Mechanisms of Gentamicin Resistance in *Listeria monocytogenes*: A Mini Review
(Mekanisme Rintangan Gentamisin dalam *Listeria monocytogenes*: Suatu Kajian Mini)

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ABSTRACT

Listeria monocytogenes is a Gram-positive foodborne pathogen capable of causing a foodborne infection known as listeriosis. There are two main types of listeriosis: a non-invasive gastroenteritis and an invasive infection which is often associated with a high mortality and hospitalization rate among susceptible individuals. Gentamicin, used as an adjunct therapy with ampicillin, remains the treatment of choice for the life-threatening invasive listeriosis. Nevertheless, there is little data on gentamicin resistance determinants in *L. monocytogenes.* Several well-controlled studies have reported that mechanisms of gentamicin resistance in this organism involve active efflux and genetic determinants that affect the uptake of the antibiotic through altered membrane potential. This mini review summarises current knowledge of genetic determinants of gentamicin resistance in *L. monocytogenes*, with the aim of contributing information that could facilitate the discovery of new therapeutic approaches to overcome, delay or avoid developments of drug resistance in this foodborne pathogen.

Keywords: *atpG2*; efflux; gentamicin resistance mechanisms; heme gene; *Listeria monocytogenes*; membrane potential

ABSTRAK

Listeria monocytogenes ialah patogen bawaan makanan Gram-positif yang mampu menyebabkan jangkitan bawaan makanan yang dikenali sebagai listeriosis. Terdapat dua jenis listeriosis utama: gastroenteritis bukan invasif dan jangkitan invasif yang sering dikaitkan dengan kadar kematian yang tinggi dan kemasukan ke hospital dalam kalangan individu yang terdedah. Gentamisin yang digunakan sebagai terapi tambahan dengan ampisilin, kekal sebagai rawatan pilihan untuk listeriosis invasif yang mengancam nyawa. Namun begitu, terdapat sedikit data tentang penentu rintangan gentamisin dalam *L. monocytogenes*. Beberapa kajian terkawal telah melaporkan bahawa mekanisme rintangan gentamisin dalam organisma ini melibatkan efluks aktif dan penentu genetik yang mempengaruhi pengambilan antibiotik melalui potensi membran yang diubah. Kajian mini ini meringkaskan pengetahuan semasa tentang penentu genetik rintangan gentamisin dalam *L. monocytogenes* bertujuan untuk menyumbang maklumat yang boleh memudahkan penemuan pendekatan terapeutik baharu untuk mengatasi, menangguhkan atau mengelakkan perkembangan rintangan dadah dalam patogen bawaan makanan ini.

Kata kunci: *atpG2*; efluks; gen heme; *Listeria monocytogenes*; mekanisme rintangan gentamisin; potensi membran

INTRODUCTION

Listeria monocytogenes

The genus *Listeria* consists of a group of Gram-positive, small rod-shaped, non-spore forming, and facultatively anaerobic bacteria of the family *Listeriaceae* (Orsi & Wiedmann 2016). Generally, members of this genus are catalase-positive, oxidase-negative, and have a low GC genome content (<50%). They are commonly found to be motile at low temperatures (Luque-Sastre et al. 2018). To date, there are up to 20 known

species in the genus *Listeria* (Nwaiwu 2020), with *L. monocytogenes* being the main pathogenic species of the genus.

L. monocytogenes is an opportunistic foodborne pathogen which is widely distributed in nature. It can be found in a variety of environmental sources, such as soil, water, sewage, silage, vegetation, waste effluent and faeces of animals and humans (Freitag, Port & Miner 2009). High-risk foods which are prone to contamination by *L. monocytogenes* include ready-to-eat vegetables, processed meat, uncooked poultry products, unpasteurised dairy products, smoked fish and raw seafood (Olaimat et al. 2018). This pathogen, which is commonly found in the environment, is able to infiltrate the food chain and food-processing facilities (Buchanan et al. 2017; Fharok 2019). As a result, governments and organisations responsible for ensuring food safety in countries, such as the United States of America (USA), Austria, Australia, New Zealand, and Italy, have implemented a zero-tolerance policy for *L. monocytogenes* (absence of the organism in 25 g of food sample) (Obaidat et al. 2015). Although *Listeria* infections are not commonly reported in Malaysia, several studies have demonstrated a high prevalence of *L. monocytogenes* in local foods (Fharok 2019; Jamali, Chai & Thong 2013; Kuan et al. 2017; Wai et al. 2020), indicating potential spread and risk of outbreaks in this country. One notable listeriosis outbreak was linked to the consumption of rock melons (cantaloupe) from a farm in Australia. This outbreak resulted in 22 confirmed cases, 1 miscarriage, and 7 deaths. Through whole genome sequencing, the isolates from patients were linked to those 37 rock melons from the farm and its processing and packaging areas. A worldwide product recall was carried out because those contaminated batches of rock melons were also distributed internationally to eight other countries, including Malaysia (Desai et al. 2019).

The prevalence of this resilient organism in the environment is aided by its ability to adapt and withstand a wide range of external stresses. It can survive and grow at a temperature ranging from 0.5 °C to 45 °C, with an optimum temperature range between 30 °C and 37 °C (Low & Donachie 1997). This is of particular concern, especially to the food industry, since it can replicate in refrigerated conditions and survive for long periods of time in frozen food products (Ramaswamy et al. 2007). It can also tolerate a wide range of pH (pH 4.3 to 9.6) and high concentrations of salt (up to 20% w/v NaCl) (Zunabovic, Domig & Kneifel 2011). Another characteristic of *L. monocytogenes* is its ability to form

biofilms on various contact surfaces, including stainless steel and plastic (Bremer, Monk & Osborne 2001; Gandhi & Chikindas 2007). These biofilms are difficult to remove as they are more resistant to disinfectants and sanitisers than free-living bacterial cells (Lewis 2001).

Apart from its versatility in adapting to a broad range of extreme environmental conditions, *L. monocytogenes* is also a facultative intracellular pathogen that can invade, survive and replicate within host cells. After gaining entry to the gastrointestinal tract via the intake of contaminated food, the bacterium attaches to the surface receptors of mucosal cells and translocates through the intestinal membrane via endocytosis. Its presence triggers the host defense mechanism which causes it to be entrapped within vacuoles in phagocytic cells. However, it is able to mediate its escape from the membrane-bound vacuoles by secreting listeriolysin O, a virulence factor encoded by the *hlyA* gene, which degrades the vacuolar membranes. It then enters the host cytoplasm where it divides rapidly and spreads to adjacent cells by using the actin polymerization as a motility force (Tilney & Portnoy 1989). Through this series of steps, this foodborne pathogen establishes an infection in humans with a combination of symptoms known as listeriosis.

LISTERIOSIS

Human listeriosis can manifest as a non-invasive or invasive infection. The non-invasive infection is a mild febrile form of gastroenteritis which affects mainly healthy individuals. The usual symptoms include fever, vomiting, diarrhoea, abdominal pain, fatigue, and myalgia. Most healthy individuals will recover without any medical intervention (Dalton et al. 1997). This self-limiting, non-invasive infection normally lasts for 9 to 32 hours after the ingestion of food contaminated with a high amount of *L. monocytogenes* (Olaimat et al. 2018).

Invasive listeriosis is a more severe disease in which infection usually spreads to the circulatory and central nervous systems of susceptible individuals, resulting in septicaemia, meningitis or meningoencephalitis (Reda et al. 2016). Cerebral listeria infections, such as rhombencephalitis, brain abscess, meningitis and meningoencephalitis, are more commonly seen in elderly patients (>50 years old) (Brouwer et al. 2006). Perinatal infections may result in abortion and stillbirth (Buchanan et al. 2017).

Invasive listeriosis is associated with a high mortality rate (20 - 30%) among populations with

underlying health conditions (Goulet et al. 2012). In comparison, other common foodborne pathogens, such as *Salmonella* spp. and *Escherichia coli* O157:H7, cause infections with much lower mortality rate of less than 1% (Scallan et al. 2011).

TREATMENT OF LISTERIOSIS

Invasive listeriosis warrants early treatment with effective antibiotics. The most common and preferred antibiotic treatment for severe listeriosis is with a β-lactam (ampicillin or penicillin) alone or in combination with an aminoglycoside (classically gentamicin). In general, *Listeria* isolates are tolerant to β-lactam antibiotics, with killing achieved only at extremely high concentrations and after a prolonged exposure to the drugs (Hof 2004; Winslow Damme & Dieckman 1983). Gentamicin is one of the most widely used aminoglycosides in the treatment of life-threatening infections. It acts by binding to the 16S rRNA of the 30S ribosomal subunit, interfering with the translation of mRNA and causing the formation of truncated or non-functional proteins which damage the membrane and other parts of the bacterial cell, leading to rapid cell death (Beganovic et al. 2018). Studies have shown that the addition of gentamicin to β-lactam treatment gives a synergistic effect that is beneficial especially to higher-risk patients with *Listeria* central nervous system infections and endocarditis (Castellazzi, Marchisio & Bosis 2018; Crum 2002; Hof 2004; Mylonakis, Hohmann & Calderwood 1998). In this combination therapy, the β-lactam antibiotic breaks down the bacterial cell wall to allow the entry of gentamicin, which is a strong bactericidal drug, into the cytoplasm of the bacterial cell (Beganovic et al. 2018; Hof 2004). However, the incorporation of gentamicin into the treatment regimen for listeriosis has also been questioned by some researchers. Some animal model studies have shown conflicting results on the effectiveness of aminoglycosides for the treatment of listeriosis, as these antibiotics are unable to pass the blood-brain barrier (Crum 2002; Temple & Nahata 2000). Additionally, due to the potential nephrotoxicity of gentamicin, this antibiotic has to be removed after 1 - 2 weeks of treatment, especially in elderly patients and patients treated alongside with other nephrotoxic drugs, such as cyclosporin A (Hof 2004).

Gentamicin Resistance in L. monocytogenes

Although rare, gentamicin resistance has been documented in *L. monocytogenes* over the years. In Greece, Tsakris, Douboyas and Antoniadis (1997) reported gentamicin resistance (minimum inhibitory concentration [MIC] > 8 mg/L) in a case of *L. monocytogenes* meningitis. In the USA, Prazak et al. (2002) isolated two gentamicin-resistant strains of *L. monocytogenes* (MIC range not reported) from cabbage farm packing sheds. In Germany, it was reported that 4.6% (12/259) of *L. monocytogenes* isolated from food, environmental and human samples to be gentamicin-resistant (MIC > 1 mg/L) (Noll, Kleta & Al Dahouk 2018). More recently, it was noted that 53.3% (73/137) of *L. monocytogenes* strains recovered from Egyptian dairy cattle farms were resistant to this antibiotic (MIC range not reported) (Elsayed et al. 2022).

On the other hand, there have been studies that demonstrated 100% gentamicin susceptibility among the *L. monocytogenes* isolates in their cohorts. For example, Srinivasan et al. (2005) found that all their *L. monocytogenes* strains (n = 38) recovered from four dairy farms in the USA were gentamicin-sensitive (MIC range = 0.5 - 4 mg/L). Another study in Poland, which performed antimicrobial susceptibility testing on 344 invasive *L. monocytogenes* isolates collected from 1997 to 2013, showed that these isolates were 100% susceptible to gentamicin (MIC range = 0.03 - 0.5 mg/L) (Kuch et al. 2018).

From our literature search (publications dated from the 1970s until 2023), we were unable to find reports on the incidence of treatment failure in patients on gentamicin therapy for listeriosis (due to resistant strains of *L. monocytogenes*) or the development of gentamicin resistance while on therapy.

One issue with the interpretation of gentamicin susceptibility results is the lack of established breakpoints for *Listeria* (CLSI 2016; EUCAST 2023). As a result, some researchers resorted to using breakpoints for other Gram-positive bacteria such as *Staphylococcus* and *Enterococcus*. This created some difficulty in the interpretation and comparison of gentamicin resistance statistics from different clinical and epidemiological reports. In this mini review, we report gentamicin resistance rates as defined by individual authors.

Mechanisms of Gentamicin Resistance in L. monocytogenes

Antibiotic resistance in *L. monocytogenes* is due to genetic determinants that are intrinsic or acquired via horizontal gene transfer or mutations. The genetic determinants of gentamicin resistance that have been described for *L. monocytogenes* are summarised in Table

1.

TABLE 1. Gentamicin resistance determinants in *Listeria monocytogenes* from well-founded studies

Gene(s)	Mode of resistance development	Mechanism	Reference(s)
Efflux gene(s)*	Over-expression	Efflux	Rakic-Martinez et al. (2011)
Heme genes (*hemA* and *hemH*)	Mutations	Reduced uptake of drug (decreasing bacterial membrane potential)	Christensen, Gram & Kastbjerg (2011), Curtis, Gram & Knudsen (2016), Kastbjerg, Hein-Kristensen & Gram (2014)
atpG2	Mutations	Reduced uptake of drug (decreasing bacterial membrane potential)	Ng et al. (2022)

*This resistance determinant was elucidated based on the results of the efflux inhibitor assay. The authors noted an over-expression of the *lde* efflux gene but did not confirm if this efflux is the determinant that contributes to gentamicin resistance

EFFLUX

Active efflux has been associated with gentamicin resistance. Rakic-Martinez et al. (2011) reported a 2- to 8-fold decrease in gentamicin susceptibility of *L. monocytogenes* after an exposure to benzalkonium chloride (BC), which is one of the most widely used disinfectants in the food processing industry, and ciprofloxacin. Susceptibility to gentamicin was, however, restored in the presence of an efflux inhibitor (reserpine), suggesting that the mechanism of gentamicin decreased susceptibility in those BC-selected and ciprofloxacin-selected strains might be associated with efflux pumps. Interestingly, it was observed that the gentamicin resistance in a BC-selected strain coincided with the over-expression of the gene encoding the multidrug-resistance efflux *lde*, which was previously shown to confer fluoroquinolone resistance in *L. monocytogenes* (Godreuil et al. 2003). Unfortunately, Rakic-Martinez et al. (2011) could neither confirm the causal role of *lde* overexpression in the gentamicin resistance phenotype nor rule out the possible involvement of other efflux pumps.

HEME GENES

It was demonstrated that short-term exposure of *L. monocytogenes* to sub lethal triclosan concentrations resulted in resistance to several aminoglycosides (including gentamicin) with an inheritable increase in MIC of 4- to 8-fold (Christensen, Gram & Kastbjerg 2011). They noted that some of these resistant isolates appeared in pinpoint-size colonies (<1 mm in diameter). Using whole-genome sequencing, this group of researchers found that all their pinpoint-sized isolates had a mutation in a heme gene (either *hemA* or *hemH*) (Curtis, Gram & Knudsen 2016; Kastbjerg, Hein-Kristensen & Gram 2014) which plays an important role in the electron transport chain. Their findings corroborated those of earlier research that identified aminoglycoside resistance in small colony variants of *Staphylococcus aureus* (McNamara & Proctor 2000). Apparently, this heme gene mutation-associated resistance is only seen with compounds that require a large membrane electrochemical gradient for uptake such as aminoglycosides. The uptake of aminoglycosides by a bacterial cell usually takes place at a higher membrane potential (Mates et al. 1982). Mutations that affect the electron transport could reduce the membrane potential, thereby decreasing the uptake of aminoglycosides and leading to the expression of resistance (McNamara & Proctor 2000). Therefore, small colony variant cells of bacteria could potentially serve as a reservoir for recurrent or prolonged infections.

atpG2

In 2022, Ng et al. characterised a gentamicin-resistant mutant (B2b) (MIC = 40 mg/L) derived from the previously susceptible *L. monocytogenes* ATCC

19115 (MIC = 2.5 mg/L). The gentamicin resistance in B2b was caused by a 10-bp deletion in *atpG2* which encodes a gamma subunit of the ATP synthase in *L. monocytogenes*. Using *atpG2* PCR, various other mutations (c367t, c388t, t689a, and del772-781) were identified in most of the gentamicin resistant mutants derived from ATCC 19115, indicating that *atpG2* mutations could be a major driving force of gentamicin resistance in *L. monocytogenes*.

When the gamma subunit of the ATP synthase is mutated, the influx of protons into the cell membrane may become unregulated, leading to a decrease in the membrane potential. Similar to the effect of mutations in a heme gene, this could prevent the uptake of the antibiotic, resulting in the development of gentamicin resistance (Mates et al. 1982; Ramirez & Tolmasky 2010).

OTHER ALLUDED (BUT UNCONFIRMED) MECHANISMS OF GENTAMICIN RESISTANCE IN L. *Monocytogenes*

AMINOGLYCOSIDE-MODIFYING ENZYMES

Aminoglycoside-modifying enzymes can be classified as aminoglycoside acetyltransferases (AACs), aminoglycoside phosphotransferases (APHs) and aminoglycoside adenyltransferases (ANTs). The gentamicin resistance gene *aadB* (encoding an ANT) was detected in a food isolate of *L. monocytogenes* showing phenotypic resistance to gentamicin (Wiśniewski et al. 2022). However, this finding was purely observational because the authors did not perform further experiments to confirm the causal relationship between this gene and gentamicin resistance in *L. monocytogenes*.

Genes encoding aminoglycoside-modifying enzymes are normally acquired by horizontal gene transfer. The gentamicin resistance gene, *aac6'-aph2*, was found to be carried by a composite transposon that occurred in the conjugative plasmid pIP501. This bifunctional, gentamicin modifying enzyme is able to mediate high-level gentamicin resistance in *Enterococcus* (MIC > 2000 mg/L) (Leclercq et al. 1992; Sparo, Delpech & Allende 2018). Interestingly, this plasmid, widely disseminated among *Streptococcus, Enterococcus*, and *Staphylococcus* (Horaud, De Céspèdes & Trieu-Cuot 1996), has been demonstrated to be transferable from *Streptococcus* to *Listeria* and re-transferable back to *Streptococcus* (Kohler, Vaishampayan & Grohmann 2018; Vicente, Baquero &

Pérez-Diaz 1988). The transfer/re-transfer mechanism involves the translocation of a single stranded plasmid DNA via a type IV secretion system (a biological 'molecular syringe' produced by many bacteria that transport DNA, proteins, or DNA-protein complexes into other cells) to a recipient cell (Kohler, Vaishampayan & Grohmann 2018). Therefore, it should theoretically be possible for *L. monocytogenes* to acquire this resistance gene from other Gram-positive organisms in the digestive tracts of humans and animals.

16S rRNA MUTATIONS

Gentamicin is a protein synthesis inhibitor that binds to A1408 and G1494 in 16S rRNA (Moazed & Noller 1987). Surprisingly, to date, no studies have reported 16S rRNA mutations that are associated with gentamicin resistance in *L. monocytogenes*. A possible explanation could be that this organism has six identical copies of the 16S rRNA gene (Somer & Kashi 2003). When amplified by the same primer set, mutations within a single allele could be 'concealed' by other non-mutated DNA of other alleles in *L. monocytogenes* (Christensen, Gram & Kastbjerg 2011). Likewise, in whole-genome sequencing, multicopy genes also present a particular challenge to identify such mutations, as most assembly algorithms seek to form a consensus sequence from sequencing reads (Su, Satola & Read 2019). Therefore, it seems plausible that these elusive 16S rRNA variants could have gone unnoticed in gentamicin-resistant strains of *L. monocytogenes* due to a lack of convenient and adequate screening procedures.

TRANSITION TO ANAEROBIC RESPIRATION

Gentamicin passes through the bacterial cell membrane in an oxygen-dependent active transport (Chaves 2023). Therefore, the use of aminoglycosides, including gentamicin, is not effective against obligate anaerobic organisms such as *Bacteroides fragilis* and *Clostridium perfringens*. Facultative anaerobes, when grown under low oxygen conditions, were found to be less susceptible to aminoglycosides (Bryan, Kowand & Van den Elzen 1979). It is known that some facultative anaerobes transitioned into the anaerobic phase to evade aminoglycoside killing. A transcriptomic study demonstrated that, when *L. monocytogenes* (a facultative anaerobe) was exposed to the sublethal concentration of four different antibiotics (including gentamicin), a switch from aerobic to anaerobic mechanisms was observed in this pathogen (Knudsen et al. 2016). However,

anaerobiosis-induced mechanism of gentamicin resistance has yet to be confirmed in *L. monocytogenes.*

CONCLUSIONS AND FUTURE PERSPECTIVES

Research on antibiotic resistance mechanisms contributes significantly to the better management of infectious diseases. In the case of invasive listeriosis, gentamicin is an important drug for treatment. Although resistance to this drug is still relatively rare among clinical strains of *L. monocytogenes*, studies in recent decades have identified multiple mechanisms and determinants of resistance. It can also be envisaged that continual or increasing exposure to gentamicin might induce the pathogen to acquire even more mechanisms or higher levels of resistance. Fortunately, the application of molecular drug susceptibility testing in the microbiology laboratory allows the inclusion of resistance determinants into routine drug susceptibility testing panels. This enables the rapid identification of resistant strains for the selection of appropriate drugs for therapy. Further investigations on resistance mechanisms might lead to the production of novel and more effective ways to overcome the problem of gentamicin resistance in the treatment of invasive listeria infections.

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