

Antibiotic resistance and prevalence of virulence and quinolone resistance genes in *Escherichia coli* strains of avian origin isolated from semi-industrial farms in Cote d'Ivoire

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ABSTRACT:

Avian colibacillosis is a serious pathology caused by extra-intestinal *E. coli* strains that cause huge economic losses for farmers. In this study, antibiotic resistance and virulence of avian *E. coli* strain isolated from a few semi-industrial farms in Côte d'Ivoire were evaluated. To do this, 75 strains of *E. coli* were isolated from chicken organs with colibacillosis by microbiological methods in agar and biochemical media. Then, the resistance of these strains to antibiotics was evaluated by the antibiogram method with disks, based on the diffusion of antibiotics in agar medium. PCR was used to search for virulence genes (PapC, Tsh and Cnf) and quinolone resistance (qnrA and qnrB) on ciprofloxacin and oxolinic acid resistant strains. The results of the antibiogram showed multi-resistant strains with high resistance for Oxacillin (100%), Amoxicillin (84%), Tetracycline (94%), Spiramycin (70%), Oxolinic acid (64%). The lowest levels of resistance were observed with Colistin (0%), Gentamicin (20%) and Ciprofloxacin (27%). The PCR revealed a remarkable presence of quinolone resistance genes, qnr A and qnr B with the same rate of 16.12%. As for virulence, only the Pap C gene was detected with a rate of 12.90%. These results are useful in the surveillance of antimicrobial resistance and in the control of avian colibacillosis.

Key words: *E. coli*, avian colibacillosis, antimicrobial resistance, virulence, Ivory Coast.

1. INTRODUCTION

Poultry has become a very important meat with a global production of 113 million tonnes in 2016 according to the **FAO (2017)**. It is expected to account for about half of world meat production in the next decade (**OECD / FAO, 2016**). In Côte d'Ivoire, poultry production has almost doubled in just four years (**Anonymous, 2016**). This boom in the poultry sector generated 240 billion CFA francs of turnover in 2016 and created 50,000 direct jobs and 170,000 indirect jobs in Côte d'Ivoire (**Côte d'Ivoire's Primature, 2017**). However, the spectacular development of Ivorian poultry farming is not without difficulties. Indeed, most farmers are not professionals and do not necessarily control the application of basic hygiene rules in poultry farms. This has led to the emergence of many diseases in these mostly bacterial farms (**Stordeur and Mainil, 2002**). Among these pathologies, there are colibacilloses

that cause a lot of damage in poultry farms (Guérin and Boissieu, 2008). It is a disease caused by strains of avian pathogenic *E. coli* (APEC). Lay and broody chicks are very vulnerable to this disease, with stunted growth, decreased feed efficiency, and increased livestock costs in terms of food and drug costs. The treatment of this disease is essentially based on antibiotic therapy. However, for some time, many resistance to quinolones has been observed with an increased risk of antibiotic resistance transfer in humans (Robineau and Moalic, 2010). To this must be added the appearance of numerous virulence factors (*PapC*, *Tsh*, *Cnf* genes etc ..) involved in the installation and development of avian colibacillosis (Stordeur and Mainil, 2002). All these elements made it difficult to fight against this disease, which must be based on an antibiogram. With this in mind, we determined the antibiotic and molecular profile of Pathogenic *E. coli* of avian origin isolated from semi-industrial farms in Côte d'Ivoire.

2. MATERIAL AND METHODS

2.1. Bacterial strains

In total, 75 strains of avian pathogenic *E. coli* (APEC) isolated from blood of various organs of chickens with colibacillosis and stored at -80 ° C at the central veterinary laboratory of Bingerville (Ivory Coast) from November 2016 to January 2017 were used for the antibiotic and molecular study. These strains came from 37 semi-industrial farms in several localities in Côte d'Ivoire.

2.2. Microbiology material

Culture media for the preparation of the strains and the production of antibiograms were used. They are: buffered peptone water (BioMérieux), TCS agar (BioMérieux), PBS broth (Bio-X Diagnostics). To this must be added antibiotic discs (BioMérieux) and all the small laboratory equipment used in bacteriology.

2.3. Evaluation of the susceptibility of strains to antibiotics

The susceptibility of strains to antibiotics was evaluated by the antibiogram test using disks according to the antibiotic agar diffusion method, following the recommendations of the Antibiogram Committee of the French Microbiology Society (CA-SFM, 2016). The isolates were tested for their susceptibility to 12 antibiotics commonly used in the veterinary field which are: Amoxicillin, Oxaciline, Cefotaxime, Cefalotine, Tetracycline, Ciprofloxacin, Oxolinic acid, Gentamicin, Streptomycin, Spiramicin, Colistin and Sulfamethoxazole-Trimethoprim. Indeed, from a pure culture obtained 18 hours on trypticase soya medium (TCS), an inoculum suspension was prepared by emulsifying a colony in 1 mL of sterile distilled water using a sterile platinum loop . Then one drop of this suspension was added to 9 mL of physiological saline. The mixture is vortexed to homogenize to have a standard 0.5 Mc Farland turbidity solution (about 108 CFU / cm³). Then, the petri dish containing Mueller-Hinton agar was flooded with 9 mL of the suspension. After homogenization to touch all the agar, the liquid was aspirated using a sterile Pasteur pipette. Subsequently, the agar was dried in an oven for 15 to 30 min. Then, the antibiotic-impregnated disks (BioMérieux) were placed on the surface of the agar by gently applying them with sterile forceps. After incubation for 18-24 hours at 37 ° C, the reading was done qualitatively by measuring the bacterial growth inhibition diameter around the discs according to the standard provided by the CASFM (2016). The resistance rate of the strains to each antibiotic was calculated by the following formula :

$$\text{Antibiotic resistance rate} = \frac{\text{Total number of resistant strains} \times 100}{\text{Total number of strains studied}}$$

2.4. Preparation of APEC strains and extraction of DNA for PCR.

The APEC strains stored at -80 ° C. were thawed at room temperature (22 ° C.) and then streaked on TCS agar media which were then incubated at 37 ° C. for 18 hours using a loop of sterile platinum. After incubation, 3 colonies were used to seed 2 mL of PBS broth into sterile Eppendorf® tubes. The extraction of chromosomal and plasmid DNA from the strains was done using bacterial DNA extraction kits and amplification of virulence genes and antibiotic resistance (QIAamp DNA Mini kit 250 tests, Cat No. 51306) according to the kit manufacturer's recommendations. The amplification kits contained Taq polymerase, dNTPs, MgCl₂, MgCl₂ buffer, and biomol water.

2.5. Detection of virulence genes

The Polymerase Chain Reaction (PCR) multiplex technique was carried out for the detection of the virulence genes *papC* (fimbriae P), *tsh* (thermo-sensistive hemagglutinin) and *cnf* (cell necrotising factor) on both types of DNA (chromosomal and plasmid) according to Vumba (2012) using primers specific to each gene and reference strains (*Escherichia coli* Laboratory from the University of Montreal, Canada) (Table I). The amplification reaction was carried out using a thermocycler (GeneAmp, PCRSystem 9700, Singapore) in a reaction volume of 25 µL of which 5 µL of each total DNA extract added to 20 µL of Master Mix comprising: 3 µL of MgCl₂ buffer (6 µM), 3.5 µL of MgCl₂ (6 µM), 2.5 µL of dNTPs (600 µM), 0.5 µL of each forward

primer (0.5 μ M) and 0.5 μ L each reverse primer (0.5 μ M), 0.3 μ L Taq polymerase (2 U) and 7.7 μ L biomol water. The amplification of the genes was done in 24 cycles with an initial denaturation at 94 ° C / 5 min. This denaturation was followed by further denaturation at 94 ° C / 30s, hybridization at 55 ° C / 30s, elongation at 72 ° C / 30s and final elongation at 72 ° C / 5. min. The amplicons obtained were stored at 4 ° C.

2.6. Detection of quinolone resistance genes

Each of the *qnrA* and *qnrB* genes was detected by PCR simplex also on the two types of DNA (chromosomal and plasmid) according to **Vounba (2012)** using specific primers and reference strains (*Escherichia coli* Laboratory. University of Montreal, Canada) (Table 1). The amplification reaction was also carried out using a thermocycler (GeneAmp, PCRSystem 9700, Singapore) in a reaction volume of 25 μ L of which 5 μ L of DNA extract added to 20 μ L of Master Mix comprising: 4 μ L of $MgCl_2$ buffer (2 μ M), 5 μ L of $MgCl_2$ (2 μ M), 3 μ L of dNTPs (200 μ M), 0.5 μ L of each Forward primer (0.5 μ M) and 0.5 μ L of each reverse primer. (0.5 μ M), 0.2 μ L of Taq polymerase (1U) and 6.8 μ L of water biomol. The amplification of the *qnr A* gene was done in 31 cycles with an initial denaturation at 94 ° C / 5 min. This denaturation was followed by another denaturation at 94 ° C / 45 s, hybridization at 48 ° C / 45 s, elongation at 72 ° C / 60s and final elongation at 72 ° C. /5 min. The amplification of the *qnr B* gene was done in 32 cycles with an initial denaturation at 95 ° C / 5 min. This denaturation was followed by further denaturation at 94 ° C / 45 s, hybridization at 53 ° C / 45 s, elongation at 72 ° C / 60 s and final elongation at 72 ° C. /5 min. All amplicons were stored at 4 ° C.

Table 1: Primers and positive control strains used for the detection of virulence and resistance to quinolones (Vounba, 2012)

Factors	Genes	Primers	Molecular Weight of Amplicon (pb)	Positive Control Strains
TSH	<i>Tsh</i>	For 5' GGTGGTGCCTGGAGTGG Rev 5' AGTCCAGCGTGATAGTG	640 pb	ECL 17088
Fimbriae P	<i>papC</i>	For 5'GACGGCTGTACTGCAGGGTGTGGCG Rev 5'ATATCCTTTCTGCAGGGATGCAATA	501 pb	ECL 13421
Cell necrotising factor	<i>Cnf</i>	For 5' TTATATAGTCGTCAAGATGGA Rev 5' CACTAAGCTTTACAATATTGAA	446 pb	ECL 13421
Quinolones	<i>qnrA</i>	For 5' TCAGCAAGAGGATTTCTCA 3' Rev 5' GGCAGCACTATTACTCCCA 3'	627 pb	J53pMG252
	<i>qnrB</i>	For 5'GATCGTGAAAGCCAGAAAGG 3' Rev 5'ACGATGCCTGGTAGTTGTCC 3'	469 pb	J53pMG293

A: adenine, C: cytosine, G: guanine, T: thymine, TSH: thermosensitive hemagglutinin, pb: base pairs, For: forward (main strand); Rev: reverse (complementary strand)

2.7. Revelation of gene amplification products

The PCR products were revealed by performing electrophoresis on 2% agarose gel (Conda, Spain) using an electrophoresis apparatus (PS Electronics). Tris-EDTA-acetate (TEA) (Carl Roth, Acros and MP Biomedicals) was used as a migration buffer. To 10 µL of PCR product, 2 µL of deposition solution was added. Then, the mixture was put on the gel in order to be subjected to an electrical voltage migration of 100 V for 60 min. To evaluate the molecular weight of each amplicon, a 100 bp DNA Ladder molecular weight marker (Thermo Scientific) was deposited on the gel along with the PCR products. After migration, the amplified DNA was observed under ultraviolet (UV) radiation by means of an intercalating agent incorporated in the gel, ethidium bromide (EtBr). The evaluation of the presence of the gene was made based on the presence of a translucent band corresponding to the molecular weight of the expected gene.

2.8. Statistical analysis of the results

The graphical representation of the data was performed with Graph Pad Prism 5.0 software (Microsoft USA). The statistical analysis of the results was made by the analysis of variances (ANOVA ONE-WAY) according to Dunnett's multiple comparison test, $p < 0.05$ is considered significant.

3. RESULTS AND DISCUSSION

3.1. Antibiotic profile of studied APEC strains

Figure 1 shows the sensitivity of APEC strains to different antibiotics. Analysis of this table reveals that the 75 strains showed variability in antibiotic resistance. Indeed, for all the strains tested, the highest levels of resistance were obtained with the β -lactams. These levels were 100% for oxaciline and 84% for amoxicillin. Resistance was also strong with tetracycline (94%), spiramicin, a macrolide (70%) and oxolinic acid (64%). The lowest levels of resistance were observed with colistin (0%), gentamicin (20%) and ciprofloxacin (27%). Cefotaxime, streptomycin and sulfamethoxazol-trimethoprim showed moderate sensitivity. The difference between the resistance of the strains to these three antibiotic groups was significant ($P < 0.05$). It should be noted that 71% of the strains were resistant to at least one quinolone (oxolinic acid and ciprofloxacin).

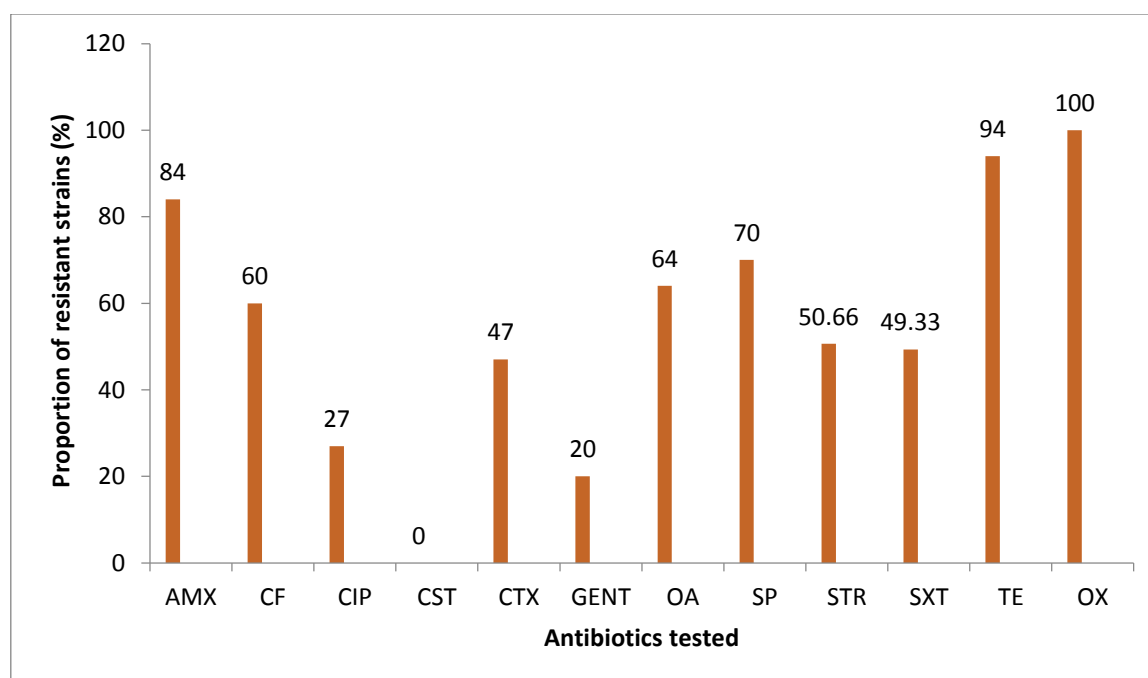


Figure 1 : Sensivity of APEC strains to the different antibiotics

AMX: amoxicillin; CF: cephalothin; CIP: ciprofloxacin; CST: colistin; CTX: cefotaxime; GENT: gentamicin, OA: oxolinic acid; SP: spiramicin; STR: streptomycin; SXT: sulfamethoxazole-trimethoprim; TE: tetracycline; OX: oxaciline

The strong resistance to these antibiotics could be explained by an increase in their use by farmers, especially in traditional farms that cohabit with semi-industrial farms. Indeed, the β -lactam antibiotics from which amoxicillin, cefotaxime and oxacillin are based are the most widely used antibiotics because of their broad spectrum of action, their safety, their effectiveness and above all their low costs (Cavallo and *al.*, 2004). However, because of their anarchic, inadequate, abusive and uncontrolled use in poultry farms, we are witnessing more and more the emergence of strong resistance in microorganisms including *E. coli* (Cavallo and *al.*, 2004). The results obtained with colistin, tetracyclines and gentamycin are similar to those of Sanogo and *al.* (2014) who reported an identical resistance pattern on *E. coli* from poultry. The very low or no resistance to colistin seems to be preserved compared to

the work of **Guessennd and al. (2014)** carried out on 27 avian septicemic strains. However, our results showed resistance growth with amoxicillin (84% vs. 74.1%) and decreased resistance to gentamicin (20% vs. 48%), ciprofloxacin (27% vs. 48%) and sulfamethoxazole-trimethoprim (49.33% vs. 96%) compared to those found by these authors. This decline in resistance may be related to an awareness of the risk of resistance and multi-resistance or a decline in the effectiveness of these antibiotics increasingly found by farmers.

The results obtained are in the same direction as those of the Senegalese researchers, **Fofana and al. (2006)** and **N'Diaye (2010)** who have previously reported similar resistance profiles for tetracyclines, streptomycin, trimethoprim-sulfamethoxazole.

3.2. Molecular profile of isolated strains

3.2.1. Rates of strains carrying virulence genes

The results of the PCR followed by electrophoresis for the detection of the virulence genes made it possible to note that only the *papC* gene was detected corresponding to a 501 bp amplicon with a presence rate of 12.90%, ie 4 strains on 31 (No 2, No 15, No 21 and No 45) (Figure 2), and the *tsh* and *cnf* genes were not detected in any of the strains studied (**Dozois and al., 2000**) found in Avian *E. coli*, the P fimbriae encoded by the genes from which the *papC* originates have been detected only in pathogens. This further confirms the pathogenicity of the strains we have studied. Various studies by many other authors on poultry have already reports a prevalence of this gene, which is close to ours in APECs, as is the case of studies by **Cortés and al. (2010)** in Spain who reported prevalences of 14% of *papC* but also 0% of *cnf* as noted in our study. The difference between our figures could be due to lack of hygiene on the mostly traditional or semi-industrial farms which favors the circulation and acquisition of plasmids by *E. coli* strains.

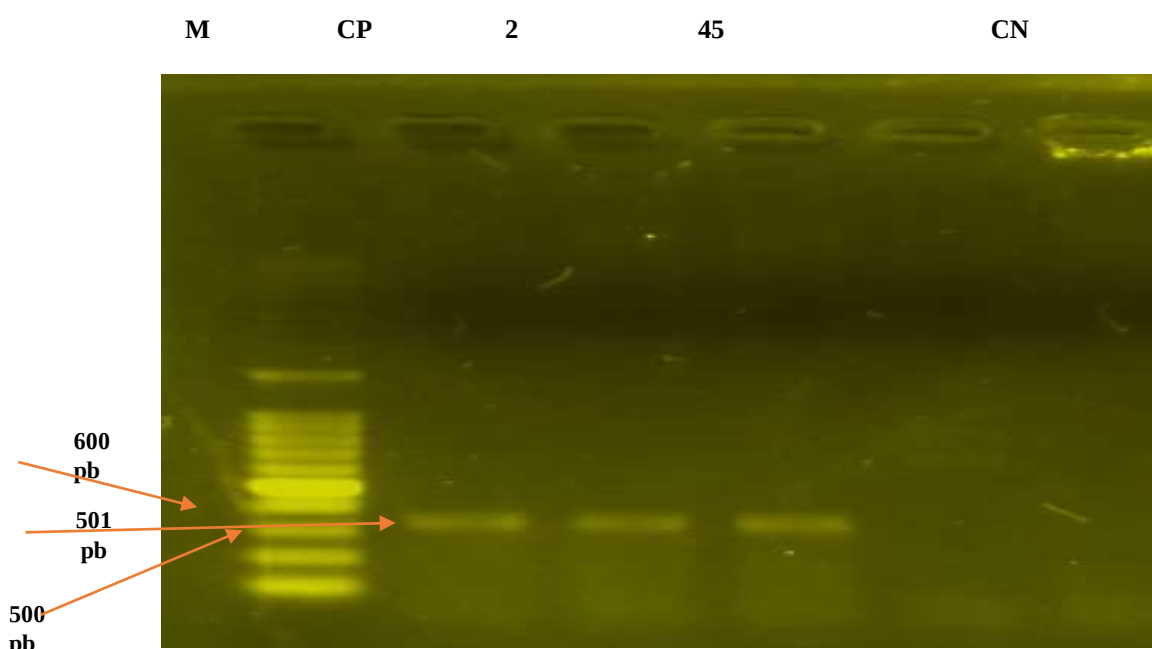


Figure 2: Electrophoretic profile of the amplification products of the virulence gene papC

Well M: molecular weight marker DNA ladder 100 bp ; Wells numbered: the samples of strains studied
CP: Positive control well ; CN: Negative control well ; Well 2, 45: positive samples

3.2.2. Strains with quinolone resistance genes

The results of the PCR revealed that out of thirty-one (31) strains studied, eight (8) carried quinolone resistance genes (*qnrA* and *qnrB*), ie 25.80%. These genes showed the same prevalence with 5 strains each of 31 or 16.12%. Indeed, examination of 2% agarose gels after electrophoresis showed 627 bp amplicons (strains No 2, No 15, No 19, No 36 and No 45) and 429 bp (strains No 16, No 24, No. 36, No 45 and No. 47) corresponding respectively to the *qnrA* (Figure 3) and *qnrB* (Figure 4) genes. It also appears that two (2) strains (No 36 and No 45) were positive for the 2 genes sought, ie 6.45%. These results confirm the phenotypic resistance to quinolones (ciprofloxacin and oxolinic acid) of the strains studied, as shown by the antibiogram test. However, this prevalence was lower than that observed phenotypically with the quinolones used in antibiograms. This difference is due to the non-expression of these genes by some strains. In addition, phenotypic resistance could be linked to other genes that we did not search for in this study. The results we obtained for these genes differ little from those obtained by **Guessennd and al. (2014)** who reported prevalence rates of 8.5% and 21.7%, respectively, for *qnrA* and *qnrB* in a study of 27 strains with septicemic colibacillosis. This difference in prevalence could be explained by the fact that these authors took samples only from farms in one

locality of Côte d'Ivoire (Bingerville) while our strains were isolated from poultry from several localities. The resistance of APEC strains to quinolones observed may be due to several factors. Indeed, the APEC being Gram negative bacteria, their mechanism of resistance could be explained by chromosomal mutations. This mutation can lead to amino acid modifications of the A and B subunits of DNA gyrase and topoisomerase (Lautenbach and al., 2004). Quinolone resistance could also be explained by the acquisition of resistance plasmids called mediated quinolone resistance (PMQR). These plasmids carry one of the following quinolone resistance genes: *qnr*, and *qep A*, (Carottoli, 2009).

Increasing this resistance may make the use of these antibiotics in avian production inappropriate because of cross-resistance with fluoroquinolones already used in human medicine as treatments of last resort (Talan and al., 2008).

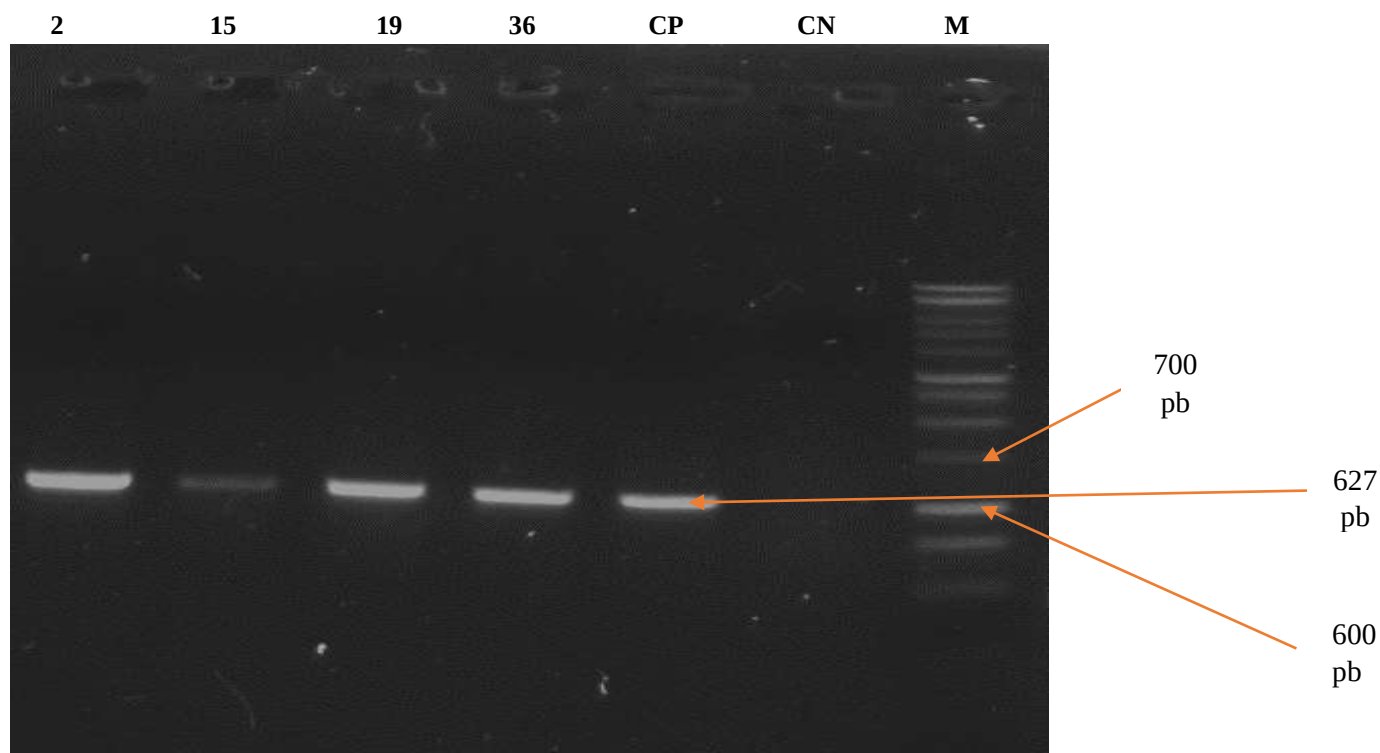


Figure 3 : Profil électrophorétique des produits d'amplification du gène *qnrA*

Well M: molecular weight marker DNA ladder 100 bp ; Wells numbered: the samples of strains studied ; CP: Positive control wells ; CN: Negative control wells ; Wells 2, 15, 19, 36: positive samples

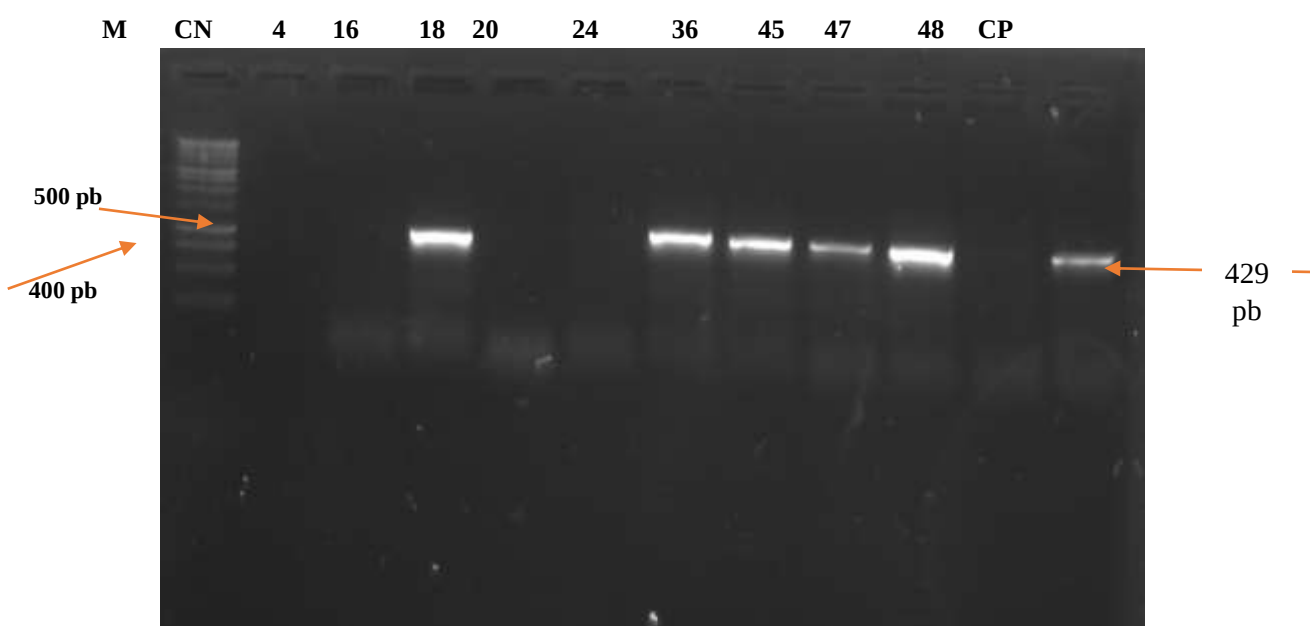


Figure 4: Electrophoretic profile of the amplicons of the *qnrB* genes

Well M: molecular weight marker DNA ladder 100 bp ; Wells numbered: the samples of strains studied ; CP: Positive control wells ; CN: Negative control wells ; Wells 16, 24, 36, 45, 47: positive samples

The analysis of PCR results also showed that antibiotic resistance (25.80%) was higher than virulence (12.90%). Indeed, only two strains possessing the virulence gene *PapC* have quinolone resistance genes. These are strains No. 2 and No. 45. However, it was found that strain No. 45 possessing the *PapC* gene carried both resistance genes to quinolones detected. These results suggest that there is no relationship between virulence and antibiotic resistance already noted by Diallo (2013).

4. CONCLUSION

At the end of this work, we retain that the strains of *E. coli* isolated from farms show phenotypic resistance to antibiotics with high representativeness for the quinolones used in the antibiogram (ciprofloxacin and oxolinic acid). These APEC strains also have a remarkable prevalence of quinolone resistance genes (*qnrA* and *qnrB*) and the *papC* virulence gene. This constitutes a major risk of transfer of resistance to humans when we know the importance of quinolones in human antibiotics. The excessive and uncontrolled use of these antibiotics for the prevention and treatment of pathologies would be the basis of the increase in resistance.

CONFLICT OF INTEREST STATEMENT

The authors declared no conflict of interest on the research work.

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