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Role of *Clostridium Perfringens* and *Escherichia Coli* in the Occurrence of Diarrhea in newborn Rabbits

Eman F Farag[1], Hassan MA[1], Asmaa Basiony A[2] and Eman Mahrous*[1]

[1]Bacteriology Department, Animal Health Research Institute, Agriculture Research Center (ARC), Giza, Egypt
[2]Infection Control Unit, Zagazig University Hospitals, Zagazig, Egypt
***Corresponding author:** eman_mahrous12@yahoo.com

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ABSTRACT

This study aimed to identify some of the bacterial causes of enteritis and death following post weaning diarrhea in rabbits. A total of 230 samples from diarrheic rabbits, including 70 rectal swabs, 100 samples of livers and intestines (50 each) from freshly dead rabbits and 60 food and water samples (30 each) were taken from rabbits of various breeds from various farms and smallholders in El-Beheira governorate, Egypt. These samples were subjected to bacteriological examination. Molecular characterization was performed to confirm the *C. perfringens* and *E. coli* isolates and to detect some virulent genes associated with their pathogenicity. The results showed that the prevalence of *C. perfringens* in rectal swabs, liver and intestinal samples was 28.5, 44.0 and 84.0%; and the prevalence of *E. coli* was 74.2, 50.0 and 70.0%, respectively. In addition, examination of 30 water and 30 feeds samples from rabbits' environment revealed that, the prevalence of *C. perfringens* was 2/30 and 5/30 while the prevalence of *E. coli* was 10/30 and 7/30, respectively. *E. coli* isolates were serogrouped into O153, O125, O27, O158 and O148 serogroup. All *C. perfringens* isolates were *C. perfringens* type A (had only alpha toxin), while beta and epsilon toxins were not detected. In *E. coli*, virulent genes (eaeA and tsh genes) were detected in 3 isolates, while the cnf1 gene was not detected in any isolate. Antimicrobial susceptibility testing of most of the isolates indicated the presence of multidrug resistant strains.

Key words: Rabbits, Diarrhea, *C. perfringens, E. coli*, Serogrouping, Virulent genes.

INTRODUCTION

Rabbit farming is a rapidly growing livestock industry worldwide. Rabbit meat is considered to be a rich source of animal proteins which can help in alleviation of the global red meat shortage. However, infections of the digestive tract are the most common pathological problems in rabbit farms, causing huge financial losses to the rabbit farmers (Aboelhadid et al. 2022).

Epizootic rabbit enteropathy (ERE) is an intestinal disease that commonly affects farm rabbits and results in high mortality rates, ranging from 30 to 95%. This disease mostly occurs within the first two weeks after weaning and is caused by various pathogens such as *Clostridium spp*., *E. coli*, *Salmonella spp*. and *Vibrio spp*. (Romero et al. 2009).

Clostridium spp. are the most common pathogens causing huge economic losses to the global rabbit industry. *C. perfringens* type A was found in the caecum of rabbits died abruptly or immediately after suffering from severe diarrhea at various rabbit farms (El-Bakey et al. 2018). Its virulence was dependent on the development of at least 20 distinct toxins and extracellular enzymes (Revitt-Mills et al. 2015). Another most important etiological agent of gastrointestinal infections in rabbits is *Enteropathogenic Escherichia coli* (EPEC). It causes dehydration, fatigue, and watery diarrhea that may be mucoid or bloody in nature (Swennes et al. 2012; Bakry et al. 2021; Bastamy et al. 2022).

This study was planned to investigate the prevalence of *E. coli* and *Clostridium spp*. in rabbits suffering from weaning diarrhea, as well as from their environment. Moreover, sensitivity of these pathogens to various antimicrobial agents was also monitored and the presence of virulent genes in these bacteria was determined using PCR.

MATERIALS AND METHODS

Ethical approval

The study was conducted at the Animal Health Research Institute, Dokki, Giza, Egypt. The experimental protocol was duly approved by the Institutional Animal Care and Use Committee, Agriculture Research Center. Cairo, Egypt.

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Collection of Samples

A total of 230 samples from diarrheic rabbits, including 70 rectal swabs, 100 samples of livers and intestines (50 each) from freshly dead rabbits and 60 food and water samples (30 each) were aseptically collected from various farms and smallholders in El-Beheira governorate, Egypt, and subjected to bacteriological examination. Rabbits between 2 and 10 weeks of age, belonging to different breeds and with a history of severe diarrhea, bloating and mortality were included in the study.

Bacteriological Examination and Antibiogram of *C. perfringens* and *E. coli*

The collected samples were subjected to bacteriological examination according to the procedure described by Quinn et al. (2011). Antibiograms of *C. perfringens* and *E. coli* isolates were performed by disc diffusion method, as described earlier (CLSI 2022), using available commercial antibiotic discs purchased from MAST Diagnostics. as the antibiotic discs used in the study were Amikacin (Ak-30 µg), Amoxicillin (AX-25µg), Ampicillin (AX-30 µ), Cefotaxime (CTX-30µg), Cephalothin (Kf-30µg), Erythromycin (E-15µg), Imipenem (IMP-10µg), Kanamycin (K-30µg), Levofloxacin (Lev-5µg), Ofloxacin (OFX-5µg), Oxytetracycline (T-30µg), Pencilline (P-10µg), Streptomycin (S-10µg), Colistin sulfate (CO-10µg), Ofloxacin (OFX-5µg), Cephadroxil (CDX-30µg) and Sulfa-trimethoprime (SXT-25µg).

Serogrouping of *E. coli* Isolates

Serogrouping of *E. coli* isolates was carried out through slide agglutination test, using polyvalent and monovalent *E. coli* antisera (Denka Seiken Co. LTD, Tokyo, Japan for antisera**).** For this purpose, the procedure described earlier by Quinn et al. (2011) was followed.

Molecular Characterization of the Isolates by PCR

DNA extraction from isolates was performed by using the QIAamp Mini-Kit for DNA (Qiagen, Germany, GmbH). For this purpose, oligonucleotide primers were obtained from Metabion (Germany) and are listed in Table 1 and 2. Amplification of PCR for *C. perfringens* and *E. coli* was done by using PCR Master Mix (Takara, Japan), the reaction was performed using Applied Biosystem 2720 thermal cycler.

Analysis of the PCR products was carried out by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1×TBE buffer, using gradients of 5 V/cm. For Gel analysis, 40µL of the products were loaded into each gel slot. Ladder of 100 bp (Qiagen, GmbH, Germany) was used to determine size of fragments. A gel documentation system (Alpha Innotech, Biometra) was used to photograph the gel and the data were analyzed by using the computer software.

RESULTS

The prevalence of *C. perfringens* and *E. coli* in the examined samples has been shown in Table 3. The data show that out of examined 170 samples including 70 rectal swabs, 50 liver and 50 intestinal samples, the prevalence of *C. perfringens* was 28.5, 44.0 and 84.0%, respectively. The corresponding values for the prevalence of *E. coli* were 74.2, 50.0 and 70.0%, respectively. These results were also supported by Fig. 1 that showed (A) carcass of a rabbit died from dehydration and severe diarrhea; (B, C) small intestine of a rabbit shows different degrees of enteritis, the intestine is distended with gases; (D) a rabbit's liver reveals congestion, enlargement, sub-capsular hemorrhage, and necrosis; (E) a rabbit with distended urinary bladder with urine congested and enlarged kidney.

Examination of feed and water samples from rabbits' environment revealed that out of examined 30 water and 30 feeds samples, the prevalence of *C. perfringens* was 2/30 and 5/30 respectively, while the prevalence of *E. coli* was 10/30 and 7/30, respectively. The serogrouping of *E. coli* isolated from water, feeds and diseased rabbits revealed that all *E. coli* isolates belonged to the O serogroup; O153, O125, O158, O27 and O148, with the frequency as 4, 2, 2, 1 and 1, respectively.

The antibiotic susceptibility of *C. perfringens* is shown in Table 4. It shows that most isolates were sensitive to Amoxicillin, Ampicillin, Penicillin, Levofloxacin, Cephalothin and Trimethoprim sulphate, while they were resistant to Amikacin, Erythromycin, Kanamycin, Streptomycin and Oxytetracycline. The antibiotic susceptibility of *E. coli* is shown in Table 5, which indicates that most isolates were sensitive to Levofloxacin, Ofloxacin, and Imipenem and resistant to Ampicillin, Amoxicillin, Cephadroxil, Colistin sulphate, Amikacin and Oxytetracycline.

Molecular characterization of *C. perfringens* isolates (Table 6 and Fig. 2) revealed that all isolates examined gave clear bands at 402 bp for the alpha toxin gene only (beta and iota toxins were not detected), indicating that all isolates examined were of the *C. perfringens* type A. Molecular characterization of *E. coli* isolates Table 7) and (Fig. 3) indicated the phoA gene was detected in all 5 isolates, in addition, the eaeA gene was detected in 3 isolates (Fig. 4). In addition, the tsh gene was detected in 3 isolates (Fig. 5), while the cnf1 gene could not be detected in any isolate (Fig. 6)p

DISCUSSION

Rabbits are particularly susceptible to enteric pathogens post weaning, which is attributed to an undeveloped gut microbiota, poor digestive capacity and a shift in gut pH Aboelhadid et al. (2022). *Clostridium spp.*, *E. coli*, *Salmonella spp*. and *Vibrio spp*. belong to the most triggering microorganisms (Romero et al. 2009). Results relating to the prevalence of *C. perfringens* shown in Table 3 appear to be similar to those of Khelfa et al. (2015), who isolated *C. perfringens* from rabbit samples with percentage values of 44, 83 and 18 from rectal swabs, intestine and liver samples, respectively. However, El-Rahman and Atwa (2006) reported that the prevalence of *C. perfringens* was 30%. According to Abdel-Rahman et al. (2006), *C. perfringens* was detected in 140 rectal swabs from apparently healthy and diarrheic post weaned rabbits in El-Menia and Assuit governorates. These differences in isolation rates could be due to differences in seasons of the study, location of the farm, feeding, housing and management systems.

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions of *C. perfringens*

Target gene	Primers sequences	Amplified segment (bp)	Primary Denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
Alpha toxin	GTTGATAGCGCAGGACATGTTAAG CATGTAGTCATCTGTTCCAGCATC	402	94˚C 5min	94˚C 30s	55˚C 40s	72˚C 45s	72˚C 10min	Yoo et al. (1997)
Beta toxin	ACTATACAGACAGATCATTCAACC TTAGGAGCAGTTAGAACTACAGAC	236						
Epsilon toxin	ACTGCAACTACTACTCATACTGTG CTGGTGCCTTAATAGAAAGACTCC	541						

Table 2: Primers sequences, target genes, amplicon sizes and cycling conditions of *E. coli*

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
tsh	GGT GGT GCA CTG GAG TGG AGT CCA GCG TGA TAG TGG	620	94˚C 5min	94˚C 30s	54˚C 30s	72˚C 30s	72˚C 10min	Delicato et al. (2003)
Cnf1	TATATAGTCGTCAAGATGGA CACTAAGCTTTACAATATTGAC	620	94˚C 5min	94˚C 30s	63˚C 45s	72˚C 30s	72˚C 7min	Kadhum et al. (2008)
eaeA	ATG CTT AGT GCT GGT TTA GG GCC TTC ATC ATT TCG CTT TC	248	94˚C 5min	94˚C 30s	51˚C 30s	72˚C 30s	72˚C 7min	Bisi-Johnson et al. (2011)
phoA	CGATTCTGGAAATGGCAAAAG CGTGATCAGCGGTGACTATGAC	720	94˚C 5min	94˚C 30s	55˚C 40s	72˚C 45s	72˚C 10min	Hu et al. (2011)

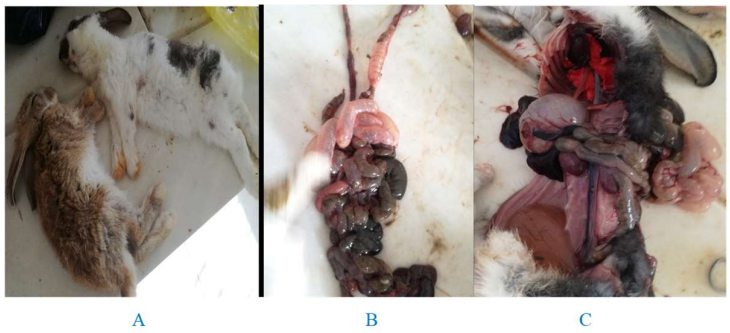

Fig. 1: Showing: (A) carcass of a rabbit died from dehydration and severe diarrhea; (B, C) small intestine of a rabbit shows different degrees of enteritis, the intestine is distended with gases; (D) a rabbit's liver reveals congestion, enlargement, sub-capsular hemorrhages, and necrosis; (E) a rabbit with distended urinary bladder with urine congested and enlarged kidney.

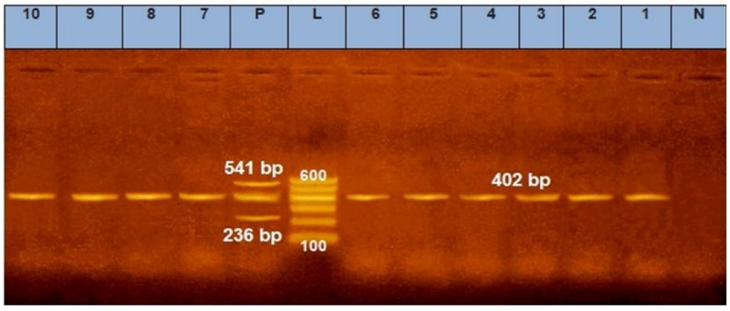

Fig. 2: Agarose gel electrophoresis of PCR products showing amplification of *C. perfringens* genes alpha, beta and epsilon toxin products at 402, 236 and 541bp, respectively. MWM-molecular weight marker (100-600bp DNA ladder), + control (Positive, Negative). Ten isolates were positive for alpha toxin only at 402bp.

The prevalence of *E. coli* recorded in the present study is similar to 75% reported by Hong et al. (2017) from freshly diseased rabbits. Sakr et al. (2019) recorded lower value of *E. coli* prevalence as 60.8%, while higher value of 84% was recorded by Eid et al. (2017). In addition, EL-Ashram et al. (2020) isolated *E. coli* from all diseased rabbits studied before and after weaning.

The prevalence of *C. perfringens* and *E. coli* recorded in water and feeds samples. Findings were like the findings (18.42%) reported by Khelfa et al. (2012). However, lower results have also been reported earlier by Heba (2012) as 5.56%, respectively. Feeds and water can become contaminated with *C. perfringens* and/or *E. coli* when they come into contact with contaminated farm effluents, animal

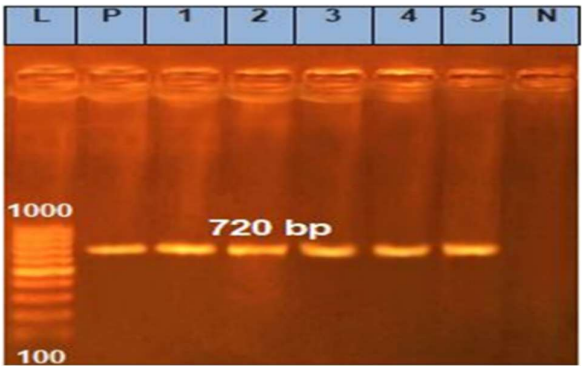

Fig. 3: Agarose gel electrophoresis of PCR products showing amplification of *E. coli phoA* gene products at 720bp. MWM-molecular weight marker (100-1000bp DNA ladder), + control (Positive, Negative); all (5) isolates were confirmed as *E. coli.*

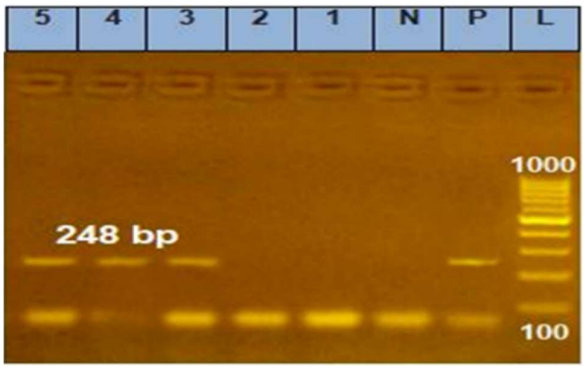

Fig. 4: Agarose gel electrophoresis of PCR products showing amplification of *E. coli* eaeA gene products at 248bp. MWM-molecular weight marker (100-1000bp DNA ladder), + control (Positive, Negative); eaeA gene was detected in 3 isolates only.

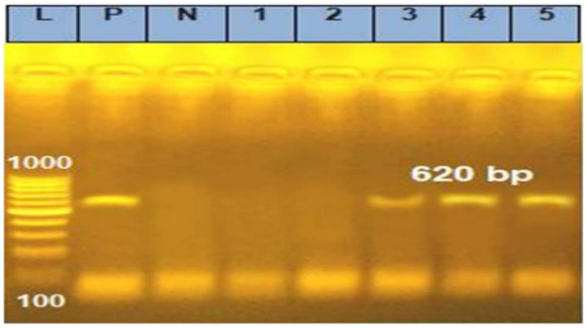

Fig. 5: Agarose gel electrophoresis of PCR products showing amplification of *E. coli* tsh gene products at 620bp. MWM-molecular weight marker (100-1000bp DNA ladder), + control (Positive, Negative); tsh gene was detected in 3 isolates only.

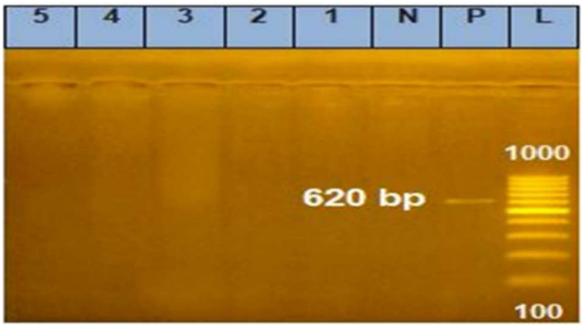

Fig. 6: Agarose gel electrophoresis of PCR products showing amplification of *E. coli* cnf1 gene products at 620bp. MWM-molecular weight marker (100-1000bp DNA ladder), + control (Positive, Negative); cnf1 gene was not detected in any isolate.

feces and surfaces harboring bacteria during plant harvesting, storage and/or transport. Therefore, it is very important to consider the role of feed and water as a source of infection for early-weaned rabbits in addition to another concomitant coccidia infection.

Similar to the results of the present study, five *E. coli* sero-groups were detected in rabbits with diarrhea by Eid et al. (2017) and Sakr et al. (2019); these sero-groups were O_{153}, O_{125}, O_{27}, O_{158} and O_{148}.

Results regarding susceptibility of *C. perfringens* shown in Table 4 are supported by those of Edham et al. (2020) who stated that *C. perfringens* was 100% susceptible to imipenem. According to Xiu et al. (2020), resistance to imipenem was less than 10%; it was 55.26% to tetracycline and 37.5% to erythromycin. Abd El-Tawab et al. (2017) reported that the sulfamethoxazole-trimethoprim combination showed moderate efficacy against *C. perfringens* isolates. El-Bayomi et al. (2020) demonstrated that all *C. perfringens* isolates examined were resistant to oxytetracycline, while 90% of the isolates were resistant to amoxicillin and 80% to ampicillin.

Results regarding susceptibility of *E. coli* shown in Table 5 are similar to those of Sakr et al. (2019), who reported that *E. coli* isolated from diarrheic rabbits showed high resistance to various antibiotics. In addition, all *E. coli* isolates were resistant to ampicillin and amoxicillin + clavulanic acid. Abd-El Rahman et al. (2005) reported that most of the *E. coli* isolates were susceptible to enerofloxacin and norfloxacin. High resistance to multiple antibiotics indicates the presence of multidrug-resistant strains that can be transmitted to humans and pose a serious public health problem.

Results on molecular characterization of *C. perfringens* isolates shown in Table 6 are supported by those of Khelfa et al. (2012), who isolated a high percentage of *C. perfringens* type A from affected rabbits. On the contrary, Solans et al. (2019) reported that *C. perfringens* type A was isolated at very low frequency in rabbits of mixed age groups.

Results regarding molecular characterization of *E. coli* isolates given in Table 7 are in accordance with those of Eid et al. (2017), who reported that all *E. coli* strains had the phoA and eaeA genes in most isolates and tsh gene was seen in only one isolate. The eaeA (intimin) gene is an *E. coli* adhesion factor that facilitates its attachment to intestinal epithelial cells, creating attaching and effacing lesions that result in enteropathogenic and enterohemorrhagic diarrhea. On the other hand, Sakr et al. (2019) detected the eaeA gene from 28.57% *E. coli* isolates, while Ashraf et al. (2014) detected the eaeA and tsh genes in three *E. coli* isolates. Higher detection rates for the eaeA gene obtained by Alton et al. (2012) and Khafagy at al. (2015) were 83 and 100%, respectively. Saad Eldin and Reda (2016) detected the tsh gene in all identified *E. coli* serogroups. According to Khafagy et al. (2015), most of the *E. coli* isolates tested were positive for the tsh gene. In addition, Sakr et al. (2019) reported that *Cnf*1 gene was not detected in any of the *E. coli* serotypes.

Table 3: Prevalence of *C. perfringens* and *E. coli* isolated from rabbits

Type of samples	No of Animals	Positive samples For *C. perfringens*		Positive samples For *E. coli*	
		No.	%	No.	%
Rectal swab (diseased)	70	20	28.5	52	74.2
Liver (freshly dead)	50	22	44.0	25	50.0
Intestine (freshly dead)	50	42	8.0	35	70.0

Table 4: Antimicrobial susceptibility of *C. perfringens* isolated from rabbits

Antibiotic class	antimicrobial agent	N=10	
		Resistant	Sensitive
Aminoglycosides	Amikacin	10	-
	Kanamycin	7	3
	Streptomycin	10	-
B – Lactams	Amoxicillin	-	10
	Ampicillin	1	9
	Penicillin	2	8
Macrolides	Erythromycin	6	4
Tetracycline	Oxytetracycline	9	1
Quinolones	Levofloxacin	1	9
	Ofloxacin	2	8
Cephalosporins	Cephalothin	-	10
Sulphonamide	Trimethoprime-sulfate	2	8

Table 5: Antimicrobial susceptibility of *E. coli* isolated from rabbits

Antibiotic class	Antimicrobial Discs	N=10	
		Resistant	Sensitive
Aminoglycosides	Amikacin	8	2
	Kanamycin	10	-
B – Lactams	Amoxacillin	10	-
	Ampicillin	10	-
	Imipenem	1	9
Cephalosporins	Cephadroxil	10	-
Polypeptides	Colistin sulphate	7	3
Quinolones	Ofloxacin	-	10
	Levofloxacin	-	10
Tetracycline	Oxytetracycline	9	1

Table 6: Molecular characterization of *C. perfringens* isolates

Sample	Alpha	Beta	Epsilon
1	+	-	-
2	+	-	-
3	+	-	-
4	+	-	-
5	+	-	-
6	+	-	-
7	+	-	-
8	+	-	-
9	+	-	-
10	+	-	-

Table 7: Molecular characterization of *E. coli* isolates

Sample	eaeA	Cnf1	Tsh	PhoA
1	-	-	-	+
2	-	-	-	+
3	+	-	+	+
4	+	-	+	+
5	+	-	+	+

From this study, it can be recommended that rabbit farmers should employ strict hygienic and preventive procedures, with proper vaccination programs, as well as regular inspection of feed components and water sources, to avoid transmission of pathogenic bacteria to rabbits. Hygienic disposal of manure and periodic disinfection of batteries is also necessary. In addition, performing a susceptibility test is very important for choosing the appropriate antibiotic to avoid the emergence of multidrug-resistant bacterial strains.

Conclusion

C. perfringens and E. coli are incriminated in the induction of enteritis in rabbits with high frequency causing huge economic losses. PCR assay was used confirmation and characterization of some virulence genes in *E. coli* isolates. All *C. perfringens* examined isolates showed involvement of *C. perfringens type A*. Antimicrobial susceptibility testing is a very important step to select the drug of choice because of the development of multidrug resistant strains.

Author's Contribution

Study design: EFF and HMA. Sample collection and processing: EFF, HMA, ABA and EM. Data analysis HMA and EM. Manuscript drafting, reviewing, and editing: HMA, ABA and EM. All authors read and approved the final manuscript.

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