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Detection of Antimicrobial Phenotypes, β-Lactamase Encoding Genes and Class I Integrons in *Salmonella* Serovars Isolated from Broilers

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Abstract: This study was conducted to determine the occurrence, antimicrobial resistance profile, β-lactamase encoding genes and class I integrons (*intI*) of *Salmonella* serovars in broiler flocks. A total of 100 diseased chickens (5 samples per bird; cloacal swab, liver, gall bladder, spleen and intestinal content) were randomly selected from different broiler farms at Dakahliya and Kafrelsheikh Governorates, Egypt, during the period from September through December 2013. Conventional isolation and serotyping, antimicrobial resistance phenotyping, PCR identification of β-lactamase encoding genes and *intI* were performed. The culturing and serotyping identified 23 (23%) *Salmonella* isolates from diseased birds that belonged to 13 serotypes. The predominant serovars distinguished in this study were *Salmonella Enteritidis*, *S. Typhimurium*, *S. Kentucky* and *S. Infantis* that constituted 52.2% (12/23) of all isolates. By antimicrobial resistance testing, 87% (20/23) of isolates exhibited multidrug resistance (MDR; resistance to 5 or more antibiotics) mostly against vancomycin, oxacillin, amoxicillin, erythromycin and nalidixic acid. For 3rd generation cephalosporins, all the isolates were sensitive to cefoxitin and only 5 (21.7%) isolates displayed resistance to ceftriaxone and cefotaxime. Using PCR, all isolates were negative for bla_{SHV}, bla_{CTX}, bla_{CMY} and bla_{OXA}, while only 5 isolates (21.7%) harbored bla_{TEM} (1080 bp). Variable amplicons with *intI* cassettes were detected by PCR from only 4 isolates (17.4%). Our findings highlighted the zoonotic potential of *Salmonella* in broilers with a possibility of antimicrobial resistance gene transmission to humans. Continuous surveillance is required to minimize the risk of human exposure to antimicrobial resistance pathogens from food producing animals.

Key words: *Salmonella*, broilers, antimicrobial resistance, β-lactamases, integrons, zoonoses

INTRODUCTION

Salmonellosis is considered one of the most important bacterial infections in poultry farms leading to high mortalities in chicken and consequently great economic losses to the poultry industry worldwide (Rostagno *et al*., 2006). Lack of biosecurity measures and the presence of various risk factors related to housing system and management has led to increasing prevalence of salmonellosis in broiler farms particularly if intensively housed (Mollenhorst *et al*., 2005; Trampel *et al*., 2014). Poultry and its products are potential sources for human infections with non-typhoidal salmonellosis (Capita *et al*., 2003) through the consumption of improperly cooked chicken meat that has been previously contaminated at any stage during slaughter, evisceration and handling of chicken carcasses (Sallam *et al*., 2014). Many *Salmonella* serovars have been implicated in human foodborne illness in both developing and developed countries (Cardinale *et al*., 2005) including *Salmonella Enteritidis*, *S. Typhimurium*, *S. Heidelberg* and *S. Newport* (Hur *et al*., 2012).

The inappropriate use of antimicrobials in treating bacterial infections has led to the substantial increase of drug resistance among foodborne pathogens including *Salmonella* (Bronzwaer *et al*., 2002). The possibility of dissemination of multidrug resistance (MDR) *Salmonella* to humans from food producing animals may occur either directly through consumption of food with antimicrobial resistant pathogens or indirectly through contact with different components of the ecosystem as water and soil (Landers *et al*., 2012). The emergence of MDR *Salmonella* especially those exhibiting resistance to cephalosporins due to the production of beta-lactamases, has attracted attention worldwide (Madhulika *et al*., 2004). Antimicrobial drug resistance in different *Salmonella* serovars has been linked to the presence of specific resistance genes (Alcaine *et al*., 2007) harbored within integrons. These mobile genetic elements facilitate the transcription and expression of these genes with a subsequent MDR distribution (Rowe-Magnus *et al*., 2002).

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Thus, the overall aim of this study was to determine the occurrence of *Salmonella* serovars in broiler flocks and associated antimicrobial resistance profiles; characterization of β-lactamase encoding genes and class I integrons (*intI*) was also performed for those isolates.

MATERIALS AND METHODS

Sample collection: A total of 100 diseased chickens (5 samples per bird; cloacal swab, liver, gall bladder, spleen and intestinal content) suffering from diarrhea were randomly collected from different broiler farms (farm size ranged from 5000-10,000 chicks) at Dakahliya and Kafrelsheikh Governorates, Egypt, during the period from September through December 2013. The cloacal swab in 2 ml sterile buffered peptone water vials and the visceral organs from each bird were packaged individually in a polyethylene bag and transferred aseptically within 2 h in an ice tank to the laboratory of Microbiology, Immunology and Mycology Department, Faculty of Veterinary Medicine, Kafrelsheikh University for the conventional bacteriological analysis under sterile conditions.

Conventional isolation and identification of *Salmonella*: One ml of each cloacal swab and 2 g of each chicken visceral organ were inoculated into 9 ml of sterile buffered peptone water (BPW; Becton Dickinson, Sparks, MD, USA), homogenized and incubated for 24 h at 37°C. Approximately 1 ml of the overnight enriched BPW was aseptically transferred to 9 ml each of Rappaport Vassilliadis (RV) broth (Becton Dickinson, Sparks, MD, USA) and 9 ml of nutrient broth (Becton Dickinson, Sparks, MD, USA) and incubated overnight at 42°C and 37°C, respectively. The culturing on Xylose lysine desoxycholate (XLD; Becton Dickinson, Sparks, MD, USA) agar and MacConkey agar (Becton Dickinson, Sparks, MD, USA) was done from the enriched RV broth and nutrient broth which were then incubated at 37°C for 24 h.

Three to five typical colonies with the morphological pattern of *Salmonella* on XLD (pink to red colonies with or without dark center) and MacConkey (pale colonies) were picked, streaked onto nutrient agar slopes and incubated overnight at 37°C for the subsequent biochemical identification.

Serotyping: The serological identification of the biochemically identified *Salmonella* isolates was done according to Kauffmann-White scheme (Kauffmann, 1974) at the Animal Health Research Institute (AHRI), Dokki, Giza by the slide agglutination technique with the polyvalent somatic (O) and flagellar (H) antisera (Welcome Diagnostic, UK).

Antimicrobial susceptibility testing: *Salmonella* isolates were phenotypically tested against 15 antimicrobials for the determination of antimicrobial susceptibility using disk diffusion assay according to the instructions described by the Clinical and Laboratory Standards Institute (CLSI, 2011). The tested antibiotics were purchased from Oxoid, UK and included ciprofloxacin (CIP; 5 µg), amoxicillin (AM; 30 µg), cefotaxime (CTX; 30 µg), cefoxitin (FOX; 30 µg), ceftriaxone (CRO; 30 µg), erythromycin (E; 15 µg), chloramphenicol (C; 30 µg), streptomycin (STR; 10 µg), nalidixic acid (NAL; 30 µg), trimethoprim/ sulfamethoxazole (SXT; 25 µg), tetracycline (TE; 30 µg), enrofloxacin (ENR; 5 µg), vancomycin (VA; 30 µg), oxacillin (OX; 1 µg) and kanamycin (KAN; 30 µg). The reference strain, *Escherichia coli* ATCC 25922, was kindly provided by the Central Diagnostic and Research Laboratory, Faculty of Veterinary Medicine, Kafrelsheikh University and used as a quality control.

DNA extraction: Three to five representative colonies of the same morphological type were taken from the slants of the previously isolated bacteria and enriched into a tube containing 2 ml of tryptic soya broth (TSB) for 18 h at 37°C. One ml of the enriched bacterial culture was centrifuged at 8000 xg for 2 min and then the sediment was homogenized with nuclease free water and heated at 95°C for 15 min. The boiled lysates were centrifuged and the supernatant was used as DNA template. All DNA samples were transferred to the Central Diagnostic and Research Laboratory, Faculty of Veterinary Medicine, Kafrelsheikh University for the identification of ß-lactamase encoding genes.

Molecular identification of ß-lactamase encoding genes and *intI*: The primer pairs for β-lactamase encoding genes and *intI* used (sequence, target gene, PCR products and PCR conditions) are summarized in Table 1. Uniplex PCR reactions were done in a volume of 20 µl consisting of 10 µl of 2X PCR Master Mix (Promega, Madison, WI), 2.5 µl DNA template and 0.2 µl of each primer (100 µM each). Positive controls were kindly provided by Central Diagnostic and Research Laboratory, Faculty of Veterinary Medicine, Kafrelsheikh University.

RESULTS AND DISCUSSION

Of the 100 diseased chickens that were tested conventionally in this study for the presence of *Salmonella* spp., 23% were biochemically identified as *Salmonella*. The overall occurrence of *Salmonella* in this study was higher than that previously recorded by Shahada *et al.* (2008) (14%), Le Bouquin *et al.* (2010) (8.6%) and Samanta *et al.* (2014) (6.1%). However, a

Table 1: PCR conditions and primers used for molecular identification of β-lactamases genes and class I integrons

Target gene	Primer sequence	PCR product	Reference	PCR cyclic conditions
bla_{TEM}. F	5'ATAAAATTCTTGAAGACGAAA-3'			
R	5'-GACAGTTACCAATGCTTAATC-3'	1080	Ahmed *et al*. (2006)	Weill *et al*. (2004)
bla_{SHV}. F	5'-TTATCTCCCTGTTAGCCACC-3'			
R	5'-GATTTGCTGATTTCGCTCGG-3'	795	Ahmed *et al*. (2006)	
bla_{OXA}. F	5'-TCAACTTTCAAGATCGCA-3'			
R	5'-GTGTGTTTAGAATGGTGA-3'	591	Ahmed *et al*. (2006)	Siu *et al*. (2000)
bla_{CTX-M}.F	5'CGCTTTGCGATGTGCAG-3'			
R	5'-ACCGCGATATCGTTGGT-3'	550	Ahmed *et al*. (2006)	Ahmed *et al*. (2007)
bla_{CMY}. F	5'-GACAGCCTCTTTCTCCACA3'			
R	5'-TGGAACGAAGGCTACGTA-3'	1007	Zhao *et al*. (2003)	
**IntI*. F	5'-GGC ATC CAA GCA GCA AG-3'			
R	5'-AAG CAG ACT TGA CCT GA-3'	Variable	Sow *et al*. (2007)	Sow *et al*. (2007)

**IntI*, Class I integron cassettes

higher prevalence of *Salmonella* from chickens was reported by Yildirim *et al*. (2011) (34%) and Srinivasan *et al*. (2014) (46%). There are many predisposing factors that have been associated with the higher occurrence of *Salmonella* in poultry farms such as increasing flock size (Namata *et al*., 2008), bad hygienic standards and improper designing of poultry farms (Asakura *et al*., 2001) and vaccination programs (Volkova *et al*., 2011).

Using serotyping, 13 serotypes were detected among the 23 *Salmonella* isolates. The predominant serovars were *S. Enteritidis* (4/23, 17.4%), *S. Typhimurium* (3/23, 13%), *S. Kentucky* (3/23, 13%) and *S. Infantis* (2/23, 8.7%) that constituted 52.2% (12/23) of the isolated Salmonella serovars (Table 2). This reflects the role of chicken as a potential source of zoonotic non-typhoidal salmonellosis in humans as *S. Enteritidis* and *S. Typhimurium* were mostly associated with human illness (Gantois *et al*., 2009; Hendriksen *et al*., 2011).

Phenotypically, *Salmonella* serovars in this study showed variable resistance to all 15 antimicrobials tested (Table 3) except cefoxitin. The highest level of antimicrobial resistance determined for the isolated serovars was vancomycin (100%; 23/23), oxacillin (91.3%; 21/23), amoxicillin (78.3%; 18/23), erythromycin (78.3%; 18/23) and nalidixic acid (78.3%; 18/23). Of the three cephalosporins tested, all the isolated *Salmonella* were sensitive to cefoxitin and 21% (5/23) of *Salmonella* isolates exhibited resistance to ceftriaxone and cefotaxime. Approximately 25% (6/23) of the isolates were resistant to kanamycin, enrofloxacin and chloramphenicol, while, less than 8.7% (2/23) exhibited resistance to ciprofloxacin.

The majority of *Salmonella* serovars (87%; 20/23) showed MDR to ≥5 of the 15 antimicrobials tested. These findings are in accordance with the previous literature from Egypt reported by Ahmed and Shimamoto (2012) (81%) and Abd-Elghany *et al*. (2015) (92.8%). Also, our findings are moderately similar to that previously recorded in many studies from different countries worldwide such as Spain (100%) (Carraminana *et al*., 2004), Brazil (90.5%) (de Oliveira *et al*., 2005), Morocco (75.4%) (Abdellah *et al*., 2009), Nepal (100%) (Shrestha *et al*., 2010), Turkey (100%) (Yildirim *et al*., 2011), Korea (87.2%) (Kim *et al*., 2012) and Romania (83.2%) (Mihaiu *et al*., 2014).

From Table 4, the antimicrobial resistance profiles (E, NAL, OX, VA, AM) and (E, NAL, OX, VA, AM, STR) were found in 52.2% (12/23) and 34.8% (8/23) of the *Salmonella* isolates, respectively. The highest resistance to penicillins (amoxicillin and oxacillin), vancomycin, erythromycin and nalidixic acid was not surprising as these antibiotics are substantially universal and are widely used in both veterinary and human medicine (Singer and Hofacre, 2006; Ahmed and Shimamoto, 2012; Abd-Elghany *et al*., 2015). Furthermore, the usage of several antibiotics of unlimited access in prophylaxis, treatment of infections and as growth promoters in chicken farms has led to overstating antimicrobial resistance (Yildirim *et al*., 2011).

Genetically, all the 23 *Salmonella* isolates were examined for the presence of β-lactamase encoding genes by uniplex PCR for the direct detection of bla_{TEM}, bla_{SHV}, bla_{CTX}, bla_{OXA} and bla_{CMY}. It was found that all *Salmonella* isolates showed no specific amplicons with any of bla_{SHV}, bla_{CTX}, bla_{CMY} and bla_{OXA} genes. However, the bla_{TEM} amplicon (1080 bp) was obtained from 5 (21.7%) *Salmonella* isolates (Fig. 1). The PCR findings of β-lactamase encoding genes were similar to that determined previously by Ahmed *et al*. (2009) who detected only bla_{TEM} in *Salmonella* isolates.

From both phenotypic antimicrobial and PCR identification results of β-lactamase encoding genes, it was clear that all of the MDR *Salmonella* that exhibited susceptibility to 3rd generation cephalosporins (e.g., cefoxitin) did not harbor bla_{SHV}, bla_{OXA}, bla_{CMY} or bla_{CTX} which are frequently present in extended spectrum ß-lactamase (ESBL)-producing bacteria. However, bla_{TEM} which hydrolyzes only penicillins and early but not later generations of cephalosporins has been molecularly

Table 2: Occurrence of *Salmonella* serotypes (n = 23) among diseased chicken birds

Salmonella serotypes	No. of serotypes	Percentage
Salmonella Kentucky	3	13.04
Salmonella Typhimurium	3	13.04
Salmonella Enteritidis	4	17.4
Salmonella Infantis	2	8.7
Salmonella Paratyphi A	2	8.7
Salmonella Ferruch	2	8.7
Salmonella Magherafelt	1	4.3
Salmonella Verginia	1	4.3
Salmonella Gaillac	1	4.3
Salmonella Atakpame	1	4.3
Salmonella Cremieu	1	4.3
Salmonella Bardo	1	4.3
Salmonella Vejle	1	4.3
Total	23	100

Table 3: Percentages of antimicrobial resistance in *Salmonella* isolates from diseased chicken birds

	---------- *Salmonella* isolates ----------		
Antimicrobial agent tested	Resistant No. (%)	Intermediate No. (%)	Sensitive No. (%)
Nalidixic acid (NAL)	18 (78.3)	1 (4.3)	4 (17.4)
Streptomycin (STR)	11 (47.8)	9 (39.1)	3 (13.1)
Kanamycin (KAN)	6 (26.1)	9 (39.1)	8 (34.8)
Ceftriaxone (CRO)	5 (21.7)	7 (30.5)	11 (47.8)
Cefotaxime (CTX)	5 (21.7)	4 (17.4)	14 (60.9)
Cefoxitin (FOX)	0 (0)	3 (13)	20 (87)
Oxacillin (OX)	21 (91.3)	2 (8.7)	0 (0)
Amoxicillin (AM)	18 (78.3)	2 (8.7)	3 (13)
Chloramphenicol (C)	6 (26.1)	5 (21.7)	12 (52.2)
Erythromycin (E)	18 (78.3)	4 (17.4)	1 (4.3)
Vancomycin (VA)	23 (100)	0 (0)	0 (0)
Tetracycline (TE)	7 (30.5)	11 (47.8)	5 (21.7)
Ciprofloxacin (CIP)	2 (8.7)	6 (26.1)	15 (65.2)
Enrofloxacin (ENR)	6 (26.1)	3 (13)	14 (60.9)
Trimethoprim/sulfamethoxazole (SXT)	11 (47.8)	9 (39.1)	3 (13)

Table 4: Antimicrobial resistance profile and resistance genes pattern in the isolated *Salmonella* serovars

Isolate No.	Isolate serovars	Antimicrobial resistance profile (Phenotypes)	Resistance genes pattern
1	*S. Enteritidis*	E, NAL, OX, VA	ND*
2	*S. Enteritidis*	E, NAL, OX, VA, AM	ND
3	*S. Enteritidis*	E, OX, VA, AM, TE, CTX, CRO	blaTEM
4	*S. Enteritidis*	C, ENR, NAL, OX, VA, AM, SXT	ND
5	*S. Typhimurium*	E, NAL, OX, VA, AM, CTX, CRO	ND
6	*S. Typhimurium*	NAL, OX, VA, CTX, CRO	ND
7	*S. Typhimurium*	E, OX, VA, AM	ND
8	*S. Kentucky*	E, NAL, OX, VA, AM, STR, TE	ND
9	*S. Kentucky*	E, NAL, OX, VA, AM, STR,KAN, C, ENR,	blaTEM
10	*S. Kentucky*	E, NAL, OX, VA, AM, KAN, C, ENR, CIP, SXT	*IntI*
11	*S. Infantis*	C, ENR, NAL, OX, STR, VA, AM, SXT, TE	ND
12	*S. Infantis*	E, NAL, OX, VA	ND
13	*S. Paratyphi* A	VA, AM, SXT, CTX, CRO	ND
14	*S. Paratyphi* A	E, VA, AM, CTX, CRO	ND
15	*S. Ferruch*	E, NAL, OX, VA, AM, STR, SXT	ND
16	*S. Ferruch*	E, NAL, OX, VA, AM, STR, TE, SXT	ND
17	*S. Magherafelt*	E, NAL, OX, VA, AM, STR, SXT, ENR	ND
18	*S. Verginia*	C, NAL, OX, STR, KAN, VA, SXT	ND
19	*S. Gaillac*	E, NAL, OX, VA, AM, KAN, TE,	blaTEM
20	*S. Atakpame*	E, NAL, OX, VA, AM, STR, KAN, SXT	ND
21	*S. Cremieu*	E, NAL, OX, VA, AM, STR, CIP, TE, ENR, SXT	*IntI* and blaTEM
22	*S. Bardo*	E, NAL, OX, VA, AM, STR, KAN, C, SXT	*IntI* and blaTEM
23	*S. Vejle*	E, OX, STR, VA, TE	*IntI*

ND*, β-lactamases genes and class I integrons not detected. E, erythromycin; NAL: nalidixic acid, OX: oxacillin, VA: vancomycin, AM: amoxicillin, KAN: kanamycin, TE: tetracycline, STR: streptomycin, CIP: ciprofloxacin, CTX: cefotaxime, FOX: cefoxitin, CRO: ceftriaxone, C: chloramphenicol, SXT: trimethoprim/sulfamethoxazole, ENR: enrofloxacin

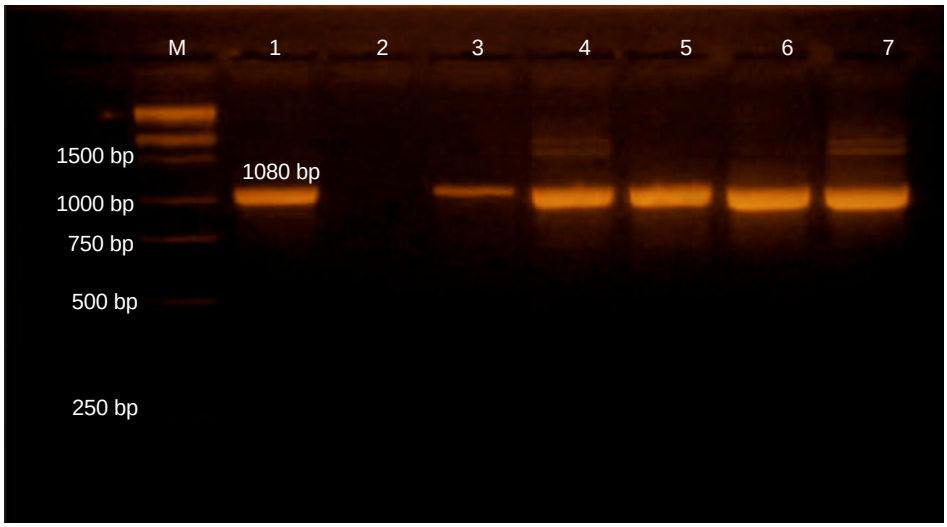

Fig. 1: PCR identification of bla_{TEM} from different *Salmonella* serovars. Lane M: 1000 bp DNA ladder. Lane 1: (Positive Control). Lane 2: (Negative Control). Lane 3, 4, 5, 6 and 7: Positive samples

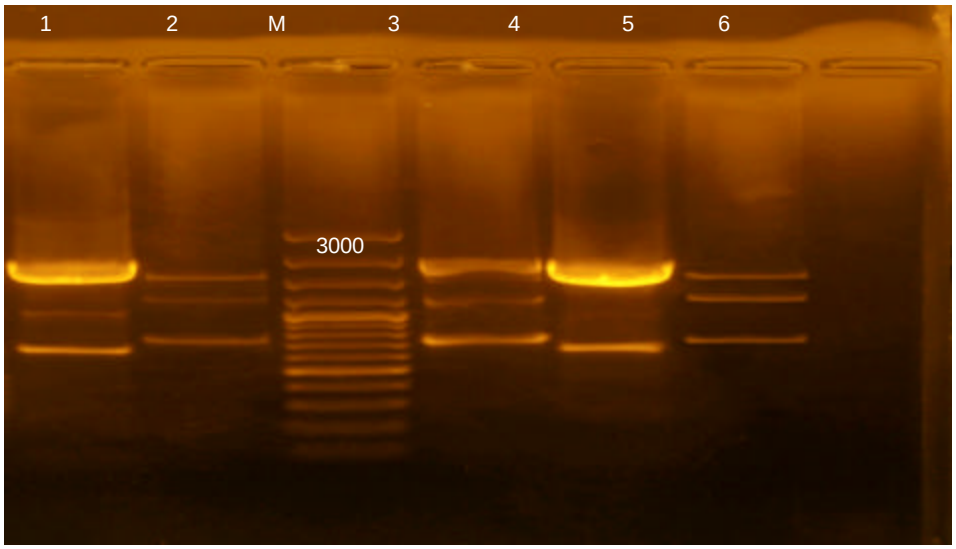

Fig. 2: PCR identification of *intI* from different *Salmonella* serovars. Lane M: 100 bp DNA ladder. Lane 1, 2, 3 and 4: Positive samples. Lane 5: (Positive Control). Lane 6: (Negative Control)

identified in 21.7% of the examined *Salmonella* isolates (Paterson, 2006; Elumalai *et al*., 2014).

From Fig. 2, variable amplicons that determined *intI* were detected by PCR from only 4 isolates (17.4%). The class I integrons has been frequently detected in *Salmonella* isolates from poultry which is responsible for the spreading of MDR (White *et al*., 2001; Mazel, 2006; Lu *et al*., 2014). The mechanism of multidrug resistance in integron-carrying bacteria comes from decreasing susceptibility not only to the antimicrobials where their respective genes were included in the integron but also to other antimicrobials even if their resistance genes were not found in the integron cassette (Malek *et al*., 2015).

Conclusion: In conclusion, this study demonstrated that *Salmonella* is still a major problem in broiler farms in Egypt with a public health concern chiefly with the predominance of *S. Enteritidis*, *S. Typhimurium* and *S. Infantis*. The identification of MDR *Salmonella* from broilers both phenotypically and genetically is another burden that necessitates the continuous surveillance

and implementation of control regimes to reduce the inappropriate use of antimicrobials which in turn lowers the dissemination of resistance genes to human.

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