Bacterial Causes of Hemorrhagic Gastroenteritis in Dogs and Cats with Detection of Some Virulence and β-lactamase Resistance Genes in *Escherichia coli* and *Salmonella* by Multiplex PCR

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Abstract

Hemorrhagic Gastroenteritis (HGE) is a life-threatening disease caused by bacteria or virus or endoparasites or irritant drugs or food allergy. Out of 202 sampled animals, the total bacterial isolates were 104. The identified bacterial isolates were 46 (٤٤.٢٣%) *E. coli*, 9 (8.65%) *Klebsiella*, 5 (٤.80%) *C. perfringens*, 22 (21.15%) *Proteus* spp., 3 (٢،٨٨%) *Salmonella* spp., 3 (2.88%) *Shigella* spp. and 4 (3.84%) *Pseudomonas aeruginosa*, 4 (3.84%) *Enterobacter* species, 2 (1.92%) *Citrobacter* species, 2 (1.92%) *Providencia rettgeri*, 1 (0.96%) *Hafnia* species, 1 (0.96%) *Serratia liquefaciens*, 1 (0.96%) *C. bifermentans* and 1 (0.96%) *C. putrefaciens*. EHEC (12/26, 46.15%), EPEC (9/26, 34.61%) and ETEC (4/26, 15.38%) strains were detected by *E. coli* serotyping. *Salmonella* isolates were serotyped as *Salmonella* Typhimurium, *S*. Heidelberg and *S*. Infantis. *E. coli* isolates from dogs were resistant to amoxicillin/clavulanic acid, cephalexin, ceftriaxone, trimethoprim/sulphamethoxazole, tetracycline, and erythromycin. Feline *E. coli* isolates had moderate resistance to amoxicillin/clavulanic acid, trimethoprim/ sulphonamides, and tetracycline. *Salmonella* isolates were highly resistant to amoxicillin/clavulanic acid, cephalexin, trimethoprim/sulphamethoxazole, tetracycline, and erythromycin. *stx*1 and *stx*2 *E. coli* virulence genes were detected in 80% and 60% of tested *E. coli* isolates,

respectively while *S.* Typhimurium was positive for *inv*A, *hil*A and *fim*H virulence genes and *S.* Heidelberg was positive for *inv*A and *fim*H genes. bla_{TEM} and $bla_{CTX\text{-}M1}$ β-lactamase resistance genes were detected in 60% and 20% of tested *E. coli* isolates, respectively. *Salmonella* Typhimurium was positive for $bla_{CMY\text{-}1}$ and $bla_{OXA\text{-}2}$ genes, *Salmonella* Heidelberg was positive for $bla_{CMY\text{-}1}$ gene. In conclusion, *E. coli, Salmonella, C. perfringens, Klebsiella* were major bacterial causes of HGE in dogs and cats. Additionally, *E. coli* and *Salmonella* isolated from companion animals can carry multidrug resistance genes encoding for extended-spectrum β-lactamases.

Keywords: Hemorrhagic Gastroenteritis, *E. coli, Salmonella*, serological identification, antimicrobial sensitivity test, virulence genes, β-lactamase resistance genes.

Introduction

Hemorrhagic Gastroenteritis (HGE) is a disease characterized by sudden vomiting and bloody diarrhea. The symptoms are usually severe, and HGE can be fatal if not treated. The most common bacterial causes of canine enteritis were *Escherichia coli* (*E. coli*), *Salmonellae*, *Clostridium perfringens* and *Campylobacter* ***(Habib et al., 2016)***. *E. coli* are Gram-negative rods belonging to the family *Enterobacteriaceae*. Domestic animals and pets act as natural reservoir for attaching and effacing *E. coli* (AEEC) strains. AEEC strains include two main groups: enteropathogenic *E. coli* (EPEC) strains and enterohemorrhagic *E. coli* (EHEC) strains. AEEC strains can cause attaching and effacing (A/E) lesions in the gut mucosa of human and animal hosts causing diarrheal disease. Identification of AEEC is performed by screening for the *eae* gene, their characteristic serotypes and virulence factors ***(Nataro and Kaper, 1998).*** EHEC strains produce shiga toxins also carry plasmids coding for enterohemolysin and other virulence functions ***(Krause et al., 2005).*** The use of specific toxin genes or molecular typing assays is the best way to differentiate pathogenic *E. coli* from non-pathogenic ***(Pass et al., 2000).*** Most cases of *Salmonella* infection in Dogs are latent and non-clinical and many dogs may be resistant to salmonellosis ***(Kozak et al., 2003).*** Salmonellosis isn't a common clinical case in cats although it

has a reported prevalence in cats by a percentage of 0.36 % to 51.4 % depending on several factors such as population size, housing condition, health status, diet, origin of the cat and method of detection ***(Paris et al., 2014).***

Clostridium perfringens (*C. perfringens*) is an anaerobic, spore-forming Gram-positive bacteria that is found in soil and gastrointestinal tract of vertebrates causing diseases for both humans and animals ***(Gohari et al., 2015).*** It has been associated with acute outbreaks of severe diarrhea in human beings, horses, dogs and cats ***(Songer, 1996).***

Companion animals treatment especially dogs with antibacterial drugs such as fluoroquinolones, potentiated sulfonamides, and β-lactams in bacterial infection, is often performed by veterinary clinicians and it may be occur by non-veterinarians in countries with no strict regulations for using these drugs in animals ***(Torkan et al., 2015)*** and prolonged exposure especially in sub-therapeutic doses of antibacterial drugs this caused *E. coli* carry multidrug resistance genes encoding for extended-spectrum β-lactamases ***(Tajbakhsh et al., 2015)*** additionally causing easy spread of antimicrobial resistance genes among bacteria can be produced by mobile genetic elements as plasmids, and transposons ***(Randall et al., 2004).*** The study aimed to detect bacterial causes of HGE in dogs and cats, serological identification, antimicrobial sensitivity test of *E. coli* and *Salmonella* and detection of some virulence (*stx*1, *stx*2 and *eae*A for *E. coli*, *inv*A, *hil*A and *fim*H for *Salmonella*) and β-lactamase resistance genes in *E. coli* (bla_{OXA}, bla_{CTX-M1} and bla_{TEM}) and *Salmonella* (bla_{CMY-1}, bla_{CMY-2} and bla_{OXA-2}) by multiplex PCR.

Materials and methods

Samples

A total of 202 rectal swab samples (143 swab samples from dogs and 59 swab samples from cats) were collected from housed dogs and cats suspected to have hemorrhagic gastroenteritis that manifested by bloody diarrhea from Governmental Pet Animals Units and private pet clinics in Damietta and Dakahlia Governorates, Egypt during the period from February 2017 till April 2020, then transferred to laboratory for bacteriological examinations.

Bacteriological examination

Isolation and identification of *E. coli, Salmonella and Klebsiella*

Fecal swabs were inoculated into buffered peptone water

(Lab M), incubated at 37°C for 24 h, then inoculum was streaked onto the surface of MacConkey's agar (Oxoid) and incubated at 37°C for 24 h, for *E. coli and Klebsiella*. The suspected lactose fermenter colonies were streaked onto eosin methylene blue (EMB) agar plates (Hi-Media). *E. coli* and *Klebsiella* isolates were identified by morphological identification on EMB agar, microscopical examination according to ***Cruickshank et al. (1975)*** and biochemically according to ***Kreig and Holt (1984);*** by indole test, methyl red test, Voges – Proskauer test, citrate utilization test, urease test, triple sugar iron (TSI) test, gelatin hydrolysis test, oxidation–fermentation test, nitrate reduction test, fermentation of sugars. In case of *Klebsiella*, mucoviscosity was detected according to ***Shon et al. (2013);*** where a loopful from the suspected culture was streaked on nutrient agar (Lab M) plates. Positive isolates were designated as hypermucoviscous *K. pneumoniae* (HVKP). Negative isolates were designated as classic *K. pneumoniae* (CKP).

For isolation and identification of *Salmonella*, samples were pre-enriched in Rappaport-Vassiliadis broth (Lab M) at 41.5°C for 24 h prior to plating on Xylose Lysine Deoxycholate (XLD) agar (Hi-Media) at 37°C for 24 h. *Salmonella* isolates were identified by morphological identification, microscopical examination according to ***Cruickshank et al. (1975)*** and biochemically according to ***Kreig and Holt (1984).***

Isolation and identification of *Proteus and Shigella*

For isolation and identification of *Proteus*, samples were pre-enriched in Rappaport-Vassiliadis broth (Lab M) at 41.5°C for 24 h prior to plating on Xylose Lysine Deoxycholate (XLD) agar (Hi-Media) at 37°C for 24 h. Identification was made depending on morphological and biochemical tests according to ***Kreig and Holt (1984).*** For isolation and identification of *Shigella*, samples were enriched in Sodium bi-selenite broth (Hi-Media) according to ***Morris, (1984)*** at 37°C for 24 h then plating on Xylose Lysine Deoxycholate (XLD) agar (Hi-Media) at 37°C for 24 h. Also, Streaking on S-S agar (Lab M) for differentiation between *Shigella* and *Salmonella*. Identification was made depending on morphological and biochemical tests according to ***Kreig and Holt (1984).***

Isolation and Identification of *C. perfringens*

For enrichment, samples were inoculated in brain heart

infusion broth (oxoid) and incubated anaerobically at 37°C for 24 h in an anaerobic jar. Enriched samples were streaked on sulphite polymixin sulphadiazine (SPS) agar plates (Hi-Media) and were incubated anaerobically. Suspected colonies were stained with Gram's stain and sub cultured on brain heart infusion (BHI) agar plates until they were free from contaminating bacteria. Biochemical identification was made as procedures described by ***Merchant and Packer (1967); OIE (2000) and Calnek et al. (1997).*** The pure colonies, suggestive of *C. perfringens* were further streaked on the 5% sheep blood agar (Hi-Media) and egg yolk agar (Hi-Media) plates and incubated anaerobically for 24 hr. The colonies producing characteristic double zone of hemolysis around them on blood agar and producing zone of opalescence around the colonies on egg yolk agar were tentatively identified as *C. perfringens*.

Isolation and Identification of *Pseudomonas aeruginosa*

Fecal swabs were inoculated into buffered peptone water, incubated at 37°C for 24h, then inoculum was streaked onto the surface of nutrient agar base medium for *Pseudomonas* (Merck, Oxoid) with cetrimide (CN). Biochemical identification was made using different biochemical tests such as oxidase test, catalase test, nitrate reduction, indole test, methyl red test, Voges-Proskauer test, citrate utilization and glucose fermentation test as per **Quin *et al.* (2011) and Carter and Wise (2004).**

Serological identification of *E. coli* and *Salmonella*

E. coli isolates were serologically identified according to ***Kok et al. (1996)***; using rapid diagnostic *E. coli* antisera sets (Set 1: O- antisera and Set 2: H- antisera) (DENKA SEIKEN Co., Japan) for diagnosis of the Enteropathogenic types. Serological identification of *Salmonellae* was carried out according to Kauffman – White scheme ***(Kauffman, 1974)*** for the determination of Somatic (O) and flagellar (H) antigens using *Salmonella* antiserum (DENKA SEIKEN Co., Japan).

Antimicrobial susceptibility test

Some isolates of *E. coli* and *Salmonella* were tested against 11 antimicrobial agents (Oxoid) including chloramphenicol (30µg), erythromycin (15µg), trimethoprim/sulfamethoxazole (1.25/23.75µg), amoxicillin/clavulanic acid (20/10µg),

ciprofloxacin (5µg), ampicillin/sulbactam (10/10µg), cephalexin (30µg), ceftriaxone (30µg), cefotaxime (30µg), gentamicin (10µg), and tetracycline (30µg). Antimicrobial susceptibility test was performed using disk diffusion method and the results were interpreted according to guidelines of ***Clinical and Laboratory Standards Institute (2015).***

Multiplex PCR for detection of virulence and antibiotic resistance genes in *E. coli* and *Salmonella*

DNA extraction from isolates

PCR was used for monitoring virulence genes of *E. coli* (*stx*1, *stx*2 *and eae*A) and *Salmonella* (*inv*A, *hil*A and *fim*H) and the antibiotic resistance genes of *E. coli* (bla_{OXA}, $bla_{CTX-M1}$and bla_{TEM}) and *Salmonella* (bla_{CMY-1}, bla_{CMY-2} and bla_{OXA-2}). Genomic DNA was extracted from five *E. coli* and 3 *Salmonella* isolates using GeneJET Genomic DNA purification kit Catalog No. K0721 according to the instructions of the manufacturer. The reaction volume was adjusted at 25-µl (3 µl of genomic-DNA, 5 µl of 5× Master Mix, and 20 pmol of each primer, the reaction volume was completed by adding distilled H_2O). Positive controls and negative controls (DNA-free) were used in all reactions. The used primers (Pharmacia Biotech) were purchased from (Sigma-Aldrich, Merk Life Science, Via Monte Rosa, Milano, Italy), primers sequences of *E. coli* and *Salmonella* virulence genes and β-lactamase resistance genes were listed in Table 1. PCR cycling conditions for *E. coli* virulence genes ***(Paton and Paton, 1998)*** and β-lactamase resistance genes ***(Ogutu et al., 2015)*** were illustrated in Table 2. PCR cycling conditions for *Salmonella* virulence genes ***(Singh et al., 2013)*** and β-lactamase resistance genes ***(Hasman et al., 2005)*** were illustrated in table 2. Finally, the separation of the obtained products was performed using the agar gel electrophoresis (1.5% agarose stained with ethidium bromide 0.5 µg/ml), and the gel was photographed.

Table (1): Primers sequences, target genes, specific amplicon size of *E. coli* and *Salmonella* virulence genes and β-lactamase resistance genes

Isolate	Target genes	Primer	Oligonucleotide sequence (5′ → 3′)	Produt size (bp)	References
E. coli	virulence genes	*stx*1 (F)	5′ ATAAATCGCCATTCGTTGACTAC ′3	180	Paton and Paton (1998)
		*stx*1 (R)	5′ AGAACGCCCACTGAGATCATC ′3		
		*stx*2 (F)	5′ GGCACTGTCTGAAACTGCTCC ′3	255	
		*stx*2 (R)	5′ TCGCCAGTTATCTGACATTCTG ′3		
		*eae*A (F)	5′ GACCCGGCACAAGCATAAGC ′3	384	
		*eae*A (R)	5′ CCACCTGCAGCAACAAGAGG ′3		
	β-lactamase resistance genes	bla_{OXA} (F)	5′ GGCACCAGATTCAACTTTCAAG ′3	564	Perez *et al*. (2007)
		bla_{OXA} (R)	5′ GACCCCAAGTTTCCTGTAAGTG ′3		
		bla_{CTX-M1} (F)	5′ TTAGGAAGTGTGCCGCTGTA ′3	655	Ogutu *et al*. (2015)
		bla_{CTX-M1} (R)	5′ CGGTTTTATCCCCCACAAC ′3		
		bla_{TEM} (F)	5′ CATTTCCGTGTCGCCCTTATTC ′3	800	Perez *et al*. (2007)
		bla_{TEM} (R)	5′ CGTTCATCCATAGTTGCCTGAC ′3		
Salmonella	virulence genes	*inv*A (F)	5′ GTGAAATTATCGCCACGTTCGGGCA ′3	284	Shanmuga-samy *et al*. (2011)
		*inv*A (R)	5′ TCATCGCACCGTCAAAGGAACC ′3		
		*hil*A (F)	5′ CTGCCGCAGTGTTAAGGATA ′3	497	Guo *et al*. (2000)
		*hil*A (R)	5′ CTGTCGCCTTAATCGCATGT ′3		
		*fim*H (F)	5′ GGA TCC ATG AAA ATA TACTC ′3	1008	Menghistu (2010)
		*fim*H (R)	5′ AAG CTT TTA ATC ATA ATC GACTC ′3		
	β-lactamase resistance genes	bla_{CMY-1} (F)	5′ GTGGTGGATGCCAGCATCC ′3	915	Hasman *et al*. (2005)
		bla_{CMY-1} (R)	5′ GGTCGAGCCGGTCTTGTTGAA ′3		
		bla_{CMY-2} (F)	5′ GCACTTAGCCACCTATACGGCAG ′3	758	
		bla_{CMY-2} (R)	5′ GCTTTTCAAGAATGCGCCAGG ′3		
		bla_{OXA-2} (F)	5′ ACGATAGTTGTGGCAGACGAAC ′3	602	
		bla_{OXA-2} (R)	5′ ATYCTGTTTGGCGTATCRATATTC ′3		

Table (2): *PCR cycling conditions for E. coli and Salmonella virulence genes and β-lactamase resistance genes*

Isolates	PCR detection for	PCR cycling conditions	References
E. coli	1- Virulence genes	35 PCR cycles, each consisting of: denaturation at 95°C for 1 min; annealing at 65°C for 2 min the first 10 cycles, decrementing to 60°C by cycle 15; elongation at 72°C for 1.5 min incrementing to 2.5 min from cycles 25 to 35	**Paton and Paton (1998)**
	2- β-lactamase resistance genes	initial denaturation at 94 °C for 10 min; 30 cycles of denaturation at 94 °C for 30 sec, annealing at 61 °C for 35 sec and extension at 72 °C for 1 min; and final extension at 72 °C for 9 min	**Ogutu *et al*. (2015)**
Salmonella	1- Virulence genes	initial denaturation at 94°C for 60 sec, followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 64°C for 30 sec and extension at 72°C for 30 sec, followed by a final extension at 72°C for 7 min	**Singh *et al*. (2013)**
	2- β-lactamase resistance genes	an initial denaturation step of 95°C followed by 35 cycles denaturation at 95°C for 30 sec, annealing for 30 sec and elongation for 1 min. at 72°C a final elongation step at 72°C for 5 min	**Hasman *et al*. (2005)**

Results

Out of the 104 total bacterial isolates, the identified isolates were 46 (44.23%) *E. coli*, 3 (2.88%) *Salmonella* spp., 5 (4.80%) *C. perfringens*, 9 (8.65%) *Klebsiella*, 22 (21.15%) *Proteus* spp., 3 (2.88%) *Shigella* spp., 4 (3.84%) *Pseudomonas* species, 4 (3.84%) *Enterobacter* species, 2 (1.92%) *Citrobacter* species, 2 (1.92%) *Providencia rettgeri*, 1 (0.96%) *Hafnia* species, 1 (0.96%) *Serratia liquefaciens*, 1 (0.96%) *C. bifermentanas* and 1 (0.96%) *C. putrefaciens*. *E. coli* was isolated from 28 (26.92%) dogs and 18 (17.30%) cats. Twenty-six isolates were subjected to

serological identification as shown in Table 3, the isolates belonged to 11 O-serogroups and were distributed as the following: O159 (2/26, 7.69%), O6 (4/26, 15.38%), O45 (2/26, 7.69%), O91 (6/26, 23.07%), O2 (1/26, 3.84%), O128 (3/26, 11.53%), O26 (3/26, 11.53%), O55 (1/26, 3.84%), O127 (1/26, 3.84%), O121 (2/26, 7.69%) and O146 (1/26, 3.84%). The percentages of isolation of EHEC, EPEC and ETEC strains were 12/26 (46.15%), 9/26 (34.61%), and 4/26 (15.38%), respectively. All serotypes of EHEC strains isolated from both canine and feline cases were non-O157. By antimicrobial susceptibility test, *E. coli* isolates from dogs were resistant to amoxicillin/clavulanic acid by a percentage of (66.67%), cephalexin (66.67%), ceftriaxone (66.67%), cefotaxime (33%), trimethoprim/ sulphamethoxazole (66.67%), tetracycline (100%), and erythromycin (100%). Feline *E. coli* isolates had moderate resistance to amoxicillin/clavulanic acid (50%), trimethoprim/ sulphonamides (50%), and tetracycline (50%). Concerning prevalence of *E. coli* virulence genes by multiplex PCR as shown in Figure 1, *stx*1 gene was detected in 80% of tested *E. coli* isolates, *stx*2 gene was detected in 60% of tested *E. coli* isolates, and two isolates carry both *stx* genes, while all tested *E. coli* isolates were negative for *eae*A gene. Concerning antibiotic resistance genes, All *E. coli* isolates were negative for bla_{OXA} gene while $bla_{\text{CTX-M1}}$ gene was detected in 20% of tested *E. coli* isolates and bla_{TEM} gene was detected in 60% of tested isolates as shown in Figure 2.

Three *Salmonella* spp. were isolated from dogs by a percentage of (2.88%) from the 104 total bacterial isolates, no *Salmonella* isolates were detected in cats. The three *Salmonella* isolates were serotyped as *S*. Typhimurium, *S*. Heidelberg and *S*. Infantis. By antimicrobial susceptibility test, *Salmonella* isolates were highly resistant to amoxicillin/ clavulanic acid (100%), trimethoprim/sulphamethoxazol e (66.67%), tetracycline (66.67%), and erythromycin (66.67%). *Salmonella* Typhimurium was positive *inv*A, *hil*A and *fim*H genes by multiplex PCR, while *Salmonella* Heidelberg was positive for *inv*A and *fim*H genes as shown in Figure 3. Concerning antibiotic resistance genes, *Salmonella* Typhimurium isolate was positive for $bla_{\text{CMY-1}}$ and $bla_{\text{OXA-2}}$ genes, *Salmonella* Heidelberg was positive for

bla$_{CMY-1}$ gene as shown in Figure 4. Results of string test for *Klebsiella* species identified 4 isolates of hypermucoviscous *K. pneumoniae* (HVKP) and 3 isolates of classic *K. pneumoniae* (CKP).

Table (3): *Serological identification of some E. coli isolates*

Serial No. (animal)	Serodiagnosis	Strain characterization
1 (Dog)	O159:H4	EPEC
2 (Dog)	O6	
3 (Dog)	O6:H11	EHEC
4 (Dog)	O45:H2	EPEC
5 (Dog)	O91:H21	EHEC
6 (Dog)	O2:H7	EPEC
7 (Dog)	O6	
8 (Dog)	O128:H2	ETEC
9 (Dog)	O6	EPEC
10 (Dog)	O91:H21	EHEC
11 (Dog)	O26:H11	
12 (Dog)	O91:H21	
13 (Dog)	O91:H21	
14 (Dog)	O55:H7	EPEC
15 (Dog)	O26:H11	EHEC
16 (Dog)	O91:H21	
17 (Dog)	O26:H11	
18 (Cat)	O91:H21	
19 (Cat)	O45:H2	EPEC
20 (Cat)	O127:H6	ETEC
21 (Cat)	O121:H7	EHEC
22 (Cat)	O121:H7	
23 (Cat)	O128:H2	ETEC
24 (Cat)	O159	EIEC
25 (Cat)	O128:H2	ETEC
26 (Cat)	O146:H21	EPEC

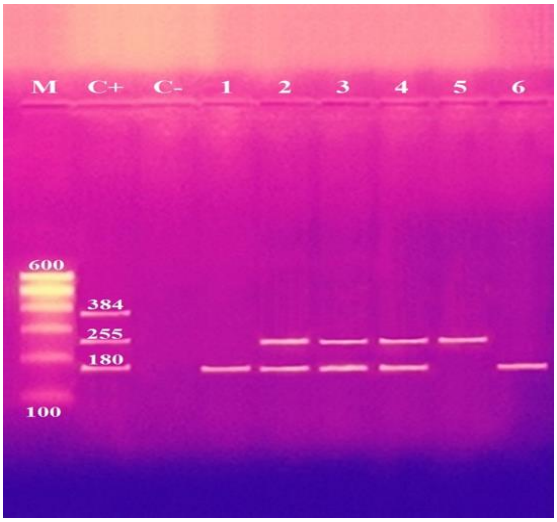

Figure (1): Agarose gel electrophoresis of multiplex PCR of *stx*1 (180bp), *stx*2 (255 bp) and *eae*A (384 bp) virulence genes for characterization of Enteropathogenic *E. coli*. **Lane M:** 100 bp ladder as molecular size DNA marker. **Lane C+:** Control positive *E. coli* for *stx*1, *stx*2 and *eae*A genes. **Lane C-:** Control negative **Lanes 1 (O45) & 6 (O128):** Positive *E. coli* strains for *stx*1 gene. **Lane 5 (O121) :** Positive *E. coli* strain for *stx*2 gene. **Lanes 2 (O55), 3 & 4 (O91):** Positive *E. coli* strains for *stx*1 and *stx*2 genes.

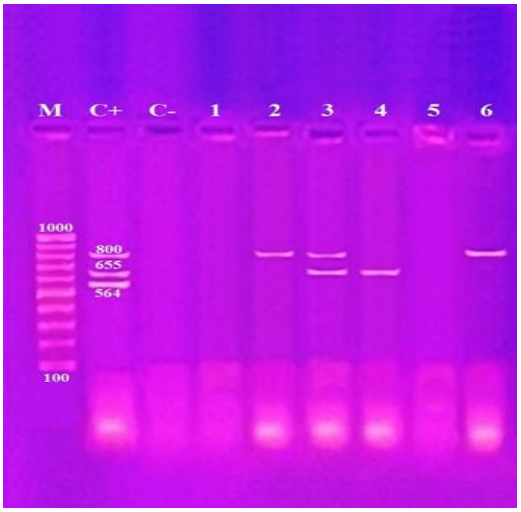

Figure (2): Agarose gel electrophoresis of multiplex PCR of bla_{OXA} (564 bp), $bla_{\text{CTX-M1}}$ (655 bp) and bla_{TEM} (800 bp) as antibiotic resistance genes of Enteropathogenic *E. coli*. **Lane M:** 100 bp ladder

as molecular size DNA marker. **Lane C+:** Control positive for *bla*$_{OXA}$, *bla*$_{CTX-M1}$ and *bla*$_{TEM}$ genes. **Lane C-:** Control negative. **Lanes 2 (O55) & 6 (O128):** Positive strains for *bla*$_{TEM}$ gene. **Lanes 4 (O91):** Positive strain for *bla*$_{CTX-M1}$ gene. **Lanes 3 (O91):** Positive strains for *bla*$_{CTX-M1}$ and *bla*$_{TEM}$ genes. **Lanes 1 (O45) & 5 (O121):** Negative strains for *la*$_{OXA}$, *bla*$_{CTX-M1}$ and *bla*$_{TEM}$ genes.

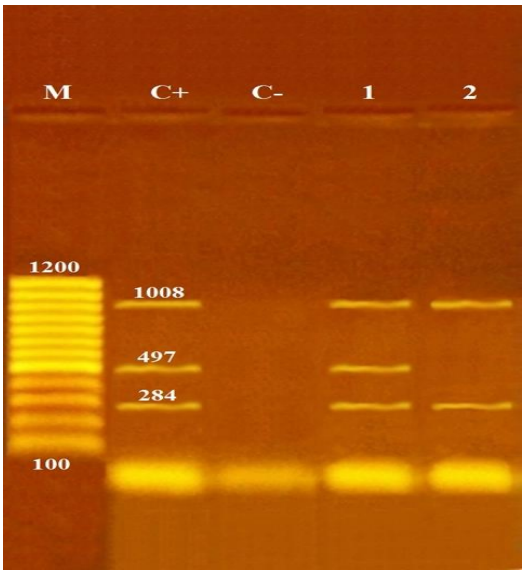

Figure (3): Agarose gel electrophoresis of multiplex PCR of *inv*A (260 bp), *hil*A (497 bp) and *fim*H (1008 bp) virulence genes for characterization of *Salmonella* species. **Lane M:** 100 bp ladder as molecular size DNA marker. **Lane C+:** Control positive strain for *invA*, *hil*A and *fim*H genes. **Lane C-:** Control negative. **Lane 1: D24 (*S.* Typhimurium):** Positive *Salmonella* for *inv*A, *hil*A and *fim*H genes. **Lane 2: D35 (*S.* Heidelberg):** Positive *Salmonella* for *inv*A and *fim*H genes.

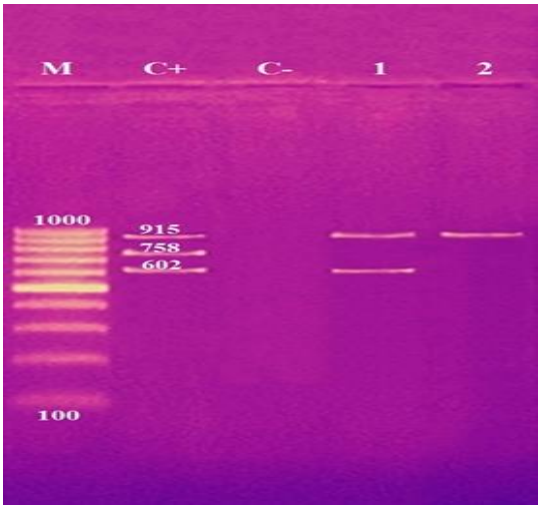

Figure (4): Agarose gel electrophoresis of multiplex PCR for identification of β-lactamase resistance genes of *Salmonella* species represented by $bla_{\text{CMY-1}}$ (915 bp), $bla_{\text{CMY-2}}$ (758 bp) and $bla_{\text{OXA-2}}$ (602 bp). **Lane M:** 100 bp ladder as molecular size DNA marker. **Lane 1:** Control positive for $bla_{\text{CMY-1,}}$ $bla_{\text{CMY-2}}$ and $bla_{\text{OXA-2}}$ genes. **Lane 2:** Control negative. **Lane 1 (*S.* Typhimurium):** Positive strain for $bla_{\text{CMY-1}}$ and $bla_{\text{OXA-2}}$ genes. **Lane 2 (*S.* Heidelberg):** Positive strain for $bla_{\text{CMY-1}}$ gene.

Discussion

The most common bacterial species isolated from dogs with Acute Hemorrhagic Diarrhea Syndrome (AHDS) was *E. coli* that is considered an enteropathogen ***(Marks and Kather, 2003)***. In the present study *E. coli* was the most prevalent isolate 46 (44.23%). Serologically, enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC) strains were the most detected strains in the present work, additionally, all serotypes of EHEC strains isolated from both canine and feline cases were non-O157; these results were in agreement with ***Marks and Kather (2003)*** who detected the three pathotypes EHEC, ETEC and EPEC in dogs and reported that there is little information demonstrating the O157:H7 role in dogs specifically. By antimicrobial susceptibility test, canine *E. coli* isolates were resistant to amoxicillin/clavulanic acid, cephalexin, ceftriaxone, trimethoprim/sulphamethoxazole, tetracycline, and erythromycin while feline *E. coli* isolates had high to moderate resistance to

cephalexin, amoxicillin/clavulanic acid, trimethoprim/sulphonamides and tetracycline. As a result of their resistance for the previous antimicrobial agents, *E. coli* isolated from dogs and cats in the current study was classified as multidrug resistant (MDR) as mentioned by ***Magiorakos et al. (2012)***. The use of PCR has become widely adopted to distinguish pathogenic *E. coli* strains from normal gut flora by detection of virulence genes (***Piccoa et al., 2015)***, additionally, PCR can identify EPEC and differentiate from EHEC (*stx*1, *stx*2, *eae*) where all *E. coli* isolates were confirmed as EPEC based on the presence of the *eae* gene and were not EHEC (absence of *stx*1 and *stx*2) ***(Kjaergaard et al., 2016)***. In the current study, EHEC was commonly detected where *stx*1 gene was detected in 80% of tested *E. coli* isolates and *stx*2 gene was detected in 60% of tested *E. coli* isolates. The bla_{TEM}, and $bla_{\text{CTX-M}}$ genes are responsible for production of TEM β-lactamases and CTX-M β-lactamases, large families of enzymes with evolutionary affinity and broad-spectrum resistance to β-lactam antibiotics ***(Al-Jassera, 2006)***. In our analysis, among *E. coli* strains, high levels of resistance to amoxicillin/clavulanic acid by a percentage of (66.67%), cephalexin (66.67%) and ceftriaxone (66.67%) were observed because bla_{TEM} and $bla_{CTX\text{-}M1}$ antibiotic resistance genes were detected in 60% and 20% of tested *E. coli* isolates, respectively.

In the present study, the prevalence of *Salmonella* isolates among dogs was 2.88%, this result was in accordance with that reported by ***(Marks and Kather (2003)***, who concluded that canine clinical salmonellosis is tremendously rare in domestic pet dogs and isolation percentage ranging from 0% to 2% in diarrheic dogs. Also, ***Ojo and Adetosoye (2009);*** recorded that the percentage of *Salmonella* isolates was 3.7% from diarrhoeic and non-diarrhoeic dogs. However, no *Salmonella* isolates were detected in cats in the present study, this result came in agreement with that documented by ***Gallaway et al. (2008)*** who reported that the clinical form of *Salmonella* infection in cats is uncommon, and cats can carry *Salmonella* asymptomatically. In the present study, the three *Salmonella* isolates were serotyped as *Salmonella* Typhimurium, *S*. Heidelberg and *S*. Infantis. ***Seepersadsingh et al. (2004);*** reported 28 different serovars of *Salmonella* in dogs while in a study performed by ***Ojo and***

Adetosoye (2009), all the *Salmonella* isolates were serotyped as *S*. Typhimurium. Dogs that fed raw chicken may be a source of environmental contamination with *Salmonella*. Since dogs can acquire *Salmonella* from their food, especially poultry products, the food should be prepared in a way that will eliminate pathogens from the food ***(Joffe and Schlesinger, 2002)***. Dogs that harbour *Salmonella* can serve as a source of *Salmonella* infection to their human companions. They can also disseminate the organism by contaminating the environment thereby exposing the general public and other animals to the risk of infection ***(Ojo and Adetosoye, 2009)***. In the current study, the antibiotic sensitivity pattern of *Salmonella* isolates revealed that *Salmonella* isolates were highly resistant to amoxicillin/clavulanic acid (100%), cephalexin (100%), trimethoprim/sulphamethoxazole (66.67%), tetracycline (66.67%), and erythromycin (66.67%). This result was near to that reported by ***Ojo and Adetosoye (2009)*** where they recorded 100% resistance to erythromycin and Cloxacillin, resistance to tetracycline (70.6%), ampicillin (47.1%), cefuroxime (52.9%), amoxicillin (35.3%), cotrimoxazole (76.5%) and gentamicin (35.3%). The multiple antibiotic resistance pattern among the *Salmonella* isolates attributed to indiscriminate use of antibiotics in animals could be responsible for emergence of resistant strains of bacteria so the use of antibiotics should therefore be well regulated and instituted only when it is absolutely indicated ***(Ojo and Adetosoye, 2009)***. In the present study, *Salmonella* Typhimurium was positive for the three genes; *inv*A, *hil*A and *fim*H genes, while *Salmonella* Heidelberg was positive for *inv*A and *fim*H genes, these results were similar to that detected by ***Salih and Yousif (2018)*** who found that all *Salmonella* isolates carried *inv*A. The previous genes considered factors of virulence as the *inv*A gene is related to host recognition and internalization of the bacterium during invasion of epithelial cells, *hil*A gene is related to the cell recognition and invasion process **(Borges *et al.*, 2013)** and *fim*H gene has an important role in adhesion of bacteria during the colonization process and host tissue invasion ***(Kuźmińska-Bajor et al., 2015)***. Concerning *Salmonella* β-lactamase resistance genes in the current work, *S*. Typhimurium was positive for $bla_{\text{CMY-1}}$ and $bla_{\text{OXA-2}}$ genes. *S*. Heidelberg was positive for

bla$_{CMY-1}$ gene. In the present work, *Salmonella* showed high levels of resistance to amoxicillin/clavulanic acid (100%) and cephalexin (100%), because *bla*$_{CMY-1}$ and *bla*$_{OXA-2}$ antibiotic resistance genes were detected in tested *Salmonella* isolates; this result was in accordance with ***Winokur et al. (2000) and Mulvey et al. (2003*)** who found that *bla*$_{CMY}$ and *bla*$_{OXA}$ genes have been found to encode ESBL resistance in *Salmonella*.

Conclusion

It may be concluded from this study that bacterial causes of hemorrhagic gastroenteritis in dogs and cats were *E. coli*, *Salmonella*, *C. perfringens*, and *Klebsiella*. *E. coli* and *Salmonella* isolates tested by antimicrobial susceptibility test were resistant to β-lactams as *E. coli* isolates had *bla*$_{TEM}$ and *bla*$_{CTX-M1}$ β-lactamase resistance genes and *Salmonella* had *bla*$_{CMY-1}$ and *bla*$_{OXA-2}$ β-lactamase resistance gene. Hence, it is recommended the proper use of antimicrobial agents in the veterinary sector.

Author contributions

MEE and ME designed the study. MEE, AW and MEA collected the samples, and applied bacteriological examinations. MEE, MEA and AW performed serological identification. MEA and MEE wrote the manuscript. ME, MEE, AW, MEA and WEH applied PCR testing. All authors have read and approved the final manuscript.

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البكتيريا المسببة لإلتهاب الأمعاء النزفي فى الكلاب والقطط والكشف عن بعض چينات الضراوة وچينات البيتالاكتاماز المقاومة للمضادات الحيوية فى ايشيريشيا كولاى والسلمونيلا باستخدام تفاعل البلمرة المتسلسل

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الملخص العربي

يُعد مرض مرض التهاب الأمعاء النزفي فى الكلاب والقطط مرض مهدد للحياة تسببه البكتيريا أو الڤيروس أو الطفيليات الداخلية أو الأدوية المهيجة أو حساسية الغذاء. تم عزل عدد ١٠٤ معزول من اجمالى عدد ٢٠٢ عينة مفحوصة ، حيث تم عزل بكتيريا ايشريشيا كولاى ٤٦ (٤٤,٢٣%)، بروتياس ٢٢ (٢١,١٥%)، الكلبسيلا ٩ (٨,٦٥%)، كلوستريديوم بيرفرنچنز ٥ (٤,٨٠%)، سلمونيلا ٣ (٢,٨٨%)، شيجيلا ٣ (٢,٨٨%)، و سودوموناس اريچينوزا (زائفة زنجارية)(٣,٨٤%)، انتيروباكتر ٤ (٣,٨٤%)، بروفيدنسيا ريتجيري ٢ (١,٩٢%)، سيتروباكتر ٢ (١,٩٢%)، هافنيا ١ (٠,٩٦%)، سيراتيا ١ (٠,٩٦%)، كلوستريديوم بيفيرمانتانز ١ (٠,٩٦%)، وكلوستريديوم بيوتريفاشينز ١ (٠,٩٦%). تم عمل اختبار الحساسية للمضادات الحيوية حيث كانت معزولات ايشيريشيا كولاى من الكلاب عالية المقاومة لكل من: أموكسيسيللين/كلاڤيولانيك، سيفاليكين، سيفترياكسون، تراى ميثوبريم/ سلفاميثوكسازول، تتراسيكللين، واريثرومايسين، بينما كانت معزولات اى كولاي من القطط متوسطة المقاومة لكلٍ من: أموكسيسيللين/كلاڤيولانيك، تراى ميثوبريم/ سلفاميثوكسازول، و تتراسيكللين. كانت معزولات السلمونيلا عالية المقاومة لكل من: أموكسيسيللين/كلاڤيولانيك، سيفاليكين، تراى ميثوبريم/ سلفاميثوكسازول، تتراسيكللين، واريثرومايسين.

أظهرت نتائج تفاعل البلمرة المتسلسل لمعزولات ايشيريشيا كولاى أنها ايجابية لتواجد چينات الضراوة *stx1 and stx2*، حيث كانت تحتوي على *stx1* ، *stx2* بنسبة ٨٠%، ٦٠%، على الترتيب. بينما كانت جميع المعزولات سلبية لچين *eaeA*. تم الكشف عن عدد من بعض چينات الضراوة فى بكتيريا السلمونيلا وهى: *invA, hilA, and fimH* ، حيث كان معزول السلمونيلا المعوية يحتوى على ال ٣ چينات، ببينما معزول سلمونيلا هايدلبرغ يحتوى على اثنان من چينات الضراوة وهى *invA and fimH*. تم عمل تفاعل البلمرة المتسلسل لعدد ٥ معزولات من بكتيريا ايشيريشيا كولاى لعدد من چينات المقاومة

blaOXA, blaCTX-M1, and blaTEM، حيث كانت إيجابية لچين *blaTEM* ، *blaCTX-M1* بنسبة ٦٠%، ٢٠% على الترتيب، بينما كانت جميع المعزولات سالبة لچين *blaOXA*. كما تم عمل تفاعل البلمرة المتسلسل ل ٢ معزول من بكتيريا السلمونيلا لعدد من چينات المقاومة وهى: *blaCMY-1 blaCMY-2 and blaOXA-2*، حيث كان معزول السلمونيلا المعوية إيجابي لچين *blaCMY-1* ، *blaOXA-2* ، بينما معزول سلمونيلا هايدلبرغ يحتوى على *blaCMY-1* چين فقط، وكان المعزولان سلبيان لچين *blaCMY-2*. من ذلك نستنتج أن أهم البكتيريا المسببة لإلتهاب الأمعاء النزفي فى الكلاب والقطط هى إى كولاي، سلمونيلا، كولستريديوم بيرفرنچنز، كلبسيلا، بروتياس، شيجيلا، و سودوموناس. بالإضافة إلى ذلك، كانت بكتيريا إى كولاى والسلمونيلا المعزولة من الحيوانات الأليفة (الكلاب والقطط) تحتوى على چينات المقاومة المتعددة للمضادات الحيوية.