

Chapter 2

Bacteriophage and Antimicrobial Resistance

Abstract The antibiotic resistance crisis is considered one of the world's major health threats. The use of antibiotic growth-promoters (AGPs) in animal agriculture over the last 70 years has been implicated in the development of antimicrobial resistance (AMR) in bacteria that cause common infections. Many studies and researchers have proposed bacteriophages as alternatives for AGPs to help maintain current animal production. The mechanisms that caused the spread of antibiotic resistance genes between bacteria occur through the gene transfer process of plasmid mediated conjugation and by phage-mediated transduction. Many studies have confirmed that phages have contributed to horizontal gene transfer (HGT) of AMR genes and virulence factors from other bacteria and would also integrate into human and animal biomes with unknown effect. This chapter will explore the complicated relationship between animal environmental factors and describe the significant role bacteriophages have played in development of AMR via HGT. The purpose of this chapter is to provide an updated overview on the use of antimicrobial agents in livestock, the pitfalls of using AGPs, and the role of bacteriophages in horizontal gene transfer between animals and humans. We wish to further expand current knowledge on the effects to both human health and animal production of using bacteriophages in animal therapy or environmental biocontrol to reduce AMR in the livestock reservoir. We also describe factors that will need consideration should we seek to overcome these obstacles in order to employ bacteriophages as an alternative or supplement to antibiotics in various applications.

Keywords Antibiotics • Antibiotic growth-promoters • Antimicrobial resistance • Animal farms • Bacteriophage • Horizontal gene transfer • Livestock • Mobile genetic elements • Phage therapy • Poultry • Transduction

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2.1 Introduction

Industrial agriculture depends heavily on the widespread use of antimicrobials to improve animal health, welfare and productivity. Antibiotics (antimicrobials at large) are also the most successful family of drugs for improving human health. The probability of premature death in humans due to infection would be 40% higher if antimicrobials were non-existent (WHO 2000). It is important to remark that several antibiotics are produced by environmental microorganisms (Waksman and Woodruff 1940), these are used to control and treat diseases as well as in sub-therapeutic doses in animal feed, to promote growth and improve production of animal products (Moore et al. 1946; Jukes et al. 1950). The last 70 years represent a period of drug innovation and implementation in human and animal health as well as in agriculture. Many antibiotic classes were produced and have been used at different periods in the life cycle of poultry, cattle, and swine including: arsenicals, polypeptides, glycolipids, tetracyclines, elfamycins, macrolides, lincosamides, polyethers, beta-lactams, quinoxalines, streptogramins, and sulfonamides (Sarmah et al. 2006). Antibiotics have been a primary defense against bacterial disease in poultry and livestock and have been used in four broad categories:

- Therapeutics: this type of treatment is given to an animal with a diagnosed illness
- Metaphylactics: the presence of clinical illness in one animal triggers drug treatment of the whole herd or flock
- Prophylactics: in the absence of clinical illness, treatment is for prevention of symptoms since both livestock and poultry share water and feed troughs and are in close contact with one another by licking, laying on each other and even rubbing snouts and noses, thus illnesses can spread rapidly. Antibiotics are used to prevent diseases at times when livestock are particularly at risk, like during weaning from the mother
- Growth promotion: antibiotics administered to food animals to enhance their growth rate and production performance.

These treatments were each associated to the emergence of bacteria resistant to antibiotics (Wright 2007; Livermore 2009).

2.2 The Problem

An antibiotic is a substance that kills or prevents bacterial growth. There are hundreds of naturally occurring antibiotics, but only a few are useful in treating conditions in animal agriculture. Bacterial infections with antimicrobial resistance (AMR) are spreading faster than the introduction of new compounds into clinical practice, causing a public health crisis. Synthetic approaches to producing antibiotics have been unable to replace this platform (Ling et al. 2015). AMR is a natural phenomenon that predates the modern selective pressure of clinical antibiotic use (D'Costa et al. 2011). AMR in humans is inter-linked with AMR in other populations, especially farm animals and in the wider environment (Woolhouse et al. 2015). Several studies suggest that antibiotic resistance found in clinical settings is intimately associated with the same mechanisms as those found in the environment (D'Costa et al. 2007). In fact, the animal farms' environment is continually exposed to a wide variety of antibiotics, cleaning agents, detergents, etc., while their metabolites are shared through wastewater treatment plant discharges, agricultural runoff and animal feeding operations. The presence of bacteriophages (or, more simply, phages), in addition to the impact of climate change and increased solar ultraviolet radiation have also contributed to the emergence and spread of antibiotic resistance genes (ARGs) (Jassim and Limoges 2013, 2014; Woolhouse et al. 2015). Moreover, the large-scale mixing of environmental bacteria with exogenous bacteria from anthropogenic sources provides the ideal selective and ecological conditions for the emergence of resistant bacteria (Wellington et al. 2013).

The use of antibiotics for growth promotion is a particularly controversial issue. First described by Moore et al. (1946) when it was observed that chicks improve in growth when fed bacterial shells of *Streptomyces aureofaciens* from which antibiotics had been extracted. In the mid-1950s, when it was discovered that small, 'sub-therapeutic' quantities (1/10 to 1/100 the amount of a therapeutic dose) of antibiotics such as procaine penicillin (a slow-acting antibiotic made from a salt of procaine and a form of penicillin) and tetracycline delivered to animals in feed, could enhance the feed-to-weight ratio for poultry, swine, and beef cattle (Stokstad and Jukes 1950). Non-therapeutic antibiotics have been used extensively on farms since the 1950s and have been reported to increase weight gain by up to 15–20%, a very significant effect (Stahly et al. 1980). The mechanism underlying the growth promotion remains uncertain (Cho et al. 2012); it works for antibacterial but not antifungal or antiviral additives and it works for a variety of animal species including human children (Gough et al. 2014).

Because the dosage of antibiotic that can provide growth enhancement was extremely small, the effect was regarded as largely nutritional by producers and authorities in the food industry (Levy 2002). These pharmaceutical products are

known as ‘growth promoters’ and these substances, as the name indicates, are antibiotics that are used in feed continuously at a low ‘sub-therapeutic’ level to improve growth and feed conversion. Unfortunately, many antimicrobial agents used for growth promotion are also used for disease prevention, including antibiotic classes ranked by the FDA as critically or highly important to human medicine, such as macrolides, streptogramins, and tetracyclines (United States FDA 2012) whereas this other use has been implicated in emerging AMR bacterial isolates in animals (Da Costa et al. 2013). In order to understand how and why this has occurred, the following example can shed some light on the problem. In 2012 approximately 80% of the overall tonnage of antimicrobial agents sold in the US was for animal use, and approximately 60% of those agents are considered important for human medicine (Paulson and Zaoutis 2015). From the above figures, around 94% of antibiotics were intended to be delivered through animal feed or water, whereas, only 4% of antibiotic drugs sold were intended to be administered by injection for animal therapeutic treatment (US FDA 2012). The administration of an antibiotic via feed or water to an entire flock or herd by farmers has given them less control over the dosage consumed by individual animals (WHO 2012), which has also led AMR to evolve (Emborg et al. 2003; Inglis et al. 2005; Diarra et al. 2007; Aarestrup et al. 2008; Alexander et al. 2008; Silbergeld et al. 2008; Varga et al. 2009; Vieira et al. 2009; Davis et al. 2011; Hammerum 2012; WHO 2012). Furthermore, many researchers have attributed the emergence of AMR on animal farms to low doses of antimicrobial agents given to healthy animals over prolonged periods to promote growth and increase feed efficiency. These antibiotic growth-promoters (AGPs) (Paulson and Zaoutis 2015), are believed to suppress sensitive populations of bacteria in the intestines which compete for food with their host. For example, it has been estimated that as much as 6% of the net energy in the pig diet could be lost due to microbial fermentation in the intestine (Jensen 1998). If the microbial population is better controlled, it is possible that the lost energy could be diverted to growth. Yet the exact modes of action of AGPs are not fully understood as they are probably multi-factorial (Gaskins et al. 2002; Dibner and Richards 2005; Niewold 2007), which justified the link with the increasing incidence of antibiotic resistance among bacterial pathogens (Gyles 2008; Prescott 2008) including bacteria from healthy animals (Yan and Gilbert 2004; Persoons et al. 2010).

In recent years, emerging issues related to methicillin-resistant *Staphylococcus aureus* (MRSA), *Clostridium difficile*, *Escherichia coli* and horizontally transferred genes, indicates that the livestock reservoir has a more significant impact on human health than was estimated 10 years ago, where the focus was mainly on resistance in *Campylobacter* and *Salmonella* (Aarestrup 2015). This has created tremendous worldwide concern over the present state of AMR among animal pathogen zoonotic agents which circulate among food-producing animals including poultry, beef, dairy cattle, goats, sheep as well as aquaculture and their impacts on the health of people on farms and potentially via the food chain (Levy et al. 1976; Holmberg et al. 1984; Hummel et al. 1986; Fey et al. 2000; Gyles 2008; Prescott 2008; Jassim and Limoges 2013, 2014). Furthermore, this has also resulted in the general

public's perception that antibiotics used by human beings have been used in food animals and the belief that this has contributed to AMR among foodborne bacteria, which could complicate public health therapies (DuPont 2007).

2.3 AGPs: Challenges and Consequences

Antibiotics have been used as a prophylactic to prevent disease, for treating animal (beef cattle and poultry) and plant infections, as well as in animal farming for promoting growth (McManus et al. 2002; Smith et al. 2002; Singer et al. 2003; Phillips et al. 2004; Shuford and Patel 2005; Cabello 2006; Castanon 2007; Khan et al. 2008). With some exceptions, the antimicrobial classes used in agricultural industries are the same as in human medicine (DuPont 2007; US FDA 2012; Paulson and Zaoutis 2015). At present, sub-therapeutic doses of antibiotics are routinely fed to livestock, poultry and fish on industrial farms to promote faster growth and to compensate for the unsanitary conditions in which they are raised (Emanuele 2010). Among these antibiotics are tetracycline, penicillin, erythromycin and other antimicrobials that are important in human clinical use. They are used extensively, in the absence of disease, for non-therapeutic purposes in today's livestock production (Mellon et al. 2001; Page and Gautier 2012), and can generally be purchased over the counter without veterinary involvement (Khachatourians 1998; Manna et al. 2006; Laxminarayan et al. 2013). Obviously, easy access to antibiotics for animal use has contributed to large amounts being released in natural ecosystems. They are freely used in the agricultural, horticultural and veterinary sectors to keep animals and plants healthy on industrial-scale farms. The worldwide total annual production of antibiotics was estimated between 100,000 and 200,000 tons, where the US used nearly 10 million kilograms (11,023 tons) per year (Mellon et al. 2001; Ternak 2005; Sarmah et al. 2006). The antibiotics fed to hogs in North Carolina each year exceed those that are clinically prescribed for the whole Country (Ternak 2005; Quarles 2006). The human population is also affected, directly or indirectly by this proliferation of antimicrobials which are associated with the increase in human height and the obesity of the population observed since the mass consumption of antibiotics began 40–50 years ago. The association between antibiotic consumption and the increase of human growth and obesity is suspected (Ternak 2005).

Not surprisingly, the current anti-infective drug market for livestock represents one of the largest markets in the world (Page and Gautier 2012). For example, in the US around 8 billion animals, (7.5 billion chickens, 300 million turkeys, and 100 million cattle) are treated by as many as 10 different antibiotics annually or during their shorter lives (Martin 2004; Page and Gautier 2012), for prevention of bacterial diseases via feed or water (Page and Gautier 2012; Laxminarayan et al. 2013). Preventive use can be anything from targeted interventions for controlling the spread of a diagnosed disease in a specific group of animals, to routine treatment of all animals during periods of stress such as weaning, after transportation, when

combining new animals with a herd or mixing animals from different sources (Laxminarayan et al. 2013). Overall the use of antibiotics for sub-therapeutic purposes in animals escalated by about 50% between 1985 and 2001 (Gerber et al. 2007), and currently approximately 80% of all antibiotics used in the US are fed to farm animals (US Congress 2011). This was primarily driven by increased use in the poultry industry, where sub-therapeutic antibiotic use increased from 2 million to 10.5 million pounds (907,185 kg–4,762,720 kg) between the 1980s and 2001, which amounted to a dramatic 307% increase on a per-bird basis (Mellon et al. 2001).

In general, it's clear that the excessive use of antibiotics in animal agriculture has contributed a considerable amount of pressure on the natural microbial environment, including beneficial bacteria, human and animal nutrition as well as immunity, resulting in the evolution of dangerous superbugs (Phillips et al. 2004; Yan and Polk 2004; O'Hara and Shanahan 2006; Buffie et al. 2011). As a consequence, over the past 15 years the carbapenemases [are β -lactamases with versatile hydrolytic capacities in which they have the ability to hydrolyze penicillins, cephalosporins, monobactams, and carbapenems (Queenan and Bush 2007)] have increasingly been reported in *Enterobacteriaceae*, which is known as carbapenem-resistant *Enterobacteriaceae* (CRE) (Livermore 2009). The CRE carries a plasmid, or mobile piece of DNA, with an enzyme that breaks down antibiotics. Their ability to transfer that plasmid, and that antibiotic resistance, to normal bacteria present in the environment makes these bacteria even more dangerous. Phages carrying β -lactamase genes were also isolated from sewage, suggesting another vector for transfer of these genes between organisms (Muniesa et al. 2004). The global spread of New Delhi metallo- β -lactamase (NDM) is a significant public health concern. Recent identification of NDM-1 producers, originally in the United Kingdom, India, and Pakistan and now worldwide, is worrisome and indicates that the world is marching at the dawn of a post-antibiotic era (CDC 2013a; Hayden 2015). Detection of infected patients and carriers with carbapenemase producers is necessary for prevention of their spread (Nordmann et al. 2011). The most serious Gram-negative infections are healthcare-associated, and the most common pathogens are *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *E. coli* and *Acinetobacter* (CDC 2013b; Rashid et al. 2015). *E. coli* was found resistant to antibiotics like ampicillin, tetracycline, and nalidixic acid, and these resistant phenotypes were common in livestock and poultry (Sørum and Sunde 2001; Hasan et al. 2011). Interestingly, multidrug-resistant extended-spectrum β -lactamase (ESBL)-producing *E. coli* isolated from aquatic sources in Bangladesh were found to be resistant to broad-spectrum antimicrobials such as aztreonam, ciprofloxacin, mecillinam, and cefuroxime sodium (Rashid et al. 2015). On the other hand, treating infections of either pan-resistant or nearly pan-resistant Gram-negative microorganisms is an increasingly common challenge in many hospitals, however, this is true as well, but not to the same extent, for some of the Gram-positive infections (e.g., *Staphylococcus* and *Enterococcus*) (CDC 2013b).

Using sub-therapeutic antibiotics in animal agriculture have consequently affected everybody with the increased antibiotic resistant bacteria and in parallel increased frequency of treatment failures (in some cases death), and the increased

severity of infections (FAO/OIE/WHO 2003). As stated by the WHO, the increasing emergence of antibiotic resistance in human pathogens is a special concern, not only for treating infectious disease, but also for other pathologies in which antibiotic prophylaxis is needed for avoiding associated infections. In this regard, WHO (2000) reported the following: “the spread of antibiotic resistance within hospitals means that commonplace medical procedures, once taken for granted, could conceivably be consigned to medical limbo; the repercussions are almost unimaginable”. Furthermore, in 2011 the Director-General of the WHO, said that “The world is on the brink of losing these miracle cures (antibiotics). In the absence of urgent corrective and protective actions, the world is heading towards a post-antibiotic era, in which many common infections will no longer have a cure” (Liljeqvist et al. 2012). It is also clear that the antibiotics used in livestock are a significant source of antibiotics released into the environment (Dolliver and Gupta 2008).

In an attempt to cure the damage already done, many governments around the world are taking some action to address this issue seriously, with an active role in research (Shute 2013). In this regard, in the European Union (EU), restricted authorisation of antimicrobial types began several decades ago and in 2006 all growth promoting use was abandoned (Laxminarayan et al. 2013; Jassim and Limoges 2014). However, this has not led to any consistent decrease in antibiotic consumption (Woolhouse et al. 2015). Typically, the growth promoter ban has prompted compensatory increases in metaphylactic and prophylactic use. The result is that in Europe, the volume of agricultural usage of antibiotics continues to rival that of medical usage (Woolhouse et al. 2015). In the US, the FDA has released draft guidelines on judicious use of antimicrobials in the rearing of animals for food production. These recommendations aim to reduce the overall use of medically important anti-microbials and include veterinary oversight and consultation. If this guidance is adhered to, a gradual phasing out of growth promoting use is to be expected (Laxminarayan et al. 2013). There have been some localized successes, for example, a more than 50% reduction in the usage of antibiotics (notably macrolides) in pigs was achieved from 1992 to 2008 in Denmark without any loss in productivity (Aarestrup et al. 2010).

2.4 AMR Shared Between Livestock and Humans

Most earlier research on human pathogens led us to ignore for decades the existence of an ecological cycle that does not directly involve humans. The emerging pathogen of growing importance for example *E. coli* O157:H7 was probably created by human activities (Quarles 2006). The author concluded that this is due to saturation of cattle in feedlots with antibiotics putting selective pressure on their microbes. In their frantic scramble to survive, bacteria may increase the frequency of mutation and genetic exchanges. These exchanges include genes for pathogenic activity and antibiotic resistance (Law 2000; Lefebvre et al. 2005) and are encoded on phage, plasmid and chromosomal genes (Law 2000). As in human medicine, the

use of antimicrobial drugs in veterinary medicine creates a selective pressure for the emergence of antimicrobial-resistant bacteria, including animal pathogens, human pathogens that have animal reservoirs and commensal bacteria that are present in animals (Singer et al. 2003; Turnidge 2004). This has led to the development of AMR that can be transmitted to humans through the animal meat, milk and egg supply, through direct contact with animals or through environmental contamination (Fig. 2.1). Increasingly, food animals are raised in large numbers under close confinement, transported in large groups to the slaughter and then processed very rapidly. These conditions can cause increased shedding of bacteria and contamination with fecal bacteria from the hide, carcass or meat. The amplified dissemination of pathogens occurs through the food chain via centralized food processing and packaging and worldwide distribution through shipment, wholesale food chains and retailers. The bacterial pathogens are transmitted from contaminated animals, meat and foods to humans through the food supply (Mølbak et al. 1999; White et al. 2001; Manges and Johnson 2012) or through direct exposure from infected or contaminated animals, such as on farms, in slaughterhouse or processing facilities (Lyons et al. 1980; Rowe et al. 1997; Price et al. 2007; Smith et al. 2007; AAP 2012). However, the farmers, farm workers, and farm families as well as casual visitors and travelers to endemic countries are at greater risk to be infected or serve as vehicles to transfer AMR (Levy et al. 1976; CDC 2001; Meltzer and Schwartz 2007; AAP 2012).

Bacteria are found everywhere in the animal farm environment, which means animals are exposed to the potential for disease whether they're raised on open range or indoors. Farm animals are an important component of resistance gene pathogens, normal flora, gut microbiota and vast numbers of soil bacteria in this

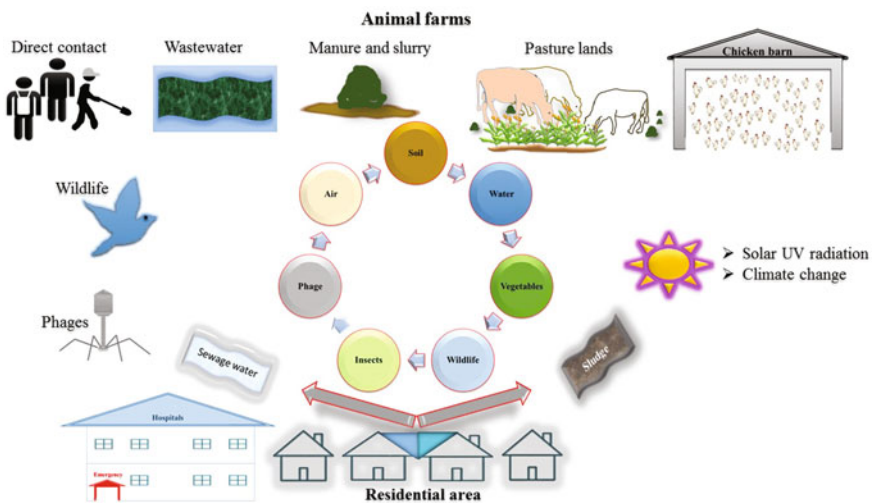


Fig. 2.1 Conceptual model of sharing AMR

complex system. They are exposed to enormous quantities of antibiotics (despite attempts at reduction) and act as another reservoir of resistance genes (Woolhouse et al. 2015). Noteworthy, most antibiotics were produced by screening soil microflora (bacteria, actinomycetes, fungi and algae) which have been producing antibiotics on a global scale for perhaps 2 billion years (D’Costa et al. 2011). They represent the original source of the majority of antibiotics used currently in medicine and veterinary medicine (D’Costa et al. 2007). For example, antibiotic penicillin came from the fungus *Penicillium* found in soil, and vancomycin is made from the *Amycolatopsis orientalis* bacteria found in soil. A newer antibiotic, teixobactin was discovered from a screen of uncultured bacteria in a soil sample. Teixobactin was found to inhibit cell wall synthesis by binding to a highly conserved motif of lipid II (precursor of peptidoglycan) and lipid III (precursor of cell wall teichoic acid) of *Staphylococcus aureus* and *Mycobacterium tuberculosis* without resistance (Ling et al. 2015). It is therefore not surprising that soil is also a major reservoir of AMR. Resistance is likely to be as natural, widespread and ancient as antibiotic production. The relationship between resistance to naturally produced antibiotics in the soil and manufactured antibiotics in the clinic remains unclear (Woolhouse et al. 2015). For instance, a metagenomics study of Fosberg et al. (2012) found multiple examples of resistance genes in the soil that had 100% homology across all major classes of antibiotics to those found in clinical isolates. That study provides clear evidence for horizontal gene transfer between soil bacteria and pathogens; however, it does not show how the gene transfer took place. Woolhouse et al. (2015) explained that, the observation that resistance determinants for synthetic quinolones (qnr genes) can be detected in soil seems to indicate transfer from an unknown source, and the clinic. The soil in farm fields often receives regular application of manure as a fertilizer and can be in direct contact with manure droppings, urine and plants which are also cycled through the animal digestive system, so it is not surprising that soil microflora antibiotics have contributed in part with AMR via vertical and HGT mechanisms for some time. Consider this a ‘call to action’ for researchers interested in understanding the dynamics of natural antibiotics produced by soil microflora on antibiotic resistance in livestock and poultry, since all livestock are living in an open environment and in direct contact with these soil microflorae most of their life. Researchers should also consider climate change, increased solar ultraviolet radiation (SUR) and the impact of other external forces on phage–host interactions in various ecosystems (Jassim and Limoges 2013).

AMR from livestock and poultry can be spread through fecal material and contaminate foods when manure containing resistant organisms is applied to agricultural soils or through direct and indirect contact. The organisms are then present in farm runoff (Heuer et al. 2011) or wastewater, leading to environmental reservoirs of pathogens and resistance genes (Chapin et al. 2005; Mackie et al. 2006) (Fig. 2.1). Cross-contamination of fruits and vegetables can occur when wastewater or contaminated water is used to irrigate crops and fish raised in contaminated water can also be exposed (Fig. 2.1). Active antimicrobial agents have been detected in surface waters and river sediments (Kümmerer 2004), and the

resistance genes identical to those found in swine waste lagoons have been found in groundwater and soil microbes hundreds of meters downstream (Chee-Sanford et al. 2001).

The damage caused to the environment at large and the effect of antibiotics used for treating infections or for farming purposes in AMR, has been studied in more detail (Witte 1998; Ferber 2003; Singer et al. 2003), still little is known on the overall effects of antibiotics on the population dynamics of the micro-biosphere (Sarmah et al. 2006). Antibiotic resistant cross contamination between humans and animals and visa versa is still unclear. There are several studies available that have shown that AMR is widespread in farm animals, for example; apramycin and ampicillin-resistant *E. coli* in newborn calves (Hoyle et al. 2004a, b, 2005; Yates et al. 2004). Nevertheless, there are equally high levels of ampicillin resistance on organic farms (Hoyle et al. 2006), and reports of carbapenem-resistant enterobacteria in livestock (Fischer et al. 2012). However, such studies do not establish the direction of movement, if any, of resistance between livestock populations and humans. Further, carbapenems are not used in livestock so resistance was presumably imported from another agent. The movements of pathogens and AMR are likely from cattle and cattle feces, can travel directly into the food supply from slaughterhouses, or more insidiously contaminate water or food crops (Swerdlow et al. 1992). The pathogens can also be spread by houseflies (*Musca domestica*), cockroaches, and even field slugs the *Deroceras reticulatum* (Rivault et al. 1993; Agui 2001; Kobayashi et al. 2002; Alam and Zurek 2004; Sproston et al. 2006). Antibiotic resistance genes have been found at feedlots, in animals, in the air, in manure, urine, and often held in lagoons and end up in water and in sediments and finally in vegetable crops via contaminated water and fertilizer (Quarles 2006) (Fig. 2.1).

Most antibiotics used for preventing or treating infections in humans or animals, as well as for promoting faster growth of livestock, are only partially metabolized and poorly absorbed in the gastrointestinal tract, resulting in excretion of parent compounds and metabolites (Wegener 2003; Shuford and Patel 2005; Boxall et al. 2006; Dolliver and Gupta 2008; Khan et al. 2008; Chee-Sanford et al. 2009). Since approximately 75% of all antibiotics given to animals are not fully digested, upon excretion these compounds pass through the body and enter the environment beyond feed yard boundaries via application of manure waste onto agricultural fields and runoff (Wegener 2003; Dolliver and Gupta 2008; Chee-Sanford et al. 2009; Roe and Pillai 2003; Chee-Sanford et al. 2009). With huge quantities of manure, 180 million dry tons of livestock and poultry waste are generated every year in the US which are routinely sprayed onto surrounding farm fields. Once in the environment, antibiotics can facilitate de novo mutation of AMR and provide a selective advantage for bacteria that acquire resistance either in treated animals or in the environment (Gilchrist et al. 2007; Silbergeld et al. 2008; Chee-Sanford et al. 2009). They can also encounter new bacteria and create additional resistant strains (Horrigan et al. 2002), also leeching into surface and ground water, contaminating drinking wells and endangering the health of people living nearby (Hrudey and Hrudey 2004; Dolliver and Gupta 2008; Clemans et al. 2011). Therefore, this has

resulted in the development of AMR (Lu 2004) which can be spread by animals, birds and insects that come in contact with animal waste (Graham et al. 2009; Page and Gautier 2012) (Fig. 2.1).

The recent advances in molecular biology techniques make it possible to track the movement of bacterial strains and AMR genes through the environment (Choi 2007). Antibiotics released at feeding operations and resistance genes generated there end up in meat products (Sunde and Norstrom 2006). AMR genes have also been found in the air, in manure and in the water at animal feedlots where manure and urine is often held in lagoons (Hrudey and Hrudey 2004; Quarles 2006; Sapkota et al. 2006). Airborne microbes are also thought to have significant impact on human and animal health including threats from pathogenic microorganisms (Behzad et al. 2015) (Fig. 2.1). Pathogens, antibiotics and AMR genes from these lagoons can end up in water and in water sediments (Pei et al. 2006; Schmitt et al. 2006). Where streams or wells are used as drinking water sources, AMR genes can end up in drinking water. They can also be found and transferred by house flies (Macovei and Zurek 2006; Petridis et al. 2006). Conversely, antibiotic resistance genes, acquired by pathogenic bacteria through HGT have been observed in environmental bacteria (Davies 1997), although they can also evolve later on under strong antibiotic selective pressure during the treatment of infections (Martinez and Baquero 2000; Martinez et al. 2007). To understand the development of resistance in full, we need to address antibiotics and their resistance genes, not just in clinics but in natural non-clinical environments (Martinez 2008).

The above findings raise questions and concerns about environmental contamination and the complex interaction that occurs between antimicrobial agents from agricultural and human use, biological vectors such as lysogenic phage, flies, birds, etc., and the effect of SUR to provoke direct DNA mutations, along with climate change stimulating rapid bacterial growth. These and other factors exerting selective pressure, could force microbial populations to stimulate horizontal and vertical gene transfer and thus amplify the number and variety of bacteria that are resistant to antimicrobial agents (Jassim and Limoges 2013, 2014) (Fig. 2.2). Consequently, we have the emergence and spread of AMR as a global health threat,



Fig. 2.2 The post-AGP effects

with agricultural use of antibiotics as a contributor to the aggregation of resistance in the environment, coupled with external forces on phage–host interactions in aquatic and other trophic ecosystems (Gilchrist et al. 2007; Levy and Marshall 2004; Jassim and Limoges 2013, 2014) (Fig. 2.2).

2.5 Resistance Genes Not in a Vacuum

AGPs usually contain a small amount of antibiotics with an average of 100 mg of antibiotic substances delivered via animal feed. This sub-therapeutic dose is consumed in animal husbandry in Europe to produce each kg of meat for human consumption (Lu et al. 2004). This regimen would have favored the selection and maintenance of rare bacterial transformants carrying the resistance genes (Lu et al. 2004). These authors have concluded that based on the large numbers of pigs and chickens that were exposed to the antimicrobial agent, the probability of gene pick-up by bacterial commensals in the animal gastrointestinal tract increased. Once incorporated into a gut commensal genome, further dissemination would have followed under antimicrobial selective pressure. Since, farm animals are raised in large numbers under close confinement, they are more likely to shed pathogens in their manures or litter. These conditions would favour such organisms enabling them to pass on their resistance genes to their offspring by replication, or to other related bacteria through “conjugation”. Plasmids carrying these genes “jump” from one organism to another or via biological vector through lysogenic phages. This process is a natural, unstoppable phenomenon causing the evolution of many zoonotic pathogens of current concern and is exacerbated by AGPs in animal husbandry. In this regard, the selective effects of antibiotic use can only be understood if considered in the context of environmental routes (e.g., water, air, soil, lagoons, manure and urine, ground water, surface waters, river sediments, soil, wastewater, environmental contact, and biological vectors such as phage vectors, flies, rodents etc.) (Fig. 2.1) and the impact of external forces that enable these bacteria and the genes they carry, to spread between different biomes leading to environmental reservoirs of pathogens and resistance genes (Fig. 2.2).

2.6 Mechanisms of Gene Transfer Between Bacteria

Bacteria have evolved numerous mechanisms to assimilate new genes to help them withstand harsh environmental conditions or settle in a new environmental niche. The same mechanisms now also enable them to develop antimicrobial resistance (Martinez et al. 2009; Wiedenbeck and Cohan 2011). Bacterial resistance to antimicrobial agents develops by a variety of mechanisms: (1) change within the existing genome of a bacterial cell (chromosomal mutations, namely, vertical evolution) and change within the proteome (phase variation) (Crumplin and Odell 1987; Mempel et al. 1994; Martinez and Baquero 2000; Woodford Ellington 2007);

(2) formation of bacterial cell interactions (mixed bacterial biofilms) (Mah 2012); and most importantly; (3) by acquiring new genes from other strains or species through HGT (Boerlin and Reid-Smith 2008; Hegstad et al. 2010a; Palmer et al. 2010).

HGT or sharing of genes between bacteria was thought to be largely, although not exclusively, responsible for the development and intervention of antibiotic-resistant bacteria through various mechanisms called the mobile genetic elements (MGEs) (Hegstad et al. 2010a). MGEs, include phage transduction; conjugation (which involves direct cell-to-cell contact and transfer of plasmids or transposons); or transformation, involving the uptake of free DNA that results from bacterial lysis (Barbosa and Levy 2000; Livermore 2003; Marti et al. 2014a). In some circumstances the presence of low levels of the antibiotic in the environment is the key signal that promotes gene transfer (Jeters et al. 2009), thereby ensuring that the entire microbial community is protected from the antibiotic (Ochiai et al. 1959). HGT may occur relatively infrequently, but once the gene is established in a successful virulent clone, the clone and the carried gene can spread in individual countries and worldwide, such as in the case of multidrug-resistant *Staphylococcus aureus* and pneumococci (*Streptococcus pneumoniae*) (Collignon et al. 2009).

The acquisition of antimicrobial resistance by phage transduction has been demonstrated in chicken meat relevant bacterial species, this mechanism in environmental settings has now been fully explored (Shousha et al. 2015), whereas, the phage can transfer bacterial DNA from one host to the other in a process known as transduction. The co-existence of various resistance genes in the same plasmid or transposon results in the incidental transfer of the whole group, even if the selective pressure is directed towards a specific gene (Summers 2002). This co-selection mechanism impacts the establishment of a linear relationship between the use of a specific antibiotic and the emergence of the corresponding resistance (O'Brien 2002). The fact that the recipient cell receives all the genetic competences mediated by a certain plasmid may result in more complex consequences, such as the transfer of virulence determinants under the selective pressure imposed by the presence of antibiotics or, in opposition, the non-selected transmission of antimicrobial resistance genes driven by the presence of heavy metals or disinfectants (Aiello and Larson 2003; Hegstad et al. 2010b). This dynamic also favours the optimization of these genetic elements, dashing initial hopes of reversing resistance by simply reducing antibiotic use (McEwen and Fedorka-Cray 2002; Enne 2010).

2.7 Phage, AMR and Virulence Factors in Bacteria Sharing

Transduction is a mechanism of genetic exchange, which is mediated by independently replicating phages (Normark and Normark 2002; Frost et al. 2005; Boerlin and Reid-Smith 2008; Michod et al. 2008; Volkova et al. 2014). Phages may typically be grouped into two categories by their life cycle: lytic 'virulent'

phages and lysogenic ‘temperate’ phages. Phages provide one of the most efficient vehicles for moving DNA sequences between bacterial cells by either; (1) multiplying inside the host cell before releasing new phage particles (lytic cycle), or (2) incorporating their viral genome into the host genome, replicating as part of the host (lysogenic ‘temperate’ cycle) (Fig. 2.3).

Lytic phages infect their host cell and begin the viral replication cycle within a short time frame, around 20 min. At the end of replication and assembly, the host bacterial cell typically lyses and releases the newly formed phage particles (Fig. 2.3), the whole lytic cycle takes around 40 min from start to finish. In contrast to lysogenic phages, lytic phages do not alter the phenotype of the host bacterial cell during a long-term genetic relationship, but they can shape the host population by eliminating susceptible cells in a population. Phage-resistant mutants lose virulence, and thereby these phage-resistant mutants will facilitate genetic exchange by transduction (Michod et al. 2008; McShan and Nguyen 2016). Therefore, transduction is the process by which DNA is transferred from one bacterium to another cell via phage vector ‘DNA’ (Hartl and Jones 1998), this can be carried-out either largely by the phage lysogenic cycle and to a lesser extent by the phage lytic cycle as described above (Fig. 2.4).

Transduction was first reported by Leonard et al. (1968) when they found both temperate and lytic phages were able to transduce streptomycin resistance. The role of phage in transduction and horizontal transfer is assumed (Christie et al. 2012). This assumption recently has been confirmed by Shousha et al. (2015). They demonstrate that lysogenic phages that infect *E. coli* (coliphages) are able to transduce important antimicrobial resistances in chicken meat. This result suggested that transduction of antimicrobial resistance is not, as has been assumed, a rare event but is rather more common for certain resistance elements. Hence this could

Transduction when bacteriophages infect a bacterial cell, their normal mode of reproduction is to harness the replication, transcriptional, and translation machinery of the host bacterial cell to make numerous virions, or complete viral particles, including the viral DNA or RNA and the protein coat.

Phage lytic cycle



Phage lysogenic ‘temperate’ cycle



■ Bacterial DNA

■ Bacteriophage DNA

Fig. 2.3 Phage life cycles

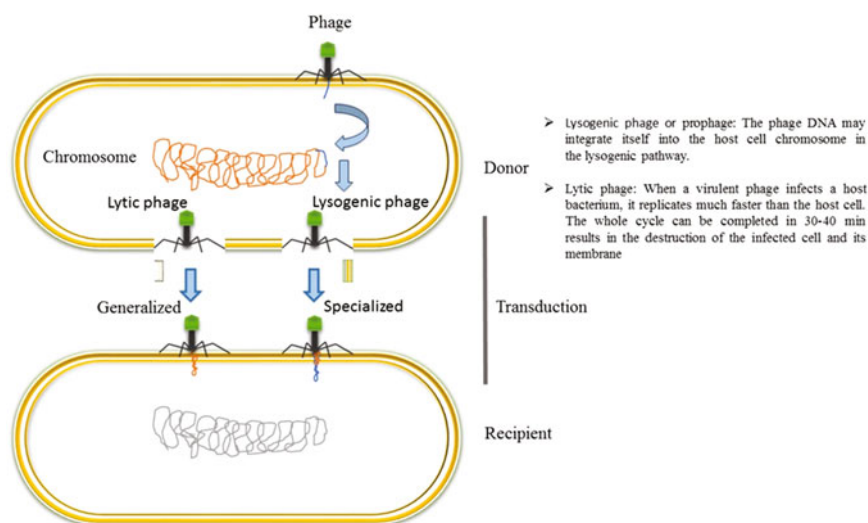


Fig. 2.4 Transfer of DNA between bacteria via phages

suggest that specialized transduction occurs frequently in the environment during lysogeny, when a prophage excises not only its own DNA but also attached bacterial DNA from the host genome. Such a process has been documented in different environments such as in environmental water and on plant surfaces (Fineran et al. 2009; Jassim and Limoges 2013). In this regard, to keep their fitness, the phages can store this DNA in the capsid (encloses the genetic material) and during the next infection cycle the bacterial host might integrate the foreign DNA by homologous recombination (Zhang et al. 2011). In hypothesis, generalized transduction, in which either a lytic or a lysogenic phage packs bacterial DNA instead of phage DNA into its capsid and delivers it to the next bacterial host, may occur without the need for homologous recombination between different species of bacteria (Volkova et al. 2014). Although there has not yet been any definitive proof of the phenomenon, broad-host-range plasmids have been transduced between bacteria of non-related species (Evans et al. 2010).

In general, if the lysogenic cycle is adopted, the phage chromosome is integrated (by covalent bonds) into the bacterial chromosome, where it can remain dormant for thousands of generations (Figs. 2.3 and 2.4). The lysogenic phage or 'prophage' will drive the adaptive evolution of bacteria to achieve more powerful virulence factors inherited from previously infected bacteria via transduction, i.e., the transfer of genetic material to a bacterial cell via phage infection (Campbell 1988; Verheust et al. 2010) (Figs. 2.3, 2.4 and 2.5). Lysogenic phage transduction serves as a driving force in bacterial pathogenesis, acting not only in the evolution of bacterial pathogens through gene transfer, but also contributing directly to bacterial pathogenesis at the time of infection and antimicrobial drug resistance (Blanchard et al. 1986; Blahová et al. 1993; Pereira et al. 1997; Willi et al. 1997; Schmieger and Schicklmaier 1999;

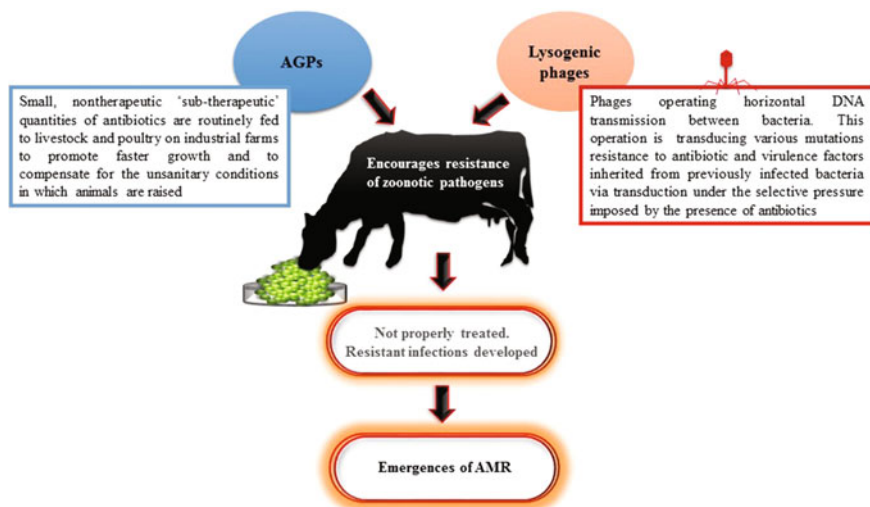


Fig. 2.5 Evolution of AMR in animal agriculture

Wagner and Waldor 2002; Frost et al. 2005; Michod et al. 2008; Verheust et al. 2010). In vitro, phages can transduce resistance to imipenem, aztreonam, and cef-tazidime in *Pseudomonas aeruginosa* (Blahová et al. 1993), methicillin in *Staphylococcus epidermidis* (Blanchard et al. 1986), tetracycline in *S. aureus* (Pereira et al. 1997) and *Actinobacillus actinomycetemcomitans* (Willi et al. 1997). It was found that phages are able to transduce resistance genes from *Salmonella enterica* serovar *typhimurium* DT104 (Schmieger and Schicklmaier 1999). Furthermore, increasing levels of resistance to antimicrobial agents in bacteria, particularly in Gram-negative rods resistant to β -lactam antimicrobial drugs, have become evident (Livermore 1995; Muniesa and Jofre 1998; Muniesa et al. 2004).

The major mechanism of resistance that causes clinically important infection in Gram-negative bacteria is the production of β -lactamases, which includes chromosome and plasmid-encoded enzymes and phages (Livermore 1995; Muniesa and Jofre 1998; Muniesa et al. 2004). The data of Vojtek et al. (2008) has indicated that horizontal transfer of lysogenic phages among group A *Streptococcus* can occur across the M-type barrier due to the extent of horizontal DNA transmission. This data also provided further support for the hypothesis that toxigenic conversion can occur via lysogeny between species (Brabban et al. 2005). Therefore, this mechanism allows more efficient adaptation to changing host challenges, potentially leading to stronger, more specialized and virulent clones (Vojtek et al. 2008) (Figs. 2.3 and 2.4), which may represent a potentially serious hazard to humans, animals and plants (Saunders et al. 2001; Verheust et al. 2010). The influence of phage transduction is evident in localized pathogenicity of *E. coli* strains endowed with at least six virulence factors: *Shigella* toxin, intimin-mediated adhesion (virulence factor 'adhesion' of Enteropathogenic *E. coli*), haemolysin, serine protease,

thermostable enterotoxin and special catalase system (Alexa et al. 2001; Hayashi et al. 2001; Sousa 2006; Vondruskova et al. 2010). This is evidence of the transductive and lysogenic capacities of this class of phages which have contributed to the evolution and shaping of emerging foodborne pathogenic bacteria through the dissemination of virulence and antibiotic resistance genes (Figs. 2.3, 2.4 and 2.5). For example, the genome sequences of *Shigella dysenteriae*, *E. coli* O157:H7 and the Shiga toxin-encoding phages demonstrate the critical role phage-mediated gene transfer events played in the evolution of these high-profile human pathogens (Brabban et al. 2005). These authors also observed the phage's role in the spread of antibiotic resistance between bacteria via interspecies transduction. Such exchanged genes include those encoding for substrate utilization, bacteriocin, exopolysaccharide and biogenic amine production, immunity to phages and antibiotic resistance (Rossi et al. 2014).

Thus through these mechanisms, phages play an important role in the evolution and ecology of bacterial species, as they have the potential to transfer genetic material between bacteria (Figs. 2.3, 2.4 and 2.5). This is evident in the study of Modi et al. (2013) who recently revealed that the viral metagenome (or virome) of antibiotic-treated mice was highly enriched for ARGs compared with that of non-treated control mice. The authors also demonstrated that ex vivo infection of an aerobically cultured naive micro-biota with phages from antibiotic-treated mice resulted in increased bacterial resistance compared to infection with phages from the non-treated control. These findings clearly show that phages have significant implications for the emergence and spread of antibiotic resistance. ARGs may be acquired and transferred among bacteria via MGEs such as conjugative plasmids, insertion sequences, integrons, transposons, and phages (Balcazar 2014). However, the contribution of phages to the spread of ARGs suggests that phages may play a more significant role in the emergence and spread of ARGs than previously expected (Parsley et al. 2010; Colomer-Lluch et al. 2011a, 2014; Balcazar 2014; Marti et al. 2014b; Shousha et al. 2015).

The increasing numbers of resistant bacteria, both commensals and foodborne pathogens, on food is a cause of concern to public health authorities, veterinarians as well as physicians and because it was linked to foods of animal origin, it represents a possible source of antimicrobial resistance in the human environment (Shousha et al. 2015). Unfortunately, the strategies to combat antimicrobial resistance have enjoyed only limited success and there are still many questions relating to how and when resistance transfer occurs (Shousha et al. 2015). Obviously there is an urgent need for new strategies in combating phage transfer of antimicrobial resistance in the food environment. The general setback in phage therapy is laid on the contribution of phages to the spread of ARGs through the horizontal transfer of genetic elements between bacteria, which is critical to the dissemination of resistance, particularly within a mixed bacterial population (e.g., skin, intestine and respiratory mucosa) in the presence of antimicrobial agents (McDermott et al. 2003; Lu and Koeris 2011; Smillie et al. 2011; Sillankorva and Azeredo 2014). This leads to another concern, that since it was found that lytic phages have been used in phage therapy for the treatment of colibacillosis caused by AMR chicken

pathogenic *E. coli* strains (Zhang et al. 2013), or for reducing *Campylobacter* loads in chickens (Kittler et al. 2013), there could be a risk of transduction of antibiotic resistance genes by these phages. Accordingly, this could put phage therapy in jeopardy since phage may be contributing through HGT of AMR genes and virulence factors from other bacteria. For this reason, it was suggested to use purified phage endolysins (lysins) directly rather than the phage itself, thus precluding unintended transfer of genetic material from the phage (Woolhouse et al. 2015). Others have suggested testing the transduction ability of these therapeutic phages as a tool for avoiding the transduction of antibiotic resistance by these phages (Shousha et al. 2015). Thus phage transduction may have a role in the exchange of antimicrobial resistance between microorganisms and thus, between the environments at large (Fineran et al. 2009; Colomer-Lluch et al. 2011a, b; Mod et al. 2013; Balcazar 2014; Shousha et al. 2015). The interactions between phage and bacterial species in the presence of antibiotics are leading to a more highly connected phage–bacterial network for gene exchange (Modi et al. 2013). Therefore, combining antibiotics with phage in animal treatment could lead to more serious AMR outbreaks which can be accelerated by the abundance of phages. With an estimated 10^{31} particles, phages are the most abundant biological entity on earth, and outnumber bacteria 10:1 (Hendrix 2002). It is likely that the phage HGT of AMR genes is on-going and more prevalent in the environmental transfer of genetic material, or transduction and could be responsible for the transfer of pathogenicity determinants and virulence factors, leading to the development of new microbes or even more resistant bacteria (O’Shea and Boyd 2002; Brabban et al. 2005; Maiques et al. 2007). Whole genome sequencing is revealing and beginning to quantify the two-way traffic of AMR bacteria between the farm and the clinic (Woolhouse et al. 2015). This will lead researchers to do further studies in order to predict new challenges to animal food safety (Fig. 2.5).

2.8 Alternative to AGPs

It is a clear fact that today the world has an urgent need for access to new effective treatments for bacterial infections (Laxminarayan et al. 2013) to replace the miracle drugs or ‘antibiotics’ of the last century. As resistance to antibiotics is becoming increasingly widespread, the sub-therapeutic use of AGPs in animal feeds was discontinued in the EU (European Union 2003). However, the potential consequences of the AGP bans on both animal and human health have been characterized (Casewell et al. 2003; Phillips et al. 2004; Castanon 2007), as follows:

- It is possible to substantially reduce the use of antimicrobial agents in livestock production without compromising animal welfare, health or production. In some cases, this should be done in combination with other measures such as biosecurity and use of vaccines (Aarestrup 2015)

- Resultant increased disease in animals and in particular in pigs, leads to an increased use of therapeutic antibiotics of importance in human medicine (Casewell et al. 2003; OIE 2007; Vigre et al. 2008; ESVAC 2013)
- Demands that farmers make improvements to the farms' hygiene
- Concerns of AMR being transmitted to humans via the food-chain (WHO 2003, 2004)
- Created problems in the international trade of animal meat and in application of the precaution principle allowed by the World Trade Organization (Castanon 2007) because the EU only imports foods obtained from animals that were not fed with antibiotics
- This has created challenges for the animal feed and feed additive industries resulting in the search for "natural" and "safe" alternatives for AGPs to help maintain current animal production levels while reducing feed costs without threatening public health (Ratcliff 2000; Millet and Maertens 2011; Lloyd 2012; Jassim and Limoges 2014)
- Antibiotics are an integral part of industrial agriculture and there are very few alternatives (Woolhouse et al. 2015).

In this regard, a variety of commercial safer non-antimicrobial substances have been studied for use in replacing antibiotics to interact with the intestinal microflora, including immunity modulating agents, phages, phage lysins, peptides, probiotics, prebiotics, synbiotics (combining probiotics and prebiotics), acidification of diets, plant extracts, clay minerals, enzymes, and the inhibitors targeting pathogenicity (bacterial quorum sensing, biofilm, and virulence) (Hooze 1999; Patterson and Burkholder 2003; Ricke 2003; Diebold and Eidelsburger 2006; Hruby and Cowieson 2006; Kocher 2006; Vondruskova et al. 2010; Cheng et al. 2014). Phages might even be able to replace the growth promoting effects of antibiotics by controlling enteric bacteria that compete for food energy thus it was suggested that if phages are to be used to target enteric bacteria, the phage must be able to remain active during the digestive processes (Huff et al. 2006). In this regard two experiments were conducted to determine the effects of dietary supplementation with phage, probiotics and their combination on growth performance, apparent total tract digestibility, fecal bacteria populations and serum immunoglobulins in growing pigs (Kim et al. 2014). The results suggest that phages and probiotics both improve different aspects of a pig's growth performance but that phages are more effective than probiotics and would appear to offer an alternative to antibiotic type growth promoters. The suggested phage level required of 0.5 g kg⁻¹ could be used as an antibiotics alternative for growing pigs (Yan et al. 2012). In agreement with previous reports, pigs fed a diet containing anti-*Salmonella typhimurium* phage, at 3 × 10⁹ plaque-forming units (pfu) kg⁻¹ of feed, showed improved performance throughout the 28-day study (Geburu et al. 2010). They suggested further work needs to be conducted to better understand their mode of action in this class of pigs.

Nevertheless, phage therapy will not prevent the use of antibiotics as growth promoters, and could only substitute those antibiotics used for animal health purposes, therefore, as such, phage therapy would also be useful in uncoupling medical

care and growth promoting (Lorch 1999). Furthermore, without an understanding of the essential problems including phage resistance, phage–host interactions, the microbial ecosystem, and the host animal, this biological pathogen control system will not be used to its fullest potential in improving swine production (Zhang et al. 2015). In spite of all the above alternatives for ARGs Cheng et al. (2014) have summarized that it is hard to conclude that the alternatives might substitute for antibiotics in veterinary medicine in the foreseeable future. The latter authors have also concluded that the prudent use of antibiotics and the establishment of scientific monitoring systems are the best and fastest way to limit the adverse effects of the abuse of antibiotics and to ensure the safety of animal-derived food and environment.

2.9 Phage Therapy

Phage therapy in animal production and in animal experimental models of human infection have been investigated in veterinary therapy since early work, 30 years ago, in cattle (Smith et al. 1987), and the studies have been intensified in the last 10 years due to the increases of AMR strains occurring both among humans and animals (Sulakvelidze 2005; Sulakvelidze and Barrow 2005; Atterbury et al. 2007; Miller et al. 2010; Völkel and Czerny 2011; Abdulamir et al. 2014; Borie et al. 2014; Cheng et al. 2014; Jassim and Limoges 2014; O’Flaherty et al. 2014; Aldoori et al. 2015). Next we review a brief concept for phage therapy; however, this is discussed in greater detail in Chap. 3.

Phages have been extremely effective at treating a number of bacterial infections in controlled animal studies, especially as a biocontrol agent in the prevention of food-borne illnesses, due to its target specificity, rapid bacterial killing and ability to self-replicate (Smith et al. 1987; Biswas et al. 2002; Hawkins et al. 2010). Phages have the potential to treat bacterial infections afflicting animals and in particular to prevent fatal *E. coli* respiratory infections in broiler chickens (Huff et al. 2002a, b, 2003a, b). Aerosol spraying and intramuscular injection have given the best results over using oral delivery of phages via direct administration or addition to drinking water and/or feed (Sillankorva et al. 2012). This may be due to gastric pH levels preventing the proliferation of phages (Spits 2009). Virulent antigen specific phages have been used in an attempt to control *E. coli* O157:H7 in batch culture (Kudva et al. 1999). Loc-Carrillo et al. (2005) and Wagenaar et al. (2005) reported that phage therapy reduces *Campylobacter jejuni* colonization of broiler chickens. Several studies have also addressed the use of phages to decrease *Campylobacter* and *Salmonella* concentrations in poultry (Goode et al. 2003; Atterbury et al. 2007; Allen et al. 2013; Kittler et al. 2013). Phage therapy can be effective, for example against *Salmonella typhimurium* in poultry and pigs, although this requires rapid selection and administration of the phage and high bacterial loads (Allen et al. 2013).

However, despite the reports of the successful use of phages in terms of reducing mortality, the severity of the clinical state and bacterial counts in vitro, in vivo, on

tissues, and *ex vivo* levels, phage therapy remains controversial (Jassim and Limoges 2014). Furthermore, many earlier studies demonstrated that the classical application of phages in bacterial therapy or biocontrol is attainable in theory but in practice, it is not so successful due to the lack of full coverage of target bacteria and the rapid emergence of bacterial mutations leading to complete resistance against phage infection (Barrow and Soothill 1997; Alisky et al. 1998; Carlton 1999; Sulakvelidze et al. 2001; Goodridge and Abedon 2003). Therefore, historically, phage therapy or phage biocontrol were unsuccessful and eventually led to the replacement of phage therapy with antibiotic treatment (Barrow and Soothill 1997). Scientific methodologies could be developed to deal with antibiotic resistance in bacteria using phage, however viral proteins would also integrate into human and animal society with unknown effect. It would be wise to approach such methodologies with caution in order to avoid repeating mistakes that were made with the improper use of antibiotics. Other authors have refuted these assumptions and concluded that the rate of developing resistance against phages can be partially circumvented by using several phages in one preparation or cocktail (much like using two or more antibiotics simultaneously) (Sulakvelidze et al. 2001). More importantly, unlike using trial and error with antibiotics, when resistance against a given phage occurs, the specialists can rapidly select through testing (in a few days or weeks) a new phage that is effective against the phage-resistant bacteria (Sulakvelidze et al. 2001).

Veterinary therapy including both animal and human applications requires the appropriate administration targeting specific bacteria, with a strategy that includes a comprehensive methodology, detailing the phage–host interactions, dose optimization and accounting for all chemical and physical factors (Jassim and Limoges 2013). In general, a deep understanding of intrinsic phage properties is critical to designing therapeutic interventions. The reduction of foodborne pathogens requires a comprehensive phage control program at the farm, where the animals are born, hatched or raised, before shipment to processing plants. Potential pre-harvest sources of foodborne pathogen contamination include breeder herds and flocks, hatcheries, contaminated feed and water, along with environmental sources and vectors, such as litter, animal caretakers, and insects (Bailey 1993; Nayak et al. 2003; this is discussed in greater detail in Chap. 3).

2.10 Phage Therapy for Animals

Phages' specific bactericidal activity gives them special therapeutic advantages over antibiotics (Sulakvelidze et al. 2001; Sulakvelidze and Kutter 2005; Loc-Carrillo and Abedon 2011; Jassim and Limoges 2014), and phages have been reported to be more effective than antibiotics in experimentally infected animals (Smith and Huggins 1982). Like bacteria but unlike antibiotics, phages mutate and therefore can also evolve to counter phage-resistant bacteria (Matsuzaki et al. 2005). Because phages attack bacteria by attaching to receptors on the bacterial cell surface,

phage-resistant mutants (which lack these receptors) are often less pathogenic than phage-susceptible bacteria (Inal 2003; Santander and Robeson 2007; Capparelli et al. 2010; Friman et al. 2011; Laanto et al. 2012). The ability of phages to recognize precisely their target hosts, rendered them as favourable antibacterial agents because broad-spectrum antibiotics kill target bacteria along with other beneficial bacteria present on the farm or in the organism, namely, animal intestinal flora (Merril et al. 2003).

2.10.1 Advantages

- Phages are very specific and do not harm the useful bacteria that live in and on the body
- Phages can kill bacteria with no known activity in human or animal cells, making them an attractive alternative to antibiotics
- Because they are harmless, phages can be used for combating harmful bacteria in fattening animals and food
- In comparison to antibiotics, phages have no side effects like diarrhoea or secondary infections
- Phages are abundant in nature; therefore, it is easy to find new phages when bacteria become resistant to them. This means that there should be an ‘inexhaustible’ supply
- Low cost: the production of phages predominantly involves growth in its host and further purification
- Possible phage transfers between individuals: this is essentially cross infection of phages from treated subjects or environments to untreated individuals, which may be potentially useful in agricultural applications
- Phages are also active against bacteria that have become resistant to antibiotics
- Lysogenic phages can be genetically modified to reverse the bacterial pathogens’ drug resistance, thereby restoring their sensitivity to antibiotics
- Purified phage endolysins (lysins) can also be used as antibacterial substances
- Phages are living drugs. They multiply at the site of the infection until there are no more bacteria
- Bacteria that have become resistant to a certain type of phage continue to be destroyed by other types

There is renewed optimism for phages as new ‘live drugs’ with the hope to overcome the AMR problem. Phages have been approved for use in food and medical industries by several international agencies FDA, GRAS, US-FSIS (Jassim and Limoges 2014), and a phage cocktail that targets *Listeria monocytogenes* contaminants on ready-to-eat foods containing meat and poultry products was granted approval in 2006, which was the first time that the US Food and Drug Administration (FDA) accepted the use of a phage preparation as a food additive (Bren 2007; this is discussed in greater detail in Chap. 3). In the US, the only phages on the market are used in the anti-bacterial treatment of food products

Intralytix (<http://www.intralytix.com>), and in EU, 2013, launched the first product called ‘Phagoburn’ to treat burn wounds infected with bacteria *E. coli* and *Pseudomonas aeruginosa* (<http://www.phagoburn.eu/>). Recently an important extension to phage therapy is the use of phage lytic enzymes that digest the bacterial peptidoglycan, especially of Gram-positive bacteria, as a novel class of alternative antimicrobials (Fischetti 2008; Nelson et al. 2012; this is discussed in greater detail in Chap. 3).

2.10.2 Disadvantages or ‘Challenges’

Despite the attractions of phage therapy, scientific and logistical challenges remain. Phages have not yet gained widespread acceptance in therapy treatment as compared to commercially proven pharmaceutical antimicrobial agents. Phage therapy approval could take years and needs substantial investment once if the key obstacles facing phage are solved. The following summary outlines the key issues in phage biocontrol and treatment that scientists have already encountered both in the literature as well as in the laboratories. These can help to frame a platform from which past mistakes with both phages and antibiotics can be avoided.

2.10.3 Obstacles

- Heterogeneity and ecology of both phages and bacteria were not understood
- Need to select highly virulent phages against target bacteria in the patient
- Single phage preparations used to treat mixtures of different bacteria
- Recognition as personalized medicine using a multivalent cocktail carefully selected to treat a patient’s specific bacterial infection(s)
- Lack of standardized lytic phages that can target only their host cell without using genetic modification
- Genetically modified phages changing the composition of colonizing bacterial flora in humans, and the risk of subsequent development of new active infections
- Lateral gene transfer of virulence factors and antibiotic resistance
- Restriction, modification, and degradation of phage DNA upon infection
- Resistance mutations in bacterial genes for adsorption, lysogeny and lysogenic conversion
- Strict safety standards for human therapies not met
- Toxigenic conversion via lysogeny between species allowing more efficient adaptation of host, potentially leading to fitter and more virulent clones
- Failure to appropriately characterize or titre phage preparations
- Phages are relatively large in comparison to chemical molecules. For this reason, the sites in the body that can be reached by them must be carefully clarified

- Most of infection agents are hidden in the interior of human and animal cells and this makes it inaccessible to phages
- In the bacterial cell envelope, for example, use of antibiotics in animal production that can cause disruption of microbial cell wall synthesis which will inhibit or defect phage attachment
- Effect of environmental factors which all contribute to the complexity and unpredictability of phage–host interactions in the field, such as SUR and visible direct sunlight, chemical disinfectants, nutrients, pollutants etc.
- Limited knowledge about the kinetics of phage
- Consumer perception problems
- The isolation and the cultivation of phages from natural sources are time consuming and problematic for producing large amounts of active inoculums
- Failure to characterize phage preparation, i.e., to determine the virulence to the target
- Failure to neutralize gastric pH prior to oral administration
- Immunogenicity antibodies developed against phage
- There are a few reports of purified phages used in therapy having side effects
- Presence of endotoxins in phage preparation leading to toxic shock in the patient
- Phages can transfer toxin genes between bacteria via HGT
- Phages are more difficult to administer than antibiotics and physicians will need special training in order to correctly prescribe and use phages
- Phage efficacy in humans or animals is unknown and needs to be tested in the lab prior to use in each treatment
- Phage therapy effectiveness must be tested in an animal model, since each phage can behave differently in vivo
- To indicate the relationship between the phage and the concentration of target bacteria present, the multiplicity of infections must be established before each treatment
- Where multiple infections or the exact species of infecting bacteria is unknown, need cocktail of phages
- Phages are less suitable for acute cases therefore, phage therapy is best suited for infected sites such as wounds, where phages can be easily applied
- Pharmacokinetics of self-replicating agents differs from those of normal drugs
- The shelf life of phages varies and needs to be tested and monitored
- In vivo susceptibility of bacterial pathogens to phages is poorly understood and future research on more phage–host cell interactions is needed to define the requirements for successful phage treatments
- Many phage experiments done in vitro need to be extrapolated to in vivo growth
- Phages can be reproduced from a commercially available phage preparation, a challenge to commercialization
- Each newly isolated phage requires approval; this procedure could become too expensive
- Intellectual property rights are challenging for the use of phage therapy in modern medicine and these can also trigger ethical discussions

- In the healthcare system phage therapy is still seen as a cost and a social program rather than an economic driver
- Phage sectors need more time to develop entrepreneurs and innovation in their sector.

2.11 Discussion and Conclusion

Phage based therapy could potentially lead to bacterial development of phage resistance mutants. It would be wise to approach such methodologies with caution in order to avoid repeating mistakes that were made with the improper use of antibiotics. In this context, this may explain why only a handful of small pharmaceutical companies are turning to phages (Thiel 2004). The grand pharmaceutical companies have shown no interest in phage therapy, in large part because phages cannot be patented and much remains to be done regarding safety, mutations, and efficacy for therapy (Pirnay et al. 2011; Brüßow 2012; Jassim and Limoges 2014). A pharmaceutical drug development strategy for phage therapy is required as described by Jassim and Limoges (2014). The big question is whether phage therapy is ever going to be used to complement or replace traditional antibiotics in western countries.

Obstacles remain before phages can be used as AGP's. Phages have been shown to contribute in HGT of AMR genes and virulence factors from other bacteria as discussed. Insufficiently virulent phages, especially against actual target bacteria, allow bacteria to survive treatment while poorly prepared phage stocks, even if using sufficiently virulent phages, lack the numbers of viable phages required for adequate treatment. Phages converted to temperate phages are problematic due to converts turning phage-sensitive bacteria into insensitive ones, and the encoding of bacterial virulence factors, including bacterial toxins adhesion, haemolysin, serine protease, thermostable enterotoxin and special catalase system (Alexa et al. 2001; Hayashi et al. 2001; Krylov 2001; Skurnik and Strauch 2006; Sousa 2006; Skurnik et al. 2007; Merabishvili et al. 2009; Gill and Hyman 2010; Kutter et al. 2010; Vondruskova et al. 2010). Phages work as biological vehicles to transfer genes between bacteria constantly in the environment. Thus, using and manipulating such phages represents a serious risk to environmentally friendly bacteria and probiotic bacteria in animal intestines. It also potentially provides them with new virulence and aggression/aggressive factors, with resistance to antibiotics which could prompt a new wave of pathogenic bacterial diseases. Therefore, it is necessary to consider fundamentally different strategies to find solutions for the antibiotics dilemma in animal agriculture. This should be an incentive to develop other novel strategies, such as the smart lytic phage which is highly relevant to the practice of phage biocontrol and can pounce on and destroy (lyse) the bacterium quickly. This is discussed in greater detail in Chap. 1. In the next Chapter we describe novel methods and applications that can use smart phages as an optimal complementary tool along with other methods for bacterial growth control including 'safer

non-antimicrobial substances’. Smart phages as an alternative to AGPs may restore the balance in microbial environments as well as enhance the farm ecology by reducing animal infections thereby reducing the use of therapeutic antibiotics. In this regard the advantage of using smart phages as a replacement for AGPs will provide the following biological characteristics:

- Improvement in feed efficiency that is economically viable
- Specific antibacterial properties targeting unwanted bacteria without harming beneficial bacteria in animal’s intestines
- They become a part of gastrointestinal ecosystems and friendly with probiotic bacteria ‘the natural friendly gut bacteria’, and
- Prevent the HGT because they are highly lytic and they will replicate in the target host cell to achieve significant phage amplification resulting in greater bacterial killing
- Negligible or no resistance mutations in bacterial genes for adsorption, lysogeny and lysogenic conversion
- Selection of strictly highly virulent lytic phages
- Wide host range: the lytic spectrum of these particles can be much broader than the spectrum of activity of a single phage
- Smart phages with wide host range may not need to be used in combination with other antimicrobial agents, including other antibiotics or chemical disinfectants.

In general, this chapter illustrated the pros and cons of using phage in animal agriculture and is also an introduction for the following chapters, in which we discuss the methods of isolation, identification and analysis of phage to prepare smart phages for phage biocontrol. We go on to discuss various novel applications for phages. Lastly, we discuss practices that can be used to prepare commercial phage biocontrol products. Phage biocontrol describes a complete disinfectant system for every environmental niche, as described in Fig. 2.1. Phage biocontrol can be an alternative to antibiotics or chemical disinfectant agents which have side effects on livestock and are implicated in AMR while increasingly ineffective against pathogenic bacteria. We expect that the use of smart phage biocontrol in animal production may help to decrease the use of antibiotics in animal agriculture, in particular, on those farms adopting better hygiene practices (Castanon 2007).

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