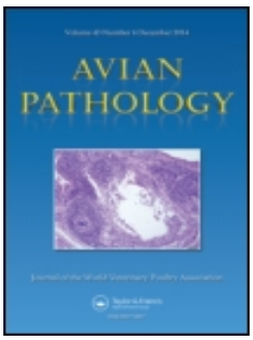

Avian Pathology

ISSN: 0307-9457 (Print) 1465-3338 (Online) Journal homepage: https://www.tandfonline.com/loi/cavp20

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To cite this article: Thotsapol Thomrongsuwannakij, Patrick J. Blackall, Steven P. Djordjevic, Max L. Cummins & Niwat Chansiripornchai (2020): A comparison of virulence genes, antimicrobial resistance profiles and genetic diversity of avian pathogenic *Escherichia coli* (APEC) isolates from broilers and broiler breeders in Thailand and Australia, Avian Pathology, DOI: 10.1080/03079457.2020.1764493

To link to this article: https://doi.org/10.1080/03079457.2020.1764493

Accepted author version posted online: 06 May 2020.

Publisher: Taylor & Francis & Houghton Trust Ltd

Journal: *Avian Pathology*

DOI: 10.1080/03079457.2020.1764493

A comparison of virulence genes, antimicrobial resistance profiles and genetic diversity of avian pathogenic *Escherichia coli* (APEC) isolates from broilers and broiler breeders in Thailand and Australia

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Running Title: APEC isolates from broilers and broiler breeders in Thailand and Australia

Abstract

Avian pathogenic *Escherichia coli* (APEC) is the causative agent of colibacillosis resulting in economic losses in the poultry industry worldwide. A total of 168 APEC isolates, equal numbers from Australian and Thai broilers/broiler breeders, were identified and tested for their susceptibility to ten antimicrobial agents. Most of the Thai APEC isolates were multidrug-resistant (MDR) (60.7%) whilst Australian APEC isolates showed MDR rate of just 10.7%. The Thai APEC isolates exhibited high resistance to tetracycline (TET) (84.5%), amoxicillin (AMX) (70.2%) and trimethoprim-sulfamethoxazole (SXT) (51.2%) whilst the Australian APEC isolates showed lower levels of resistance (TET 36.9%, AMX 29.8%, SXT 17.86%). The 34 Thai APEC and 4 Australian APEC isolates which were resistant to nalidixic acid were characterised for their carriage of mutations in the quinolone resistance determining region of *gyrA*, *gyrB*, *parC* and *parE*. While no mutations were detected in *gyrB* in the Thai isolates, the Ser83Leu and Asp87Asn substitutions in *gyrA* and Ser80Ile in *parC* were common (n=9/34). In regard to the Australian isolates, the Ser83Leu and Asp678Glu substitution in *gyrA*, Pro385Ala and Ser492Asn in *gyrB* and Met241Ile and Asp475Glu in *parC* were identified (n=3/4). Rep-PCR analysis of the 84 Thai and 84 Australian APEC isolates showed 16 main clusters that mostly contained isolates from both countries. Our results suggest that the emergence of MDR is a major concern for the Thai APEC isolates and that more prudent use of antimicrobial agents in Thai poultry production is required.

Keywords: avian pathogenic *Escherichia coli*, colibacillosis, virulence gene, antimicrobial resistance, whole genome sequencing, poultry

Introduction

Avian pathogenic *Escherichia coli* (APEC) is the causative agent of colibacillosis in poultry, a disease which can cause a major economic impact due to high morbidity and mortality. The disease occurs as systemic or localised infections such as airsacculitis, septicemia, pericarditis, perihepatitis, salpingitis and cellulitis in chickens (Nolan *et al.*, 2013). Colibacillosis is often the most frequently reported disease linked to condemnations at the processing plant; for example, 33% of broiler carcasses which were condemned for the disease at processing in Canada had lesions involved in colisepticemia (Kumor *et al.*, 1998).

The molecular characterisation of APEC has been studied by several researchers in recent years (Gai *et al.*, 2015; Johnson, Wannemuehler, Doetkott, *et al.*, 2008; Kemmett *et al.*, 2013). Virulence-associated genes (VAGs) encode a range of properties such as adhesins, hemagglutinins, iron-acquisition systems, and an enteroaggregative heat-stable toxin (Ewers *et al.*, 2005). It has been demonstrated that these genes are more predominant in APEC isolates than non-pathogenic *E. coli* isolates, providing a rapid tool for the identification of presumptive APEC (De Carli *et al.*, 2015; Johnson, et al., 2008).

Antimicrobial resistance (AMR) is a serious concern in both human medicine and the poultry industry. In recent years, similarities between extra-intestinal pathogenic *E. coli* (ExPEC) isolated from humans and APEC from broilers have suggested that there may have been an exchange of transferable mobile genetic elements between pathogenic *E. coli* isolates from animals and humans (Johnson *et al.*, 2012; Johnson, Wannemuehler, Johnson, *et al.*, 2008). This potential link increases the importance of surveillance of AMR in broiler chickens. In Thailand and Australia, veterinarians only prescribe antimicrobials for the treatment of chickens and use of these agents for growth promotion is prohibited in both countries (Mehdi *et al.*, 2018).

The detailed characterisation of *E. coli* isolates can be done by several phenotypic and genotypic methods (Al-Kandari & Woodward, 2019; Mohapatra *et al.*, 2007). These techniques can be used to infer the phylogenetic relatedness of strains which, in the case of closely related isolates, can indicate a shared epidemiological origin. Regarding genotypic methods, one of the easy-to-use and cost-effective techniques to find genetic relatedness among isolates is repetitive extragenic palindromic-PCR (rep-PCR) which is a genotypic method using oligonucleotide primers complementary to repetitive sequences dispersed throughout the genome of *E.coli* (Versalovic *et al.*, 1991).

In this study, *E. coli* isolates were obtained from broilers and broiler breeders with systemic forms of colibacillosis in Thailand and Australia. The aims of this study were to compare the isolates from both countries in regard to 1) the VAGs harboured by APEC isolates; 2) antimicrobial resistance profiles of the isolates; 3) the presence of mutations in the quinolone resistance determining regions (QRDRs) of the *gyrA, gyrB, parC* and *parE*; and 4) the genetic diversity of Thai and Australian APEC by using rep-PCR.

Materials and Methods

Isolates

With regards to Thai *E. coli* isolates, 200 suspect colibacillosis cases from broilers and broiler breeders located in Central, Eastern and North-eastern areas of Thailand were sent to the Avian Health Research Unit, Faculty of Veterinary Science Chulalongkorn University, Thailand during December, 2016 and January, 2018. At the time of the study, antimicrobial agents have been banned as growth promoters in Thailand meaning that veterinarian can prescribe them for treatment of bacterial infections only. For the Australian *E. coli* isolates, 89 *E. coli* isolates from clinical cases of colibacillosis in broilers and broiler breeders were sourced from diagnostic laboratories which were located in the states of Queensland, New South Wales

and Victoria. These isolates were collected initially between February, 2011 and January, 2018. In Australia, there is no agent with a claim for "growth promotion" registered for chickens (see https://portal.apvma.gov.au/pubcris). All Thai and Australian isolates were from affected internal organs of broiler or broiler breeders showing typical colibacillosis signs such as perihepatitis, pericarditis, peritonitis, salpingitis and/or cellulitis.

E. coli isolation and identification

All samples from the Thai birds were plated onto 5% sheep blood agar (SBA) and MacConkey agar plates and were aerobically incubated at 37ºC for 24-48 h. Presumptive pink colonies on MacConkey agar were sub-cultured onto eosin methylene blue (EMB) agar and were aerobically incubated at 37ºC for 24 h. Presumptive colonies, which showed a metallic green sheen on EMB agar, were sub-cultured onto SBA and aerobically incubated at 37ºC for 24 h. Biochemical tests including oxidase, indole and triple sugar iron (TSI) tests were performed from the incubated SBA. Confirmed *E. coli* isolates were then stored in tryptone soya broth (TSB) with 15% glycerol at -80ºC for further study. All media were obtained from OXOID (Basingstoke, Hampshire, England).

The Australian isolates were obtained as pure cultures from the various source laboratories in Australia.

DNA extraction and detection of VAGs

Genomic DNA was extracted from pure bacterial cultures as previously described (Thomrongsuwannakij *et al.*, 2017). Five VAGs including *iroN*, *ompT*, *hlyF*, *iss* and *iutA* were detected by using pentaplex PCR as previously described (Johnson, et al., 2008) and strains exhibiting carriage of ≥4 of such genes were classified as APEC. Positive samples of each gene were confirmed by sequencing using the relevant PCR primers after that these DNA were used as positive controls for PCR.

Antimicrobial susceptibility test

Minimum inhibitory concentrations (MICs) tests were done using Muller Hinton agar (MHA) (OXOID) and a two-fold agar dilution technique according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2013). The ten antimicrobials used and the breakpoints for determining resistance were as follows: amoxicillin (AMX, 32 µg/ml), ceftiofur (CEF 2 µg/ml), chloramphenicol (CHL 32 µg/ml), ciprofloxacin (CIP 4 µg/ml), enrofloxacin (ENR, 2 µg/ml), gentamicin (GEN, 16 µg/ml), nalidixic acid (NAL 32 µg/ml), florfenicol (FLO 16 µg/ml), trimethoprim-sulfamethoxazole (SXT 4/76 µg/ml), tetracycline (TET, 16 µg/ml). CHL and ENR were used for Thai APEC isolates only whilst CEF and FLO were used for Australian APEC isolates only. The agar plates were incubated for 16-20 h at 37^{o}C under aerobic condition. The interpretive criteria used were those recommended for *Enterobacteriaceae* according to the CLSI standards (CLSI, 2015) except CEF was based on the EUCAST epidemiologic cut-off values (EUCAST, 2019). The control organism was *E. coli* ATCC 25922. All antimicrobials were obtained from Sigma-Aldrich (St Louis, MO, USA).

Detection of mutation(s) in the QRDRs of *gyrA, gyrB, parC* and *parE*

Thirty-four of the Thai APEC isolates, which were resistant to NAL, were subjected to nucleotide sequence analysis in the QRDRs of *gyrA, gyrB, parC* and *parE* using the forward and reverse primers as previously described (Yang *et al.*, 2004). The PCR products were purified by using GenepHlow^{TM} gel/pcr kit (Geneaid Biotech, Taiwan) and were submitted for nucleotide sequencing at First Base Laboratories (Seri Kembangan, Selangor, Malaysia). The DNA sequences obtained were compared and aligned with wild-type *E. coli gyrA* (Genbank accession number AE000312), *gyrB* (AE000447), *parC* (AE000384) and *parE* (AE000385), all obtained from GenBank database (available at: www.ncbi.nlm.nih.gov).

Screening for the *qnr* genes responsible for plasmid-mediated quinolone resistance (PMQR)

Two Thai APEC isolates, which were resistant to both nalidixic acid and enrofloxacin but were not found to have any mutation in the QRDRs from *gyrA, gyrB, parC* and *parE,* were subjected to screening for *qnr* genes by PCR amplification of *qnrA* (Wang et al., 2004), *qnrB* (Gay *et al.*, 2006) and *qnrS* (Gay, et al., 2006) as previously described. Positive samples of all *qnr* genes were confirmed by sequencing using the relevant PCR primers.

Whole genome sequencing (WGS) for nalidixic acid-resistance Australian APEC isolates

Four Australian APEC isolates (BR 2602, BR2636, BR 2640 and BR2641), which were resistant to nalidixic acid, were subjected to WGS. Genomic DNA was extracted using the DNeasy UltraClean Microbial Kit (Qiagen GmbH, Hilden, Germany) as per the manufacturer's instructions from overnight cultures grown on 5% SBA. The DNA quality was assessed and quantified using a NanoDrop (Thermo Fisher Scientific) spectrophotometer. A total of 200 ng of DNA was used for the library preparation and sequencing using the Illumina NextSeq 500 platform (150 bp paired ends). The sequencing was performed by the Australian Center for Ecogenomics at the University of Queensland.

Four pairs of short-read data of the Australian APEC isolates sequenced for this study have been deposited in the NCBI Short Read Archive under Study ID 563768 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA563768) with accession numbers from SRX6799142 to SRX6799145 for BR 2602, BR2636, BR 2640 and BR2641, respectively. The raw Illumina sequencing data were quality filtered to remove Illumina adaptor sequences and low quality reads using Trimmomatic (Galaxy version 0.36.6) on the Galaxy platform (https://usegalaxy.org.au/) (Afgan *et al.*, 2015; Bolger *et al.*, 2014). The qualities of the reads were assessed using FastQC (Galaxy version 0.72) (Andrews, 2010). Pre-processed paired-end

reads were then assembled using SPAdes (Galaxy version 3.11.1). The assembled consensus of the contiguous sequences (contigs) of each bacterial genome was compared and aligned with wild-type *E. coli gyrA*, *gyrB*, *parC* and *parE* as mentioned earlier.

Rep-PCR

The genetic diversity of the 84 Thai and 84 Australian APEC was characterised by using rep-PCR as previously described (Mohapatra, et al., 2007). Electrophoresis was performed at 70 V for 90 min through 1% agarose gel. Gels were then stained with 1 µg/ml ethidium bromide and were visualised by ultraviolet transilluminator. TIFF image files were imported into Bionumerics 6.5 (Applied Maths, Sint-Martens-Latem, Belgium). The similarity matrix was computed using the Dice similarity coefficient and clustering by the unweighted paired group method with arithmetic mean values (UPGMA). Band position tolerance and the optimisation coefficient were both set to 2%. Clusters were determined at the 80% similarity level using Bionumerics.

Results

VAGs found in the Thai and Australian *E. coli* isolates

Two hundred Thai and 89 Australian *E. coli* isolates were investigated for the presence of 5 VAGs. Using the criterion that *E. coli* which harboured at least 4 out of 5 VAGs is classified as APEC, the results showed that 84 *E. coli* isolates from each country could be classified as APEC isolates. As shown in Table 1, the most frequently detected genes found in the Thai and Australian APEC isolates were *hlyF* (100%) and *ompT* (100%), which regulate putative avian hemolysin (Morales *et al.*, 2004) and an episomal outer membrane protein, respectively. Moreover, *iroN,* a gene that regulates the salmochelin siderophore receptor (Johnson *et al.*, 2006) was also found in all the Thai APEC isolates. In contrast, *iutA* that

regulates aerobactin siderophore receptor (Johnson, et al., 2008) was found only 78.6% in the Thai APEC isolates compared to 98.8% of the Australian APEC isolates.

Phenotypic antimicrobial resistance

According to CLSI protocols (CLSI 2013), *E. coli* ATCC 25922 was used as a control organism, and all MIC values were within the MIC quality control ranges in all batches. Overall, the Thai APEC isolates were more resistant to various classes of antimicrobial agents compared to the Australian APEC isolates. The rate of multidrug-resistant (MDR), defined as being resistant to three or more antimicrobial classes, of the Thai APEC isolates was at 60.7% whilst Australian APEC isolates was only at 10.7% (Figure 1). Regarding the Thai APEC isolates, they demonstrated a high resistance rate to TET (84.5%), AMX (70.2%), SXT (51.2%) and NAL (40.5%). In contrast, while the Australian APEC isolates were most commonly resistant to the same classes of antimicrobial compounds was to similar agents as the Thai isolates, the rates were much lower - TET (36.9%), AMX (29.8%) and SXT (17.9%). In this study, all of the Australian APEC isolates were sensitive to CEF and CIP. The most common resistance patterns found in this study were AMX-ENR-NAL-TET (14.3%) for the Thai APEC isolates and TET (17.9%) for the Australian APEC isolates (Table 2).

Genetic characterisation of mutation(s) in the QRDRs of *gyrA, gyrB, parC* and *parE*

The DNA sequence of the *gyrA, gyrB, parC* and *parE* genes of the 34 Thai and 4 Australian APEC isolates, which were resistant to nalidixic acid, were submitted to Genbank. Genbank accession numbers of all submitted sequences are shown in a supplementary Table 1.

With regards to the 34 Thai APEC isolates, the MIC values for NAL ranged between 64 and >256 µg/ml and between 0.25 and 64 µg/ml for ENR. Regarding the selected 4 Australian APEC isolates, the MIC values for NAL ranged between 64 and >128 µg/ml and between 0.25 and 0.5 µg/ml for CIP. Amino acid substitutions were found in QRDRs except

in GyrB for the Thai APEC isolates and except in ParE for the Australian APEC isolates. A serine-to-leucine mutation of the 83rd amino acid (Ser83Leu) and aspartate-to-asparagine, aspartate-to-glycine and aspartate-to-tyrosine mutations of the 87th amino acid (Asp87Asn, Asp87Gly and Asp87Tyr) of the GyrA protein were detected in the Thai APEC isolates. A serine-to-leucine mutation of the 83rd amino acid (Ser83Leu) and an aspartate-to-glutamate mutation (Asp678Glu) of the GyrA protein were detected in the Australian APEC isolates. A serine-to-isoleucine mutation of the 80th amino acid (Ser80Ile) of the ParC protein was dominant in the Thai APEC isolates (n = 18). A serine-to-alanine mutation of the 458th amino acid (Ser458Ala) was detected in the 4 out of the 34 Thai APEC isolates. With regards to the Thai APEC isolates, the most frequent pattern of mutations (n = 9) included a double mutation in GyrA (Ser83Leu, Asp87Asn) and a single point mutation in ParC (Ser80Ile). With regards to the Australian APEC isolates, a proline-to-alanine mutation of the 385th amino acid (Pro385Ala) and a serine-to-asparagine mutation of the 492nd amino acid (Ser492Asn) of the GyrB protein were detected. In addition, a methionine-to-isoleucine mutation of the 241st amino acid (Met241Ile) and an aspartate-to-glutamate mutation of the 475th amino acid (Asp475Glu) of the ParC protein were detected in the Australian APEC isolates. Interestingly, two Thai APEC isolates which were resistant to both nalidixic acid and enrofloxacin had no mutation in *gyrA*, *gyrB*, *parC* or *parE*.

Screening for the *qnr* genes

Two Thai APEC isolates which were resistant to both nalidixic acid and enrofloxacin and were not found to have any mutation in the QRDRs from *gyrA, gyrB, parC* and *parE* were both positive for *qnrA*, *qnrB* and *qnrS,* the genes responsible for PMQR.

Genotypic diversity by rep-PCR

All of the 84 Thai and 84 Australian *E. coli* isolates were genotyped by rep-PCR. Sixteen distinct genotypic clusters were identified with 80% similarity (Figure 2), meaning that APEC isolates in this study were diverse. Clusters 2, 5, 7, 8, 12 and 16 contained APEC isolates from both countries. Cluster 4 was the largest group with all of the isolates being from broiler and broiler breeders of Australian origin. In addition, clusters 6, 9, 10, 11 and 14 also contained only Australian APEC isolates. In contrast, in clusters 1, 3, 13 and 15 all APEC isolates originated from Thailand. Overall, APEC isolated from broiler breeders cannot be completely separated from broiler isolates by using rep-PCR.

Discussion

This study was conducted to investigate APEC isolated from broiler and broiler breeders in Thailand and Australia in terms of VAGs, antimicrobial resistance patterns and genetic diversity. Moreover, the presence of mutations in the QRDR of the *gyrA, gyrB, parC* and *parE* was assessed.

APEC are *E. coli* isolates that cause systemic disease in poultry. The most general lesions associated with colibacillosis are perihepatitis, airsacculitis and perihepatitis (Nolan, et al., 2013). The APEC pathotype can be defined by using phenotypic or genotypic techniques, but there is no single trait or group of traits that can give a definition of APEC (Dziva & Stevens, 2008). Many researchers have tried to identify VAGs that are mostly found in APEC isolates, with 5 to 13 genes being used (Ewers, et al., 2005; Johnson, et al., 2008; Varga *et al.*, 2018). In this study, we followed the protocol of a previous study (Johnson, et al., 2008) to define an APEC as an *E. coli* with at least 4 of the 5 most commonly recognized VAG. Typing of APEC by using VAGs is a simple and practical approach in a routine laboratory compared to other methods such as serotyping which has limited application in Thailand and Australia as there is no central reference laboratory that offers this technology in both countries. While there

is a PCR-based technique to subtype O-serogroup of *E. coli* (Iguchi *et al.*, 2015), the method requires over a hundred primers for complete serotyping, a requirement which is not practical.

The 5 VAGs used in this study were related to adhesion, iron acquisition and serum resistance which are characteristic of the APEC pathotype (Delicato *et al.*, 2003). Both the Thai and Australian APEC isolates harboured these VAGs at a high prevalence with a lowest prevalence of *iutA* found in the Thai APEC isolates at 78.6%. This finding agrees with studies from Canada (Varga, et al., 2018), Australia (Cummins *et al.*, 2019); Brazil (Barbieri *et al.*, 2013), Korea (Jeong *et al.*, 2012) and the USA (Johnson, et al., 2008).

After testing the presence of 5 VAGs, the 84 APEC isolates from both Thailand and Australia were examined for their antimicrobial resistance profiles. We found that the Thai APEC were generally more resistant to various antimicrobial agents such as TET, AMX, SXT, NAL, ENR and CHL than the Australian APEC isolates. In addition, the MDR rate of the Thai APEC isolates was 60.7% compared to 10.7% of the Australian APEC isolates.

AMR is a serious concern in both human medicine and the poultry industry. It is accepted that antimicrobial use at the farm level can increase the risk of antimicrobial resistance development in both commensal and pathogenic enteric bacteria in food animals (Ozawa *et al.*, 2008; Varga *et al.*, 2009). *E. coli* infections are commonly found as secondary bacterial infections in chickens and in both countries only veterinarians can prescribe therapeutic antimicrobials to chickens. In particular, in Australia, there is no agent with a claim for "growth promotion" registered for chickens (see https://portal.apvma.gov.au/pubcris) meaning that at the time of sample, the antimicrobial agents were not being used as growth promoters. However, the AMR rate of the Thai APEC was high for some antimicrobial agents. In Thailand, the drug of choice for the treatment of colibacillosis is typically a drug in groups of beta-lactams, tetracyclines or sulfonamides; it is therefore unsurprising that these are the classes of

antimicrobial compounds to which isolates were most commonly resistant. This finding is in agreement with previous studies from China (Dou *et al.*, 2016) and Japan (Ozawa, et al., 2008) where a high prevalence of AMR was found to antimicrobial agents that frequently used to treat colibacillosis. In addition, CHL has been banned in food animals in both countries for decades, but resistance to this agent could be found, presumably because of the effects of co-selection of resistance from antimicrobial agents that are currently available and approved (Harada & Asai, 2010). The highest prevalence of AMR in the Australian APEC isolates was most common in TET (36.9%), AMX (29.8%) and SXT (17.9%). Although TET, AMX and SXT are also common antimicrobial agents for treating colibacillosis in Australia, the resistance rates were clearly lower than the rate of the Thai APEC isolates. This evidence might suggest that there is very prudent use of antimicrobial agents in Australia and highlights the need for judicious and sustainable use of antimicrobial agents in Thailand. In addition, all of the Australian APEC isolates in this study were susceptible to CEF and CIP which were similar to a previous study conducted in Australia showing that they do not carry AMR genes conferring resistance to these compounds (Cummins, et al., 2019). The results suggest that antimicrobial use in Australian poultry is more limited than occurs in poultry in Thailand. It would appear important that government regulators, veterinarians and farmers in Thailand address the issue of a more limited use of these agents to minimise the promotion of antimicrobial resistance.

Fluoroquinolone resistance (FQR) has rapidly increased in many countries worldwide and is a major public health concern (Ozawa, et al., 2008; Yang, et al., 2004). FQR is typically caused by alterations in the target enzymes (DNA gyrase and topoisomerase IV) and by changes in drug entry and efflux (Jacoby, 2005). A previous study (Bagel *et al.*, 1999) identified that all quinolone-resistant isolates possessed typical mutations in the topoisomerase genes, *gyrA* and *parC*. In this study, PCR and Sanger sequencing were used to identify the

DNA sequence of the *gyrA, gyrB, parC* and *parE* genes of the Thai APEC isolates using a short sequence (<250 base pairs, bp) for each gene. In contrast, for the Australian APEC isolates, WGS was used to compare these genes compared with a wild type of *E. coli*. The size of the *gyrA, gyrB, parC* and *parE* genes of wild type *E. coli* ranged between 1,893 and 2,628 bp meaning that the use of WGS for detecting point mutations possibly give more opportunity to find novel mutations on these genes.

With regards to the Thai APEC isolates, isolates which possessed double point mutations in *gyrA* together with a single or double mutations in *parC* or *parE* exhibited resistance to both NAL (MIC values > 256 µg/ml) and ENR (MIC values = 8-64 µg/ml). Yang et al. (2004) reported that double *gyrA* mutations together with mutations in *parC* conferred high-level resistance to FQs. Interestingly, there were two Thai APEC isolates which were resistant to NAL and ENR and that lacked any mutations in the *gyrA, gyrB, parC* and *parE* genes; but they were positive for *qnrA*, *qnrB* and *qnrS* which are responsible for PMQR. This confirms that, besides QRDRs mutations, PMQR also plays an important role in FQ resistance as previously reported (Gay, et al., 2006; Wang, et al., 2004). Four Australian APEC isolates that were resistant to NAL but susceptible to CIP, had double point mutations in *gyrA*, *gyrB* and *parC* meaning that these point mutations might not affect CIP resistance characteristics. Although mutations in *gyrB* and *parE* have been associated with FQ resistance, the frequency of mutation is much lower compared to those for *gyrA* and *parC* (Yang, et al., 2004).

In conclusion, screening for the VAGs discussed serves as a useful and rapid approach in the identification APEC. Even though only veterinarians can prescribe antimicrobial agents for bacterial infections in chickens in both countries, a high antimicrobial resistance rate was found in Thai APEC. As APEC may cause cross-resistance with human enteric pathogens, prudent use of the antimicrobial agents in veterinary medicine is highly recommended.

Acknowledgement

This research was funded by Chulalongkorn University; Government Budget No. GB_B_60_101_31_12 and also were supported by Ratchadapisek Somphot Endowment Fund for Postdoctoral Fellowship, Chulalongkorn University and Endeavour Fellowship program supported by Australian government. We would like to thank Dr Conny Turni and Dr Lida Omaleki for their critical suggestions. The support of all the farmers involved in sample collections was an important contribution to this work.

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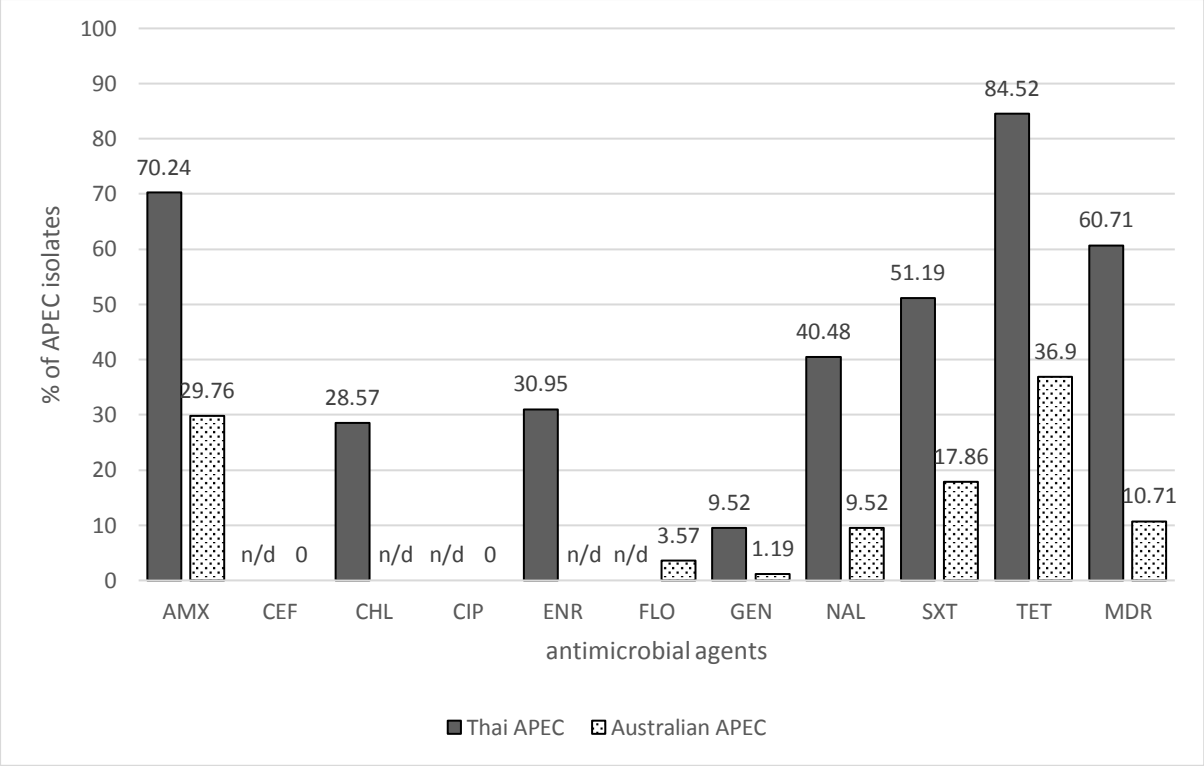
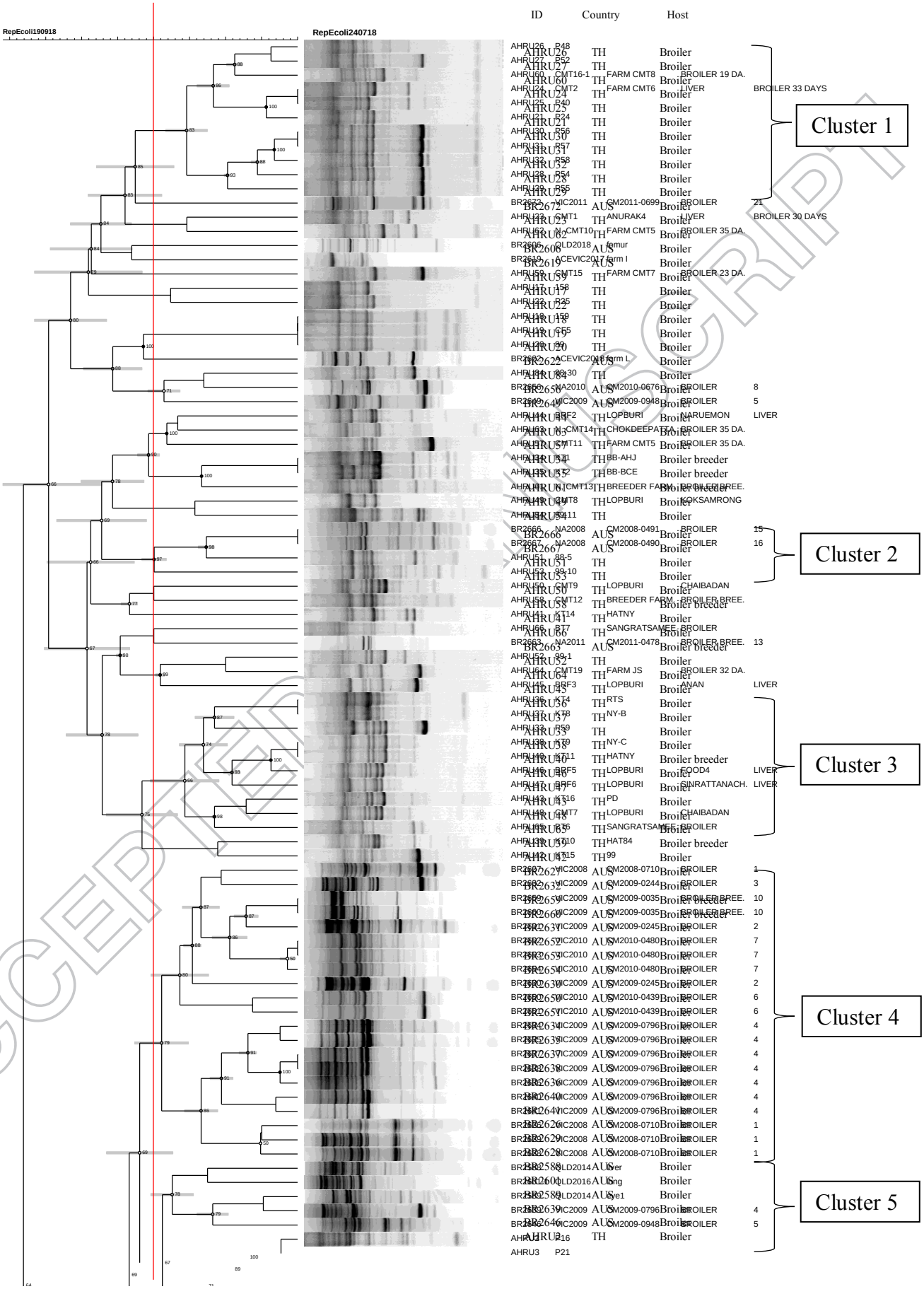


Figure 1. The frequency of resistance to ten antimicrobial agents in Thai APEC (n = 84) and Australian APEC isolates (n = 84). AMX, amoxicillin; CEF, ceftiofur; CHL, chloramphenicol; CIP, ciprofloxacin; ENR, enrofloxacin; GEN, gentamicin; NAL, nalidixic acid; FLO, florfenicol; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; MDR, multidrug resistance; n/d, not done.



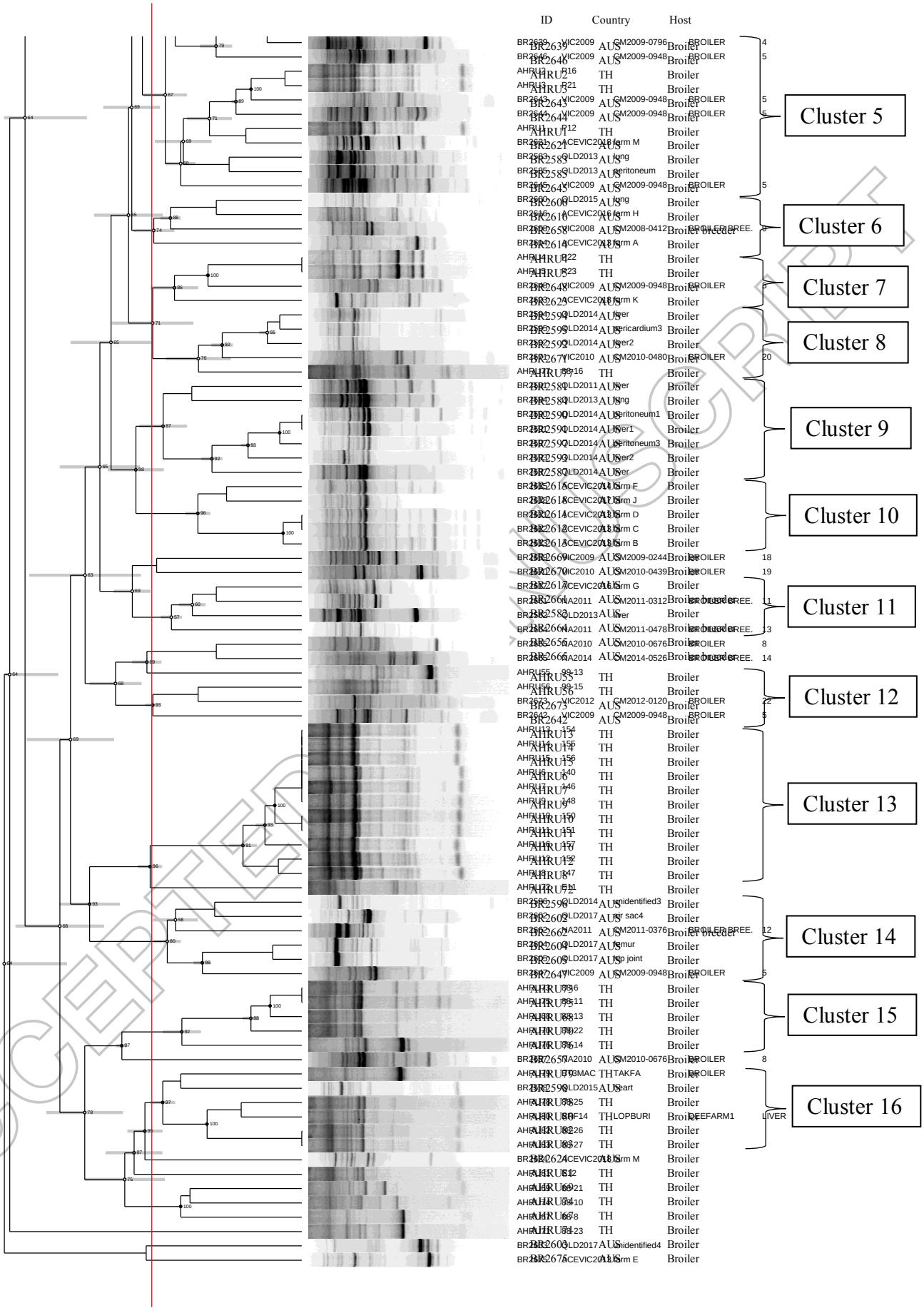


Figure 2. Phylogenetic analysis representing rep-PCR of the Thai (n = 84) and Australian APEC (n = 84) isolated from broilers and broiler breeders at the 80% cutoff genetic similarity. The similarity matrix was computed by using Dice similarity coefficient and clustering by the UPGMA. Band position tolerance and the optimization coefficient were both set to 2% using Bionumerics.

Table 1. Prevalence of VAGs in the Thai and Australian APEC strains.

Samples	% isolates harboured 5 major VAGs				
	iutA	*iss*	*hlyF*	*ompT*	*iroN*
Thai APEC (n = 84)	78.57	98.81	100	100	100
Australian APEC (n = 84)	98.81	100	100	100	97.62

Table 2. Antimicrobial resistance pattern of the Thai (n = 84) and Australian (n = 84) APEC strains.

Source	Antimicrobial resistance pattern	No. of strains (%)
Thai APEC	AMX-ENR-NAL-TET	12 (14.29)
	AMX-TET	11 (13.10)
	SXT-TET	9 (10.71)
	AMX-CHL-SXT-TET	8 (9.52)
	AMX-SXT-TET	5 (5.95)
Australian APEC	TET	15 (17.86)
	AMX-SXT-TET	7 (8.33)
	AMX-NAL	7 (8.33)
	SXT-TET	6 (7.14)

[A]Only the antimicrobial resistance patterns represented by at least five isolates are shown. AMX, amoxicillin; CHL, chloramphenicol; DOX, doxycycline; ENR, enrofloxacin; TET, tetracycline; SXT, trimethoprim–sulfamethoxazole.

Table 3. Amino acid substitutions in the QRDRs and the MIC for corresponding FQ resistance profiles among the Thai (n = 34) and Australian (n = 4) APEC strains.

No. of isolates	Amino acid substitution(s)*				MIC range (µg/ml)		
	GyrA	GyrB	ParC	ParE	NAL	ENR	CIP
Thai APEC							
2	-	-	-	-	128->256	4-32	n/d*
7	Ser83Leu	-	-	-	64->256	0.25-8	n/d
1	Asp87Gly	-	-	-	32	0.5	n/d
2	Asp87Tyr	-	-	-	64	0.25	n/d
1	Ser83Leu	-	Ser80Ile	-	>256	4	n/d
1	Ser83Leu	-	-	Asp475Glu	256	0.5	n/d
1	Ser83Leu, Asp87Asn	-	-	-	>256	8	n/d
9	Ser83Leu, Asp87Asn	-	Ser80Ile	-	>256	16-32	n/d
2	Ser83Leu, Asp87Tyr	-	Ser80Ile	-	>256	16	n/d
1	Ser83Leu, Asp87Asn	-	Ser80Arg	-	>256	8	n/d
1	Ser83Leu, Asp87Asn	-	-	Ser458Ala	>256	64	n/d
3	Ser83Leu, Asp87Asn	-	Ser80Ile	Ser458Ala	>256	16-64	n/d
1	Ser83Leu, Asp87Asn	-	Ser80Ile	Ile464Phe	>256	32	n/d
1	Ser83Leu, Asp87Asn	-	Ser80Ile, Glu84Gly	-	>256	64	n/d
1	Ser83Leu, Asp87Asn	-	Ala56Thr, Ser80Ile	-	>256	16	n/d
Australian APEC							
3	Ser83Leu, Asp678Glu	Pro385Ala, Ser492Asn	Met241Ile, Asp475Glu	-	64->128	n/d	0.25-0.5
1	Asp678Glu	Pro385Ala, Ser492Asn	Met241Ile, Asp475Glu	-	128	n/d	0.25

*n/d, not done.