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Assessment of antimicrobial resistance in avian pathogenic *Escherichia coli Strains* isolated over four years in Tunisian poultry

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Avian pathogenic *Escherichia coli* strains (APEC) are responsible for major economic losses in poultry farms. The use of antibiotics has led to the emergence of resistant bacteria having direct impact on the food industry. In order to evaluate the resistance of 191 Tunisian APEC strains, we determined the antimicrobial resistance profile of these bacteria to 18 antibiotics by disk diffusion method. This study revealed high resistance towards most of the tested antibiotics. Indeed for 13 antibiotics over 50% of strains were resistant. The results also showed significant increase in time of resistance percentage and multidrug resistance; which may be related to the selection pressure due to the overuse of antimicrobial agents for treatment and as growth factors in poultry. Statistical tests revealed several statistical descriptive values, reflecting scattered distribution of resistance with normality dispersion. Phylogenetic analyses showered clustered strains. Data converge towards a heterogeneous distribution of resistance with increasing rates, suggesting considerable overlap between APEC strains.

Key words: APEC strains, colibacillosis, antimicrobial agents, resistance profile, Tunisia.

INTRODUCTION

The poultry industries worldwide suffer great financial losses every year because of the high morbidity and mortality rates caused by colibacillosis, common bacterial infection (Guerin and Boissieu, 2008) are mostly important in avian pathology. Colibacillosis is caused by avian pathogenic *Escherichia coli* (APEC) (Lau et al., 2010; Oh et al., 2011) with a broad spectrum of clinical outcomes. APEC strains are endowed with different properties that allow them, for example: to enter the bloodstream, overcome to host defense mechanisms or colonize deep organs and is a subset of extra intestinal pathogenic *E. coli* (ExPEC) (de Pace et al., 2011). They share virulence traits with strains isolated from human cases of neonatal meningitis, urinary tract infections, and septicemia. Thus, APEC strains represent a high risk of zoonotic infection (Bauchart et al., 2010) and their virulence gene pool may contribute to the emergence of other ExPEC strains (Bertrand et al., 2010). Avian

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colibacillosis takes various shapes with a general process of respiratory or genital input (Bertrand et al., 2010) and can affect digestive, biliary and urinary tracts, which are major source of contamination in poultry farms (Oyetunde et al., 1978). APEC strains commonly cause airsacculitis, pericarditis, perihepatitis, peritonitis, salpingitis, and subsequently the most acute form, septicemia, resulting in sudden death (Mellata et al., 2003; Ask et al., 2006; Zhao et al., 2009 ; Giovanardi et al., 2013). It has been shown that from 10 to 15% colibacillary population belongs to potentially pathogenic serotypes (Dho-Moulin and Fairbrother, 1999).

In Tunisia the poultry industry is an important part of the economy and treatment strategies which are based on the use of antibiotics and control environmental factors. Antimicrobial treatments against colibacillosis are usually given to the whole flock via the drinking water or feed over several days and thus may impact the equilibrium and susceptibility of bacteria present in the intestinal flora. The poorly controlled use of broad-spectrum antibiotics has favored the emergence of highly resistant bacteria, which place the treatment of certain infections in therapeutic impasses. The acquired resistance of APEC strains to several antimicrobial drugs is becoming a major issue in intensive poultry farming (Furtula et al., 2010). Furthermore, the risk of consuming chicken meat contaminated with resistant *E. coli* consists mainly of the possible transfer of resistance genes to other, potential pathogenic bacteria, present in the human intestinal tract (Markland et al., 2015).

The increasing incidence of antibiotic resistance in APEC strains and the high risk of transmission to humans and potential effect on the environment, especially because litter from farms is commonly used as fertilizer, is an area of growing concern (Furtula et al. 2010). The purpose of this study was to investigate antibiotic resistance of APEC strains isolated at the Veterinary Research Institute of Tunisia (IRVT) from poultry in Tunisian commercial poultry farms in order to study resistance dynamics and transfer. This may give new insight in improving treatment strategies. The focused on this study is on 18 antimicrobials, administered over four years.

MATERIALS AND METHODS

Isolation of *E. coli* strains

A total of 191 APEC strains were collected over a four-year period (from April 2010 till April 2014) from Tunisian poultry and isolated from different organs (livers, hearts and spleens) of sick chickens exhibiting clinical symptoms of avian colibacillosis in «diagnostic bacteriology laboratory of the Veterinary Research Institute of Tunisia». Number of strains and sites of isolation are depicted in Table 1.

Table 1. Number of strains and corresponding site of isolation.

Number of strains	Site of isolation
128	Livers, Spleens
63	Livers, Spleens, Hearts

For 128 strains, samples were isolated from living organs: livers and spleens at the same time For 63 strains samples were isolated from living organs: livers, spleens and heart at the same time All tests were performed aseptically to avoid contamination by non-pathogenic bacteria.

Growth conditions of APEC strains

The samples, which were collected from affected organs, were grown in Bromocresol Purple Lactose Agar (BCP) medium aerobically for 18 to 24 h at 35 to 37°C. Specimens must be directly streaked onto the medium not later than 2 h after collection or must be kept refrigerated (not longer than 24 h) to avoid overgrowth of the infectious agents or contaminants. Differentiation of APEC isolates from other specimens was performed by Gram stain followed by appropriate standard biochemical tests (oxidase test, urease, B-galactosidase, Kligler iron agar, citrate permease etc) and commercial API 20E antisera test according to the manufacturers' instructions (Biomérieux).

Antibiotic susceptibility testing assay

Several assays for estimating antimicrobial susceptibility of 18 antibiotics belonging to most known families with direct interest to human health were conducted for the 191 APEC strains using the disk diffusion method recommended by Antibiogram Committee of the French Society for Microbiology CA-SFM, according to the French Veterinary Benchmark Standards (Haenni et al., 2011). Mueller Hinton agar plates were inoculated with an inoculum of *E. coli* strains and disks impregnated with antimicrobial agents were filed on the inoculated agar plates. After incubation at 37°C for 18 to 24 h, the study of the bacteriostatic effect of antibiotics was determined by measuring the diameter of the inhibition zone around the disk. Details regarding families of antibiotics tested are listed in Table 2.

Reference strains (E. coli ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853) were used as susceptibility testing quality control in order to ensure the validity of the results.

Statistical analysis

Statistical analyses were performed with R programming language and software environment for statistical computing and graphics (version3.0.2). R functions are executable through command lines and scripts. Our data were analyzed using statistical tests of R package that calculate resistance rates to antibiotics in APEC strains and determine significant differences between them. We also determined several statistical descriptive measures such as variance and SD (deviation) of resistance dispersion. The Shapiro-Normality test was used to analyze the normality of resistance distribution. This test is based on *W* statistic that offers a *w* value associated to *P*-value. A *P*-value less than 0.05 are considered statistically significant supporting that the resistance does not follow a normal distribution.

Phylogenetic analysis

Phylogenetic analysis was performed with R program in order to

Table 2. List of antibiotics tested associated to their families.

Family	Subfamily	Group	Antibiotics or chemotherapeutic agent
Beta-Lactam	Penicillin (Penams)	Group A	Ampicillin (AM)
			Amoxicillin (AMX)
			Amoxicillin-clavulanic acid (AMC)
	Cephalosporins (cephams)	First generation	Cephalexin (CN)
			Cefalotin (CF)
		Second generation	Cefoxitin (FOX)
		Third generation	Ceftiofur (XNL)
Aminoglycosides			Streptomycin (STR or S)
			Neomycin (N)
			Gentamicin (GM)
			Spectinomycin (SPT)
Tetracyclines	First generation		Tetracycline (TE)
Polypeptides	Overactive (detergents)		Colistin (Polymyxin E) (CS50)
Quinolones	First generation		Nalidixic acid (NA)
	Second generation		Flumequine (UB)
	Third generation (fluoroquinolones)		Enrofloxacin (ENR)
			Marbofloxacin (MAR)
diaminopyrimidines			Trimethoprim-sulfamethoxazole (SXT)

test phylogenetic links between resistant strains. Input data were translated from excel table to a matrix in binary format to be correctly treated by R commands.

RESULTS

Assessment of antibiotic resistance rate

The results of resistance testing to all antibiotics showed variable rates ranging from 8% (intermediate resistance level) of strains resistant to ceftiofur (XNL) to 86% (high resistance level) of strains resistant to tetracycline (TE). Among strains tested, more than 50% exhibit resistance to 13 of the 18 tested antibiotics, ampicillin, amoxicillin, amoxicillin-clavulanic acid, cephalexin, streptomycin, neomycin, spectinomycin, tetracycline, trimethoprim-sulfamethoxazole, nalidixic acid, flumequine, enrofloxacin and marbofloxacin. For the other five antibiotics the rate of resistant strains was comprised between 8% and 39%. Thus, for the majority of samples resistance rate was described as high. Resistance and susceptibility rates for all antibiotics are plotted as histograms and depicted in Figure 1.

Comparison between antibiotic resistances in different periods of time was conducted and an increase in number of resistant APEC strains from a year to another was noticed with the highest global level in 2013. For nine antibiotics: cephalexin, cefalotin, cefoxitin, ceftiofur, neomycin, spectinomycin, trimethoprim-sulfamethoxazole, enrofloxacin and marbofloxacin has the highest resistance level and was observed in 2013; for five antibiotics: ampicillin, amoxicillin, tetracycline, colistin and flumequine, has the highest resistance level which was observed in 2010; for two antibiotics: amoxicillin-clavulanic acid and nalidixic acid, resistance was the highest in year 2011 and 2012, respectively, and for the two latest antibiotics: the highest level was observed once in both 2010 and 2012 (for streptomycin) and again in both 2011 and 2013 (for gentamicin). A slight decrease in global resistance rate was observed in 2014. Moreover, it seems that this spread does not depend on the families of antibiotics tested. Indeed, antibiotics belonging to the same family could be prevalent each in different period. Only one case of extremely related antibiotics those of the cephalosporin subfamily (Beta-lactam family), predominant in 2013 with four patterns, was observed. These findings are summarized in Figure 2.

Statistical tests were performed with R language software and revealed several statistical descriptive values informing on distribution and correlation between variables characterizing evolutionary trends of APEC strains drug resistance. The average of resistance rate for all antibiotics, obtained by dividing the sum of all resistant rates by their number, was 67.83 with a standard deviation of 43.57 (67.83±43.57). The coefficient of variation (cv) representing the dispersion of drug

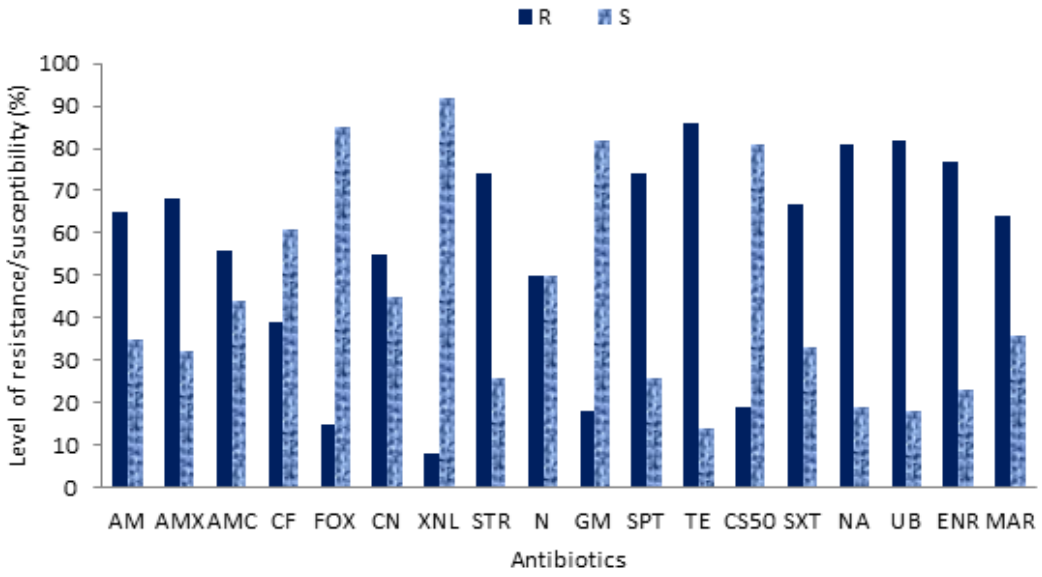

Figure 1. Resistance and susceptibility levels of APEC isolates to antibiotics of interest.

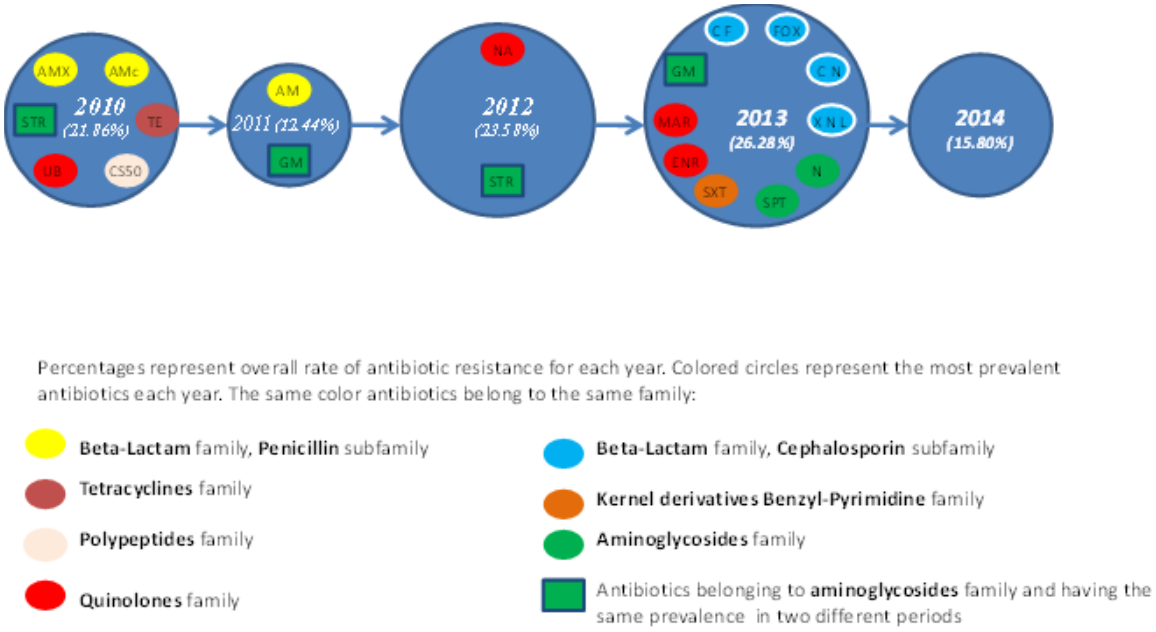

Figure 2. Evolution of overall drug resistance in APEC strains during the study period.

resistance rates versus the average value, was also determined (64.23) and showed that the variation of resistance to all antibiotics tested, tended to be scattered as compared to their average, meaning that different drug resistant rates were statistically distant since cv > 50. *W* and *P*-values were also determined to study the nature of different antibiotics resistance distribution; *w* = 0.93 and *P*-value = 0.24. Thus, the distribution of resistance was normal since *P*-value was found to be > 0.05.

Most isolates exhibit multidrug resistance

Among the 191 isolates studied, 168 (88%) specimens were resistant to at least three antibiotics at the same time and so have multidrug resistance profiles. Whereas, the other 23 strains (12%) were resistant to one or two antibiotics each. These results showed an increase in multidrug pathogenic *E. coli* that could be related to the overuse of antibiotics in the veterinary field. In fact, it has

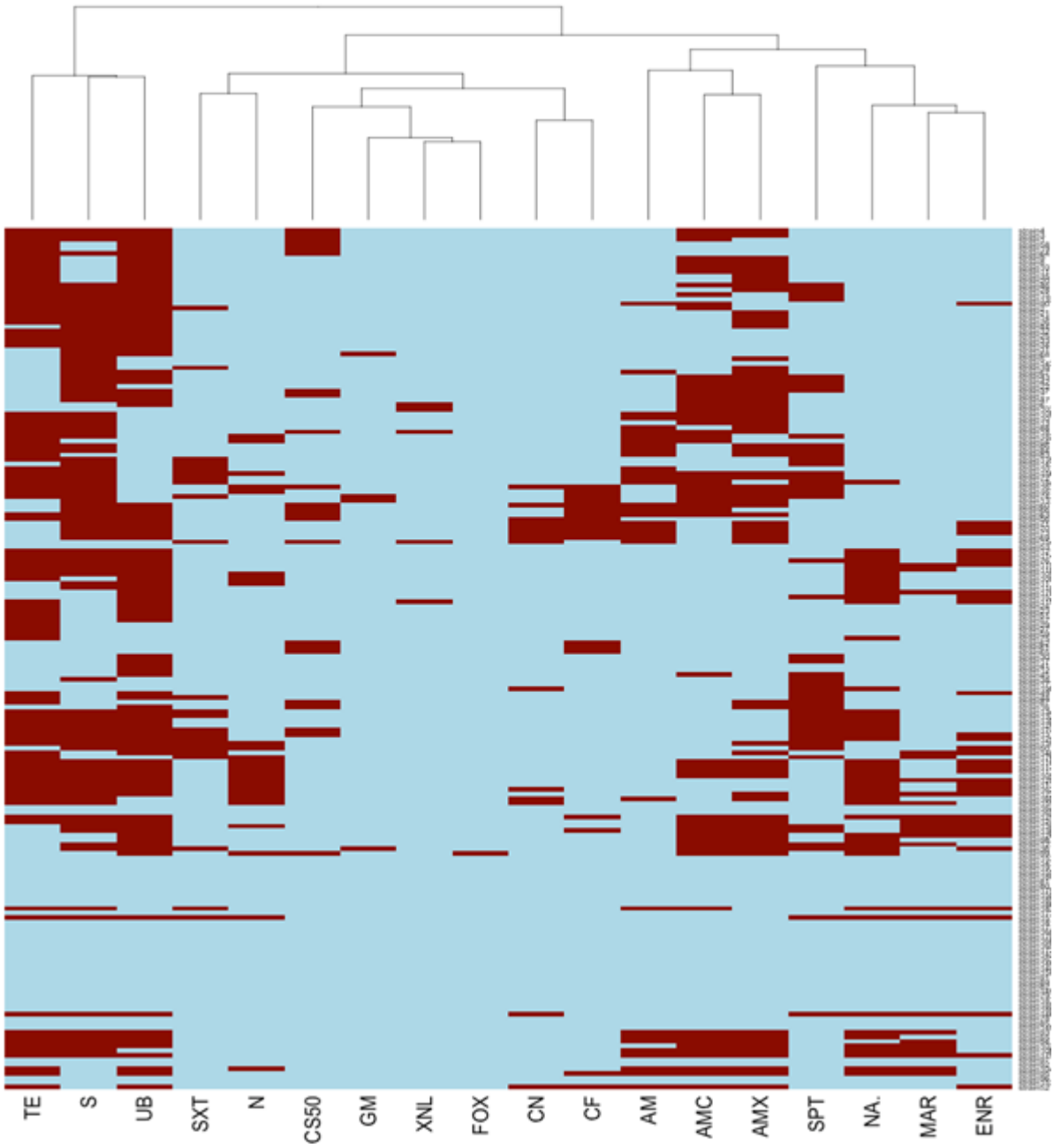

Figure 3. Hierarchical clustering rate based on antibiotics studied.

been shown that the use of antimicrobial agents is associated with antimicrobial resistance and even leads to human health consequences (Anqulo et al., 2004; Zhao et al., 2012).

Phylogenetic construction and clustering rate

Phylogenetic analyses were conducted with R programming software in order to determine relatedness link between isolates on the base of resistance that exhibit different antibiotics. Phylogenetic tree was constructed and visualized with distance matrix method. Comparing drug resistance profiles strains were subject to our phylogenetic study which revealed the presence of twenty-two clusters, fourteen clusters which composed of two strains, five clusters composed of three strains, two clusters composed of four strains and finally one cluster composed of five strains showing the same drug resistance profile (Figure 3). Clusters were mainly associated to antibiotics for which, the resistance was high.

DISCUSSION

In the present study we explored the resistance of pathogenic *E. coli* strains isolated from Tunisian poultry to 18 antimicrobial agents belonging to the most common antibiotics families used in Tunisia to treat avian colibacillosis. This has been achieved according to the standards adopted by the French Society for Microbiology Committee (Haenni et al., 2011). For this purpose, we focused on the study of 191 isolates.

Based on the resistance profile, we noted that highly polymorphous resistance rates have been displayed for one antibiotic to another with global high level in most cases studied. The level of resistance to ampicillin observed in our study (65%) was rising continuously which is consistent with results previously reported. A previous work conducted in diagnostic bacteriology laboratory of IRVT revealed ampicillin resistance rate which is close to our findings (52.5%) (Data not published). Another study conducted by Zhao et al (Zhao et al., 2012) showed an increase in ampicillin resistance over time. Similar resistance profiles to amoxicillin and amoxicillin-clavulanic acid were observed which seems to be quite expected seeing that these antimicrobials were widely used in various respiratory infections treatment (Gaillat et al., 1987). Resistance to nalidixic acid was also high (81%) which could be explained by cross-resistance with that to oxolinic acid as they have the same regulating functional role of blocking the same enzymes during DNA synthesis.

As regular monitoring of antibiotic resistance is a key to effective and appropriate therapeutic strategies limiting the emergence and the spread of multidrug-resistant strains, we looked for the antibiotic resistance combination exhibited by each strain. Our data showed the grouping of strains in clustered profiles suggesting horizontal transfer of antibiotic resistance.

Taken together, our data converge towards a heterogeneous distribution of resistance with increasing rates that revealed a considerable overlap between APEC strains reminding clonal expansion. In fact, resistance genes are known to spread via two phenomena, horizontal gene transfer and clonal expansion. Such variability seems reasonable as a result of the overuse of antibiotics in the treatment of colibacillosis, a treatment that is sometimes inappropriate and not controlled when administrated in poultry farms (Salehi and Bonad, 2006).

It has been previously shown that genes encoding antibiotic resistance are commonly found in *E. coli* from different hosts (Venturini et al., 2013). Thus, APEC strains probably serve as a reservoir of genes encoding resistance proteins which could explain the rapid dissemination of antibiotic resistance. On the other hand, the use of antimicrobial agents as growth promoters in poultry feed has an important implication on the emergence of antimicrobial resistance in bacteria (Smith et al., 1999; Shuford and Patel., 2005). Fortunately this practice is banned in Tunisia since 2007. However, the misuse of these antimicrobials preventively remains a concern. Combination of these antimicrobials and resistant *E. coli* could be risk factor for environmental contamination that could be transferred to human. In fact, it has been shown that the same type of *E. coli* carrying an identical gene encoding sulphonamide resistance (*sul2*) can colonize both animals and humans, and that strains which can be found among animals which may be implicated in human infections such as septicaemia (Trobos et al., 2009). APECs probably serve as source of human infection by pathogenic *E. coli* through transmission via the food chain (Zhao et al., 2009) of several known drug resistance genes (de Pace et al., 2011), such as those encoding siderophores and capsules. Thus, zoonotic potential of animal-derived strains need to be more explored specially with increasing knowledge of molecular genetics and pathotypes of ExPEC of human and animal origin. Controversy continues to be needed to determine the pathogenicity of APEC strains as well as the potential effect of antimicrobial residues analysis on public health.

Conclusion

Since infections referred to avian colibacillosis are responsible for large financial losses to the poultry industry each year due to mortality, lost production and condemnations, antimicrobial treatment has become a common practice which has however several implications affecting the poultry sector mainly in relation to the emergence of antibiotic resistant strains. In this study, we plotted the epidemiological distribution and evolution of antimicrobial resistance dynamics in Tunisian APEC strains that evolve exponentially during these last few years. It has been concluded that, such epidemiological studies provide effective tools in antibiotic resistance studies; nevertheless further work is needed to define additional biological features.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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