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ANTIBIOTIC RESISTANCE IN ENTERIC INFECTIONS – EXPLORING A NEW APPROACH –

REZISTENȚA LA ANTIBIOTICE ÎN INFECȚIILE ENTERICE – EXPLORAREA UNEI NOI ABORDĂRI –

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ABSTRACT | REZUMAT

Antibiotic resistance is now regarded as a major existential threat to mankind. Extensive use, abuse and overuse in human and veterinary medicine and for growth promotion purposes in livestock has resulted, in some countries, with nearly 100% of strains of *Escherichia coli* and *Staphylococcus aureus* being multi-resistant. International Institutions have called for greater regulation of antibiotic use in human and veterinary medicine and cessation of their use for growth promotion. However, levels of resistance will remain high unless new approaches are developed and adopted which will reduce the currently high levels of carriage of antibiotic resistance plasmids. We have developed one of these approaches. We have used phages experimentally in animals for controlling different types of infection for many years. There is always concern about the development of resistance to the phage during therapy. This can be avoided by selecting phages which target surface virulence determinants such that any phage-resistant bacterial mutants that develop will be attenuated. We used phages which target the sex pili produced by self-transmissible antibiotic plasmids. With plasmids which are highly transmissible this type of phage not only specifically kills bacteria harbouring the plasmids, but phage resistant mutants which arise during therapy do not produce the sex pili and most of these have lost the plasmid and so become antibiotic sensitive. This has been shown *in vitro* and also *in vivo* in young chickens. With further development, this approach offers new opportunities for reducing levels of carriage of antibiotic resistance plasmids in enteric bacteria.

Keywords: antimicrobial resistance, swine, phages, plasmids

Actualmente, rezistența la antibiotice este privită ca o amenințare existențială majoră pentru omenire. Folosirea neadecvată, intensivă și abuzul în medicina umană și veterinară precum și utilizarea antibioticelor drept promotori ai creșterii animalelor au făcut, în unele țări, ca aproape toate tulpinile de *Escherichia coli* și *Staphylococcus aureus* să fie multirezistente. Instituțiile internaționale solicită o reglementare riguroasă a utilizării antibioticelor și încetarea utilizării acestora ca promotori de creștere. Cu toate acestea, nivelurile de rezistență vor rămâne ridicate, cu excepția cazului în care vor fi dezvoltate și adoptate noi abordări care vor reduce transmiterea plasmidelor rezistente la antibiotice. Noi am dezvoltat una dintre aceste abordări. Timp de mulți ani, pentru a controla diferite tipuri de infecții s-au utilizat fagi la animale. Totuși, și aici există îngrijorări privind dezvoltarea rezistenței la fagi, care poate surveni în timpul terapiei. Acest lucru poate fi evitat prin selectarea fagilor care vizează determinanții membranari ai virulenței, astfel încât mutațiile bacteriene care conferă rezistenta la fagi să fie atenuate. Ca urmare, s-au folosit fagi cu șase pili, produși de către plasmidele auto-transmisibile cu gene ale rezistenței la antibiotice. Acest tip de fag nu numai că ucide în mod specific bacteriile care conțin plasmidele transmisibile, dar mutațiile bacteriene rezistente la fagi care apar în timpul terapiei, nu mai produc pili și cele mai multe dintre aceste bacterii pierd plasmida și, astfel, devin sensibile la antibiotice. Acest lucru a fost demonstrat *in vitro* și, de asemenea, *in vivo* la puii broileri. Odată cu dezvoltarea ulterioară, această abordare oferă noi oportunități pentru diminuarea transferului plasmidelor rezistente la antibiotice în bacteriile enterice.

Cuvinte cheie: rezistență antimicrobiană, bacteriofagi, plasmide, suine

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One of the consequences of rearing animals, especially young animals, in close proximity to others and at high density is enteric infections, particularly neo-natal and post-weaning diarrhoea. This is most frequently bacterial (especially Enterotoxigenic *E. coli*, ETEC) or viral (rotaviruses, coronaviruses) but sometimes involving both. This is not unique to livestock and occurs in

young children in many countries. For decades, these have been addressed in veterinary medicine by oral antibiotic administration, both therapeutically and prophylactically. They also remain in use in many countries for stimulating the growth of livestock although this has been banned in Europe for several years. Use, overuse and abuse in human medicine both in the west and other countries has also resulted in very high levels of antimicrobial resistance (AMR) including, most worryingly, to some of the Critical Use Antibiotics such as 3rd and 4th generation cephalosporins, fluoroquinolones, polymyxins and certain glycopeptides. Zoonotic transfer of AMR bacteria is also a major source for humans, either by physical contact or by ingestion. Their use in veterinary medicine is now being scrutinised very closely (20). Quinolone and polymyxin resistance were always historically a chromosomal mutation but both are now found as characteristics mediated by self-transmissible plasmids, the latter first found in China in 2016(15) on a plasmid harbouring 18 additional genes encoding antibiotic resistance. This gene, *mcr-1*, has now spread globally as a result of movement of humans, livestock and food products. Multi-resistance has reached the stage where a small number of human infections have been completely untreatable with fatal consequences.Globally, antimicrobial resistance (AMR) is now recognised as a major existential problem for mankind (17).

ANTIMICROBIAL RESISTANCE AND SELF-TRANSMISSIBLE PLASMIDS

Most bacterial AMR is now mediated by small extra-chromosomal circular pieces of DNA called plasmids. These frequently code for important characteristics which are frequently important for the bacteria and may, for example, increase virulence, encoding adhesins, allowing *E. coli* to stick to the small intestinal epithelial cells, or toxins that induce the diarrhoea. They are clearly important in the evolutionary ecology of enteric and other bacteria. They can be classified by their ability or not to co-exist with other plasmids (Incompatibility) which is related to their replication mechanism. The first plasmid discovered in the 1940s was F and the majority of AMR plasmids in human medicine belong to this same Incompatibility group F (IncF) (9,13,14, 19)and it is also one of the major types in veterinary medicine. A key feature of plasmids is that most are self-transmissible which means that they can spread rapidly through a bacterial population and between other genera within the *Enterobacteriaceae*. This is clearly a major concern. The transmission process is called conjugation mediated by a large set of genes which in IncF plasmids involves >30 genes, one of which encodes a protein which coalesces on the bacterial surface to produce a sticky pilus which attaches to potential recipient bacteria. This sex pilus is also the attachment site for a range of bacteriophages (more on this below). In the original F plasmid every bacterial cell, which contains a plasmid, is able to transmit it (=highly de-repressed). In contrast, most wild-type plasmids show degrees of repression of self-transmissibility. It is thought that this provides a balance between low levels of transmission and resistance to these sex pilus-specific phages (9, 10, 31, 32).

AMR PLASMIDS AND GUT ECOLOGY

AMR plasmid transmission rates are lower between bacteria in the intestine (18, 21). The main reason for this is that enteric bacteria in the gut are outnumbered by other bacteria by a factor of more than 10^5:1 producing inert barriers to the initial bacterial contact. Removal of these "inert" bacteria by some oral antibiotics not only increases contact between enteric bacteria but can also select for a plasmid which encodes resistance to the antibiotic being used. In the gut it is the commensal organisms such as *E. coli* which are subject to the greatest selective exposure to oral and other antibiotics. AMR plasmids can thus, under some circumstances, transfer from commensal *E. coli* strains to enteric pathogens such as ETEC (Enterototoxigenic *E. coli*) and APEC (Avian Pathogenic *E. coli*) and *Salmonella* (26) with serious consequences for therapy. There is concern about the zoonotic transfer of AMR enteric bacteria to man by carcass contamination.

It is also worth mentioning that maintaining plasmids represents asignificant metabolic load on the bacteria and in the absence of appropriate selective pressure the plasmids may be gradually lost (25, 27).

WHAT IS TO BE DONE ABOUT THIS ?

The international community does understand the significance and enormity of the AMR problem and several international institutions (8, 30) have attempted to develop a strategy which might lead to reduced levels of infection. This has included (i) prohibition of all antibiotics as growth promoters, (ii) reduced use and improved management of antibiotic use, (iii) improved and more rapid diagnosis to enable more targeted antibiotic administration, (iv) improved biosecurity and, also (v) the development of new approaches to reducing antibiotic resistance. It is understood that simply reducing use will not affect bacterial strains which are already resistance and the epidemiological decline of resistance will be slow (25).

Bacteriophage (phage) is one of the newer approaches to infection control that have been explored increasingly during the last thirty years as a result of the great global concern over AMR.

PHAGE THERAPY

Phages are viruses which are specific for bacteria and since they do not infect humans or animals offer great potential for disease therapy in a post-antibiotic world. Unlike antibiotics, which can target many different types of bacteria, phages are very specific as they attach to and infect only bacteria which have specific surface structures to which the phages attach. They were discovered during the first world war and were soon proposed for use against bacterial infections (7) but the work done to assess them was poor and when antibiotics were gradually introduced nearly everyone lost in interest in phages (1, 2). They were still used in Russia and some satellite countries and are still used there to this day (28). In the west they were "rediscovered" in the 1980s by an imaginative Welsh bacteriologist who showed that they could be very effective against *E. coli* enteritis in pigs and calves (22-24). They have huge potential for treating bacterial infections but the conditions under which they can be used must be carefully defined. Therefore, should we not be using these to treat bacterial infections, especially those that are antibiotic resistant ? One of the issues raised by those with concerns over their use is that during treatment of a bacterial infection with phage, resistance to the phage can develop in the bacteria – certainly not a good thing ! This can be avoided. The surface of bacteria is what interacts with the animal host and contains some of the structures involved in disease, including capsules, some of which are also attachment sites for phages. Treatment with phages kills the pathogens and may select for resistance to the phages by the existence of random mutants in the culture that do not possess the surface virulence structure. So, they may be resistant to the phage but they are also attenuated.

SEX PILUS-SPECIFIC PHAGES – THE NEW APPROACH – EXPERIMENTAL WORK

This is where some lateral thinking proved to be useful to specifically tackle AMR in Gram-negative enteric bacterial pathogens. Phages were isolated in the 1960s which attach specifically to the sex pili produced by self-transmissible plasmids for the conjugation process (6). These phages were used to identify different types of plasmids (3, 4) and to explore bacterial genetics (11). We speculated that these phages might be used specifically to kill plasmid-containing bacterial cells, including bacteria containing AMR plasmids. One fascinating corollary of this idea is that any phage-resistant mutant that might exist in the bacterial population, and which might take over and predominate, should be phage resistant because they do not produce the pili and in most cases would do so because they had lost their plasmid and, into the bargain, had become antibiotic sensitive. Large plasmids are a metabolic drain on the bacterial cells and their elimination enables faster rates of growth and multiplication of the plasmid-negative bacteria. The consequence is that the plasmid-negative derivative will come to predominate in a culture *in vitro* and also *in vivo*. The contrast with typical phage therapy is that the development of resistance to the phage is actively sought. This approach is also highly specific for bacterial cells containing transmissible plasmids irrespective of the bacterial genus involved or of the genes encoded by the plasmid.

PRELIMINARY FINDINGS

The work, published by Colom et al. (2019), was initiated under purely experimental *in vitro* conditions (5). A bacterial lawn was made on a MacConkey plate of a lactose-non-fermenting, laboratory J62 strain of *E. coli* which possessed a derivative of the F plasmid containing the *lac* operon (thereby conferring on the lactose-negative J62 strain the ability to ferment lactose) and a transposon Tn*3*, conferring ampicillin resistance on the bacterial strain. 50uL drops of a high titre preparation of phage MS2 were added to the surface of the lawn. This phage is specific for the ability of bacteria possessing the F and F-like plasmids (6). Cultivation of the lawn resulted in discrete areas of bacterial lysis (Fig. 1, left image). Secondary bacterial growth was vi-

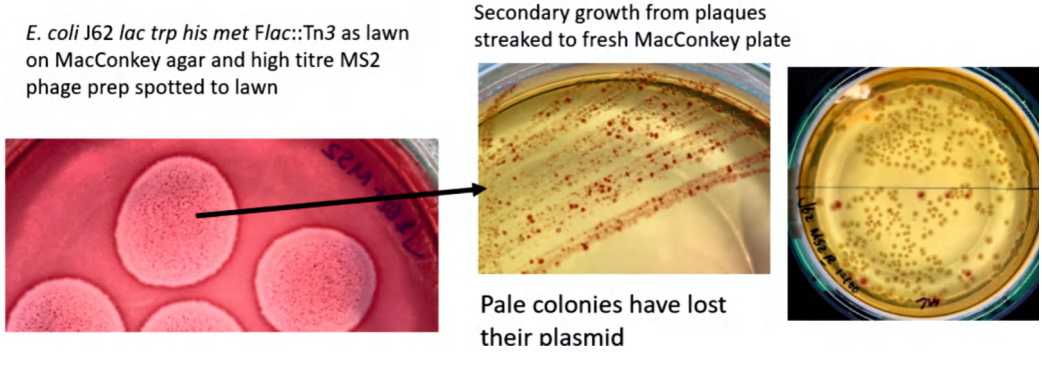

Fig. 1. The effect of bacteriophage MS2 on the carriage of a plasmid encoding ampicillin resistance and the ability to ferment lactose by a non-lactose-fermenting E. coli on MacConkey agar

sible within the areas of lysis most of which presumably were phage resistant mutants. Bacterial growth from the centre of the plaque was picked and streaked out and also diluted to a fresh MacConkey plate (Fig. 2, middle and right images) and it was clear that the majority of the phage-resistant mutants were now lactose -non-fermenting i.e. the bacterial cells had lost their plasmid and were phage-resistant. They were now also antibiotic (ampicillin)-sensitive.

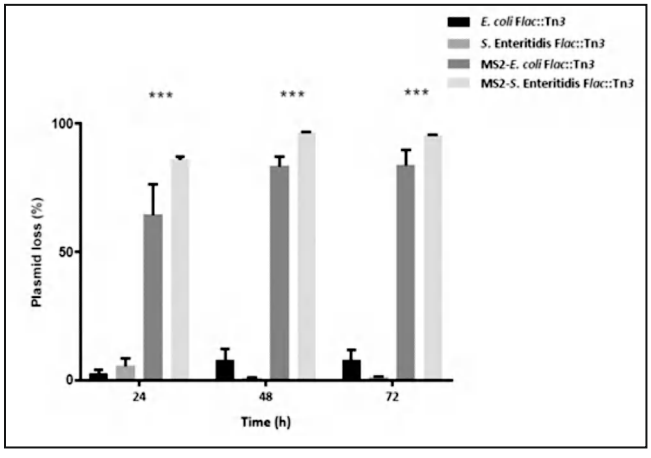

Fig. 2. The effect of passage in nutrient broth on the loss of plasmid F*lac*::Tn*3* from *E. coli* and *S*. Enteritidis induced by phage MS2

When they were checked, all the lactose-non-fermenting colonies had lost their plasmid and were phage resistant. The small number of lactose-fermenting colonies were also phage resistant, tested by their inability of the MS2 phage to attach to the bacteria. These phage-resistant mutants retained their plasmid and were resistant to ampicillin but they possessed mutations in the *tra* region of genes responsible for conjugation and had become non-self-transmissible.

IN VITRO EFFECT

We transferred the F*lac*::Tn*3* plasmid to a *Salmonella* Enteritidis strain by conjugation and looked at the effect of the MS2 phage on the J62 and *S*. Enteritidis strains containing the plasmid during prolonged passage in nutrient broth. Fig. 2 shows that the rate of plasmid loss was greatest in the first 24 hours but continued to increase thereafter. We also found out that the phage prevented the conjugation process presumably by binding to the pili and preventing attachment to recipient bacteria (data not shown).

The F plasmid is completely de-repressed meaning that it is highly transmissible from every bacterial cell and we wanted to see how the phage behaved with AMR plasmids that are more repressed. Measuring plasmid loss was more difficult and done by using replica plating. This showed that on prolonged culture the percentage of plasmid loss increased gradually to cca. 5% (Fig. 3).

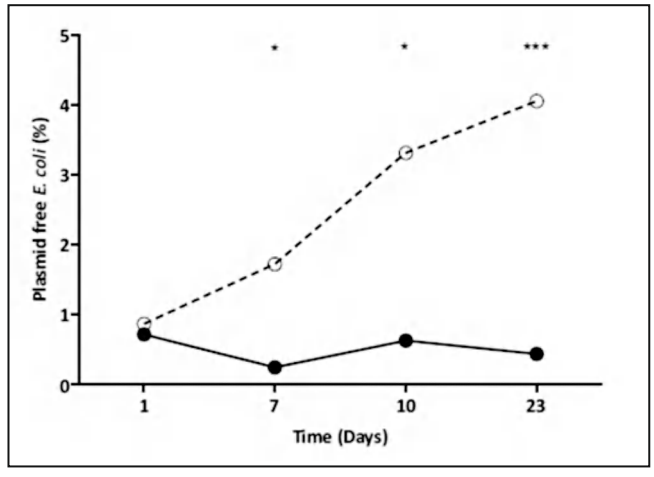

Fig. 3. The effect of phage MS2 on AMR plasmid loss in a wild-type poultry strain of E. coli resistant to streptomycin and sulphonamide.

We then assessed the effect during colonisation of the intestines and spread between very young chickens. Two groups of 15 newly-hatched chickens were housed separately and 5 birds of each group were inoculated orally with an avirulent *S*. Enteritidis strain possessing the F*lac*::Tn*3* plasmid. Spread of the infection from the 5 inoculated birds to the remaining 10 was monitored daily by microbiology and all the birds in one of the groups of 15 were inoculated orally daily and via the drinking water with a high titre MS2 phage preparation. Fig. 4 shows that spread of the S. Enteritidis to the group of initially uninoculated chickens was delayed by the phage treatment (Fig. 4a). When loss of the AMR plasmid was measured in the groups of 5 birds which were inoculated with the *Salmonella* very high levels of loss were detected after treatment with the phage (Fig. 4b). Interestingly, gradual increases were also observed in the birds not treated with phage indicating that random loss does occur and plasmid-negative derivatives clearly spread more rapidly. In the "in-contact" birds a similar picture of massive plasmid loss was seen but with a delay in increase in colonisation by the plasmid-negative strain (Fig. 4c).

DISCUSSION

We have shown that phage MS2 targets an *E. coli* and *Salmonella* strain harbouring a highly self-transmissible AMR plasmid and selects for increases in numbers of the random mutants in the bacterial population which have lost their plasmid. The results observed supported the initial hypothesis that use of phages which are specific to the sex pili produced by self-transmissible plasmids can have this beneficial effect.

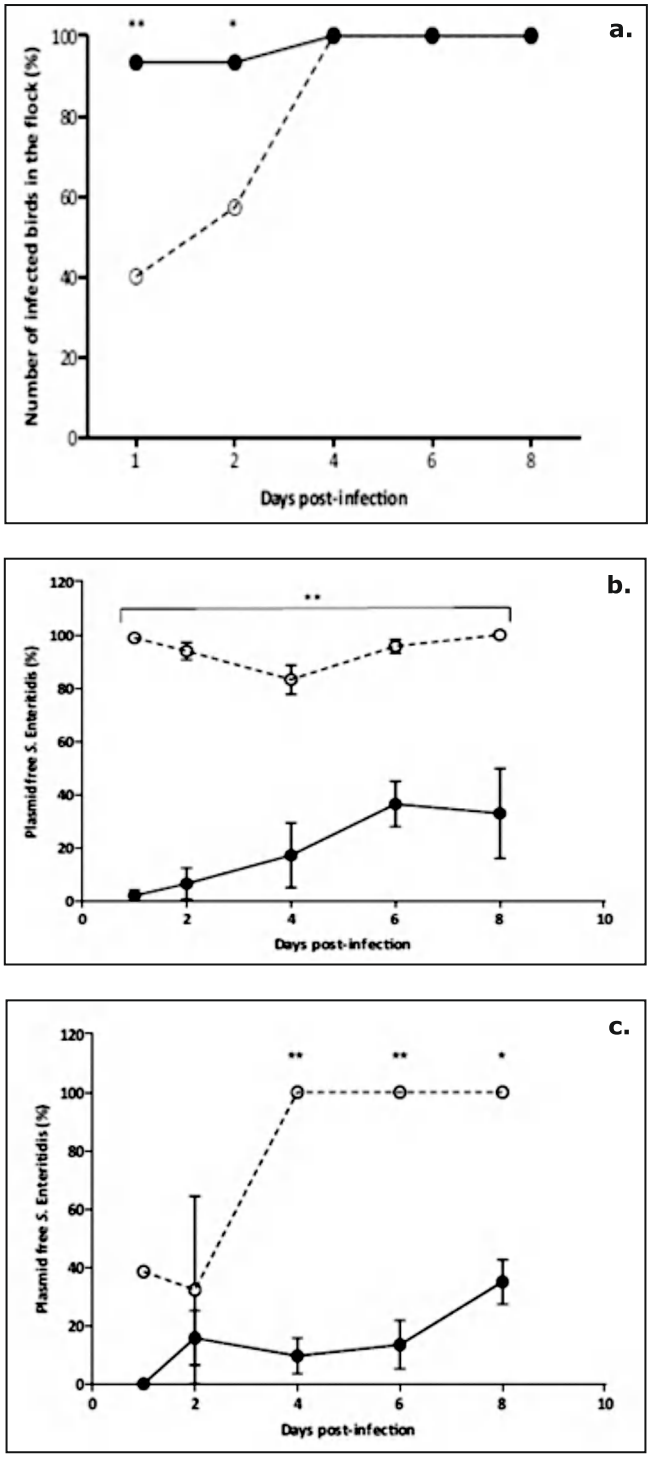

Fig. 4. Effect of phage MS2 on spread of *S.* Enteritidis between chickens in a group and on loss of plasmid Flac::Tn3 without phage treatment, with phage treatment. (a) Transmission of the Salmonella within the groups with time. (b) Plasmid loss in the groups of 5 chickens inoculated with the Salmonella. (c) Plasmid loss in the groups of 10 chickens not inoculated with the Salmonella and into which it had spread.

This effect has also been found with sex pilus-specific phages and plasmids of different Incompatibility groups (12, 16) but this was the first time that this has been demonstrated *in vivo*. This is clearly a general phenomenon which is related to the interaction between bacteria, plasmids and phage and is likely to be a major feature ecologically whether this occurs in the intestines of animals or in the environment, in current times in sewage and surface water. The advantage of carrying out this work with Incompatibility group F AMR plasmids is that these represent the majority of wild-type plasmids (9, 13,14,19) although some plasmids now occur with more than one replicon. There is thus clearly scope to apply this sort of technology in veterinary medicine.

It has been mentioned above that this effect is greatest with plasmids where self-transmissibility is not-repressed and also has a positive effect for plasmids which are partially-repressed. However, many wild-type plasmid are highly repressed with rates of plasmid transfer of less than 10^{-6} pre recipient (with F the rates are greater than 50%) and it seems unlikely that using these phages alone will have a sufficiently positive effect on plasmids such as these. Additional approaches are therefore required to increase phage susceptibility. However, these phages reduce conjugation and since, even for repressed plasmids, transfer is followed by a short burst of transmissibility, they should at least reduce transfer of repressed plasmids.

It is well known that plasmids have a mechanism which facilitates their maintenance in a bacterial population by killing those bacterial cells which have lost their plasmid during cell division (29). Therefore, in theory the extent of plasmid loss that we have seen here should not be possible. Further investigation of this aspect is therefore recommended.

CONCLUSION

Sex pilus-specific bacteriophages are effective in killing bacteria with highly transmissible plasmids, including antibiotic resistance plasmids. We have shown this *in vitro* and *in vivo* in young chickens. These phages also increase plasmid loss in semi-repressed plasmids after prolonged passage.

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