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Antibiogram, virulotyping and genetic diversity of *Escherichia coli* and *Salmonella* serovars isolated from diarrheic calves and calf handlers

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ABSTRACT

Antimicrobial resistance profile of *E. coli* and *Salmonella* serovars isolated from diarrheic calves and handlers in Egypt is unknown due to the absence of monitoring. Therefore, this study aimed to determine the virulence, genetic and antimicrobial resistance profiles of *E. coli* and *Salmonella* serovars associated with diarrhea in calves and handlers in intensive dairy farms in Egypt. A total of 36 bacterial strains (20 *E. coli* and 16 *Salmonella*) were isolated from fecal samples of 80 diarrheic Holstein dairy calves (10 *E. coli* and 13 *Salmonella*) and hand swabs of 35 handlers (10 *E. coli* and 3 *Salmonella*) in two intensive dairy farms in Sharkia Governate in Egypt. *E. coli* strains belonged to six different serogroups and O114:K90 was the most prevalent serogroup (30%). However, *Salmonella* strains were serotyped into four different serogroups and *S. Kiel* was the most prevalent serotype (50%). Thirteen (65%) *E. coli* isolates were harbouring either *stx2*, *eaeA* and/or *astA* virulence-associated genes. However, *stn* and *spvC* virulence genes were detected in 2 (12.5%) and 4 (25%) of *Salmonella* isolates, respectively. *E. coli* isolates showed marked resistance to ampicillin (75%), while *Salmonella* strains exhibited high resistance to amikacin (100%), gentamicin (93.75%) and tobramycin (87.5%). Results of the present study showed that *E. coli* and *Salmonella* serovars isolated from diarrheic calves and handlers in intensive dairy farms in Egypt exhibited resistance to multiple classes of antimicrobials, which may pose a public health hazard. Thus, the continuous monitoring of antimicrobial resistance is necessary for both humans and veterinary medicine to decrease the economic losses caused by antimicrobial-resistant strains in animals as well as the zoonotic risk.

1. Introduction

Calf diarrhea associated with infectious pathogens is a major problem for dairy producers worldwide [1,2]. *Escherichia coli* (*E. coli*) and *Salmonella* are among the most important bacterial causes of diarrhea-associated mortality in calves [3], which cause severe economic loss if the appropriate antimicrobial and supportive therapies are not administrated early in the disease [3,4]. Generic *E. coli* are abundant commensal enteric bacteria in animals and humans and are ubiquitous in the environment [5]. Diarrheagenic *E. coli* were classified on the basis of their virulence properties into six pathotypes including Enteropathogenic *E. coli* (EPEC), Enterohaemorrhagic *E. coli* (EHEC), Shiga toxin-producing *E. coli* (STEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC) and Enteroaggregative *E. coli* (EAEC) [6]. Different pathotypes (ETEC, EHEC, STEC and EPEC) were associated with diarrhea in calves [4,7,8]. *Salmonella* is also one of the major pathogens associated with enteric diseases in animals [9,10]. *Salmonella* were divided into two main species *S. bongoni* and *S. enterica* which is further divided into several subspecies using somatic O and flagellar H antigens [11]. Salmonellosis in calves has been associated with non-typhoidal serotypes and manifested clinically by diarrhea, pneumonia, septic arthritis, meningitis, gangrenous necrosis of the distal extremities, fever, anorexia, dehydration, endotoxemia and death [12].

Virulence genes which play a significant role in *E. coli* (e.g. *stx1*, *stx2*, *eaeA*, and *astA* genes) and *Salmonella* (e.g. *stn* and *spv* genes) pathogenicity, associated with diarrhea in animals and humans have been described [13,14]. For tracking sources of *E. coli* and *Salmonella* contamination, molecular typing based on PCR techniques including enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) has been developed [15]. ERIC-PCR is a simple, highly reliable, cost-effective and powerful tool for studying the genetic relationship of the isolates from different sources [16].

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Antimicrobials are an essential part of the treatment plans of *E. coli* and *Salmonella* infections in animals and humans [17]. Several studies have shown that the use of antimicrobials may reduce mortality to 10% compared with 75% in untreated animals [18]. The choice of antimicrobials for the treatment of *E. coli* and *Salmonella* would ideally be based on sensitivity test [17]. However, this is not performed in Egypt due to the long time it takes to get the results, the price and the availability of the antimicrobials over-the-counter (without a prescription). Furthermore, antimicrobials were widely used as a growth promoter, which leads to the emergence of antimicrobial-resistant bacteria in animals as well as humans through direct contact or food chain [19].

Growing demands for livestock products have been the main driver of intensive production system in developed countries [20]. In Egypt, the intensive system includes farms of various sizes and types, ranging from ten to thousands of heads of cattle and represent over 7% of total bovine population in the country [21]. Several studies have investigated the virulence and genetic profiles of *E. coli* and *Salmonella* isolated from calves under extensive production system in Egypt [22–24]. However, the information from calves under intensive production system in Egypt is scarce. In addition, the routine monitoring of antimicrobial resistance is absent and the prevalence and antimicrobial resistance profile of *E. coli* and *Salmonella* in food animals, humans and environment are unknown. Therefore, the objective of this study was to determine the virulence, genetic and antimicrobial resistance profiles of *E. coli* and *Salmonella* strains associated with diarrhea in calves and handlers in intensive dairy farms in Egypt.

2. Materials and methods

2.1. Bacterial isolates

A total of 36 bacterial strains including 20 *E. coli* (10 from calves and 10 from handlers) and 16 *Salmonella* (13 from calves and 3 from handlers) were tested in the present study. Calves' strains were previously isolated from fecal samples of 80 Holstein dairy calves (one day-3 months old) raised under intensive production system in two large dairy farms in Sharkia Governate, Egypt. Calves were diagnosed with diarrhea based on clinical examination and haemato-biochemical analysis. Human strains were isolated from hand swabs of 35 calf handlers. Swabs were aseptically collected by rolling sterile swab over the palm of the hand area between fingers, fingertips and nails [25] immediately after calf handling. Swabs inserted into 10 mL sterile buffered peptone water (BPW; Oxoid, UK) then transported directly in an insulated ice box to the laboratory for further analysis. Isolation of *E. coli* and *Salmonella* was carried out according to protocol described by Edwards and Ewing [26]. Briefly, a loopful from each enriched sample in BPW was streaked on Levins eosin methylene blue agar (EMB; Oxoid, UK) for *E. coli* and Xylose- lysine-Desoxycholate (XLD; Oxoid, UK) for *Salmonella*. The colonies showed typical *E. coli* and *Salmonella* morphology were picked up and then sub-cultured onto nutrient agar (Oxoid, UK). Biochemical identification of *E. coli* and *Salmonella* was done using API 20E kits (bioMérieux, USA) following the manufacturer instructions. Serotyping of the strains was performed using agglutination polyvalent and monovalent O, K, and H antisera (Sifin diagnostics GmbH, Berlin, Germany; Denka Seiken Co., Japan). Each serotyped isolate was purified on nutrient agar and maintained in brain heart infusion broth (Oxoid, UK) containing 20% glycerol, then preserved at −80 °C until later analysis.

2.2. Bacterial DNA extraction

Each preserved isolate in glycerol containing broth was refreshed overnight on sterile brain heart infusion broth and cultivated on EMB agar for *E. coli* and XLD for *Salmonella*. A loopful from the colony was inoculated in an Eppendorf tube containing 1.5 mL of brain heart infusion (BHI) broth and incubated at 37 °C for 24 h. The incubated broth was centrifugated at 6,000 rpm for 2 min and the supernatant was discarded. Bacterial DNA was extracted from the pellet using a QIAamp DNA mini kit (QIAGEN GmbH, Hilden, Germany) according to manufacturer's recommendations.

2.3. Virulotyping

For *E. coli* isolates, the extracted DNA was screened by uniplex PCR for identification of *stx2* [27], *eaeA* [28] and *astA* [29] virulence associated genes. The DNA extracted from *Salmonella* isolates were subjected to uniplex PCR for detection of *spvC* [14] and *stn* [30]. The reactions were carried out in a T3 Biometra thermal cycler (Biometra, Göttingen, Germany). The PCR products, 100 bp DNA ladder (Fermentas, Maryland, USA), positive and negative controls were run on 1.5% ethidium bromide-stained agarose gel for 30 min. The gel was photographed using a gel documentation system (Alpha Innotech, Biometra, Göttingen, Germany).

2.4. Genotyping

Genotyping and genetic relationship between *E. coli* or *Salmonella* isolates from diarrheic calves and handlers were determined by ERIC-PCR using a single amplification profile [15]. The oligonucleotide primers ERIC-DG111-F (5`-ATGTAAGCTCCTGGGGATTCAC-3`) and ERIC-DG112-R (5`-AAGTAAGTGACTGGGGTGAGCG-3`) from Metabion (Martinsried, Germany) were used [15]. The ERIC-PCR reaction was performed in two separate trials. Samples from each isolate were loaded on electrophoresis and results were compared. Clear and reproducible bands displayed by each electrophoresis were recorded for the ERIC-PCR analysis. The ERIC-PCR fingerprinting data were transformed into a binary code depending on the presence or absence of each band. Data were analyzed using Jaccard measure to generate similarity coefficient and a dendrogram was constructed using unweighted pair group method with arithmetic average (UPGMA), sequential hierarchical and nested clustering routine. The cluster analysis and dendrogram construction were performed with SPSS ver. 21 (IBM Corp., Chicago, IL, USA). The discrimination index (D) of ERIC-PCR was calculated based on Simpson's index of diversity [31].

2.5. Antimicrobial susceptibility testing

Antimicrobial susceptibility of *E. coli* and *Salmonella* isolates was performed with Vitek-2 system (BioMérieux, Marcy-l'Étoile, France), using AST-GN71 cards following the manufacturer's instructions. Briefly, bacterial isolates obtained from BHI broth were suspended in 3 mL of 0.45% saline solution, vortexed, and adjusted to the turbidity of 0.5 McFarland standard and then inoculated into the AST card. The 17 tested antimicrobial agents (MIC ranges) were ampicillin (2–32 μg/mL), ampicillin-sulbactam (2/1–32/16 μg/mL), cefazolin (4–64 μg/mL), ceftriaxone (1–64 μg/mL), cefepime (1–64 μg/mL), aztreonam (1–64 μg/mL), ertapenem (0.5–8 μg/mL), imipenem (0.25–16 μg/mL), meropenem (0.25–16 μg/mL), amikacin (2–64 μg/mL), tobramycin (1–16 μg/mL), gentamicin (1–16 μg/mL), ciprofloxacin (0.25–4 μg/mL), moxifloxacin (0.25–8 μg/mL), Trimethoprim-sulfamethoxazole (20–320 μg/mL) tigecycline (0. 5–8 μg/mL) and nitrofurantoin (0.5–16 μg/mL). The isolates were categorized as sensitive, intermediate, or resistant to tested antimicrobials according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [32]. The multiple antibiotic resistance (MAR) index was calculated as the ratio of the number of antimicrobials to which the isolate displayed resistance to the number of antimicrobials to which the isolate had been evaluated for susceptibility [33]. However, multidrug resistance was defined as resistance to at least three antimicrobial classes [34].

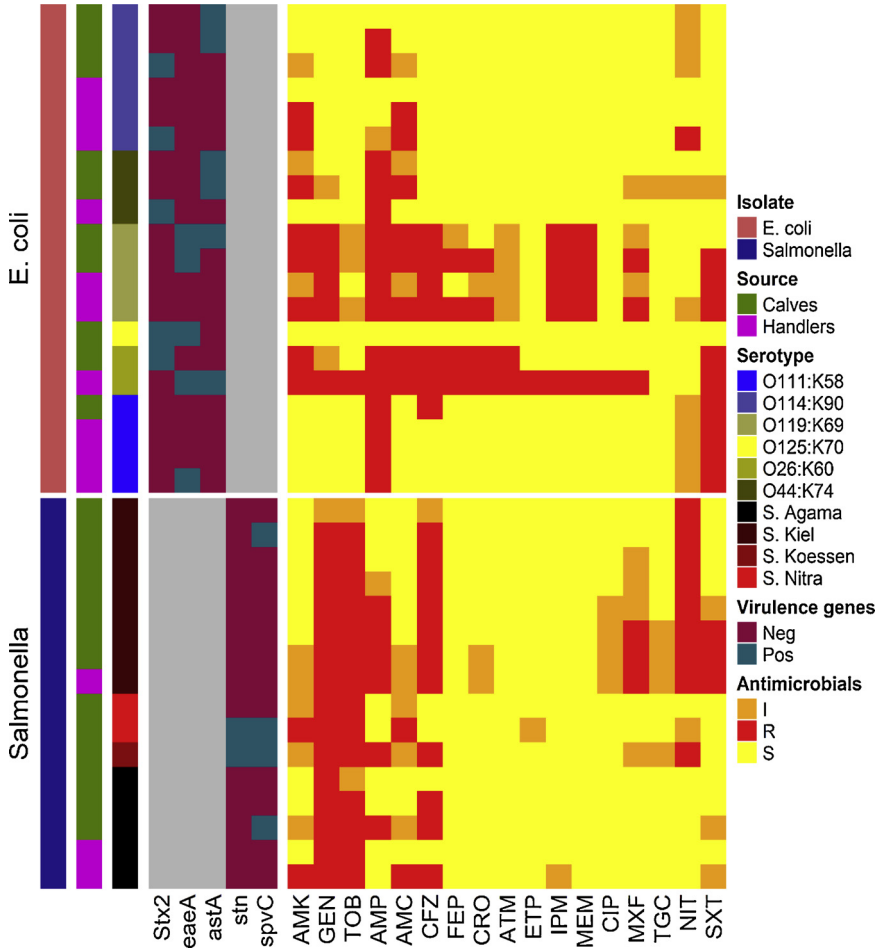

Fig. 1. Heat map representation of the virulence and antimicrobial resistance profiles of the 20 *E. coli* and 16 *Salmonella* isolates recovered from diarrheic calves and handlers.

2.6. Data analysis

Data were introduced into R software (R Core Team, 2019; version 3.5.3) for descriptive and statistical data analysis, with results considered significant at P-value < 0.05. The R package "ComplexHeatmap" [35] was used to build a heatmap based on the virulence genes and antimicrobials tested.

3. Results

3.1. Serogrouping

Serogrouping of *E. coli* and *Salmonella* isolates showed high variability. Six different *E. coli* serogroups and four different *Salmonella* serotypes were detected (Fig. 1). Serogrouping of *E. coli* isolates revealed that O114:K90 was the most prevalent serogroup (30%), followed by O119:K69 (20%) and O111:K58 (20%). Other serogroups of O125:K70, O26:K60 and O44:K74 were also identified (Table 1). However, EPEC was the most prevalent pathovar (65%), followed by EHEC (20%) and EAEC (15%). Serogrouping of *Salmonella* isolates showed that *S. Kiel* was the prevalent serotype (50%), followed by *S. Agama* (31.25%), *S. Nitra* (12.5%) and *S. Koessen* (6.25%) (Table 2).

3.2. Virulotyping

Of the 20 *E. coli* isolates, 13 (65%) showed the presence of *stx2*, *eaeA* and *astA* virulence-associated genes (Table 1). However, the remaining 7 (35%) isolates didn't harbour any researched virulence genes. Ten (50%) of the *E. coli* isolates harboured only one virulence gene and only three (15%) isolates were positive for more than one virulence genes. Virulence-associated genes (*stn* and *spvC*) were detected respectively in 2 (12.5%) and 4 (25%) out of the 16 *Salmonella* isolates. Both genes were identified simultaneously in 2 (12.5%) isolates; however, the two genes were not detected in 12 (75%) isolates (Table 2).

3.3. Genotyping

The genetic similarity of 20 *E. coli* and 16 *Salmonella* isolates from diarrheic calves and handlers were assessed using ERIC-PCR. The banding patterns of *E. coli* and *Salmonella* revealed multiple amplified fragments, ranged in size from 130 to 2113 bp and 149 to 3439 bp, respectively. The *E. coli* and *Salmonella* isolates were classified into nine (E1–E9; Table 1), and seven (E1–E7; Table 2) distinct ERIC profiles, respectively. The discriminatory power of ERIC-PCR (D value) in typing the *E. coli* and *Salmonella* isolates were 0.86 and 0.90, respectively.

The dendrogram analysis of ERIC-PCR fingerprints of *E. coli* isolates showed that 7 (I–VII) main clusters were generated at a linkage distance of 12.5 (Fig. 2A). Using the Jaccard coefficient, the similarity between the *E. coli* isolates from diarrheic calves and their handlers was 100% in cluster III, IV, V and VI. Fig. 2B shows the dendrogram analysis of ERIC-PCR fingerprints of *Salmonella* isolates also generated 7 (I–VII) clusters. Cluster I included *S. Kiel* isolates from diarrheic calves (n = 2), and handlers (n = 1), while Cluster II included *S. Agama* isolates from diarrheic calves and handlers (one each) with 100% similarity.

Table 1
Serotypes, clusters, ERIC, virulence and antimicrobial resistance profiles of 20 *E. coli* isolates recovered from diarrheic calves and handlers.

ID	Serotype (pathotype)	Source	Cluster	ERIC profile	Virulence profile			Resistance patterns	MAR index[a]
					stx2	*eaeA*	*astA*		
18	O114:K90 (EPEC)	Handlers	I	E1	–	–	–	–	0
19	O114:K90 (EPEC)	Handlers	I	E1	–	–	–	SAM	0.06
20	O114:K90 (EPEC)	Handlers	I	E1	+	–	–	SAM, NIT	0.12
17	O125:K70 (EPEC)	Calves	I	E2	+	+	–	–	0
14	O114:K90 (EPEC)	Calves	II	E3	–	–	+	–	0
15	O114:K90 (EPEC)	Calves	II	E3	+	–	–	AMP	0.06
16	O114:K90 (EPEC)	Calves	II	E3	–	–	+	AMP	0.06
8	O44:K74 (EAEC)	Handlers	III	E4	+	–	–	AMP	0
9	O44:K74 (EAEC)	Calves	III	E4	–	–	+	AMP, SAM, AMK	0.18
2	O26:K60 (EPEC)	Handlers	IV	E5	–	+	+	AMP, SAM, TOB, CFZ, FEP, CRO, ATM, ETP, IPM, MEM, GEN, CIP, MXF, SXT	0.82
3	O26:K60 (EPEC)	Calves	IV	E5	+	–	–	AMP, SAM, CFZ, FEP, CRO, ATM, SXT	0.41
1	O119:K69 (EPEC)	Calves	IV	E6	–	+	+	AMP, SAM, CFZ, IPM, MEM, GEN	0.35
10	O111:K58 (EHEC)	Calves	V	E7	–	–	–	AMP, CFZ, SXT	0.18
11	O111:K58 (EHEC)	Handlers	V	E7	–	–	–	AMP, SXT	0.12
12	O111:K58 (EHEC)	Handlers	V	E7	–	–	–	AMP, SXT	0.12
13	O111:K58 (EHEC)	Handlers	V	E7	–	+	–	AMP, SXT	0.12
4	O119:K69 (EPEC)	Handlers	VI	E8	–	+	–	AMP, SAM, CFZ, FEP, CRO, IPM, MEM, MXF, GEN, SXT	0.59
6	O119:K69 (EPEC)	Calves	VI	E8	–	–	–	AMP, SAM, CFZ, FEP, CRO, IPM, MEM, MXF, GEN, SXT	0.59
7	O119:K69 (EPEC)	Handlers	VI	E8	–	–	–	AMP, CFZ, IPM, MEM, GEN, SXT	0.35
5	O44:K74 (EAEC)	Calves	VII	E9	–	–	+	AMP, AMK	0.12

[a] MAR, Multiple antibiotic resistance index.

3.4. Antimicrobial susceptibility test

Antibiotic susceptibility profiles of the 20 *E. coli* and 16 *Salmonella* isolates are illustrated in Fig. 1. *E. coli* isolates exhibited high frequencies of resistance to AMP (75%) and SXT (45%), whilst the lowest number of *E. coli* resistant isolates were observed for TGC (0%), ETP (5%), TOB (5%), CIP (5%) and NIT (5%) (Table 3). The highest frequencies of *Salmonella* resistant isolates were observed for aminoglycosides (AMK (100%), GEN (93.75%), and TOB (87.5%)). However, all *Salmonella* isolates were susceptible to FEP, ATM and MEM (Table 3). Multidrug resistance was observed in 8 (40%) *E. coli* and 10 (62.5%) *Salmonella* isolates. The mean MAR index for multidrug resistant *E. coli* isolates (resistant to at least 3 antimicrobials) was 0.43 (range 0.18–0.82) and *Salmonella* isolates was 0.33 (range 0.18–0.47).

4. Discussion

Escherichia coli and *Salmonella* are the most common bacterial pathogens associated with diarrhea in calves [3]. In Egypt, *E. coli* serovars O125 [22,36] and O26 [23,37] and *Salmonella* serotypes (*S. Enteritidis* and *S. Typhimurium*) [38] were the most prevalent serovars in healthy and diarrheic calves. However, to the author's knowledge, there is no reports about the isolation of *E. coli* (O114, O119 and O111) and *Salmonella* (*S. Kiel*, *S. Agama*, *S. Nitra* and *S. Koessen*) serovars from intensive dairy farms in Egypt. Thus, the present study aimed to determine the virulence, genotype and antimicrobial resistance profiles of these strains which isolated recently from diarrheic calves and their handlers in two large intensive dairy herds in Egypt.

In the present study, virulence-associated genes (*stx2*, *eaeA* and *astA*) were detected in *E. coli* strains isolated from diarrheic calves and handlers. This finding was consistent with previous studies in which these virulence genes were identified in *E. coli* isolated from diarrheic calves and human cases [39–42]. However, a recent study in Egypt has detected only *stx1* in *E. coli* strains isolated from dairy farms, dairy workers and milk consumers [22]. Furthermore, other studies have reported that *stx1* was more prevalent than *stx2* in *E. coli* from diarrheic

Table 2
Serotypes, clusters, ERIC, virulence and antimicrobial resistance profiles of 16 *Salmonella* isolates recovered from diarrheic calves and handlers.

ID	Serotypes	Source	Cluster	ERIC profile	Virulence profile		Resistance patterns	MAR index[a]
					stn	*spvC*		
7	*S. Kiel* (O1,2,12: H1 g,p:H2:-)	Handlers	I	E1	–	–	AMP, AMK, GEN, TOB, CFZ, MXF, NIT, SXT	0.47
15	*S. Kiel* (O1,2,12: H1 g,p:H2:-)	Calves	I	E1	–	–	AMP, AMK, GEN, TOB, CFZ, MXF, NIT, SXT	0.47
16	*S. Kiel* (O1,2,12: H1 g,p:H2:-)	Calves	I	E1	–	–	AMP, AMK, GEN, TOB, CFZ, MXF, NIT, SXT	0.47
5	*S. Agama* (O4,12: H1 i:H2 1,6)	Calves	II	E2	–	–	AMK, GEN	0.12
8	*S. Agama* (O4,12: H1 i:H2 1,6)	Handlers	II	E2	–	–	AMK, GEN, TOB	0.18
13	*S. Agama* (O4,12: H1 i:H2 1,6)	Handlers	III	E3	–	–	SAM, AMK, GEN, TOB, CFZ	0.29
10	*S. Nitra* (O2,12: H1 g,m:H2:-)	Calves	IV	E4	–	–	AMK, GEN, TOB	0.18
11	*S. Nitra* (O2,12: H1 g,m:H2:-)	Calves	IV	E4	+	+	SAM, AMK, GEN, TOB	0.24
12	*S. Kiel* (O1,2,12: H1 g,p:H2:-)	Calves	V	E5	–	–	AMP, AMK, GEN, TOB, CFZ, NIT	0.35
2	*S. Koessen* (O2,12:H1 l,v:H2 1,5)	Calves	VI	E6	+	+	AMP, AMK, GEN, TOB, CFZ, NIT	0.35
4	*S. Kiel* (O1,2,12: H1 g,p:H2:-)	Calves	VI	E6	–	–	AMK, GEN, TOB, CFZ, NIT	0.29
9	*S. Agama* (O4,12: H1 i:H2 1,6)	Calves	VI	E6	–	–	AMK, GEN, TOB, CFZ	0.24
14	*S. Agama* (O4,12: H1 i:H2 1,6)	Calves	VI	E6	–	+	AMP, AMK, GEN, TOB, CFZ	0.29
1	*S. Kiel* (O1,2,12: H1 g,p:H2:-)	Calves	VII	E7	–	–	AMK, GEN, TOB, CFZ, NIT	0.29
3	*S. Kiel* (O1,2,12: H1 g,p:H2:-)	Calves	VII	E7	–	–	AMK, NIT	0.12
6	*S. Kiel* (O1,2,12: H1 g,p:H2:-)	Calves	VII	E7	–	+	AMK, GEN, TOB, CFZ, NIT	0.29

[a] MAR, Multiple antibiotic resistance index.

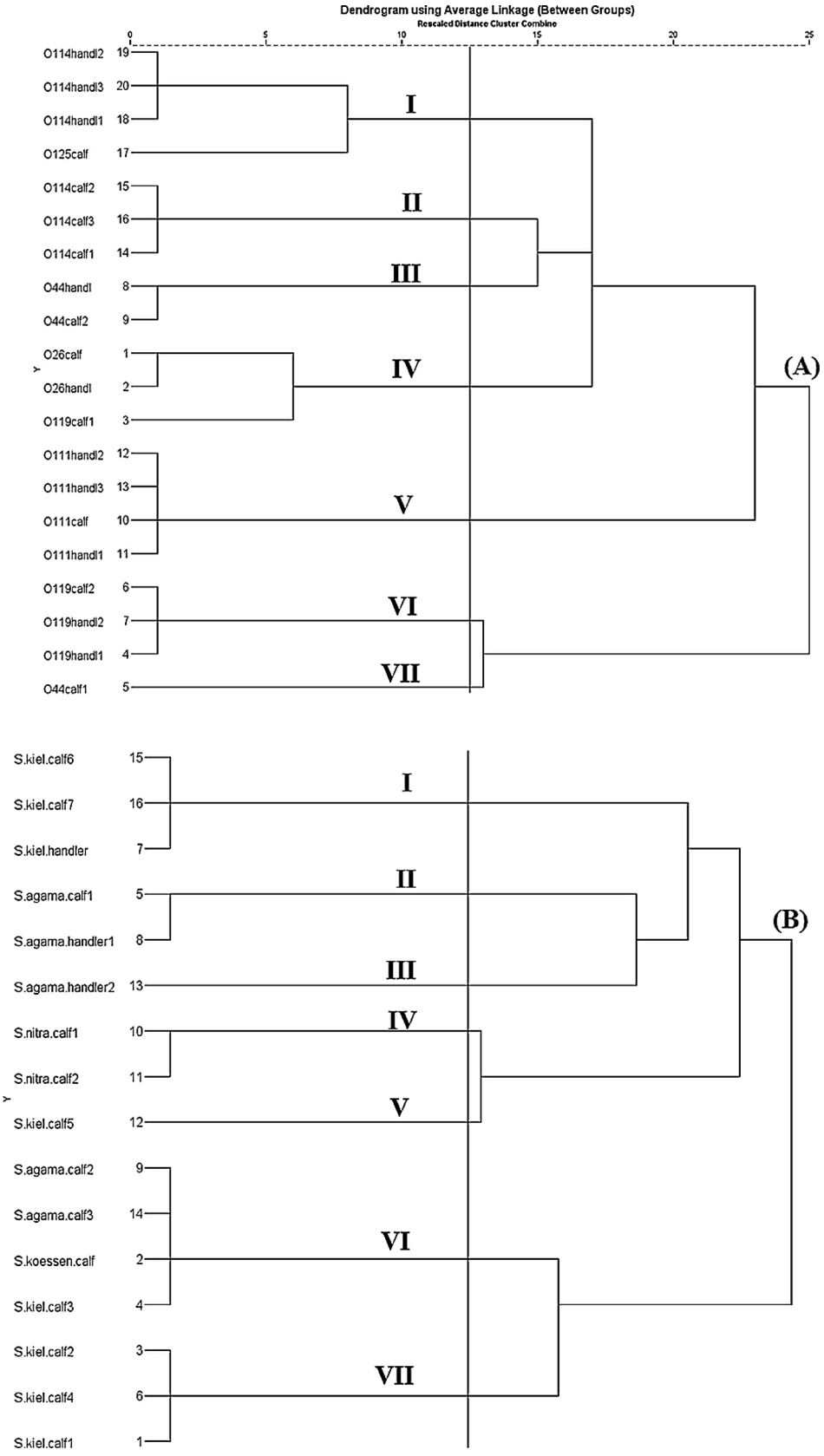

Fig. 2. ERIC-PCR dendrogram for *E. coli* (A) and *Salmonella* (B) isolates recovered from diarrheic calves and handlers. Percentage similarity of profiles for isolates was calculated using Jaccard coefficient and UPGMA. The 12.5 linkage distance was used and seven main clusters were observed.

Table 3
The antimicrobial resistance profile of the 20 *E. coli* and 16 *Salmonella* isolates recovered from diarrheic calves and handlers.

Class of antimicrobial	Antimicrobial	MIC range (μg/mL)	No. of *E. coli* isolates (%)[a]			No. of *Salmonella* isolates (%)[a]		
			R	I	S	R	I	S
Penicillins	Ampicillin (AMP)	2–32	15 (75)	1 (5)	4 (20)	6 (37.5)	1 (6.25)	9 (56.25)
β-Lactams	Ampicillin-sulbactam (SAM)	2/1–32/16	8 (40)	3 (15)	9 (45)	2 (12.5)	5 (31.25)	9 (56.25)
Cephalosporins	Cefazolin (CFZ)	4–64	7 (35)	0 (0)	13 (65)	11 (68.75)	1 (6.25)	4 (25)
	Ceftriaxone (CRO)	1–64	4 (20)	1 (5)	15 (75)	0 (0)	2 (12.5)	14 (87.5)
	Cefepime (FEP)	1–64	4 (20)	1 (5)	15 (75)	0 (0)	0 (0)	16 (100)
Monobactams	Aztreonam (ATM)	1–64	2 (10)	4 (20)	14 (70)	0 (0)	0 (0)	16 (100)
Carbapenems	Ertapenem (ETP)	0.5–8	1 (5)	0 (0)	19 (95)	0 (0)	1 (6.25)	15 (93.75)
	Imipenem (IPM)	0.25–16	5 (25)	0 (0)	15 (75)	0 (0)	1 (6.25)	15 (93.75)
	Meropenem (MEM)	0.25–16	5 (25)	0 (0)	15 (75)	0 (0)	0 (0)	16 (100)
Aminoglycosides	Amikacin (AMK)	2–64	2 (10)	1 (5)	17 (85)	16 (100)	0 (0)	0 (0)
	Tobramycin (TOB)	1–16	1 (5)	3 (15)	16 (80)	14 (87.5)	2 (12.5)	0 (0)
	Gentamicin (GEN)	1–16	5 (25)	2 (10)	13 (65)	15 (93.75)	1 (6.25)	0 (0)
Fluoroquinolones	Ciprofloxacin (CIP)	0.25–4	1 (5)	0 (0)	19 (95)	0 (0)	4 (25)	12 (75)
	Moxifloxacin (MXF)	0.25–8	3 (15)	3 (15)	14 (70)	3 (18.75)	4 (25)	9 (56.25)
Folate pathway inhibitors	Trimethoprim-sulfamethoxazole (SXT)	20–320	9 (45)	1 (5)	10 (50)	3 (18.75)	3 (18.75)	10 (62.5)
Glycylcyclines	Tigecycline (TGC)	0. 5–8	0 (0)	1 (5)	19 (95)	0 (0)	4 (25)	12 (75)
Nitrofurans	Nitrofurantoin (NIT)	0. 5 – 16	1 (5)	9 (45)	10 (50)	9 (56.25)	1 (6.25)	6 (37.5)

[a] R, resistant; I, intermediate; S, sensitive.

calves in Brazil [43] and bovine samples in the USA [44]. Previous study has reported an association between detection of *stx2*, *eaeA* genes and diarrhea in calves and humans [42]. Furthermore, it was also reported that *Salmonella stn* gene has a role in inducing diarrhea in calves [45].

Molecular genotyping by ERIC-PCR has been used to assess the genetic similarity of *E. coli* and *Salmonella* strains from different sources [46,47]. In the present study ERIC-PCR fingerprinting of the 20 *E. coli* and 16 *Salmonella* strains isolated from diarrheic calves and handlers showed high genetic diversity, which classified into 9 (E1-E9) and 7 (E1-E7) distinct profiles, respectively. The high genetic diversity of the *E. coli* and *Salmonella* strains examined in the present study, indicates the absence of an endemic strain in the investigated area [48]. The dendrogram analysis of *E. coli/Salmonella* strains revealed 7 clusters. All clusters contained *E. coli* isolates from calves and handlers except cluster II and VII (Fig. 2A). However, cluster I and II were the only two clusters contained *Salmonella* isolates from calves and handlers (Fig. 2B). The high genetic similarity between *E. coli/Salmonella* isolates from calves and handlers suggested a high level of cross-contamination between calves and their handlers in dairy farms in Egypt and confirmed the zoonotic potential of such serotypes. Similar findings have been reported in previous studies in China [49,50] and India [51].

Antimicrobials have been used for the treatment of *E. coli* and *Salmonella* infections in animals and humans [52,53]. The extensive use of antimicrobials agents in animals resulted in the appearance of antimicrobial resistance strains [41]. Since some antimicrobials used in animal production are also used for the treatment of human infections, the emerging of resistant and multidrug-resistant *E. coli* and *Salmonella* among animal isolates is a significant public health concern [54].

The antimicrobial susceptibility test of the 20 *E. coli* isolates revealed that resistance to ampicillin (75%) were the most prevalent types of resistance, followed by SXT (45%) and SAM (40%). The high resistance of *E. coli* isolates from diarrheic calves and handlers was previously reported in Egypt [36,37] and worldwide [45,55], which have been attributed to the excessive use of penicillin's in veterinary medicine in Egypt and elsewhere. The emergence of penicillin and B-lactam resistant *E. coli* are of clinically important since those groups of antimicrobials are frequently used in the treatment of *E. coli* urinary infection in human [56]. Although, *E. coli* isolates showed resistance to antimicrobials commonly used for the treatment of *E. coli* infection, all isolates were susceptible to tigecycline and showed high susceptibility to ertapenem, ciprofloxacin and amikacin. Therefore, the results of this study would suggest the use of tigecycline as the first choice for the treatment of *E. coli* infection, in accordance with another report [57]. The high susceptibility of *E. coli* isolates from diarrheic calves and handlers to tigecycline, ertapenem and ciprofloxacin have been reported in other studies in Egypt [58] and India [45]. Furthermore, the use of those antimicrobials in food-producing animals and humans is restricted in Egypt due to expensive, not easily affordable and only used with a prescription.

In the present study, all the *Salmonella* isolates were resistant to at least one of the tested antimicrobials, a result that is equal to or higher than in studies performed in dairy calves in Egypt [37,38] and other countries [17,55]. A potential explanation for the high antibiotic resistance in this study is that calves were raised under the intensive system, which increases the risk of pathogen exposure and concurrent illness. *Salmonella* isolates in the present study showed a high level of resistance to aminoglycoside (amikacin, gentamicin and tobramycin), in agreement with previous studies which reported high gentamicin resistance *Salmonella* isolates from cattle [59,60] and humans [60]. Nonetheless, our results indicate that *Salmonella* isolates were susceptible to many antimicrobials including cefepime, aztreonam, imipenem and meropenem, ertapenem, ceftriaxone, ciprofloxacin and tigecycline. These results were consistent with previous studies which reported high susceptibility of non-typhoidal *Salmonella* isolated from calves and human to ciprofloxacin [59–61]. Furthermore, *Salmonella* isolates in this study showed a low level of resistance to antimicrobials (ampicillin and sulfamethoxazole/trimethoprim) that have historically been used in the treatment of bovine salmonellosis and this could be attributed to the shift in antimicrobials selection toward cephalosporins and fluoroquinolones [62], although cephalosporins and fluoroquinolones are the drugs of choice for treatment of human *Salmonella* infections [62,63]. Antimicrobials (ceftriaxone and ciprofloxacin) are used in the treatment of *Salmonella* infections in children and adults, respectively [62,63]. No resistance to ceftriaxone, cefepime and ciprofloxacin was detected in the *Salmonella* isolates in this study, in consistence with other studies in Egypt [37], Ethiopia [64,65], Brazil [55] and Australia [17].

Multidrug resistance has been reported in *E. coli* and *Salmonella* isolates from different sources [17,38,55]. In the present study, 40% of *E. coli* isolates showed multidrug resistance, which is higher than the prevalence of multidrug resistance *E. coli* isolated from diarrheic calves in Egypt [58] and Romania [66] and humans in Mexico [67]. Further, there were two isolates resistant to at least three of the antibiotics tested, whereas resistance to 6, 7, 10 and 14 antimicrobials agents was harboured by two, one, two and one isolates, respectively. On the other

hand, 62.5% of *Salmonella* isolates showed multidrug resistance, which is higher than those reported in *Salmonella* isolates from diarrheic calves in Australia [17] and USA [68], cattle in Ethiopia [60,65] and USA [59,61] and humans in Ethiopia [60,64] and USA [61]. *Salmonella* isolates showed resistance to 5, 6 and 8 antibiotics were 5, 2 and 3 isolates, respectively. The multiple antibiotic resistance (MAR) index is an effective method for risk assessment and determining the extent of antibiotic resistance. MAR index higher than 0.2, indicates that the isolates came from sources where antibiotics are extensively used [69]. In the present study, 30% of *E. coli* and 81.3% of *Salmonella* isolates had a MAR index higher than 0.2 and most of theses isolates were recovered from hand swabs of handlers. Due to the uncontrolled use of antibiotics in dairy cows, antimicrobial residues in cow's milk may result in development of resistance towards several antibiotics in calf's intestine that can be transmitted to handlers through direct contact with calves, possibly faecal contamination or food chain [70,71].

5. Conclusions

The present study demonstrates that *E. coli* and *Salmonella* serovars isolated from diarrheic calves and handlers in intensive dairy farms in Egypt exhibited resistance to multiple classes of antimicrobials. The emergence of such multidrug resistance *E. coli* and *Salmonella* attributed to the overuse of antimicrobials in veterinary medicine which may pose a public health hazard. Thus, the continuous monitoring of antimicrobial resistance is necessary for both humans and veterinary medicine to decrease the economic losses caused by antimicrobial-resistant strains in animals as well as the zoonotic risk.

Declaration of Competing Interest

All authors declare that they have no conflict of interest on any data published in this manuscript.

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