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Review

Phage-mediated dissemination of virulence factors in pathogenic bacteria facilitated by antibiotic growth promoters in animals: a perspective

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Abstract

Addition of sub-therapeutic antibiotics to the feed of food-producing animals for growth promotion and disease prevention has become a common agricultural practice in many countries. The emergence of antibiotic-resistant pathogens is a looming concern associated with the use of antibiotic growth promoters (AGPs) around the world. In addition, some studies have shown that AGPs may not only affect antibiotic resistance but may also stimulate the dissemination of virulence factors via bacteriophages. Although only a few studies are currently available in the literature regarding this topic, in this article we endeavor to provide a perspective about how AGPs would impact the transmission of virulence factors by horizontal gene transfer via phages in a few pathogenic bacterial species significant to livestock production.

Keywords: antibiotic growth promoters, bacteriophages, virulence factor, horizontal gene transfer.

The introduction of antibiotics is one of the most significant achievements in the past century playing a substantial role in the reduction of the burden of infectious diseases in humans and animals worldwide (Aarestrup, 2015). Shortly after the introduction of antibiotics, their growth-promoting effect in chickens was discovered when it was observed that birds fed with streptomycin exhibited increased growth compared with those not receiving streptomycin (Moore *et al.*, 1946). Since the beginning of the 1950s, several antibiotics have been added to the feed of food-producing animals. In many countries, it has become a hallmark of modern animal husbandry to use sub-therapeutic levels of antibiotics as antibiotic growth promoters (AGPs) to enhance growth rate and feed efficiency (Castanon, 2007). Although the mechanisms for these effects have not been fully elucidated, Cho *et al.* (2012) demonstrated that growth promotion involves the modification of gut microflora populations and changes in the host metabolism. In the USA, in 2015, medically important antimicrobials accounted for 62% of the nation's annual domestic sales of all antimicrobials approved for use in food-producing animals, with tetracycline constituting the largest volume (i.e. 71%) (FDA, 2016). In China, similarly, approximately 52% of the total antibiotic consumption in the country was used for animals in 2013 (Zhang *et al.*, 2015). A recent modeling study estimated that worldwide antibiotic consumption in food animal production was approximately 63,000 tons in 2010, and this number is projected to increase by two-thirds to 105,000 tons by 2030 (Van Boeckel *et al.*, 2015).

Despite the prevalent use of AGPs in agriculture, their effects on antibiotic resistance by pathogens and their contribution to environmental contamination are serious public health concerns worldwide. In addition, a few recent articles suggest that AGPs are likely to affect the spread of virulence factors in pathogens. Since the impact of AGPs on antibiotic resistance and the environment have been extensively reviewed elsewhere (Wegener, 2003; Pruden *et al.*, 2013; Aarestrup, 2015), we will only briefly mention these issues. In this article, we focused on

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presenting potential mechanisms by which AGPs could be implicated in the dissemination of virulence factors. We will base our discussion on information currently available regarding phage-encoded virulence factors and the molecular mechanisms of phage-mediated transduction in a few pathogenic bacterial species significant to livestock production.

Effects of AGPs on the development of antimicrobial resistance

Widespread use of AGPs in livestock has contributed to the emergence of antibiotic-resistant bacteria through natural selection and has an adverse impact on public health. Despite controversial debates, a number of studies have demonstrated that the use of AGPs in food animals is implicated in the development of antimicrobial resistance in bacteria (Bager *et al.*, 1997; Wegener, 2003). Many studies suggest that a long-term exposure to low doses of AGPs may have greater selective potential than a short-term and full-dose therapeutic use (Andersson and Hughes, 2014; You and Silbergeld, 2014). The former ideally stimulates the emergence, mobilization, and persistence of antibiotic-resistant bacteria in both livestock and humans (You and Silbergeld, 2014). An epidemiological study in chickens and pigs demonstrated a strong association between the use of avoparcin, a glycopeptide antibiotic, as a growth promoter and the occurrence of vancomycin-resistant enterococci (VRE) in food-producing animals in Denmark (Bager *et al.*, 1997). A ban of avoparcin as a growth promoter in the European Union in 1997 resulted in a decrease in VRE in poultry (Klare *et al.*, 1999). Resistance of *Salmonella* Heidelberg to extended-spectrum cephalosporins is strongly correlated with the *in ovo* use of ceftiofur, a third-generation cephalosporin for animals, to prevent *Escherichia coli*-induced omphalitis (i.e. yolk sac infection) in broiler chickens in Canada. Voluntary withdrawal of ceftiofur from broiler chicken hatcheries reduced the resistance to the next-generation cephalosporins (Dutil *et al.*, 2010). Moreover, a recent study from seven European countries strongly correlated veterinary antimicrobial consumption levels of eight classes of antimicrobials with the prevalence of resistance in non-pathogenic commensal *E. coli* isolates from pigs, poultry, and cattle (Chantziaras *et al.*, 2013). The public health concern is that the resistant bacteria that are circulated in food-producing animals can be transmitted to people through a number of pathways, such as the food chain and environmental sources, and by occupational contact with animals (Casey *et al.*, 2013; Founou *et al.*, 2016).

Environmental contamination with antibiotics

Subsequent to the use of antibiotics in intensive animal production systems, a substantial quantity of antibiotics or their active metabolites is excreted by the animals in feces, which leads to environmental contamination with sub-lethal concentrations of antimicrobials (Li *et al.*, 2013). For example, most (ca. 75%) of the dietary chlortetracycline is not digested in cattle intestines and is excreted in manure (Elmund *et al.*, 1971). Resistant bacteria and active antibiotics can spread from the farm to the environment when farmland is spread with manure containing antibiotic residues. An increase in the prevalence of tetracycline-resistant bacteria from soils amended with pig manure slurry was observed (Sengeløv *et al.*, 2003). The tetracycline-resistance gene *tet*(M) was also detected in the soil treated with pig manure, and residues of chlortetracycline and oxytetracycline remained highly stable in the soil (Agersø *et al.*, 2006). Furthermore, a significant proportion of excreted antimicrobials ends up remaining in livestock wastewater (Pruden *et al.*, 2013). In China, antibiotics can be detected in residents' tap water in some provinces due to the contamination of major rivers, presumably from hospitals, pharmaceutical companies, and farms (Huang *et al.*, 2015). Moreover, bacteria exposed to sub-lethal concentrations of antibiotics in the environment can develop resistance, and the resistance phenotype or genotype may be disseminated by horizontal gene transfer. Thus, a resistome may be established in the environment by the release of antimicrobials in wastewater effluents and animal waste (Finley *et al.*, 2013).

Phage-mediated dissemination of virulence factors by AGPs

Horizontal gene transfer may occur in gastrointestinal microflora (Liu *et al.*, 2012). Horizontal gene transfer is mediated by three major mechanisms, including conjugation, transformation, and transduction. Conjugation transfers DNA through a conjugation pilus from a donor to a recipient cell, requiring a cell-to-cell contact (Koraimann and Wagner, 2014). Transformation enables bacteria to take up exogenous DNA from the environment. In order for natural transformation to occur in the gastrointestinal tracts, extracellular DNA should be protected from DNA-damaging agents, such as nucleases. Furthermore, the recipient bacteria need to be naturally transformable. However, only some, not all, bacteria are naturally competent for DNA transformation (Johnston *et al.*, 2014). Transduction is mediated by bacteriophages (simply phages), viruses specifically infecting bacteria (Salmond and Fineran, 2015). Since phage DNA is located within a capsid, phage DNA can be protected by DNA-damaging agents in the intestines.

A significant number of virulence factors are encoded in mobile genetic elements, including phages (Frost *et al.*, 2005). Whereas lytic phages vigorously produce progeny phage particles and are released by bacterial cell lysis, temperate phages may be integrated into the bacterial chromosome as a prophage, and the prophages replicate together with the bacterial genome (Feiner *et al.*, 2015). When exposed to stresses (e.g. ultraviolet (UV) light and antibiotics), the SOS response system of bacteria triggers prophages to enter a lytic cycle (Fajardo and Martinez, 2008; Loś *et al.*, 2011). Despite the well-established mechanisms of phage-mediated horizontal gene transfer and the induction of phages by antibiotics, few studies have examined the impact of AGPs on the phage-mediated horizontal transfer of virulence genes between pathogens. We will therefore discuss this topic

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mainly by extrapolating from our recent publication (Kim *et al.*, 2016) in combination with currently available information about phage-encoded virulence factors in a few pathogenic bacterial species found in food-producing animals.

AGP-mediated transmission of Shiga toxin-encoding phages in STEC

A number of previous studies reported that antibiotic treatment of *E. coli* significantly increased the propagation of Shiga toxin (Stx) phages in Shiga toxin-producing *E. coli* (STEC) by the stimulation of the SOS response system in *E. coli* (Zhang *et al.*, 2000; Cornick *et al.*, 2006; McGannon *et al.*, 2010). The SOS system represents a ubiquitous response to DNA damage that upregulates genes involved in DNA repair. The SOS response is regulated by the RecA and LexA proteins (Michel, 2005). Induction of SOS response *in vitro*, which is characterized by the activation of the RecA protein, has been demonstrated with various antibiotics, including β-lactams (Maiques *et al.*, 2006) and fluoroquinolones (Bearson and Brunelle, 2015). The *stx* genes are encoded in a λ prophage in the chromosome of the STEC (Allison, 2007). Thus, antibiotic treatment induces the production of Stx and is not recommended for patients infected with enterohemorrhagic *E. coli* (Davis *et al.*, 2013). The induction of Stx-encoding phages by antibiotics has been a controversial issue in human medicine, but has not been considered a serious issue in livestock because cattle, the major reservoir for STEC, are resistant to Stx (Pruimboom-Brees *et al.*, 2000). However, antibiotic-mediated induction of phages encoding virulence factors in the animal hosts for STEC may have a different impact on public health, since phages mediate horizontal gene transfer (Touchon *et al.*, 2017). The transduction of Stx-encoding phages to non-pathogenic commensal *E. coli* may convert non-pathogenic *E. coli* (i.e. non-STEC) to STEC in the intestines of cattle after treatment with antibiotics or in the manure contaminated with residual antibiotics (Fig. 1).

Our recent study demonstrated that AGPs used in beef production may facilitate the transmission of virulence factors in *E. coli* even at extremely low concentrations. The induction and subsequent transmission of Stx-encoding phages to non-pathogenic *E. coli* were significantly affected by oxytetracycline and chlortetracycline even at a concentration as low as 0.01 μg ml^{-1}; these antibiotics significantly induced the SOS response system by increasing *recA* expression (Kim *et al.*, 2016). Although antimicrobials are less frequently used for cattle compared with chickens and swine, a significant amount of antimicrobials is still used for cattle globally (Van Boeckel *et al.*, 2015). Feedlots use medications in feed and water to preserve animal health and improve production. According to a survey conducted by the USDA in 2011, more than 73% of all feedlots in the USA administered at least one antimicrobial in the feed to cattle for prophylaxis or growth promotion (USDA, 2013). Ionophores, tylosin, chlortetracycline, and oxytetracycline are commonly given to feedlot cattle. Ionophores, such as monensin, are used to improve production and control coccidia (Giguère *et al.*, 2013; USDA, 2013). Tylosin is used to promote growth and control the occurrence of liver abscesses in cattle (Nagaraja and Chengappa, 1998; USDA, 2013). Chlortetracycline and oxytetracycline are used as feed supplements mainly to prevent bovine pneumonia and bacterial enteritis at sub-therapeutic levels, but they are also used at therapeutic levels as metaphylactics to prevent infections in some feedlots, particularly when calves are first introduced into feedlots (Gustafson and Kiser, 2012; Giguère *et al.*, 2013). Our research demonstrated that tetracyclines significantly increased the propagation of Stx phages and stimulated the transmission of the *stx* genes to non-pathogenic *E. coli*, thus converting them to STEC by transduction. A previous study reported that *stx*-positive commensal *E. coli* are more frequently isolated from cattle after using chlortetracycline in the feed (O'Connor *et al.*, 2004); this suggests that antibiotic treatment may affect the prevalence of *stx*-positive commensal *E. coli*. It can be speculated that the use of AGPs in cattle may partly explain the diversification of serotypes of STEC.

Botulinum toxins in Clostridium botulinum

Clostridium botulinum is a Gram-positive, spore-forming bacterium and an etiological agent of botulism, a progressive flaccid paralysis. Spores of *C. botulinum* are frequently isolated from the environment, such as soil, animal carcasses, and sediments in lakes, and the intestinal tracts of animals (Huss, 1980). Animals can be intoxicated by the ingestion of pre-formed toxins or by the production of toxins by germinated spores in the intestines (Critchley, 1991). Among the seven toxinotypes (i.e. A, B, C, D, E, F, and G), C and D toxins are generally involved in botulism outbreaks in animals, such as cattle, horses, sheep, and wildfowl (Bohnel and Gessler, 2005). In *C. botulinum*, the production of toxins C and D is dependent on the presence of *tox*$^{+}$ prophages, and the toxinotypes C and D were interconvertible by the type of infecting phage (Eklund and Poysky, 1974). Curing of a prophage renders *C. botulinum* Type C unable to produce a toxin, and the phage-cured *C. botulinum* Type C is converted to type D and even to *Clostridium novyi* Type A depending on the type of the phage used for transduction (Eklund *et al.*, 1974). This strongly suggests that phage-mediated transduction may enable *C. botulinum* to produce neurotoxins. Whereas ruminants and poultry are sensitive to botulism, swine are relatively resistant. *Clostridium botulinum* has been detected in 3% of the swine intestinal samples in Finland (Myllykoski *et al.*, 2006) and 62% of the fecal samples of pigs in Sweden (Dahlenborg *et al.*, 2001). A study in Japan reported that *C. botulinum* type C was detected in swine liver, feces, and environmental samples from swine farms at high rates (ca. 76%), suggesting that healthy swine could be a carrier of this pathogen (Yamakawa *et al.*, 1992).

The phage responsible for toxin production in *C. botulinum* Type C, CEβ, is induced by UV light and acridine orange (Eklund *et al.*, 1971); information about antibiotic-mediated induction of the phage is not available. However, given the prevalence of *C. botulinum* Type C in pigs and the induction of the phage lytic cycle by stresses, it can be hypothesized that

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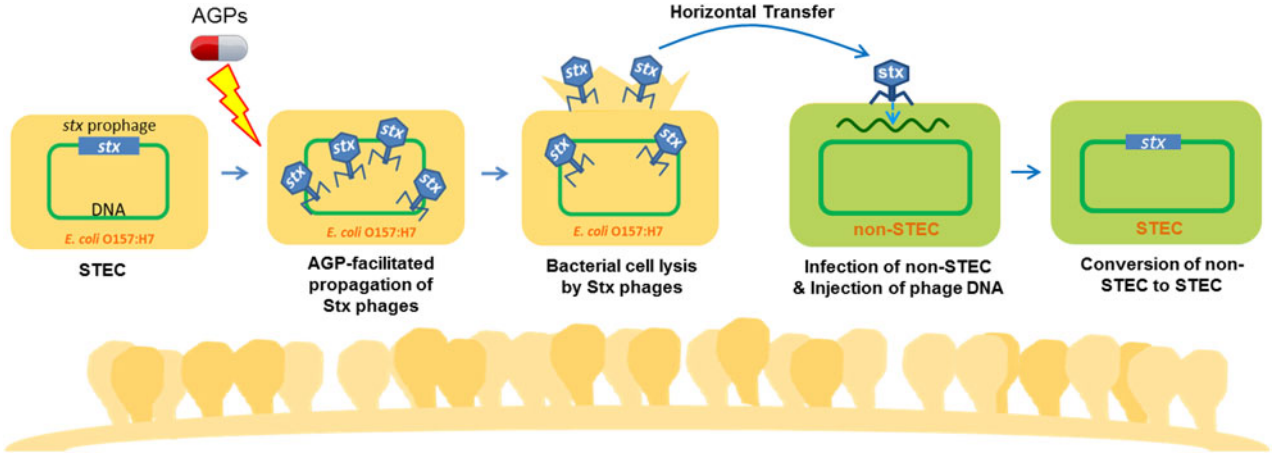

Fig. 1. Schematic diagram for posited horizontal transfer of the *stx* gene via bacteriophages from STEC to non-pathogenic *Escherichia coli* (i.e. non-STEC) after exposure to AGPs.

botulinum phages could be induced by antibiotics used for swine production, and the phages may be transmitted to *C. botulinum* in the gastrointestinal tracts of pigs. Allen *et al.*, showed that the supplementation of feed with ASP250 (chlortetracycline, sulfamethazine, and penicillin) increased the abundance of phage integrase-encoding genes. This demonstrates that in-feed antibiotics may affect the virome in the swine gastrointestinal tracts (Allen *et al.*, 2011). The phage-mediated transduction would be unlikely to occur in manure in the environment due to the oxygen sensitivity of *C. botulinum*. However, no experimental data are currently available to test this hypothesis.

Phage-encoded virulence factors in Staphylococcus aureus

Staphylococcus aureus is isolated from skin, nasopharynx, and gastrointestinal tracts of human beings and animals, mainly causing skin and soft-tissue infections (Weese, 2010). In people, *S. aureus* co-colonizes the intestinal tracts of 62% of vancomycin-resistant *Enterococcus faecium* (VRE)-colonized patients (Ray *et al.*, 2003). It has been reported that several virulence factors in *S. aureus* are encoded by phages, such as toxic shock syndrome toxin 1 (Lindsay *et al.*, 1998), Panton–Valentine leukocidin (Diep *et al.*, 2006), enterotoxin A (Betley and Mekalanos, 1985), and exfoliative toxin (Yamaguchi *et al.*, 2000). The genomic island νSaβ that encodes multiple virulence factors, such as staphylococcal superantigens, proteases, and leukotoxins, is transmissible in *S. aureus* strains from human beings and animals by phages (Moon *et al.*, 2015). Prophages are frequently activated by environmental signals activating the SOS response, such as antibiotics (Wagner and Waldor, 2002). Ubeda *et al.* (2005) reported phage-induced excision and replication of bovine-specific pathogenicity island SaPIbov1 after SOS induction by antibiotics. It also promoted horizontal dissemination of virulence factors in the pathogenicity islands of staphylococci. Recently, it was demonstrated that SOS response activation by β-lactam antibiotics triggered prophage induction in *S. aureus* lysogens, which in turn resulted in the replication and high-frequency transfer of the pathogenicity islands, indicating that such antibiotics may have an unintended consequence of promoting the spread of bacterial virulence factors (Maiques *et al.*, 2006). Although responses of staphylococcal phages to AGPs at sub-lethal concentrations have not been investigated, the existence of multiple types of phages in *S. aureus* and the prevalence of *S. aureus* in animals would possibly increase chances of transmission of virulence factors in *S. aureus* by AGPs in livestock.

Effect of AGPs on Salmonella *prophages encoding virulence factors*

Non-typhoidal *Salmonella* is a major foodborne pathogen of human beings, accounting for approximately 153 million illnesses worldwide per year (Kirk *et al.*, 2015). *Salmonella* is isolated from a wide range of food-producing animals. According to a recent study of FoodNet Canada, *Salmonella* is prevalent in manure samples from broilers (55%), swine (30%), dairy cattle (13%), and beef cattle (10%) (Flockhart *et al.*, 2017). The ubiquitous nature of *Salmonella* in food-producing animals may enable *Salmonella* to be transmitted to people through the food chain and by direct contact with animals. A number of virulence genes are encoded in prophages in *Salmonella*. For instance, *S.* Typhimurium phages SopEϕ and Gifsy-1, -2, and -3 encode type III secretion system effector proteins, such as SopE, GogB, SseI and SspH1, and the superoxide dismutases SodC-I and SodC-III involved in intracellular survival (Figueroa-Bossi and Bossi, 1999; Mirold *et al.*, 1999; Figueroa-Bossi *et al.*, 2001). The Gifsy-1, -2, and -3 phages can infect and lysogenize serovars other than Typhimurium, such as Typhi, Abortusovis, and Gallinarum (Figueroa-Bossi *et al.*, 2001).

Phages in *Salmonella* can be induced by antibiotics. Fluoroquinolones (e.g. enrofloxacin and danofloxacin) induce phage replication in two multidrug-resistant *S.* Typhimurium strains, DT104 and DT120, and facilitate phage-mediated

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horizontal gene transfer in *Salmonella* (Bearson and Brunelle, 2015). Carbadox is a veterinary antibiotic and is widely used in the USA for swine production, to improve feed efficiency and prevent enteric diseases. Carbadox induces phage propagation in *S*. Typhimurium LT2, even at low concentrations (e.g. 0.5 μg ml^{-1}), and mediates the transfer of virulence and antibiotic-resistance genes in the Fels-1 prophage from LT2 to a susceptible *Salmonella* strain (Bearson *et al.*, 2014). *Salmonella* colonizes the intestines of various food-producing animals, such as poultry, pigs, and cattle and will be exposed to AGPs in the gastrointestinal tracts of these animals. This may stimulate the propagation of phages in *Salmonella*.

Thus far, studies regarding the AGP-mediated transmission of virulence factors by phages have been conducted *in vitro*, and further verification is required *in vivo*. However, a recent study demonstrated that the transduction of the phage SopEΦ in *S*. Typhimurium occurs at high frequencies during inflammation of murine intestines, through the induction of the bacterial SOS response by stressors elicited by inflammatory immune defenses (e.g. reactive nitrogen species and reactive oxygen species) (Diard *et al.*, 2017). Because antibiotics rely on the same induction mechanisms (i.e. the SOS response) for phage induction as reactive oxygen species, AGPs would similarly mediate the phage propagation and transmission in animals. In addition, manure from food-producing animals contains antibiotic residues (Youngquist *et al.*, 2016) and phage-harboring pathogens excreted from the intestines of food-producing animals treated with AGPs. It has been shown that even environmental stresses in cattle feedlots, such as UV light (i.e. sunshine) and high temperature, may synergistically mediate the transduction of Stx phages to non-pathogenic *E. coli* (Yue *et al.*, 2012). Therefore, it is highly plausible that AGPs in manure may facilitate phage induction and transmission in combination with the environmental stresses affecting phage transduction. Even though the scientific data about phage physiology and horizontal gene transfer strongly support the idea that AGPs would affect the expansion of pathogenic bacterial populations, this still awaits future experimental verification.

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