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Emergence of Tobramycin *Escherichia coli* resistance in poultry meat linked to biocides overuse during COVID-19

Aparición de resistencia a la tobramicina en *Escherichia coli* en la carne de aves de corral vinculada al uso excesivo de biocidas durante COVID-19

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ABSTRACT

The effect of excessive use of biocides during the COVID-19, on the resistance of *Escherichia coli* to Tobramycin in poultry, meat was examined in this observational epidemiological study (Before and after COVID-19). Tobramycin *E. coli* resistant strains isolated from poultry meat before COVID-19 appearance were compared with those isolated after COVID-19 emergence. Univariable analyses were performed using *t*-test and chi-squared test. Odds ratios and 95% confidence intervals were used for statistically significant risk factor. Multivariate analysis was done with the binary logistic regression to detect an independent predictor, and with the principal component analysis (PCA), to analyze whether the Tobramycin resistance in *E. coli* was linked with the COVID-19 outbreak. Statistical significance was set at $P<0.05$. The frequency of Tobramycin *E. coli* resistant isolates was more important after COVID-19 emergence (12.5%) than before COVID-19 (2.1%). Graphical representation of PCA qualitative variables shows the interfactor relationship. A significant relationship between Tobramycin *E. coli* resistance and COVID-19 emergence (P=0.014), and the effect of the emergence of COVID-19 on the Tobramycin *E. coli* resistance was OR = 6.57 (95% Confidence interval (CI) 1.61-7.94). The probability of Tobramycin *E. coli* resistance linked with poultry meat bought after COVID-19 was 1.88 times more than before COVID-19 emergence. Poultry meat purchased after COVID-19 found related to Tobramycin resistance in *E. coli*. It seems possible that the overuse of biocides during COVID-19 increased the risk of Tobramycin *E. coli* resistance in poultry meat.

Key words: Antibiotic; antimicrobial-resistance; biocide; cross-resistance; risk-factor

RESUMEN

En este estudio epidemiológico observacional (Antes y después del COVID-19) se examinó el efecto del uso excesivo de biocidas, durante la pandemia de COVID-19, sobre la resistencia de *Escherichia coli* a la tobramicina en la carne de aves (CA). Las cepas resistentes a la tobramicina *E. coli* aisladas de la CA antes de la aparición de la COVID-19 se compararon con las aisladas después de la aparición de la pandemia COVID-19. Los análisis univariables se realizaron mediante la prueba t y la prueba de Ji-cuadrado. Se utilizaron razones de probabilidad e intervalos de confianza del 95 % para el factor de riesgo estadísticamente significativo. El análisis multivariante se realizó con la regresión logística binaria para detectar un predictor independiente y con el análisis de componentes principales (PCA) para analizar si la resistencia a la tobramicina en *E. coli* estaba relacionada con el brote de COVID-19. La significación estadística se fijó en $P<0{,}05$. La frecuencia de aislamientos de *E. coli* resistentes a la tobramicina fue más importante después de la aparición de la COVID-19 (12,5 %) que antes de la COVID-19 (2,1 %). La representación gráfica de las variables cualitativas de PCA muestra la relación interfactorial. Una relación significativa entre la resistencia a la tobramicina *E. coli* y la aparición de COVID-19 (valor de P=0,014), y el efecto de la aparición de COVID-19 en la resistencia a la tobramicina *E. coli* fue Odds Ratio (OR) = 6,57, intervalo de confianza (IC) del 95 % : (1,61-7,94). La probabilidad de resistencia a la tobramicina *E. coli* vinculada con la CA comprada después de la COVID-19 fue 1,88 veces mayor que antes de la aparición de la COVID-19. La CA comprada después de COVID-19 se encontró relacionada con la resistencia a la tobramicina en *E. coli*. Parece posible que el uso excesivo de biocidas durante el COVID-19 haya aumentado el riesgo de resistencia a la tobramicina *E. coli* en la CA.

Palabras clave: Antibiótico; biocida; factor de riesgo; resistencia antimicrobiana; resistencia cruzada

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INTRODUCTION

Repercussions of Coronavirus disease 2019 outbreak (currently known as COVID-19) on future global health are still being investigated, including the pandemic's potential effect on the emergence, and spread of global antimicrobial resistance (AMR) [3]. According to World Health Organization (WHO) data, AMR causes about 700,000 deaths annually, which has been estimated to increase reaching 10 million deaths worldwide by 2050 [24].The post-COVID-19 study is expected to alter this data, and the death attributed to AMR is expected to approach a much higher number due to a global change in antibiotic consumption patterns [27].Further, concerns such as biocides (disinfectants, sanitizing agents, and cleaning chemical agents) use during COVID-19 pandemic could also increase AMR [3]. Massive additional quantities of disinfectants have been applied during the COVID-19 pandemic as infection preventive and control measures [1].

Antimicrobials are valuable therapeutics whose efficacy is seriously compromised by the emergence and spread of antimicrobial resistance [18]. However, recent data from food animal studies showed that limiting the use of antibiotics in food animal production resulted in modest reductions in the prevalence of resistance, instead of elimination of resistance, in certain bacteria [37]. Several articles exemplify resistance stabilization and induction mechanisms, independent of a corresponding antibiotic exposure [33].

Long-term sub-lethal exposure to biocides can exert a selective pressure leading to the emergence of microbial strains with a reduced susceptibility to the used antimicrobials, which can colonize food-processing environments and recurrently contaminate food [25]. Microbial tolerance to biocides does not only compromise food safety but also threatens Public Health (PH) mainly because the mechanisms that convey tolerance to biocides are similar to those observed in antimicrobials of clinical importance in a phenomenon known as cross-resistance [8, 25].

There is now considerable evidence that transfer of AMR from food-producing animals to humans directly via the food chain is a likely route of spread [36].Bacteria tolerant to a wide range of antimicrobial compounds (including biocides) are becoming more frequent in the food chain [20]. According to the Food and Agriculture Organization (FAO) statistics, poultry has become the most widely consumed meat worldwide [12]. The use of antimicrobials in animal farming is likely to accelerate the development of antimicrobial resistance in pathogens, as well as in commensal organisms [21]. Animal products may contain antimicrobial resistant bacteria as a result of fecal contamination during slaughter [32]. Commercial broilers can be reservoirs of virulent and resistant genes as well as *Escherichia coli* causing extra-intestinal infections, which can be a potential threat to humans via direct contact and food [16]. During the processing of meat, the bacteria from animal origin can contaminate other food items, the processing plant, or workers. On the other hand, it is possible that resistant organisms are introduced from the outside, e.g. by food handlers into the production line [36]. Sanitizers are used in the disinfection process to control, reduce, and inactivate foodborne pathogens. They reduce microorganisms of PH importance to levels considered safe, based on established parameters, without adversely affecting either the quality of the product or its safety [9]. Due to the current COVID-19 pandemic, the use of biocides has hugely increased in private, community and hospital settings [3]. The role of biocides or sanitizing agents in food production and manufacturing facilities, as well as in hospital settings, has been linked to the risk of increasing antibiotic resistance [6].

Many *in vitro* studies have investigated genetic changes in standard culture collection strains following biocide exposure [17]. Unsurprisingly, this preconditioning reduces the susceptibility of these strains, often resulting in reduced susceptibility to biocides and increased minimal inhibitory concentrations for antimicrobial agents [6]. However, lifestyle modifications due to pandemics are changing the usage pattern of these disinfectants and have a realistic potential to enhance AMR development [27].

The objective of this study was to examine the effect of excessive use of disinfectants and biocides as a means of controlling the spread of the virus during the COVID-19 pandemic on the resistance of *E. coli* to Tobramycin in poultry meat (PM).

Tobramycin is an aminoglycoside antibiotic, used in the treatment of systemic and ocular infections, mainly caused by Gram-negative bacteria and *Staphylococcus aureus*. It is especially effective against species of *Pseudomonas* [22]. However, is not allowed for poultry use in Algeria [19]. Hence the risk of antibiotic resistance linked to its use in poultry farming is thus eliminated and the confusion bias is controlled.

MATERIALS AND METHODS

Sample collection

The study lasted from January 2018 to July 30, 2020. A total of 159 different cuts of PM samples were bought from ten butcher shops in the Algerian City, Biskra. All samples were subjected to a bacterial examination. Following the Food and Drug Administration's (FDA) Bacteriological Analytical Manual [9], 134 *E. coli* isolates were identified from PM samples. Ninety four *E. coli* strains were isolated from PM samples purchased from the butcher's shop prior to the COVID-19 emergence, from January 2018 to January 2020, and 40 *E. coli* strains were isolated from PM samples purchased from the same butcher's shops (10 shops) after the COVID-19 outbreak, from June 15 to July 30, 2020.

E. coli isolation and identification

Positive lactose isolated bacteria from MacConkey agar (Liofilchem, Italy), were investigated by microscope (OPTIKA-B150 microscope, Optika, Italy) after Gram stain, the identification of characteristic Gram-negative bacilli was then confirmed by biochemical tests:

- Brilliant Green Bile Broth with Durham Tube (BGLB) : positive gas production,
- Triple Sugar Iron agar (TSIA) : Acid/Acid, gas Positive, H_2S negative,
- Citrate : negative,
- Urease : negative,
- Indole : positive.

Finally, *E. coli* was confirmed using API-20E strips (Biomerieu, France).

E. coli antimicrobial susceptibility testing

The disc diffusion method was used to assess antibiotic susceptibility of all identified isolates (n=134). Mueller-Hinton agar (Himedia, India), was used to distribute bacterial suspensions. Tobramycin (TOB, 10 microgram -µg-) was utilized as the antibacterial. The antibiotic discs were incubated for 18 to 24 h at 37°C. The diameter of the inhibitor zone was determined in millimeters (mm). The interpretations were made in accordance with the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST); Recommendations 2019 V.2.0 Mai [29] and Recommendations 2020 V.1.1 Avril [30].

Statistical analysis

Tobramycin resistance in *E. coli* isolated from PM, before COVID-19 appearance, was compared with those isolated after COVID-19 emergence.

Means ± standard error mean (SEM) of the inhibited zone was calculated and data distribution was checked with boxplot diagram. Univariable analyses were performed using the Student's *t*-test (t) for continuous variables while the chi-squared test (X^2) for categorical variables. Odds ratios (OR) and 95% confidence intervals (CI) were used for statistically significant risk factor. Multivariate analysis was done with the principal component analysis (PCA), to examine whether the Tobramycin resistant in *E. coli* was linked with COVID-19 outbreak. The binary logistic regression used to detect the independent predictor. The goodness of fit for the logistic regression model was assessed with Nagelkerke test. A receiver operating characteristic (ROC) analysis was used to determine the area under the curve (AUC). A *P-value* of 0.05 was considered to indicate statistical significance. The statistical package for the Social Sciences (SPSS, version 21) [14] was used for descriptive and regression statistics while principal component analysis (PCA) was performed with R software version 3.1.3 [26].

RESULTS AND DISCUSSION

The objective of this study was to examine the effect of excessive use of biocides, during the COVID-19, on the resistance of *E. coli* to Tobramycin in PM.

Independent *t*-test

A disc diffusion assay was used to estimate the antibiotic susceptibilities to Tobramycin (10 µg) of *E. coli* isolated from PM. Results showed that inhibition zones before COVID-19 emergence were significantly larger than those of after COVID-19 appearance (*P-value*=0.023, *t*-test of differences, TABLE I). Indicating that *E. coli* isolated from PM purchased after the emergence of COVID-19 were more resistant to Tobramycin than those isolated before the appearance of COVID-19. Boxplot was used to present the distribution of inhibitor zone size obtained before and after the appearance of COVID-19 (FIG. 1).

TABLE I
Comparison of mean zone size of inhibition in Tobramycin *E. coli* resistance isolated in poultry meat before and after COVID-19 emergence

Variable class	N (%)	Mean ± SEM	SD	T	*P*-value
Before COVID-19	94 (70.15)	16.07 ± 0.17	1.64	2.30	0.023
After COVID-19	40 (29.85)	15.37 ± 0.24	1.53		

N: Sample size. SEM: Standard Error Mean. SD: Standard Deviation. t : Student's *t*-test

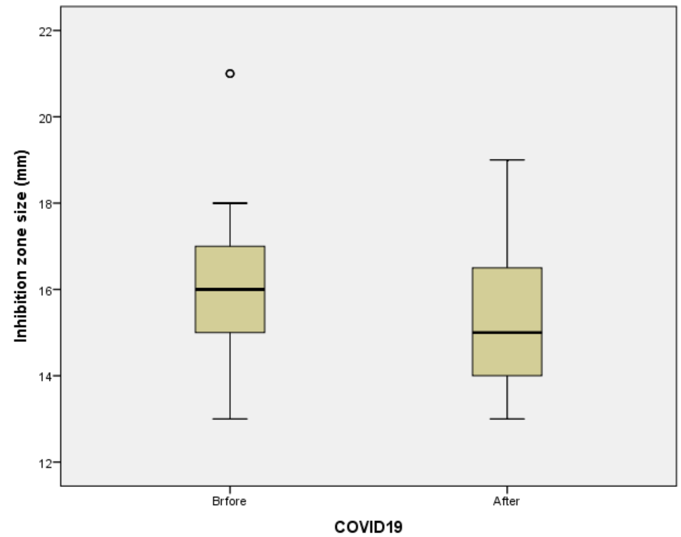

FIGURE 1. Boxplot of Tobramycin *E. coli* resistance profiles estimated from inhibition zone deviations

Chi-squared test (X^2) and Odds ratio (OR)

FIG. 2 shows that the frequency of Tobramycin *E. coli* resistant isolates is more important after COVID-19 emergence (12.5%) than before COVID-19 (2.1%). From the TABLE II, there was a significant relationship between Tobramycin *E. coli* resistance and COVID-19 emergence (Pearson's Chi-squared test *P*=0.014), furthermore the effect of COVID-19 time enlapsed (period) on the Tobramycin *E. coli* resistance was OR=6.57 (95% CI 1.22 – 35.45).

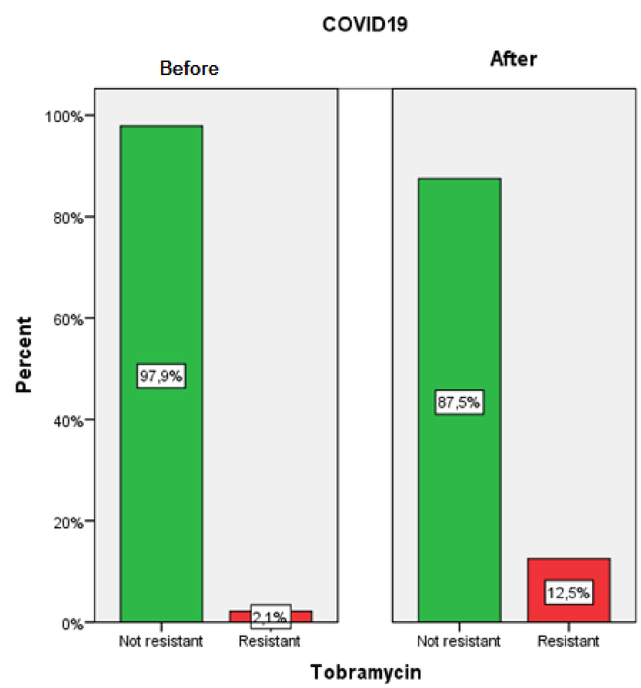

FIGURE 2. Frequency histogram showing the percentage of the Tobramycin *E. coli* resistance before and after COVID-19 emergence

TABLE II
Results of Chi-square analysis on Tobramycin *E. coli* resistance

Variable	X^2	Df	*P*–value	OR	95% CI
Befor/After COVID-19	6.10	1	0.014	6.57	[1.22 – 35.45]

Df : Degrees of freedom. CI: Confidence Interval

Principal component analysis (PCA)

PCA was carried out to show relationship between Tobramycin resistance in *E. coli* strains and the appearance of COVID-19.

The distribution of qualitative factors according to the dimensions of the first (80.02% of the total variance) and second (19.98% of the total variance) was shown in FIG. 3.

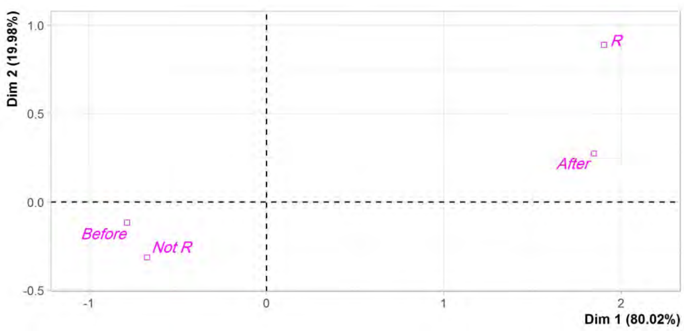

FIGURE 3. Principal component analysis (PCA) of Tobramycin resistance in *E. coli* strains obtained before and after COVID-19

PM purchased after the appearance of COVID-19 was associated with Tobramycin *E. coli* resistant strains isolated from PM (FIG. 3).

PCA of qualitative variables reveals that the first two principal components (Dim1 and Dim2) explained 80.02 and 19.98% of the total variance. The PCA results show the relationship between Tobramycin *E. coli* resistance and the period of COVID-19. Before = PM purchased before the appearance of COVID-19, After = PM purchased after the appearance of COVID-19,Not R = not resistant to Tobramycin, R = resistant to Tobramycin.

Binary logistic regression

A binary logistic regression was used to estimate and to predict the probability of Tobramycin resistance in *E. coli*, associated to the overuse of biocides after COVID-19 emergence. From the Omnibus Tests of Model Coefficients, Chi-square $X^2 = 5.45$ (*P-value*=0.02), it means the logistic model was significant. Nagelkerke $R^2 = 0.12$, suggests that the model explains roughly 12% of the variation in the outcome. Sensitivity and specificity analyses (ROC curves) were performed in order to assess the ability of overuse biocides (during COVID-19) to predict Tobramycin resistant in *E. coli* isolated in PM. The area under the ROC curve (AUC) was = 71.9% (95% CI: 0.52-0.92, *P-value*=0.05), indicating that the model discriminates well (FIG. 4). The estimates of regression coefficients of the predictors *B*, Wald statistic and P-values were presented in TABLE III.

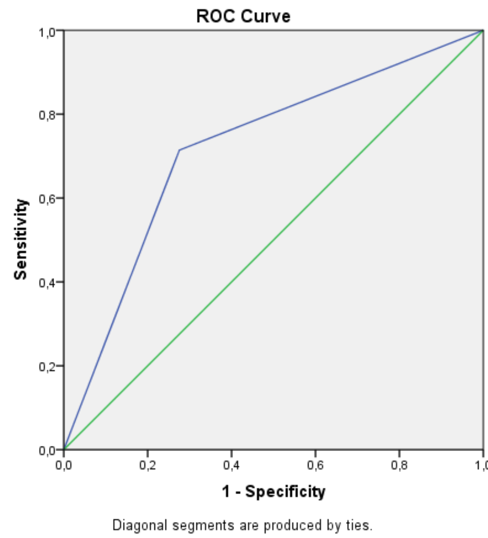

FIGURE 4. Receiver operating characteristic (ROC) curves with respective area under the curve (AUC) binary logistic validation

Results of logistic regression indicated that after COVID-19 emergence (OR 6.57, 95%CI: 1.22-35.45; *P*-value=0.029) was found to be independent risk factor associated with Tobramycin resistant in *E. coli* isolated in PM (TABLE III).

TABLE III
Binary logistic regression

Equation variables	Regression coefficient B	Wald	Significance	Exp (B)	95%CI for Exp (B)	
					Lower limit	Upper limit
Before/After COVID-19	1.88	4.79	0.029	6.57	1.22	35.45
Constant	-3.82	28.69	0.001	0.022		

E. coli strains isolated from PM purchased after COVID-19 outbreak were 5.57 times more likely to be Tobramycin resistant than *E. coli* strains isolated from PM purchased before COVID-19.

The probability of resistance to Tobramycin in *E. coli* linked with PM bought after COVID-19 was 1.88 times more than before COVID-19 emergence: *Logit P = (-3.82) + 1.88 (After COVID-19)*

Tobramycin *E. coli* resistance before COVID-19 appearance

Resistance rates to Tobramycin of *E. coli* strains isolated from PM, before COVID-19 appearance, found in the current study was 2.13% (FIG. 2). The results agree with those of Kocúreková *et al.* [16] in Slovakia who found Tobramycin *E. coli* resistance from broilers in 1.96%. The most common mechanism of acquired resistance to aminoglycosides is the modification of the chemical structure of the antibiotic by bacterial enzymes. This mechanism of resistance is thought to be more frequent in the presence of Tobramycin and Gentamicin than for other aminoglycosides [7]. Furthermore, Tobramycin and Gentamicin are not allowed for aviculture use in Algeria [19]. As a consequence, the risk of antibiotic resistance linked to its use in poultry farming is thus reduced and the confusion bias is reduced.

Tobramycin *E. coli* resistance after COVID-19 appearance

After COVID-19 emergence, Tobramycin *E. coli* resistance in PM, found in the current study, increased to 12.5% (FIG. 2). Even so,Tobramycin is always not allowed for aviculture use.Similar result of Tobramycin *E. coli* resistance in raw PM samples collected from retail PM market of Bhubaneswar, India was 11.16% [28].

Antibiotic resistance persists in spite of the restricted use of several key antibiotics, which indicates that there are components governing the evolution, dissemination, and perpetuation of these resistance systems, many of which are independent of antibiotic usage [4, 31].

TABLE I and FIG. 1, indicate that *E. coli* isolated from PM purchased after the emergence of COVID-19 were more resistant to Tobramycin than those isolated before the appearance of COVID-19 (*P-value*=0.023, TABLE I). In that study, PM purchased after COVID-19 was statistically related to Tobramycin resistance in *E. coli* (*P*=0.014, OR= 6.57 (95% CI 1.22-35) in TABLE II. Graphical representation of PCA qualitative variables shows the interfactor relationship (FIG. 3). The results of this study show that PM purchased after COVID-19 (OR=6.57, 95% CI 1.22-35) was an independent predictor of Tobramycin resistance at logistic regression analysis (TABLE III). And the probability of resistance to Tobramycin in *E. coli* linked with PM bought after COVID-19 was 1.88 times more than before COVID-19 emergence.

The explanation for this increase in Tobramycin *E. coli* resistance could be attributed to the excessive use of biocides in Algeria, during the COVID-19 outbreak, according to WHO recommendations. The Algerian government has issued several decrees laying down additional measures to prevent the spread of Coronavirus. These measures include making disinfectant products available to users and customers, in particular hydro-alcoholic gels, and daily cleaning and disinfection of business premises [23]. Hydroalcoholic solution was available for employees in 85% and for customers only in 4%. In more than 70% of cases, disinfection of surfaces, floors and door handles took place frequently according to survey of 115 service sector companies carried out in the prefecture of Setif City (Algeria) in order to evaluate the preventive measures taken by the service sector companies against the spread of the virus [13].

The Algeria Press Service stated, on March 21, 2020, that several companies in the public and private sectors of the sanitation, disinfection and personal hygiene products sector have doubled their production capacity with the spread of the Corona virus (COVID-19) in Algeria. The production capacity of the public sector companies specialized in the production of disinfectants and personal hygiene products is 1,000 units·days^{-1} (U·d^{-1}) for disinfectant gel and liquid soap, 4,000 liters (L)·d^{-1} for surface cleaners as well as 4,500 U of bleach. This should increase its production capacity to 3,000 U·d^{-1} of disinfectant gel and liquid soap, 20,000 L of floor cleaner as well as 10,000 bottles of bleach [2].

Constant selective pressures exerted on microbiota not only can increase their tolerance to biocidal agents but their resistance to certain antibiotics. Such risks are likely to be exacerbated by the non-diverse portfolio of active ingredients used in current 535 disinfectant products approved for COVID-19 [5]. Recent studies suggest that exposure to sub-inhibitory biocide concentrations facilitates the evolution of resistance to the biocide and may also lead to co-resistance and cross-resistance to other antimicrobial agents such as antibiotics [11]. Repeated exposure of bacteria to certain microbicides (biocides) *in vitro* can result in decreases in antimicrobial susceptibility [10]. In accordance with the present results, Westgate *et al.* [34] observed unstable clinical resistance to Tobramycin (10 µg) in *E. coli*, after exposure to the cationic biocide and oxidizing agent. Westgate *et al.* [35] demonstrated that exposure of *E. coli* to Triclosan (0.00002%) altered the antibiotic susceptibility profile to Tobramycin sufficiently to change the clinical interpretation from susceptible to intermediate. Biocidal agents used for disinfection in healthcare, Veterinary Medicine, food production, food handling or in the domestic setting may also have a risk of enhancing antibiotic resistance, especially during low level exposure [15].

CONCLUSIONS

Many *in vitro* experimental studies have often resulted in reduced susceptibility to biocides and increased minimal inhibitory concentrations for antimicrobial agents in a phenomenon known as cross-resistance. This observational epidemiological study showed the association between the overuse of biocides during COVID-19 emergence and the increase in Tobramycin resistance in *E. coli* isolated in PM. The resistance to Tobramycin level of *E. coli* in PM purchased before the emergence of COVID-19, found in this study was 2.1%. This rate increased after COVID-19 emergence to 12.5%. PM purchased after COVID-19 found related to Tobramycin resistance in *E. coli*.It seems possible that the excessive use of biocides during COVID-19 increases the risk of Tobramycin *E. coli* resistance in PM.

Conflict of interest

The authors declare that they have no conflicts of interest in the research.

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