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Comparison of antimicrobial resistance phenotypes and genotypes in enterotoxigenic *Escherichia coli* isolated from Australian and Vietnamese pigs

M. Smith [a], T.N. Do [b], J.S. Gibson [a,*], D. Jordan [c], R.N. Cobbold [a], D.J. Trott [d]

[a] The University of Queensland, School of Veterinary Science, Gatton, QLD 4343, Australia
[b] National Institute of Veterinary Research (NIVR), 86 Truong Chinh, Hanoi, Vietnam
[c] New South Wales Department of Primary Industries, Wollongbar, NSW 2477, Australia
[d] School of Animal and Veterinary Sciences, The University of Adelaide, Mudla Wirra Road, Roseworthy, SA 5371, Australia

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ABSTRACT

This study aimed to compare the antibiogram phenotype and carriage of antimicrobial resistance genes (ARGs) of 97 porcine multidrug-resistant (MDR) enterotoxigenic *Escherichia coli* (ETEC) isolates obtained from Vietnam and 117 porcine MDR-ETEC obtained from Australia, two countries with different antimicrobial regulation systems. An antimicrobial resistance index (ARI) was calculated to quantify their potential significance to public health. Both Vietnamese and Australian isolates had moderate to high levels of resistance to commonly used antibiotics (ampicillin, tetracycline and sulphonamides). None of the Australian isolates were resistant to fluoroquinolones or third-generation cephalosporins and none possessed associated plasmid-mediated ARGs. However, 23.1% of Australian isolates were resistant to gentamicin owing to ARGs associated with apramycin or neomycin resistance [e.g. *aac(3)-IV*] that impart cross-resistance to gentamicin. Whilst Vietnamese isolates carried aminoglycoside ARGs, 44.4% of commercial pig isolates were resistant to gentamicin in comparison with 0% of village pig isolates. The plasmid-mediated fluoroquinolone ARG *qnrB* was commonly detected in Vietnamese isolates (52.3% commercial, 44.1% village), but phenotypic resistance was low (3.2% and 11.8%, respectively). The mean ARI for Vietnamese isolates (26.0) was significantly different ($P < 0.001$) from the mean ARI for Australian isolates (19.8), primarily reflecting fluoroquinolone resistance in the former collection. This comparison suggests the effectiveness of regulations that slow the dissemination of 'critical' resistance by restricting the availability of important classes of antimicrobials.

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is an important pathogen in swine production, causing neonatal diarrhoea and post-weaning diarrhoea in young piglets. Although a wide range of therapeutic products is effective, the availability of specific drugs varies between countries [1,2] owing to regulatory differences for use in animals. The most commonly used antimicrobials include ampicillin, neomycin, apramycin, spectinomycin, colistin, zinc oxide and potentiated sulphonamides [3]. Long-standing reliance on these drugs has resulted in selection for antimicrobial resistance, and multidrug resistance has become common in porcine ETEC [4–7].

Australian regulations governing antimicrobial use in livestock are conservative [8], with a restricted range of antimicrobials registered [9]. Neither fluoroquinolones nor gentamicin can be administered [10], and the third-generation cephalosporin ceftiofur has strict label constraints. However, it can be used 'off-label' to treat colibacillosis caused by multidrug-resistant (MDR) strains of *E. coli* [5]. Fluoroquinolone drugs and the third-generation cephalosporins are regarded as 'critically important' in human medicine and should be considered as drugs of last resort in food animals [11]. The most recent survey into Australian swine herds reported strong reliance on antimicrobials not considered to be as critically important to human health (such as penicillins, tetracyclines and sulphonamides) [12]. Nevertheless, the same study revealed that 25% of herds had used ceftiofur in the previous

* Corresponding author. Tel.: +61 7 5460 1830; fax: +67 7 5460 1922.
E-mail addresses: gibson.j@uq.edu.au, justine.gibson@uqconnect.edu.au (J.S. Gibson).

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year, suggesting difficulties in managing infections such as MDR-ETEC.

In a recent study of Australian porcine ETEC [4], no resistance to ceftiofur or fluoroquinolones was found. However, a large number of isolates were MDR to 'lower importance' antimicrobials. Some isolates were resistant to the aminoglycosides, apramycin and neomycin (ca. 30% for each), presumably as a consequence of their recent use in ETEC management.

Aminoglycoside resistance may pose a potential risk as some resistance genes can impart cross-resistance to gentamicin, which is still highly valued in human medicine [13]. Whilst controlled use of antimicrobials in Australian pigs has probably slowed the onset of resistance to some 'critical' drugs [4], it may come at the cost of more rapid selection of resistance to other groups such as the aminoglycosides. One approach for studying this is to compare the resistance attributes of ETEC isolates originating from different regulatory environments.

Vietnam is representative of many developing countries. It has high levels of antimicrobial use both in humans and animals and resistance among bacterial pathogens is widespread [1]. Use of antimicrobials in commercial pig production is largely unregulated and access to veterinary advice is limited. In village-based enterprises, pigs may be treated with any available antibiotics [14,15]. Consequently, in Vietnam and countries with similar regulation systems, there is potential for rapid emergence of antimicrobial resistance to a wide variety of drugs. However, whilst one study has focused on resistant phenotypes in pathogenic *E. coli* isolates from swine in Vietnam [1], no studies have been conducted to correlate the drug resistance phenotype with genotypes.

Pigs in Australia are virtually all raised under the same system of intensive production common to the developed world, albeit with a conservative approach to antimicrobial registration. Pigs in Vietnam are managed in two distinctly different production systems, which are both relatively unregulated with regard to antimicrobial use: village pigs raised by smallholder farmers; and commercial pigs raised using an intensive approach similar to Australia.

In a previous study, Smith et al. characterised a collection of Australian porcine ETEC isolates to determine the frequency of occurrence of single resistance attributes and associated antimicrobial resistance genes (ARGs), allowing an antimicrobial resistance index (ARI) to be attributed to each isolate [4]. The current study applied the same methodology to a collection of ETEC isolates obtained from village and commercial pigs from Vietnam and then compared these results with the data previously obtained from the Australian ETEC porcine collection, with the aim of investigating relationships between levels of regulatory control of antimicrobial use with phenotypic and genotypic resistance levels amongst production porcine isolates.

2. Materials and methods

2.1. Bacterial isolates

A total of 97 MDR-ETEC isolates (2001) were obtained from Vietnamese pig faecal samples as described previously [1]. Animals' symptoms were consistent with porcine colibacillosis. In total, 63 and 34 isolates were collected from commercial piggeries and village piggeries, respectively, in northern Vietnam.

Veterinary diagnostic laboratories throughout Australia provided 117 MDR-ETEC isolates (1999–2005), as described in a previous study [4]. The isolates originated from Queensland ($n = 52$), Victoria ($n = 28$), South Australia ($n = 27$), Western Australia ($n = 8$) and New South Wales ($n = 2$). All isolates were obtained from the faeces or intestine of pigs with symptoms of post-weaning diarrhoea and were stored in brain–heart infusion broth (Oxoid Australia Pty Ltd., Thebarton, SA, Australia) plus 20% glycerol (ChemSupply Pty Ltd., Gillman, SA, Australia) at −80 °C and as freeze-dried specimens.

2.2. Antimicrobial susceptibility testing

The ETEC collection was screened for resistance to 12 antimicrobial agents from seven different chemical classes (ampicillin, ceftiofur, gentamicin, apramycin, neomycin, spectinomycin, streptomycin, florfenicol, chloramphenicol, enrofloxacin, tetracycline and trimethoprim/sulfamethoxazole) using the disc diffusion susceptibility testing method performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines [16]. Isolates with resistance to three or more antimicrobials by disc diffusion were then subjected to minimum inhibitory concentration (MIC) susceptibility testing (using the same antimicrobial agents listed above) performed by the broth microdilution method in 96-well microtitre plates (Sarstedt Australia Pty Ltd., Technology Park, SA, Australia) as described in the CLSI standards manual [16]. Inhibition zones and MICs were interpreted using CLSI recommended inhibition breakpoints for enteric pathogens where available. As there are no CLSI breakpoints for florfenicol and ceftiofur applicable to *E. coli* of animal origin, breakpoints for florfenicol ($\geq$16 μg/mL) and ceftiofur ($\geq$8 μg/mL) were sourced from the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP) [17,18]. Inhibition zone sizes used for florfenicol [resistant (R) $\leq$ 14 mm, susceptible (S) $\geq$ 19 mm] and ceftiofur ($R \leq 17$ mm, $S \geq 21$ mm) were CLSI breakpoints established for bovine respiratory disease isolates [16]. Neomycin ($\leq$12 mm and $\geq$16 μg/mL) (Zoetis, West Ryde, NSW, Australia) and apramycin ($\leq$10 mm and $\geq$32 μg/mL) (Bayer Australia Ltd., Pymble, NSW, Australia) breakpoint information was obtained directly from the respective manufacturers. *E. coli* reference strain ATCC 25922 was used as a control.

2.3. Identification of antimicrobial resistance genes by PCR

Single PCR was used to detect the presence of 32 ARGs in the Vietnamese isolate collection as described previously [4]. Single PCR was also use to detect the presence of the plasmid-mediated quinolone resistance genes *qnrA*, *qnrB* and *qnrS* in all isolates [19]. All targeted ARGs have previously been identified in enteric gram-negative organisms and encode resistance or reduced susceptibility to one or more of the 12 antimicrobial agents tested. The *int* primers were used to detect the presence of class 1 integrons, with positive amplicons subjected to a second PCR using the *intVR* primers [20].

2.4. Antimicrobial resistance index calculation

An ARI was calculated for each Vietnamese isolate using a previously described algorithm developed to calculate the ARI of the Australian isolates [4]. Briefly, the ARI accounts for the measured extent of phenotypic resistance to each of 12 drugs used to assess phenotypic resistance, the antimicrobial resistance genes present, and the number and type of integrons present. Within the index, phenotypic or genetic resistance to drugs of public health significance is given extra weighting. Importance weightings were based on the Expert Advisory Group on Antimicrobial Resistance (EAGAR) recommendations [21]. Comparisons between populations (Australian and Vietnamese commercial and village piggeries) in the proportion of isolates testing positive to various traits were assessed for significance using Fisher's exact test. The ARI for each collection of isolates were graphically summarised and *t*-tests were used to assess the equality of means between source