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Fatal Attraction: How the Overuse of Colistin led to the Evolution of Colistin Resistance

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ABSTRACT

Antibiotics resistance is a worldwide problem, with numerous bacterial infections having already become very challenging to treat. Colistin, a last-resort antibiotic, is often used in case of infection by a multiple resistant bacterial strain. However, an increasing amount of bacteria previously susceptible to colistin have been showing resistance against it, which endangers human and animal health. This essay aimed at reviewing existing literature about colistin resistance and using this information to clarify the evolution of colistin resistance, how it started, what mechanisms are responsible for it and how it can be transferred to humans. Furthermore, literature was also used to investigate what can be done to reduce colistin resistance and how to prevent it from spreading in the future. It was found that the excessive use of colistin in human medicine and animal husbandry practices are the main reasons for the increase in colistin resistance. Bacteria can evolve colistin resistance in their genome, but it can also be acquired through horizontal gene transfer. The horizontally transferred genes are most common in animal husbandry practices, and, by consuming contaminated food, humans can be infected or colistin resistance can be transferred to humans via the human gut microbiome. Most important now is avoiding the further evolution and dispersal of colistin resistant bacterial strains by reducing the use of colistin and limiting it to the essential cases.

Key words: Colistin, Antibiotic resistance, Animal Husbandry Practices, Evolution of resistance, Horizontal Gene Transfer

1. INTRODUCTION

The resistance of bacterial strains to antibiotics is becoming an increasing issue in both high and low income countries (Davies & Davies, 2010; Laxminarayan *et al.*, 2013). Resistance can evolve at a higher rate under selection pressure on resistance genes - both chromosomal and extrachromosomal - due to excessive use of antibiotics. Increased global trade and travel, poor sanitation or hygiene, and the possibility for interspecies gene transmission are all factors contributing to the evolution of antibiotics resistance (Laxminarayan *et al.*, 2013). This poses a great threat to human health, as it can lead to patients suffering from more serious, prolonged infections, which result in higher mortality rates (Ashbolt *et al.*, 2013).

Colistin, a last resort antibiotic also known as polymyxin B, was discovered in 1947 (Storm *et al.*, 1977; Pogue *et al.*, 2017; Barlaam *et al.*, 2019). It is a polypeptide antibiotic that was originally retrieved from *Bacillus polymyxa.* It functions by altering the bacterial membrane structure of Gram-negative bacteria (Storm *et al.*, 1977; Barlaam *et al.*, 2019). It shows only very narrow ability against Gram-positive bacteria due to a limited ability to bind to their cytoplasmic membranes (El-Sayed Ahmed *et al.*, 2020). Therefore, colistin is most often specifically used in case of infections caused by Enterobacteriaceae or other multiple-resistant Gram-negative bacteria (Storm *et al.*, 1977; Kempf *et al.*, 2016)

The use of antibiotics in animal husbandry practices is very common for promoting animal health and growth of such healthy animals. It is estimated that the use of antibiotics in animal husbandry practices strongly exceeds that of human medicine (Barlaam *et al.*, 2019). In Belgian veal calves for example, it was shown that on an average of 1000 calves, around 417 are treated with any type of antimicrobials on a daily basis (Hoelzer *et al.*, 2017). This excessive use also happens with colistin, which is often utilized in veterinary practices to promote the growth of the animals or to treat infections (Kempf *et al.*, 2016). The excessive use of colistin in animal husbandry practices may have played a large role in the evolution of colistin resistance (Barlaam *et al.*, 2019).

Colistin serves as a last-resort antibiotic that is only used in very specific and highly necessary cases, namely in infections caused by multiple-drug resistant bacteria or in patients suffering from cystic fibrosis (Kempf *et al.*, 2016). The emerging resistance against it could therefore have disastrous consequences for human and animal health care. This essay will review available literature on the evolution of colistin resistance, specifically examining the role of animal husbandry. Furthermore, it aims to determine which mechanisms in bacteria are responsible for establishing this resistance and how we can try to prevent the evolution of further colistin resistance.

2. COLISTIN USE AND FUNCTIONING

Colistin was originally discovered in 1947 and has been used in human and animal medicine ever since (Storm *et al.*, 1977). Colistin functions by targeting the lipopolysaccharide component (LPS) of the outer membrane. It interacts with the lipid A component of this membrane. The interaction between the negatively charged phosphate groups of lipid A and the positively charged acid residues of colistin displaces the divalent cations: magnesium (Mg^{2+}) and calcium (Ca^{2+}). This renders the cell destabilized and vulnerable (Velkov *et al.*, 2010).

In the 1970s, it was discovered that colistin had disastrous side-effects, with the potential to cause sever renal and neurological problems. Therefore, its use on human patients decreased drastically and it was replaced by less toxic antibiotics (Rhouma *et al.*, 2016; Lima *et al.*, 2019). However, after the increasing prevalence of multi-drug resistant bacteria by the mid-1990s, it was once again applied in the treatment of infections (Spapen *et al.*, 2011; El-Sayed Ahmed *et al.*, 2020). Two forms of colistin are administered to humans: colistin sulphate and colistin methanesulphate (CMS). Colistin sulphate

is commonly used for either selective digestive tract decontamination or the treatment of skin infections. CMS is administered as a last resort drug against bacteria with resistance against multiple other antibiotics (Catry *et al.*, 2015). Most commonly, it is used in case of an infection by Gram-negative multiple-resistant bacteria like *Pseudomonas aeruginosa, Acinetobacter baumannii* and *Klebsiella pneumonia* or *Enterobacter* spp (El-Sayed Ahmed *et al.*, 2020), but it can also be applied against infections of other bacteria such as *Acinetobacter or Klebsiella* spp, *Escherichia coli*, *Salmonella* spp, *Shigella* spp, *Haemophilus influenzae*, *Bordetella pertussis*, *Prevotella* spp and *Fusobacterium* spp (Balaji *et al.*, 2011).

Colistin has been commonly used in veterinary practices since the 1950s, mainly to treat infections caused by Enterobacteriaceae in many different animals (e.g. pigs or cattle). Furthermore, it is also used in aquaculture to prevent infections of Gram-negative bacteria (Catry *et al.*, 2015). Lastly, it also often serves as a growth promotor (Kempf *et al.*, 2016). Colistin is often used in combination with other antibiotics to ensure optimal impact (Catry *et al.*, 2015). Though the use of colistin in animal husbandry practices is often legally restricted to only applying the antibiotic in case of an infection that is otherwise non treatable (Barlaam *et al.*, 2019), this is challenging to actually enforce and follow up on. Furthermore, in several countries (e.g. China, Brazil or Sudan) the sales of pharmaceuticals like colistin are unregulated, making them easily accessible. Therefore, it is very well possible many animals are still administered colistin as a growth enforcer, though it is difficult to estimate exactly how common it is (Morgan *et al.*, 2011; Rhouma *et al.*, 2016).

The excessive use of colistin in animal and human medicinal practices has led to the large issue of colistin resistance, as an increasing fraction of previously susceptible bacteria are acquiring resistance (Lima *et al.*, 2018; Elbediwi *et al.*, 2019). For example, when patients are infected by *A. baumannii*, there is a high probability of mortality. Treating the infection effectively is thus of vital importance for human health. However, this poses a challenge as this bacterium is already resistant to a wide range of antibiotics including: carbapenems, tetracyclines, and β-lactams. Therefore, colistin is used as a last-resort antibiotic in a final attempt to save patients. However, *A. baumannii* strains that are resistant against colistin have recently been discovered, meaning further options to treat these infections are currently lacking (Abdelkader *et al.*, 2020).

Colistin resistance can either occur due to chromosomal mutations or due to mobilized colistin resistance (mcr) genes on the extrachromosomal DNA of bacteria (Laxminarayan *et al.*, 2013; Gharaibeh & Shatnawi, 2019), though the exact mechanisms remain unknown for certain bacteria (Poirel *et al.*, 2017).

3. CHROMOSOME-MEDIATED RESISTANCE MECHANISMS

Though resistance of bacteria can occur and develop naturally, the overuse and misuse of colistin has been identified as the leading cause for the high level of colistin resistance genes discovered in the bacterial chromosome (Barlaam *et al.*, 2019; Gharaibeh & Shatnawi, 2019).

3.1 Intrinsic resistance

Some Gram-negative bacteria have intrinsic mechanisms making them resistant against colistin. For instance, *Proteus mirabilis* and *Serratia marcescens* have a higher amount of phosphoethanolamine (PetN) and/or 4-amino-4-deoxy-L-arabinose (L-Ara4N) groups attached to their LPS, which causes the charge of the LPS to be higher, making it impossible for colistin to bind to it (Poirel *et al.*, 2017). In *K. pneumoniae* an intrinsic regulator called RamA can cause alterations in the LPS without need for any mutations in the genome. It works by binding to genes involved in the lipid A synthesis, which leads to

reduced susceptibility to colistin as it has a harder time interacting with the LPS (De Majumdar *et al.*, 2015).

3.2 LPS modification systems

Mutations on the chromosome can lead to resistance of Gram-negative bacteria against colistin. Most mechanisms focus mainly on avoiding the devastating effect colistin can have on the LPS component of the bacterial outer membrane (Velkov *et al.*, 2010; El-Sayed Ahmed *et al.*, 2020). The strategy applied here focusses on reducing the negative charge of the outer membrane of the bacteria by adding one or both cationic components L-Ara4N and PetN. In doing so, the negative charge of the outer membrane is reduced, blocking the functioning of colistin as it can no longer interact with the LPS component of the membrane (Kaye *et al.*, 2016; Poirel *et al.*, 2017; El-Sayed Ahmed *et al.*, 2020). The exact pathway responsible for activating this resistance mechanism can differ slightly between bacterial species, but, to a large extent, the same genes and operons are involved. Mutations in these genes or operons can lead to colistin resistance as described in Table 1 (Kaye *et al.*, 2016).

In many bacteria, a combination of mechanisms have been identified that all contribute to colistin resistance, such as in case of *K. pneumoniae* (Fig. 1) (Poirel *et al.*, 2017; El-Sayed Ahmed *et al.*, 2020). Sometimes though, the exact resistance mechanisms are still uncertain. Lee *et al.* (2014), for example, investigated colistin resistance in *P. aeruginosa* and identified three genes (PA1980, PA4541 and PA5447) that allowed the bacteria to be more resistant to colistin. However, their exact functioning and contribution to resistance remains unclear.

Table 1 *Overview of most important genes and operons known to contribute to colistin resistance. Mutations in these genes that can cause repression or overexpression can lead to resistance. The information to make this table was retrieved from Poirel et al., 2017.*

Gene or operon	Role of the gene or operon
pmrC gene (phosphoethanolamine phosphotransferase)	The gene is encoded by the *pmrCAB* operon, which also codes for *pmrA* and *pmrB*. *PmrC* adds a PetN group to LPS.
pmrHFIJKLM operon	Both are responsible for the synthesis of L-Ara4N and its attachment to lipid A.
pmrE gene	
pmrA gene	Together, they encode the *PmrAB* two-component system. In this system, *pmrB* is responsible for activating *pmrA*, which in turn activates the *pmrCAB* operon, the *pmrHFIJKLM* operon and the *pmrE* gene.
pmrB gene	
phoP gene	The two genes encode for the *PhoPQ* two component system. *phoP* is activated by *phoQ* and then activates the *pmrHFIJKLM* operon, and it can also activate *pmrA*.
phoQ gene	
mgrB gene	This gene has the capacity to negatively regulate the *PhoPQ* two component system, so when it is inactivated (by a mutation for example), the overexpression of the *PhoPQ* operon is a consequence.
crrAB operon (colistin resistance regulation)	Encodes for *crrA* and *crrB*, but exact physiological role is still unknown. Inactivation of *crrB* leads to overexpression of the *pmrAB* operon.

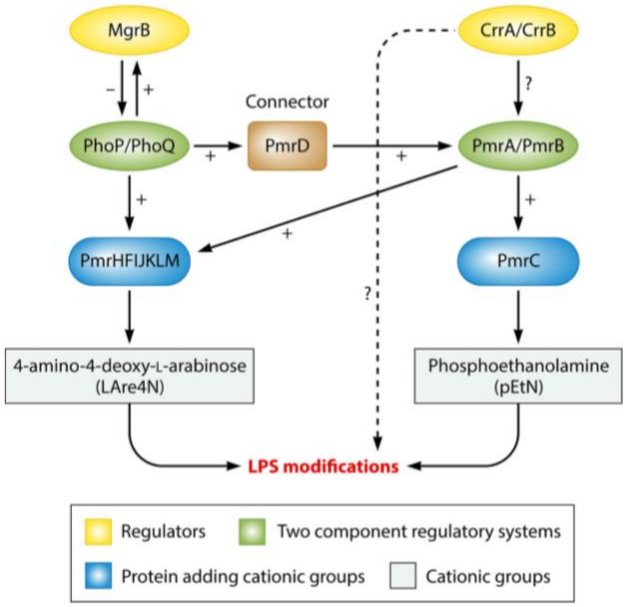

Figure 1 *Retrieved from Poirel et al. (2017). Regulation of colistin resistance by modifying the LPS component in K. pneumoniae.*

3.3 Overproduction of capsular polysaccharide

Another mechanism that can aid in the resistance of bacteria to colistin is the overproduction of capsular polysaccharide (CPS), which is located on the bacterial surface. This resistance strategy is mostly known to be used by *K. pneumoniae* (Fresno *et al.*, 2006). An increased production of CPS can lead to the overexpression of the *pmrAB* operon by inactivating *crrB* (Fresno *et al.*, 2006; El-Sayed Ahmed *et al.*, 2020). Furthermore, CPS also has the capacity to trap colistin, preventing it from binding (El-Sayed Ahmed *et al.*, 2020).

3.4 Efflux pump systems

Colistin has both hydrophilic and hydrophobic molecular parts, meaning efflux pumps can play a role in acquiring resistance against it (Lima *et al.*, 2018). However, these systems have not yet been well studied (El-Sayed Ahmed *et al.*, 2020). Evidence has however been found that efflux pumps have the capacity to promote the elimination of colistin and thus increase colistin resistance (Lima *et al.*, 2018).

4. PLASMID-MEDIATED RESISTANCE MECHANISMS

The excessive use of colistin in animal husbandry practices is held responsible for the emergence of plasmid mediated colistin resistance (Rhouma *et al.*, 2016).

Horizontal gene transfer is the process in which organisms' genetic material is transferred between two organisms, but in a non-genealogical way (Boto, 2010). It is often considered the main mode of antibiotics resistance transfer (Gyles & Boerlin, 2014). There are many cases of horizontal gene transfer between bacteria. The process is even known to occur even in eukaryotes, for example *Drosophila* (Brown, 2003). Horizontal gene transfer between bacteria is often facilitated by plasmids - which are extrachromosomal DNA molecules - or by phages (Brown, 2003; Gyles & Boerlin, 2014). The first mechanism responsible for horizontal gene transfer is transduction. In this case, genetic material is injected into a bacterium by a phage. Secondly, conjugation eases the transport of a plasmid through the use of a pilus from donor to recipient bacterium. Finally, transformation can also be a method of

horizontal gene transfer by allowing the uptake of loose DNA from the environment (Gyles & Boerlin, 2014).

Mcr genes are responsible for plasmid-mediated colistin resistance. They have all been identified as PetN transferases, and thus function by adding PetN to the outer membrane to reduce its negative charge so colistin can no longer act on it. Since the first discovery in 2015, 10 different mcr genes have been identified worldwide (Table 2; Fig. 2; Velkov *et al.*, 2010; El-Sayed Ahmed *et al.*, 2020; Wang *et al.*, 2020). Though the mcr genes function comparably, their degree of amino acid sequence similarity varies and they all have a different percentage of genetic similarity. The mcr-1 gene shows for example 82% genetic similarity to mcr-2, but only 34% similarity to mcr-4. Though genetic similarity has been researched between some of the mcr genes, it has not been investigated between all resistance genes. To do so has become very complex due to all mcr gene variants - that differ from the original mcr gene by only a small number of amino acids - available of the mcr genes. These genetic differences point towards them having different genetic origins (El-Sayed Ahmed *et al.*, 2020).

In 2015, the mcr-1 gene was discovered in China, representing the first case of horizontally transferred colistin resistance. The gene was harboured on the plasmid, which was dispersed via conjugation. Though it was officially found in *E. coli*, it was discovered to be able to reside in *K. pneumonia* and *P. aeruginosa* as well. The mcr-1 gene was identified in raw meat (15% of the samples), animals (21% of the samples), and patients with an *E. coli* infection (1% of the samples) (Liu *et al.*, 2016). Since then, a total of 22 variants of mcr-1 have been identified. It has also been found in the environment and vegetables. The mcr-1 gene is the mcr gene most commonly isolated from human samples (Barlaam *et al.*, 2019; El-Sayed Ahmed *et al.*, 2020). One year later, in 2016, mcr-2 was found in Belgium. They identified the gene in bovine and porcine colistin resistant *E. coli*, with mcr-2 found to be more prevalent than mcr-1. Though mcr-2 was found in *E. coli*, genetic analysis suggests that it actually originates from *Moraxella* spp (Xavier *et al.*, 2016). The gene has now also been identified from vaginal swap samples from China. So far, 3 genetic variants are known (El-Sayed Ahmed *et al.*, 2020). The mcr-3 gene was also retrieved first in China in 2017 from swine isolates and it was discovered in *E. coli* plasmids. The gene has since then been identified in a human *Salmonella enterica* sample from the United States (Yin *et al.*, 2017). It has already been found in Asia, Europe and North-America and a total of 30 genetic variants have been identified (El-Sayed Ahmed *et al.*, 2020). In 2017, mcr-4 was discovered in Italy in *S. enterica* isolates sampled from a pig, and the gene was also isolated from *E. coli* that same year. The used sample was however from 2013, meaning the resistance gene had been circulating far longer. Furthermore, in both Spain and Belgium, the mcr-4 gene was also identified in samples from 2015 and 2016 respectively (Carattoli *et al.*, 2017). It is hypothesized that the mcr-4 gene originates from the *Shewanella* bacteria and 6 genetic variants of the gene have been found (El-Sayed Ahmed *et al.*, 2020). Mcr-5 and mcr-6 were also discovered in 2017. Mcr-5 was first found in Germany and was retrieved from *S. paratyphi* samples from poultry (Borowiak *et al.*, 2017). So far, four genetic variants have been identified (El-Sayed Ahmed *et al.*, 2020). The mcr-6 gene on the other hand was found in *Moraxella* spp retrieved from pig samples. Its first discovery was in Great Britain and so far only one variant has been identified (Lima *et al.*, 2019). In 2018, the mcr-7 gene was discovered in *K. pneumoniae* chicken isolates in China, and mcr-7.1 is the only known variant so far. It can be horizontally transferred to *E. coli* (Yang *et al.*, 2018). Also in China, the mcr-8 gene was first found. It was identified in *K. pneumonia*e isolates from both pig and human samples in 2018 (Wang *et al.*, 2018). Since then, a total of 4 genetic variants have been discovered (El-Sayed Ahmed *et al.*, 2020). Mcr-9 was discovered in 2019 in the USA from a *S. typhimurium* strain and a total of two variants have been found since first discovery (Caroll *et al.*, 2019). Lastly, mcr-10 was found in China in 2020, though it was actually recovered from a strain of the *Enterobacter roggenkampii* that was isolated from a patient in 2016. Mcr-10 shows high genetic resemblance to mcr-9, and 82.93% amino acids are identical between

the two. Though the gene was only recently discovered, it has already been identified all over the world (Wang *et al.*, 2020).

The mcr-1 gene - which is the most studied mcr gene - has already been found on many types of plasmids with various sizes, which indicates its capacity to rapidly spread amongst bacteria (Anyanwu *et al.*, 2020). Furthermore, all mcr genes can be found on a large diversity of plasmids, like IncP, IncE and IncY for example. They are however most likely to appear on the Incl2, IncX4 or IncHI2 plasmids, which are spread all over the world (Fig. 2; Elbediwi *et al.*, 2019; Anyanwu *et al.*, 2020). All mcr genes are most commonly found on highly mobile plasmids. This poses a big threat as they thus have the capacity to spread across the world at a very fast rate (Elbediwi *et al.*, 2019). Due to the large diversity of plasmids upon which mcr genes are found, a detailed discussion of all the known variants is outside the scope of this essay.

Table 2 *Table retrieved from Gharaibeh & Shatnawi (2019) and slightly adapted, additional information retrieved from El-Sayed Ahmed et al. (2020). Table summarizing the year of first publication of each mcr gene, country in which it was found, bacterial species in which it was identified and sample origine where each mcr gene was first discovered, and number of variants that have already been discovered so far.*

Gene	Year	Country	Bacteria	Sample origin	Variants
MCR-1	2015	China	*E. coli*	Animal, human, food	22
MCR-2	2016	Belgium	*E. coli*	Animal	3
MCR-3	2017	China	*E. coli*	Animal	30
MCR-4	2017	Italy, Spain and Belgium	*E. coli, Salmonella typhimurium*	Animal	6
MCR-5	2017	Germany	*S. typhimurium*	Animal, food	4
MCR-6	2017	UK	*Moraxella pluranimalium*	Animal	1
MCR-7	2018	China	*K. pneumoniae*	Animal	1
MCR-8	2018	China	*K. pneumoniae*	Animal	4
MCR-9	2019	USA	*S. typhimurium*	Human	2
MCR-10	2020	China	*Enterobacter roggenkampii*	Human	1

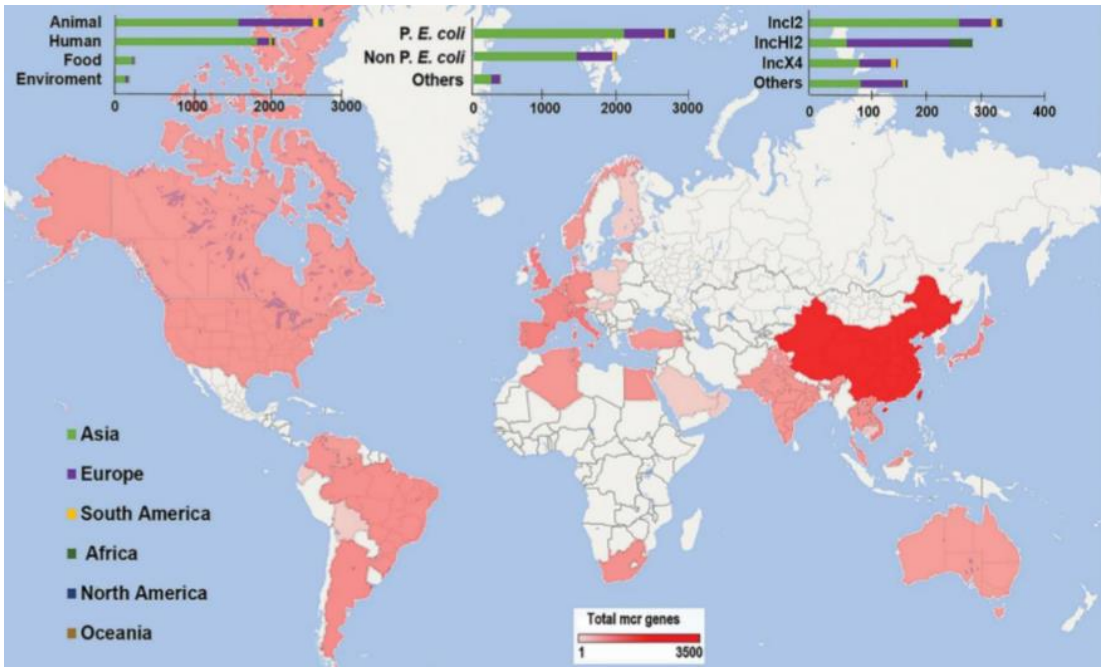

Figure 2 *Retrieved from* Elbediwi *et al.*, 2019*. Frequency of the occurrence of mcr genes, simultaneously showing the hosts, bacteria, and plasmid types the genes are mostly associated with for each of the continents. Countries coloured white do not yet have any cases on mcr genes reported.*

5. RESISTANCE TRANSFER TO HUMANS

It is clear that the excessive use of colistin brings about the resistance against it amongst pathogenic bacteria, after which these food-related microorganisms can contaminate meat or dairy products commonly consumed by humans, which can cause illness (Hoelzer *et al.*, 2017). However, an additional problem presents itself when any form of contact with the infected foods may lead to the transmission of antibiotic resistance (Barlaam *et al.*, 2019), which is why understanding how this happens is of pressing importance.

The mechanisms of horizontal gene transfer of the mcr-1 gene, found in *E. coli* and isolated mostly from retail meat, have been extensively studied and the ways on how it can then infect humans are therefore the most well-known. After extensive use of colistin in animal farms, selection pressure favours colistin resistance in bacteria. Following the spread of such resistance in the animal populations, the farmers working on that farm are exposed to the contaminated animals and resistant strains. Furthermore, the faeces produced by these animals can reach wastewater, and travel further in the aquatic environment. Contaminated water is an immense issue as it can be used on the agricultural fields for irrigation, allowing crops to be exposed to the resistant bacterial strains. It can also be consumed by farm animals, further aiding in the spread of colistin resistance in animal husbandry farms. The water can also be consumed by wild animals, although it remains unknown how the resistant strains may then be passed on to humans. Finally, the people handling the raw meat, such as slaughterhouse operators or veterinarians, are also at risk of exposure (Barlaam *et al.*, 2019). Via all these paths, plasmid-mediated resistance to colistin can be passed on to humans (Fig. 3).

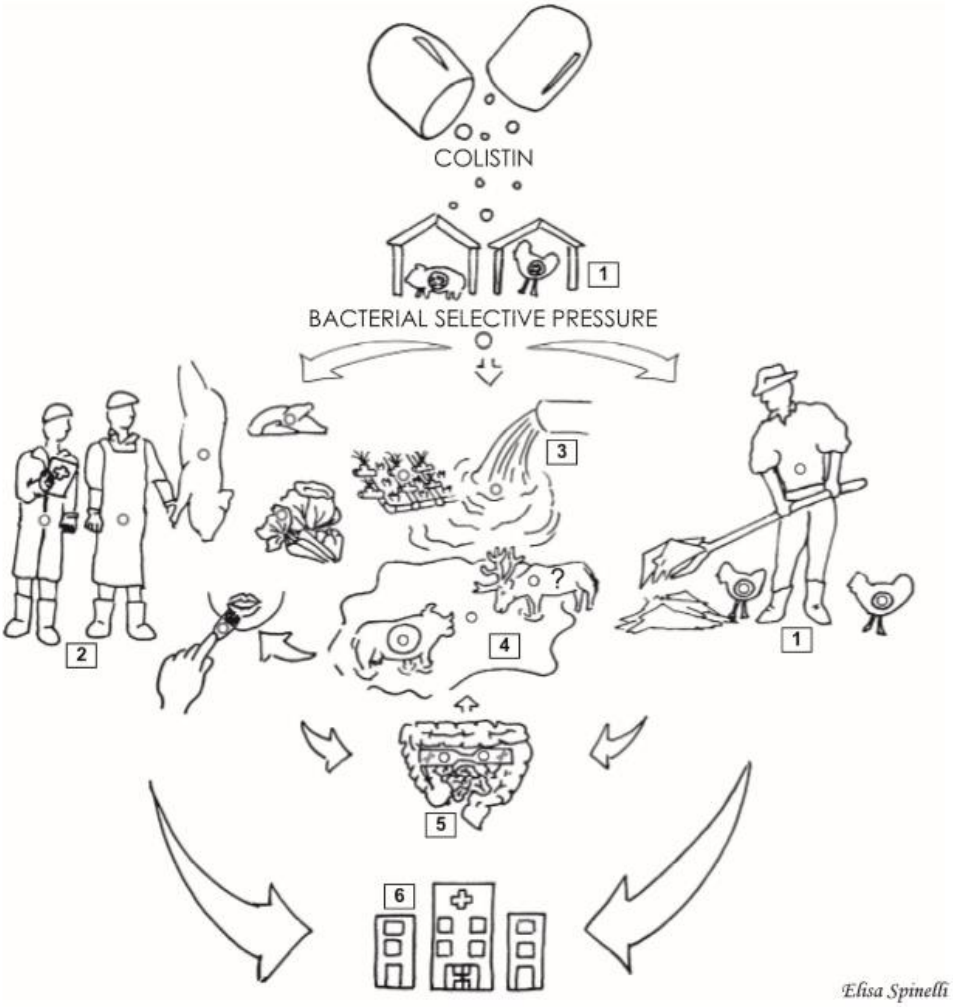

Figure 3 *Retrieved from* Barlaam *et al.* (2019). *Summarized figure of the pathways via which colistin resistant E.coli bacteria carrying the horizontally transferred mcr-1 gene, are selected for and spread in the population. 1. The selection pressure takes place on a farm, where colistin can be intensively applied and people working on farms are exposed to the threat. 2. In the slaughterhouse, both veterinarians and slaughterhouse operators come into close contacted with the possibly contaminated meat. 3. Via the faeces of the animals, the E. coli strains can reach wastewater and 4. from this water, the colistin-resistant bacteria can be transferred to wild animals, farm animals or even crops. 5. The mcr-1 gene can be passed on to humans in the gut microbiome. 6. All contact with resistant strains is dangerous to humans and can lead to them having to be administered to the hospital.*

The gastrointestinal tract plays a key role in resistance transfer as it harbours the gut microbiota. Due to the large amount of bacteria that can be found there, horizontal gene transfer can easily take place, making it a reservoir for genes allowing resistance to antibiotics (Carlet, 2012; Van Schaik, 2015). Though many bacteria only pass through the gut and do not reside there, they are still capable of horizontally transferring resistance genes to the residing microbiome (Barlaam *et al.*, 2019). Furthermore, the gut also contains opportunistic pathogens that may cause issues and become infectious under the right conditions, i.e. they can cross the intestinal barrier of patients already struggling with reduced immunity. These pathogens can obtain resistance genes and possibly become infectious when they are translocated across the intestinal barrier (Van Schaik, 2015). The transfer of antibiotic resistance genes in the gut mostly happens via conjugation or transduction (Fig. 4) (Van Schaik, 2015; Von Wintersdorff *et al.*, 2016). Conjugation is the most common, and is thus expected to contribute most to the dispersal of antibiotics resistance in the human microbiome. Research has shown that conjugation more often takes place between two distantly related bacteria rather than when bacteria are closely related (Van Schaik, 2015). Due to the important role the gut microbiome

plays in the dispersal of horizontally transported resistance genes, there is a high risk of further transmitting the multiple resistant bacteria in hospital settings too. An immense amount of bacteria can be contained in human faeces, and, in the case of poor hospital hygiene, these can be transmitted further via the hands of the health care personnel (Carlet, 2012).

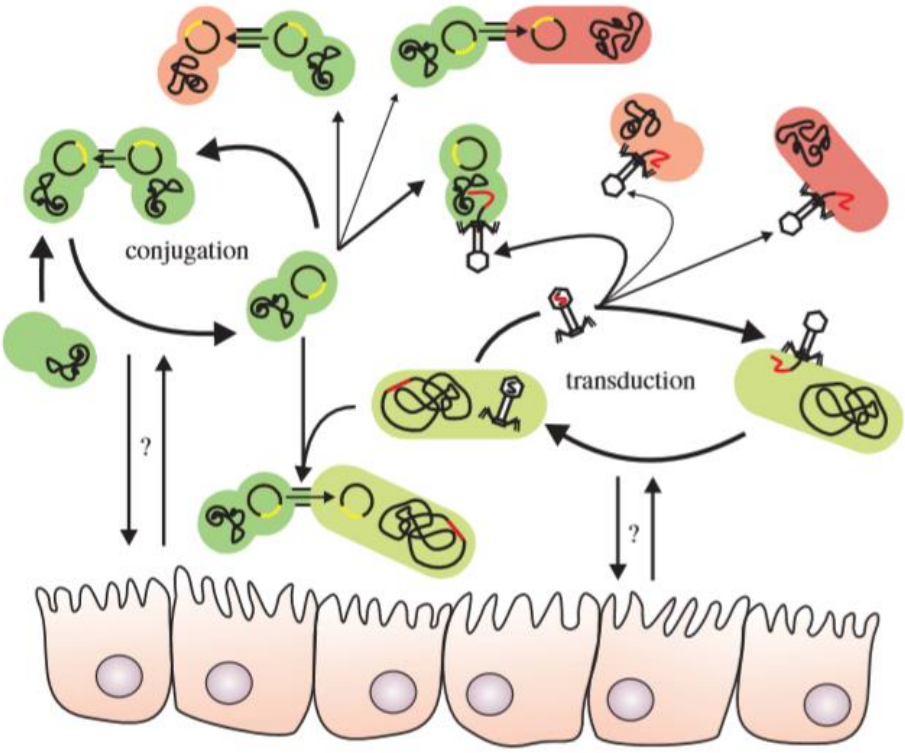

Figure 4 *Retrieved from Van Schaik (2015). Mechanism of horizontal gene transfer that can take place in the human gut. The resistance gene can be acquired by a transient bacterium that obtained colistin resistance. Green bacteria are the anaerobic commensal bacteria, which can serve as a reservoir for antibiotics resistance genes. The opportunistic gut-dwelling pathogenic bacteria are depicted in red.*

6. THE LOSS OF COLISTIN RESISTANCE

Genetic reversion can lead to the loss of colistin resistance in *P. aeruginosa*. The resistance is lost as it decreases the fitness of the resistant bacteria when no colistin is present. Therefore, when colistin is not present, non-resistant strains can outcompete the resistant ones (Lee *et al.*, 2016). Furthermore, genetic fluidity can be displayed by resistant bacterial strains, meaning they lose their colistin resistance in the absence of the antibiotic. *A. baumannii* displays a lower growth rate, and thus reduced fitness, when its DNA mutates to be resistant against colistin via an increased expression of *pmrC*. These bacterial strains of the *A. baumannii* have been shown to have a significant competitive disadvantage that is associated with their colistin resistance. Therefore, eliminating selection pressures towards resistant strains that occurred due to mutations is highly important (Lesho *et al.*, 2013). However, a mutation in *pmrB*, which can also lead to colistin resistance in the *A. baumannii*, is not associated with loss in fitness. Furthermore, many resistant strains did not display a decreased virulence (Durante-Mangoni *et al.*, 2015). This is of great concern as, depending on what the mutation that causes colistin resistance is, it could mean that bacterial strains may not suffer from fitness loss in case of colistin resistance. A consequence of this is that resistant strains are not outcompeted and remain in the population, meaning a decrease in the use of colistin will not eliminate these strains.

Resistance genes located on plasmids are in some cases also associated with fitness loss. Horizontal gene transfer contributes to persistence of antibiotics resistance as conjugation - the most important form of gene transfer - happens at a sufficiently high rate that transfer of plasmids is maintained. This is also the case for costly plasmids, even when antibiotics are not used (Lopatkin *et al.*, 2017). The exact fitness cost of having plasmid-mediated colistin resistance might differ between bacterial species. It has been found that mcr-1 significantly decreases the growth rate of *K. pneumonia*, while *E. coli* does not experience any loss of fitness when carrying mcr-1 (Tietgen *et al.*, 2018). Chromosome-mediated resistance in *E. coli* is costly and would thus be selected against, but, as there is no cost to being resistant to colistin when it is plasmid mediated, the dispersal of colistin resistance might still easily spread in *E. coli* (Choi *et al.*, 2020).

The loss of colistin resistance is more complex due to resistance genes that occur in heteroresistant bacterial populations. This means that their population consists of several subpopulations, each having a different degree of colistin resistance. If colistin is then applied, the subpopulation resistant to a higher concentration of colistin is able to survive and reproduce and take over the entire population. Hereafter, a colistin resistant population has to be dealt with. This principle is most often detected in *A. baumannii* and *K. pneumonia*, while it is more scarce in the *P. aeruginosa* (Kaye *et al.*, 2016; El-Sayed Ahmed *et al.*, 2020).

This information tells us that a temporary reduction in the use of colistin will not provide the solution to fully eliminate colistin resistance, and there is thus an urgent need to find alternative treatments or resistance-reversal methods.

7. FUTURE PROSPECTS

The first and most important step for the future should be reducing the amount of colistin used. Colistin use should be limited to those cases where there are no other options. As both chromosome-mediated and plasmid-mediated resistance are ultimately caused by the intensive use of colistin (Rhouma *et al.*, 2016; Gharaibeh & Shatnawi, 2019), reducing the use of colistin could both limit the further evolution of resistance and could also reduce the spread of those existing resistance genes that are associated with a fitness cost. This strategy is already being adopted in some regions (Barlaam *et al.*, 2019). Due to the risks regarding increased colistin resistance, Europe is limiting the use of colistin both in human and veterinary medicine. Colistin is now only used on humans when patients suffer from infections for which only limited treatments options are still available, and in animals it can only be administered when there are no alternative antibiotics that can be used (Catry *et al.*, 2015; Barlaam *et al.*, 2019). Beyond this though, there need to be stronger controls on the distribution of colistin so it can no longer be illegally distributed and there is thus less colistin in circulation (Rhouma *et al.*, 2016).

Secondly, finding mechanisms to avoid colistin resistance or reverse the evolution of it are necessary to safeguard the future use of it. One suggestion to reverse colistin resistance is an adjuvant molecule that can supress colistin resistance in *A. baumannii* and *K. pneumoniae*. This adjuvant downregulates the *pmrCAB* operon and, in doing so, hinders PetN in modifying lipid A, allowing colistin to function again and thus reversing colistin resistance (Harris *et al.*, 2014). Certain drugs can sever as adjuvant, like Niclosalmide - an anthelmintic drug – for example. Interesting about the drug is that it has been shown to be effective in combination with colistin against several Gram-negative bacteria, namely *A. baumannii, K. pneumonia, E. coli* and *Enterobacter cloacae*. Niclosamide can aid the functioning of colistin by disrupting the efflux pump of bacteria, but it can also supress the development of resistance against colistin (Domalaon *et al.*, 2019), though more research is required to better understand how it functions exactly. Another suggestion is using phages to combat antibiotics resistance. Evidence has been found that an infection by *A. baumannii*, which is a highly resistant and mortal bacteria, can be

treated with a combination of colistin and the bacteriophage PKM34. PKM34 has an intrinsic antibacterial activity caused by the endolysin LysMK34. Administering LysMK43 together with colistin can safeguard colistin's function. An added bonus is that a lower dose of colistin can be given to a patient, limiting the harmful side-effects (Abdelkader *et al.*, 2020).

Lastly, the colistin resistance genes have the capacity to distribute, and this process needs to be kept in check as well. The mcr genes already have a wide distribution (Fig. 2) in water, animal, food and human samples (Elbediwi *et al.*, 2019). They can further disperse via international food trade and travel (Anyanwu *et al.*, 2020). If possible, quality control of food products before shipment could be done to avoid spreading contaminated food and consuming it. Furthermore, it is important to investigate the presence of resistant strains in the environment, and if mcr genes are discovered, research is needed to attempt to remove them from the environment. There is also a pressing need to better understand all the possible pathways that mcr genes can use to disperse.

8. CONCLUSION

Resistance against colistin is an increasing threat to human health. Both excessive use and misuse in human medicine and animal husbandry practices are responsible for the increase in colistin resistance. Several mechanisms, both chromosome- and plasmid-mediated, have been identified as being responsible for this. The resistance is mostly caused by genes that allow the bacteria to modify their LPS component of the membrane, which is the main target of colistin, in such way that colistin is no longer able to interact with it. Though some of these mechanisms are lost in the absence of colistin due to their fitness cost, research has shown that a decreased use of colistin is not sufficient to fully reverse colistin resistance, meaning there is an urgent need for new ways to avoid resistance and make bacteria susceptible to colistin again. Some mechanisms have been discovered to reverse colistin resistance or to avoid it from evolving, but most are still relatively recent studies and thus in a preliminary phase. Further research is required to understand how exactly molecules or bacteriophages contribute to resistance and what to do in case they fail. Therefore, most importantly now is to avoid further spread and rise of colistin resistant strains by immediately minimizing its use in human medicine and animal husbandry practices. The attractiveness of colistin as a growth promotion factor and an easy tool to treat infections has fatal consequences.

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