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Apramycin-resistant *Escherichia coli* isolated from pigs and a stockman

J. E. B. HUNTER[1,2]*, M. BENNETT[1], C. A. HART[2], J. C. SHELLEY[1] AND J. R. WALTON[1]

Departments of Veterinary Clinical Science[1] and Medical Microbiology[2], University of Liverpool, PO Box 147, Liverpool L69 3BX

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SUMMARY

Escherichia coli serotype O147:K89:K88a,c was found to be associated with outbreaks of diarrhoea in preweaner pigs of up to 4 weeks of age on a pig unit. Resistance to apramycin, gentamicin, netilmicin, tobramycin and other antibiotics was associated with conjugative plasmids of approximately 62 kb. The presence of a gene which encoded for the aminoglycoside acetyltransferase enzyme AAC(3)IV was confirmed by DNA hybridization.

Samples collected during the following 12 months revealed widespread dissemination of these resistance plasmids in non-serotypable, non-haemolytic *E. coli* throughout the farm. Apramycin-resistant *E. coli* were also isolated from a stockman and it appeared from plasmid profile analysis and antibiotic sensitivity testing that the human isolates carried the same plasmid as that carried by the porcine *E. coli*. *Klebsiella pneumoniae*, with a slightly smaller conjugative plasmid and similar resistance pattern, was isolated from the stockman's wife.

INTRODUCTION

Some *Escherichia coli* strains are known to be pathogenic while others are regarded as commensal strains found normally in the intestinal flora. It has been well documented that certain toxigenic *E. coli* strains or serotypes are more likely to be the cause of preweaning and postweaning diarrhoea in pigs [1] and that these toxins can be plasmid-encoded [2]. It is not known if the presence of antibiotic resistance plasmids in toxigenic *E. coli* has an effect on pathogenicity, although antibiotic resistance is considered to hinder treatment [3].

Resistance to the aminoglycoside apramycin has been found in salmonellas and *E. coli* isolated from farm animal species [4–6] and in *Salmonella typhimurium* 204c and other enterobacteria isolated from humans [7–9].

Apramycin is used in the United Kingdom in calves and young pigs for the treatment of diarrhoea and septicaemia caused by Gram-negative bacteria. We describe here the distribution of apramycin-resistant *E. coli* on a pig unit where

* Present address: Scottish Agricultural College Veterinary Services, Greycrook, St. Boswells, Roxburghshire TD6 OEU.

apramycin had been used regularly for several years previously although not for over a year before the study began.

Farm history

Repeated outbreaks of neonatal and postweaning colibacillosis had occurred on a breeding and finishing pig unit for several years. The unit comprised approximately 180 breeding sows and the total number of pigs on the farm varied according to the stage reached in a policy to eradicate swine dysentery. The buildings consisted of two farrowing houses, flatdeck accommodation and arks for postweaner pigs of 4–10 weeks of age and a flattening house for grower pigs from 10 weeks to 4 months of age. The pig unit was managed in combination with a dairy cattle herd, several sheep and also boarding kennels.

E. coli possessing the fimbrial antigen K88 had been isolated from young pigs on several occasions previously and it was recognized that these bacteria were associated with recurring episodes of diarrhoea and poor weight gain. The veterinary surgeon involved found the disease difficult to control as there had been poor response to both a variety of antibiotics and hygiene measures taken to improve the situation. The *E. coli* isolates were usually resistant to several antibacterial agents. Investigation of this farm for the purposes of this study began where apramycin-resistant *E. coli* O147:K89:K88a,c was isolated in pure culture from rectal faeces of young pigs.

MATERIALS AND METHODS

Samples of faeces were collected from pigs and a cat by inserting a cotton-wool tipped swab into the rectum. These were placed immediately in containers containing transport medium and stored for up to 18 h at 4 °C. For longer-term storage swabs were stored in 15% glycerol broth at −20 °C. Days on which samples were collected are listed in Table 1.

Samples of slurry, mud and voided pig faeces were collected by submersing the cotton-wool tip of a bacteriology swab and storing as described above. Water samples were collected in 20 ml volumes in plastic screw-topped containers and cultured by flooding an agar plate with approximately 1 ml.

Human faecal specimens were collected in plastic screw-topped containers. The stool was swabbed for bacteriological examination. Swabs were stored as described above and the faecal specimens at −20 °C.

Swabs were used to inoculate MacConkey Agar No. 3 (Oxoid CM115) supplemented with 32 μg/ml apramycin sulphate (Dista). Swabs from human samples where there was no growth from this culture were also preincubated overnight in nutrient both. Colonies were confirmed as *E. coli* and *Klebsiella* sp. by using an API 20E kit (API System). The majority of *E. coli* were serotyped using slide agglutination with *E. coli* antisera (including polyvalent A, K91, K89 and K88) supplied by the Central Veterinary Laboratory, Addlestone. The serotype of representative isolates was confirmed by the Central Veterinary Laboratory, Addlestone.

Antibiotic sensitivity was assessed using a controlled disk diffusion test with Isosensitest Agar (Oxoid CM471) and Oxoid disks [10]. *E. coli* NCTC 10418 was

Table 1. *Isolations of apramycin-resistant* Escherichia coli

Day	Total number of pigs sampled	Number of pigs with *E. coli* apr+*	Other animal species with *E. coli* apr+
1	4	4	NT
38	18	18	NT
46	8	6	NT
130	10	6	Stockman, cat
210	NT	NT	Stockman's wife†
280	19	16	Calves

* *E. coli* apr+, apramycin-resistant *E. coli* isolated.
† *Klebsiella pneumoniae* isolated, not *E. coli*.

used as a sensitive control organism. Disks, containing the following amounts of antibiotic (μg), were used: amikacin 30 (Ak), amoxycillin and clavulanic acid 30 (Ac), ampicillin 10 (Am), apramycin 15 (Ap), chloramphenicol 10 (Cm), ciprofloxacin 5 (Cp), compound sulphomanides 500 (Su), furazolidone 15 (Fr), gentamicin 5 (Ge), kanamycin 5 (Kn), nalidixic acid 30 (Nx), neomycin 10 (Ne), netilmicin 10 (Nt), oxytetracycline 30 (Tc), spectinomycin 25 (Sp), streptomycin 25 (Sm), tobramycin 10 (Tb) and trimethoprim 5 (Tm). Isolates were deemed resistant if the zone of inhibition around the disks was $\leqslant 3$ mm width or the zone was $\geqslant 3$ mm less than the control zone.

Minimal inhibitory concentrations (MIC) of apramycin and gentamicin were also determined [6].

Plasmids were transferred by conjugation in broth [6]. Bacterial DNA was extracted [11] and the plasmids separated by electrophoresis in 0·7% agarose gels and their molecular weights determined by comparison with four reference plasmids carried in *E. coli* strain 39R861 [7]. The plasmid pWP701 [12] was provided by W. Piepersberg (University of Munich, Germany). A 1·65 kb Pst-1 fragment of pWP701 containing the gene for AAC(3)IV was purified from agarose using glass beads (Geneclean, Bio 101, California, USA) radiolabelled by nick translation and used as a probe in colony hybridizations [13].

RESULTS

Swabs of rectal faeces were taken on day 1 of the study from 2 unweaned pigs in a farrowing house and 2 weaned pigs during an outbreak of diarrhoea and apramycin-resistant *E. coli* O147:K89:K88a,c was isolated from 3 of the 4 pigs. Non-serotypable apramycin-resistant *E. coli* were isolated from the fourth pig. Both O147 and untypable *E. coli* carried plasmids and both transferred a similar 62 kb apramycin resistance plasmid to recipient *E. coli* K12. Resistance patterns were determined for some of these plasmids (Table 2). Resistance patterns varied although the molecular weight of the plasmids did not. The O147 isolates carried resistance to kanamycin and neomycin, unlike the untypable isolates on this occasion.

Apramycin-resistant untypable non-haemolytic *E. coli* were isolated from pigs on four occasions during the following 7 months (Table 1). Isolates conjugated successfully and plasmid profiles of the transconjugants revealed plasmids of

Table 2. *Resistance patterns of apramycin resistance plasmids from* Escherichia coli *isolated from pigs with diarrhoea sampled on the first day of the survey*

E. coli serotype	Antibiotic resistance conferred by plasmids									
O147:K89:K88a,c	Ap	Ge	Kn	Ne	Nt			Tb	Tc	Tm
	Ap	Ge	Kn	Ne	Nt			Tb	Tc	
Untypable	Ap	Ge			Nt	Sm	Sp	Tb	Tc	Tm
	Ap	Ge			Nt	Sm		Tb	Tc	Tm
	Ap	Ge			Nt			Tb	Tc	Tm

Ap, apramycin; Ge, gentamicin; Kn, kanamycin; Ne, neomycin; Nt, netilmicin; Sm, streptomycin; Sp, spectinomycin; Tb, tobramycin; Tc, oxytetracycline; Tm, trimethoprim.

Table 3. *Resistance patterns of 18 apramycin-resistant* E. coli *isolates from 18 pigs on day 38*

Resistance Pattern code														Age of animals*			
														A	B	C	D
1	Am	Ap	Cm	Ge			Nt	Sm	Sp	Su	Tb	Tc	Tm				1
2	Am	Ap	Cm	Ge			Nt	Sm	Sp	Su	Tb	Tc		3	1		3
3	Am	Ap		Ge	Kn	Ne	Nt	Sm	Sp	Su	Tb	Tc				1	
4		Ap	Cm	Ge			Nt	Sm		Su	Tb	Tc	Tm	1			1
5		Ap		Ge			Nt	Sm	Sp	Su	Tb	Tc			1		
6		Ap		Ge			Nt			Su	Tb		Tm	1			
7		Ap	Cm	Ge			Nt	Sm	Sp	Su	Tb	Tc	Tm			1	
8		Ap		Ge			Nt		Sp	Su	Tb					1	1
9		Ap		Ge	Kn	Ne	Nt	Sm		Su	Tb	Tc	Tm		1		
No. of patterns with resistance for each antibiotic	3	9	4	9	2	2	9	7	6	9	9	7	5				

* A, less than 4 weeks; B, 4–10 weeks; C, 10 weeks–4 months; D, sows.

Key to abbreviations: Am, ampicillin; Ap, apramycin; Cm, chloramphenicol; Ge, gentamicin; Kn, kanamycin; Ne, neomycin; Nt, netilmicin; Sm, streptomycin; Sp, spectinomycin; Su, compound sulphonomanides; Tb, tobramycin; Tc, oxytetracycline; Tm, trimethoprim.

approximately 62 kb. An example of resistance patterns found from isolates collected on day 38, from 18 pigs, is shown in Table 3.

On day 46 samples were collected from the environment and isolations of apramycin-resistant *E. coli* were made from the following: sow trough, piglet faeces, piglet feeding area, diarrhoeic piglet faeces on the floor, piglet water trough, pathway near fattening unit, walkway in dry sow house, rainwater puddle in yard, feed passageway in sow house, slurry, entrance to farrowing house, ground at feed dispenser, and a puddle in the calf house.

Between days 130 and 286, 11 faecal samples were collected from 7 humans on the farm. Apramycin-resistant *E. coli* were isolated from a pighandler only on day 130 and not on day 210 and not from any other humans. However, apramycin-resistant *Klebsiella pneumoniae* was isolated from the sample from his wife. A 57 kb plasmid from this isolate transferred to *E. coli* K12 and also to *Salmonella typhimurium* 204c. The resistance pattern conferred by the plasmid in the K12

Table 4. *Apramycin-resistant isolates showing source, plasmid MW and resistance transferred*

Origin of *E. coli* isolate	Plasmid (kb) transferred	Antibiotic resistance transferred*
Piglet	62	Ap Cm Ge Nt Sm Sp Tb
Postweaner	62	Ap Ge Ka Ne Nt Su Tb
Grower	62	Ap Ge Nt Sm Sp Su Tb
Stockman	62	Ap Ge Nt Sm Sp Su Tb
Wife†	57	Am Ap Ge Nt Sm Tb

* Am, ampicillin; Ap, apramycin; Cm, chloramphenicol; Ge, gentamicin; Ka, kanamycin; Ne, neomycin; Nt, netilmicin; Sm, streptomycin; Sp, spectinomycin; Su, compound sulphomanides; Tb, tobramycin.

† *Klebsiella pneumoniae*.

transconjugant and also to the *S. typhimurium* isolates is shown in Table 4. In addition a non-serotypable apramycin-resistant *E. coli* was isolated from a farm cat on day 130.

Plasmid profiles revealed that non-serotypable *E. coli* isolates collected on day 130 from 2, 6 and 12-week-old pigs and the pighandler all contained a similar plasmid of approximately 62 kb which encoded apramycin resistance and was transmissible by conjugation to *E. coli* K12 (Table 4). Three resistance patterns were demonstrated but the plasmid from the pighandler had the same pattern as that from the grower pig (Table 4). The plasmid from the cat isolate was of heavier molecular weight (154 kb).

Approximately 7 months (day 280) after the first apramycin-resistant isolate had been collected the farm was revisited. Apramycin-resistant non-serotypable *E. coli* were isolated from 16 of 19 pigs (Table 1). These pigs included some from each age group tested: farrowed sows and their piglets in two houses, 10-week-old fatteners, dry sows, 4–6-week-old weaners and gilts. Water and sediment from a stream which ran near to the farm were sampled and apramycin-resistant *E. coli* were isolated. Five out of six swabs taken from various puddles of water on the farm were also positive as well as two calves a few days old and a dung channel in the calf unit.

All *E. coli* isolates listed in Tables 2, 3 and 4, the *Klebsiella pneumoniae* isolate and transconjugants of donor isolates in Table 4 hybridized with the AAC(3)IV DNA probe.

DISCUSSION

There have been few reports of the ecology of apramycin-resistant coliforms in farm animals. Ose and colleagues reported in 1976 that less than 1% of *E. coli* from farm animals in one survey produced the AAC(3)IV enzyme [14]. The present study has show that apramycin-resistant *E. coli* could be isolated from pigs on a farm on a regular basis although, because a selective media was used, the proportion of *E. coli* resistant to apramycin was not determined.

Dissemination of apramycin-resistant *E. coli* was demonstrated in pigs of different age groups and in the environment, both inside and outside buildings. It is possible that the use of apramycin 3 years previously had established a resistant

E. coli population which was being maintained by the use of other antibiotics including oxytetracycline and chloramphenicol since apramycin had not been used during the previous year. Persistence of pathogenic porcine *E. coli* for several months in an empty pig house has been reported [15]. In the present study apramycin-resistant *E. coli* were isolated from rainwater puddles and a stream allows the possibility of water draining from the farm and contaminating the local waterway. Distribution of slurry on fields could also allow dissemination of these resistant *E. coli*.

Both potentially serotypable/pathogenic and non-serotypable apramycin-resistant *E. coli* strains were found in both healthy and diseased pigs and it may be that the resistant non-serotypable *E. coli* act as a reservoir of multiple resistance for pathogenic serotypes or *vice versa* since plasmids were readily transferred in the laboratory to *E. coli* and *S. typhimurium*. There is a possibility, therefore, of *in vivo* transfer of resistance plasmids between *E. coli* and also salmonella as described by Hunter and colleagues in the intestine of calves [6] or in the environment.

The results showed that different resistance patterns were transferred by plasmids of similar molecular weight. This demonstrated that the apramycin resistance plasmids were not identical and further studies to characterize them more fully are in progress.

Jorgensen and Sorensen found that an 80 kb plasmid encoding resistance to chloramphenicol and several other antibiotics was common to three porcine *E. coli* serotypes [16]. These authors suggested that this demonstrated the ability of a plasmid to transfer successfully in natural conditions. It would appear that this is likely with apramycin-resistant plasmids as this resistance associated with plasmids of 62 kb was often found in non-serotypable as well as pathogenic *E. coli* isolates.

The isolation of apramycin-resistant *E. coli* with similar plasmid profiles and identical antibiotic resistance pattern from both the stockman and a pig suggest transmission of apramycin-resistant *E. coli* from pig to man while the isolation of apramycin-resistant *K. pneumoniae* was made from the stockman's wife despite her having no direct contact with pigs. The faecal sample tested had to be pre-incubated for apramycin-resistant colonies to be isolated, suggesting that low numbers of these bacteria were present. It is unlikely that *K. pneumoniae* itself originated in the pigs as this was not isolated from pigs using apramycin selective agar on any of 20 pig farms studied during a 3 year project [3, 18]. That no apramycin-resistant *E. coli* were isolated at the same time does not exclude the possibility that *E. coli* of porcine origin may have been ingested previously and been the source of a transferable plasmid for *K. pneumoniae*. The apramycin-resistant plasmid in the *K. pneumoniae* had a slightly lower molecular weight (57 kb) compared with that of *E. coli* (62 kb) and some genetic information may have been lost in transfer, if transfer had indeed occurred. Another possibility is that *K. pneumoniae* could have been of hospital or other origin as the stockman's wife had been hospitalized on several occasions. At the time of isolation she was receiving treatment with cephalexin but *K. pneumoniae* was found to be sensitive to this antibiotic. Apramycin-resistant *K. pneumoniae* has been reported previously in humans [19] and we have previously shown apramycin-resistant

E. coli to be prevalent in a hospital [9]. Plasmids carrying trimethoprim resistance in *E. coli* have previously been reported as identical in both pigs and humans indicating an overlap of bacterial strains in man and other animals [17].

This study has shown that apramycin-resistant *E. coli* can persist on a pig farm without direct selection by the use of apramycin. Previous work has shown that such resistance is widespread [3, 17] and that transfer of resistance can occur from non-serotypable *E. coli* to other pathogenic enterobacteria during antibiotic treatment [6]. Langlois and colleagues [20] indicated that tetracycline resistance can persist for over 10 years in pig *E. coli* in the absence of any antibiotic selection pressure but where tetracycline had been used previously. Similarly Gellin and colleagues [21] concluded that reversion to lower levels of antibiotic resistance and multiple resistance will take a long time once all use of antimicrobial agents has ceased. The findings from the present study indicate that transferable apramycin resistance occurs and that in some instances may lead to inefficacy of a valuable antibiotic in treating serious infections. One likely mechanism involved in the maintenance of apramycin resistance may be the use of other antibiotics which select for plasmids which also encode for apramycin resistance. The increased prevalance of apramycin resistance in human *E. coli* [9], and the isolation of apramycin-resistant *E. coli* from humans in this and other studies, is of note. However, more work is required to understand the mechanisms by which resistant bacteria are maintained in a host population, particularly the roles of direct ingestion of faeces and environmental contamination. Larger-scale studies of humans in contact with animals or animal products and comparison of resistance plasmids from human and animal isolates may provide a clearer understanding of the importance of the transmission of multi-resistant commensal *E. coli* from animals to man. The persistence of apramycin-resistant *E. coli* in the environment would also appear to be worthy of further investigation.

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