. The "I. R." typically stands for Integron-encoded Resistance, indicating that the gene encoding this enzyme is located within an integron, a genetic element in the bacterial genome. The association of GES-5 with IR-GES-5 enhances its ability to spread rapidly across different bacterial species, making it a concern in the treatment of bacterial infections. The active center of the IR-GES-5 enzyme, like other GES-type β-lactamases, possesses a catalytic serine residue (Ser70). The structure of the active site allows GES-5 to bind and hydrolyze carbapenems, which are often resistant to degradation by other β-lactamases (Labuschagne *et al*. 2008).

6.4. GES-6

The active center of the GES enzyme 6 (GES-6), like other GES-type enzymes, features a serine-based mechanism typical of class A β-lactamases. The key residues in the active site are Lys and Ser at the 104 and 170 positions, respectively. The active center also includes other residues, such as Lys73, Ser130, Glu166, and Asn170, which are involved in substrate binding, catalysis, and stabilization of the transition state (Kotsakis *et al*. 2010).

Compared to GES-1, GES-6 has more significant activity against carbapenems and ceftolozane and reduced susceptibility to β-lactamase inhibitors (except avibactam). It is worth noting that when ceftolozane was combined with tazobactam, the MIC decreased slightly compared to the value for ceftolozane without the inhibitor, demonstrating the reduced effectiveness of the inhibitors. However, the activity of GES-6 towards imipenem was higher than in GES-1, which confirmed the involvement of Ser170 in the higher activity of the enzyme. It should be noted that the substrate profile of GES-6, in some sense, reflects MBLs with activity against carbapenems and some resistance to inhibitors (Poirel *et al*. 2018).

Botelho et al. (2015) showed that the blaGES-6 gene in the *P. aeruginosa* strain is accompanied by the *aacA7* gene encoding aminoglycoside acetyltransferase type 1, conferring resistance to amikacin, netilmicin, and tobramycin.

6.5. GES-11

This enzyme was first discovered in 2008 in France in *Acinetobacter baumannii* (Moubareck *et al*. 2009). Substitution of the glycine at position 243 in GES-11 was associated with increased activity toward aztreonam, as had been observed for GES-9. GES-11 did not have a substitution of the Gly170 residue, resulting in increased hydrolysis of imipenem as in GES-2, GES-4, GES-5, and GES-6. Research conducted by Moubareck et al. (2009) showed that expression of the blaGES-11 gene in porin-deficient cells may lead to resistance to imipenem. The active site of GES-11 is similar to other serine carbapenemases, including serine at position 70 (Ser70), lysine at position 73 (Lys73), glutamate at position 166 (Glu166) and glycine at position 170 (Gly170). The lack of substitution of the Gly170 residue increases the hydrolytic properties of this enzyme. GES-11 has not been fully classified, and research is still ongoing on whether it belongs to β-lactamases or carbapenemases (Moubareck *et al*. 2009).

6.6. GES-14

The active site of GES-14, considering key amino acid positions, is similar to other carbapenemase-active GES variants: Ser 70, Lys 73, Glu 166, Gly 170. The exact sequence of the amino acids surrounding these residues defines the enzyme's active site. Unlike GES-11 carbapenemase, GES-14 contains additional hydrogen bonds in the active site formed by oxygen in the side chain of Ser170 with the carboxyl group of Glu166. It is worth noting that GES-11 has a serine at position 170 and, similarly to the previous variants, it shows activity towards carbapenems. Another feature of the amino acid chain of this enzyme is that it has an alanine at position 243, which confers increased resistance to classic β-lactamase inhibitors. Ala243 also makes the enzyme effective against aztreonam, ceftazidime, and cefotaxime (Moubareck *et al*. 2009).

IR-GES-14 enzyme is a variant of the GES-type β-lactamases, specifically associated with integrons, which enhances GES-14's ability to spread antibiotic resistance genes. Catalytic serine residue (Ser70) is crucial for the enzyme's ability to hydrolyze β-lactam rings. It acts as a nucleophile in the hydrolysis reaction. The active site of GES-14, like other GES enzymes, is flexible enough to accommodate a wide range of β-lactam antibiotics (Bonnin *et al*. 2011).

6.7. GES-16

This enzyme was first identified in *S. marcescens* in Brazil. The carbapenemase activity of GES-16, like that of other GES variants, is mainly determined by the presence of specific amino acids in its active site. These residues typically include Ser 70, Lys 73, Glu 166 and Gly 170. Based on changes in the amino acid chain, i.e. Gln38Glu and Gly170Ser, GES-16 and GES-5 have been distinguished. In a comparative study regarding the activity of GES-16 against imipenem, ertapenem, and meropenem conducted by Streling et al. (2018), it has been shown that this enzyme has the highest effectiveness against imipenem (compared to other carbapenems). GES-16, apart from hydrolyzing carbapenems, also inhibits the action of other antibiotics, i.e. penicillin, cephamycin, and cephalosporins, but importantly, it does not hydrolyze aztreonam (Escandón *et al*. 2017; Streling *et al*. 2018).

6.8. GES-18

The amino acid sequence in the GES-18 active center is similar to GES-1 and GES-2; the substitution of Gly170Ser and Val80Ile causes a change in the location of the hydrolytic water molecule and the amino acid essential in hydrolysis – glutamic acid (position 166), which may partially explain the differences in enzyme specificity and action. Like GES-5, it has low effectiveness against ceftazidime. Also, it hydrolyzes imipenem and cefotaxime with similar kinetic parameters, while the difference concerns the presence of Val80Ile (in GES-18), but this change does not significantly affect the substrate profile. It is worth noting that GES-18, unlike GES-1, is less susceptible to classic inhibitors, i.e. clavulanic acid and tazobactam (Bebrone *et al*. 2013).

6.9. GES-20

This type of enzyme was first identified in 2011 in a strain of *P. aeruginosa* in Mexico (Garza-Ramos *et al*. 2015). The *blaGES-20* gene has two single nucleotide substitutions, translating into amino acid chain changes. Studies have shown that GES-20 resistance often co-occurs with OXA-2 (oxacillinase-2). GES-20-producing isolates studied by Recio et al. (2022) showed the replacement of aspartic acid with serine (position 165). In the place that encodes leucine, a sequence change resulted in the STOP codon (position 237), thus shortening the amino acid chain, translating into resistance to CZA (ceftazidime/avibactam).

7. Identification of GES carbapenemases

Identification of GES carbapenemases called "minor class A carbapenemases" poses a particular challenge due to the low level of carbapenem hydrolysis that characterizes these enzymes (Bonnin *et al*. 2021), which contributes to an increase in the percentage of false-negative tests in phenotypic methods (biotyping, serotyping, assessment of drug susceptibility profiles, protein analysis methods). Biochemical tests only enable the detection of carbapenem resistance but without determining the specific type of resistance. Therefore, it is necessary to confirm the result by molecular tests. They mainly involve the amplification of nucleic acids using multiplex diagnostics such as PCR (Polymerase Chain Reaction), LAMP (Loop-mediated Isothermal Amplification), or RPA (Recombinase Polymerase Amplification) (Ortiz-Cartagena *et al*. 2023). They detect the presence of known carbapenemases genes on plasmids, porin channel mutations, or efflux pump mutations.

However, these methods, despite the possibility of accurate and simultaneous identification of individual GES carbapenemase genes, are quite limited due to the need for specialized equipment, costs, and reduced speed; additionally, it is possible to detect only known genes, which significantly limits the spectrum of gene detection (Tenover 2021).

Laboratories often use a combination of phenotypic and genetic tests against the risk of false results.

Diagnostics increasingly seek alternative methods to identify bacterial enzymes that hydrolyze the β-lactam bond in the carbapenem molecule. These include commercially available the EntericBio CPE test (Serosep Ireland), a multiplex real-time PCR reaction. In research conducted by Vanstone et al. (2018), this test showed both 100% specificity and susceptibility.

The CIM (Carbapenem Inactivation Method) test is a diagnostic tool used to detect carbapenemases' activity in Gram-negative bacteria. This test is a phenotypic test, which employs an indirect method for the detection of carbapenem production. The presence of resistance is determined by the interpretation of the enzymatic hydrolysis of a meropenem disc following exposure to strain producing carbapenemases, including GES-5, OXA-372, GIM-1 (German Imipenemase-1), FRI-1 (Florence Imipenemase-1), SME-1/-2 (*Serratia marcescens* Enzyme-1), NMC-A (Non-metallo Carbapenemase-A) and IMI-1/-2/-3. The test also detects the following carbapenemases: KPC-2, GES-5, SME-1/-2 (*Serratia marcescens* Enzyme-1), NMC-A (Non-metallo Carbapenemase-A), IMI-1/-2/-3. A positive result is indicated by the growth of the indicator *E. coli* strain on the Muller-Hinton medium. As this test detects a multitude of different carbapenemases, it is not feasible to ascertain with absolute certainty the specific resistance that has been identified (Aguirre-Quiñonero *et al*. 2017; Bonnin *et al*. 2021). In a study involving 124 *Enterobacteriaceae* strains, Aguirre-Quińonero et al. (2017) evaluated the CIM assay for its effectiveness in detecting different types of carbapenemases, including GES-6. While the test demonstrated efficacy in detecting carbapenemases of the KPC, NDM, VIM, IMP and OXA-48 types, it exhibited relatively lower sensitivity for GES-6 (79.3%). Of the 22 strains with the gene encoding GES-6, only eight were positive for CIM, while 11 exhibited a false negative result. This result may be attributable to the low hydrolytic level of GES-6. Nevertheless, despite the necessity for additional confirmation tests to identify GES antibiotic resistance, this test's simplicity and low cost render it a valuable tool. One modification of the CIM test, the rapid Carbapenem Inactivation Method (rCIM) assay, was found to facilitate rapid detection of carbapenemase activity, including, but not limited to, GES-5. This assay employed a nephelometer to accelerate the detection of carbapenemases (Muntean *et al*. 2018).

The MAST Carba PacE test is a colorimetric test based on the hydrolysis of a chromogenic cephalosporin analogue. A change in colour from yellow to orange or red is observed in the presence of an enzyme belonging to the carbapenemases. A study by Rezzoug et al. (2023) revealed that the MAST Carba PacE test exhibits insufficient sensitivity towards GES-type carbapenemases (the test did not detect any of the strains tested that produce GES-type enzymes), leading to the conclusion that the test is not effective in detecting this resistance.

Lateral flow immunoassays (e.g. NG-Test CARBA-5) represent a rapid and straightforward method for identifying carbapenemases, with a detection time of less than 15 minutes. The test exhibits high sensitivity and specificity in detecting carbapenemases in *Enterobacterales* strains, rendering it an efficacious diagnostic instrument within the hospital environment. In NG-Test CARBA 5 studies, the test demonstrated a sensitivity of 98% and a specificity of 100% for *Enterobacterales*, indicating its high compatibility with molecular methods. The test is valued for its rapidity and ease of use, crucial for managing life-threatening infections (Mende-Sotelo *et al*. 2023).

The modified Hodge test (MHT) is a phenotypic test for detecting GES-type carbapenemases in bacteria belonging to the *Enterobacteriaceae* family. Regrettably, this test has low sensitivity and a high incidence of false positives, resulting in its limited use for detecting carbapenemases. The principal advantages of this test are its cost-effectiveness, ease of implementation in standard medical laboratories and simplicity of performance (Ramana *et al*. 2013). Another modification of this assay, involving the addition of Triton-X-100 (Triton Hodge assay), has been shown to have good sensitivity in detecting carbapenemases such as GES-5, SME-1 and NMC-A (Pasteran *et al*. 2016).

8. Future perspectives in the rare carbapenemases detection

Therefore, there is an urgent need to look for methods that would be equally susceptible and specific, and at the same time fast, simple, and cheap, in short, methods that would not require DNA extraction. In a study conducted by Concha Ortiz-Cartagena et al. (2023), an assay based on LAMP CRISPR-Cas13a (Clustered Regularly Interspaced Short Palindromic Repeats) has been adapted. It is not a commonly available test used in diagnosing GES carbapenemases, but due to its advantages, it seems particularly valuable and worth comments. It enables the detection of OXA-48 and GES carbapenemases in *Enterobacterales* and *Pseudomonas* spp. This technique is free from purification and concentration of nucleic acids. It allows the detection of *blaOXA-48* and *blaGES* genes responsible for carbapenem resistance. As the authors claim, this test costs less than EUR 10 per reaction, takes less than two hours to complete, and is 100% specific and susceptible to identifying both OXA-48 and GES carbapenemases. It is easily accessible because it does not require specialized equipment or trained personnel. It is currently one of the fastest

and most effective tests on the market, and it can be routinely introduced in clinical microbiology laboratory tests to detect multidrug-resistant pathogens.

9. Treatment of infections caused by carbapenem-resistant pathogens:

9.1. New drugs in the treatment of carbapenemases-producing strains

In 2024, the World Health Organization (WHO, 2024) published a list of antibiotic-resistant bacteria that pose a considerable threat to public health to indicate the direction for research and development initiatives. WHO has classified bacteria into three risk groups – see Table II.

Bacterial strains exhibiting GES-type antibiotic resistance are treated analogously to other carbapenem-resistant strains. Currently, there are no pharmaceutical agents available that are specifically designed to target this resistance. The following are examples of drugs that can be employed in the treatment of infections caused by carbapenem-resistant bacteria with GES-type resistance, among others.

Given the increasing antibiotic resistance, scientists should develop new drugs that could be used to treat multidrug-resistant strains that threaten humans. A recently developed drug is a siderophore cephalosporin – cefiderocol, which binds to iron, and its action is compared to the mechanism of a "Trojan horse". Iron is an essential element for the synthesis of bacterial DNA, energy production, and other processes necessary for life. Thus, once this drug binds to iron, it is absorbed by bacteria and then binds to PBP (penicillin-binding protein), its target (Wernicki 2018; European Medicines Agency 2020a).

Other new drugs are eravacycline, a tetracycline, and lefamulin, the first pleuromutilin approved for human use. Lefamulin works by inhibiting the synthesis of bacterial proteins – it blocks the 23S rRNA molecule of the 50S ribosomal subunit (European Medicines Agency 2020b). Eravacycline, a third-generation tetracycline, changes the conformation of ribosomes, preventing protein elongation. This drug has been approved for the treatment of complicated abdominal infections caused by strains producing GES carbapenemases (and NDM /New Delhi metallo-β-lactamase/, VIM /Verona integron-encoded metallo-β-lactamase/, OXA) (European Medicines Agency 2024a). It is also worth noting that eravacycline is in phase III clinical trials for use in complicated urinary tract infections. *In vitro* studies conducted by Grossman et al. (2015) showed its promising activity against *E. coli*. If, in *in vivo* studies, the effectiveness of this tetracycline on the biofilm formed by the bacteria mentioned above is confirmed. In a second phase III clinical trial, it was discovered that the administration of eravacycline at a dose of 1.5 mg/kg body weight via intravenous infusion every 24 hours, commencing on day 3 with a gradual reduction in dosage to 200 mg administered every 12 hours, was comparable to the use of levofloxacin in the treatment of complicated UTIs (Zhanel *et al*. 2016).

Carbapenems can also be used in combination with carbapenemase inhibitors against carbapenemase-producing strains. However, bacteria can cope with this by producing enzymes not susceptible to inhibitors. An example is GES-5 type resistance, characterized by the

Table II
World Health Organization (WHO) bacterial priority pathogens list

Bacteria	Priority group	Resistance type
Enterobacterales	critical	carbapenem-resistant
Enterobacterales		third-generation cephalosporin-resistant
Acinetobacter baumanii		carbapenem-resistant
Salmonella Typhi	high	fluoroquinolone-resistant
Shigella spp.		fluoroquinolone-resistant
Enterococcus faecium		vancomycin-resistant
Non-typhoidal *Salmonella*		fluoroquinolone-resistant
Neisseria gonorrhoeae		third-generation cephalosporin and/or fluoroquinolone-resistant
Staphylococcus aureus		methicillin-resistant
Pseudomonas aeruginosa		carbapenem-resistant
Group A Streptococci	medium	macrolide-resistant
Streptococcus pneumoniae		macrolide-resistant
Haemophilus influenzae		ampicillin-resistant
Group B Streptococci		penicillin-resistant

decreased susceptibility to carbapenemase inhibitors. Therefore, new inhibitors are looked for. For example, the following combinations have recently been developed: relebactam used in combination with imipenem, vaborbactam used in combination with meropenem (Hayden *et al.* 2020) and ceftazidime with avibactam (Vázquez-Ucha *et al*. 2020). These three drug combinations have proved safe and effective and should be considered an alternative treatment for infections caused by carbapenem-resistant pathogens (Bouchet *et al*. 2020).

Relebactam is a DBO (diazobicyclooctane) inhibitor of a second generation (non-β-lactam molecules). It binds to the active site of serine β-lactamases, which effectively inhibits class A (including GES) and C β-lactamases. The drug restores the activity of β-lactams despite bacteria being resistant to these drugs (Bouchet *et al*. 2020).

Imipenem, in combination with relebactam, has broad activity against many Gram-negative bacteria, including Enterobacterales, *P. aeruginosa*, and *Bacteroides* spp. (belonging to anaerobic bacteria) producing enzymes that inhibit the action of carbapenems. The combination of imipenem and relebactam also shows effectiveness against multidrug-resistant strains resistant to, e.g. fluoroquinolones (Bouchet *et al*. 2020).

Another important inhibitor is vaborbactam, which is a derivative of boronic acid. Studies have shown that the combination of vaborbactam and meropenem is effective in the treatment of UTIs, nosocomial pneumonia, ventilator-acquired pneumonia, intra-abdominal infections, or bloodstream infections associated with carbapenem-resistant bacteria. Microbiological experiments have shown that adding vaborbactam to meropenem restores the minimum inhibitory concentration to a level comparable to the wild strain of *Enterobacterales* (Vázquez-Ucha *et al*. 2020).

When describing new drugs against carbapenemase-producing strains, the combination of ceftazidime and avibactam should also be mentioned. Avibactam is the first synthetic DBO showing activity against clinically important resistance mechanisms, such as GES, KPC, SHV, CTX-M and OXA-48. The mechanism of action of avibactam, which is based on binding to the active site of the bacterial enzyme, is reversible. After deacylation, an unchanged drug is released, which can inhibit another β-lactamase (including carbapenemase) (Vázquez-Ucha *et al*. 2020).

9.2. Drugs against GES carbapenemases producing strains in development

GES carbapenemases represent a significant challenge in the treatment of bacterial infections due to their ability to hydrolyze a range of antibiotics, including penicillins, cephalosporins, monobactams and carbapenems (Bonnin *et al*. 2011). Carbapenems are among the most important antibiotics used to treat multidrug-resistant infections. Developing new drugs against bacterial strains is currently a topic of significant interest. Many drugs are currently under investigation, including durlobactam + sulbactam, taniborbactam and cefepime+enmetazobactam. These drugs have demonstrated activity against strains resistant to carbapenems, including strains with GES-type resistance (Soszyńska-Morys 2023; European Medicines Agency 2024a). Table III shows drugs under investigation for the treatment of infections with carbapenemase-producing strains of the GES type. The table provides information on the active substances, their class, spectrum of action, testing phases, and additional information on their efficacy and therapeutic areas.

Table III
Drugs against GES carbapenemases producing strains in development (Soszyńska-Morys 2023; European Medicines Agency 2024a)

Substance	Class	Spectrum	Research phase	Additional information
durlobactam + sulbactam	Inhibitor of β-lactamases of classes A, C and D, according to Ambler	*Acinetobacter baumanii*	Phase 3 clinical trials, completed	The combination shows greater activity against MDR
taniborbactam	Non-β-lactam inhibitor of β-lactamases of classes A, B, C, and D, according to Ambler	*P. aeruginosa, Enterobacterales*	Phase 3 clinical trials of taniborbactam with cefepime in UTIs, completed	Enables the use of cefepime against carbapenem-resistant strains
cefepime + enmetazobactam	β-lactam (cephalosporin) + β-lactamase inhibitor	ESBL-producing bacteria, Enterobacterales resistant to 3rd generation cephalosporins, Carbapenem-resistant *K. pneumoniae*	CHMP issued a marketing authorization for a medicinal product containing cefepime and enmetazobactam (2024)	Therapeutic area: pyelonephritis, UTI, HAP and VAP

CHMP – Committee for Medical Products for Human Use, ESBL – Extended-Spectrum β-Lactamase, HAP – Hospital-Acquired Pneumonia, MDR – Multidrug Resistant Strains, UTI – Urinary Tract Infection, VAP – Ventilator Associated Pneumonia

Table IV
Summary of antibiotics that can be used against carbapenemase-producing strains (Rejestr produktów leczniczych 2012; 2014; 2015a; 2015b; Electronic Medicines Compendium 2024; European Medicines Agency 2018; 2020a; 2020b; 2022; 2024b; 2024c)

Substance	Spectrum	Indications
Colistin	*P. aeruginosa, K. pneumoniae, E. coli, A. baumanii*	Sepsis, lower RTI, UTI, RTI in CF patients
Fosfomycin	*K. pneumoniae, E. coli, Citrobacter* spp., *Proteus* spp.	Acute, uncomplicated cystitis; profuse, asymptomatic bacteriuria; UTI prevention before surgery and transurethral diagnostic procedures
Nitrofurantoin	*E. coli,* enterococci, staphylococci, *Citrobacter* spp., *Klebsiella* spp., *Enterobacter* spp.	Acute or recurrent lower UTI; inflammation of the renal pelvis (spontaneous or after surgery)
Tobramycin	*P. aeruginosa, Corynebacterium* spp., MSSA, *Citrobacter* spp., *Haemophilus* spp., *Salmonella* spp., *Shigella* spp, *P. vulgaris*	HAP (incl. severe pneumonia), exacerbations of lower RTI in CF patients, complicated and recurrent UTI; intra-abdominal infections; skin and soft tissue infections (incl. severe burns)
Amikacin	*P. aeruginosa, S. aureus, Citrobacter freundii, E. coli, K. pneumoniae, P. mirabilis, P. vulgaris*	HAP (incl. severe pneumonia), abdominal infections (incl. peritonitis and post-operative infections), complicated and recurrent UTI, skin and soft tissue infections and burns; bacterial endocarditis
Cefiderocol	*E. coli, K. pneumoniae, P. mirabilis, P. aeruginosa, Enterobacter cloacae complex*	Infections caused by aerobic Gram-negative bacteria, complicated UTI, pyelonephritis
Eravacycline	*E. coli, K. pneumoniae, S. aureus, E. faecalis, E. faecium, Streptococcus* spp.	Complicated intra-abdominal infections in adults
Lefamulin	*S. pneumoniae, S. aureus, L. pneumophila, M. pneumoniae, C. pneumoniae*	Community-acquired pneumonia (in case of ineffective treatment with recommended drugs)
Imipenem with relebactam	*E. coli, H. influenzae, K. pneumoniae, P. aeruginosa, S. mercescens,*	HAP, VAP; bacteremia in HAP, infections with aerobic Gram-negative bacteria in case of limited treatment options
Meropenem with vaborbactam	*E. coli, K. pneumoniae, Enterobacter cloacae complex, Citrobacter* spp., *P. aeruginosa, S. mercescens, S. aureus, S. epidermidis, S. agalacitiae, B. fragilis, C. perfringens, Prevotella* spp.	Complicated abdominal pneumonia, complicated UTI, pyelonephritis, HAP and VAP
Ceftazidime with avibactam	*C. freundii, E. cloacae, E. coli, K. oxytoca, K. pneumoniae, P. aeruginosa, P. mirabilis, S. mercescens*	Complicated intra-abdominal infection, complicated UTI, pyelonephritis, HAP, VAP, infections caused by aerobic Gram-negative microorganisms in adults and children > 3 months of age
Plazomycin	*E. coli, K. pneumoniae, P. mirabilis, E. cloacae*	UTI and pyelonephritis

CF – cystic fibrosis, CSF – cerebrospinal fluid, HAP – hospital-acquired pneumonia, incl. – including, MS – multiple sclerosis, MSSA – Methicillin-Susceptible *Staphylococcus aureus*, RTI – respiratory tract infection, UTI – Urinary tract infection, VAP – ventilator associated pneumonia

9.3. Summary of antibiotics that can be used against carbapenemase-producing strains

Table IV provides essential data on the antibiotics that can be employed to treat infections caused by carbapenemase-producing strains. The table offers comprehensive information on the active substances, their spectrum of action, and their indications for use. It serves as a valuable reference in daily medical practice to facilitate the selection of an appropriate antimicrobial treatment.

10. Conclusions

Individual GES carbapenemases have different amino acid sequences resulting from mutations in the bacterial DNA chain, thus allowing their differentiation by PCR and electrophoresis methods. Knowledge about specific resistance allows one to make the right treatment decisions. The use of appropriately selected drugs against GES will reduce the ineffectiveness of the therapy, which will prevent the spread and emergence of further resistance mechanisms. Currently, there are drugs against strains with GES resistance that are widely used, e.g. colistin or fosfomycin, as well as newly developed combinations of carbapenems with inhibitors (e.g. meropenem with vaborbactam) and many drugs under investigation. However, it should be noted that if rational antibiotic therapy is not followed, they will stop being active against some strains.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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THE ROLE OF VOLUNTARY COUNSELLING AND TESTING POINTS (VCTs) IN HIV DIAGNOSTICS – ANALYSIS OF VCTs ACTIVITY IN POLAND IN THE CONTEXT OF LOCAL AND EUROPEAN EPIDEMIOLOGY

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Abstract. Screening tests are now readily accessible, quick and highly sensitive. Diagnostics of HIV infections involves serological testing followed by confirmation tests by molecular biology methods (NAAT). Fast, accurate, anonymous and free HIV testing has been provided by Voluntary Counselling and Testing Points (VCTs) for over 20 years to any adult in Poland, regardless of nationality. Additionally, the educational activities are conducted aimed at informing people from various social backgrounds about HIV prevention and diagnostic possibilities, as well as other sexually transmitted infections (STIs). The aim of the article was to present the procedures for the operation of VCTs in Poland, their location, testing scheme and a summary of the activities that promote such points in the medical community. Between 2019 and 2023, 157,833 people registered for HIV testing in VCTs in Poland, of which 10,177 (6.45%) were foreigners. In the analyzed period, the number of tests among foreigners visiting VCTs in Poland almost doubled, from 1,552 in 2019 to 2,827 in 2023. In addition, a higher percentage of positive results was recorded among foreigners – 5.08% compared to Poles – 1.24%. The frequency analysis of positive results detection reported in VCTs vs. National Institute of Public Health NIH – National Research Institute showed that, on average 26% (2,342/8,891) of positive results in Poland from 2019–2023 were detected in the framework of tests carried out in VCTs. The operation of VCT points in Poland enables the reaching of a larger group of people, especially key adult populations. It is a valuable complement to routine diagnostics outside the health care system.

Keywords: Check Point, diagnostic, HIV, testing, voluntary counselling and testing points (VCTs)

1. Introduction

Despite advancements in medicine, HIV (Human Immunodeficiency Virus) infections continue to be a global issue. It is estimated that globally in 2022, the number of people living with HIV was 39 million (33.1–45.7 million), with two-thirds (25.6 million) in African countries and approximately 2.3 million in European countries. Globally, in 2022, 630,000 (480,000–880,000) people died from HIV-related causes, and 1.3 million (1.0–1.7 million) became newly infected (WHO 2023).

According to a European Centre for Disease Prevention and Control (ECDC) report, 110,486 people were diagnosed with HIV across 49 European countries in 2022. The average for Europe was 12.4 per 100,000 population; the highest HIV incidence rate per 100,000 inhabitants in 2022 was noted in the Russian Federation – 38.4 (55,573 diagnosed HIV infections), and the lowest rate was in Bosnia and Herzegovina – 1.7 (54 HIV infections) [Table I]. To compare some examples, in Western European countries the rates were as follows in alphabetical order: Austria

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Table I
Diagnosis rate per 100,000 population and diagnosed HIV infections in countries with the highest and lowest rates vs Poland in 2022 (ECDC 2023).

Country	Diagnosis rate per 100,000 population	Diagnosed HIV infections
The highest HIV incidence rates (over 15)		
Russian Federation	38.4	55,573
Ukraine	29.8	12,212
Moldova	28.4	929
Cyprus	24.1	218
Kazakhstan	20.7	4,006
Armenia	19.2	535
Estonia	18.8	250
Ireland	17.5	887
Belarus	17.2	1,644
Georgia	16.5	617
Kyrgyzstan	16.5	1,094
The lowest rates (2.0 and below)		
Slovenia	2.0	42
North Macedonia	2.0	41
Bosnia and Herzegovina	1.7	54
Poland	**5.4**	**2,050**

2.1 (189 HIV infections), Belgium 9.1 (1,060), Denmark 4.4 (258), Finland 4.9 (273), France 6.1 (4,158), Germany 3.9 (3,239), Greece 5.4 (565), Italy 3.2 (1,888), Portugal 7.8 (804), Spain 6.2 (2,937), Sweden 4.3 (446), United Kingdom 6.0 (4,040) (ECDC 2023).

For Poland, this rate in 2022 was 5.4 per 100,000 inhabitants, resulting in 2,050 new HIV cases in 2022 (ECDC 2023). According to national data from the National Institute of Public Health NIH – National Research Institute (NIPH NIH – NRI) – an institution collecting and reporting infections in Poland, summarising reports from 2022, 2,384 HIV infections were registered. However, among these cases, three were diagnosed in 2018, 3 in 2019, 22 in 2020, 285 in 2021, and the remaining 2,071 in 2022 (NIZP – PZH 2023).

From 1985 to the end of 2022, a total of 30,092 HIV infections were identified in Poland among Polish citizens and foreigners residing in Poland. According to NIPH NIH – NRI statistics, at least 6,462 infections were related to drug use, 2,288 through heterosexual contact and 4,872 through homosexual contact (NIZP – PZH 2023).

It is essential to note that late detection of infection is associated with high healthcare costs, an increase in AIDS (*Acquired Immunodeficiency Syndrome*) cases, a shorter average lifespan, higher mortality rates and a higher incidence of HIV-related comorbidities (Martin-Iguacel *et al.* 2022).

The number of new AIDS cases and related deaths is decreasing globally. According to an ECDC report, the number of reported AIDS cases in 2022 remained at the same level as in 2021 (0.6 per 100,000 inhabitants), but compared to 2019, there was a 14% decrease. Regarding AIDS-related deaths, there was a 2.0% decrease compared to 2021 and a 44.5% decrease compared to 2019. These changes are likely significantly influenced by better access to treatment and patient care policies (ECDC 2023).

According to the ECDC report, in 2022, 7,642 people in 44 European countries were diagnosed with AIDS (diagnosis rate 1.1 per 100,000 inhabitants). Overall, 64.7% of AIDS cases were diagnosed in Eastern Europe (diagnosis rate 4.4 per 100,000 inhabitants), 24.5% in Western Europe (0.5 per 100,000 inhabitants), and 10.8% in the Central Region (0.4 per 100,000 inhabitants). For Poland, this rate, according to the ECDC, was 0.3, resulting in 114 cases in 2022 (ECDC 2023).

According to NIPH NIH – NRI data for 2022, of the 135 registered AIDS cases, 1 case was diagnosed in 2020, 19 cases in 2021, and 115 cases in 2022 (NIZP-PZH 2023). The discrepancies between the ECDC and NIPH NIH – NRI reports in 2022 (21 HIV cases and 1 AIDS case) likely result from the one-time release and publication of the ECDC report based on NIPH NIH – NRI data, which were subsequently continuously updated with incoming reports.

Global examples, mainly from African countries, show that with good detection, diagnosis, prevention, education, support from non-governmental organizations, and state involvement, pandemics like HIV can be overcome. The Joint United Nations Programme on HIV/AIDS (UNAIDS 2023) presents data indicating that eliminating AIDS by 2030 is possible. The President's Emergency Plan for AIDS Relief (PEPFAR), implemented worldwide, has had a significant impact on reducing the number of new HIV infections. From 2010 to 2022, the number of new HIV infections decreased by

57% and the number of AIDS-related deaths decreased by 59% in the supported countries (UNAIDS 2023).

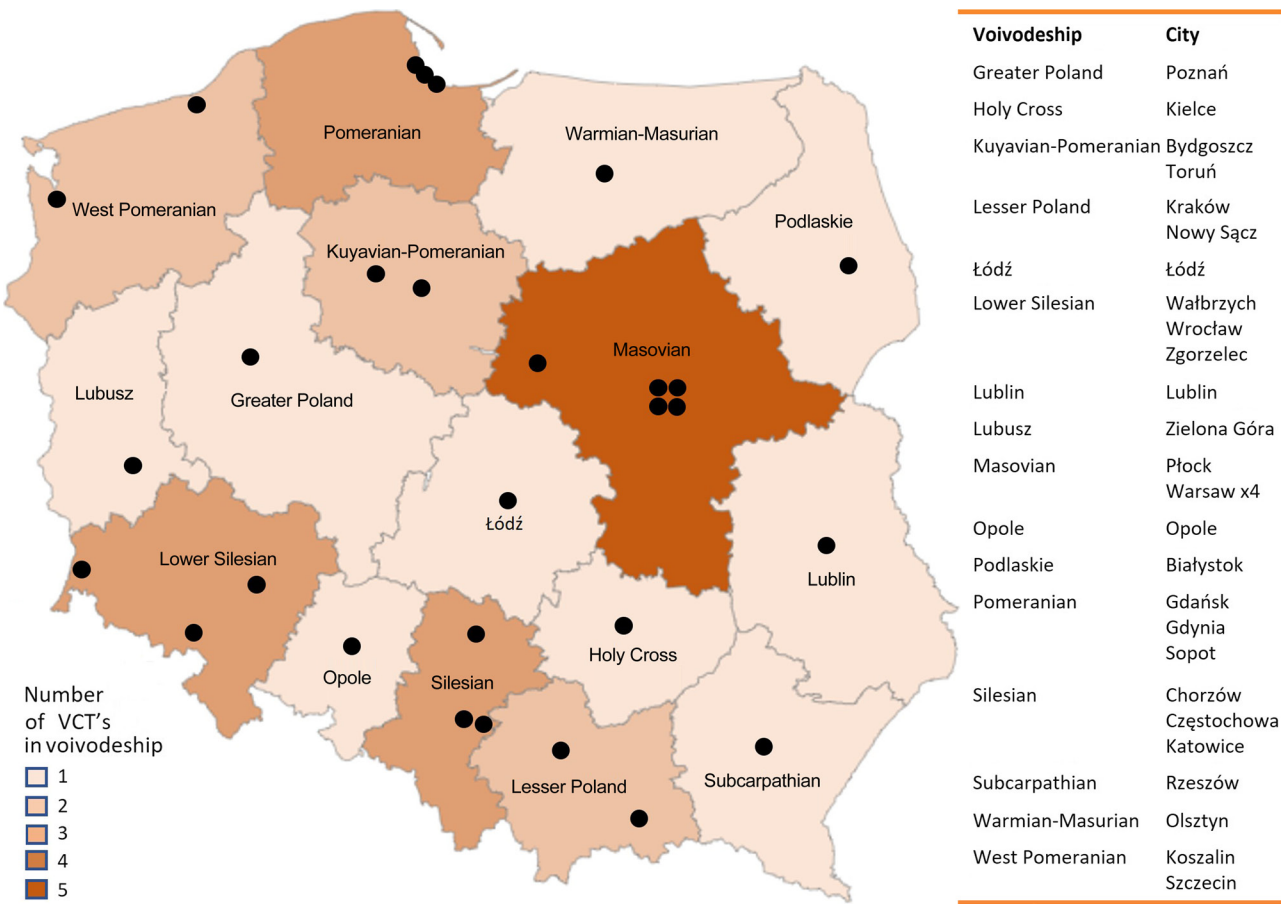
In Brazil, over the past few decades, there has been a significant decline in AIDS incidence due to the introduction of widespread and free access to antiretroviral therapy (ART), harm reduction policies aimed at reducing or eliminating the health effects of psychoactive substance use, prevention efforts and extensive diagnostic testing (Ribeiro *et al.* 2020).

The Polish Scientific Society of PTN AIDS recommends testing every person at risk of HIV infection using HIV 1/2 antigen/antibody combination immunoassays (Szetela *et al.* 2022; 2023; 2024). Such testing is possible in medical and non-medical settings, including Voluntary Counselling and Testing Points (VCTs).

2. Organization of Voluntary Counselling and Testing Points in Poland

Voluntary Counselling and Testing (VCT) points are coordinated by the National AIDS Centre (NAC), which has operated under the Ministry of Health since 1993, implementing the National Programme for HIV Prevention and AIDS Control following the Council of Ministers' regulation dated February 15, 2011 (PKD AIDS Misja 2022). The first VCT point was established in Szczecin in 1997, initiated by Anna Nowak, and has been operating continuously for over 20 years. Until 2011, this Point was funded by the Polish Society for Health Education O/T Szczecin through projects, and since 2012, by the "DA DU" Volunteer Association (Kłys-Rachwalska 2018).

The objective of VCT points is the early detection of HIV infections, limiting the spread of the virus, promoting HIV infection prevention and assisting people living with HIV and their close ones (Kłys-Rachwalska 2018). Over the years, a network of VCT points has been created nationwide. Currently, there are 29 points in 26 cities, located in all 16 voivodeships [Fig. 1]. In the Masovian Voivodeship, where the highest number of HIV tests are conducted, there are as many as five points, four located in Warsaw and one in Płock. In the Lower Silesian (Wrocław, Wałbrzych, Zgorzelec), Silesian (Chorzów, Częstochowa, Katowice), and Pomeranian (Gdańsk, Gdynia, Sopot) voivodeships, there are currently three active points each. Two VCT points operate in the Lesser Poland (Kraków, Nowy Sącz), Kuyavian-Pomeranian (Bydgoszcz, Toruń), and West Pomeranian (Koszalin, Szczecin) voivodeships. In other voivodeships, there is one VCT point located in the capital cities of these voivodeships: in Greater



Voivodeship	City
Greater Poland	Poznań
Holy Cross	Kielce
Kuyavian-Pomeranian	Bydgoszcz Toruń
Lesser Poland	Kraków Nowy Sącz
Łódź	Łódź
Lower Silesian	Wałbrzych Wrocław Zgorzelec
Lublin	Lublin
Lubusz	Zielona Góra
Masovian	Płock Warsaw x4
Opole	Opole
Podlaskie	Białystok
Pomeranian	Gdańsk Gdynia Sopot
Silesian	Chorzów Częstochowa Katowice
Subcarpathian	Rzeszów
Warmian-Masurian	Olsztyn
West Pomeranian	Koszalin Szczecin

Fig 1. Map of the distribution of 29 Voluntary Counselling and Testing Points in Poland as of May 2024. Own elaboration.

Poland in Poznań, in Świętokrzyskie in Kielce, in Łódź Voivodeship in Łódź, in Lublin Voivodeship in Lublin, in Lubusz Voivodeship in Zielona Góra, in Opole Voivodeship in Opole, in Podlaskie Voivodeship in Białystok, in Subcarpathian Voivodeship in Rzeszów, and Warmian-Masurian Voivodeship in Olsztyn. All VCT points in Poland are funded or co-funded by the National AIDS Centre from municipal budgets and private funds. Annually, reports from each Point's activities are sent to the National AIDS Centre, which also oversees the activities of these units. Some points may be closed for financial reasons or difficulties in finding adequately trained personnel, as was the case with the VCT in Jelenia Góra at the end of 2023. New VCT points are also being opened, such as in Katowice (May 2024) (KC AIDS PKD 2024).

VCTs provide the possibility for every adult individual visiting the facility to perform a quick, anonymous, and free HIV test. According to the applicable law in Poland, the Act of December 5, 1996, on the professions of physician and dentist (Journal of Laws 2019.537 consolidated text, article 32, paragraph 1, and the Code of Medical Ethics, article 15, paragraph 1), and the Patient Rights and the Commissioner for Patients' Rights Act (Journal of Laws 2017.1318 consolidated text with later amendments), article 17, paragraphs 1 and 2, informed consent from the patient is necessary to conduct an HIV test. For minor patients under 16, permission must be given by a parent/legal guardian or a court. For patients aged 16–18, dual consent is required from both the patient and the parent/legal guardian (HIV/AIDS 2024; Dz. U. z 2023 r. poz. 1516; Dz. U. z 2024 poz. 581).

Among the tasks performed by VCTs are educational and advisory activities. Educational activities are conducted directly for persons reporting to VCTs and in the form of promotional campaigns, such as World AIDS Day, celebrated on December 2 each year, or the World Testing Week, organized twice a year – in spring and autumn. These campaigns aim to increase public awareness about HIV, improve access to rapid testing, and support people living with HIV (Treston 2023, European Testing week 2024). Educational activities for individuals reporting to VCTs are conducted as pre-test and post-test counselling by qualified counsellors.

A person can become a counsellor if they have higher medical, psychological, pedagogical, or related education and hold a valid counsellor certificate authorizing them to provide counselling issued by the National AIDS Centre. The role of a VCTs counsellor is to educate clients about the modes of HIV transmission and methods of prevention. Additionally, the counsellor informs about the risk of contracting other sexually transmitted diseases (STDs) (Standardy PKD 2024).

From the individual reporting to the VCTs, after assigning an identifier number and a password invented by them, a detailed interview is collected to assess the risk of infection, enabling their characterization in terms of age group, gender, area of residence, and sexual preferences. Data are collected through electronic questionnaires conducted during the consultation visit. The questionnaire includes a series of questions to decide on the need for testing, including the number of HIV tests performed in the past and their results, the reason for reporting for testing, the number of sexual partners during life, the presence of a steady partner and whether they have been tested, whether there were unprotected sexual contacts (oral active/passive, vaginal, anal active/passive) and the time elapsed since such risky contact, the individual's sexual orientation, whether they used pre-exposure prophylaxis (PrEP), psychoactive substances (which/when), occupational/non-occupational blood contact, information on sexually transmitted diseases (STDs) contracted in the last year (VCTs electronic questionnaire 2024). Each patient undergoing the test gives informed consent for the test and voluntarily participates in the questionnaire. If the person finds the questions too intimate and personal, they can refuse to answer. After qualifying the patient for the test, they are directed to the screening test.

3. Diagnosing HIV infection in Voluntary Counselling and Testing Points in Poland

VCT points operate according to the national guidelines for functioning and testing defined by the National AIDS Centre. In most VCTs, it is possible to perform both third and fourth-generation HIV tests. The choice of the test is made by the VCTs counsellor based on the data collected in the interview. The third-generation cassette test allows for quick, up to several minutes, detection of anti-HIV-1/2 antibodies. The limitation of this test is a diagnostic window of 12 weeks from contact (Szetela *et al*. 2024). According to the recommendations for 2023 and 2024 of the Polish Scientific Society for AIDS (PSS AIDS) (Szetela 2023; 2024), the use of fourth-generation serological tests detecting p24 antigen (possible detection as early as two weeks after infection) and specific antibodies for HIV-1 (group M and O) and HIV-2 envelope antigens is recommended (the diagnostic window is six weeks) (Fig. 2). Obtaining a negative result with a fourth-generation test performed six weeks after exposure concludes the diagnosis of HIV infection. Confirmation of HIV infection is based on obtaining a reactive result with the fourth-generation test, verified with a confirmation test using molecular methods (NAAT, Nucleic Acid Amplification Test) to eliminate potentially possible cross-reactive results. The recommended diagnostic scheme aims to identify individuals infected with HIV

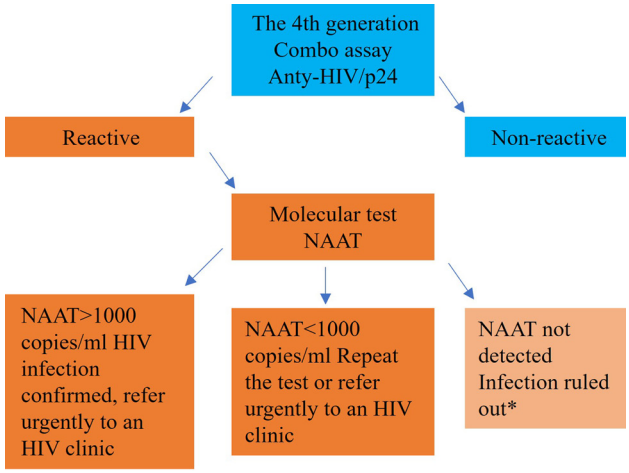


Fig. 2. Schematic of screening laboratory diagnostics, developed based on the recommendations of PSS AIDS 2024 (Szetela *et al.* 2024).

while simultaneously removing the risk of obtaining false-positive results. Factors that may cause a falsely reactive screening test result include pregnancy, vaccinations in the past 4–6 weeks, autoimmune diseases, immunosuppressive, oncological, and antiviral treatment. A particular group includes patients receiving antiretroviral therapy (ART) as well as those covered by pre-exposure prophylaxis (PrEP) and post-exposure prophylaxis (PEP) for HIV infection (Szetela *et al.* 2022; 2023; 2024; Standardy PKD 2024).

It is worth mentioning that in June 2023, changes were made in the recommendations for confirming HIV infection in Poland. Molecular tests replaced the Western Blot (WB) test as the method of choice for infection verification. In VCTs, adjusting to the new guidelines lasted until the end of 2023 (Szetela *et al.* 2022; 2023; 2024).

The National AIDS Centre's recommendations mandate that all tests be conducted from a single blood sample because the individual's identity is anonymous, making it impossible to verify their identity and recall them for a confirmation test sample collection (Standardy PKD 2024).

A person who has received a reactive result in an HIV screening test and a positive result in a confirmation test has the option to voluntarily decode such a test result and obtain a personal certificate confirming HIV infection. This procedure facilitates more straightforward and quicker access to Acquired Immunodeficiency Syndrome Clinics and allows for the possibility of receiving ARV treatment in the shortest possible time (Standardy PKD 2024). Moreover, each patient who gets a positive result is informed about the criminal liability according to Article 161 of the Penal Code: "*Whoever, knowing that they are infected with HIV or suffering from a venereal, infectious, severe incurable, or life-threatening illness, exposes another person to direct risk of infection with that virus or disease, shall be subject to imprisonment from 3 months to 5 years*" (Dz.U. 2020.1444 t.j. ze zm.; KC AIDS 2021).

In most VCT points in Poland, a rapid immunochromatographic test for the presence of anti-HCV antibodies, confirming hepatitis C, and anti-syphilis antibodies, assessed in the diagnosis of syphilis, can also be performed. In the case of a reactive result, the patient is directed to a specialized facility to verify the tests.

4. Summary of Voluntary Counselling and Testing Points activities in Poland 2019–2023

VCT points allow testing for Polish citizens and foreigners, with counselling provided in English or other languages upon prior arrangement. In 2019–2023, 157,833 individuals visited VCTs in Poland, of which 10,177 (6.45%) were foreigners. During the 2019–2023 period, there was almost a twofold increase in the number of tests conducted among foreigners visiting VCTs in Poland, from 1,552 in 2019 to 2,827 in 2023 [Table II]. This might be attributed to the political situation in Eastern Europe, including the armed conflict in Ukraine and the influx of migrants through the Belarusian border. Among non-Polish nationals, a higher percentage of positive results was also noted. In total, in the years 2019–2023, the percentage of positive results in the Polish population was 1.24% (1,825/147,656), whereas among individuals of other nationalities, it was 5.08% (517/10,177) [Fig. 3]. The higher percentage of positive results among foreigners indicates the need to test this population, especially during periods of significant migration and unrestricted movement.

The frequency analysis of positive results detection reported in VCTs vs. NIPH NIH – NRI showed that on average 26% (2,342/8,891) of positive results in Poland from 2019–2023 were detected in the framework of tests carried out in VCTs: from 33% (537/1,615) in 2019, 37% (309/840) in 2020, 38% (448/1,173) in 2021, 22% (520/2,384) in 2022 to 18% (528/2879) in 2023 (NIZP – PZH 2023; Niedźwiecka *et al.* 2022; 2024).

4. Summary

In summary, the operation of Voluntary Counselling and Testing Points in Poland, offering free and anonymous testing for HIV infection, is a valuable complement to the diagnostics conducted within the healthcare

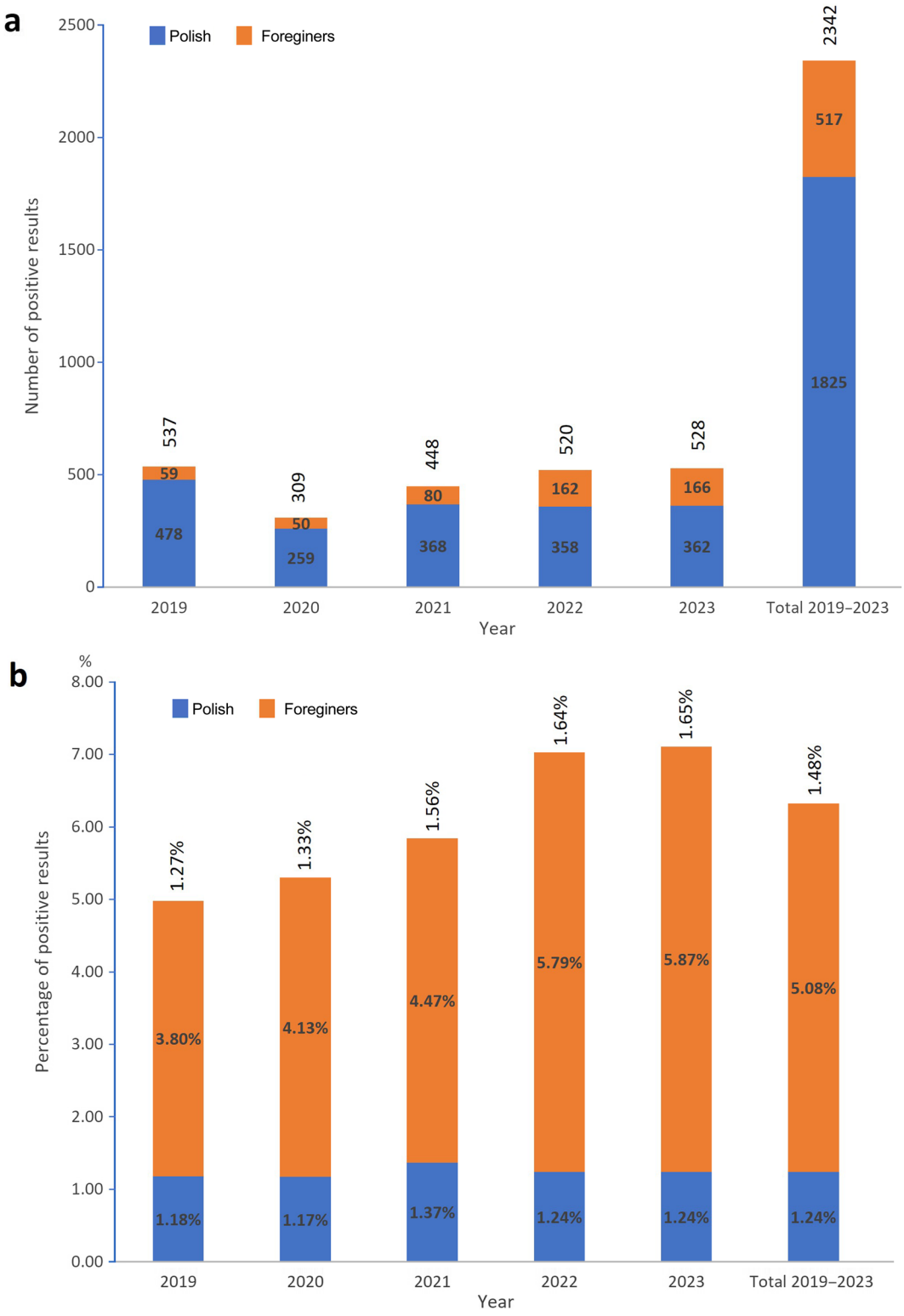


Fig. 3. Number and percentage of positive results obtained among Poles and foreigners tested for HIV infection in Voluntary Counselling and Testing Points in Poland in 2019–2023.

Source: own elaboration based on data from NAC electronic surveys (2019–2023).

system in Poland. It enables outreach to a group of people who, for various reasons, cannot or do not want to use the services offered by the state healthcare system. The operation of VCTs raises awareness of the issue. It increases accessibility to HIV diagnosis in key adult populations, such as men who have sex with men, sex workers, and people using psychoactive substances. Despite the popularisation of knowledge, the development of medicine, and diagnostic possibilities, the topic of HIV still evokes fear, concerns, and controversy.

Table II
Number of tests conducted and positive results obtained among individuals tested for HIV infection in Voluntary Counselling and Testing Points in Poland from 2019–2023, considering nationality.

Year	Nationality	Number of tests performed	Number of positive results	% of positive results
2019	Polish	40,602	478	1.18%
	Foreigners	1,552	59	3.80%
	Total	**42,154**	**537**	**1.27%**
2020	Polish	22,064	259	1.17%
	Foreigners	1,212	50	4.13%
	Total	**23,276**	**309**	**1.33%**
2021	Polish	26,856	368	1.37%
	Foreigners	1,790	80	4.47%
	Total	**28,646**	**448**	**1.56%**
2022	Polish	28,905	358	1.24%
	Foreigners	2,796	162	5.79%
	Total	**31,701**	**520**	**1.64%**
2023	Polish	29,229	362	1.24%
	Foreigners	2,827	166	5.87%
	Total	**32,056**	**528**	**1.65%**
2019-2023	Polish	147,656	1,825	1.24%
	Foreigners	10,177	517	5.08%
	Total	**157,833**	**2,342**	**1.48%**

Source: own elaboration based on data from electronic surveys by NAC (2019–2023).

However, it is essential to expand promotional activities and rapid HIV testing continuously. Therefore, there is consideration of the development and creation of additional VCT points in Poland to ensure the possibility of free and anonymous HIV testing for the largest possible group of recipients, considering groups of people engaging in risky sexual behaviour or intravenous drug users. In Poland, the low level of knowledge about HIV and STDs remains an issue, making the counselling provided within VCT points an important education component that allows for delivering basic knowledge about HIV and the latest forms of prevention.

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Ethic approval
Our report is a retrospective analysis using anonymous survey data collected during routine practice at each VCT. Ethic approval was obtained from the Jagiellonian University Collegium Medicum Ethic Commission (118.0043.1.158.2024).

Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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ZOONOTIC DISEASES IN NORTHEN CYPRUS: CURRENT AND FUTURE THREATS

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Abstract. Diseases transmitted naturally between animals and humans are referred to as zoonoses. Zoonotic diseases are responsible for many pathogenic infections in humans, especially in endemic regions. In recent years, emerging and re-emerging zoonotic infections have become widespread and pose a threat worldwide. Transmitted such bacterial, viral and parasitic infections have been detected in Northen Cyprus over the years. Climate change and human migration are increasing essentially, making such infections potentially more dangerous. To quickly detect these pathogens and limit their spread, it is crucial for the island to control their animal reservoirs. In addition, continued and expanded research is essential for global surveillance.
This review aimed to provide an overview of the etiology of the most recorded zoonotic diseases in Northen Cyprus, their impact on human health, and measures to control their spread better.

Keywords: bacterial zoonosis, viral zoonosis, parasitic zoonosis, northern Cyprus, control of diseases/outbreaks

1. Introduction

The word zoonosis was first used by Rudolph Virchow (1821–1902) in his "Handbook of Communicable Diseases" in 1855 (Singh *et al.* 2023). Zoonoses have been significant among infectious diseases since ancient times and have caused various public health problems. "Zoonotic diseases" or "zoonoses" are terms commonly used to refer to infectious diseases transmitted from animals to humans. Many microorganisms such as bacteria, viruses, parasites, fungi and prions, whose original hosts were animals, are responsible for human zoonoses (https://www.emro.who.int/about-who/rc61/zoonotic-diseases.html). It is estimated that there are one billion cases of disease and millions of deaths caused by zoonoses worldwide each year. Zoonoses account for about 60% of emerging infectious diseases. In the last three decades, 30 new human pathogens have been discovered, 75% of which were transmitted from animals (Jones *et al.* 2008). According to the World Health Organization, the threat of zoonoses is increasing in the eastern Mediterranean region due to increased global interactions and international trade (Malik *et al.* 2013). Some endemic zoonoses, including brucellosis, anthrax and rabies, have not been eliminated. Increasing problems result from infections with viruses causing yellow fever, chikungunya, West Nile fever, Q fever, Cream-Congo hemorrhagic fever, Ebola hemorrhagic fever, Rift Valley fever, highly pathogenic H1N1 influenza virus, monkeypox, sand fever, MERS-CoV. Infections occur in various countries, including Sudan, Yemen, Tunisia, Afghanistan, Iraq, Pakistan, sub-Saharan Africa, Saudi Arabia, Egypt, Iran, Jordan, the Kingdom of Saudi Arabia, Kuwait, Lebanon, Oman, Qatar and the United Arab Emirates. They pose a threat there due to their epidemiological potential, high mortality risk and lack of treatment and vaccines to control their spread (https://www.emro.who.int/about-who/rc61/zoonotic-diseases.html)

According to the 2022 report of the European Centre for Disease Prevention and Control on zoonoses, many pathogens including *Yersinia* spp., *Toxoplasma gondii*,

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rabies, *Coxiella burnetii* (Q fever), West Nile virus, tularemia, *Bacillus* spp., *Chlamydia* spp., *Clostridium* spp., *Cronobacter* spp., *Klebsiella* spp., *Enterococcus* spp., pathogenic *Escherichia coli*, *Proteus* spp., *Staphylococcus* spp., *Streptococcus* spp., *Vibrio* spp., *Leptospira* spp. Caliciviruses, Flaviviruses, Hepatitis virus, *Cysticercus* spp. and Sarcocystis spp. were monitored according to the epidemiological situation in the European Union countries in 2022 (EFSA and ECDC 2023). While the most commonly reported infections are campylobacteriosis, salmonellosis, yersiniosis, infections of Shiga toxin-producing *Escherichia coli* (STEC) and *Listeria monocytogenes*, the highest hospitalizations and mortality rates were due to listeriosis and West Nile virus infections (EFSA and ECDC 2023). Cyprus is located in the eastern part of the Mediterranean Sea and is part of Western Asia. The island is divided into two parts: northern and southern. Turkish Cypriots live in the northern part, while Greek Cypriots live in the southern part of the island. Legally, citizens of both countries can travel across the border after passing security checks. Therefore, there is interaction between citizens living in Cyprus and global connectivity with other countries. There is a problem of cross-border disease outbreaks, including those of animal origin. In Northern Cyprus, the country's economy is based on agriculture and small-scale farming, which also increases the risk of human infections spread from animals. This review presents zoonotic infections detected in Northern e that are dangerous to the island citizens in three sections: bacterial, viral, and parasitic.

2. Bacterial zoonoses

The brucellosis affects livestock and humans worldwide and causes economic losses. The genus *Brucella* includes three highly virulent species that have become endemic in many countries, especially those with low economic levels. These species are *Brucella abortus*, which infects mainly cattle; *Brucella melitensis,* which infects sheep and goats; and *Brucella suis*, which infects mainly pigs (Laine *et al*. 2023). Infection of humans by *Brucella canis* can occur through contact with infected dogs' contaminated secretions or improper laboratory handling (Krueger *et al*. 2014). It is estimated that in 2021, 29 EU/EEA countries reported 0.04 cases per 100,000 people. According to the annual epidemiological report (ECDC 2023a), no cases of brucellosis were reported in 2017, 2018, 2019, 2020 and 2021.

Due to various eradication programs in Cyprus, animal brucellosis has remained at the level of 0.1% since 2007 (Sayı 2013). A study by Süer showed that the seroprevalence was 3.6% with RSAT *B. canis* M (–), 4.4% with RSAT *B. abortus* S99, 5.3% with ELISA *B. canis* M (–) and 9.8% with ELISA *B. abortus* S99 in healthy individuals without brucellosis. These studies are insufficient to present the current situation of *B. canis* infections in Cyprus, but the seropositivity rates can help assess the risk (Süer *et al*. 2023).

Despite the endemic nature of brucellosis in livestock in Northern Cyprus, there is a lack of published scientific data on the disease. Özdoğaç et al. (2018) published the only available information on brucellosis. He found that seropositivity in humans ranged from 3.1% to 6.5% among professionals such as veterinarians, animal breeders and butchers. Regardless of the causative agent, these numbers are consistent with the low to moderate seroprevalence data in livestock reported earlier (Özdoğaç *et al*. 2018).

Francisella tularensis, which causes tularemia, is spread through direct animal-human contact and via vectors, food, water or infected aerosols (Kosker *et al*. 2013). The incubation phase usually lasts three to five days, although it can extend up to 21 days (Gurcan *et al*. 2014). The first case of tularemia in Northen Cyprus was reported in a 5-year-old girl by Uncu M. et al. in 2017. Symptoms included fever, pharyngitis, bilateral periorbital swelling and congestion, cervical and mesenteric lymphadenopathy (LAP), liver and spleen enlargement, and diarrhea. In this case, it was reported that the patient recovered without complications within two weeks of antibiotic treatment (Uncu *et al.* 2017).

Rickettsiae are Gram-negative, obligate intracellular bacteria that include several zoonotic pathogens that are widespread worldwide. The host of *Rickettsiae* typhi infection is rats (*Rattus rattus* and *R. norvegicus*), and the vector is the oriental rat flea (*Xenops cheopis*) (Güvenir *et al.* 2022). According to the study, three species of *Rickettsia* have been found in Southern Cyprus: *Rickettsia conorii*, *R. typhi* and *R. felis* (Psaroulaki *et al*. 2006; Koliou *et al.* 2007). The results of this study show that the geographical distribution of fleas coexists with the geographical distribution of the pathogens they may carry, indicating a potential risk of flea-borne infections in Southern Cyprus (Christou *et al*. 2010). Güvenir et al. reported that although there is not enough information about rickettsiae infections in Northen Cyprus, they are aware of the increase in rickettsiae infections during the SARS-CoV-2 pandemic (Güvenir *et al*. 2022).

Listeriosis is a disease caused by *Listeria monocytogenes*. It mainly causes infections in pregnant women, infants and adults with weakened immune systems. Healthy adults do not develop any symptoms except pregnant women. According to the Annual Epidemiological Report for 2021 distribution of confirmed listeriosis, there were no cases in Cyprus in 2017, one case in 2018, one case in 2019, two cases in 2020 and one case in 2021 (ECDC 2022).

The Shiga toxin-producing strains of *Escherichia coli* can produce toxins that are virtually analogous to those produced by *Shigella dysenteriae* type 1. Two types of these toxins have been described: Shiga 1 (Stx1), which differs from the true Shiga toxin by one to seven amino acids, and Shiga toxin 2 (Stx2), which has about 60% homologies to Stx1. Both Stx1 and Stx2 toxins belong to the Shiga toxin family. Shiga toxin-producing *Escherichia coli* are called STEC. Functionally active Shiga toxins can be detected by the Vero cell toxin test. Therefore, these bacteria are also called verotoxin-producing *Escherichia coli* (VTEC). According to the ECDC/EFSA joint technical report, only two cases were reported in 2008 (ECDC 2011).

3. Viral zoonotic infections

A significant percentage of disease-causing pathogens (70%) known to cause human infections are viruses. Viral zoonoses are a severe problem due to high mortality and morbidity (Marie *et al*. 2023). These agents are transmitted mainly from vertebrate animals, including domestic and wild animals, by insects and arthropods. They can be transmitted by direct contact, aerosols, congenital routes and, in some cases, from person to person (Glud *et al*. 2021; Socha *et al*. 2022). Environmental changes resulting from, among others, the combustion of fossil fuels, increased deforestation and livestock farming may cause the spread of vectors from different geographical regions and contribute to the increase in the number of viral zoonotic infections (Arikan *et al.* 2023). Although there are no scientific publications on the presence of vectors in the island's northern part, various studies on mosquitoes have been conducted in the southern part since 1946. According to these studies, *Aedes* mosquitoes, including *Aedes aegypti*, *Aedes detritus*, *Aedes m*ariae, and *Aedes caspius*, have been detected in the Republic of Cyprus (Violaris *et al.* 2009; Abushoufa *et al*. 2021). Therefore, Northern Cyprus may also be exposed to infections with dengue, yellow fever, chikungunya, and Zika viruses, which are transmitted by *Aedes* mosquitoes. Considering that people from many countries and regions migrate to the island for various purposes, such as education, tourism, and work, the need to consider these diseases is revealed. Moreover, due to the arrival of many foreigners from endemic countries, other significant vector-borne infections, including Ebola virus, West Nile virus, Rift Valley fever, and Cream-Congo hemorrhagic fever, should also be considered in Northern Cyprus. Therefore, this part of the review will discuss some of the main critical viral zoonoses that may pose a high risk to Northen Cyprus.

Currently, it is estimated that half of the world's population is exposed to dengue fever, and about 100–400 million dengue infections occur each year, as it is one of the fastest-spreading arboviral diseases (Sah *et al.* 2023). The disease is transmitted by Aegypti mosquitoes and can cause clinical conditions ranging from asymptomatic/mild infections to life-threatening severe conditions such as dengue hemorrhagic fever and dengue shock syndrome (Sah *et al.* 2023). Dengue virus (DENV) infections are endemic in tropical and subtropical regions worldwide. Still, infections have been reported in many countries, including the Americas, Africa, the Middle East, Asia, and the Pacific Islands (CDC 2023). The European Centre for Disease Prevention and Control (ECDC) reported more than 2 million cases of dengue and more than 500 deaths worldwide between December 2023 and February 2024. This represents a 249% increase compared to the same period in 2023 and a 354% increase compared to the average of the last five years (ECDC 2024). In Southern Cyprus, only two sporadic cases of dengue fever have been reported so far, while in the northern part of the island, no cases have been reported.

Chikungunya is another disease transmitted by vectors, mainly through the bites of mosquitoes, mainly *Aedes aegypti* and *Aedes albopictus*. The disease occurs primarily in Africa and Asia, but cases imported from abroad have also been reported. Since 2005, more than two million cases have been reported in more than 110 countries in Asia, Africa, Europe and America (WHO 2022). Only by February 2024, 70,000 cases and 15 deaths have been reported worldwide. These cases have been detected most frequently in Brazil, Paraguay, Argentina and Bolivia. Although Cyprus has not reported any data for 2017–2021 (ECDC, 2024a), chikungunya fever may pose a significant future threat to Northern Cyprus.

Another emerging infectious disease transmitted mainly by *Aedes* mosquitoes is Zika virus (ZIKV). Since the first human outbreak on Yap Island in 2007, Zika virus outbreaks have occurred in French Polynesia, Easter Island, Cook Islands, New Caledonia, and Brazil from 2013 to 2015 (ECDC 2021). As a link between ZIKV infections and microcephaly and other neurological disorders was demonstrated, WHO declared a public health emergency of international concern in 2016 (WHO, 2016). Mosquito-borne Zika virus infections have been reported in 86 countries to date, but global surveillance is insufficient. As a result of the projection studies, the potential spread of *Aedes* mosquitoes and the risk of ZIKV infection in South Africa, Africa, Oceania, Asia and Northen America was estimated at 16.6% of the land area or 78.6% of the world's population. The study predicts that people living in the risk area, especially in South Asia, tropical Africa, Southern America, North America and the Mediterranean, are at risk. Still, climate change may change these projections

(Xu *et al*. 2022). Another modelling study predicted that global warming could expose more than 1.3 billion new people to ZIKV by 2050 (Ryan *et al*. 2021). In Northern Cyprus, the first and only study presented by Fathi et al. showed no Zika virus infections (Abushoufa *et al*. 2021). However, the increased risk of mosquito spread indicates that ZIKV infections will also occur on the island, and necessary precautions should be taken in advance. Although everyone is at risk for this infection, people with weakened immune systems, people who have close contact with mosquito or tick habitats, and healthcare workers who have direct contact with infected individuals should be trained and educated on the measurements (CDC 2024).

Although none of the above vector-borne diseases have been reported in Cyprus, West Nile virus (WNV) has spread here. The first WNV infection in humans in the Republic of Cyprus was detected in 2016, while the first three cases in Northern Cyprus were described in 2019 (Paphitou *et al*. 2017; Balaman *et al*. 2020). As of December 13, 2023, 707 human cases of WNV have been reported. These infections occurred in Italy (n = 336), Greece (n = 162), Romania (n = 103), France (n = 43), Hungary (n = 29), Spain (n = 17), Germany (n = 6), Croatia (n = 6) and Cyprus (n = 5) (ECDC 2023). The detection of a new case in the capital of Nicosia in Northern Cyprus in October 2023 and the presence of *Culex pipiens*, *Culex perexiguus* and *Culex torrentium* (Orshan *et al*. 2008; Benbetka *et al*. 2018; Vilibic-Cavlek *et al*. 2019) demonstrate the need for vector management and continuous monitoring of all arboviruses in Cyprus (Ministry of Health TRNC 2023; Yetismis *et al*. 2022).

4. Parasitic zoonoses

It was estimated that in 2022, 249 million cases of malaria worldwide caused 608,000 deaths. An infected female *Anopheles* mosquito transmits malaria to humans by biting. Malaria can also be transmitted through blood transfusions and contaminated needles. A person can become seriously ill and die within 24 hours of contracting *Plasmodium falciparum* malaria if left untreated. Five species of *Plasmodium* cause human malaria. Two of them – *P. falciparum* and *P. vivax* are the most dangerous. *P. falciparum* is prevalent on the African continent. Outside of sub-Saharan Africa, *P. vivax* infections predominate. Three other malaria species can also infect humans: *P. malariae*, *P. ovale* and *P. knowlesi* (WHO 2023a). The island of Cyprus was one of the most malaria-affected areas for centuries. However, between 1946 and 1950, the island underwent a "Malaria Eradication Project" that successfully eradicated malaria by eliminating *Anopheles* mosquitoes, which are carriers of *Plasmodium* parasites, and by draining marshlands. There are currently no local cases of malaria in Northern Cyprus. However, imported cases have begun to be observed. This is due to the increased number of people coming to the island to study or to work from regions where the disease is endemic. These patients already bring antimalarial drugs from home (Güler *et al*., 2023). According to the data of the Ministry of Health of the TRNC, 56 cases of imported malaria were reported in Northern Cyprus between 2014 and 2022 (Ministry of Health of the TRNC, 2023). Güler *et al*. link this to the fact that since 2011, the number of students in higher education institutions in Northern Cyprus has increased significantly. Due to these population changes in Northern Cyprus, the Ministry of Health has modified malaria control strategies, increasing the supply of antimalarial drugs (Güler *et al*., 2023).

Leishmaniasis is another significant vector-borne disease affecting about one million people annually (WHO 2023). Female flies of the *Phlebotomidae* family transmit the disease. It affects people living in low-income countries worldwide, mainly in Africa, Asia and Latin America, and is associated with malnutrition, population displacement, poor housing conditions, and weak immune systems. In 2022, more than 5,000 cases of cutaneous leishmaniasis were reported in eight countries, including Afghanistan, Algeria, Brazil, Colombia, Iran (Islamic Republic), Iraq, Peru and the Syrian Arab Republic. Of these cases, 337 were imported. Globally, 69 imported cases of visceral leishmaniasis were also reported in 2022 (WHO 2023). In Cyprus, cases in humans and dogs have been reported since 1990. They are becoming a severe threat as sand flies and dogs have also been reported as vectors and main reservoirs (Demir *et al*. 2010; Ergunay et al. 2014, Ruh *et al*. 2019). Emrah et al. revealed that the rate of positive canine leishmaniasis (canL) and borderline positive results were 3.61% and 15.66%, respectively, in 2004 in Northern Cyprus. In 2012, Tözel et al. reported three more cases of canL in the northern part of the island (Töz *et al*. 2013). In 2016, two different studies were conducted, and the seropositivity of canL was 1.9% and 3.55%, respectively (Beyhan *et al*. 2016). Cases of CanL were also detected in the southern part of Cyprus. The overall seropositivity among dogs in the south part of the island was 1.7% in 1996 (Deplazes *et al*. 1998). However, this rate increased compared to 10 years ago and was 14.9% in the following years (Mazeris *et al*. 2010). Cases of human infection with *Leishmania donovani* and *L. infantum* have been reported in both parts of Cyprus. The first cases in humans in the northern part of Cyprus were detected in 1935. In 1990, 10% and 35% of people tested positive for the parasite in the Kyrenia and Lapithos provinces (Deplazes *et al*.

1998). In the following years, in 2016, three cases of visceral leishmaniasis in children were reported (Sayılı *et al.* 2016). Emrah et al. (Ruh *et al.* 2017) also published that the seropositivity rate for leishmaniasis was 1.2% in 2017. Three human cases were reported in Southern Cyprus in 2006 (Antoniou *et al.* 2008). Furthermore, CL was reported in a family of four in 2014 (Koliou *et al.* 2014). Over the years, zoonotic bacterial, viral and parasitic infections have been detected in Northern Cyprus. Factors such as vectorial mobility due to the effects of global warming and human migrations indicate that such infections may become increasingly dangerous. Vector and reservoir management should be implemented on the island to detect possible agents and limit their spread rapidly. Additionally, continuous and extended studies should be conducted for global surveillance. It may also benefit countries by creating infrastructure for diagnosis and treatment, effective control programs for such infections, and community education programs in case of natural disasters such as earthquakes, floods, etc.

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KLEBSIELLA PNEUMONIAE – TAXONOMY, OCCURRENCE, IDENTIFICATION, VIRULENCE FACTORS AND PATHOGENICITY

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Abstract. Gram-negative bacilli *Klebsiella pneumoniae* are among the most important pathogens responsible for healthcare-associated infections (HAIs). These bacteria often have high pathogenic and epidemic potential, contributing to infection outbreaks worldwide. *K. pneumoniae* is part of the natural microbiota of humans. At the same time, as an opportunistic microorganism, when the host organism is weakened, it can cause serious infections such as pneumonia, urinary tract infections, septic infections and intra-organ abscesses. Widespread distribution in nature and exceptional adaptability provide *K. pneumoniae* with the opportunity to master new niches in the hospital environment, which poses a threat to hospitalized patients. Also, the bacteria are increasingly causing life-threatening infections in the non-hospital environment. The pathogenicity of *K. pneumoniae* is determined by the presence of many virulence factors such as capsular polysaccharide (CPS, K antigen), lipopolysaccharid (LPS, O antigen), fimbrial and non-fimbrial adhesins, siderophores (aerobactin, enterobactin, salmochelin and yersiniabactin), heat-stabile and heat-labile enterotoxins, cytotoxins and biofilm-forming ability. Currently, hypervirulent strains of *K. pneumoniae* (hvKp) equipped with new virulence traits constitute a significant danger. The paper presents these bacteria concerning the global threat arising from the dynamic spread of hvKp strains in hospitals in Poland and worldwide.

Keywords: identification, *Klebsiella pneumoniae*, occurrence, pathogenicity, virulence factors

1. Introduction

The Gram-negative bacilli *Klebsiella pneumoniae* are an opportunistic pathogen with high pathogenic and epidemic potential, contributing to infection outbreaks worldwide. *K. pneumoniae* are the etiological factors of respiratory tract infections, mainly pneumonia, meningitis, septic infections and difficult-to-treat urinary tract infections. Increasing drug resistance, high mortality among patients infected with this pathogen and difficulties in treating the infection resulted in the World Health Organization (WHO) including these bacteria on the list of one of the most dangerous pathogens in the world (WHO 2024).

1.1. General characteristics of *Klebsiella* genus

1.1.1. Nomenclature and taxonomy

The generic name *Klebsiella* comes from the surname of the German microbiologist Edwin Klebs (1834–1913). Bacteria was first isolated in 1882 by Carl Friedländer from a patient who died of pneumonia (Grimont and Grimont 2015). The first species belonging to the genus *Klebsiella* described by Karl von Frisch was the bacterium *Klebsiella rhinoscleromatis* isolated from a patient with scleroma (Grimont and Grimont 2015).

In routine microbiological practice, exploitation of the 16S rRNA gene as a molecular marker led to the correction of previous findings regarding the taxonomy of bacilli belonging to the genus *Klebsiella* (Ma *et al.* 2021). This gene contains conserved regions (regions common to many bacteria) and species-specific regions, which allows precise identification of the genus or species of an isolated bacterial strain based on comparing determined sequences with sequences available in public databases (Srinivasan *et al.* 2015). According to the current state of scientific knowledge acquired in the course of many molecular studies, it has been shown that other genes with evolutionarily conserved sequences, for example, selected housekeeping genes, including the *rpo*B gene encoding the β subunit of RNA

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polymerase, are also characterized by a high potential differentiating (He *et al.* 2016). Currently, the identification of various groups of bacteria is carried out based on phenotypic and genotypic features based on rRNA coding sequences or sequenced genomes, additionally supplemented with information obtained from the amino acid structure of proteins performing critical functions in cells (Kim *et al.* 2021). On this basis, changes in the taxonomy of gamma-proteobacteria were proposed in 2016, the name of the *Enterobacteriales* order was modified, and a new division of families separated from the *Enterobacteriaceae* family, which included genus *Klebsiella* was introduced within the *Enterobacterales* order novum (Adeolu *et al.* 2016). Based on the findings, the monotypic order *Enterobacteriales*, containing one family of *Enterobacteriaceae*, was transformed into the polytypic order *Enterobacterales* order novum, consisting of seven new families, including *Enterobacteriaceae*, *Erwiniaceae*, *Pectobacteriaceae*, *Yersiniaceae*, *Hafniaceae*, *Morganellaceae* and *Budviciaceae* (Adeolu *et al.* 2016). It was also agreed that bacteria previously classified to the *Enterobacteriaceae* family, including species of the *Klebsiella* genus, will now be classified as a taxon in the order *Enterobacterales* (Fig. 1) (Adeolu *et al.* 2016; Schoch and Karsch-Mizrachi 2020).

In the course of the conducted phylogenetic studies, considering the latest divisions of microorganisms within the *Klebsiella* genus, a new species, *Klebsiella aerogenes,* appeared, referred to as a nomenclature (homotypic) synonym as *Klebsiella mobilis* (Szewczyk 2019). These bacteria were first described in 1885 by Theodor Escherich as "*Bacterium lactis aerogenes*", then renamed "*Bacillus aerogenes*" in 1896 by Walther Kruse, then "*Aerobacter aerogenes*" and finally named in 1960 by Estenio Hormaeche and Peter Geoffrey Edwards as *Enterobacter aerogenes* (Tindall *et al.* 2017). *Klebsiella mobilis* is an opportunistic pathogen responsible for nosocomial infections (Szewczyk 2019).

The genus *Klebsiella* also contains species previously counted in other taxonomic groups, including *Klebsiella oxytoca* and *Klebsiella ozaenae* (Tachibana *et al.* 2022; Yang *et al.* 2022). The first was isolated from sour milk and first described in 1886 by Carl Flügge as "*Bacillus oxytocus perniciosus*", then renamed in 1923 by David Hendricks Bergey as "*Aerobacter oxytocum*" and finally named *K. oxytoca* by Hans Lautrop in 1956 (Yang *et al.* 2022). The species name *K. oxytoca* comes from the Greek language and consists of the two elements "*oxus*", meaning "*sour*", and "*tokos*", meaning "*production*" (Yang *et al.* 2022). The other species included in the genus *Klebsiella* was *K. ozaenae*. These bacteria were observed in 1893 by Rudolf Abel in the nasal discharge of patients with ozena, or chronic atrophic malodorous rhinitis. Initially, these capsulated bacteria were known as "*Bacillus mucosus ozaenae*" and finally changed its name to *K. ozenae* (Tachibana *et al.* 2022). Another species included in the *Klebsiella* genus was *Klebsiella granulomatis* with the former name "*Donovania granulomatis*", otherwise called "*Calymmatobacterium granulomatis*" – the etiological agent of inguinal granuloma (donovanosis), i.e. an infectious granulomatous disease affecting the genitals and groin (Belda Junior 2020). The genus *Klebsiella* has also been enriched with a new species, *Klebsiella variicola*, isolated mainly from elements of edible plants such as roots, leaves and banana stem (Latin *Musa* spp.), corn shoots (Latin *Zea mays L.*), rice roots (Latin *Oryza sativa L.*) (Ma *et al.* 2021). In 2001, three other species of *Klebsiella*, namely *Klebsiella ornitynolytica*, *Klebsiella planticola*, and *Klebsiella terrigena* isolated from the environment previously classified as "*Klebsiella-like organisms*" were transferred to the newly created genus *Raoultella* (Kimura *et al.* 2014; Ma *et al.* 2021).

There are several taxonomic classification systems of rods belonging to the genus *Klebsiella* in use in the world, including the Cowan classification system introduced in 1960, the Bascomb classification system introduced in 1971, and the Ørskov classification system introduced in 1984 (Grimont and Grimont 2015). Most scientific teams rely on the classification developed by Ørskov (Grimont and Grimont 2015). Currently, the genus *Klebsiella* includes 22 species (Table I).

Domein: *Bacteria*
Type: *Proteobacteria*
Class: *Gammaproteobacteria*
Order: *Enterobacterales*
Family: *Enterobacteriaceae*
Genus: *Klebsiella*
Species: *Klebsiella pneumoniae*

Fig. 1. The current taxonomic position of the species *K. pneumoniae*.
Own graphic design according to (Adeolu *et al.* 2016; Schoch and Karsch-Mizrachi 2020; Dong *et al.* 2022).

Table I.
Clinical significance of selected *Klebsiella* species presented in alphabetical order

Species	Special features	References
1. *K. aerogenes*	The opportunistic pathogen, an etiological agent of nosocomial infections, present in various sewage wastes, chemicals and soil. Commercially important bacterium, „preeminent producer of hydrogen" produced by anaerobic fermentation, used as a substrate in molasses experiments, and a common cause of spoilage in maple sap and syrup.	(Tindall *et al.* 2017)
2. *K. africana*	The bacillus isolated from the asymptomatic carriage of the inhabitants of Kenya and Senegal, mainly an opportunistic pathogen.	(McDougall *et al.* 2021)
3. *K. granulomatis*	The etiological agent of inguinal granuloma (donovanosis), an infectious disease occurring in tropical and subtropical regions of Southeast Asia, India, Africa and Central America. The diagnosis of donovanosis is based on the history taking, the characteristic clinical picture (no changes in the lymph nodes) and the detection of the presence of vacuole in the tissue smear, the so-called Donovan bodies surrounding bacteria.	(Belda Junior 2020)
4. *K. grimontii*	A relatively common human pathogen isolated mainly in France, Germany and South Africa. It mainly causes bacteraemia and soft tissue infections.	(Passet and Brisse 2018)
5. *K. huaxensis*	The opportunistic pathogen. The etiological agent of urinary tract infections (UTIs).	(Hu *et al.* 2019)
6. *K. indica*	The opportunistic pathogen. Relatively little described in the scientific literature.	(Gujarati *et al.* 2020)
7. *K. kielensis*	The opportunistic pathogen. Relatively little described in the scientific literature.	(Schoch and Karsch-Mizrachi *et al.* 2020)
8. *K. michiganensis*	The opportunistic pathogen. First detected in Michigan. The bacterium was first isolated in Europe from blood and rectal swabs from an immunosuppressed patient.	(Seiffert *et al.* 2019)
9. *K. milletis*	The opportunistic pathogen. Bacillus mainly transmitted by food.	(Alves *et al.* 2006)
10. *K. oxytoca*	The second important species pathogenic for humans after *K. pneumoniae.* Isolated from pneumonia, and UTIs. Common cause of nosocomial infections in neonatal wards.	(Neog *et al.* 2021)
11. *K. pasteurii*	The opportunistic pathogen. Isolated from human and animals stool samples such as cows and turtles.	(Merla, Brisse *et al.* 2019)
12. *K. pneumoniae* subsp. *ozaenae*	The etiological factor of ozena - chronic, atrophic rhinitis, causing halitosis.	(Tachibana *et al.* 2022)
13. *K. pneumoniae* subsp. *pneumoniae*	The most frequently isolated in about 95% of all *Klebsiella* strains. An opportunistic pathogen. Isolated from: sepsis, endotoxic shock, pneumonia, lung abscesses, infections of the urinary, digestive and biliary tracts. In addition, it causes inflammation of the sinuses, middle ear, inflammation of soft tissues, osteomyelitis, and meningitis in newborns.	(Ali *et al.* 2022)
14. *K. pneumoniae* subsp. *rhinoscleromatis*	Frisch's bacillus, the etiological agent of heart disease („rhinoscleroma") known as „Slavic leprosy", a chronic infectious granulomatous disease of the respiratory tract covering mainly the nasal cavity, as well as the oral cavity, pharynx, larynx, trachea, and bronchi; is now very rare in Poland.	(Fusconi *et al.* 2018)
15. *K. quasipneumoniae*	Originally thought to be largely confined to agriculture. However, it may be responsible for causing disease in humans.	(Mathers *et al.* 2019)
16. *K. quasipneumoniae* subsp. *quasipneumoniae.*	The name derives from „*quasipneumoniae*" which means almost like „*pneumoniae*". The opportunistic pathogen. Pathogenicity as in *K. pneumoniae*, mainly the etiological agent of pneumonia.	(Brisse *et al.* 2014)
17. *K. quasipneumoniae* subsp. *similipneumoniae*	Name derived from „*similis*" which means similar to „*pneumoniae*". The opportunistic pathogen. Pathogenicity as in *K. pneumoniae*, mainly the etiological agent of pneumonia.	(Brisse *et al.* 2014)
18. *K. quasivariicola*	The opportunistic pathogen. First time isolated from a wound.	(Long *et al.* 2017)
19. *K. senegalensis*	The opportunistic pathogen. First detected in Senegal. Mainly foodborne pathogen.	(Alves *et al.* 2006)
20. *K. spallanzanii*	The opportunistic pathogen. Mainly isolated from human urine, cow feces and farms. cow feces and farms.	(Merla, Brisse *et al.* 2019)
21. *K. steroids*	The opportunistic pathogen. Relatively little described in the scientific literature.	(Schoch, Karsch-Mizrachi *et al.* 2020)
22. *K. variicola*	These rods account for less than 10% of *Klebsiella* clinical isolates previously classified as *K. pneumoniae*. Hypervirulent isolates have been identified, and colistin-resistant isolates of this species are also reported. Abundant in the environment (mainly rivers), edible plants, e.g. root, leaves, banana stem, sugar cane stem, corn shoots, rice roots. The etiological agent of mastitis in cattle.	(Rodriquez-Medina *et al.* 2019)

1.1.2. Occurrence

Bacteria of the genus *Klebsiella* are microorganisms widely distributed in the natural environment (Navon-Venezia *et al.* 2017; Khan *et al.* 2019; Huang *et al.* 2020). Moreover, *Klebsiella* is part of the microbiota in humans and various animals (dogs, cats, horses and pigs) (Navon-Venezia *et al.* 2017). *Klebsiella,* excreted in human and animal feces, is commonly found in soil, groundwater, surface and seawater, and on various plants such as banana, corn, rice and sorghum (Khan *et al.* 2017; Huang *et al.* 2020). These bacteria are also a component of industrial sewage (Navon-Venezia *et al.* 2017). The high adaptability of many species of *Klebsiella* bacilli, mainly *K. pneumoniae*, enables them to colonize hospital environments where multidrug-resistant hospital strains are selected. Recent studies show these strains present in the hospital environment, primarily in anesthesiology, intensive care, cardiology, neurosurgery, and neonatal departments. In patients and medical staff, these bacteria are part of the physiological permanent or transient microbiota (Ali *et al.* 2022). Outside the hospital, premature infants, newborns, older adults, as well as immunocompromised patients and alcoholics, are most at risk for infections caused by *Klebsiella* bacilli, in particular *K. pneumoniae* (Chang *et al.* 2021).

2. Characteristics of the *Klebsiella pneumoniae* species

2.1. Morphology, growth conditions, culture and biochemical profile

Klebsiella pneumoniae (formerly called Friedländer's bacilli) is a cylindrical, capsulated, ciliated, non-spore-forming bacterium measuring 0.3 to 1 µm in width and 0.6 to 6 µm in length (Fig. 2) (Ali *et al.* 2022). Some clinical strains of *K. pneumoniae* may be equipped with a single flagellum, which determines motility. The presence of these flagella is considered a virulence factor (Carabarin-Lima *et al.* 2016). *K. pneumoniae* in culture microscope preparations are arranged in pairs or short chains, the cells generally joining poles (Szewczyk 2019).

K. pneumoniae are facultative anaerobes that grow at an optimal temperature of 37°C. These bacilli survive in the inanimate environment in a wide temperature range (12°C–42°C). The ability to break down glucose, especially at high temperatures (up to 44.5°C), gives bacteria an advantage in non-living environments by providing energy for key life processes, supporting biofilm production and enabling adaptation. As a result, bacteria can survive longer in harsh environments, enhancing their ability to infect new hosts (Mason and Ztirich 1987; Centeleghe *et al.* 2023; Horng *et al.* 2023).

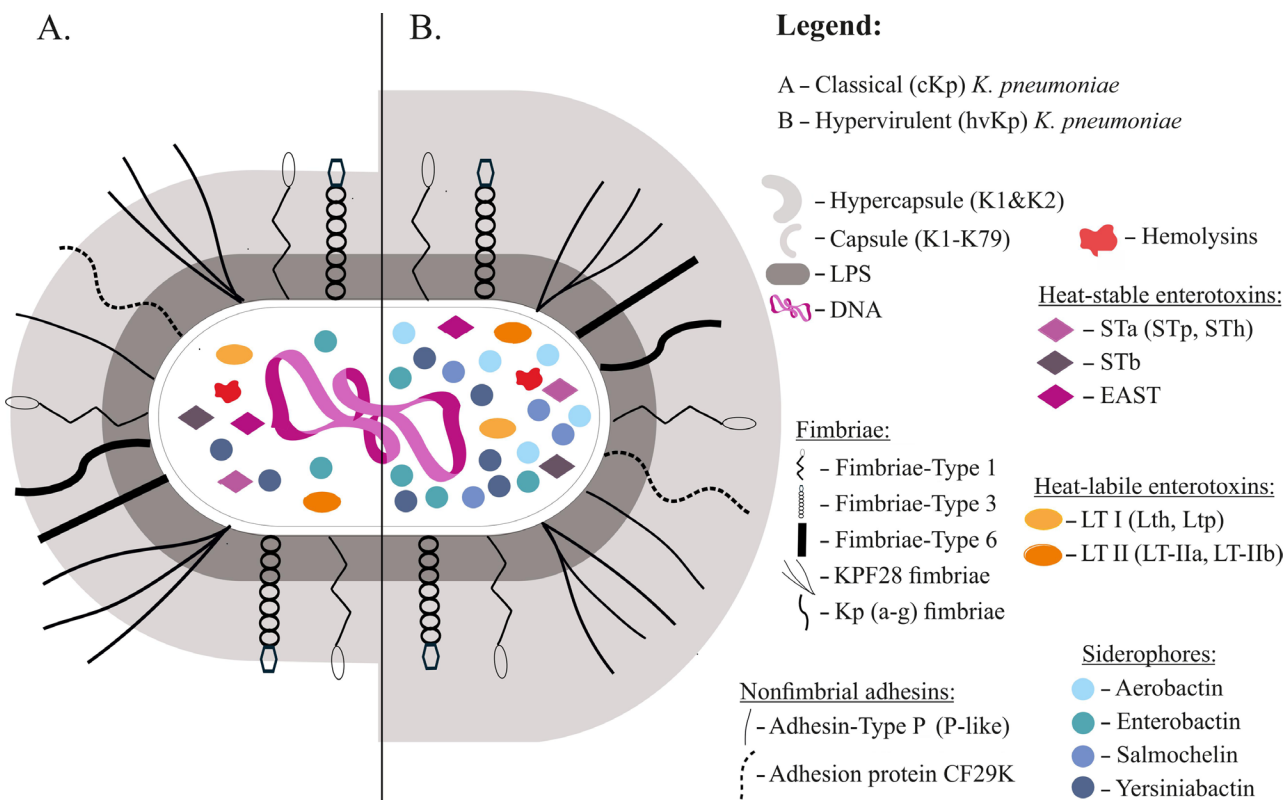

Fig. 2. Schematic representation of the differences in cell morphology of classical (cKp) and hypervirulent (hvKp) *K. pneumoniae,* taking into account virulence factors.

Own graphic design according to (Paczosa & Mecsas 2016; Ali *et al.* 2022; Dai and Hu 2022).

These bacteria are catalase-positive and indole-negative, produce urease, ferment lactose, produce lysine decarboxylase, do not produce ornithine decarboxylase, reduce nitrates to nitrites, do not produce deoxyribonucleases (DNases), use malonic acid and citrate as a carbon source, are oxidase-negative, do not cause deamination of phenylalanine (Brisse *et al.* 2014; Mączyńska 2015; Szewczyk 2019). Broth cultures of *K. pneumoniae* are uniformly turbid with a ring or a characteristic film located on the surface of the culture. Like other species of *Enterobacterales*, *K. pneumoniae* grow well and abundantly on solid substrates, forming characteristic mucous, shiny, convex, smooth, grey-white colonies (Murray *et al.* 2022). *K. pneumoniae* bacilli cultures can be performed on non-selective media such as tryptic soy agar (TSA) and blood agar (BA), as well as on selective-differentiating (selective) media such as: (1) MacConkey agar containing selective factors (crystal violet and sodium deoxycholate) and a differentiating factor (lactose) – *K. pneumoniae* form pink colonies, (2) eosin-methylene blue agar (EMB), medium containing selective factors (eosin and methylene blue) and differentiation factors (glucose and/or sucrose) – *K. pneumoniae* form blue-black colonies, (3) as well as Drigalski medium containing selective factors (crystal violet and sodium deoxycholate) (4) and bromothymol blue agar (BTB), differentiation medium on which the distinguishing of bacilli from the family Enterobacteriaceae is based on the ability to ferment the lactose in the presence of bromothymol blue – *K. pneumoniae* form yellow colonies (Brisse *et al.* 2014; Szewczyk 2019).

2.2. Species identification

Various microbiological methods are used to identify the species of *K. pneumoniae* bacilli, from microscopic techniques through traditional phenotypic methods to advanced molecular analyses (Grimont and Grimont 2015; Cheng *et al.* 2018; Froböse *et al.* 2020). During the cultivation of bacilli on solid media containing carbohydrates, the isolation and initial classification of bacteria into the *Klebsiella* genus is facilitated by the visible mucous appearance of bacterial colonies as a result of the production of a multi-sugar bacterial coating capsular polysaccharide (CPS) by *K. pneumoniae* strains (Szewczyk 2019; Murray *et al.* 2022). The bacterial capsule of *K. pneumoniae* can be visualized using various staining techniques, including the negative-positive method (Burri-Gins) using Chinese ink and alkaline fuchsin. Identification of *K. pneumoniae* strains characterized by the ability to create a hypermucoviscosity (HM) phenotype typical of highly pathogenic isolates is carried out using the string test (Eisenmenger *et al.* 2021). Identification of *K. pneumoniae* is based on traditional bacteriological methods involving the analysis of biochemical features using manual methods or standardized sets, e.g. API® 20E strips, or automatic methods using compact systems for identifying bacteria with the simultaneous determination of antimicrobial susceptibility of microorganisms, for example using the VITEK® 2 Compact system (Master *et al.* 2013). According to Monnet *et al.* (1991), for conventional methods, incorrect identification was made in 13% of the *K. pneumoniae* strains tested (Monnet *et al.* 1991). Precise identification to the species level can be performed using highly specialized techniques using matrix-assisted laser description/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Váradi *et al.* 2017).

Among the molecular methods used for species identification of *K. pneumoniae*, the polymerase chain reaction (PCR) method is commonly used (Järvinen *et al.* 2009). For the molecular identification of *K. pneumoniae*, as well as typing of the most pathogenic strains, numerous genetic methods based on the multiplex-PCR technique have been developed, aiming to detect genes encoding pathogenicity factors, as well as to determine the serotype of the envelope (Chen *et al.* 2014; Fonseca *et al.* 2017). Multiplex-PCR has a high sensitivity and test specificity of over 90% (Dessajan and Timsit 2024). PCR also forms the basis of other techniques used in *K. pneumoniae* identification/differentiation based on regions of the *rrn* operon. These techniques use a variety of methods, including amplification of a variable region within the gene encoding 16S or 23S rRNA, amplification of polymorphic sequences located between the genes encoding 16S and 23S rRNA (Internal Transcribed Spacer-PCR, or ITS-PCR) (Liu *et al.* 2008) and Real-Time PCR for detecting *K. pneumoniae* with *rmp*A or *mag*A genes associated with the hypermucoviscosity phenotype (Hartman *et al.* 2009). The Real-Time PCR technique has high sensitivity and specificity (Hartman *et al.* 2009). Droplet digiatal (ddPCR) is used to detect *K. pneumoniae* in stool samples (Feng *et al.* 2024).

Another innovation is the Loop-Mediated Isothermal Amplification (LAMP) method (Poirier *et al.* 2021). LAMP (like the method with PCR) uses technology based on amplification and detecting specific DNA sequences. It relies on the isothermal amplification reaction of nucleic acids. The LAMP method is highly specific, as six primers (3 pairs) are used in the reaction, and amplification of genetic material occurs only if the primers recognize 6 to 8 specific DNA sequences of the pathogen under study (Poirier *et al.* 2021). The LAMP technique found particular application in a study by Poirier *et al.* (2021), who identified three target genes (*yha*I, *eps*L and *xcp*W) common to *K. pneumoniae* isolates from both China and Europe and designed LAMP assays for detecting *K. pneumoniae* in clinical

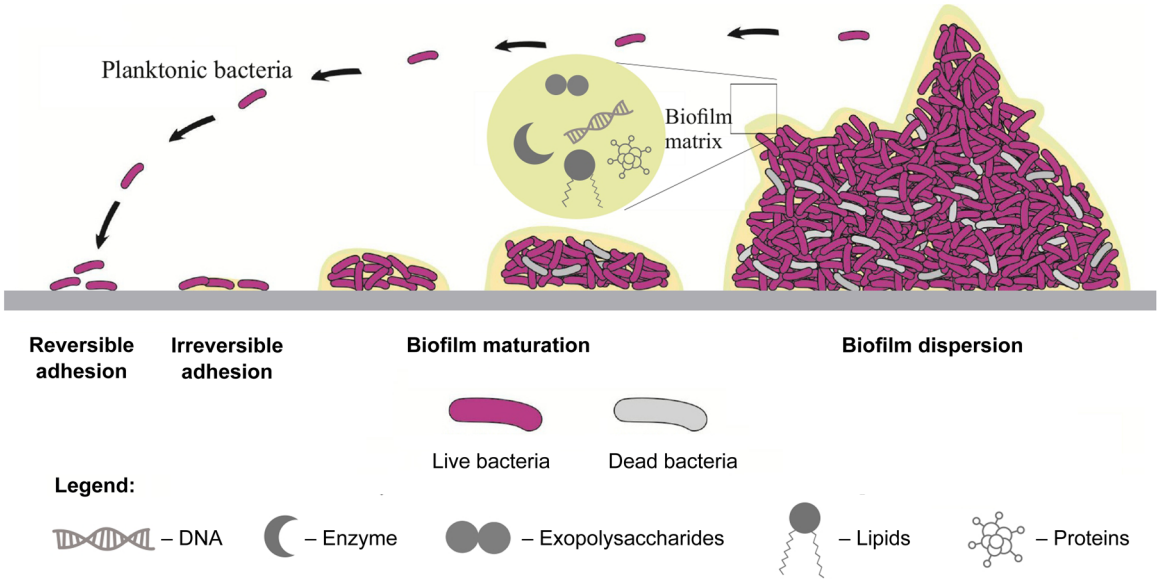

Fig. 3. Schematic representation of the subsequent stages of bacterial biofilm formation and its extracellular polymeric substance (EPS).
Own graphic design according to (Zhao *et al.* 2023).

samples (Poirier *et al.* 2021). In turn, Dong *et al.* (2015) described the LAMP method for rapid detection of the synthesis of the envelope polysaccharide regulating the *rcs*A gene from *K. pneumoniae* (Dong *et al.* 2015). The LAMP method is also being used to detect carbapenem resistance genes (bla_{KPC}, bla_{NDM-1}, $bla_{OXA-48-like}$, $bla_{IMP-1\ group}$, and bla_{VIM}) in *K. pneumoniae* (Poirier *et al.* 2021; Kim *et al.* 2022).

In addition to PCR-based methods, methods on Sanger sequencing, next-generation sequencing (NGS) and whole-genome sequencing (WGS) are used for proper molecular identification of *K. pneumoniae* species (Nafea *et al.* 2024). The main advantage of NGS over the conventional method is the simultaneous use of many genetic markers with high-resolution genetic data (Nafea *et al.* 2024). In turn, the high resolution of WGS analyses can provide information on the origin of bacteria, their routes of transmission, and biological traits (e.g., serotype). WGS also enables the identification of virulence genes and antibiotic-resistance genes. WGS analyses are suitable for introduction into routine laboratory testing. With comparative analysis of the entire genome, WGS could become the primary typing method used for early detection of epidemic outbreaks and monitoring the dynamics of the spread of a given pathogen (Nafea *et al.* 2024).

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR-Cas) is increasingly used in microbiology. One of the first applications of this method was the typing of bacterial strains. CRISSP-Cas can also help develop new antimicrobial strategies (Barrangou *et al.* 2016; Ding *et al.* 2020). These techniques may find application in advanced studies of *K. pneumoniae.*

Molecular methods are increasingly being used in the identification of *K. pneumoniae* over classical phenotypic methods because they are independent of culture conditions, are more reproducibly sensitive and allow for shorter waiting times for results. The versatility of these methods is due to their applicability to the examination of virtually any biological material with minor adjustments to laboratory procedures. The limitations of these methods are limited availability and the inability to distinguish whether specific genetic material comes from live or dead bacteria.

2.3. Pathogenicity

Klebsiella spp. constitute a heterogeneous group of closely related enteric bacilli. *Klebsiella* rods are commonly found in hospital and non-hospital environments. They are part of the KESC subgroup (*Klebsiella*, *Enterobacter*, *Serratia* and *Citrobacter* subgroup), which collects genera with the closest relatedness and a similar biochemical profile (Szewczyk 2019). Microorganisms classified as KESC are characterized by multidrug resistance (MDR), and the presence of factors that enable them to survive freely in the hospital environment makes them a frequent cause of nosocomial infections (Szewczyk 2019). The species of the *Klebsiella* genus that most often causes infections in humans is *K. pneumoniae*. The second most frequently isolated species from clinical materials is *K. oxytoca*. The remaining *Klebsiella* species, much less frequently, may also be the etiological factor of infections (Chang *et al.* 2021).

K. pneumoniae is responsible for most (about 95%) severe human infections (Murray *et al.* 2022). Risk fac-

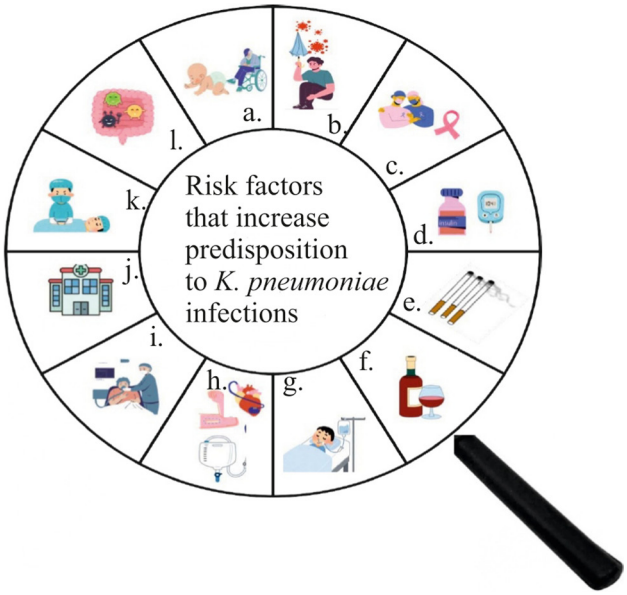

Fig. 4. Schematic representation of the risk factors that increase predisposition to *K. pneumoniae* infection: a – age (premature babies, newborns, elderly people), b – lowered immunity, c – debilitating diseases (cancer), d – concomitant diseases (e.g. diabetes), e – smoking, f – alcoholism, g – frequent or long-term hospitalization, h – use of vascular and urological catheters, drains and other implants, i – assisted breathing, j – stay in nursing homes, k – surgical interventions in the abdominal cavity, l – colonization of the gastrointestinal tract by hospital strains.

Own graphic design according to (Mączyńska 2015).

tors predisposing to *K. pneumoniae* infections include age (premature infants, newborns, the elderly), reduced immunity, debilitating diseases (cancer), frequent or prolonged hospitalization, assisted breathing, surgical interventions in the abdominal cavity, use of catheters (vascular, urological) drains and other implants, alcoholism, smoking, residence in nursing homes, colonization of the gastrointestinal tract by hospital strains (Fig. 4) (Mączyńska 2015).

Klebsiella can cause both healthcare-acquired infections (HAIs) and community-acquired infections (CAIs) (Chang *et al.* 2021). HAIs usually affect premature and frail neonates, older adults, and immunocompromised patients and include pneumonia, urinary tract infections (UTI), septic infections, endocarditis, central nervous system infections, purulent infections, wound infections, gastrointestinal infections associated with toxin production (Chang *et al.* 2021). CAIs include pneumonia, primary liver abscesses (PLA) combined with a characteristic invasive syndrome characterized by blood-borne infections spreading to other organs (bones and joints, eye, brain, lung, prostate, spleen) and rare infections occurring endemically (ozena, scleroderma and donovanosis) (Table I) (Fig. 5) (Fusconi *et al.* 2018; Belda Junior 2020; Tachibana *et al.* 2022).

K. pneumoniae is crucial in primary hospital-acquired pneumonia (HAPs) and community-acquired pneumonia (CAPs). In hospitalized patients, *K. pneumoniae* strains cause 7–14% of HAPs. The frequency of HAPs depends on the ward and condition of the patients and the intensity of invasive medical procedures (intubation, tracheotomy) associated with respiratory support (Mączyńska 2015). These factors increase the risk of pulmonary infections, including those associated with ventilator-acquired pneumonia (VAP), for which *K. pneumoniae* is also a significant etiologic agent. *K. pneumoniae* is generally the only *Enterobacteriaceae* causing 4–5% of out-of-hospital respiratory tract infections occurring most often in patients over 60 years of age, in poor health, often accompanying another severe underlying disease (e.g., diabetes, cardiovascular disease) but also much more common in smokers and alcohol abusers. These infections are characterized by a sudden onset, a severe course and a relatively high mortality rate. The characteristic symptom of the disease is the expectoration of a large amount of purulent, thick secretion, often colored by blood (Mączyńska 2015; Murray *et al.* 2022). The most important virulence factor of *K. pneumoniae* causing pneumonias is the polysaccharide envelope, which protects the bacteria from phagocytosis, and lysis by the complement system. It plays a crucial role in pathogenesis. Adhesins (especially fimbriae types 1 and 3) facilitate colonization of the airway epithelium

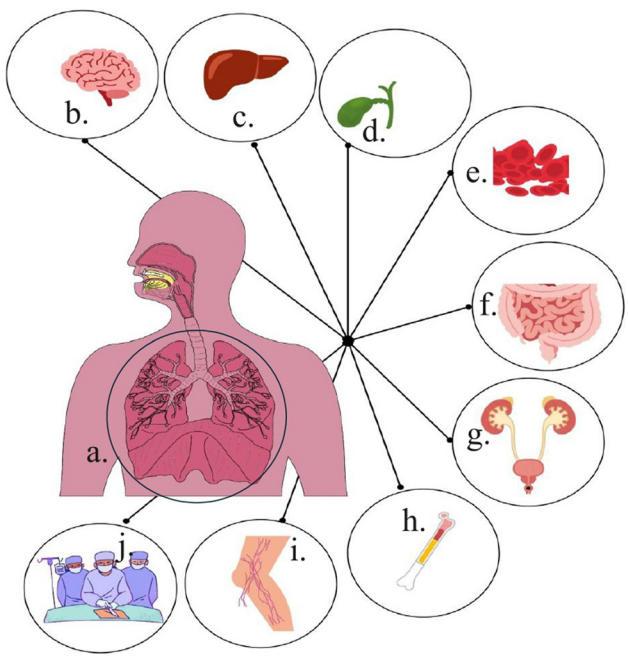

Fig. 5. Schematic representation of the pathogenicity of *K. pneumoniae*: a – pneumonia, b – central nervous system infections, c – primary liver abscess, d – cholecystitis, e – septic infections, f – gastrointestinal infections associated with toxin production, g – urinary tract infections, h – bone and joint infection, i – soft tissue infections, j – purulent and wound infections.

Own graphic design according to (Martinez and Baquero 2002; Chang *et al.* 2021; Ali *et al.* 2022).

and the production of mucus (exopolysaccharide) that is part of the biofilm structure that *K. pneumoniae* can form, for example, in the patient's lungs and endotracheal tubes or tracheostomy tubes, are also important in pneumonia (Alcántar-Curiel *et al.* 2013; Mączyńska 2015; Cader *et al.* 2020; Ochońska *et al.* 2021; Murray *et al.* 2022).

In addition to pneumonia, *K. pneumoniae* is often responsible for causing urinary tract infections – UTIs. *K. pneumoniae* causes 6–17% of hospital-acquired UTIs (Campana *et al.* 2017; Murray *et al.* 2022). UTIs can progress as pyelonephritis, typical bladder infections, but can also be recurrent and lead to permanent kidney changes. Among the most critical pathogenicity factors of uropathogenic *K. pneumoniae* strains are fimbriae, responsible for bacterial adhesion to urinary tract epithelial cells, which prevents the washout of microorganisms during micturition. The ability to produce urease and form biofilm structures on urinary catheters are also important virulence factors (Campana *et al.* 2017).

Septic infections caused by *K. pneumoniae* can manifest as asymptomatic bacteremia; these bacilli can also cause sepsis. The most common causes of sepsis are untreated urinary tract infections, respiratory tract infections and inflammation and obstruction of the intestines in immunocompromised patients (Carabarin-Lima *et al.* 2016).

A primary liver abscess (PLA) and the characteristic invasive syndrome are mainly caused by hvKp strains. The ability of hvKp strains to cause PLA with a tendency to spread to multiple tissues and organs (central nervous system, kidney, bones, eyes, lungs, prostate, skin and subcutaneous tissues, pancreas) is significant. The most common route of infection is bacterial translocation from the intestine. PLA caused by *K. pneumoniae* is localized in the right lobe of the liver and is limited in nature. Multiple abscesses may occur at different locations with greater or lesser frequency, commonly as liver abscesses, lung abscesses, biliary tract abscesses, skin and soft tissue abscesses, pleural abscesses, peritonitis, and inflammation of the external coating of the eye (*endophtalmitis*) (Ali *et al.* 2022). The rapid course of infection, having a poor prognosis and a tendency to develop generalized infection, is referred to as "*characteristic invasive syndrome*" (DIS) (Mączyńska 2015).

Among the isolates of *K. pneumoniae* are distinguished between classical *K. pneumoniae* (cKp) strains and hypervirulent *K. pneumoniae* (hvKp) strains (Fig. 2) (Russo *et al.* 2024). The cKp strains commonly cause infections in immunocompromised individuals. These are community-acquired pneumonia, UTIs, bacteremia or meningitis (Dai and Hu 2022). In addition, cKps strains are isolated from elderly patients and those with risk factors such as alcohol abuse and smoking (Russo *et al.* 2024). cKp is a group of *K. pneumoniae* that lacks hypercapsule, macromolecular exopolysaccharide or excessive siderophores and rarely causes disease in healthy individuals (except for UTIs), although it is MDR (Dai and Hu 2022). In contrast, hvKp are highly virulent strains responsible for community-acquired pneumonia characterized by a severe course and a high mortality rate reaching up to 40% in some regions of the world (Russo *et al.* 2024). As a result of the translocation process from the gut, strains of hvKP can spread to other organs and tissues (central nervous system, lungs, bones, prostate, skin and subcutaneous tissue) and also contribute to the formation of PLAs (Russo *et al.* 2024). hvKp is another type of *K. pneumoniae* that harbors hypercapsule, macromolecular exopolysaccharide, or highly active siderophores and induces infections in both immunocompromised and otherwise healthy individuals (Dai and Hu 2022).

2.4. Virulence factors

The pathogenicity of *K. pneumoniae* is determined by many virulence factors (Compain *et al.* 2014; de Souza *et al.* 2024). Their presence can lead to infection and antibiotic resistance. The major virulence factors playing an essential role in the pathogenesis of infections caused by *K. pneumoniae* are capsule polysaccharides (CPS, K-antigen) and lipopolysaccharides (LPS, O-antigen). These critical virulence factors help to enter the bloodstream and cause septic shock in the host. Fimbrial and non-fimbrial adhesins, siderophores (aerobactin, enterobactin, salmochelin and yersiniabactin), heat-stable and heat-labile enterotoxins, cytolysins and the ability to form a biofilm are also important (Fig. 2) (Ali *et al.* 2022). The genes encoding many virulence factors are located in the large mosaic virulence plasmid (pLVPK) in *K. pneumoniae* isolates (Mączyńska *et al.* 2015; Clegg and Murphy 2016).

2.4.1. Capsule polysaccharide (CPS)

The capsule polysaccharide (CPS) is the essential, well-known virulence factor of *K. pneumoniae,* forming a layer hermetically surrounding the bacterial cell wall (Ali *et al.* 2022). It is responsible for the initial interaction between bacteria and host. Moreover, the CPS is vital for the survival of *K. pneumoniae* in the host tissue, allowing the pathogen to escape phagocytosis (Ali *et al.* 2022). Structurally, *K. pneumoniae* CPS is a heteropolymer comprised of repeated sugar moieties of hexoses (D-glucose, D-galactose and D-mannose), deoxyhexoses (D-fucose and D-rhamnose) and glucuronic and galacturonic acids. The capsule may also include non-sugar components such as O-acyl, succinate, formate and pyruvate residues (Ali *et al.* 2022). The CPS of *K. pneumoniae* is characterized by the diversity of the structure of polysaccharides that build them and

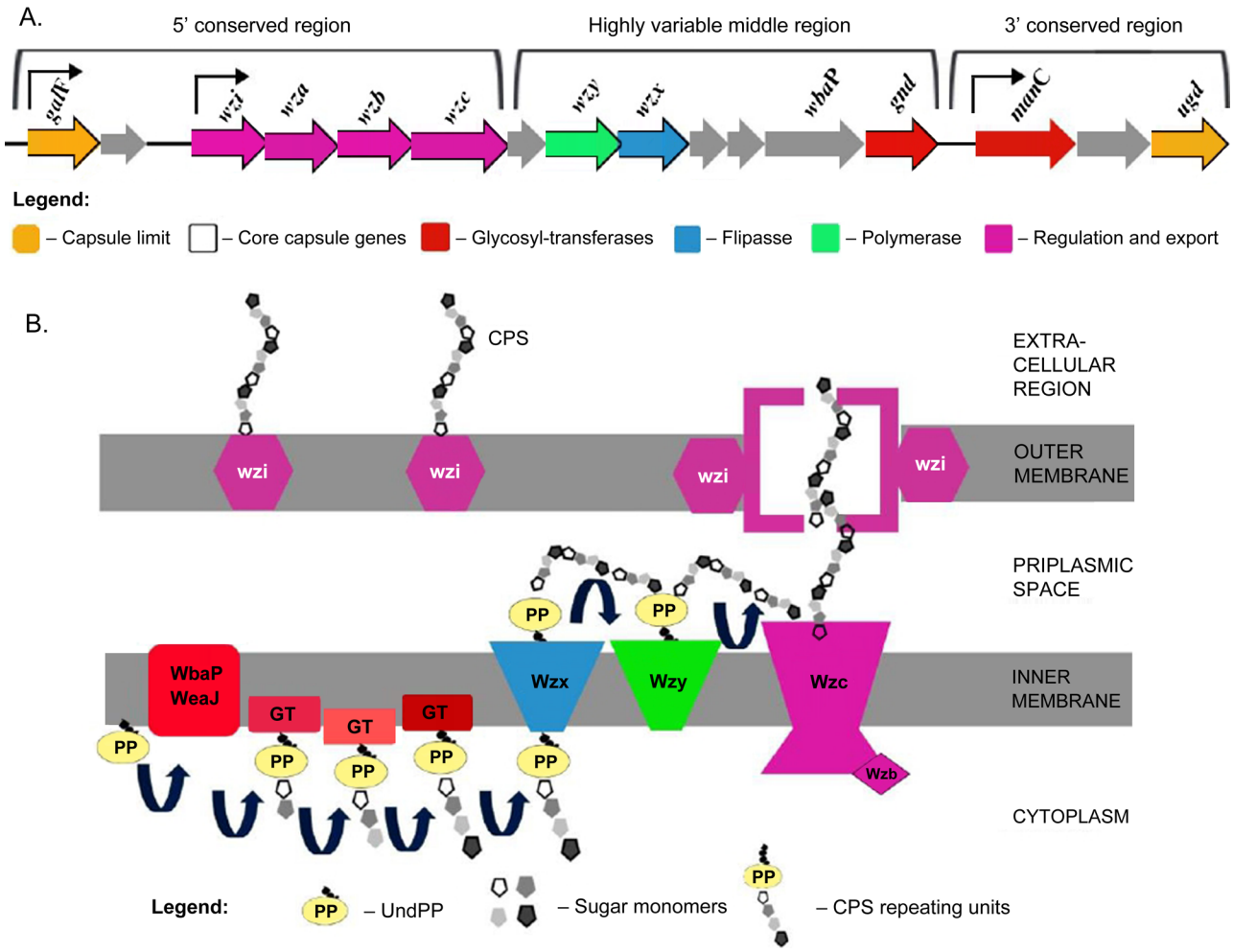

Fig. 6. (**A**) Schematic representation of the capsule (CPS) biosynthetic pathway in *K. pneumoniae.* Own graphic design according to (Patro and Rathinavelan 2019, Rendueles 2020, Patro *et al.* 2020),

(**B**) Scheme of the representative *locus* cps system of *K. pneumoniae* using the K1 serotype as an example. Own graphic design according to (Rendueles 2020, Patro *et al.* 2020).

high serological variability. Genes involved in capsule production are located on the chromosome's capsular polysaccharide synthesis (*cps*) region. The region of the *cps* cluster (from *gal*F to *ugd*) harbors over 20 genes, mainly driven by three promotors located upstream of genes, *gal*F, *wzi*, and *man*C, respectively (Fig. 6) (Zhu *et al.* 2020). A group of six genes mainly carries out the synthesis of the capsule at the 5' end of the *cps* operon (*gal*F, *cps*ACP, *wzi, wza, wzb, wzc*) and the *ugd* gene located at the 3' end (Shu *et al.* 2009; Wyres *et al.* 2016). The diversity of serotypes is the result of the action of various glycosyl-transferases (GTs), whose genes *wba*P, *wba*Z, *wca*N, wcaJ and *wca*O, are located in the middle part of the *cps* operon (Shu *et al.* 2009). The protein encoded by *wba*P mediated the first step in capsule biosynthesis. Glycosylation of the repeating unit is initiated by WbaP (when the initializing sugar linked to the undecaprenol-pyrophosphate – Und-PP – is galactose) or by WcaJ (when the initializing sugar linked to Und-PP is glucose). Current research has shown that some *wba*P mutations increased pathogenicity by increasing biofilm formation and invasion of bladder epithelial cells in urinary tract infections (UTIs) (Zhu *et al.* 2020). Several genes that regulate the biosynthesis of CPS sugar, *rml*A, *rml*B, *rml*C, *rml*D, *man*B and *man*C, are also located towards the end of the *cps* locus (Ali *et al.* 2022). Differences in *K. pneumoniae* capsule types result from changes in the nucleotide sequences of the *cps* locus and genes involved in CPS biosynthesis, assembly and translocation (Ali *et al.* 2022).

Enhanced capsule production in *K. pneumoniae* may result from the activity of other genes in addition to those located in the *cps* cluster: the capsular synthesis gene B (*rcsB*), the mucoid phenotype regulators A and A2 (*rmpA* and *rmpA2*) and *Klebsiella* virulence regulators (*kvrA* and *kvrB*) and *wzy-K1* (Zhu *et al.* 2020). Different combinations of these genes can result in the production of capsules with different structures. The *rmp*A or *rmp*A2 genes are found in 55–100% hvKp strains, while they are less frequently found in cKp

strains. RmpA regulates mucoid phenotype in pK100 and RmpB (Dai and Hu 2022). The expression of *rmp*A depends on RcsB, KvrA, and KvrB. The newly described regulators, *kvr*A and *kvr*B, affect the virulence of K1/K2 hvKp strains due to the activation of capsule gene expression, which is not present in cKp strains. Different from *rcs*B found in chromosome, both *rmp*A and *rmp*A2 could be located in plasmid or chromosome. Chromosomal *rmp*A (*c-rmp*A and *c-rmp*A2) are located in an integrative and conjugative element (ICEK*p1*) and are only found in < 50% *K. pneumoniae* strains of serotype K1 (Zhu *et al.* 2020; Dai and Hu 2022). Plasmidic *rmp*A (*p-rmp*A and *p-rmp*A2) are more prevalent. The *wzy*-K1 gene is specific to the K1 serotype of *K. pneumoniae*. The function of a *wzx* gene product is to transport the polymer from the cytoplasm to the periplasm. The Wzy protein is involved in the polymerization *via* a catch-and-release mechanism (Zhu *et al.* 2020). Proteins encoded by *wza* and *wzc* genes form a translocation complex responsible for assembling capsular polysaccharides and transporting them from the periplasm to the surface of the bacteria (Pan *et al.* 2013). The Wzb protein, as the cognate phosphatase of Wzc, combines with the catalytic domain on Wzc and, in turn, dephosphorylates Wzc (Zhu *et al*. 2020).

Hypercapsules can be regulated by the capsule A (*cps*A) and B (*cps*B) genes (Dai and Hu 2022). 70% of hvKp strains produce a hypercapsule composed of types K1 and K2. This kind is more stable than the typical capsule found in cKp strains, contributing to their increased virulence in hvKp strains. Other capsule types occur in cKp strains (Marr and Russo 2019). In *K. pneumoniae*, the capsule binds to the surface protein Wzi. Loss of this protein can reduce or lose virulence (Ali *et al.* 2022). There are 79 serotypes of capsulated *K. pneumoniae* strains (K1 to K79). The eight most common types have been described in hvKp strains: K1, K2, K5, K16, K20, K54, K57, and KN1 (Zhu *et al.* 2020). Recently, a classification scheme has been proposed based on the sequence of conserved *wzi* and/or *wzc* genes in the *cps* locus (Ali *et al*. 2022).

In *K. pneumoniae*, the WGS method is specifically used to identify *cps* locus variants (Wyres *et al*. 2016). In a study conducted by Wyres *et al*. (2016), among 2503 *K. pneumoniae* genomes, the diversity of capsid fusion loci (K-loci) was examined. The study included analysis of full-length K-locus nucleotide sequences and clustering protein-coding sequences to identify, annotate and compare K-locus structures. A total of 134 distinct K-locuses were identified, including 31 new types. Comparative analyses revealed 508 unique clusters of protein-coding genes that appear to reassort through homologous recombination. In addition, a high diversity of intra- and inter-locus nucleotides was detected among *wzi* and *wzc* genes. Based on the results, a standardized nomenclature for K loci was proposed, a reference database was presented, and a new software tool – Kaptive, was developed to automate the process of identifying K loci based on complete locus information extracted from the whole-genome sequence (https://github.com/katholt/Kaptive) (Wyres *et al.* 2016).

Assessment of the prevalence of specific serotypes in the *K. pneumoniae* population is valuable for epidemiological investigations. The prevalence of individual serogroups/serotypes depends on the geographical location, patient age, and changes over the years. Strains of different serotypes differ in their resistance to phagocytosis *in vitro* and their ability to activate the humoral response. Some serotypes are more frequently associated with human diseases and epidemics. Hypervirulent strains of *K. pneumoniae* (hvKp) with high virulence usually have the K1 or K2 envelope antigen (Ali *et al.* 2022). *K. pneumoniae* strains represent the same epidemic clone and have the same capsule type (Choi *et al.* 2020). Serotyping is not sufficient for epidemiological purposes due to its poor resolution. However, the synthesis of the bacterial capsule is determined by a set of *cps* genes located in the chromosome and plasmid. Thanks to knowledge of the allele sequence in the *cps* locus, the PCR method is now more widely used for their detection (Walker and Miller 2020; Ali *et al.* 2022).

2.4.2. Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS), also called endotoxin, is an integral and essential component of the cell membrane of Gram-negative bacteria (Ali *et al.* 2022). LPS has strong cytotoxic, immunomodulatory and proinflammatory properties. LPS remains one of the most important pathogenic factors and the main antigen of the *K. pneumoniae* cell wall. It plays a role in the pathomechanism of infection, especially in endotoxic shock accompanying central nervous system infections, blood infections and pneumonia (Choi *et al.* 2020). It is connected with a massive release of LPS after bacterial cells lysis. Increased release of LPS can occur under the influence of various groups of antibiotics that cause lysis of bacterial cells or interfere with their function, including β-lactams (Eng *et al.* 1993; Kirikae *et al.* 1997; Holzheimer 2001).

LPS is a substance with a conservative structure consisting of three fundamental components, i.e. lipid A, the core oligosaccharide and a polysaccharide that determines antigenic specificity (chain O, somatic antigen O). Serological typing of *K. pneumoniae* is based on two main groups of antigens, i.e. the somatic polysaccharide O and the typical-specific capsular antigen K (Choi *et al.* 2020).

Lipid A is a crucial virulence factor responsible for the endotoxic effects of LPS (Navon-Venezia *et al.*

2017). It is recognized as the most structurally conserved region. Several enzymes encoded by the *lpx* gene cluster are involved in the synthesis of lipid A components. The host immune cell receptor, Toll-like receptor 4 (TLR4), recognizes and binds lipid A of LPS, which initiates a cascade of host immune reactions. Although modifications of the lipid A component help the pathogen escape recognition by the host immune cells by favoring the pathogen to establish the infection successfully (Ali *et al.* 2022). Lipid A is the hydrophobic part of endotoxin, responsible for anchoring the heteropolymer in the outer membrane of the host, thanks to which it creates a specific barrier that inhibits the penetration of substances, including antibiotics and detergents, into the microorganism while generating resistance to these compounds. In *K. pneumoniae*, the ineffective antimicrobial effect of colistin can occur through plasmid-mediated transfer of *mcr-1*, a resistance gene, causing modification of LPS lipid A and disruption of the interaction between polymyxins and lipid A (MacDermott-Opeskin *et al.* 2022). Acylation of lipopolysaccharides plays a key role in providing Gram-negative bacteria with some resistance to structural and intrinsic defense mechanisms, particularly the antibacterial properties of detergents (e.g., bile) and cationic defensins (Clements and Strugnell *et al.* 2022).

The core oligosaccharide component of the LPS connects lipid A to the terminal side chains called the O antigen. The genes encoding core oligosaccharides are located in the *waa* locus, and the ligase enzyme WaaL, which links the core structure to the antigen O chain, is encoded by *waa*L. The outer part of the LPS structure, O antigen, comprises multiple repeating units of oligosaccharides: glucose, galactose, mannose and ribose residues. Epidemiological investigations within a species are based on their structure. The *wb* gene cluster regulates the O antigen's synthesis, assembly and translocation. The variation in the oligosaccharide repeats underlies the LPS diversification structurally and functionally. So far, up to nine O *K. pneumoniae* antigens have been identified based on the composition of the sugar molecules (Ali *et al.* 2022).

2.4.3. Fimbrial and non-fimbrial adhesins

The adhesive properties of *K. pneumoniae* are also due to the possession of fimbrial and non-fimbrial adhesins. The fimbrial adhesins include type 1 mannose-sensitive (MS) fimbriae, type 3 mannose-resistant (MR) fimbriae, type 6 fimbriae, KPF-28 fimbriae. *Klebsiella pneumoniae* fimbriae with a fimbrin molecular mass of 28 kDa) and Kp (a-g) fimbriae (Klemm *et al.* 2000; Struve *et al.* 2009; Chen *et al.* 2011; Alcantal-Curiel *et al.* 2013; Mączyńska 2015; Alcantal-Curiel *et al.* 2018; Khonsari *et al.* 2021). Non-fibrillar adhesins include the non-fibrillar P-type adhesion factor and CF29K adhesion protein (CF29K adhesion factor) (Staniszewska *et al.* 2000; Chan *et al.* 2012; Hennequin *et al.* 2016).

Type 1 fimbriae are among the best characterized. They are expressed in about 90% of *K. pneumoniae* strains (Mączyńska 2015). They are mannose-sensitive hemagglutinins (MSHAs), forming long, thick, stiff filaments 1 to 2 μm long and about 7 nm in diameter (Chen *et al.* 2011). Type 1 fimbriae are protein heterocomplexes of the major fimbriae subunit (FimA) that form the protuberance's structure. Smaller subunits (FimB, FimC, FimD, FimE, FimF, FimG, FimH, FimK, FimS and FimX), in addition to adhesion functions, are responsible for protuberance elongation and stability (Alcantal-Curiel *et al.* 2013; Mączyńska 2015). Receptors for FimH are mannosides. The protein determining adhesion properties can be located at the top of the fimbriae and distributed along the spear's entire length (Alcantal-Curiel *et al.* 2013, Mączyńska 2015). The individual subunits of the fimbriae are linked by hydrophobic bonds and form a right-handed helix stabilized by hydrogen bonds. The structural and functional integrity of the elements formed is called the "*fimbriae-adhesin complex*" (Mączyńska 2015). A set of *fim* genes located in a chromosome or plasmid is responsible for the expression of type 1 fimbriae. Synthesis of type 1 fimbriae follows the "all-or-nothing" principle (Mączyńska 2015). Recent evidence shows that the expression of fimbriae's subunit genes responsible for turning on or off fimbriae synthesis can be directly influenced by oxygen availability, elevated temperature but also by the presence of sub-minimal inhibitory concentrations (sub-MICs) of an antibiotic (e.g. streptomycin), which can affect the production of longer fimbriae lacking the ability to bind mannose (Shibl *et al.* 1985; Klemm *et al.* 2000; Struve *et al.* 2009; Mączyńska 2015). Streptomycin induces fimbriae formation that is both functionally and morphologically abnormal. This may have resulted from amino acid substitutions in fimbrial proteins due to the misreading of mRNA by ribosomes (Shibl *et al.* 1985).

Type 3 fimbriae are expressed on the surface in more than 80% of *K. pneumoniae* strains (Murphy and Clegg 2012; Khonsari *et al.* 2021). These are protein heterocomplexes and are mannose-resistant hemagglutinins (MRHA). They form short and thin filaments about 2–4 nm wide and 0.5–2 μm long (Murphy and Clegg 2012). At least nine genes from the *mrk* cluster are required to express type 3 fimbriae. The *mrk* gene cluster can be located in chromosomal or plasmid DNA. The *mrk*A gene encodes the main structural subunit of the fimbriae, while the *mrk*D gene encodes the actual adhesin. This protein determines the specific interaction of the protuberance with the receptor. Smaller

fimbriae subunits MrkB, MrkC, and MrkD form the characteristic structure, and their genes *mrk*B, *mrk*C and *mrk*D regulate the spears' expression. The product of the *mrk*F gene stabilizes the fimbriae structure on the bacterial cell surface (Murphy and Clegg 2012; Alcantal-Curiel *et al.* 2013; Mączyńska 2015).

Type 6 fimbriae are the longest, thick spears present in small numbers on the bacterial surface. Type 6 fimbriae have only been confirmed in the species *K. pneumoniae* subsp. *ozenae* and their role in pathogenicity is little understood (Darfeuille-Michaud *et al.* 1992; Mączyńska 2015).

KPF28 fimbriae (*Klebsiella pneumoniae* fimbriae with a fimbrin molecular mass of 28 kDa) are a long, thin, and flexible, about 4 to 5 nm in diameter and 0.5 to 2 mm long (Di Martino *et al.* 1996). The N-terminal amino acid sequence of the KPF-28 major fimbrial subunit showed no homology with type 1 and type 3 pili of *K. pneumoniae*. Still, it showed 61.7% identity with residues 6 to 19 of the N-terminal amino acid sequence of PapA, the Pap major pilus subunit expressed by uropathogenic *Escherichia coli* strains (UPEC) (Di Martino *et al.* 1996). In a study of *K. pneumoniae* responsible for nosocomial infections, KPF-28 was shown to be present in strains producing the extended-spectrum β-lactamase CAZ-5/SHV-4 (current name SHV-4) (Di Martino *et al.* 1996). KPF-28 fimbriae are plasmid-encoded, specifically in plasmid R, which contains $bla_{\text{SHV-4}}$ gene (Di Martino *et al.* 1996). A study by Di Martino *et al.* (1996) involving *K. pneumoniae* strain CF914-1 isolated from urine from a patient in ICU and 78 other *K. pneumoniae* isolates involved in nosocomial infections showed that fimbriae KPF-28 were present in *K. pneumoniae* strain CF914-1, as well as in vast majority (83%) of clinical *K. pneumoniae* strains producing SHV-4 extended-spectrum β-lactamase (DiMartino *et al.* 1996). Further studies on the occurrence of KPF28-type fimbriae in *K. pneumoniae* strains causing UTIs are needed.

Kp-type fimbriae (Kpa, Kpb, Kpc, Kpd, Kpe, Kpf and Kpg) are another seven types of fimbriae detected in *K. pneumoniae* (Wu *et al.* 2010). A study by researchers in Taiwan showed that Kp-type fimbriae are only found in *K. pneumoniae* strains with the K1 capsule antigen and increase the ability of strains to form a biofilm (Wu *et al.* 2010).

A non-fimbrial P-type adhesion factor is a protein heteropolymer that exhibits hemagglutination properties similar to the analogous properties of P fimbriae in *E. coli*. It has no fimbrial filament-forming subunits. The receptor for the non-fimbrial P-type adhesion factor is the globoside receptor α – D – Galp – (1 4) – D – Galp. The P-type factor involves bacterial adhesion to the epithelium of the urinary, gastrointestinal and respiratory tract (Staniszewska *et al.* 2000).

CF29K (nonfimbrial protein of 29 kDa) (CF29K adhesion factor) – nonfimbrial adhesion protein was found in *K. pneumoniae* strains characterized by high adhesion capacity to intestinal cell lines. It is encoded by the *cf*29A gene located on a plasmid that also contains the gene encoding TEM-5 β-lactamase. It shows high homology to the CS31A-L protein encoded by the *clp*G gene and produced by enterotoxigenic *E. coli* (ETEC) strains (Hennequin *et al.* 2016).

2.4.4. Siderophores

Siderophores such as aeroactin, enterobactin, salmochelin and yersiniabactin are virulence factors synthesized by *K. pneumoniae* (Farzand *et al.* 2021). Bacterial siderophores, called iron carriers, are low-molecular, organic chemical compounds of a non-protein and non-porphyrin nature, chelating iron ions and secreted extracellularly by some microorganisms to capture this element (Farzand *et al.* 2021). In bacterial cells, iron is an element necessary for the synthesis of cytochromes and ribonucleotide reductase, which are involved in the DNA synthesis process, as well as other enzymes. The survival of *K. pneumoniae* in the environment depends on siderophores to meet the demand for iron. They compete with the host for the available iron pool (Chhabra *et al.* 2020).

Enterobactin is the primary iron uptake system in *K. pneumoniae* and is the most commonly but not only siderophore synthesized in this bacterial species. Studies show that hypervirulent hvKp strains quantitatively produce more siderophores than cKp strains (Dai and Hu 2022). Enterobactin is catecholate. The chromosomal gene cluster *ent*ABCDEF and *fep*ABCDG encode its biosynthesis and transport. *ybt* and *fyu* genes encode transporters for the secretion of enterobactin, and *ybt*O encodes the uptake receptor of enterobactin. Lipocalin-2 (LCN2), known as neutrophil gelatinase-associated lipocalin (NGAL), is extremely important in immune processes and can bind and neutralize enterobactin. LCN2 participate in the regulation of cell aging, cell differentiation and modeling of the immune response (Xiao *et al.* 2017). In *K. pneumoniae* respiratory tract infection, LCN2 is up-regulated by the host. This lipocalin also has pro-inflammatory effects, leading to IL-8-mediated recruitment of neutrophils to the site of infection (Dai and Hu 2022).

Aerobactin is a citrate-hydroxamate siderophore found mainly in more than 90% hvKp strains, while it is less common (6%) in (cKp) strains. Aerobactin was present in hvKp-caused lung infections and is the dominant siderophore in hvKp strains. Aerobactin production is usually associated with hypercapsule, while *K. pneumoniae* with hypercapsule does not always contain aerobactin. The *iuc*ABCD gene cluster controls aerobactin synthesis, while its transport is determined

by *iut*A. They are often present in the same pLVPK-like plasmids carrying *p-rmp*A (Dai and Hu 2022). LCN2 does not neutralize aerobactin (Xiao *et al.* 2017).

Salmochelin is a c-glucosylated form of enterobactin and another siderophore in *K. pneumoniae*. Glucosylation is carried out by the *iro* gene cluster, *iro*ABCDE, which can be localized in a chromosome or plasmid. IroN contributes to the transport of iron-carrying salmochelin. Salmochelin is not neutralyzed by LCN2. This siderophore induces colonization of the nasopharyngeal cavity by *K. pneumoniae*, leading to pneumonia. Salmochelin, like aerobactin, is usually found in hvKp strains with a frequency of over 90% and only 2–4% in cKp strains (Dai and Hu 2022).

Yersiniabactin is another siderophore in *K. pneumoniae* whose production is likely due to horizontal gene transfer (HGT) genes from *Yersinia*. Yersiniabactin was found in 18% of cKp strains and 90% hvKp. Located in chromosome *irp* gene cluster encodes proteins for yersiniabactin synthesis. Yersiniabactin and enterobactin are highly expressed during lung infection, and LCN2 does not inhibit it *in vivo*. However, yersiniabactin alone cannot acquire the iron for *K. pneumoniae,* and the lack of the other three siderophores would prevent *K. pneumoniae* from colonizing the lungs (Holden and Bachman 2015; Dai and Hu 2022).

2.4.5. Heat-stable and heat-labile enterotoxins

Important extracellular pathogenicity factors of *K. pneumoniae* are also enterotoxins – protein toxins similar to enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST 1), heat-stabile (ST) and heat-labile (LT) enterotoxins (O'Ryan 2011). Plasmids carrying enterotoxin-coding genes acquired by multidrug-resistant strains may contribute to the emergence of epidemics. Bacterial diarrhea occurring in the hospital environment can cause the spread of bacteria, and strain ability to produce enterotoxins can be a factor that predisposes to cause an epidemic outbreak (O'Ryan 2011).

2.4.6. Hemolysins

Until recently, *Klebsiella* spp. were considered non-hemolytic, and only single papers of one research group from the 1980s described the hemolysin produced by *K. pneumoniae* and *K. oxytoca* (Barberis *et al.* 1986). According to them, these hemolysins are thiol-activated cytolysins and are supposed to belong to the TACY group. The cytolytic activity of the TACY group toxins is observed upon the addition of thiol compounds, for example, 2-mercaptoethanol or dithiothreitol (DTT). The hemolysin produced by these bacteria was named klebolysin, and it was established that it is inactivated in the presence of cholesterol and cross-reacts with antibodies directed against streptolysin O (Szramka 2001).

2.5. *K. pneumoniae* biofilm

Over the past few years, there has been a growing problem of infections caused by *K. pneumoniae* due to the bacteria's ability to form a biofilm (Dsouza *et al.* 2019). *K. pneumoniae* exhibits many pathogenic properties that facilitate their survival, spread in the hospital environment, and adhesion to biotic or abiotic surfaces (Piperaki *et al.* 2017). *K. pneumoniae* is characterized by a high ability to adhere and form biofilm structures, which plays an essential role in the colonization and persistence of these microorganisms on mucous membranes of the body and artificial surfaces of catheters, implants and others. Important pathogenic features of *K. pneumoniae* involved in biofilm formation include overproduction of mucus (hvKp strains), selection of strains with a specific type of envelope and transfer of adhesin genes in plasmids (Guerra *et al.* 2022).

Biofilm is defined as a complex organized multicellular, single- or multi-species structure in which bacterial cells are embedded in a matrix made of extracellular polymeric slime (EPS), where they adhere to each other and/or show adhesion to various surfaces (Piperaki *et al.* 2017). The phenomenon of biofilm formation is a process that occurs in several stages: reversible adhesion, irreversible adhesion, maturation and dispersion (Fig. 3) (Piperaki *et al.* 2017). Then, as a result of the movement of bacterial cells along with blood and other body fluids, the colonisation process of new niches begins, giving rise to a new biofilm. Mature biofilm structures are characterized by bacterial persister cells (PCs), which enable the renewal of the biofilm population (She *et al.* 2022). The biofilm formation process is controlled by "*quorum sensing*", a unique intercellular communication system regulated by chemicals called signaling molecules (Piperaki *et al.* 2017).

Bacteria in biofilms, including pathogens such as *K. pneumoniae*, display highly developed adaptive capabilities that enable them to survive under challenging conditions, colonize new environments, and evade the host immune system (Piperaki *et al.* 2017; Dsouza *et al.* 2019; Thoraninsdottir *et al.* 2020; Guerra *et al.* 2022; Centeleghe *et al.* 2023). Mature biofilm provides a protective barrier against the effects of antibiotics and disinfectants. One of the possible mechanisms of antibiotic resistance is limited penetration of antibiotics into the bacterial cell and reduced metabolism of cells located inside the biofilm. Even if antibiotics reach the bacteria, the cells inside the biofilm are less metabolically active, making antibiotics that target rapidly dividing cells (e.g., β-lactams) less effective. Biofilm constitutes a physical barrier for immune cells such as neutrophils and macrophages, hindering their access to the bacteria inside (Piperaki *et al.* 2017; Dsouza *et al.* 2019; Thoraninsdottir *et al.* 2020; Guerra *et al.* 2022; Centeleghe

et al. 2023). The biofilm matrix can also bind components of the complement system, which hinders its activation and limits the effectiveness of the immune response. Bacteria in a biofilm can survive in conditions lethal to planktonic (free-swimming) bacteria. Low nutrient or oxygen availability is compensated by bacteria differentiating into different metabolic states. Currently, the phenomenon of biofilm formation is related mainly to the rapid development of biomaterials engineering (biomedical materials) and their extensive use in various fields of modern medicine (Chung *et al.* 2016; Piperaki *et al.* 2017; Zheng *et al.* 2018; Dsouza *et al.* 2019; Thoraninsdottir *et al.* 2020; Ochońska *et al.* 2021).

Biomaterial surfaces with biofilm formation are an essential reservoir of etiological agents of biomaterials associated infections (BAIs) (Chung *et al.* 2016; Piperaki *et al.* 2017; Thoraninsdottir *et al.* 2020). Targeted at improving patient comfort and function, the comprehensive use of biomaterials contributes to an increase in the frequency of the risk of developing BAIs. It is currently estimated that BAIs are responsible for approximately 65–80% of all infections occurring in humans and animals (Garcia and Percival 2011; Guerra *et al.* 2022). BAIs include device-related and non-device-related infections due to streptococci, staphylococci, Gram-negative bacteria and/or fungal infections (Jamal *et al.* 2018). A study of clinical strains of *K. pneumoniae* reported that 72.7% of tested isolates detected on medical devices were biofilm producers. However, they remained susceptible to different classes of antibiotics (Folliero *et al.* 2021). Another study confirmed the survival of *K. pneumoniae* on dry surfaces in biofilm (Centeleghe *et al.* 2023). The presence of viable but non-culturable (VBNC) bacteria indicated that *K. pneumoniae* could survive on surfaces for up to 4 weeks. It was possible to remove these bacteria from surfaces by mechanical wiping. The study proved the need for robust cleaning regimens in the hospital (Centeleghe *et al.* 2023). Another research showed that 44.4% of the tested clinical strains of *K. pneumoniae* could form biofilm on the surfaces of tracheostomy tubes made of polyethylene and polyvinyl chloride. The biofilms formed on the inner part of these surfaces were observed using scanning electron microscopy (SEM) after only 48 hours of exposure to a bacterial suspension at a concentration of 10^6 CFU/ml (Ochońska *et al.* 2021). A varied degree of biofilm formation by clinical *K. pneumoniae* strains was also found on venous catheters made of polyurethane and urinary catheters made of latex, polyvinyl chloride and silicone. In a study of the penetration of antibiotics (β-lactams, quinolones, aminoglycosides and trimethoprim) and disinfectants (chlorhexidine, ethacridine lactate, hydrogen peroxide, polyhexanidine, povidone-iodine and octenidine) into the biofilm formed by *K. pneumoniae*, the minimum inhibitory concentration (MIC) for bacteria in the biofilm was higher than in the planktonic form (Bartoszewicz *et al.* 2011). Preliminary analyses of the effect of erythromycin on the biofilm formed by *K. pneumoniae* strains showed that macrolides affect the synthesis of the AI-2 autoinducer system, for example, by reducing the expression level of *lux*S genes, blocking the autoinducer synthase enzyme or the signal molecule itself (Martínez and Baquero 2002). The search for agents that would prevent the formation of *K. pneumoniae* biofilm or cause its breakdown is still ongoing, e.g., studies involving attempts to coat catheters with various substances, e.g., silver (Mousavi *et al.* 2023) or to use specific antimicrobial preparations such as octenidine hydrochloride or sodium hypochlorite (Stoffel *et al.* 2020; Huang *et al.* 2022). Attempts are also being made to use some enzymes that eliminate slime or block the metabolic pathways of bacteria, leading to their multiplication and biofilm formation (e.g. DNA-ze, oxindole-L-alanine, a tryptophanase inhibitor regulating the disintegration of tryptophan to indole) (Mączyńska *et al.* 2015). In addition, research is being conducted on regulating *K. pneumoniae* biofilm production. New genes related to this process are being discovered, such as *lux*S – synthesis of autoinducer, *lux*R – coordination of biofilm formation steps, e.g. regulation of synthesis of various virulence factors (including "*quorum sensing*"), *fim*A – regulation of specific adhesion, *mag*A, *rmp*A, *rmp*A2 – regulation of mucus production in hvKp strains (Widmer *et al.* 2007; Mączyńska 2015).

Fimbrial and non-fimbrial adhesins also play a vital role in BAIs by *K. pneumoniae* (Alcántar-Curiel *et al.* 2013). These bacterial structures actively participate in adhesion to epithelial cells, which facilitates colonization, which is the first stage of infection. A thorough understanding of the structures involved in bacterial adhesion and invasion may contribute to the discovery of effective inhibitors of these processes, which will allow for effective treatment at the beginning of the disease, which will enable effective suppression of the disease development (Davies *et al.* 2009; Kalia 2013; Gopu *et al.* 2015; Ribeiro *et al.* 2015; Wang *et al.* 2022).

The main approaches to reduce biofilm development involve modifying the surface of materials to reduce microbial adhesion. In recent years, many research teams have focused on low-molecular-weight compounds (small molecules) capable of inhibiting biofilm development. In a study by Davies and Marques (2009), it was shown that cis-2-decylenic acid, produced by a strain of *Pseudomonas aeruginosa*, can eradicate the mature biofilm of various bacterial species including *K. pneumoniae* (Davies *et al.* 2009). In another study, new chemical entities (NCEs) with activity against *K. pneumoniae* and *Acinetobacter baumannii* could be used in new therapies for drug-resistant infections (Blasco and Piddock

2024). A promising strategy to combat BAIs is application coatings that exhibit bacteriostatic or bactericidal properties (Siddique and Muzammil 2020). Siddique and Muzammil (2020) demonstrated the efficacy of silver nanoparticles (AgNPs) as safe antimicrobial and antibiofilm compounds against MDR *K. pneumoniae* (Siddique and Muzammil 2020). Among the intensively researched biological methods of *K. pneumoniae* biofilm eradication, phagotherapy appears promising (Zurabov *et al.* 2023). Another strategy for biological control of *K. pneumoniae* biofilm is using enzymes targeting the polysaccharide matrix (matrix-targeting enzymes) (Ribeiro *et al.* 2015). Interference with the structure or degradation of the extracellular polymeric matrix of the biofilm can effectively weaken it or lead to its dispersal (Ribeiro *et al.* 2015).

Inhibition of quorum sensing (QS) systems, called Quorum Quenching (QQ), is now considered another promising strategy to combat biofilm-forming bacteria (Kalia 2013). Many substances of natural and synthetic origin with the function of quorum sensing inhibitors (QSIs) have become known and may have potential therapeutic applications. Plant compounds are considered one of the most important groups of QSIs due to their chemical structure similarities to acylated homoserine lactone (AHL) and their ability to degrade protein transcriptional regulators (LuxR/LasR) (Kalia 2013). A study by Gopu *et al.* (2015) showed the quorum quenching activity of anthocyanin malvidin from *Syzygium cumini* (L.) Skeels against *K. pneumoniae* (Gopu *et al.* 2015). Promising results were obtained for two molecules (3-methyl-2(5H)-furanone and 2-hydroxycinnamic acid) that can be developed as a complement to antibiotics (Cadavid and Echeverri 2019). A study by Ahmad *et al.* (2020) attempted to identify new inhibitors of SdiA (a homolog of the transcriptional regulator LuxR) of *K. pneumoniae* using various computational techniques (Ahmad *et al.* 2020). A study by Liu *et al.* (2020) showed that tea polyphenols can act as an effective QS inhibitor, enhance resistance to *K. pneumoniae* infection in a *Caenorhabditis elegans* model, and may serve as a novel antiviral agent to combat bacterial pathogens (Liu *et al.* 2020). A study by Wang *et al.* (2022) showed that chlorogenic acid (CA) may be an effective antimicrobial and antiviral compound that can target QS in hvKp infections, thus providing a new therapeutic direction for treating bacterial infections (Wang *et al.* 2022).

3. Conclusion

K. pneumoniae is an example of a microorganism that has evolved from a common opportunistic microorganism to one of the most dangerous pathogens causing serious healthcare-acquired infections – HAIs. Infections caused by this bacterium are characterized by a severe and progressive course, requiring prolonged hospitalization of patients. They are often challenging to treat due to the ease of acquiring new virulence and antibiotic-resistance traits by *K. pneumoniae.* The accumulation of virulence factors in bacterial strains of *K. pneumoniae* significantly impacts their ability to cause disease and survive in the host. Through mechanisms of horizontal gene transfer, regulation of gene expression, biofilm formation and increased envelope production, these bacteria can effectively evade the host immune system. In addition, the spread of virulence mechanisms is facilitated by the development of civilization and the faster and unrestricted movement of highly virulent *K. pneumoniae* strains. In the face of these threats, knowledge about *K. pneumoniae* should be continuously updated to capture the changing pathogenicity characteristics to prevent future infections.

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